LECTURES IN NEUROBIOLOGY

SERC SCHOOLS SERIES, DEPARTMENT OF SCIENCE AND TECHNOLOGY

LECTURES IN NEUROBIOLOGY

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Foreword

The Science and Engineering Research Council (SERC) is a high-level body which advises the Department of Science and Technology on matters concerning frontline research and development activities in various science and engineering disciplines.

SERC has adopted a two-pronged strategy for fulfilling its objectives. First, it supports time-bound R & D projects which are carefully referred and processed through a peer review mechanism. Second, in order to meet the long-term manpower requirement, SERC has also initiated a major programme of organising summer, and winter schools in selected thrust areas to encourage young scientists to take up research at various academic institutions and laboratories in the country. Under the programme, several series of such schools are being organised in various identified thrust areas, like Theoretical High Energy Physics (THEP), Condensed Matter Physics, Neurobiology, and Immunology. It is proposed that in each of these areas there should be a five year cycle of such schools. The prime concern here is to have enough coordination and continuity between successive schools as to be able to assess the impact of these schools on the manpower development. Isolated workshops may not be expected to have that much impact.

One of the first series to be organised was in the area of Theoretical High Energy Physics (THEP). A series of five schools was started in 1985. The second series of SERC Schools was in Neurobiology. This was a series of five courses which commenced in 1987. Four such courses have been held to update the knowledge of postgraduate students specially those belonging to clinical neurosciences with recent developments and concepts in basic neurosciences. Realising the continuing need for young and energetic manpower suitably trained to address the contemporary and frontline problems, neuroscience community took considerable interest in developing these courses.

It was felt that the quality, nature and relevance of the lectures delivered at the schools were such that the lectures, if published, would be useful for a much wider readership even at the international level, also if made available to the students and research community in India at an affordable price, it would further aid in manpower development – a primary objective of these schools. It was, however, decided that the lectures be suitably written, reorganised (if required, not necessarily schoolwise) and published in the form of books/monographs to serve as material for training young and aspiring researchers. Conventional conference proceedings do not have this character. The present monograph is an outcome of the fourth course in the Neurobiology series. Five monographs will complete this series. It is hoped that this effort will be useful for neurobiologists in general and young Indian researchers in particular.

On behalf of the Department of Science and Technology, let me place on record our grateful appreciation to Professor P.N. Tandon, in particular, for taking the difficult responsibility of being the Series Editor of these monographs. The authors of individual chapters also deserve our special thanks for timely completion of their manuscripts with meticulous care and proficiency.

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Preface

Keeping in mind the need to update the knowledge of postgraduate students, specially those pursuing clinical courses, about the recent developments and concepts in the fast expanding frontiers of basic neurosciences, the Fourth Course in Neurobiology was held at the All India Institute of Medical Sciences, New Delhi from February 18-March 1, 1991.

This volume, fourth in series on Lectures in Neurobiology is a compilation of some of the selected lectures delivered during this course. While providing the state of the art information on these topics efforts have been made to make the book relevant, comprehensible and easily readable for the clinical postgraduates. The subjects covered are different from those included in the previous three volumes although repetition of some topics is inevitable. The generous contribution and untiring efforts of the distinguished members of the faculty of the course, drawn from the different parts of the country has made this volume a reality. Our special thanks are due to them. To the Department of Science and Technology, Govt. of India, which has been financially supporting these courses and publication of the monographs, we are ever grateful.

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Neurobiology Education for Clinicians: A Rationale

P.N. TANDON

Few years ago the Programme Advisory Committee on Neurobiology constituted by the Department of Science & Technology, recognized the prevailing deficiency in teaching of basic neurosciences for postgraduates in clinical disciplines of Neurology, Neurosurgery and Psychiatry. It was realized that during the past two decades or so a number of departments have been established in these clinical fields. They are providing modern service facilities and have initiated postgraduate courses in these disciplines. However, the development of basic neurosciences departments has not kept pace. There were indeed very few institutions where even a modest nucleus existed. The clinical teachers, who had been responsible for the comprehensive training and teaching of the postgraduates, burdened with ever increasing demands for patient care, were unable to keep pace with the explosive developments in basic neurosciences.

To compensate for this deficiency, atleast partly, it was decided to organise annual courses, to provide atleast some "capsulized" exposure to clinical trainees, (to which was later added young faculty members). The present course is the fourth in this series. Based on the experience of running these courses and discussions held both with the trainees, the faculty of the courses, (many derived from non-medical faculties) and those responsible for the clinical training programmes has raised some basic issues. Attempt is made to discuss some of these in this presentation. Do postgraduates enrolled in professional courses (M.D. Psychiatry, D.M. Neurology, M.Ch. Neurosurgery) require teaching of basic neurosciences? This might sound a very naive question, afterall if it was not necessary, why should various academic bodies include basic neurosciences, neuroanatomy, neurophysiology, neurochemistry, neuropathology etc. in the cur-

ricula prescribed for such courses. Atleast one out of three theory papers for D.M. or M.Ch. is restricted to basic neurosciences. However, a survey of the existing practice all over the country reveals that the total teaching of basic neurosciences, (even where it was part of the teaching programme) generally consisted of some "Seminars" prepared by the student himself and "read out" during these teaching sessions, with only minimal inputs by the participating faculty. The topics of these seminars are generally examination oriented, cramming information rather than concepts and lacking in an understanding of their implications for clinical applications. Relying on "standard" text books, often out of date, these seminars generally lack state of art knowledge. Keeping in mind the oft quoted principle of modern medicine, "As is your basic science so is your practice", it would be obvious that this lip service to basic science can only lead to professional mediocrity.

In the past basic science research was purely an intellectual exercise, pursued by some "absent minded" professors, "out of contact with the real world", having no immediate relevance for its practical application. Thus Harvey expounded his theory of circulation at the second Lumleian Lecture on 17th April 1616, but waited for 12 years (till 1628) to publish it and it took decades for its clinical application. Today the time taken for the conversion of findings of basic research to its practical use is in months and years and not in decades or centuries. It is, therefore, all the more important for clinicians to be atleast aware of the current scenario in the allied sciences, if not an indepth knowledge which undoubtedly is beyond the reach of even the most motivated clinicians. Lord Walton, President of the World Federation of Neurological Societies recently commented, "we live at a time when development in neuroscience and the consequential benefits for clinical neurology have never been greater".

Let me give a few examples to illustrate the above statements. DNA was first isolated by a German Chemist directly in 1869, and its chemical composition was already established in the early years of this century. However, upto 1950 no one understood its physical structure and therefore how it coded and transmitted information regarding heredity. It was a few years after my graduation from the medical school that Watson and Crick constructed a molecular model of DNA (1953). The significance of this information for transfer of genetic information in living material was obvious to them and acclaimed by the award of the Nobel Prize in 1962. Already in 1968 Khorana (alongwith Robert Holley and Marshall Nirenberg) received his Nobel Prize for the interpretation of the genetic code and its function in protein synthesis. Soon after that listening to Prof. Khorana at the Institute, not only most clinicians, including myself, could not understand his lecture, but failed to appreciate that it had any practical relevance for neurosurgeons or neurologists. Today any clinician who does not have reasonable familiarity with molecular genetics will not only be unable to understand a sizeable number of papers published in Journals of Neurology and Neurosurgery but find himself at a loss to apply this knowledge to his practice. Just to take an example, the locus for the gene of Duchenne Muscular Atrophy (DMD), the most common inherited human muscle disease, was localized to Xp21 region of

the X-chromosome; this was cloned by Kunkel and his colleagues in Boston in 1987. This was followed by the discovery of dystrophin, the missing gene product in 1988. And already immunohistochemistry based on this protein is being used for diagnosing and classifying cases of DMD. To take it a step further, the recent observation of Karpati and his group in Montreal that genes directly injected into adult muscle fibres have a long functional half life opens interesting perspectives for therapy. Likewise the loci for the genes for Charcot-Marie-Tooth, spinocerebellar degeneration, Friedreich's ataxia, familial manic-depressive psychosis, familial Alzheimer's disease have all been identified during the last few years. The gene for human tyrosine hydroxylase (TH), the enzyme concerned with synthesis of dopamine and norepinephrine has been cloned though it is yet to be established if it is related to development of a clinical syndrome. It is true that the eventual aim to identify the mutated gene and to characterise its protein product necessary for developing therapeutic strategies remains to be achieved for several of these diseases but the genetic linkage results obtained so far are useful for possible antenatal diagnosis as has already been done for DMD or Huntington's disease. Today gene therapy may still be only a theoretical possibility, its practical application requiring lot more work, but it is no more science fiction but a practical reality. For the neurosurgeons, it may be of interest to know the role of oncogenes in the pathogenesis of brain tumors. It has recently been demonstrated that the product of expression of a well known oncogene has some homology to the platelet derived growth factor, which in turn has striking resemblance to glial growth factor. The role of such factors in the development or biological behaviour of glial tumors-which constitute the greatest challenge to them may soon acquire practical importance.

To take another example i.e. of ion-channels: At the face of it the very mention of ion-channels, the techniques to study them, the proteins constituting their receptor may evoke a response from most clinicians "leave this to physiologists or biochemists". Though the existence of ion channels was postulated many years ago but it was Erwin Nehrer's discovery and development of the patch clamp technique in later part of 1970's on one hand and radioactive ligand binding techniques on the other that have revolutionized their study. A variety of ion channels – $Na+$, $K+$, $Co2+$ and their subtypes; voltage dependent, ion dependent and ligand gated, have been identified. New molecular biological methods have resulted in determining complete primary sequencing of some of the receptor proteins e.g. acetylcholine receptor and sodium channel. Most of this knowledge is no more than a decade old, but we already are using calcium channel blockers in the day to day management of our patients. At the sametime the biologists are hoping that these studies one day in near future, may help to explain higher nervous functions e.g. memory, learning etc. Should it not be of interest to neurologists, neurosurgeons and psychiatrists alike?

The same could be said of the receptors on the surface of the neuronal membrane. It is now clear that much of the information processing in the brain involves synaptic transmission mediated by neurotransmitters acting at specific receptors. Major advances in understanding receptor-ligand interaction have

taken place only during last 10-15 years, with the development of new tools and techniques like autoradiography, radio-immunoassay, immunohistochemistry and other chemical and pharmacological methods. I am sure it will be of interest to clinicians to know that in 1982, Synder predicted, "in the next decade, I suspect that we will be able to observe in living humans receptors for all known neurotransmitters and drugs. We will probably be able to measure the activity of neurons throughout the brain in awake, active thinking and feeling humans". It has already been achieved atleast partly. The current generation of PET and SPECT radiotracers permit study of neurotransmitter properties from a number of different perspectives including their pre and postsynaptic sites and the activity of the enzymes which regulate their concentration. Although dopamine system has been the most extensively investigated in human beings, the possibility of its use for the study of other neurotransmitters including acetylcholine, serotonin, benzodizapine, opiate, NMDA etc. is actively being pursued. The clinical applications of these range from the study of normal function and the characterisation of neurotransmitter activity in neurological and psychiatric diseases to the study of the binding of newly developed drugs to treat such diseases. This knowledge has already helped in developing new psychiatric drugs and several others are on the anvil. I trust you know that for those who cannot afford a PET scan, there is a hope that study of neurotransmitter receptors on the peripheral blood platelets may provide an indication of their status in the brain. Using biochemical ligand binding technique a group of workers at Lucknow (Sethi, Nag, Dhawan, Seth) have observed that the platelets appear to be a useful target for monitoring Parkinson's disease and Schizophrenia by assaying 3H spiperone and 3H-5HT binding sites respectively. It is thus obvious that with the tools and techniques available to us, it is now possible to explore the molecular basis of neuronal functions and as a consequence the basis of neurological and psychiatric disorders ultimately leading to effective therapeutic strategies. Richard Thomas of Stanford University recently commented that "much of the new biology, of course, is science fiction becoming fact. We are only beginning but the new biology has already contributed substantially to our understanding of life and its complex processes". A report by some distinguished neuroscienctists from National Institutes of Health, USA have stated that 90 percent of what we know about the brain has been learnt during the last decade. Should the clinical postgraduates not be exposed to this knowledge, this excitement?

Let me take just one more example i.e. fetal neural transplant. Attempts to transplant neural tissue into the brain started 100 years ago, some success was already achieved little over 50 years ago but it was only in early 1970's, following the use of fetal neural tissue that Das and Altman, Bjorklund and his colleagues and a few others (See Lectures in Neurobiology Vol. 1) firmly established that a viable, functional graft is possible. Already in 1982 Backlund and his colleagues applied the knowledge gained from such purely basic experimental studies for treatment of human disease-Parkinsonism. Without going into the details, in less than a decade several hundred patients have received various types of neural grafts including fetal substantia nigra. No doubt a great deal needs to be learnt

and worked out before it could be recommended as a safe, effective, reliable mode of therapy, not only for Parkinson's disease but several others, nevertheless it has tantalizing promises. Although by the end of 1990, more than 400 patients have been submitted to this procedure, for it to be routinely used a number of basic neurobiological problems need to be resolved. These relate to developmental biology on one hand e.g. cell maturation, multiplication, neuron-glial interaction, effect of trophic factors, development and growth of dendritic processes, establishment of host-graft neuronal circuitry etc., and those related to immune mechanism like extent of immune privilege of the brain, the role of blood-brain-barrier, the affector and effector immune mechanisms involved. If ultimately it is the responsibility of the neurosurgeon to advise and perform the transplant, is it not necessary that he is familiar with these basic considerations! The basic scientists have already gone ahead. Recognising the practical and ethical problems associated with transplantation of the fetal tissue, they are exploring other alternatives. Thus utilising techniques of tissue culture and genetic engineering, they have engineered autologous fibroblasts to secrete nerve growth factor (attempts to make them secrete a specific neurotransmitter may be on the horizon) which could be transplanted to correct disorders of neurons or promote the growth of their processes.

Many more examples of clinical application of researches in basic neurosciences can be easily provided. However, let us look at from purely clinical standpoint where inspite of phenomenal advances in diagnostic and therapeutic capabilities during the last decade or so (the availability of CT, MRI, SPECT, PET, microscope, bipolar cautery, lasers, ultrasonic suction etc.), we have reached our wits end to improve any further the results of treatment of most of the neurological and psychiatric disorders. I would just refer to a few of those with which neurosurgeons are primarily concerned.

Head injuries constitute a major cause of morbidity and mortality, specially affecting the young. Inspite of all efforts, use of CT for prompt diagnosis, efficient surgical management of compressive hematomas and compound injuries, careful monitoring in intensive care units, administration of drugs and other measures to counteract brain edema, reduce cerebral metabolism, it has not been possible to reduce the mortality of severely head injured patients (Glasgow Coma Scale 8 or below) to less than 30 to 35 percent. It is obvious that advances in diagnostic and surgical techniques are unlikely to improve these results. It has not been so inspite of very aggressive efforts by a large number of competent teams around the world during the last decade or so. One must, therefore look for an answer by improving our understanding by applications of tools of basic science, of the various pathogenetic mechanisms involved, study of biochemical alterations, release of vasoactive substances and free radicals, changes in hemodynamics, alterations in regional metabolism etc.

Tuberculosis of the central nervous system, still a common disease in our country, has been the subject of large number of clinical, radiological, pathological and therapeutic studies. No doubt a great deal has been achieved. Nevertheless early and reliable diagnosis eludes us even today. Application of modern techni-

ques of molecular biology and immunology alone would help in identifying the specific antigen against which suitable immunodiagnostic test can be developed. An intimate interaction between the clinicians and basic scientists is essential to achieve the desired goal.

Gliomas of the brain continue to defy all attempts for a satisfactory therapy. With all the advances in diagnosis, surgical techniques, radio and chemotherapy, there have been only marginal gains in the five year survival rates, and the quality of survival of patients with malignant gliomas. It is obvious that unless the basic biology of these tumors is understood better no dramatic improvement is likely.

Similar situation prevails in respect of most degenerative disorders of the central nervous system, stroke, epilepsy etc. Most advances in relation to these disorders are likely to come from researches in basic sciences. The clinicians, therefore, should not passively await these results. The least one could do is to be aware of the current developments. The intellectual and technical barriers that traditionally existed between basic neurosicentists—the neuroanatomists, neurophysiologists, neurochemists, neuropharmacologists etc.—are breaking down.

While no one denies the need for basic science education for clinicians, there are many practical difficulties. Obviously one cannot expect the already overburdened clinical residents to find time to delve in details of the development in the vast and rapidly extending field of neurobiology. Their service work load, need for attaining a certain standard of knowledge and skills in the field of their specialization, the limited time span of the academic course, make it nearly impossible for them to devote the necessary time required to gain even a superficial familiarity of these allied basic science disciplines. It is, therefore, necessary that those responsible for their training should first decide as to what could be legitimately expected from the trainees. While the principle of "learn while you work" is easily applicable to the professional course, it could not be so, for the basic science component of their training. The vexed question of how much basic science teaching is desirable needs to be carefully discussed and debated by the academic community responsible for the training programmes. Once this is decided the next question will be "by whom". From long experience as a teacher in Neurosurgery, I dare say the existing practices are unsatisfactory. There is need for involving, though not fully depending upon, basic scientists for this purpose. In UK, Europe and USA special courses, like the present one, are organised at regular intervals. It would be desirable to assess the utility of the courses organised by us and decide if these should be continued in future.

Developmental Plan of the Central Nervous System

V. BIJLANI

The central nervous system develops from the surface ectoderm of the primitive embryonic disc in the third week of intrauterine life in man. Based on the evidence of comparative studies on the development of nervous system in lower animals it is presumed that chordamesoderm induces the overlying surface ectoderm to form the neural plate – the forerunner of central nervous system. As the plate grows, its margins become raised forming the neural folds with a longitudinal midline groove between them. The neural folds become prominent at the cranial end and the walls of the groove become expanded. This appears to be the first indication of brain formation. Two transverse constrictions mark the division of this cranial region into prosencephalon, mesencephalon and rhombencephalon.

Neural Tube

The neural groove deepens and the edges approximate forming the neural tube. In the later part of third week the fusion starts in the region of rhombencephalon and extends both cranialwards and caudalwards. A small opening is left at each end of the neural tube. The cranial opening called anterior neuropore closes in the middle of fourth week. In adult brain the lamina terminalis indicates the site of anterior neuropore. Posterior neuropore closes by the end of fourth week (Fig. 1). The neural crest cells which give rise to several structures including dorsal root ganglia etc. lie along the line of fusion of the dorsal edges of the neural plate. The walls of the neural tube are all along bathed by the fluid in the amniotic cavity. The closure of neuropores coincides with the establishment of blood vascular circulation for the neural tube, formation of the choroid plexuses and circulation of CSF. Subsequent to the closure of rostral neuropore gentle

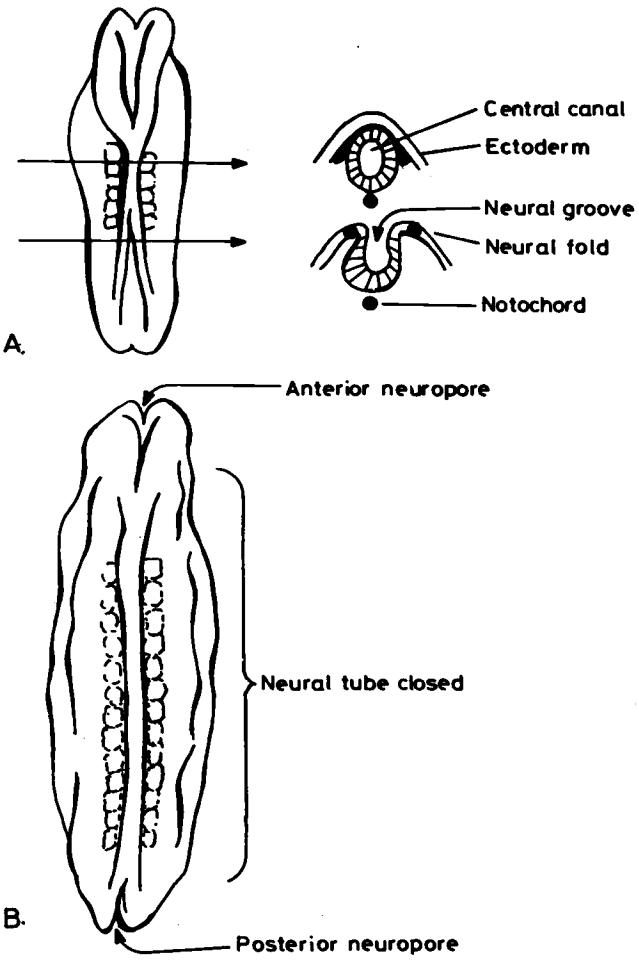

Fig. 1 Formation of neural tube in the third week. Closure of anterior neuropore occurs around 23-25th day (18-20 somite stage). Posterior neuropore closes two days later.

fusiform expansions of three primary vesicles of the brain are recognisable. The hind brain is continuous with the neural tube which forms the spinal cord.

Flexures

With the formation of head-fold of the embryo there is bending of the forebrain vesicle forwards which continues till the forebrain is almost parallel to the hind brain. This bending is called mesencephalic flexure and it makes the midbrain as the most prominent dorsal structure in the embryo at this stage. Soonafter, towards the end of fourth week, the cervical flexure appears at the junction of hind brain and spinal cord. This flexure increases from the 5th to 7th week by which time the hind brain is almost at right angle to the spinal cord. After the 7th week, extension of the head of the embryo occurs causing reduction in the

cervical flexure till it disappears. Cervical and mesencephalic flexures are considered important in shaping the head. Pontine flexure is seen in 4th week at the level of pons and is in the opposite direction to the above mentioned flexures. This flexure continues to become acute till its cranial (metencephalic) and caudal (myelencephalic) slopes oppose each other. This happens by about 8th week of intrauterine life. In this process the roof of pons stretches and becomes very thin.

Spinal Cord

The primitive neural tube is lined by single layer of columnar neuroepithelial cells. This cell layer undergoes proliferation and gives rise to the wall of the tube which can be histologically distinguished to be composed of the ventricular, intermediate and marginal zones from inside-outwards. The ventricular zone consists of cells which proliferate and the intermediate zone contains cells which have migrated out with some in certain areas undergoing a second bout of proliferation. In the adult spinal cord this histological pattern of three zones is retained with the marginal zone representing the ascending and descending tracts of the white matter. The lumen of neural tube is the future central canal of the spinal cord. At first the lumen is narrow and slit-like, as the lateral walls thicken the lumen widens in its dorsal part and becomes diamond shaped in a cross-section. By 20 weeks of gestation it appears circular (Rath *et al* 1984). Widening of the canal is associated with the identification of sulcus limitans on each side dividing the ependymal and mantle layers in each lateral wall into a basal and an alar lamina. The cells in the basal lamina develop into motor cells while those of the alar lamina mostly form interneurons. By using combined Golgi-electronmicroscopic method, Wadhwa *et al* (1986) have shown symmetric and asymmetric synaptic profiles on a single interneuron in 16-17 weeks old foetus. Rath (1982) has described identification of groups of neurons in the ventral horn at 10 weeks. By 26 weeks these groups were comparable to the adult in their topology. Bijlani *et al* (1990) reported development of dorsal grey in the cervical region of 60 foetuses ranging in age from 8 to 37 weeks of gestation. Rexed's laminae were identifiable at 13-14 weeks and the dorsal grey acquired an adult shape by 30 weeks of gestation. According to these investigators the neurons in the deeper laminae developed first followed by the neurons in lamina I. The neurons in the substantia gelatinosa were the last to mature. They have correlated the differentiation of the laminae, neurons and dendritic arborization with the growth of afferent trajectory. Rizvi *et al* (1986) have described in detail the synaptogenesis in the lamina I of the dorsal grey. Bijlani *et al* (1988) reported the appearance of substance P, GABA, enkephalin and serotonin in the dorsal horn of spinal cord at the different foetal ages in man.

In the caudal region description of the development of spinal cord is controversial. Lemire (1975) has discussed secondary caudal neural tube formation. The notochord in contact with the caudal end of the neural tube merges with the tube and together they undergo transformation with the appearance of multiple vacuoles. The final stage of caudal part of neural tube formation consists of degeneration and then reorganization to form the adult structure. There is

complicated interaction with nearby mesonephric and hind gut derivatives. These observations are significant for the understanding of congenital defects associated with spina-bifida. It is well known that there is a high incidence of urogenital defects in cases of lumbo-sacral meningomyeloceles, and congenital absence of sacrum and coccyx. The central canal caudally seen in adult as a fusiform dilation is called terminal ventricle.

In the third month of intrauterine life spinal cord extends the entire length of the embryo and the nerves pass out through the intervertebral foramina at their levels of origin. Rath *et al* (1984) have described the measurements of the cervical enlargement, lumbar enlargement and total length of the spinal cord in twenty-eight human foetuses of 8 to 26 weeks of gestational age. With increasing age the terminal end of spinal cord gradually shifts and at birth is located at the level of third lumbar vertebra. Finally in adults it terminates at the upper border of second lumbar vertebra. The cervical and lumbar enlargements are first seen with the development of respective limb buds in the second month. The spinal cord at this age is about 2.5 cm long. The cervical enlargement measures 0.5 cm and the lumbar enlargement 0.3 cm.

Failure of closure of neural tube may be due to several reasons. The defect may occur due to failure of primary closure, due to overgrowth of neural tissue or hyperplasia and focal necrosis. There may be rupture of the neural tube due to improper flow of CSF in the canal. All these abnormalities may result in what are grouped as "dysraphic states" resulting in craniorachischisis, anencephaly and meningomyelocoele etc. These observations are based on the examination of abnormalities seen in aborted foetuses. Abnormal angiogenesis of the cranial vessels is reported to be another cause of the dysraphic states. Abnormal secondary caudal neural tube formation is related to the occurrence of diastematomyelia, diplomyelia, sacral agenesis and a wide spectrum of pathology presenting skin covered caudal spine masses in infancy and childhood including meningocoele, teratomas, lumbosacral lipomas and pilonidal cysts.

Medulla, Cerebellum, Pons

In the fourth week of gestation when the mesencephalic flexure is seen, the length of rhombencephalon exceeds that of entire brain and it lies between the isthmus rhombencephali and the first cervical nerve. Pontine flexure divides it into metencephalon and myelencephalon. Myelencephalon develops into the medulla oblongata. The caudal part of myelencephalon develops like the spinal cord. The flexure stretches the thin roof. The lateral walls are separated and the canal of the neural tube becomes rhomboid shaped with the lateral angles forming lateral recesses of the fourth ventricle. In the caudal part the gracile and cuneate nuclei develop from the dorsolateral laminae. At about 4 months of age descending corticospinal pathways are seen to invade the medulla to form the pyramids ventrally. On the dorsal side ascending fibres from the spinal cord olivary nuclei, vestibular nuclei form the inferior cerebellar peduncle. On the whole the lateral walls of this region differentiate into dorsolateral and

ventrolateral laminae. The ventrolateral laminae differentiate into three elongated but interrupted columns (Fig. 2A). Most ventral (medial) column is con-

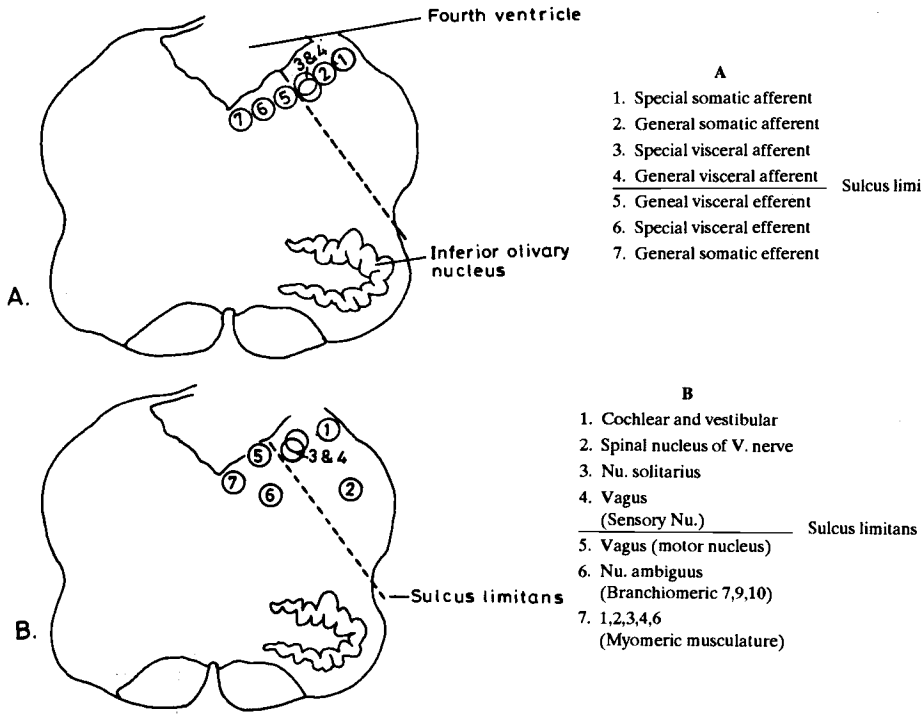

Fig. 2 Schematic diagram showing disposition (A) and displacement (B) of cell columns giving rise to cranial nerve nuclei. Names of the columns and cranial nerve nuclei are given against the numerical figures.

tinuous with the ventral grey of spinal cord and is represented in the brain stem by hypoglossal, abducent, trochlear and oculomotor nuclei. The intermediate column differentiates into nucleus ambiguus (glossopharyngeal, vagus, accessory nerves). It is continuous in the spinal cord as the spinal accessory nerve. The lateral grey of the spinal cord is represented in the brain stem as dorsal nucleus of vagus and salivatory nuclei. Although it appears that the functional columns in the brain stem formed by the cranial nerve nuclei are a continuation of the spinal grey but some of these nuclei migrate during development and get displaced by differential growth patterns (Fig. 2B). A well known example is seen in the movement of facial nerve nucleus which in 10 mm embryo (age 5-6 weeks) lies in the floor of fourth ventricle. Its change in position has been described to occur caudally then dorsally and finally to the ventral part at the pontomedullary junction in adult. Nucleus ambiguus is also initially immediately deep to the ventricular floor and is displaced more ventrally in adult. Cells of the inferior rhombic lip migrate out actively into the marginal zone of ventrolateral lamina burying the oval bundle of tractus solitarius. Dorsolateral lamina rhombic lip cell migration gives rise to arcuate nuclei, olivary nuclei and scattered nuclei pontis. These neurons also form corpus pontobulbare.

The superior rhombic lips are at first parallel to each other, subsequent to flexure formation they become oblique with upper ends coming close to each other. Later when the cranial and caudal parts of rhombencephalon approximate the laminae become horizontal. These form the analage of cerebellum. During the second month the superior rhombic lips thicken and bulge in the fourth ventricular cavity (Fig. 3A). In the third month, the cerebellar mass everts out, fusion occurs forming the vermis and the posterolateral fissure is identifiable (Fig. 3B). Fissura prima appears later towards the end of third month. The cerebellar cortex grows faster than the deeper layers between 3 to 5 months and it attains the final configuration of folia etc. by 7th month. Histologically detailed descriptions are available about the development of cerebellar cortex and it has been a favourite model to study the normal and abnormal neural developmental processes (Deo *et al* 1978, Bijlani *et al* 1980, Mishra *et al* 1983). In man the cerebellar cortex has two layers from 3 to 8 weeks, three layers at 10-11 weeks and five layers by 20-21 weeks. Five layer stage is characteristic of human. The lamina dessicans appears to be a significant layer during development. The adult pattern of three layers is established by second postnatal year (Rakic and Sidman 1971). The primordium for dentate nucleus appears at 8 weeks and the other nuclei are seen around 14 weeks (Wadhwa *et al* 1985). The dorsal part of the dentate nucleus differentiates earlier than the ventral part. Development of dentate nucleus, gyration, change in position and neuronal differentiation have been studied in detail in our laboratory (Hayaran *et al* 1991).

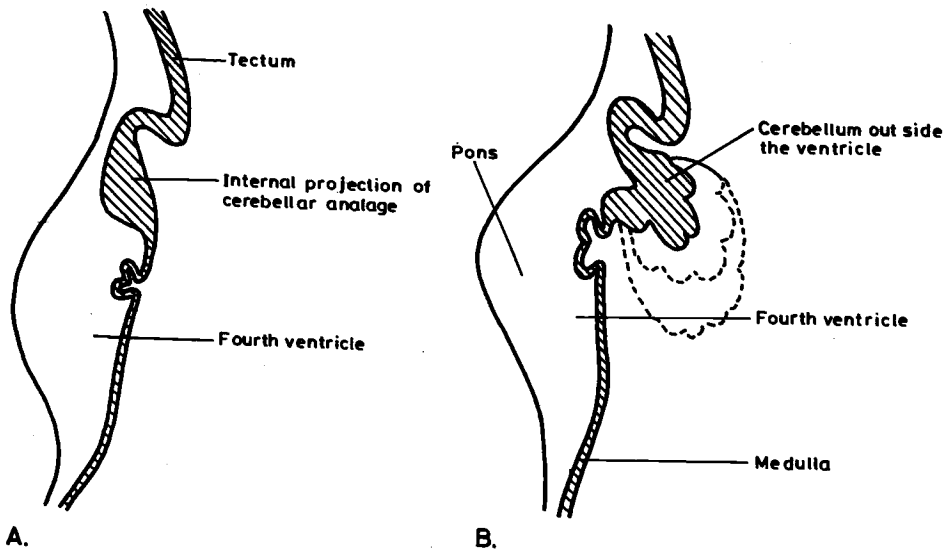

Fig. 3 Growth of cerebellum in relation to fourth ventricle.

Midbrain

Ventrolateral laminae of the midbrain enlarge rapidly after fourth month and form the adult cerebral peduncles. Oculomotor, trochlear and mesencephalic nucleus of trigeminal nerve also develop from this lamina. There is a rostral migration of trochlear and mesencephalic nucleus due to developmental change at the isthmus of rhombencephalon. The dorsolateral laminae proliferate and form the tectum which in the adult are seen as corpora quadrigemina. The red nucleus, substantia nigra and tegmentum are identifiable by the end of third month and probably arise from ventro- and dorsolateral laminae. Development of the tectum has been extensively investigated in several experimental models. Neurobiological studies illustrating chemospecificity, effects of environment, behaviour etc. have been carried out. The region of isthmus undergoes changes which are rather hard to interpret. The greater part becomes absorbed into the caudal end of midbrain, roof plate is the site of formation of anterior medullary velum and the dorsolateral laminae form superior cerebellar peduncles. Trochlear nucleus and decussation are displaced rostrally.

Several clinical syndromes have been identified due to early failure of formation and proper orientation of cranial nerve nuclei. In Mobius syndrome the cranial nerve nuclei are dysplastic and absent. Cases of cerebellar agenesis or severe hypoplasia have been described. Subtle loss of cerebellar tissue is a common finding on autopsy in patients not suspected of neurological disease. Experimen-

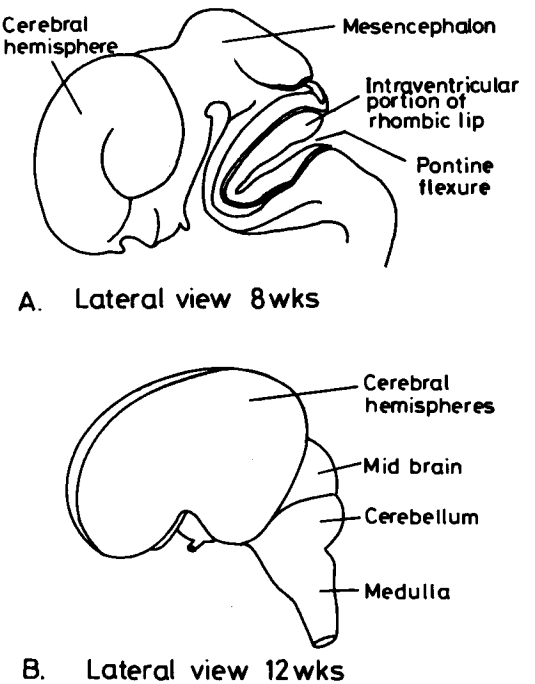

Fig. 4 Schematic diagram to show development of brain.

tal studies have demonstrated genetic and nutritional factors causing abnormal layering of cerebellar hemispheres in experimental animals (Deo *et al* 1978, Mishra *et al* 1983). This must be occurring in humans also but enough work has not been done in examining histopathologically the cerebellum from postmortem specimens. The cystic dilation of the fourth ventricle in Dandy-Walker syndrome has been attributed to the failure of regression of posterior medullary velum. Underdevelopment of vermis and heterotopias have been reported in several malformations. Beaking of quadrigeminal plate has also been reported along with other defects in Arnold-Chiari malformations.

Cerebral Hemispheres

The forebrain divides into prosencephalon and diencephalon. These regions have the same differentiation at early stages as the spinal cord – thin roof and floor plates, and thick lateral walls divided into dorsal and ventral areas by a sulcus. The sulcus is analogous to the sulcus limitans and is represented in the adult brain by hypothalamic sulcus extending from the interventricular foramen to the aqueduct. The part of diencephalon dorsal to the sulcus develops into thalamus, metathalamus and epithalamus. Ventral to the sulcus the lateral wall of diencephalon forms hypothalamus and subthalamus. In the beginning the lateral aspect of the thalamus is separated from the medial side of developing cerebral hemisphere by a cleft which is obliterated later and corticofugal and corticopetal fibres of the thalamus become related to internal capsule. The optic vesicles arise from the lateral wall of prosencephalon prior to the differentiation of telencephalic vesicle. Two diverticula expand from the primitive forebrain rostrolateral to the optic stalk and give rise to cerebral hemispheres which are formed at the beginning of the 5th week. By middle of second month the basal part begins to increase and bulge into the lumen of the lateral ventricle. This part appears striated in a transverse section and is called corpus striatum. The hemisphere gradually expands covering the lateral aspect of diencephalon, mesencephalon and cranial part of metencephalon. The caudate nucleus and thalamus come into close contact with the medial wall of hemisphere; the lateral aspect of thalamus is in continuity with medial aspect of corpus striatum and a secondary union between the diencephalon and telencephalon occurs providing a route for passage of fibres to and fro to cerebral cortex. The continuous growth of cerebral hemispheres in anterior, dorsal and inferior directions results in the formation of frontal, temporal and occipital lobes. The corpus callosum appears during tenth week of development as a small bundle in the lamina terminalis. With the expansion of cerebral hemispheres it extends over the roof of the diencephalon. The anterior commissure is the first one to appear followed by hippocampal commissure called fornix. These commissures initially pass through the lamina terminalis.

Studies on the histogensis of cerebral cortex have revealed certain interesting features. The neurons arising first in the neuroepithelial layer are the earliest to migrate and form deeper layers. Neurons born later bypass the deeper layers to give rise to superficial layers (Berry and Rogers 1965). The molecular cues for

this "inside out" layering of cortex are not understood. Rakic (1988) has described the migration process in electronmicroscopic preparations, and has discussed the formation of ontogenetic columns.

Abnormalities of histogenesis of cerebral cortex results in lissencephaly, pachygyria, ectopias etc.

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Cell-Adhesion Molecules and their Mechanism of Action

GOMATHY GOPINATH

Introduction

Three major steps in the development of the nervous system are (1) cell multiplication (2) cell migration and (3) differentiation.

Cell multiplication takes place at the neuropithelial lining of the central canal of the neural tube. Cells make and break contacts during mitosis. The daughter cells destined to become neurons (neuroblasts) migrate to the final position in the nervous system.

Migration of neurons is an important event during the development of the nervous system for spatial positioning of the neurons of same or similar functions. In the central nervous system neuronal migration to a large extent is along the radial glial cells. Neural crest cells in the periphery migrate to form ganglia with the help of peripheral tissues along the way.

Differentiation is the process by which specific neuronal aggregations are formed and connections are established with the appropriate target areas according to the functions: what is called cell sorting and synaptic connectivity. For connections neural processes travel varying distances in the CNS or in the periphery.

Thus it can be seen that there is considerable mobility of cell and its processes during cell migration and differentiation. These events overlap; but each event proceeds according to strictly defined timing and kinetics so that a complex neuronal network with a high degree of synaptic specificity is established. Thus functionally distinct neurons exhibit stereotyped anatomic locations and extension of axons to prospective targets over novel terrains. All these highly specific developmental processes, require diversity of cellular interactions with other cells or substratum.

Cell adhesion molecules are cell surface glycoproteins that play important roles in organogenesis and in defining territories of discrete cell populations. Specific adhesion molecules are important for regulation of cell motion and adhesion that lead to pattern formation. Migration, cell sorting and synaptic connectivity are regulated by an additional group of molecules – the extracellular matrix molecules.

Cellular interactions during development can be divided into two general classes: those which occur at a distance mediated by diffusible molecules and those as a consequence of direct cell contacts through cell surface molecules. Contacts between cells can mediate two types of events. The behaviour of the cell may alter in the absence of any strengthening in their physical association. This could be alteration in the differentiation programme, cell mobility or orientation. On the other hand the contact site may get stabilized with the adhesion of the interacting cells leading to a long range of intimate cellular interactions.

An impressive amount of literature is available on the adhesion molecules. A large number of such molecules have been identified and a bewildering array of names are attributed to them. Adhesion molecules undergo developmental changes in their distribution which support the increasingly held belief that these molecules play important roles in neural development and regeneration. As the molecular identities, localization and functions became better known, these molecules were divided into two groups, cell surface molecules which mediate cell-cell interaction (CAMs) and extracellular molecules mediating cell-substratum interaction. Under each group a number of molecules are identified according to the structure and binding qualities.

Cell-Cell Adhesion Molecules (CAMs)

Cell adhesion molecules can be categorised in two groups: general CAMs and restricted CAMs. General CAMs are seen on cells of many tissues including neural tissue. These are calcium independent CAMs which include NCAMs (neuronal CAMs) and NgCAMs (neuron-glia CAMs) and a number of calcium dependent CAMs called N-cadherins. The latter are resistant to proteolytic digestion in the presence of calcium ions. Restricted CAMs are found in distinct tissue sub-regions and are likely to mediate more specialised and localised functions. Molecules like myelin-associated glycoproteins (MAG) and axon-associated cell adhesion molecules (AxCAMs) belong to this category. There is likely to be some overlap between molecules of these two classes. General CAMs may take over more restricted functions whereas restricted CAMs are less likely to do the reverse.

Distribution of CAMs in the nervous system

NCAM is distributed in substantial amount in the neural plate. After neurulation both N-cadherins and NCAMs are expressed uniformly in the neural tube and in the adjacent somite mesoderm. Because of their global distribution in the nervous system these molecules are termed as general "glue" to be used as and

when required. Radial glial cells also express NCAM. After differentiation motor neurons, motor axons, spinal cord and dorsal root ganglia are associated with high levels of NCAM.

Developing brain contains both high and low forms of NCAM depending on the carbohydrate moiety. Binding capacity of the molecule is thought to be determined by the carbohydrate moiety. The low carbohydrate form (L form) plentiful in the early embryo probably stabilizes the neuroepithelial stage. Subsequent rise in the high carbohydrate form (H form) may help in destabilization of the contacts and thus help migration of cells. Rise once again in L form in adults may help stabilize neuronal localization and circuitry.

NgCAM, termed as L1 in rat is expressed mainly on postmitotic central neurons as well as on neurons and Schwann cells in the periphery. NCAM is also expressed by these cells, as also some neural tumours.

N-cadherins show almost the same distribution as NCAM. N-cadherins are concentrated towards the terminal or ventricular surface of the neuroepithelial cells, whereas NCAMs are located towards the outer region. High concentration of N-cadherins is seen at morphological cell junctions. This may be directly or indirectly associated with cytoskeleton.

In the peripheral nervous system the fasciculating and non-myelinating Schwann cells are NCAM and NgCAM positive at the time of contact; NgCAM disappears from both axons and Schwann cells as soon as myelination starts. NCAM expression also declines as the myelination proceeds. As NgCAM declines MAG starts appearing at the axon-Schwann cell interface and also at the apposing membrane surface of the loops forming myelin. MAG later disappears from the compact myelin and in its place myelin basic protein (MBP) becomes evident.

MAG is observed in Schmidt-Lantermann incisures, outer and inner mesaxon and paranodal loops. MAG has also been seen to mediate neuron-oligodendrocyte interaction and oligodendrocyte – oligodendrocyte adhesion. Co-existence of different AxCAMs are demonstrated on the same axon. Some of them occupy distinct domains.

Structure of cell surface molecules

CAMs are glycoproteins with considerable heterogeneity in both protein and carbohydrate moieties. Most of the CAMs manifest structural similarities. These have intracellular, transmembrane and extracellular domains (Fig. 1). Intracellular domains consist of proteins of different sizes depending on the form of NCAM. Intracellular domain is large in 180 KD-NCAM and smaller in 140 KD-NCAM. The 120 KD form has no intracellular or transmembrane domain and is anchored to the membrane by a covalent link. The transmembrane protein is inserted into the lipid bilayer. The extracellular domain has both polypeptide and carbohydrate in the form of polysialic acids. 30% sialic acid is present in the high carbohydrate form of NCAM whereas only 15% in the low carbohydrate form. Sialic acids are more in volume than the polypeptide and occupy the hydrated region around the polypeptide and thus may put constraints

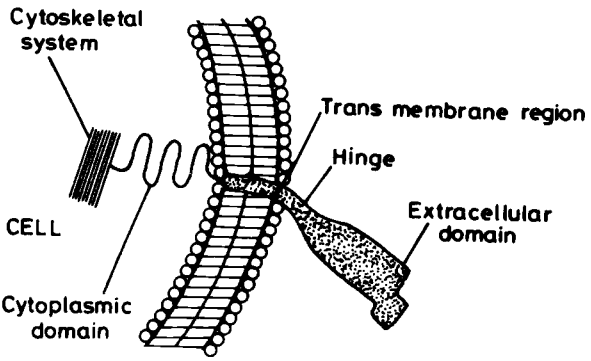

Fig. 1 Diagrammatic illustration of a cell-surface molecule. Different domains are labelled. Transmembrane region lies across the cell membrane. Hinge is reported to be the mobile part of the molecule.

on adhesive capability. Immunoglobulin domains which are constant can be seen in relation to the extracellular domain.

Ca+2 dependent N-Cadherins lack immunoglobulin like domains, but show similarity to other Ca+2 dependent molecules of non-neural tissue.

MAGs have a common carbohydrate epitope with NCAM and NgCAM. Molecular variations of MAG exist. Their intracellular domains are shorter. MAG and NgCAM are soluble in the absence of detergents, a feature not encountered with integral membrane proteins. This feature supports the observation of detecting NCAM, NgCAM and MAG in the extracellular matrix. AxCAMs are structurally distinct glycoproteins of 130-190 KD. These are seen to the released from membranes alongwith the neurotransmitter release following stimulation. So MAGs are also seen in soluble and membrane bound forms.

Binding of cell surface molecules

In vitro experiments show that cells expressing various CAMs tend to sort out from one another aggregates expressing a particular CAM. This segregation depends on the specificity and quantity of each CAM expressed on the cell surface. Further, interaction of a particular CAM with the cytoskeleton also must be playing a major role in the cell sorting and segregation. It is logical to assume that a relatively large number of CAMs closely situated at a given region of the cell surface and stabilized by cytoskeletal attachments, are necessary for effective cell adhesion. Thus specificity of surface molecules and their concentration and freedom of the hinge regions, especially in cells where the cell membrane is rapidly changing shape, the type of cytoplasmic domain and its specific interaction with the cytoskeletal components and the readjustment of all the cell components as cell process extension and movements occur, are all factors involved in binding and subsequent sorting of the cells.

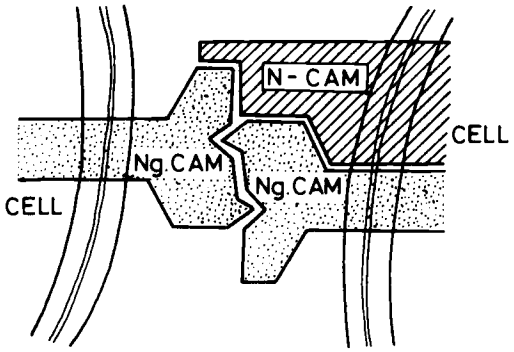

Fig. 2 Diagram to illustrate homophilic and heterophilic bindings of N-CAM and Ng-CAM molecules and also multiple binding of the molecules.

Considering the variety of cellular interactions encountered in the nervous system, the nature of adhesions must be of different type. Thus it is possible that adhesion molecules act either individually or in conjunction leading to a concomitant change in the adhesive properties. This may allow different combinations and economic use of the molecules.

Cellular receptors primarily involved in binding some of the adhesion molecules are already identified. Accordingly it is seen that some molecules are homophilic in their binding interaction (same molecules), whereas some heterophilic (between different molecules) and a few show both homophilic and heterophilic binding property. NCAM, NgCAM and N-Cadherins have homophilic binding property. NgCAM expresses homophilic binding mechanism and also to NCAM, a heterophilic property. It appears that NCAM in this instance assists the homophilic interaction between NgCAMs (Fig. 2). It is shown that NgCAM binds homophilically to neurons and heterophilically to astroglia.

N-Cadherin binding is mostly homophilic, but a few studies indicate that binding could take place between different cadherin molecules.

So far the receptor for MAG has not been identified.

An interesting point has emerged from the *in vitro* studies of retinal cells. It is shown that the retinal cell – NCAMs must interact with a heparan sulfate proteglycan on neuronal surface for cell adhesion to occur. Proteoglycans are a large class of glycoproteins present on the surface of any cell. Probably binding to any of the proteoglycans may bring about the conformational changes in the NCAM, facilitating the homophilic bindings. Conformational change in the extracellular molecule, fibronectin, has been partially proved.

Suggested functional roles of cell surface molecules

NCAMs – Junctional communication in the neural plate is mediated by NCAMs. NCAMs are implicated in the stereotyped migration of neurons to specific regions. Migration of granule cells from the external granular layer to

the internal granular layer in the developing cerebelllum can be cited as an example. These molecules also have a role in holding the axons in fascicles and in guiding each axon to its specific destination. An example could be axons from specific region of retina reaching and making contact with a particular part of the tectum. Establishment of neuromuscular contact seems to be also influenced by NCAMs as both axons and muscle cells express NCAMs during the developmental stage.

N-Cadherins-These molecules help in closing of neural plate and the retina. They probably mediate separation of neural tube from the N-cadherin-negative ectoderm. Presence of N-cadherins on growing sensory fibres indicates that these may help in extension of these fibres.

AxCAMs-These may have the same type of functions as N-Cadherins; like elongation and growth of axons on pre-existing fascicles. These molecules also help in distinct and stereotyped patterns of fasciculation.

Extracellular matrix molecules

Diffusible molecules have a major role in directing the neuronal processes to their distant targets. Laminin and fibronectin are seen to promote neurite outgrowth through neuron-substratum interaction. The others identified are tenascin or cytotactin or Jl and thrombospondin. All these were first identified *in vitro* and the antibodies against them were used to localise these in the brain. Laminin and fibronectin are seen in a number of restricted areas in the CNS during development, whereas they have a wider and more concentrated distribution in the peripheral nervous system.

Tenascin is identified during embryogenesis. It has an unusual site-restricted distribution and is thought to be involved in neuron-glia interaction. It appears to be made up of multiple polypeptides with HNK-1 carbohydrate antigenic determinant. Just as the other extracellular molecules, tenascin also binds specifically to proteoglycans. It is noted to have a role in cell rounding and migration. There may be alteration in the molecular form and distribution during development.

Thrombospondin, a large glycoprotein is synthesised by a variety of peripheral cells and glia. The wide spread synthesis, the role in adhesion and control of proliferation and the rapid incorporation and removal from the extracellular matrix, all suggest that thrombospondin is a major extracellular component in the regulation of development. Thrombospondin is highly positive around neuroepithelial cells during neural tube formation, and around growing axons from neuroepithelium and ventral motor neurons. It is also seen to have a role in the migration and aggregation of neural crest cells.

On certain occasions many of these extracellular molecules can be localised on the cell surface, just as some NCAMs can be identified in the extracellular space. Schwann cells show surface laminin, fibroblasts fibronectin and glial cells tenascin and thrombospondin. Their presence on the cell surface may reflect their association with cell surface receptors. However, many of the cells which syn-

thesise or bind these molecules are unable to express them on the cell surface normally. It may be that the cells control the maintenance of these molecules on the surface according to the functional necessity.

Binding of extracellular molecules

Most of the extracellular molecules have heterophilic binding property, mainly to the proteoglycans on the cell surface. Laminin and fibronectin also bind homophilically to the respective molecules on the cell surface. The extracellular molecules interact with cell surface through a group of cell surface receptors known as integrins. These have two non-covalently associated transmembrane polypeptides; alpha and beta. Binding is of low affinity and hence multiple binding seems to be required. The ability to bind to one or more specific extracellular molecules is determined by the type of alpha/beta combination in the integrins. Evidence suggests that integrins are involved in mediating the effects of laminin and fibronectin on neuronal attachment and neurite out-growth. Many of them also bind to proteoglycans or polysaccharides derived from them such as heparin, heparan sulfate, chondroitin sulfate or dermatan sulfate. This type of binding is supposed to stabilize the extracellular matrix molecules.

Consequence of cell-cell or cell-substrate interactions

It is seen that many of the cell surface molecules are intimately related to the cytoskeleton. Further the molecules bind to more than one receptors.

Cell interaction say set up conformational changes that could potentially transmit signals to cell interior affecting the gene expressions and second messenger system resulting in reorganization of the cytoskeletal network. Organization of the cytoskeletal network is different in stationary and migrating cells and hence the reorganization may facilitate redistribution of cell surface molecules for motility. Production of biosynthetic enzymes of neurotransmitters is seen to be enhanced following laminin-heparin binding on chromaffin cells.

Gene localisation of cell surface molecule

In man NCAM gene has been localised on band q23 of chromosome number 11 near the thy-1 locus. In mouse the gene has been seen linked to thy-1 in chromosome 9. Each type of NCAM has its own mRNA.

An overview

Though considerable details are already available on cell-cell and cell-substratum interactions, a lot still remains to be learnt about the synthesis, structure, localisation and function of the molecules involved. Even the details presently available are complex and confusing due to the overlapping structure and binding properties of the molecules. Recognition of genes and mRNAs which regulate these and molecules may throw more light on the cell-cell and cell-substratum interactions during development and repair in the nervous system.

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Extracellular molecules

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Cytoskeletal Proteins in the Development and Disease of the Nervous System

T.R. RAJU

Different types of eukaryotic cells have distinct shapes and a high degree of internal organisation. They are capable of undergoing changes in shapes and have the ability to migrate from point A to point B. The cells also exhibit surface movements of the membrane, extension and retraction of microvilli etc. Within the cells the organelles migrate from one place to another. These properties of shape, internal organisation and movement depend on complex network of protein filaments which fill the entire cytoplasm and form the 'bone and muscle' of the eukaryotic cell. Accordingly these filaments together are called the cell's cytoskeleton (Alberts *et al* 1983).

Composition of cytoskeleton

Cytoskeleton in any eukaryotic cell is composed of three elements namely microfilaments (5-7 nm) intermediate filaments (10 nm) and microtubules (24 nm) (Bray and Gilbert, 1981). While microfilaments and microtubules are basically made up of same proteins in different cell types, intermediate filaments, although morphologically similar, are made up of different proteins depending on the cell type.

Microfilaments are composed of actin; several actin binding proteins are also present in these filaments. Intermediate filaments are composed of neurofilament (NF), triplet peptides (neurons), glial fibrillary acidic protein (GFAP) (astrocytes), keratin (epithelial cells), vimentin (mesenchymal cells and immature astroglia, neurons) and desmin (muscle) (Franke *et al* 1978). Antibodies against various purified intermediate filament proteins are useful in identifying

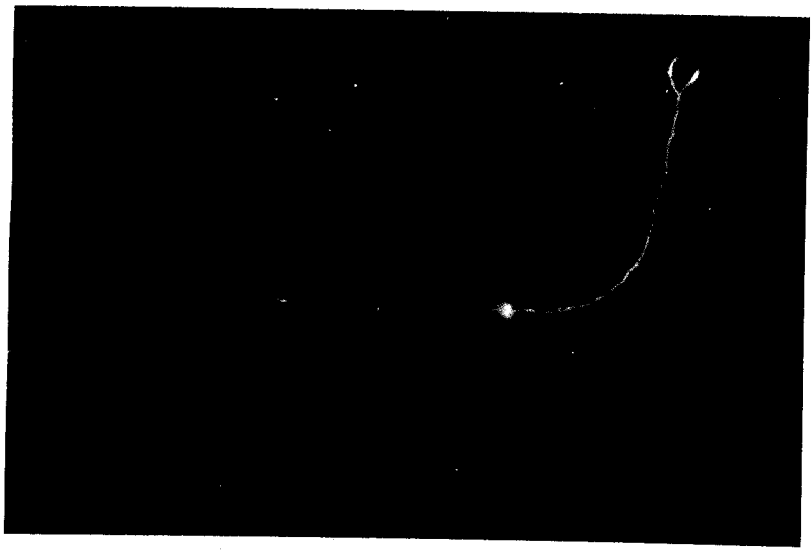


Fig. 1 Immunofluorescence micrograph of a cultured neuron labeled with antibody against NF peptides. Note the soma as well as the processes of the neuron labeled with this antibody X 320.

different cell types (Figs. 1 and 2). Development and differentiation of neurons and astrocytes have been studied both *in vitro* and *in vivo* using antibodies against

Fig. 2 Immunofluorescence micrograph of cultured astrocytes labeled with antibody against glial fibrillary acidic protein (GFAP), which forms the intermediate filaments in these cells X 480.

NF and glial filament proteins (Raju *et al* 1981). Microtubules are basically polymers of alpha and beta tubulin; several microtubule associated proteins (MAPS) as well as "Tau" are also present along with tubulin.

Role of cytoskeleton in neurite growth

Cytoskeletal role in neurite growth has been studied in cultured neurons. Neurons initially appear as spherical round cells. Due to polymerization of actin filaments several membranous projections called microspikes are generated by the neurons. There are several microtubule organizing centres throughout the cytoplasm.

Then one of the microvillus process extends further to form a presumptive neurite. Microtubule organizing centres migrate towards the base of the newly formed neurite and conglomerate together. Then tubulin starts polymerizing and extends along the length of the neurite. Microtubule associated proteins such as MAP 2 stabilises the newly formed tubules. NFs are the last cytoskeletal structures to enter the neurites. A comparison of the ultrastructure of 1-d and 29-d postnatal optic nerve reveals that while NFs are sparse in 1-d axon, the 29-d axon is filled with NFs cross linked to each other (Hirokawa *et al* 1984). In axons NFs are evenly spaced with numerous cross bridges; however they are tightly packed in dendrites.

Microtubules and their role in developmental plasticity of the nervous system

Microtubules formed by the polymerization of tubulin are not only important for the structural integrity of neurons, but also play a crucial role in the axoplasmic transport of materials (Smith *et al* 1975). Neuronal process can exist without NFs but microtubules are extremely important for neuronal stability. Although microtubules formed by the polymerization of tubulin alone are very sensitive to cold temperatures and drugs like colcemid, microtubules which are present in neuronal processes apart from those in the growth cones are resistant to the treatment (Bamburg *et al* 1981). In Purkinje cell dendrite of rat cerebellum at postnatal day 10, less than 25% of microtubules were cold stable but by 35th day over 90% of microtubules were resistant to cold treatment, indicating that there is a striking increase in the stability of neuronal microtubules during this period (Faivre *et al* 1985). This would mean that the overall neurite structure which accommodates to the external forces that mould axonal and dendritic morphology during early phase of development becomes more fixed and less accommodating once the development is over (Matus 1987). These results also focus attention on microtubules as a key element in the regulation of neuronal plasticity during development. Less stable microtubules would result in neurons capable of more growth, forming more synaptic contacts as well as elimination of unwanted synapses. Stable microtubules will result in a mature established process and synapses. The stability of microtubules in neuronal process is brought about by several proteins which are associated with microtubules known as "Microtubule Associated Proteins" (MAPS) and "Tau".

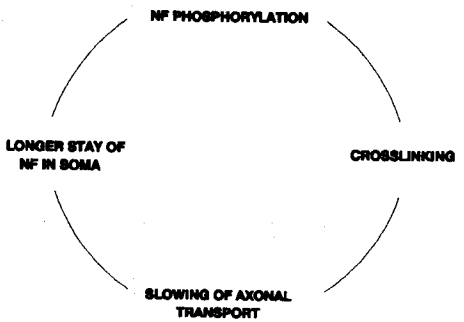
Microtubule associated proteins

As the name implies these proteins are associated with microtubules and they fall into two categories namely 'MAPS' and 'Tau'. MAPS are composed of heterogeneous group of proteins which are termed as MAP1-MAP5 (Matus, 1988). There is a regional specificity in the distribution of certain types of microtubule associated proteins. For example MAP2 is present in the cell body and dendrites in mature neurons whereas 'Tau' is exclusively present in the axon. However in immature neuron MAP2 is present both in dendrites and axons. Neurotrophic factors and gangliosides induce the synthesis of MAP2 (Brugg and Matus 1988, Ferreira *et al* 1990). These proteins function as regulators of microtubule assembly through promoting tubulin polymerization and later stabilizing the tubules formed. Microtubule associated proteins also crosslink microtubules with other cytoskeletal elements.

Cytoskeleton in nervous system disease

Cytoskeletal pathology involving NFs in the form of neurofibrillary tangles is commonly seen in Amyotropic Lateral Sclerosis, Parkinsonism, dementia of Guam and Alzheimer's disease (for a review see Schlaeppfer 1987). Experimental injection of aluminium intrathecally into rabbit results in the formation of neurofibrillary tangles (Bizzi *et al* 1984). Translation of NF mRNA into NF peptides occurs in the cell soma where they are also assembled into filaments. The phosphorylation of NFs occurs when they enter into the axon. They are then transported down the axon to the terminal region where entry of calcium results in the activation of neutral proteases like calpain which degrade the NFs. Catabolised materials from NF are retrogradely transported back to soma (Fig. 3). Phosphorylation of NF peptides leads to crosslinking of NF with cytoskeletal elements.

The neurofibrillary tangle starts off with excessive phosphorylation of NF in the cell soma region. Extensive crosslinking amongst NF and with other cytoskeletal elements lead to slowing down of NF transport. The longer the NF stays in the soma, the more phosphorylated it gets, and thus a vicious cycle develops as follows:



This condition results in massive accumulation of NF in the soma virtually choking the neuron and impairing its function. Agents like aluminium may act

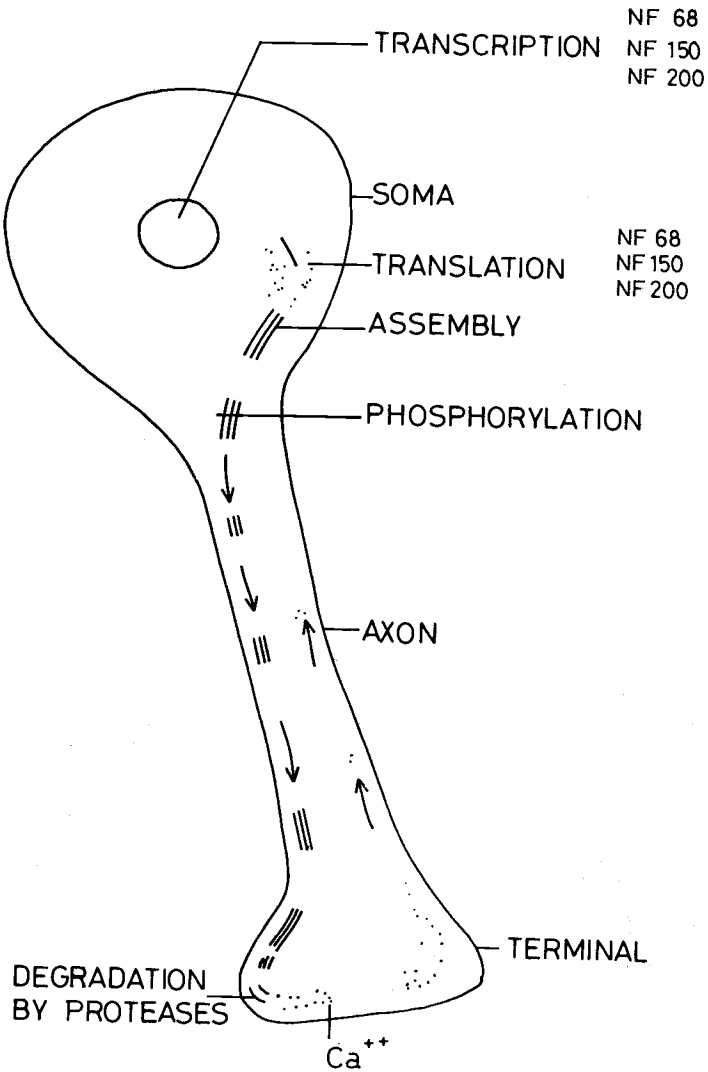


Fig. 3 Schematic diagram showing the retrograde transport of catabolised neurofilaments back to the soma.

by direct inhibition of calpain and also by binding to calpain active sites on NF leading to stearic inhibition. This results in reduced proteolysis of NF causing its accumulation within the neuron (Nixon *et al* 1990). The neurofibrillary tangles seen in various neurodegenerative diseases are by themselves probably not the underlying cause of a given neurological disease. NF pathology may be just an epiphenomenon reflecting on some biochemical abnormalities associated with NF metabolism commonly occurring in several neurodegenerative diseases.

While accumulation of NF results in neurofibrillary tangles, depolymerization of NF in axons also results in certain types of pathology. The calcium activated

proteases are not active in the cell soma or axon since free calcium level is low. However due to neuronal membrane damage free calcium can be increased in these regions. This would activate the proteases leading to the proteolysis of NF resulting in the collapse of axon as seen in Wallerian degeneration and acute axonal degeneration in several neuropathies. Phosphorylated "Tau" also forms a major component of the neurofibrillary tangle of Alzheimer's disease. Abnormal phosphorylation of 'Tau' may disrupt the neuronal cytoskeleton contributing to cell injury and death (Baum *et al* 1990).

Cytoskeleton of the nervous system which gives form and shape to neural cells plays a vital role in development and differentiation. They are also important for sophisticated molecular biology techniques, induction of cytoskeletal protein gene expression may enable us to overcome developmental disorders and also achieve successful CNS regeneration.

While cytoskeletal proteins have been strongly implicated in degenerative disorders of the nervous system, unraveling the nature of the cytoskeletal lesion is still a formidable and distant goal. Nevertheless characterization of cytoskeletal proteins in the sera or CSF from patients will provide one of the clues for the diagnosis and prognosis of neurodegenerative diseases.

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Glia Neuron Interactions

SHASHI WADHWA

The two main cellular components of the nervous system – neurons and glia – are intimately packed and closely associated with each other. It is known that glial cells outnumber the neurons tenfold in the CNS and contribute to more than half the volume of the brain. While the neurons have been the focus of attraction for decades, it is only now that technological developments have allowed glial physiology to capture the imagination of scientists and clinicians. It is now becoming clear that neurons and glial cells work as a functional unit interacting in a complex and interdependent manner to control each others' development, differentiation and physiological processes. We are now beginning to understand the brain-its development, function and pathologic manifestation from the perspective of neuron-glial relationship.

Loci of glia neuron interactions

The intimacy of relationships between the neurons and glia are vividly demontrable at the ultrastructural levels. The glia may modulate the morphology and activity of dendritic spine and shaft, influence the function of dendritic bundles and physiology of the nodal as well as myelinated regions of the axon. The glial cells and their processes also surround the neuronal soma and may contribute to its growth and maintenance as well as participate in the neuronal synaptic physiology. Another site of neuronal glial interactions is the blood-brain barrier where control on transport and exchange between vascular and brain compartments is exerted.

The neurons and glia are able to influence each other through modifications in the composition of the extracellular space between them by modulating the exchange of ions, neuroactive substances and metabolites. There is a spatial organisation of the neural elements and parcellation of the neuropil. This compartmentation of the neural elements brought about by the glial cells is of

importance. The coupling of glia into syncytial networks may establish within and between the compartments electrical, ionic and macromolecular gradients capable of altering the activity of neuronal assemblies rather than cell to cell interactions.

Interactions between the neurons and glia can be considered under two situations – that during development and those in the adult.

Glial cell proliferation and glial growth factors

Although the exact nature of the astrocyte-oligodendrocyte lineage is not clear, it has been shown by Raff and his colleagues (1983) that in the rat optic nerve glial cells which originate from the preprogenitor cells are protoplasmic Type I astrocytes. This preprogenitor stem cell lacks A_2B_5 antigen. Another bipotential $A_2B_5^+$ progenitor cell gives rise to Type II fibrous astrocytes and if grown in serum deprived conditions develops into oligodendrocytes. Control of glial cell proliferation is not only important in development but also in respect of the response of the mature CNS to disease and trauma. Epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), fibronectin, cAMP, prostaglandins and hormones stimulate astrocytic DNA synthesis and mitosis (Heldin *et al* 1980, Morrison and de Vellis 1981). Studies *in vitro* and *in vivo* indicate that even mature oligodendrocytes have proliferative potential and may be induced to remyelinate following CNS trauma or disease (Willis *et al* 1976).

Guidance of migratory neurons

Rakic in 1971, was the first to propose that young postmitotic neurons migrate along radial glia using them as props to reach their final destination in the CNS. This received further support by the immunocytochemical identification of radial glia by GFAP antiserum throughout the embryonic brain and spinal cord in the monkey at very early stages of embryogenesis, prior to the onset of neuronal migration (Levitt and Rakic 1980, Eckernhoff and Rakic 1984). Even in the rat spinal cord, Hockfield and Mckay (1985) have provided direct evidence for the early appearance (embryonic day 11) of radial glia using monoclonal GFAP antibody.

During the process of migration of cohorts of neurons along the radial glial guides there is directional growth of leading neuronal process, translocation of nucleus to new positions and maintenance of close contact between the neuron and glial surface. Surface appositions between neurons and glial cells with intermembranous spaces of 200-300A *in vivo* (Rakic 1971, 72) and *in vitro* (Walker and Hild 1969, Hatten *et al* 1984) and increase in surface area of migrating cells by addition of new membrane components support the mechanisms of migration proposed by Rakic (1981).

The migration of neuron precursors along the radial glia helps the formation of laminar patterns in the cortex. In the reeler mouse, an autosomal recessive mutant there is abnormal lamination in the cerebral cortex probably due to

defect in neuron glia adhesion which results in a jamming effect for the younger neurons migrating from beneath them (Pinto Lord *et al* 1982). The migration of neurons from the ventricular zone to the cortical plate was proposed by Rakic to form radial ontogenetic columns. However, studies of cell lineage in the cerebral cortex using retrovirus mediated gene transfer do not demonstrate strictly radial grouping of the clonally related neurons (Walsh and Cepko 1988, Luskin *et al* 1988, Price and Thurlon 1988).

Neurite elongation and growth

There is growing evidence that the outgrowth and elongation of neuronal processes is under the control of dynamic interplay between genomic factors and environmental signals which bring about endogenous electrical, physical and chemical gradients in the CNS. Some reports suggest that neurite outgrowth can be influenced by a steady *electrical* field. It has been observed that neuronal activity can be altered by weak electromagnetic fields. A large number of *chemical* factors have also been demonstrated which affect neurite growth and extension. Nerve growth factor (NGF) (Varon 1975), glial released proteins (GRP's) and soluble release factors from glia as well as matrix molecules such as fibronectin, glycoprotein and glycosaminoglycans (Schubert and La Corbiere 1982) are increasingly important in brain development and activity as well as in understanding a number of neuropathological conditions. Furthermore, there is evidence for involvement of neurotrophic factors in neurite pruning. In neuronal regenerative events their role is, however, not completely understood.

Formation of organised neuropil structure

Involvement of glial cells in induction of particular patterns of neuronal morphogenesis has been demonstrated in both the PNS (Mudge 1984) and CNS (Banker 1980, Denis-Donini *et al.* 1984). In the developing insect antennal system, projection of sensory axons to the antennal lobe causes changes in glial shape and disposition one day before the glomeruli are identified. Early removal of glial cells prevents development of glomeruli despite the presence of afferent axons. Thus the glial cells appear to play a role as intermediaries in the induction of glomeruli by afferent axons (Oland *et al.* 1988). Similarily, in the development of the cerebellar dentate nucleus in man, we have observed a sheet of glial cells around the nucleus just before it undergoes gyrification. Later the glial cells disappear.

In the vibrissal system of the mouse, development of vibrissae matched barrel fields in the somatosensory cortex has been studied. It has been observed that the glial cells mark the sites where neuronal bodies will come to lie in layer IV, thereby demarcating the prospective barrels one day before they are visible in Nissl stained material. Cooper and Steindler (1986 a, b) have demonstrated the co-localisation of glial cells marked by GFAP and the surface glycosylated molecules such as J1/tenascin, on the margins of the barrels. They have thus hypothesized that by regulating the expression of these glycosylated molecules on the surface of the glial cells to which the neurons adhere, the glia bring about the characteristic disposition of neuronal cells bodies into the barrels. It is proposed

that J1/tenascin adhesive substrate may be directing the afferent thalamocortical fibres into the barrel fields. A gradient of J1/tenascin has been observed from the periphery to the centre of the barrel. This, it has been suggested to be involved in shaping the relationship between the pre and postsynaptic partner cells in the barrel fields and thereby provide the cells with positional information. These anti-adhesive properties of J1/tenascin may probably be due to its association with other extracellular matrix molecules and could help in (i) cordoning off of the neurons within the barrel from those of another, (ii) inducing cessation of axonal growth into the barrel and (iii) restricting the dendritic elements from arborising outside of their functional domains.

Biochemical differentiation

Non-neuronal cells through their ability to alter the microenvironment play a major role in shaping the neuronal developmental event that determines the final neurotransmitter phenotype. The most widely used model is the superior cervical ganglion (SCG). Neuronal cells constituting the SCG are predominantly adrenergic (ADR) and 5% cholinergic (CHOL). Transplanting the neural crest cell population which gives rise to SCG neurons to another area that gives rise to vagal neurons and vice versa results in the change in the biochemical nature of the cells thus demonstrating that the environment exerts a selective influence on cell differentiation (Le Dourain *et al* 1977). In vitro studies on monoaminergic cells reveal the influence of growing these cells on astrocytic cultures as compared to fibroblast cultures (Lauder 1986). It is now known that neuronal cells are responsive to a number of environmental signals and can integrate these signals in order to make the appropriate choice of the neurotransmitter they will secrete. In general, the glucocorticoids appear to play a critical role in the normal development of sympathetic nervous system by inhibiting the non-neuronal release of a potent EGF inducible cholinergic promoting factor and/or by stimulating the release of an adrenergic promoting factor (Edgar and Thoenen 1978). It may explain certain neurologic deficits attributed to maternal stress. This is supported by the experiments in which steroid treated pregnant rats develop embryos having neuroblasts with an altered neurotransmitter phenotype (Jonakait *et al* 1981). There is also evidence that dysautonomia may be linked to an altered metabolism or sensitivity to NGF, perhaps of glial origin (Schwartz and Breakefield 1980).

Myelination

The relationship between oligodendroglia and Schwann cells with nerve fibres has been known for a long time. It is only now that factors regulating the steps in myelinogenesis are being identified. Some important observations are mentioned below:

(1) Contact with axons is necessary for induction of Schwann cell proliferation, differentiation, production of extracellular matrix and myelin.

(2) Composition of tissue culture medium can influence the response of Schwann cell to nerve fibre contact and pattern of polypeptides released by the Schwann cells in absence of neuronal elements. It has been suggested that this modulation in the ability of Schwann cells to secrete extracellular products may be associated with their ability to undergo correct migration into nerve bundles *in vivo* and *in vitro*. The alterations in secretory function of Schwann cells could help explain the neuropathology observed in dystrophic mice.

(3) Intrinsic abnormalities of Schwann cells have been shown to be responsible for the neurological dysfunction seen in the trembler mutant mouse. This has been demonstrated using simple experiment of normal nerve section followed by donor grafting from trembler mutant or reverse experiments of normal donor nerve grafts in trembler nerves (Aguayo, Bray and Perkins 1979).

(4) Glia neuron interactions influence the fundamental properties of nerve fibre-ion channel distribution, impulse conduction velocity and ectopic impulse generation.

(5) After myelination the glia continue to play an essential role in proper maintenance of structure and function of nerve fibre. Glial produced proteins are transferred to axoplasm which may influence the synaptic function and neuronal cell body activity.

(6) The nodal region with no myelin wrapping is specialised to permit saltatory passage of current and ion exchange. Glia and extracellular matrix may influence and regulate the microenvironment of the node and electrophysiology of the axon.

(7) Two proteins NI 35 and NI 250 have been obtained from the CNS myelin and oligodendrocyte membrane. These have been shown to strongly inhibit the growth of neurites (Caroni and Schwab 1980a). Monoclonal antibodies IN-1 and IN-2 against these proteins neutralise the inhibitory effect and permit growth of fibres in adult rat optic nerve explants and *in vivo* corticospinal tract lesions in young rats (Caroni and Schwab 1988b). Thus similar to J1/tenascin antiadhesive function during early development, these inhibitor proteins may be associated with negative guidance or channeling function for late growing CNS tracts and stabilizing the pathways by preventing the sprouting and side branching throughout adult life. These inhibitors may also be the factors preventing regeneration in the adult CNS.

Receptor mediated events in glia

Cultured glial cells exhibit receptors which possess transport systems to remove neurotransmitters and release trophic factors. Thus chemical signals like neurotransmitters, ions and macromolecules may affect astroglial functions such as enzyme activities, membrane potential, release of macromolecules, protein

phosphorylation dependent on cAMP, Ca, phosphoinositide, cell morphology and neurotransmitter uptake.

Glia can influence information processing in the adult by their involvement in metabolic activities of neurons, ionic activity in the extracellular space and regulating the neurotransmitters.

Metabolic cooperation

Functional linkage between the glia and neuronal metabolism dates back to the pioneering work of Hyden wherein association of increased neuronal cell activity to shifts in glia and neuronal metabolism were thought to be indicative of energy coupling. Experiments demonstrating the stimulation of glial adenyl cyclase system with norepinephrine released from neurons is suggestive of metabolic cooperation between these cells. The glucose so mobilised being utilised by the surrounding neuronal population. These observations support the concept that neurons and glial cells support the physiologic needs of each other (Newburgh and Rosenberg 1973).

Glial involvement in ionic activity

Glial cells are actively involved in regulation of ions in the extracellular space. Whether this glial neuronal biochemical exchange reflects active participation of the glia in information processing in the brain is undetermined.

Regulation of extracellular potassium

The glial cell membrane is permeable to potassium ions passively and with involvement of an active mechanism of transport. Slow depolarisation waves have been recorded from glia.

Glia probably participate with neurons in clearing the extracellular potassium by using the Na/K ATPase system and thereby influence brain activity by compartmentalising K^+ (Cordingley and Somjen 1978). Direct evidence for an extended redistribution of K^+ by glia and the influence of glial released K^+ on neuronal activity are however required. The ability of glia to participate in K^+ metabolism has also been said to play a role in pathological conditions of epileptic seizures and neoplastic events. It is felt that more information would be needed on differential sensitivity of interneurons, dendrodendritic coupling sites and somatic synapses to the glial ionic mechanisms and on the effects of glial coupling through gap junctions forming a syncytium, by employing wider and simultaneous sampling of the properties of glial cells in physiological and pathological states.

Regulation of other ions

Besides K^+, Na^+, Cl, HCO^{-3} and H_2O are known to be transported across the glial membrane. The flux of these ions is interdependent and may contribute to (i) changes in the neuronal excitability and (ii) water flow into the glia inducing swelling of the astrocytes. Chloride transport and K^+ dependent HCO_3 stimu-

lated swelling in glia are blocked by acetazolamide which inhibits the enzyme carbonic anhydrase.

Regulation of neurotransmitters

In the metabolism of glutamate and glutamine, the neurons and glia are intimately involved in regulating the glutamate transmitter available at the synaptic sites. This interaction provides a safety mechanism whereby detoxification of injurious elements and inactivation of excess neurotransmitter takes place to control neuronal synaptic activity (Stewart and Rosenberg 1979). These mechanisms are known to already exist in relation to GABA, taurine, catecholamines, serotonin, prostaglandins, acetylcholine, adenosine and cAMP also. Thus the capability of glial cells for uptake, release, synthesis and degradation of neuroactive substances is significant for the normal *in vivo* operation of neural networks.

Synaptic remodelling

Recent *in situ* observations on neurons and glial cells in sympathetic ganglion of adult living mice by video-enhanced microscopy have shown that over a period of several months there are changes in the location of glial cells in relation to the neuronal surface and in the number of glial nuclei associated with each neuron. (Pomeroy and Purves 1988). It is also observed that presynaptic nerve terminals are more prevalent in the vicinity of glial nuclei than elsewhere on the neuronal surface. The rearrangements have led the authors to suggest that glial cells may be involved in synaptic remodelling in the adults.

CNS immune interactions in trauma and disease

Patients with neurological disorders such as viral encephalitis, subacute sclerosing panencephalitis, progressive multifocal leukoencephalopathy and multiple sclerosis have areas of glial hyperplasia and perivascular cuffing infiltrated with lymphoid cells including T cells (Hofman *et al* 1986). Such infiltrates have been seen in animal models of CNS trauma and demyelination (Raine and Traugott 1984). From the studies on factors influencing the glial proliferation, Merrill (1987) has proposed a mechanism of interaction of the immune response with macroglia in the CNS, in conditions of trauma or disease. Macrophage derived and T cell derived factors activate astrocytes to proliferate which leads to hyperplasia of astrocytic cells. The active astrocytes produce prostaglandin E and suppress interleukin-2 formation from T cells. These then prevent the remyelination of diseased neurons by oligodendrocytes which otherwise are capable of remyelination.

While the importance of the role of glia in neuronal function has been realised, it is a long way before the nature of the coupling between neurons and glial cells in health and disease would be completely delineated and a full understanding of the interactive nature of the components of nervous tissue be available to explain the functioning of the brain.

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Role of Thyroid Hormones in Brain Development

P.K. SARKAR, A. DE AND S. CHAUDHURY

The profound role of thyroid hormones in promoting the development of vertebrate brain was recognised in the late 19th century when it was established that endemic goitre and cretinism are diseases related to thyroid hormone deficiency or hypothyroidism and that they could be cured by thyroid extract therapy (Murray 1891). The miraculous effect of such therapy was that children otherwise doomed to helpless idiocy could be restored to almost normal mental and physical health within six weeks of treatment (Osler 1898). It was important to initiate treatment within the first few months after birth to maximize the probability of having normal IQ for congenitally hypothyroid children. Thus, in human, there is a critical period of thyroid hormone action in brain development and this period happens to be during the fetal life. In India, about 150 million people suffer from hypothyroidism or iodine deficiency disorders with almost 2 million cretins and 8 million with subnormal brain function (Pandav *et al* 1989). Pioneering work in this field which led to the implementation of National Goitre Programme was carried out by Ramalingaswami and his associates (Sorch and Ramalingaswami 1965, Sorch *et al.* 1973).

Much of our current knowledge concerning the mode of action of thyroid hormones in brain development is based on various histochemical studies on hypothyroid rat brain. A classical example is that of Legrand and his coworkers (for review see Legrand 1979) who showed that at the morphological level, the primary effect of hypothyroidism on rat cerebellum is retarded maturation of different 'circuits' leading to the development of adult 'wiring' pattern. The number of Purkinje cells remain unaffected but their differentiation, i.e. axodendritic outgrowth, is severely affected. The migration of cells from the external granular layer is delayed and the number and density of synaptic contacts is decreased resulting in a permanent impairment of neuronal connectivity.

Relationship between the critical events of brain development and thyroid ontogeny

Fig. 1 depicts the early events of brain development in human and rat along with the ontogeny of thyroid function. The critical events of early brain development

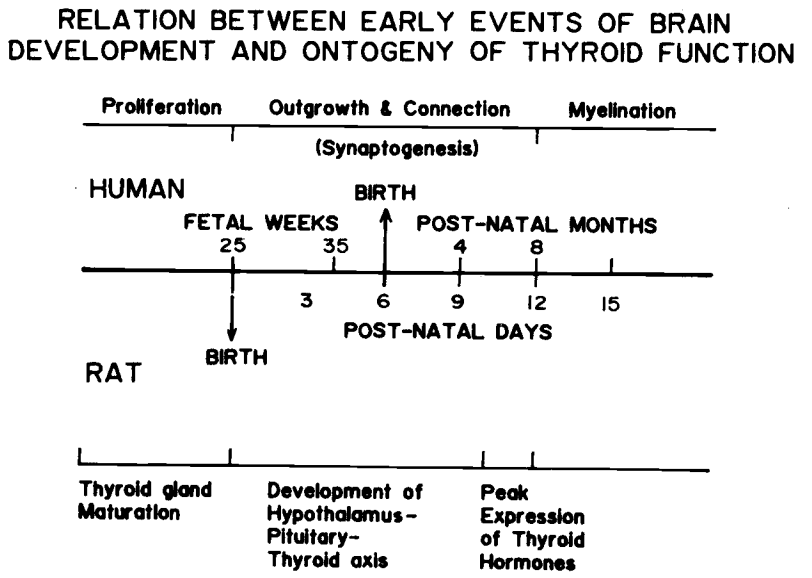

Fig. 1 Early events of brain development and their temporal relationship with thyroid ontogenesis and function.

can be visualized as cell proliferation, neurite outgrowth, connectivity, synaptogenesis and myelination. An interesting correlation can be seen between the timing of these events with that of maturation of the thyroid gland, development of the hypothalamus-pituitary-thyroid axis and the peak expression of thyroid hormones. Of particular interest is the fact that although the length and period of these events vary significantly between human and rat, in both cases, the maturation of the thyroid gland occurs during the fetal stage and the peak expression of T_3 is seen during synaptogenesis. In between, the neuroendocrine control for secretion of thyroid hormone matures.

Thyroid hormone receptors are present in both neuronal and glial cells

Thyroid hormone action is initiated by its binding to specific nuclear receptor. Early experiments by Schwartz and Oppenheimer (1978) on the distribution of T_3-receptors in whole rat brain showed that maximum levels of receptor are during 2–5 days after birth. Table 1 shown that excepting the results observed with cultured neuron (N) glial (G) cells, T_3 receptors are generally present at relatively high concentrations during the first postnatal week and decline thereafter. The binding affinity is very high with a K_α of 10^9-10^{10} M^{-1}. The highest concentration is found in the amygdala, hippocampus and the cortex, while the

lowest concentration is found in the brain stem and cerebellum (Hubank *et al* 1990). These results presented in Table 1 show that T_3 receptors of high binding affinity and binding capacity are present in both neuronal and glial cells.

Table 1: Thyroid Hormone Receptors in Developing Rat Brain

Cell/tissue	Age of study	Period of maximum level	K_a	MBC (fmole/mg DNA)	Ref
Whole brain	Postnatal	2 day			Schwartz and Oppenheimer (1978)
Cerebrum	Postnatal	2 day	Not determined	550-1250	
Cerebellum	Postnatal	14 day			
Whole brain	Fetal and Postnatal	5-7 day	$4\text{-}9\times 10^9\ M^{-1}$	600	Perez Castillo *et al* 1985
Neuronal and glial cells	Neonatal Rat brain	—	$2\text{-}3\times 10^{10}\ M^{-1}$	514-629	Chatterji and Sarkar 1986
Neuronal and glial cells	Postnatal upto day 21	G 5 day N 15 day	$0.4\text{-}0.5\times 10^9 M^{-1}$	557-1774	Hubank *et al* 1990
Neuronal and glial cells	Fetal and Postnatal	N 25 day G 23 day	$1.8\text{–}3.2\times 10^{10} M^{-1}$	140-937	Luo *et al* 1986
			$1\text{-}2\times 10^{10} M^{-1}$	271-566	

Involvement of tubulin in thyroid hormone action in developing brain

Although a few reports are available on the effects of thyroid hormones on microtubule proteins of developing brain (Legrand 1979, Nunej 1984), little is yet known about the molecular basis of thyroid hormone action. Initial experiments in our laboratory (Choudhary and Sarkar 1983, Chowdhary *et al* 1983) were directed to determine the effect of thyroid hormones on tubulin levels in developing brain since the primary effect of the hormone in hypothyroid rat brain was reported to be retarded axodendritic outgrowth (Legrand 1979) and since tubulin represents the major soluble protein in the brain cells (Bamburg *et al* 1973). Polymerization of tubulin into neurotubules is also an essential factor for axon development (Yamada *et al* 1970). These experiments, which were conducted with organ cultures of rat and chick brain first revealed the sensitivity of the developing brain to induction of tubulin by T_3. This sensitivity was limited to a short period after birth corresponding to the onset of synaptogenesis. The disappearance of the hormonal sensitivity soon after birth is likely to be due to the increasing level of endogenous T_3, which rises rapidly after birth.

Effect of T_3 on fractionated neuronal (N) and glial (G) cells from developing rat brain

To determine more specifically the effect of T_3 on brain development at the cellular and molecular level, fractionated N and G cells were employed. Using ^{3}H-colchicine binding assay for quantitation of tubulin, it was found that exposure of organ cultures of newborn rat brain to T_3 followed by cell fractionation or direct exposure of N and G cells pre-fractionated using ficoll gradients, led to

almost selective induction of tubulin in glial cells. Treatment with T_3 elicited a 40-50% stimulation in G cells within 2 hours without any significant increase in the N cells (Chatterji and Sarkar 1986).

In view of the large need of tubulin for neurite outgrowth during synaptogenesis (Daniels 1972), the absence of induction of tubulin in the N cells by T_3 could conceivably be due to the use of Tyrode's balanced salt solution for culturing cells which does not promote morphological differentiation of the neurons. In this context, the effect of T_3 on the induction of tubulin as well as other proteins was investigated using primary cultures of N and G cells where neurite outgrowth occurred in a manner similar to that *in vivo*.

Neuronal and glial cells were fractionated using poly-l-lysine coated culture dishes as described earlier (Schwarz and Oppenheimer 1978). The purity and identity of the fractionated cells was determined not only on the basis of morphological differences, but also on the basis of their positive immunofluorescence with respective N and G cell markers. Fig. 2 shows the positive immunofluorescence of the neuronal cells with antitetanus toxin and an-

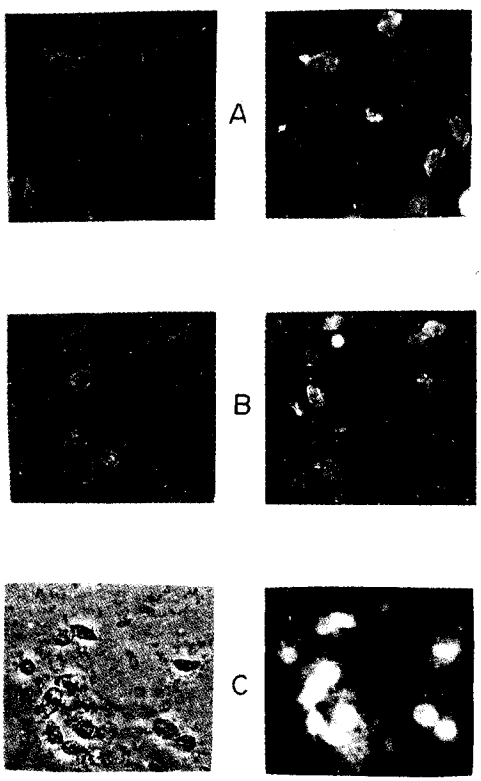

Fig. 2 Identification of N and G cells by immunofluorescence assay. Phase-contrast (left) and immunofluorescence patterns (right) of neuronal cells stained with anti-tetanus serum (A), antineurofilament (68K) protein (B) and of glial cells stained with anti-glial fibrillary acidic protein (C).

tineurofilament protein; likewise the glial cells gave a positive reaction with anti-GFAP.

The effect of T_3 on the tubulin content of the 30,000 g supernatants from primary cultures of N and G cells from 1 day rat brain was examined first by quantitation of tubulin with ^{3}H-colchicine binding assay. The results showed that in 2 hour cultures, treatment with 0.5-5 nM hormone had no significant effect, whereas in 18 hour cultures, a 35-40% increase in tubulin content relative to untreated controls was observed specifically in the G cells with little or no increase in the N cell (De *et al* 1991).

These results were intriguing since they did not reveal any effect of the hormone on the N cells, whose morphological differentiation is severely affected in hypothyroid brain (Legrand 1979). To further investigate the effect of T_3 at the molecular level, the primary cultures of N and G cells were labeled with 1 μci/ml of ^{14}C-leucine for 18 hours and 30,000g supernatants from these labeled cells were assayed for incorporation into total protein and SDS-page electrophoresis. Incorporation of leucine into total protein was stimulated only 5-15% by T_3 in both N and G cells. Likewise the stimulation of the tubulin bands by T_3 in the SDS-gels by Coomassie blue staining was marginal in the case of both N and G cells, although the band corresponding to the mobility of actin was clearly stimulated (Fig. 3A). More interestingly, in the autoradiographic pattern of the same gels, where equal amounts of TCA-insoluble radioactivity was loaded in each lane, 1.5-2 fold stimulation of the intensity of tubulin bands in the G cells along with a 2.3 fold stimulation in N cells was observed. Furthermore, these

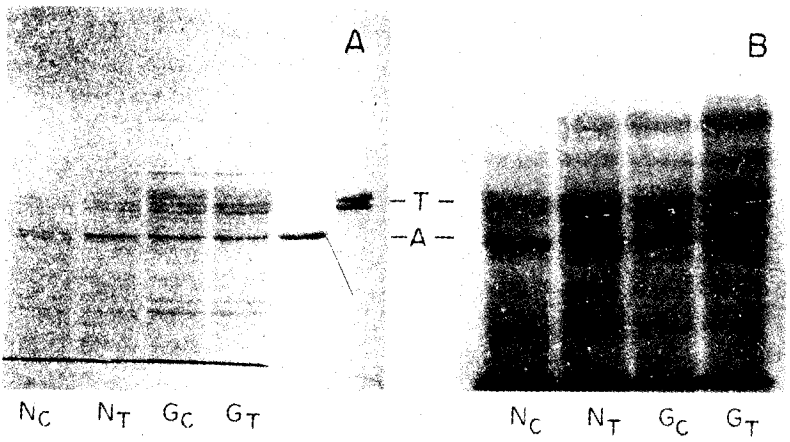

Fig. 3 Coomassie blue pattern (Fig. 3A) and autoradiographic analysis (Fig. 3B) of the effect of thyroid hormones on the soluble proteins of neuronal (N) and glial (G) cells by SDS-PAGE procedure. Equal amounts of radioactivity from 30,000g supernatants of control and T_3-treated N and G cells labelled with 1 μCi/ml ^{14}C leucine for 18 hours were loaded on 10% gels. N_C, N_T and G_C, G_T represent control and T_3-treated N and G cells respectively. T and A represent positions of authentic tubulin and actin respectively.

autoradiograms displaced a 1.5-2 fold stimulation of action by T_3 in both N and G cells with a relatively greater effect on N cells (Fig. 3B). The identity of actin was not only found on the correspondence of the mobility of the band with standard actin but also by its purification from the 30,000g supernatant using affinity chromatography on a DNAse-sepharose column (Zechel and Weber 1978).

Thus comparison of the labelled protein profiles in the 30,000g supernatants for control and T_3-treated cells provided the first clear evidence for the stimulation of tubulin as well as actin in both N and G cells. Despite a greater stimulation of labelled tubulin in N cells relative to G cells such induction was not detectable by ^{3}H-colchicine binding assay. The failure to detect the stimulation of tubulin in the N cell supernatants could be due to (a) rapid turnover of tubulin in these cells, (b) rapid translocation of soluble tubulin into the insoluble membrane bound form or (c) the presence of relatively greater amount of oligomeric forms of tubulin which are insensitive to ^{3}H-colchicine binding assay. This would be anticipated in view of the continuous need for assembly of tubulin i.e. conversion of dimeric to polymeric form during neurite outgrowth and axon development (Yamada *et al* 1970). Indeed, the results obtained from the SDS-PAGE analysis of the solubilized 30,000g pellet containing the membrane bound proteins (data not shown) from control and T_3-treated cells support this contention. Autoradiograms of these gels show that T_3 enhances both labelled tubulin and actin to a relatively greater extent in the N cells compared to the G cells suggesting a role of the hormone in the translocation of the newly synthesized cytoskeletal proteins from the soluble fraction of the membrane fraction.

The molecular and cellular basis of thyroid hormone action in brain development have been investigated. Primary cultures of neuronal and glial cells from neonatal rat brain have been employed to determine the effects of T_3 on the level of tubulin and other cytoplasmic proteins. While ^{3}H-colchicine binding assay revealed induction of tubulin by T_3 almost selectively in the G cells, autoradiographic analysis of labeled proteins in the 30,000g supernatants as well as 30,000g pellets from control and T_3-treated cells revealed that T_3 not only elicits a stimulation of the labelled tubulin and actin in the soluble fraction but also plays a role in their translocation to membrane fraction thus facilitating morphological differentiation. These effects of T_3 have been found to be more pronounced in the N cells relative to G cells.

Acknowledgement

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Effect of Environmental Factors on Neural Development

U. SABHERWAL AND T.S. ROY

Environmental factors play a significant role in production of congenital defects in offsprings. It has been estimated that 25% of human congenital malformations are due to known genetic and chromosomal defects, 10% defects have been traced to known environmental agents, 2-3% have been identified to be due to drug exposures. For 62-63% of malformations no specific teratogenic agent has been identified and are grouped under the category of malformations of unknown etiology. These are possibly due to the interactions between the genetic and environmental factors.

The extent of damage produced by a teratogenic agent depends upon the stage of embryonic development at which the exposure occurs. During the preimplantation stage the teratogenic insult results in death of the embryo. During the period of organogenesis which is from gestational days 7 to 16 of rodent development and 3 to 8 weeks of human embryonic period, the exposure would cause birth defects. Teratogenic influences during fetal period cause growth retardation and functional deficits. Embryonic period is the stage of highest susceptibility to the teratogenic influences and is the stage of organogenesis.

Developing nervous system is susceptible to teratogenic influences. During the period of organogenesis and histogenesis vulnerability is very high and major defects of nervous system result during this period. In the human developing brain there are two periods of rapid growth spurt. The first phase is from 15-20th week of gestation and the second phase is after 25th prenatal week which continues into second year postnatally. This first stage of nervous system development is the most vulnerable period, the brain is growing at a fast rate with migration of neuroblasts, formation of glial cells, myelination and establishment

of functional connections. Environmental insult at this stage would cause mental retardation and persistent behavioural deficits in later life.

In the most commonly investigated laboratory animal, rat, the period of susceptibility corresponding to the human growth spurt phase is in the first 2-3 postnatal weeks with the peak at 6-9 postnatal days. Teratogenic effects of environmental agents have been investigated in this animal model after administration of these agents during the period of susceptibility to elucidate the mechanism of neuronal development and relationship between morphological alterations and behaviour. The effects of some commonly used substances and drugs on developing nervous system are described here.

Alcohol Abuse

Alcohol is a substance of social abuse and despite antenatal advise on adverse effects of drinking many women continue alcohol intake during pregnancy. Fetal alcohol syndrome consists of malformations associated with growth retardation, intellectual and behavioural deficits resulting from heavy alcohol exposure in the prenatal period. A characteristic facies with microcephaly, mental retardation, hyperactivity and associated skeletal and cardiac anomalies are frequently observed. The central nervous system is vulnerable to prenatal alcohol exposure and the exposed children often manifest neurobehavioural deficits without any morphological abnormalities.

General growth retardation is a commonly reported effect of alcohol exposure during development. A reduction in brain weight of offsprings has been observed after alcohol vapour exposure during embryonic period. Neuroglial heterotopias, neuronal and glial dysplasias, ectopic and disorganised neuronal arrangements have been observed in histopathological studies on autopsy material obtained from such newborns. In experimental studies on rats general growth retardation, delayed neuronal development and premature onset and termination of myelination has been observed in pups exposed to alcohol in prenatal period. Action of alcohol on developing brain also appears to be at the stage of synaptogenesis where alcohol causes reduction in synaptosomal sialic acid concentration. Further, the reduced sialic acid content of neuronal cell-adhesion molecules might result in inhibition of neuronal migration, premature aggregation and adhesion of neurons. Low level alcohol exposure during pregnancy and lactation may result in degeneration of fibres in the deep layer of somatosensory cortex (Murray *et al* 1981).

Morphological alterations in cerebellum and hippocampus observed in experimental models after prenatal alcohol exposure may provide explanation for the coordination and learning deficits observed in children of mothers indulging in alcohol abuse during pregnancy. A reduction in the number of pyramidal cells of hippocampus and cerebellar Purkinje cells has been noted after prenatal and early postnatal alcohol exposure. Alcohol exposure in the third trimester during the brain growth spurt phase, has deleterious effect on glial cells. A dose related effect of alcohol has been seen on glial proliferation and maturation. Delay in

myelination reported by Jacobson *et al* (1979) may be explained by reduction in number of glia which produce myelin in the central nervous system. Sulik and associates (1984) have described reduction in hippocampal primordium after intraperitoneal alcohol injections during prenatal period in rats.

Alterations in dendritic growth and branching pattern have been demonstrated following pre and postnatal alcohol exposure. In hippocampal pyramidal neurons of CA1 region stunted basal dendrities have been noted. The dendritic tree of pyramidal neurons of layer V of parietal cortex has been observed to be decreased with a reduction in the dendritic spines after prenatal alcohol exposure.

Nicotine abuse

Cigarette smoking during pregnancy results in intrauterine growth retardation (Butler and Goldstein 1973, Eriksson *et al* 1979). Behavioral changes, neonatal hyperactivity and learning deficits later have been reported in offsprings of smoking women (Longo 1977). Animal models have been developed to identify specific factors which produce growth retardation and nervous system alterations after prenatal nicotine exposure. Nicotine causes significant reduction in fetal brain DNA, RNA content and protein concentration (Slotkin *et al* 1987). Significant reduction in body weight and brain weight has been observed up to day 40 postnatal in our studies after prenatal intraperitoneal nicotine from day 1 to day 20 of gestation. Brain weight, body weight ratio is observed to be altered in the studies conducted up to postnatal day 40. Studies on rat embryos following exposure to nicotine alone and alcohol and nicotine in combination, have shown significant reduction in crown rump length and gross morphological changes in rat embryos.

A number of studies have suggested that nicotine acts directly on immature neurons through nicotinic receptors present in early fetal and neonatal development. Prenatal exposure to nicotine has been shown to inhibit DNA synthesis, upto postnatal day 15, in all brain regions but the effect is more marked in the brain stem and mid brain region as compared to the cerebral cortex, these being the regions which are rapidly maturing at this time. The overall pattern of cell replication and differentiation is disrupted in the entire brain leading to abnormalities in cell number and size, in synaptogenesis and synaptic activity (Lichtensteiger *et al* 1988, Navarro *et al* 1989).

However, it has been shown by morphological studies, conducted in our laboratory, on neonatal rat brain after prenatal nicotine exposure that at day 40 the cell density in pyramidal cell layer of somatosensory cortex is more and neuronal diameter is significantly less. The thickness of cortical layer is also significantly less. The effects on hippocampus are more marked.

Our studies on hippocampal region of rat after 40 postnatal days following prenatal nicotine exposure in the dosage of 3mg/kg body weight have shown delay in maturation of dentate gyrus. The thickness of granule cell layer of the hippocampus, the neuronal diameter and the density of neurons is more as

compared to the age matched controls. The dendritic tree is less extensive in the granule cells of nicotine treated animals. The apical as well as basilar dendritic branching is less and the axonal process is also smaller. Ultrastructure of these granule cells shows increased nuclear indentations, dilated cisternae of rough endoplasmic reticulum and of the Golgi apparatus. The pyramidal cell layer of CA_3 and CA_1 hippocampal region shows smaller diameter of neurons. The dendritic arborizations of pyramidal neurons of CA_3 and CA_1 region are less in the nicotine exposed pups in comparison to the controls. Dendritic intersections were counted (by Sholl analysis) and in the nicotine exposed brains the number of intersections is significantly less. The apical dendrite is shorter and shows early branching (Figs. 1, 2). The basal dendrites are less extensive and shorter with reduced number of intersections in Sholl analysis.

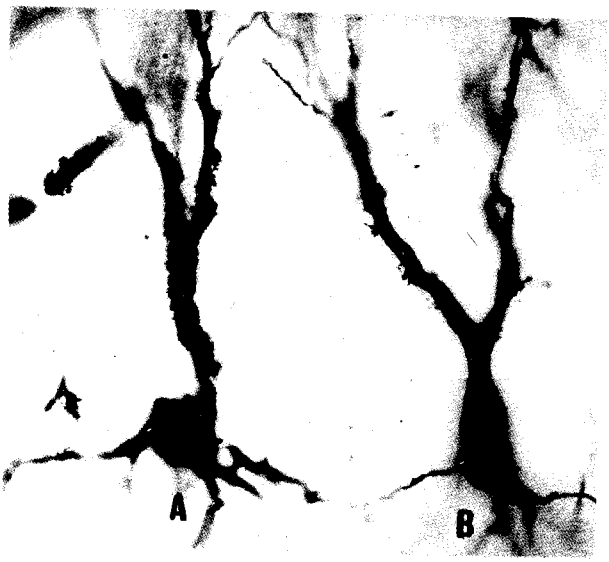

Fig. 1 Photomicrograph of pyramidal neuron of CA_1 region of hippocampus showing early branching of apical dendrite of prenatal nicotine exposed animal (B), in comparison to apical dendrite of pair fed control rat (A) at postnatal day 40. Golgi impregnated cerebral cortex, X1800.

The ultrastructure of pyramidal neurons of CA_1 as well as CA_3 regions shows markedly dilated cisternae of rough endoplasmic reticulum and Golgi apparatus also has large irregularly dilated cisternae (Figs. 4, 5). The mitochondria do not show any alteration in morphology. The synaptic input to the apical segments of CA_3 and CA_1 pyramidal neurons is from the mossy fibres, which are the axons of the dentate granule cells (Fig. 3). Our studies have shown ultrastructural alterations in the morphology of granule cells as well as less extensive dendritic arbor after nicotine treatment. Reduction in the mossy fibre input to the pyramidal neurons of CA_1 and CA_3 regions may be responsible for the reduced dendritic branching of these neurons. The direct effect of nicotine on differentiating CA_1

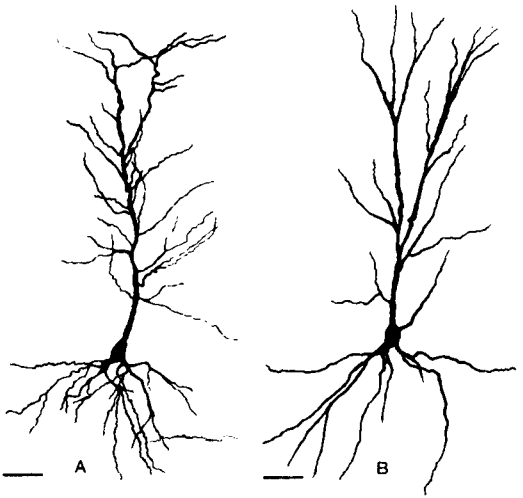

Fig. 2 Camera lucida drawings of pyramidal neurons of CA_3 region of hippocampus showing branching pattern of apical and basilar dendrites in pair fed control rat (A) and in prenatally nicotine exposed rat (B) at 40 postnatal day. Bar represents 20 um.

and CA_3 neurons may be responsible for the morphological changes observed in our studies at the ultrastructural level. It appears that the nicotine effects are

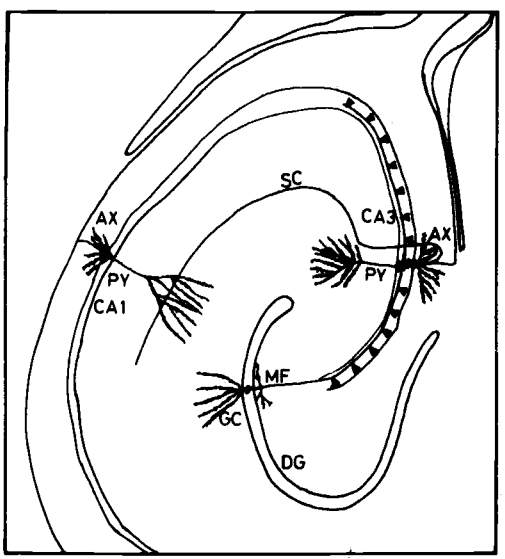

Fig. 3 Schematic drawing of hippocampus of adult rat showing the afferent input to pyramidal neurons (PY) of CA_3 and CA_1 regions of hippocampus, MF-mossy fibre from dentate gyrus (DG) granule cell (GC), AX-axon of pyramidal neurons, SC-Schaffer collaterals.

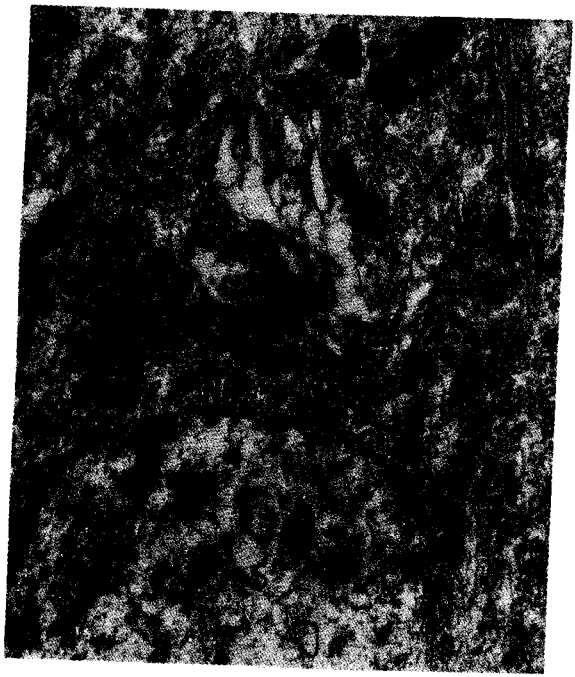

Fig. 4 Electron micrograph of pyramidal neuron of CA_1 region of hippocampus in prenatal nicotine exposed animals at postnatal day 40 showing markedly dilated (G) cisternae of Golgi apparatus. NE-nuclear envelope, X13950.

more on the late developing brain region (hippocampus) as compared to relatively early maturing neurons of the somatosensory cortex.

Vascular effects of nicotine also contribute to the overall structural alterations produced by nicotine because nicotine reduces the vascularity. The decrease in total body weight and brain weight observed after nicotine exposure in prenatal life may be resulting from lower nutritional status of these animals as food intake is reduced in these animals.

Valproic acid

Valproic acid (VA) and its salt-Sodium Valproate (SV) is used in treatment of epilepsy, alcohol suppression syndrome and delirium tremens and is continued as maintenance dose during pregnancy. In a study reported from United States 7000-10000 pregnant women were found to be on Valproate therapy. Several reports associating certain birth defects with the drug have been published. DiLiberti *et al* (1984) have observed a consistent craniofacial phenotype and psychomotor delay in a series of children exposed to VA during intrauterine life.

Neural tube defects-hydrocephalus, microcephaly and spinal bifida have been observed in infants of mothers who were given sodium valproate during pregnancy (Jeavons 1982). Kelly (1984) has calculated that maternal use of SV in first trimester was associated with 1% risk of spinal bifida.

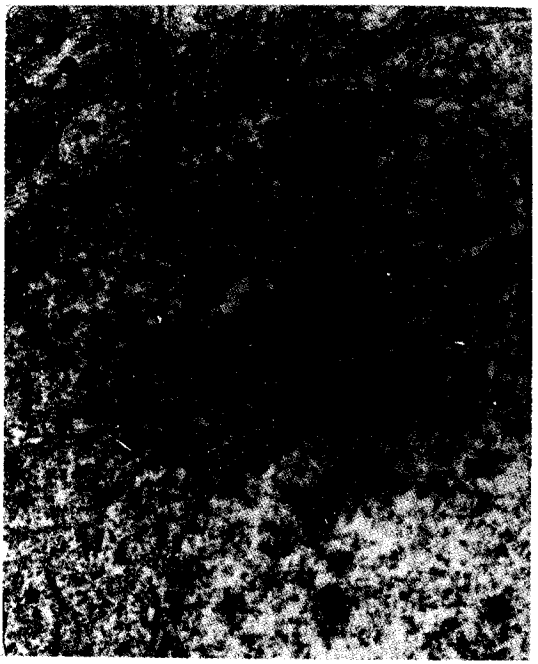

Fig. 5 Electron micrograph of pyramidal neuron of CA_3 region of hippocampus at postnatal day 40 after prenatal nicotine exposure. R-dilated cisternae of rough endoplasmic reticulum NE-Nuclear envelope, X10400.

VA crosses placental barrier freely and serum concentrations in new born exceed maternal serum level. Animal studies have shown VA to have developmental toxicity in several animal species. Whittle (1976) had reported a dose related teratogenicity in VA treated rat and mouse embryos with increased resorptions, vertebral fusions and renal agenesis. Kao *et al* (1981) also reported dose dependent teratogenicity after *in utero* exposure to VA in mice. Growth retardation, exencephaly, craniofacial deficits have been noted by several investigators after prenatal SV exposure.

Failure of neural tube closure is comparable to the spinal bifida defect observed in humans. Neural tube closure being a complex staged process, each stage is controlled by mechanisms which differ in susceptibility to teratogenic agents. This may explain the difference in teratogenic response of VA which produces spinal bifida in human and exencephaly in animal studies.

Our studies on Swiss mice after administration of SV during the period of organogenesis have shown a dose related teratogenicity. SV was given to pregnant mice from day 4 to day 19 of pregnancy in dose of 150-300 mg/kg body weight. There is an increase in maternal mortality and the abortion rate is high as compared to the pair fed controls. There is significant reduction in birth weight of drug exposed pups. Skeletal defects ranging from abnormal shape of vertebral bodies, forelimb defects, reduction in number of digits are noted in new

born pups. Neural tube defects are also seen. At highest dose level (330 mg/kg), meningomyelocoele and enlarged cerebral aqueduct is observed in 4% of drug exposed pups.

The morphological and behavioural alterations observed in embryos and newborns and later in adults due to prenatal environmental influences, represent the overall result of interaction of several teratogenic factors on the genetically susceptible embryos at the critical stages of development. Further the duration and dose of the teratogenic insult also have an important role to play in producing morphological defects.

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Neural Nets

JITENDRA C. PARIKH

The ultimate aim of all studies of the brain, is to develop a theory, based on a few fundamental principles, that will provide a comprehensive understanding of how the brain works. Such a theory ought to "explain" motor coordination, sense perception, memory, learning, psychological aspects such as feelings and emotions and also consciousness. At the present time, one is obviously nowhere near achieving this goal, but all the same one has,

(i) a rapidly growing body of experimental evidence concerning brain structure and function,

(ii) some ideas, conjectures and partly successful models which deal with limited aspects of brain function, and

(iii) many unanswered questions of fundamental nature.

It ought to be realized that modern technology, in the form of sophisticated computers, has revolutionized theoretical studies of the brain, since simulations of brain activity are possible. Such simulations provide "laboratories" to test various ideas and models, which can very often be implemented in practice to carry out useful tasks. Basically, this represents the area of artificial intelligence (AI) and neural network studies. These studies are useful for various reasons (Penrose 1989). The most immediate practical use relates to the development of robotics, where mechanical devices carry out "intelligent" tasks. Clearly robots are to be preferred for carrying out dangerous tasks, but in addition, they are necessarily more efficient and reliable, compared to human beings, for executing certain types of routine jobs. Thus, robots may be employed, for handling dangerous chemical and radioactive materials, for exploration of deep sea and outer space and on assembly lines. A less "physical" use of AI studies is the development of expert systems where knowledge and information of a given profession – say legal, medical – can be made available on a computer and

updated regularly to help these professionals to give state of the art advice to their clients.

As we shall see, AI and neural network studies imitate certain behaviour of the human brain, and hence they provide models for understanding specific brain activity as well. This will be the main theme of the discussion here. In addition, such studies may also provide clues about deep questions of philosophy, and insights into the meaning of the concept of "mind" (Penrose 1989). For instance, one may envisage getting answers to questions like, what does it mean to think or to feel? What is mind? If there is such a thing as mind is it dependent upon the physical structures (brain in living beings) to which they are associated? etc. These are difficult and controversial issues and I will have very little to say about them here, but I strongly urge you to read these recent articles (Churchland and Churchland 1990, Searle 1990 and Penrose 1990).

Structure and Electrochemical Activity of a Neuron

In order to discuss neural nets, it is necessary to first review some basic features of a real nerve cell.

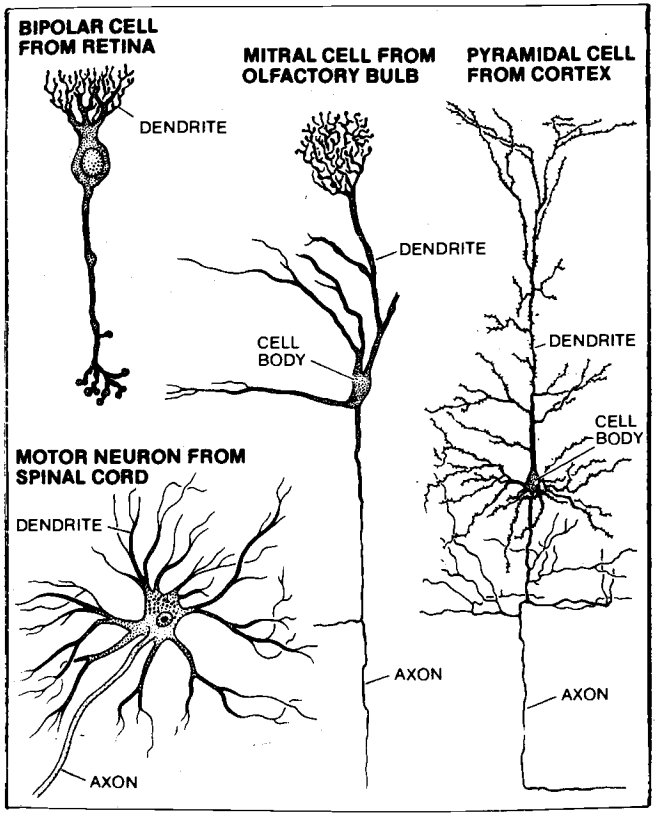

Fig. 1. Different types of neurons (from Kuffler and Nicholls, 1976)

The brain consists of a large number ($\sim 10^{10}$ in human beings) of cells called neurons and they come in a wide variety of shapes and sizes as shown in Fig. 1. Each neuron has a cell body (or soma), dendrites and an axon, surrounded by a cell membrane. The electric input to a neuron is carried out by the dendrites and the output from it is carried by the axon. Neurons receive signals from and transmit signals to other neurons at junctions called synapses (Fig. 2a).

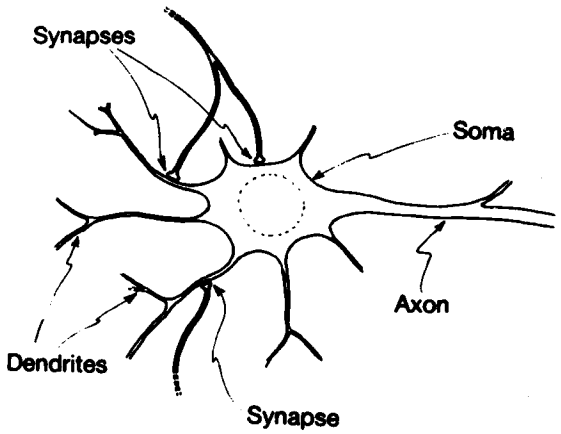

Fig. 2a. Synapses in a nerve cell (from Penrose, 1989)

There is actually a gap between the surfaces of the two neurons at these synapses (Fig. 2b). There are positive (Na^+, K^+) and negative (Cl^-) ions inside and outside the nerve fibre or the axon. The propagation of a signal along a nerve fibre is a consequence of an imbalance between the positive and the negative ions inside and outside of the nerve fibre. A nerve cell can be considered to be in one of two possible states, resting and propagating. In the resting state (Fig. 3a) the inside is negatively charged (due to an excess of Cl^- ions) and the outside is positively charged. Also there are more K^+ ions inside (less outside) relative to Na^+ ions. If in a given region of the nerve fibre this charge imbalance is reversed – i.e. inside is positively charged and outside is negatively charged and if this reversal

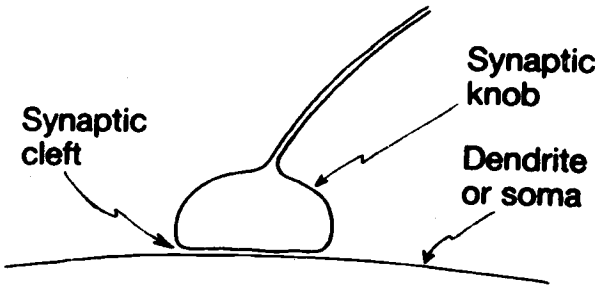

Fig. 2b. Gap at a synapse (from Penrose, 1989)

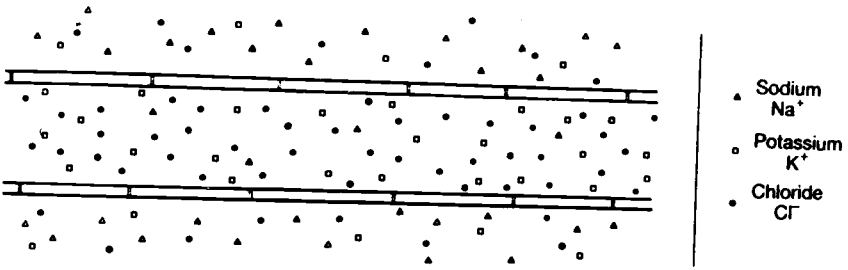

Fig. 3a. Ionic position in the resting state of a neuron (from Penrose, 1989)

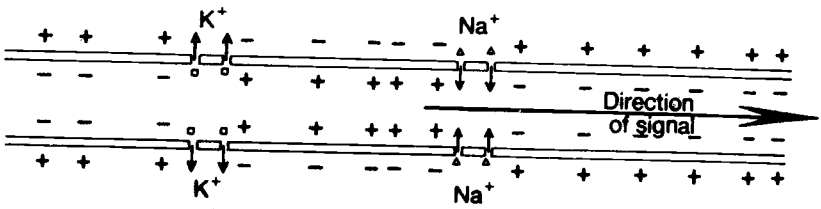

Fig. 3b. Ionic composition in the propagating state from a neuron (from Penrose, 1989)

travels along the fibre then one has a propagating nerve signal (Fig. 3b). This is the other state of a neuron.

When a signal arrives at a synapse the communication from a neuron to the next one across the gap (see Fig. 2b) is carried by chemicals called neurotransmitters. These can be either excitatory or inhibitory. If the sum total of all the signals, arriving at the dendrites of a neuron, has a net excitatory effect and exceeds a critical threshold value, then the neuron fires or gives an output signal (action potential) which propagates along the axon – otherwise there is no signal. The action potential does not increase with an increase in the strength of the excitatory input signal but the frequency with which the neuron fires increases. Thus, the neurons exhibit threshold excitability, with an all or none electric response, suggesting thereby, that an idealized neuron in a neural network may be modelled as a binary (0-resting state, 1-firing state) unit.

Going beyond a single neuron, it appears that, information processing and execution of higher functions in the brain, are a consequence of both the architecture of the brain and the connectivity between the neurons. Also, over the years, one has learnt that the connectivity amongst neurons is not at all random, but is highly organized. In addition, this connectivity exhibits plasticity both at the developmental level and the adaptive level.

The basic features of a single neuron and its electrochemical activity together with what has been learnt about the structure and function of some part of the brain (e.g. cerebellum, hippocampus or cerebral neocortex) have motivated workers in the field to model specific activities of the brain. Such an approach may be termed as a microscopic (neural network) approach. It will be discussed at some length in the following sections.

Assembly of neurons — from simple to complex model brain

Modelling a Single Neuron

Consider first a single neuron. During the last forty years considerable work has been done to model its activity (Cowan and Sharp 1988). In particular, mathematical equations, which accurately represent generation and propagation of action potential, were proposed by Hodgkin and Huxley. The transmission and reception of signals across synapses, was modelled involving electrical circuits, having capacitors and conductors, by Eccles. The role of dendrites and the importance of geometrical structure was initiated by Rall. The outcome of all these studies is that even a single neuron is a complex object and requires sophisticated treatment. As a consequence, studies of neural nets generally employ 'idealized' neurons which can be in either of the two binary states mentioned earlier.

The McCulloch-Pitt Net

Very simple assemblies of neurons were considered by McCulloch and Pitt (1943) using idealized neurons. Figure 4 shows a network of three idealized (binary) neurons X, Y and M. The neurons X and Y each have an excitatory synapse with the motor neuron M and the strength at each synapse (or synaptic weight) is + 1. The synaptic weight is a measure of the strength of connectivity between two neurons. If it is excitatory (in the present context positive in sign), then a greater strength would imply a higher conductance across the synapse, leading to a higher firing frequency for the neuron receiving the signal. The motor neuron M has a threshold $t = -2$. It is clear therefore that, if both X and Y are in the active state, the input to the motor neuron will be able to overcome the threshold t, and the motor neuron M will fire. For all the other 3 states of the two neurons X and Y, M will not be activated. This is readily seen from the following "truth" table (Table 1). It shows, for all possible combinations of states of neurons X and Y, the state of the motor neuron M.

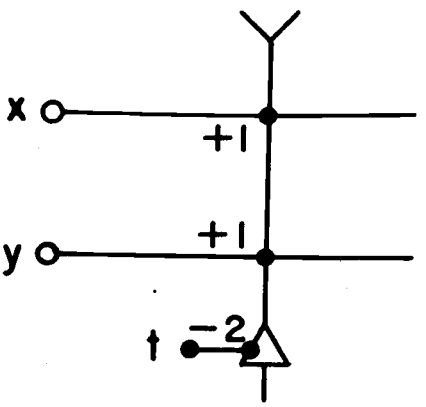

Fig. 4: A simple McCulloch-Pitt net (from Cowan and Sharp, 1988)

Table 1: States of Neurons X, Y and M, 0: inactive, 1: active

X	Y	M
0	0	0
1	0	0
0	1	0
1	1	1

In terms of logic, the network of Fig. 4 embodies the statement "X and Y". Another example is shown in Fig. 5 which represents the statement "not X". It is clear that nets are basically simple logical switches.

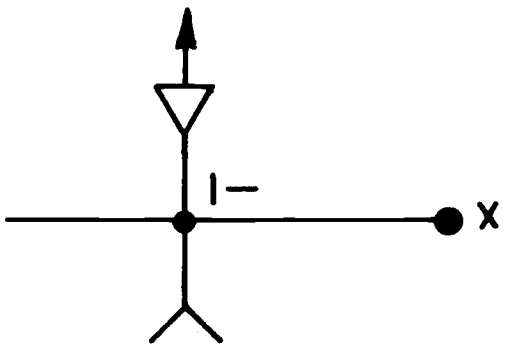

Fig. 5: A simple McCulloch-Pitt net (from Cowan and Sharp, 1988)

These examples illustrate the main features of McCulloch and Pitt nets 1943). Actually, these authors proved that nets of such idealized neurons (i) can carry out simple arithmetic calculations (ii) can classify, store and retrieve finite sets of data and (iii) can be used to apply simple logical rules.

A severe limitation, of this kind of net, is related to its reliability (Cowan and Sharp 1988), if it incurs damage. It is clear that the network would not function if any part of it is damaged. In order to overcome this shortcoming, Von Neumann introduced the notion of redundancy. Basically, it uses many neurons to do the job of one. For example, the choice between firing (+ 1) or not firing (0) for a neuron would not be signalled by a single neuron but by a synchronous activation of (say) more than half the number of neurons. In this way, one bit of information is distributed over a large number of neurons, and simultaneously, each neuron (partially) carries many bits of information. This idea of redundancy provided valuable insight into how neural nets may function in the brain despite damage, and was in agreement with the observations made by Lashley in rats.

From more recent studies, it appears that, this distributive method of functioning is valid for the more peripheral regions of the brain but may not be appropriate for the central regions of the brain. In addition, it must be recalled that, in the early processing of the visual information, there are neurons (feature detectors) which carry out very specific functions such as detecting a bright centre dark

surrounding, or an edge etc. (Kuffler and Nicholls 1976). For such neurons the notion of redundancy does not apply.

Adaptive McCulloch-Pitt Net and Learning

It was suggested by Hebb (1949) that, the connectivity in a brain changes as learning takes place, and this happens by synaptic modifications. According to him, this change occurs by repeatedly activating one neuron by another, such that, in the process the conductance across their synapse increases. By such a mechanism, it would be possible to transform, a group of weakly connected cells, into an assembly of strongly connected ones.

This basic suggestion of Hebb (1949), has been used to devise, adaptive McCulloch-Pitt nets (Cowan and Sharp 1988) which have the property of plasticity. so that they can be "taught" and learning can take place.

This is illustrated by an example taken from Cowan and Sharp (1988) and shown in Fig. 6.

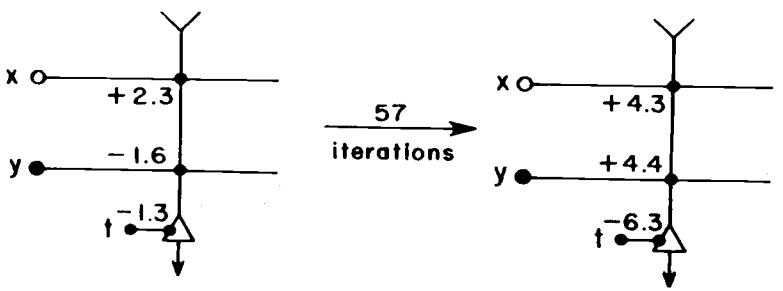

Fig. 6: An adaptive McCulloch-Pitt net (from Cowan and Sharp, 1988)

Table 2: "Truth" tables for the nets shown in Figure 6

X	Y	"X and not Y"	X	Y	"X and Y"
0	0	0	0	0	0
1	0	1	1	0	0
0	0	0	0	1	0
1	1	0	1	1	1

The objective is to modify the network (and the "truth" table) representing the statement "X and not Y" shown at left into a network having the "truth" table "X and Y" shown at right. The modification is to be carried out by setting up a learning algorithm which will modify the synaptic weights. As discussed by Cowan and Sharp (1988) a possible learning algorithm is as follows.

(i) Start with the synaptic weights shown in Fig. 6 at left and consider each of the four stimulus patterns for the neurons X and Y in the "truth" table (Table 2).

(ii) If for a given stimulus the response is what is desired then make no changes in the weights.

(iii) If the response in incorrect, increase the weight at each synapse by 1, if the "truth" table at the right indicates that the net would be activated. On the other hand, if the "truth" table indicates, that the net should not be activated, then the weight at each synapse should be decreased by 1.

(iv) A finite number (57 in the present example) of such modification of synaptic weights generates the net at right (Fig. 6) representing "X and Y" from the one representing "X and not Y" at left.

From the example, we note that the memory of learning is distributed in the synaptic weights at all the connections that are modified in the training phase. Also since this memory is distributed over many connections it is less likely to be disrupted by damage.

It is known, however, that human memory is associative. Neural nets having this feature have also been studied. The basic idea is that the net learns, by modifying the synaptic weights, to associate two (or more) sensory inputs by repeated presentations. To begin with, only one of the two sensory inputs gives a motor response, but at the end of the training phase, the other one also gives a motor response. It is clear that this association is Pavlovian in nature. This type of memory is termed as associative content addressable memory (ACAM) and has been studied extensively (Taylor 1956, Anderson 1968, Cooper 1973, Kohonen 1977, Parikh and Pratap 1984).

These studies have shown that such an ACAM can discriminate between patterns and also have associations between them. It has also been demonstrated (on computers) that for ACAMs a partial stimulation of memory leads to a full motor response.

Marr's Model of the Cerebellum (ACAM)

The discussion so far, has been confined to progressively incorporating more and more features in neural nets, so that they reflect increasingly more complex aspects of memory and learning of the real brain. In a very significant development, Marr (1969) shown that the functioning of the cerebellum in vertebrates can be understood by considering it to be an ACAM.

The cerebellum is an organic unit in the vertebrate brain, which learns to perform motor skills, and having learnt them controls and coordinates voluntary movements. The cerebellum has a very simple architecture and consists of five different types of neurons. These are granule, Golgi, basket, stellate and Purkinje cells. In this model, Marr (1969) emphasized the importance of this architecture and also assigned a specific function for each type of neuron, thus relating structure with function. The structure of the cerebellum, its architecture and connectivity are schematically shown in Fig. 7.

According to Marr, during the learning phase, the Purkinje cells receive information from climbing fibres (CF) and mossy fibres (MF). The information arriving via the CF concerns the elemental sequence of motor movements taking place, and that arriving via the mossy fibres (MF) is about the context in which the firing patterns of nerve cells took place, which led to the appropriate se-

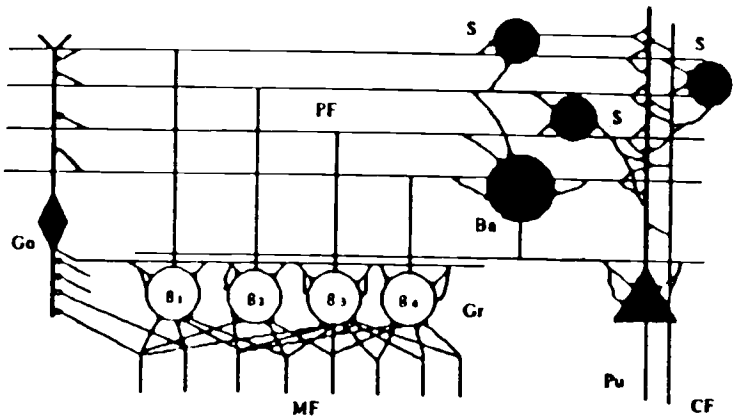

Fig. 7. A schematic illustration of the connectivity of different types of neurons in the cerebellum (from Cowan and Sharp, 1988)

quence of movements. As a result, associations are established between MF and CF activity patterns. These patterns are stored at synapses between the granule and the Purkinje cells. Once a movement is learnt, the context alone through the MF activity stimulates the Purkinje cells to initiate the sequence of movements. The role of the granule cells is to decorrelate the activity pattern coming from the MF, whereas the Golgi cells control the threshold of granule cells to avoid overloading of too many patterns in the cerebellum. Furthermore, in order to correctly retrieve the patterns, the threshold of Purkinje cells must be set high enough to suppress unwanted patterns. This function is carried out by the basket and stellate cells which have inhibitory synapses with the Purkinje cells.

Some other authors have suggested different models for the cerebellum, and attempts have been made to test the various models. There is as yet no conclusive evidence in favour or against any of the models.

It should be mentioned that Marr (1970, 1971) has also developed models for cerebral neocortex and for the hippocampus.

Hopfield Net and Extensions

During the '80s a different kind of network was proposed which has been extensively studied. This is the Hopfield (1982, 1984) net. It consists of a net of idealized neurons having the property that each neuron must excite as well as inhibit its neighbours. In this respect, it is even more unrealistic than the neural sets considered earlier. As we shall see, there are, however, some extremely pleasing features in a Hopfield net.

Let $\beta_i = \pm 1$ denote the state of the i^{th} neuron and ω_{ij} the synaptic weight between i^{th} and j^{th} neuron. Let us also assume that the connection is symmetric so that $\omega_{ij} = \omega_{ji}$. Consider an 'energy' function defined as

$$E = -\sum_{i<j} \omega_{ij}\, \beta_i\, \beta_j$$

It is easy to see that if all $\omega_{ij} > 0$ (all excitatory synapses) or all $\omega_{ij} < 0$ (all inhibitory synapses) then there is a single state (specified by all β_i) of the neural system which minimizes the function E. These are shown in Fig. 8.

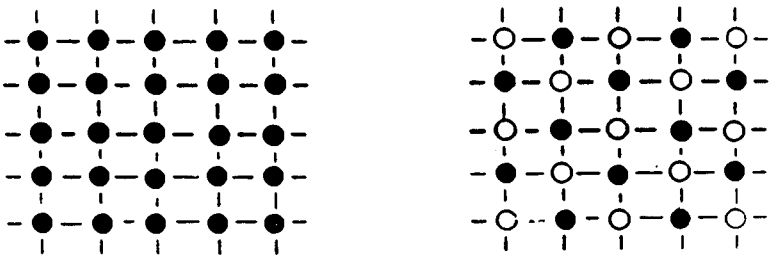

Fig. 8. A single global minimum of E is reached if all $\omega_{ij} > 0$ (at left) or if all $\omega_{ij} < 0$ (at right) (from Cowan and Sharp, 1988)

However, if ω_{ij} are random, with about half having values > 0, and the remaining having values < 0, then there are may different states which minimizes the function E locally. This is schematically indicated in Fig. 9.

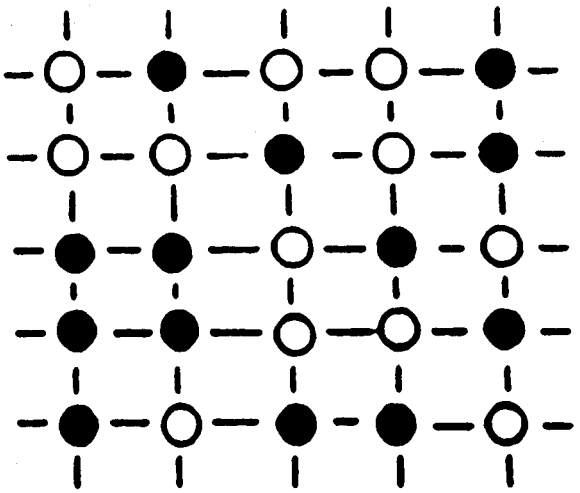

Fig. 9. Many local minima for E exist if ω_{ij} is random (from Cowan and Sharp, 1988)

It turns out that if the different minima correspond to sufficiently different states then starting from an arbitrary initial state the minimization procedure leads to the minimum state closest to it.

In view of this, if we want to store in the network M patterns corresponding to M neural states $\{\beta_i^s\}$ (s = 1, 2,...,M), then this can be done by choosing,

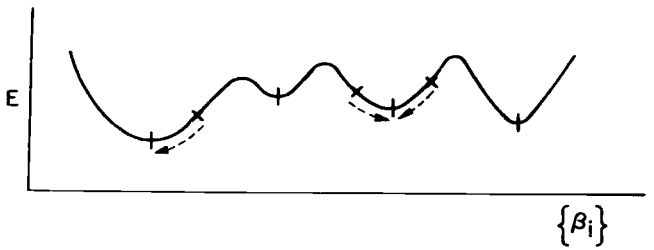

Fig. 10. Schematic illustration of local minima

$$\omega_{ij} = \sum_{s=1}^{M} \beta_i^s \beta_j^s$$

The minima of the energy function then correspond to the M patterns we want to store and any initial guess $\{\beta_i^{initial}\}$ would on minimization lead to the nearest 'local' minimum.

The beauty of this type of connectivity amongst neurons is that, information or patterns that are stored are in dynamically stable configurations. This is of immense value in artificial intelligence studies. There is a limit however to the number of patterns that can be stored. For N neurons, the number of patterns M $< N/4 \ln_2 N$ so that if $N = 10^6$ M ~ 13000. This is not very satisfactory and there are other kinds of ACAMs which have better performance as regards storage and retrieval of patterns (Cowan and Sharp 1988).

Adaptive Hopfield nets, having more complex structure that include "hidden" units (neurons) have also been studied. Hidden units, are those neurons in the net, which connect to other neurons, without directly receiving inputs from or providing outputs to the external environment. Thus, they are neither sensory units nor motor units. This is schematically shown in Fig. 11.

In such adaptive Hopfield nets learning can take place by modification of the synaptic weights. A crucial and significant recent advance is that using a new algorithm (Cowan and Sharp, 1988), the weights of the hidden units can also be

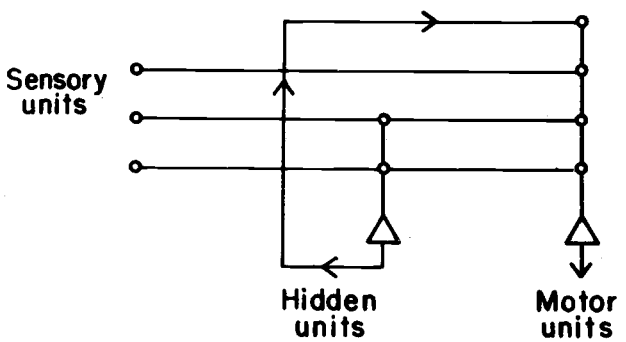

Fig. 11: A neural net with hidden units (from Cowan and Sharp, 1988)

modified. This had remained an outstanding problem in AI studies for many years, but with its solution many new applications have been made. Further in another very important recent development, it has been shown that, through learning, the weights ω_{ij} that are created, provide a distributed representation of correlations that exist between the various stimulus patterns. This implies that through these correlations the notion of a 'concept' – or abstraction – can be defined. Using this definition of an abstract concept, one can classify the various patterns and obtain relations between classes of patterns – clearly a major advance in AI studies.

It turns out that such adaptive Hopfield nets (or multilayer perceptions) are still quite slow and hence the algorithms work for small problems. It also has a problem of trapping, wherein a given pattern of activity is repeated without solving the given task. Sometimes, it so happens that, after training on a large number of patterns, one more pattern leads to a catastrophe – the net loses memory. Hence there are still unresolved questions related to the stability and reliability of the net. An interesting solution to some of these difficulties has been proposed where the rules for generating adaptive nets are themselves subject to adaptation (Cowan and Sharp 1988).

Summary and Outlook

The important themes that have emerged from the studies of neural nets may be listed as follows.

- McCulloch-Pitt (MP) net – logic and simple arithmetic
- distributed information – redundancy
- plasticity, learning, memory (adaptive MP nets, perceptrons)
- associative content addressable memory (ACAM) – model of cerebellum
- Hopfield net – information in dynamically stable configuration
- adaptive Hopfield nets (multilayer perceptrons) – abstraction.

A fundamental question which arises out of all these developments is "How far can one continue in this manner?" In other words, can there eventually be machines (computers) that can think, feel and have a mind?". These questions are not easy to answer and actually at the present time there is a raging controversy going on with regard to them.

One viewpoint, termed the strong AI viewpoint argues that higher brain functions involve implementation of suitable algorithms, and hence these functions actually reside in appropriate software packages. Thus, if one has the right software packages for machines, then it would not be possible, by operational tests, to distinguish between computers and human beings, and hence if human beings think, feel and have a mind so do computers.

The opposite viewpoint claims that hardware (in the case of human beings the "brain") is actually very important. The 'hardwiring' in the brain, according to them, reflects the basic intent and logic of the "designer". According to this

opinion, evolution (of life) or natural selection has primarily acted as a 'critic' to hardwire the network and not as trainer to software them.

If appears however that this controversy will continue for quite some time, but I am sure it will be an exciting one to follow.

There is another question, of greater relevance, for understanding of higher brain functions. This is – whether the present day physics, mathematics, chemistry are adequate for "explaining" the mind. Penrose (1949) very eloquently presents his ideas on this matter. He suggests that quantum aspects, limitations imposed by Gödel's theorem and many such considerations may be necessary but even then we would need to discover a new physics of the mind for the purpose.

Acknowledgement

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Membrane Ionic Channels and Neurological Disorders

S.K. SIKDAR

Membrane ionic channels

Membrane ionic channels are transmembrane proteins with hydrophilic transmembrane segments through which solutes of appropriate size and charge can pass by simple diffusion.

There are broadly two classes of ionic channels; some being continuously open, and others which open transiently, and these are said to be "gated". Of the gated channels, some respond to an extracellular ligand binding such as a neurotransmitter (example, acetylcholine, GABA) to specific receptors on cell surface and are called "ligand-gated" channels. Others open in response to changes in transmembrane potential and are called "voltage-gated" ionic channels (Fig. 1).

The opening and closing of channels involve conformational changes of the transmembrane channel protein which occur in response to ligand binding or by changes in transmembrane electric field (Hille 1984).

Voltage-dependent gating of Na^+ and K^+ channels

The voltage-dependent gating of membrane ionic channels is studied by a direct electrical method called the "voltage-clamp". In this method, the membrane potential is controlled electrically by a feed-back amplifier, while the ionic movements through the channels are recorded directly as electric currents flowing across the membrane. This method was applied to the squid giant axon membrane in the classic experiments of Hodgkin and Huxley (1952 a,b).

By resolving the recorded currents into individual ionic components by changing the ionic solutions that bathe the membrane, two types of ion selective channels-

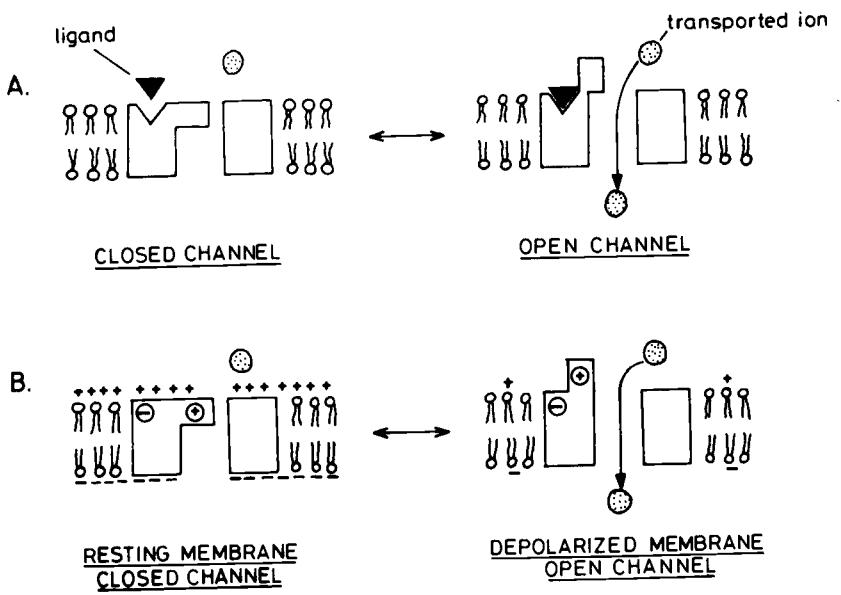

Fig. 1 Two types of channels in biological membranes. A. Ligand-gated ionic channel, and B. Voltage-gated ionic channel showing transitions from closed to open state.

Na^+ channels and K^+ channels which show voltage-dependent gating were identified. The kinetic model of the opening and closing states explained the current waveforms obtained on application of a voltage step (V) in the positive direction (depolarization) from the resting state (R).

The Na current is inward (0→ –ve) and is characterized by a rapid rise in the current amplitude (activation) from a closed state, followed by return of the trace to baseline value (inactivation) during the pulse application (Fig. 2A).

The K current on the other hand is an outward current (0→ +ve) and shows a slow activation from a closed state but no inactivation follows (Fig. 2B).

The conductance of the channel is given by the Ohm's law relation,

$$I = g(E-E)_{ion}$$

where, I = ionic current amplitude
g = conductance
E = membrane potential
E = Equilibrium potential for the particular ion

The Na and K channel conductance changes adequately explain the action potential waveform of the nerve fibre, caused by a depolarizing stimulus. Essentially, it begins with a transient opening of voltage-gated Na^+ channels that allows the Na^+ ions to enter the fibre and depolarize the membrane, followed by voltage gated opening of K^+ channels that repolarize the membrane.

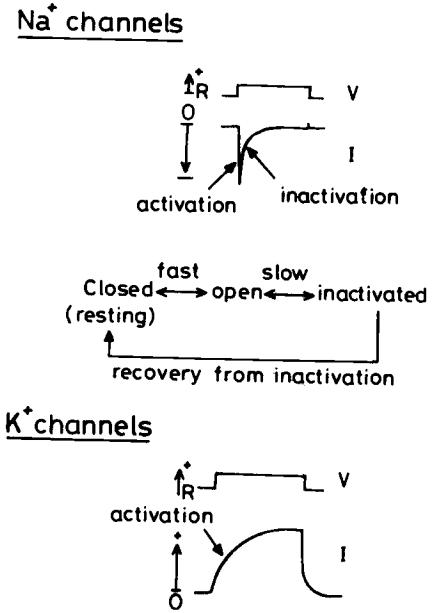

Fig. 2 Ionic current characteristics of voltage-gated Na^+ and K^+ channels. On application of a depolarizing voltage pulse (V) from the resting state (R) the current through the Na^+ channels is inward showing rapid activation and slow inactivation, followed by recovery from inactivation. The different states are represented schematically.
The current through K^+ channels is outward and shows slow activation and almost no inactivation on application of a similar depolarizing voltage pulse.

Apart from the delayed rectifier K^+ channel whose current shape has been described, there are other K^+ channel subtypes such as the aminopyridine-sensitive transient K^+ channel, and the $Ca2^+$ sensitive K^+ channel.

In addition to the Na^+ and K^+ channels, neuronal membranes also contain voltage-gated $Ca2^+$ channels. Three subtypes have been identified i.e., L, T and N type depending on voltage activation threshold, inactivation kinetics and pharmacological blockade by dihydropyridines.

The voltage-clamp technique has undergone much improvisation since the initial application to squid giant axon. Following the description of the ionic currents in the squid giant axon, attempts to record similar currents from single neurons were made by introducing two glass microelectrodes onto a single neuron under voltage-clamp. Such studies were however possible only in large sized invertebrate neurons and could not be applied to the small sized vertebrate neurons. This became possible with the recent advent of the "patch-clamp" technique (Hamil *et al* 1981). This technique allows voltage-clamp of small neurons with a single electrode. The most important aspect of the technique is that its sensitivity allows the measurement of current flow in a single ionic channel. In this technique, a glass micropipette with a fine polished tip of diameter less than 1 μm or

less is pressed against the membrane of the cell. An electrical seal in the giga-ohm range is created by the tight contact between the annular zone of contact of the cell membrane with the microelectrode tip, defining a tiny patch of the cell surface, and the ionic channels present in the patch can be detected by the current flowing through it. This is the "cell-attached" mode. The membrane spanning the microelectrode tip can be disrupted by application of suction through the electrode, and this permits access to the cell interior, and the intracellular ionic content is dialyzed by the aqueous salt solution in the micropipette. This is called the "whole-cell" recording mode.

In the following paragraphs an application of the patch-clamp technique to understand the etiology of a neurological disorder attributed to effects on ionic channels will be discussed. For this the Lambert-Eaton myaesthenic syndrome will be taken as an example to illustrate the dysfunction at the ionic channel level associated with this condition (Vincent *et al* 1989).

Lambert Eaton myaesthenic syndrome

Lambert Eaton myaesthenic syndrome (LEMS) is a neuromuscular transmission disorder with clinical presentation of proximal muscle weakness and a reduction or absence of tendon reflexes. It is also associated with dysfunction of the autonomic nervous system characterized by dry mouth, sexual impotence and constipation. In about 60% of the cases it is associated with small cell lung carcinoma (SCLC). LEMS is an example of paraneoplastic disorder i.e., a disorder which is due to the remote effect of cancer. One of the causative mechanism for a paraneoplastic disorder is immunological cross-reactivity between the tumour and components of the self. A neurological paraneoplastic syndrome manifests due to sharing of antigens between the tumour and the nervous system.

Physiological features of LEMS

Microelectrode recordings from biopsied LEMS intercostal muscles by Elmqvist and Lambert (1968), to understand the electrophysiological basis of clinical observations, revealed that the number of ACh-containing quanta released per nerve impulse was markedly reduced, reducing the end plate potential (EPP) amplitude, resulting in failure to achieve the threshold depolarization required for generating an action potential in the muscle membrane.

Immunological basis for LEMS

Evidence for an immunological basis for LEMS came from passive transfer of LEMS. Purified IgG from plasma of LEMS patients when injected daily at 10 mg/day for 30-99 days resulted in significant reduction in the mean quantal content of the EPPs in diaphragms of mice (Wray *et al* 1984).

Role of cancer and antibodies to voltage gated Ca channels in the etiology of LEMS

Patients with small cell lung carcinoma (SCLC) manifesting LEMS show the presence of voltage gated calcium channels in the tumour cells, and these cells

can generate action potentials (Tischler *et al* 1977). SCLC cells incubated with LEMS IgG show significantly low K^+- stimulated $Ca2^+$ influx, suggesting that LEMS patients have circulating antibodies against voltage-gated $Ca2^+$ channels (Robert *et al* 1985). The antibodies directed at the voltage gated Ca channels in the presynaptic nerve terminal are responsible for the clinical features of neuromuscular transmission disorder in LEMS. The cyclical course of events is given schematically in Fig. 3.

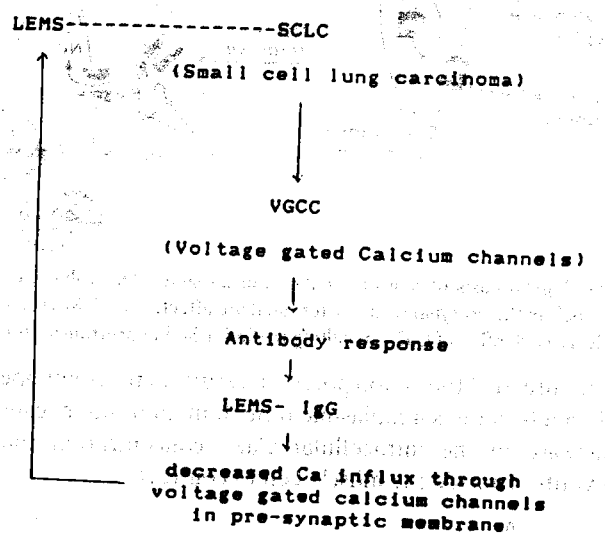

Fig. 3 Cyclical course of events leading to neuromuscular transmission disorder in Lambert Eaton Myasthenic Syndrome.

Physiological basis of neuromuscular transmission disorder in LEMS

To understand the physiological basis of the defect we shall first consider transmission across a neuromuscular junction where an electrical impulse travelling down the nerve stimulates a muscle to contract.

The steps as shown schematically in Fig. 4 are:

1. When the nerve impulse reaches the nerve terminal, the decrease in membrane potential transiently opens the voltage gated $Ca2^+$ channels in the presynaptic nerve terminal membrane causing influx of $Ca2^+$ and stimulating release of the transmitter-acetylcholine (ACh).
2. Acetylcholine thus released acts on the ACh receptor of the muscle membrane. ACh receptor is a ligand-gated channel permeable to Na^+ resulting in localized depolarization of the muscle membrane.
3. Following this localized depolarization, voltage gated Na^+ channels in the muscle membrane open, allowing Na^+ entry, resulting in further depolarization of the muscle membrane which causes further opening of Na^+ channels resulting in action potential generation which then spreads across the whole cell membrane.

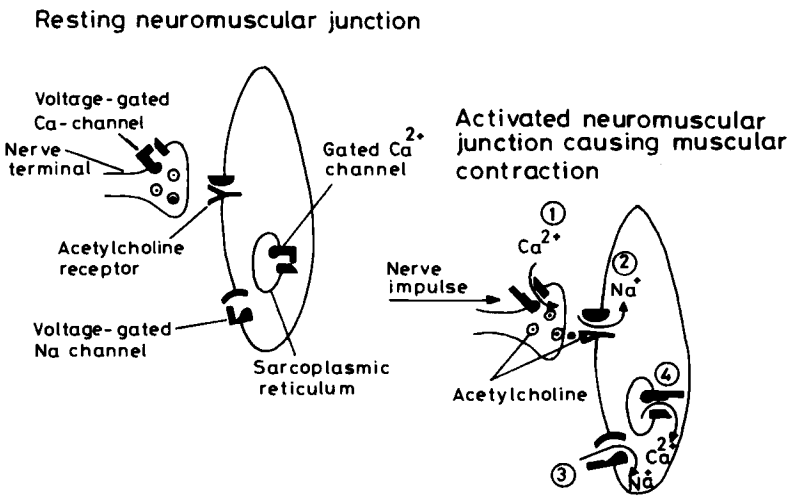

Fig. 4 Physiological basis of neuromuscular transmission. The voltage gated $Ca2^+$ channels in the presynaptic nerve terminal are affected in LEMS such that there is decreased influx of $Ca2^+$, resulting in diminished neurotransmitter release.

4. The spread of the action potential results in transient opening of $Ca2^+$ channels in the sarcoplasmic reticulum membrane, causing a sudden increase in the intracellular $Ca2^+$ concentration that causes the myofibrils inside the muscle cell to contract.

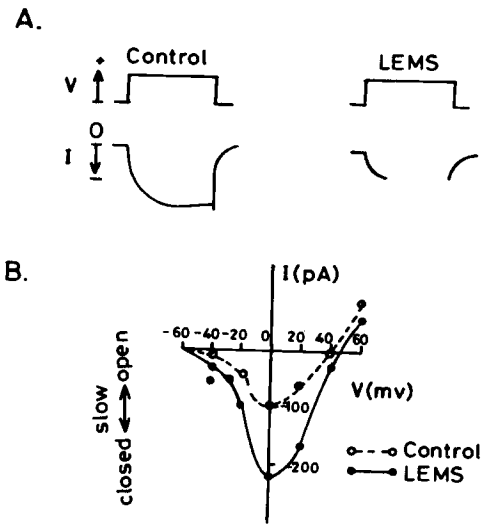

Fig. 5 Schematic diagram showing the whole-cell recording of $Ca2^+$ current in adrenal chromaffin cells with control and LEMS IgG. A. Upper traces, voltage pulse, lower traces are inward $Ca2^+$ currents. V, voltage; I, current. Treatment with LEMS IgG results in diminished $Ca2^+$ current amplitude, suggesting that LEMS IgG block $Ca2^+$ influx through voltage-gated $Ca2^+$ channels. B. Current-voltage plot of $Ca2^+$ current at different potentials in control and following LEMS IgG treatment to show the decreased current amplitude over a range of potentials.

Circulating antibodies in LEMS seem to recognize an epitope that is present in the voltage gated $Ca2^+$ channels, and reduce the $Ca2^+$ currents characteristic of L-type $Ca2^+$ channels. Fig. 5 is a schematic representation of the suppression of $Ca2^+$ current in bovine adrenal chromaffin cells treated with control and LEMS IgG, studied with the patch-clamp technique (Kim and Neher 1988).

The study illustrates the potency of the patch-clamp technique to elucidate the molecular mechanism of a paraneoplastic disorder resulting from an autoimmune response directed against the voltage-gated $Ca2^+$ channels.

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Pathophysiology of Cerebral Ischaemia

JACOB ABRAHAM AND D. R. THEODORE

The phenomenon of "signalling" in cerebral ischaemia is vital and an example of one signal which I would like to dwell upon, and which highlights the point that there are inbuilt safeguards in most threatening situations, is that which follows hemodynamic changes in an artery.

We know that the perpendicular force exerted by blood flow in an artery is borne mostly by the proteins elastin and collagen in the vessel wall and the tangential or shear stress falls entirely on the endothelial cell. Lansman (1988) postulated that the endothelial cell is both a flow sensor and a pressure sensor and Olesen *et al* (1988) reported a K^+ selective shear-stress activated ionic current producing hyperpolarisation not only of the endothelial cell but also of the underlying vascular smooth muscle, induced by flow change. This represents the earliest and fastest stimulus-response coupling of haemodynamic forces to endothelial cells yet found, this vasodilatation occurs without the release of the relaxing factor-Nitric Oxide. On the other hand, ionic channels which are sensitive to pressure have been demonstrated on the surface of aortic endothelial cells. These pressure sensitive channels allow most cations including Ca^{2+} to pass through into that cell resulting in the release of a vasoconstrictor endothelin. The difference in the reaction of blood vessels, dilatation or vascoconstriction, is determined by signals generated through the two different types of ionic channels activated on the endothelial surface. We found that signalling of this kind holds true also for the regional vascular bed. 30 minutes following right MCA occlusion in the monkey, widespread alkaline phosphatase activation was demonstrated in the endothelium of the regional capillaries (Theodore and Abraham 1990a, 1990b). This enzyme activation may have occurred even earlier mediated through K^+ activated channels and cAMP. This state of "recruitment" or "preparedness" of

the endothelial cells of the capillary bed is reflected in the increase in number of capillaries and maximal dilatation. Coinciding with the ischaemic but maximal dilated state of the capillaries in the ischaemic side, a significant activation of alkaline phosphatase on the opposite normal side was also seen. We are unable to conclude whether this 'recruitment' or 'preparedness' of the opposite side was due to haemodynamic changes or is a result of neuronal and neurotransmitter signalling from the ischaemic side (Theodore Abraham 1990c). Before we leave the interesting topic of signals we should be aware that some signals are protective but others signal events which if initiated will be suicidal for the cell.

To move on to events which cause brain damage in focal cerebral ischaemia. The reason I have chosen the more difficult of the two subjects – viz focal rather than global ischaemia for the present discussion is only because focal cerebral ischaemia is clinicaly more relevant than global ischaemia and delineation of mechanisms of damage could throw light on strategies which could be investigated in respect to reversal or salvage of the endangered regions.

In 1971 we detected the prevalence rate of hemiplegia to be 57 per 100,000 population (Abraham and Daniel 1972). Furthermore a prospective consecutive study of 850 cases of strokes undertaken at that time revealed a 71% incidence of focal ischaemic lesions. Based on clinical imperatives, a laboratory model of focal ischaemia was perfected in primates. The biochemical changes in our primate stroke model have already been described (Nagarajan and Balasubramanian 1989). I shall confine myself to general basic observations on the pathophysiology of cerebral ischaemia.

By virtue of the almost total dependence of the brain, on adequate circulation and substrate delivery, the slightest reduction in either oxygen or glucose is immediately associated with some failure of function. To demonstrate this precarious dependence the following experiment can be done on oneself. Close one eye, now gently compress the open eye by pressing on the eyelid and count. Usually in 6 seconds or so the vision 'greys'. This is about the time it takes for neurons to be affected if blood supply and consequently oxygen supply is reduced-the brain contains a very small oxygen reserve.

Oxidation of one molecule of glucose supplies approximately 35 molecules of ATP and it is ATP hydrolysis which maintains the electrochemical disequilibrium across the nerve membrane. Anaerobic glycolysis yields only 2 molecules of ATP per molecule of glucose whereas glycogen yields 3 molecules of ATP. ATP regeneration quickly declines and is completely arrested after two minutes (Hossmann and Kleihues 1973).

The total energy consumption of the brain, expressed as the cerebral metabolic rate (CMR) of either oxygen ($CMRO_2$) or glucose (CMR gluc) can be considered as a sum of the activation metabolism and the residual or basal metabolism. Activation metabolism supports synaptic transmission, whereas residual metabolism equals the oxygen and glucose consumption in the brain after synaptic transmission has been abolished, i.e. after the EEG is flat. It was found that 50% of energy was used for Na^+ K^+ ATPase activity and another 50% for

unidentified processes. Therefore we have a situation of an ischaemic flow threshold of synaptic transmission failure which is around 16-20ml/100gm/min (Sharbrough 1973, Trojaborg 1973) and a critical flow threshold of membrane failure at around 8-10ml/100gm/min: at these flows the oxygen supplies are 3ml/100gm/min and 1.4ml/100gm/min respectively. It is at this lower flow rate that 'infarction' develops (Astrup 1982).

The first basic concept: critical flow thresholds

Regional CBF (MCBF) less than 20ml/100gm/min will cause electrical silence, with the cellular membranes intact but with ATP reduced. This area is designated the penumbra region.

Regional CBF less than 10ml/100gm/min causes membrane disruption and this is the core ischaemic area which will become an infarct.

The second basic concept: time

The effect of rCBF a little less than 20ml/100gm/min for less than 2-4 hours is reversible while the effect of rCBF less than 10ml/100gm/min for more than 2 hours is irreversible.

The third basic concept: reflow

Reflow is both useful and harmful.

The fourth basic concept: diaschesis

Areas remote from the area of ischaemia are affected by a phenomenon called diaschesis.

The fifth basic concept: factors

There are factors which modify extent of infarction and irreversibility. Mitigating factors are reflow, hypothermia, immaturity of brain and inhibition of cerebral metabolism. Factors which worsen outcome are reflow, hyperglycemia, increased metabolic demand and inadequate energy production.

The sixth basic concept: mitochondrial viability

Mitochondrial viability determines whether ischaemia becomes infarction. This is reflected by the fractional extraction of oxygen (OEF) and $CMRO_2$. As $CMRO_2 = CBF \times OEF$, $CMRO_2$ is determined by the degree of ischaemia and the capacity of a rising OEF to compensate for it. Once the threshold of $CMRO_2$, approximately 67 μmol/min/100gm has been breached, irreversible infarction occurs.

Following arterial occulsion, there is a core region with no flow and hypometabolism which eventually dies. Around this area, a penumbra region with reduced flow, hypermetabolism (Shiraishi 1989) and increased cerebral glucose utilization (Nedergaard 1988) is observed. This increased glucose utilization may be due to continued depolarization. In this region of "misery

perfusion" (Baron 1981) there is a reduction in oxygen and glucose supply inspite of a preserved near normal oxygen consumption (Powers and Raichle 1985). This results in gradual and progressive energy depletion (Siesjo 1978). The extent of depletion depends on the severity of ischaemia. The flow threshold for ATP reduction is 12 to 14ml/100gm/min and when it reaches 4-5ml/100gm/min, ATP and Pcr almost completely disappear. With extensive reduction of ATP there is a Na^+ pump disruption (Naritomi 1988). A simultaneous increase in extracelular K^+ ensues, indicating membrane failure (Astrup *et al* 1977). During this time of depolarization extracellular Ca^{2+} enters the neurons through voltage dependent Ca^{2+} channels (Hansen 1985) and NMDA operated channels (MacDermot 1986). Since the ATP dependent calcium pump does not function adequately during ischaema there is an elevation in intracellular Ca^{2+}.

In addition, the increased intracellular sodium ion and free fatty acids stimulate release of Ca^{2+} from mitochondria (Cheung *et al* 1986). This results in a further increase in intracellular calcium which can increasingly bind to calmodulin (Picone *et al* 1989) and activate target enzymes such as kinases, proteases and phospholipase which destroy the membranes, intracellular organelles and liberate lysosomal enzymes.

If this flow level persists for 2 hours or more a large infarct is produced (Jones *et al* 1981). However, Yanagihara *et al* (1985) demonstrated an alteration in cytoskeletal sturctures even as early as 5 minutes following occlusion of common carotid artery.

Simultaneously, due to the energy crisis, there is accumulation of metabolites like lactate, adenosine, inosine, hypoxanthins, aspartate, glutamate, taurine, GABA, alanine and DA in the extracellular fluid (Hillered *et al* 1989). Acidosis and adenosine can improve blood flow and prevent cell death by inhibiting neuronal firing. On the other hand the released neurotransmitters depending on the prevalence of the type of receptors on the cell can excite the cell and stimulate calcium inflow and deplete the meagre amount of ATP available or inhibit the activity of the cells and protect it against ischaemic assault. Thus the selective vulnerability of the cells to ischaemia depends on the type of receptors they have.

Now that the ground plan is outlined a closer look at specific deleterious factors is necessary. About 72 years ago Haldane (1919) observed that anoxia not only stops the machine but wrecks the machinery.

Free radicals

A free radical is an atom, a group of atoms or a molecule with an unpaired electron in its outermost orbit accounting for its extreme reactivity (Cord 1985). The free radicals of importance in ischaemia include superoxide ($O_2^{\bullet}$) and hydroxyl ($OH^{\bullet}$). The latter is more toxic. Free iron or other transition metals allow hydrogen peroxide (which readily crosses membranes) to generate the more toxic $OH^{\bullet}$ from $O_2^{\bullet}$. ($H_2O_2 + O_2^{\bullet} \rightarrow O_2 + OH^- + OH^{\bullet}$). As free radicals

* denotes free radicals

are produced in small amounts by normal cellular processes a variety of defence mechanisms have also evolved. Alpha tocopherol (Vit. E), Vitamin C and superoxide dismutase have been accepted as the most common neutralisers of free radicals. Two additional protective enzymes, catalase and glutathione peroxidase destroy the H_2O_2 which superoxide dismutase produces when neutralising $O_2^{\bullet}$ molecules. Glutathione peroxidase also detoxifies lipid hydroperoxides.

The lack of O_2 leads to reduction of components of the mitochondrial electron transport system like flavin adenine dinucleotide and co-enzyme Q(CoQ) which reacts with the minimal O_2 available to autoxidise and produce $O_2^{\bullet}$ radicals. CoQ (reduced) + $O_2 \rightarrow$ CoQ + $O_2^{\bullet}$. The free radicals thus produced react with and damage proteins, nucleic acids, lipids and extracellular matrix. The polyunsaturated fatty acids (PUFA) are particularly vulnerable and membranes, which have a high concentration of PUFA are among the earliest of casualties.

The free radical attacks the blood vessels of the ischaemic area with as much devastation as the brain parenchyma. The arachiodonic acid released from membrane phospoholipids during ischaemia would be acted upon by O_2 which may become available from a trickle flow of blood to produce $O_2^{\bullet}$. As ATP is depleted hypoxanthine accumulates. In the meantime xanthine dehydrogenase (XDH) is converted to xanthine oxidase (XO), probably by Ca^{2+} activated protease calpain, which catalyzes the conversion of hypoxanthine to uric acid forming $O_2^{\bullet}$ as a byproduct. The $O_2^{\bullet}$ is immediately scavenged by superoxide dismutase but with the creation of H_2O_2, which can react with $O_2^{\bullet}$ as a byproduct. The $O_2^{\bullet}$ is immediately scavenged by superoxide dismutase but with the creation of H_2O_2, which can react with $O_2^{\bullet}$ in the presence of iron, to form the more lethal $OH^{\bullet}$ Xanthine dehydrogenase is abundantly present in the microvessel of the blood brain barrier (Betz 1985). The relevance of these free radical mechansims to human hypertensive encephalopathy, loss of autoregulation in ischaemic tissue, ischaemic brain edema or vasospasm following subarachnoid haemorrhage is uncertain but of potential interest (Schmidley 1990).

Ionic events

In the few minutes following ischaemia there is a dramatic increase of potassium and a lowering of sodium, chloride and calcium concentrations in the core region, whereas in the penumbra, there may be repeated episodes of the above changes (like a spreading depression) which may induce energy failure by stimulating metabolism-a sort of flip-flop phenomenon. Details of ionic events will be mentioned in the subsequent discussion.

Acidosis

Earlier studies permit the following generalisations to be made (Siesjo 1988)

(a) When ischaemia or hypoxia is accompanied by excessive accumulation of lactate, the outcome of the insult is clearly aggravated. Such accumulation is observed if the plasma (and tissue) glucose con-

centrations are raised in the period proceeding complete ischaemia, or if a continued blood supply delivers additional glucose to the tissue during the insult, as is the case during incomplete ischaemia or hypoxia/ischaemia in fed or glucose infused subjects.

(b) Deleterious hyperglycaemia can be elicited in the well fed individual simply through mobilisation of glucose from the body glycogen stores during the stress response triggered by ischaemia.

(c) Tissue damage associated with excessive lactate accumulation is characterised by the appearance of post ischaemic seizures, edema and extensive neuronal necrosis. This damage can be immediate or delayed.

In most experiments involving hyper and hypoglycaemia in anoxia/ischaemia, brain damage may take one of the two extreme forms, selective vulnerability and pan-necrosis. In the former only neurons are injured. In the latter both glia and vascular cells are affected as well. Selective neuronal damage is seen in hypoglycaemia and occurs without acidosis. On the other hand acidosis rather than lactic acid is perhaps responsible for the pan-necrotic lesion.

The actual mechanisms involved are; (i) edema formation, (ii) calcium related damage and (iii) iron catalysed free radical changes. Cellular edema occurs either because new osmoles like lactate and H^+ are formed within the cell, or osmotically active substances Na^+ and Cl^- enter from the extracellular fluid (Kimelberg *et al* 1982). The increased intercellular osmolarity (by 50-80mOsm/kg) (Hossmann 1982, 1985) could cause rapid swelling during reperfusion, with stretch induced rupture of plasma membranes. Furthermore swelling of vascular endothelium and perivascular glial cells would hinder re-perfusion and produce a "no-reflow phenomenon" (Ames *et al* 1968).

Influx of osmotically active substances: Na^+ can enter through conductance channels gated by glutamate and other excitatory amino acids. 'Symporter' i.e., a membrane device that translocates Na^+ and Cl^-, the ion pair into the cell. Symporters of this kind can either transport $1Na^+$ and $1Cl^-$, or $1Na^+$, $1K^+$ and $2Cl^-$. Since it forms a gate for entry of Na^+ plus Cl^-, it represents a threat to the cell, a potentially troublesome "osmotic leak". The third mechansim is made up of two coupled antiporters, exchanging external Na^+ for interval H^+, and external Cl^- for internal $HCO3^-$. The net result of ion translocation by coupled antiporters is the influx of Na^+ and Cl^-, with osmotically obliged water, i.e. swelling (Boron 1983).

Cell volume therefore is the resultant of 2 variables, pump and leak. Molecular mechanisms exist that provide tentative explanations for the association between acidosis and edema. These encompass stimulation of coupled Na^+/H^+ and Cl^-/HCO_3^- ion exchangers (increased leakage) and inhibition of oxidative phosphorylation (decreased pumping) by low pH.

Seisjo (1988) suggested that the adverse effect of a low pH was due to denaturing of proteins and nucleic acids. This seems a viable suggestion, but the question

which requires to be decided is-what are the adverse reactions triggered by acidosis per se? These may be (i) allowing a greater Ca^{2+} influx, (ii) triggering free radical reaction by decompartmentalising of protein bound iron. There are fairly good reasons to accept that ischaemic brain damage is primarily due to a lowering of the pH, though it still remains to be explained why a lowering of pH from 7.0 to 6.5 causes dramatic exacerbation of free radical formation *in vitro*, whereas accentuation of brain damage *in vivo* appears to require even more acidosis.

Role of Ca2$^+$

It is established that calcium (Ca^{++}) has a pivotal role to play in neuronal function. Therefore abnormalities of Ca^{++} homeostasis will jeopardise cellular integrity considering that extracellular Ca^{++} is 10^{-2}-versus-an intracellular concentration of $<10^{-7}$. The many roles, Ca^{++} may play in pathophysiology of ischaemic brain damage is being slowly unravelled. One major role is the production of neuronal hyperexcitability which would enhance the mismatch between energy demands and substrate delivery.

The intracellular Ca^{++} is rigidly controlled by a variety of mechanisms, because an increase of intracellular Ca^{++} from $<10^{-10}$ to $10^{-7}/10^{-6}$ ascribes messenger status to ionized calcium. The major control mechansims are given below.

(i) Na^+/Ca^{++} antiport pump (electrogenic with ratio of approximately 3:1). The direction of Ca^{++} flow is dependent on Na^+, which is again dependent on Na^+/K^+ ATPase pump-which is jeopardised in ischaemia.

(ii) Ca^{++}-ATPase enzyme which pumps out intracellular Ca^{++} and is regulated by calmodulin which is a high affinity calcium binding protein (Penniston 1983).

(iii) Mitochondria: the glycoproteins of the outer plasma membrane, phospholipids along the inner aspect and endoplasmic reticulum bind large amounts of calcium (Inesi 1985). The endoplasmic reticulum appears to be the major source of Ca^{++}. 1,4,5 triphosphate is the most likely candidate as the primary signal for the release of intracellular Ca^{++}. Phosphatidyl inositol 4,5-biphosphate breakdown into diacylglycerol also serves to activate protein kinase C which may allow Ca^{++} to enter through ion channels. The importance of protein kinase C route is because neurotransmitters like acetylcholine, histamine, serotonin may also utilize this channel (Meyer 1989).

The operating of voltage or receptor operated Ca^{++} channels is one of the major mechanisms allowing Ca^{++} influx from the extracellular compartment and hence the activation of Ca^{++} dependent processes.

In ischaemia calcium related cell damage is the final common pathway to explain irreversibility. Under anaerobic conditions the rapid decline in ATP would result in failure of Na^+/K^+ pump which ultimately causes membrane depolariza-

tion which opens voltage dependent Ca^{++} channels. There is also an associated role of excitatory aminoacids which cause early cellular death attributable to an influx of Na^+, Cl^- and H_2O and a delayed death secondary to intracellular Ca^{++} release. There is evidence to suggest that Ca^{++} may be responsible for some of the pre-injury changes which may take place in the initial stage. It precipitates metabolic cascades which include activation of phospholipase A_2 resulting in gross increase of free fatty acids and membrane disruption allowing neurotransmitter exocytosis and altering receptor function. The free-fatty acid will become a source of free radicals. Due to the increased intracellular Ca^{++} the mitochondria will accumulate Ca^{++} which is one of its functions, but due to the lack of energy its final binding cannot be effected, resulting in mitochondrial damage. This may be the beginning of the end of the cell. Ischaemic seizures may be related to Ca^{++} influx especially in the neurons of CA1 (Meyer 1989).

Furthermore, calcium triggered events may influence vascular activity, causing spasm at the level of the major vessels and inducing changes in the permeability of the blood-brain barrier.

Role of neurotranmsitters and modulators

Further interest in neuronal self-destruction has been fostered by the observation in a number of models that a process of delayed neuronal death can progress for days after brief ischaemia (Pulsinelli *et al* 1982, Smith *et al* 1984) and may involve excitotoxins, such as glutamate and aspartate acting via N-methyl-D-aspartate (NMDA) receptor to initiate an accumulation of calcium ion in the cytosol via agonist operated channels (Rothman and Olney 1986). Because the transition of glutamate from neurotransmitter to neurotoxin may depend on the elimination of the voltage-dependent Mg^{++} block of the NMDA receptor channel by local interference with glucose and oxygen delivery as shown in cultured cerebellar neurons (Novelli *et al* 1988), progressive microcirculatory flow shut down could act in concert with excitotoxins to produce delayed neuronal death. That is, these two potential mechanisms for delayed death may not be mutually exclusive (Hallenback *et al* 1990).

G Protein

The family of guanine nucleotide binding proteins on the membrane dissociates to GTP bound α subunits which act on voltage sensitive ion channels and *adenylate cyclase*. If the GTPase is prevented from converting GTP to GDP a persistently activated effector system is produced. I believe that much of the cellular damage that occurs in ischaemia is due to 'signals' such as these which have lost their ability to be switched off!.

The vascular endothelium

Under normal circumstances, the endothelium presents to the blood an actively anticoagulant and antithrombotic surface, the major contributor to this being the thrombomodulin-protein C-protein S system: prostacyclin, adenosine, heparin

like molecules and a general protease inhibitor macroglobulin. All available on the endothelial surface are vasodilators and antiplatelet aggregation factors. However, in ischaemic conditions the endothelial cells which are both a source and target for IL-1 (Libby *et al* 1986) induce expression of a procoagulant tissue factor (Bevilacqua *et al* 1984), formation of platelet activating factor (PAF) (Bussolino *et al* 1986) and inhibition of protein S membrane binding interfering with the thrombomodulin-protein C-protein S system. It also produces leukocyte-endothelium adhesion molecules (Fajardo 1989). Thromboxane A2 a potent vasoconstrictor and platelet aggregator may exceed the prostacycline produced. Furthermore ischaemic endothelial cells may produce superoxide causing further detrimental effects. Again following endothelial damage, the unopposed mediators of vessel contraction may further reduce blood flow. A recently discovered 21 residue vasoconstrictor peptide isolated from endothelium and called endothelin, the most potent vasoconstrictor known to date, may also be involved (Hallenback *et al* 1990).

Thus, the intricate and interwoven mechanisms that are being elucidated in endothelial cell tissue culture confer on the blood endothelial interface the capacity for a variety of cell damaging reactions during ischaemia and reperfusion (Hallenback *et al* 1990).

Reperfusion injury

The role of Ca^{++} and free radicals have been briefly alluded to above. The role of leukocytes and cholesterol will be mentioned here:

Leucocytes

Leucocyte accumulation during ischaemia and reperfusion has been demonstrated. The leucocytes produce reperfusion injury to the blood vessels and brain parenchyma by blocking capillaries and producing rheologic effects. Weiss (1989) described an intricate interaction between nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase-derived oxygen metabolites and granule-based toxins that would have been established millions of years ago during evolution. Superoxide, generated by the neutrophil, is rapidly converted to H_2O_2. The H_2O_2 is converted by myeloperoxidase to hypochlorous acid ($H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$). HOCl can combine with granule based proteinases and enable neutrophils to subvert all the intrinsic and host-erected barriers that normally serve to protect host tissue from injury. By inactivation of a series of key proteinase inhibitors and the simultaneous activation of latent proteinases, neutrophils can create an environment in which elastase, collagenase and gelatinase are able to exert destructive effects more efficiently and with greater specificity than could be achieved by oxidants alone.

Cholesterol

Golino *et al* (1987 a,b) have demonstrated in acutely hypercholesterolemic, nonatherosclerotic rabbits that the infarct size and areas of non-reperfusion were considerably larger. They subsequently demonstrated that there was a four-fold

increase of platelets in the injury zone. The work suggests that the adverse effects of acute hypercholesterolemia on reperfusion injury are platelet dependent. Monocyte adherence to endothelium has been observed to increase upto 50 fold during acute hypercholesterolemia, raising the possibility that this cell type also participates (Joris *et al* 1983).

This sketchy outline from the literature of the myriad happenings at the cellular level following ischaemia perhaps underscrores the last point which requires emphasis, which is, any therapeutic intervention must be 'holistic' in approach to be effective. And unless we can determine all the specific 'wreckers' at work in all the constituents of the ischaemic area and the sequence and site at which they are most destructive, our efforts at altering the inexorable path of the cell to self-destruction may at best be minimal.

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Cerebral Ischaemia: Methods of Brain Protection

G.K. AHUJA

The current review will be restricted to brain protection in focal cerebral ischaemia which differs from global ischaemia in several ways due to major difference of the existence of the collateral circulation in focal cerebral ischaemia. The delivery of glucose under anaerobic conditions leads to complex metabolic changes. The existence of collateral circulation creates a 'penumbra'- an area around the infarcted area in which the neurons are not completely damaged and are capable of recovery if circulation is promptly restored. The concept of such a penumbra has been validated by PET and SPECT studies.

Pathophysiology

Meyer *et al* (1987) provide an excellent review of the subject. The normal cerebral blood flow (CBF) is approximately 53ml/100gm/minute. Brain electric failure occurs below 15-18ml/100gm/mt. At this level there is attenuation in EEG. Somatosensory evoked potentials are suppressed at CBF below 15ml/100gm/mt. At CBF below 10ml/100gm/mt. ionic failure occurs. There are alterations in extracellular potassium, intracellular calcium, liberation of free fatty acids, disturbances of water content of brain and rapid depletion of ATP. There is intracellular acidosis and irreversible neuronal damage. It is presumed that in ischaemic penumbra electric failure occurs but not ionic failure and hence there is potential for recovery.

The metabolic changes occur when CBF falls below 10ml/100gm/mt. There is depletion of ATP and accumulation of lactic acid. Increase in extracellular potassium depolarises neuronal membranes. Intracellular calcium increases and is believed to be the final pathway leading to cellular death. Increase in intracellular calcium activates phospholipase A and C which attack membrane phos-

pholipids. This in turn results in accumulation of free fatty acids, prostaglandins, leukotrienes and free radicals. The end result is vasoconstriction, increased membrane permeability and further degradation of cell membranes. Accumulation of lactic acid and intracellular acidosis leads to denaturing of proteins, glial oedema and production of free radicals.

Ischaemia and resultant metabolic changes produce cerebral oedema. Cytotoxic oedema which is due to involvement of perivascular glial cells develops early and further reduces blood flow. Vasogenic oedema follows cytotoxic oedema and is due to endothelial damage. There is break down of the blood-brain barrier. Extravasation of plasma into extracellular space leads to oedema. Lactic acidosis plays an important role in production of cytotoxic oedema. Cerebral oedema produces increase in intracranial pressure with all its secondary effects like compression and displacement of brain structures and further reduction in CBF.

Methods of brain protection

The overall incidence and mortality from stroke has declined (Whisnant 1983) but mortality in the acute phase has not changed significantly (Garraway *et al* 1983). The clinicians have also been concerned about morbidity and disability in those who survive an acute ischaemic stroke. Therapeutic measures which are aimed at reducing the brain damage after an ischemic insult and those which aid in the recovery of reversibly damaged neurons in 'penumbra' and thus reduce the morbidity will be reviewed. Some of these measures are of historic importance others have only minimal effect and still others are currently in investigative stage undergoing clinical trials.

Systemic measures

Many patients are unconscious and measures required for the care of comatose should be instituted. Special care should be taken to keep airway patent. Oxygen inhalation by nasal catheter or mask is not needed. Optimal cardiac output should be maintained. Davis and Sundt (1980) demonstrated that increasing collateral blood flow to the ischaemic area by inducing an increase in the systemic blood pressure may be beneficial. However, experimental studies have provided controversial results (Fenske *et al* 1978). The measure has not found clinical application for fear of converting an ischaemic infarct into a haemorrhage infarct and increasing cerebral oedema. If a patient with cerebral ischaemia is detected to have hypertension, the pressure should be lowered gradually (unless very high) and systolic pressure maintained at mildly elevated level. There is experimental evidence to suggest that hyperglycaemia increases intracellular acidosis (March *et al* 1986). Pulsinelli *et al* (1983) have demonstrated that patients with ischaemic stroke who have hyperglycaemia with or without established diabetes do not do as well as those with normal blood sugar levels. A corrollary of these findings would be that patients with acute ischaemic stroke should not be infused with glucose solutions-a common practice in many hospitals.

Barbiturates

Barbiturates have been shown to exert a protective action in experimental cerebral ischaemia. The proposed mechanisms through which barbiturates act include selective decrease in active metabolism, reduction of cerebral oedema, scavenging of free radicals, prevention of production of free fatty acids and improvement in microcirculation. From the work of Selman *et al* (1982) it is apparent that to be effective barbiturates must be given within an hour after occlusion of a vessel. The problem of respiratory depression, altered sensorium and equivocal results in clinical setting have precluded routine use of these agents. The optimal dose has also not been worked out for review see Michenfelder (1981).

Mannitol

It has been demonstrated that mannitol attenuates ischaemic damage to neurons if given before and after occlusion of middle cerebral artery (Little 1979). Neurosurgeons make use of intraoperative mannitol before vessel occlusion. Mannitol is believed to exert protective action through reduction of cerebral oedema, improvement in microcirculation and scavenging of free radicals. In experimental model of focal ischaemia it has been demonstrated that mannitol stabilises cortical blood flow in ischaemic penumbra.

Corticosteroids

Since cerebral oedema is considered to reduce CBF and produce secondary effects like herniation, corticosteroids have been used to reduce oedema. Donley and Sundt (1973) have shown that corticosteroids do not attenuate cerebral oedema of focal cerebral ischaemia. Corticosteroids failed to show any beneficial effect in patients with acute stroke in a double blind study (Patten *et al* 1972).

Free radical scavengers

The subject has recently been reviewed by Schmidly (1990). A free radical is any molecule with unpaired electron in its outer orbit. Because of an "open" or "half" bond, the free radicals are highly reactive and have a tendency to initiate and participate in chain reactions. Free radicals damage nucleic acids, lipids and extracellular matrix. Polyunsaturated fatty acids found in high concentration in CNS are particularly vulnerable. Superoxide and hydroxyl radicals are important in cerebral ischaemia. Consequences of cell damage by free radicals can be serious. Free radicals are produced mainly during reperfusion. This fact must be considered while planning or interpreting the results of medical or surgical procedures for revascularisation. The appreciation of potential role of free radicals in causing tissue damage in cerebral ischaemia has resulted in a search for agents which could mop up free radicals. Vitamin E is a natural free radical scavenger. Superoxide dismutase has been used to protect lungs and gut from reperfusion injury. However, it does not cross blood brain barrier and has a short half life. Alternative delivery systems by which the enzyme could reach CNS are being worked out. Using liposome trapped enzyme is one such method. The

21-aminosteroids are other agents under study. These are molecules which cross blood brain barrier after systemic administration and reduce free radical generation.

Calcium channel antagonists

Increased intracellular calcium concentration is implicated as a cause of neuronal damage and death in cerebral ischaemia. Calcium antagonist nimodipine has been reported to have beneficial effect in patients with subarachnoid haemorrhage in several controlled and uncontrolled trials. Nimodipine crosses blood brain barrier easily. In experimental studies it has been shown to reverse constriction in isolated arteries produced by serotonin, thrombin or whole blood (White *et al* 1982). Gelmers *et al* (1988) in a controlled trial of nimodipine concluded that patients with acute ischaemic stroke may benefit from early treatment with nimodipine but the therapeutic effect was limited in man. Gelmers and Hennerici (1990) reviewed pooled results from 5 randomised trials in the literature. There were 781 patients of acute stroke out of which 335 received nimodipine and 346 were controls. All patients had CT scans to exclude haemorrhage. Nimodipine 120mg per day was begun within 72 hours of acute stroke and continued for 28 days. There was less neurological impairment and death in nimodipine group than in controls. The patients above 65 years of age, with duration of stroke less than 12 hours and those who had moderate to severe stroke did better. The value of oral nimodipine 120mg per day for acute stroke was studied in a randomised double blind placebo controlled trial in the U.K. (Trust Study 1990). In this multicentre trial, 1215 patients were included. 608 patients received nimodipine and 607 placebo. The primary end point was independence after 6 months. Fifty five percent of nimodipine and 57 percent of placebo group were independent at 6 months. The authors concluded that the results do not support the case of oral nimodipine in acute stroke.

Naloxone

Claims have been made that naloxone is effective in reversing ischaemic neurological deficit (Baskin and Hosobuchi 1981). The presumed mechanisms of action include inhibition of neutrophil superoxide release, antioxidant action, antiplatelet aggregating effect, vasodilation and attenuation of cerebral oedema. Though the drug is safe the data for efficacy are inconclusive (Olinger *et al* 1990).

Anticoagulation

Seventy to eighty percent strokes are due to thromboembolism. The rationale for use of anticoagulant is their ability to interfere with thrombus formation.

The subject has been reviewed by Estol and Pessin (1990). The use of anticoagulants goes back to half a century when Hedenius (1941) reported favourable results in patients of cerebral thrombosis treated with heparin. Both heparin and oral anticoagulants have been used in all forms of acute ischaemic stroke including TIA's, stroke in progression and completed stroke. Anticoagulation as a treatment in acute stroke has remained a controversial subject.

Numerous studies, both controlled and uncontrolled, have appeared in the literature and there are strong proponents and opponents of this form of therapy. One thing which becomes clear on review of the literature is that benefit is more often seen in open, uncontrolled trials than in rigidly controlled ones. Though shrouded in controversy, one area where anticoagulants have been shown to be effective in reducing recurrent stroke is when cerebral embolism is from a cardiac source. Haemorrhage is a serious and often life threatening complication of anticoagulation therapy. It occurs despite optimal laboratory control. In authors personal view, the risks of anticoagulation are real while the benefits are doubtful and their use is not recommended except in preventing recurrence of cardiogenic embolism.

Haemodilution

Blood flow is inversely proportional to viscosity which in turn is influenced by haematocrit. Haemodilution therapy is based on presumption that reducing blood viscosity will improve cerebral perfusion and thus prevent irreversible damage to ischaemic penumbra. There is experimental evidence that haemodilution leads to increase in cardiac output and regional CBF and reduces size of infarct. Haemodilution could be hypovolumic, isovolumic or hypervolumic. Strand *et al* (1984) demonstrated beneficial effect of isovolumic haemodilution in a randomised prospective study in patients with acute stroke. Haemodilution was effected by venesection and infusion of dextran. In a more recent randomised multicentre trial, it has been observed that patients who were given this therapy within 12 hours of onset of stroke improved. The authors concluded that hypervolumic haemodilution needs further evaluation (Scan Stroke Study Group 1988).

Perfluorocarbons

These are low viscosity oxygen carrying agents and improve microcirculation and oxygen supply to ischaemic areas (Peerless *et al* 1981). Infusion of 20 percent Flusol DA has been shown to improve ischaemic deficit in patients with subarachnoid haemorrhage (Handa 1982). High concentration of oxygen in the inspired is required for saturation of these agents. The question of toxicity has not yet been resolved.

Thrombolytic therapy

It is reasonable to conclude that early recanalisation occluded vessel and restoration of blood flow will lead to significant recovery. Recanalisation can be achieved by clot lysis. In 1960s fibrinolytic agents like streptokinase and urokinase were used for the purpose. These agents are not clot specific and their use had to be abandoned because of high incidence of systemic and intracranial haemorrhage. Now with improved understanding of pathophysiology of reperfusion, availability of clot specific fibrinolytic agents and their success in limiting the size of myocardial infarction, interest in these agents has been rekindled. In an open multicentre trial using recombinant tPA of 71 patients, it has been demonstrated that complete or partial recanalisation can be obtained in all

patients (del Zoppo 1990), Three out of 71 patients studied died due to parenchymatous haemorrhage and haemorrhagic transformation of ischaemic infarct occurred in 27 per cent patients.

Del Zoppo (1990) has after an exhaustive review of the subject concluded that though early intervention with recombinant tPA may result in rapid thromobolysis, the true clinical relevance of these agents must await further human studies. Another point which must be borne in mind that establishment of perfusion will lead to generation of free radicals which have a deleterious effect. To obtain optimal results recanalisation may have to be combined with use of free radical scavengers.

NMDA antagonists

Recently excitatory amino acid neurotransmitters like N-methyl-D-aspartate (NMDA) have attracted a great deal of attention. It is believed that these transmitters play a role in pathophysiology of several neurological disorders including ischaemic damage and their antagonists may have a protective role. Simon *et al* (1984) have produced some evidence that NMDA antagonists may protect against ischaemic brain damage. However, more work is needed to define the value of these agents.

From the foregoing it is clear that as yet no satisfactory method is available for protection of brain against ischaemic insult. However, many new and exciting ideas and hypotheses are being tested and hopefully in not too distant future clinicians will have more effective therapy for patients with acute ischaemic brain damage.

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Imaging for Cerebrovascular Disease

R.D. LELE

Cerebrovascular disease is the third leading cause of death after heart disease and cancer in developed countries. In addition, the economic impact of stroke survivors in the form of loss of work and extended hospitalization is devastating.

Modern imaging techniques help in demonstrating the effects of reduced or absent blood flow to regions of the brain, as well as the source of the trouble (commonly extracranial). This article will discuss cerebral angiography with contrast media, MRI angiography, CT, MRI, SPECT, and PET.

Cerebral angiography

Cerebral angiography performed by selective extracranial injection after trans-femoral catheterization remains the most reliable method of assessing the cerebrovascular system. It can:

(1) Detect ulcerative lesions, severe stenosis, and formation of mural thrombus at the carotid bifurcation,

(2) Directly visualize atherothrombotic disease or dissection in the siphon and intracranial vessels,

(3) Demonstrate collateral circulation around the Circle of Willis and the cortical surface,

(4) Show embolic occlusion of cerebral branch vessels.

Although angiography cannot measure blood flow directly, it reflects relative pressures in the major vessels and can suggest compromised flow in the internal carotid system. Computerized enhancement techniques have improved the resolution of angiography and reduced the amount of dye injection necessary.

The risks of cerebral angiography are, aortic or carotid dissection and embolic strokes. Various large series report complication rates ranging form 2-12%. Rarely cholesterol microemboli from aortic arch atheroma can cause watershed cerebral infarction and renal failure. A skilled angiographer and careful attention to hydration can reduce these risks. The value of glucocorticoids in preventing ischaemic complications in patients with recurrent headaches or history of migrainous phenomena is not established. Some experts prefer the less risky technique of brachial artery injection instead of selective intracranial angiography from transfemoral catheterization.

Intravenous Digital Subtraction Angiography (DSA) is developed to circumvent problems inherent to arterial catheterization. However, it's poor resolution and the need for large amounts of contrast material have made this technology obsolete.

The ultimate aim of investigations is definitive treatment. In future interventional radiologists will navigate into small vessels in the distribution of the anterior, middle and posterior cerebral, vertebral, basilar, external carotid or spinal circulation to treat lesions; for instance microvascular embolization treatment of AVM with polymerizing glues, occlusion of aneurysms or fistulae (carotid-cavernous or vertebral-venous) with detachable balloons attached to microcatheters. Non detachable balloons may be used to detect and treat certain cases of vasospasm secondary to subarachnoid hemorrhage. Pre-operative embolization is often used to reduce vascularity in tumours (e.g. meningioma, glomus jugular, angiofibromas) prior to surgical removal.

Transcranial Doppler

Recently, Doppler technology has been developed to allow analysis of intracranial blood flow in the middle and anterior cerebral artery stems, the distal internal carotid and ophthalmic arteries and in the basilar and vertebral arteries (transcranial doppler). Such studies of intra-cranial arterial flow are particularly helpful in assessing collateral flow in the Circle of Willis, identifying middle cerebral artery stem stenosis or vasospasm, and documenting the direction and velocity of blood flow in the vertebral and basilar arteries.

Non-invasive testing is most helpful in assessing the carotid artery in patients with middle or anterior cerebral artery stroke or TIA of uncertain cause, in following the progress of carotid stenosis. Transcranial Doppler can give an assessment of intracranial pressure (ICP) by deriving it from blood flow velocity to which it is inversely proportional. Head injury and stroke patients can be monitored for raised ICP and thereby ensured against undertreatment or overtreatment for raised ICP.

MRI angiography

Flowing blood in vessels gives a different MRI signal than other tissues. This provides the basis for MRI angiography without the need for injecting iodine contrast material. The state-of-the-art MRI machines are capable of giving good images of the carotid, vertebral and basilar arteries and their intracranial branches (Fig. 1).

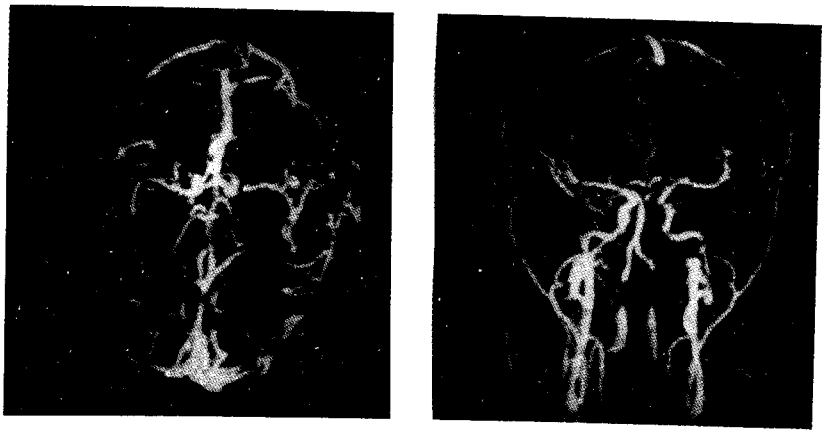

Fig. 1 Vascular structures in head and neck depicted by MR angiography-cross sectional and coronal planes.

Stenosis of vessels with turbulent flow artifact above the stenosis, vascular lesions including encroachment upon the lumen by atheromatous plaques in the carotid and vertebrobasilar system can be well delineated by this technique.

Cerebral aneurysm and arteriovenous malformations can also be well delineated by MRI angiography.

MRI and CT imaging

Computed X-ray Tomography (CT) provides a sensitive method for evaluating suspected lesions in the CNS. It is often the procedure of choice whether or not MRI is available and may also be complementary in circumstances where MRI is available. It's principal utility is when rapid information about the state of the CNS is desired and it is particularly important for decisions related to emergent surgical versus medical management of patients with the sudden onset of a neurological deficit. Such conditions include acute head or spinal trauma, stroke where a differentiation between haemorrhage and infarction is important and other instances where a decision as to immediate operative intervention is important. CT continues to have an advantage over MRI in emergency settings in patients with acute neurologic deterioration. It has high specificity particularly for the demonstration of acute haemorrhage where its imaging capacity exceeds that of MRI. CT is also the better imaging method for fractures of the face, temporal bone and the base of the skull.

Imaging in stroke

The extent and location of infarcted brain tissue can be assessed by CT. In the supratentorial regions small 0.5-1 cm lacunar infarcts can be visualized. In addition, CT scans immediately exclude haemorrhage as the cause of focal stroke

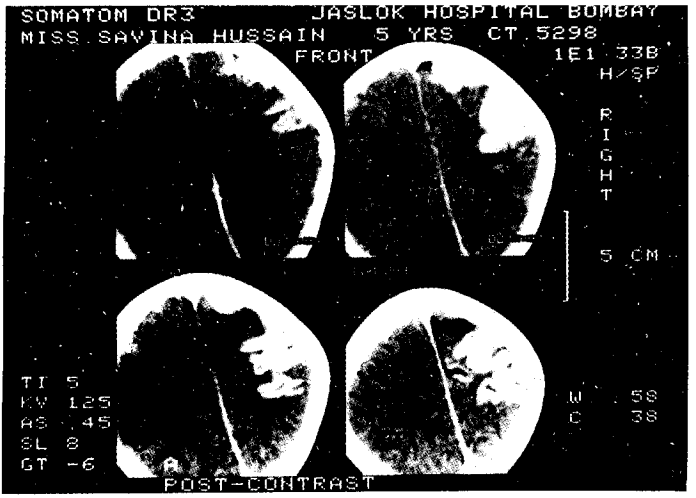

Fig. 2 Post-contrast study of high parietal brain cuts shows finger-like gyral type of enhancement in the right high parietal area, showing infarction.

and can detect surrounding edema, and less consistently, haemorrhagic infarction (Fig. 2).

It must be remembered that CT *cannot* detect most ischemic infarcts for *atleast 48 hours* after the onset. It does not reliably identify infarction of the cortical surface gray matter at any age. CT is even less reliable in detecting ischaemic infarction in the brainstem (vertebrobasilar territory) because of bone and motion artefact and the small size of many infarcts.

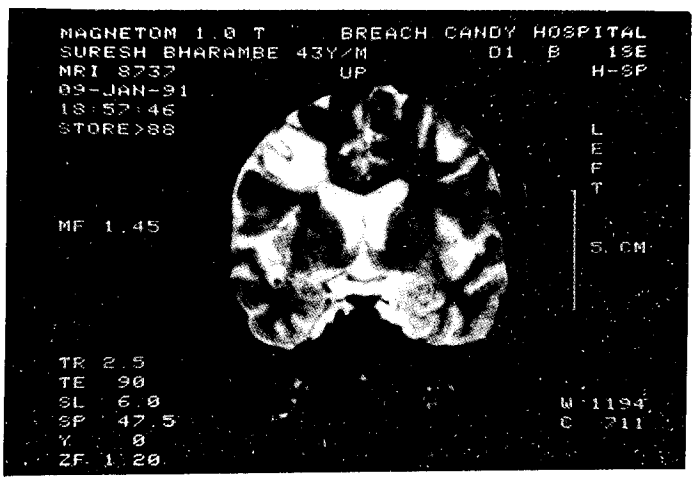

Fig. 3 Coronal T2 weighted image shows hyperintense lesion in the right parietal area, showing recent infarct.

MRI imaging reveals more than CT in patients with stroke within 6 hours of onset. MRI can identify the extent and location and infarction on the cortical surface (Fig. 3) and small lacunar infarcts in the posterior fossa. Due to its ability to differentiate between degradation products of hemoglobin viz., reduced hemoglobin, met-hemoglobin, hemosiderin etc. MRI can differentiate between haemorrhagic and non-haemorrhagic infarcts much earlier than CT (Fig. 4).

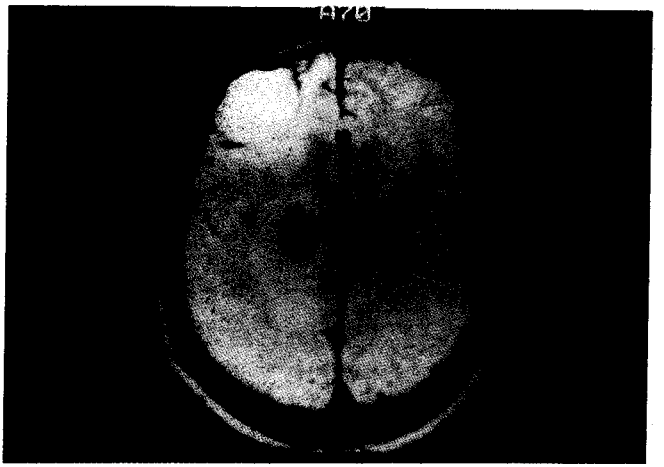

Fig. 4 Axial T2 weighted image shows anterior hyperintense lesion (fresh infarct) with a posterior hypointense lesion surrounded by hyperintense area (old infarct) in the parietal region.

Due to the very high contrast resolution of MRI, focal ischemic lesions in cases of TIA are seen which regress totally on follow-up studies. Subcortical areas of reversible ischemia are identified in suspected cases of migraine (Fig 5a, 5b) so also white matter infarcts (Fig. 6).

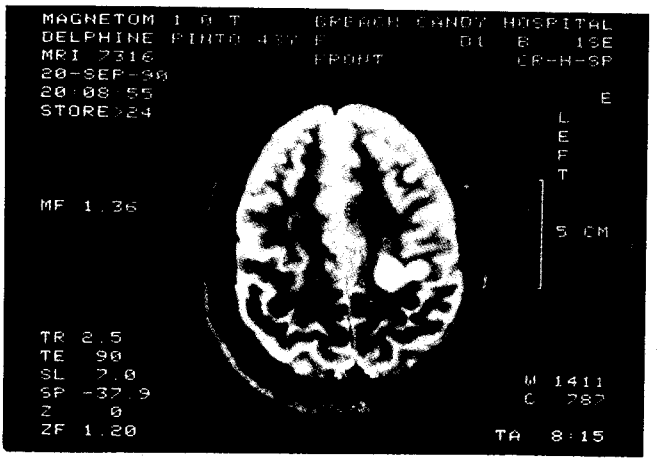

Fig. 5a Axial T2 weighted image shows hyperintense lesion in the left parietal area in a case of migraine, during an attack.

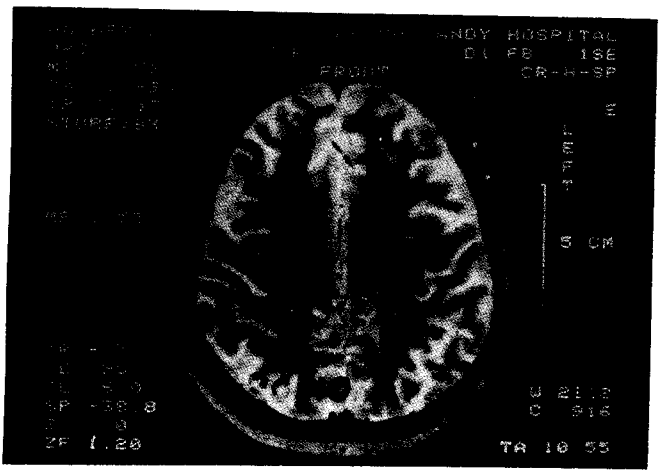

Fig. 5b Axial T2 weighted image shows complete resolution of the left parietal lesion, in the same patient within one month.

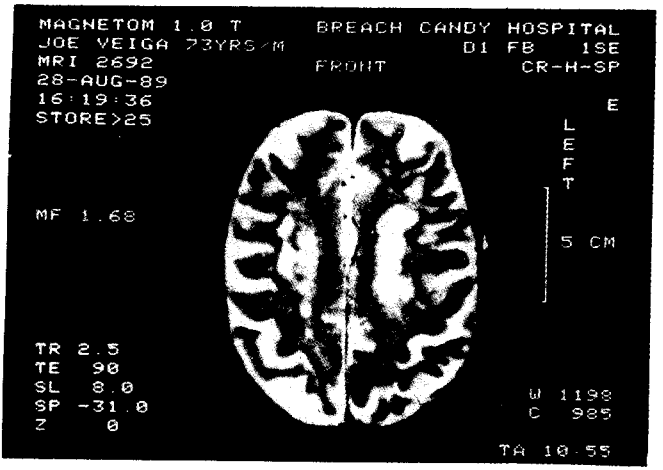

Fig. 6 Axial T2 weighted image shows multiple hyperintense areas bilaterally in the periventricular region-white matter lacunar infarcts.

SPECT imaging

Single Photon Emission Computed Tomography (SPECT) is a new imaging technique wherein a rotating gamma camera (single head or 2 or 3 heads) acquires data of the distribution of a gamma-emitting radiotracer like Technetium-99m HMPAO (Hexa Methylene Propylene Amine Oxime), and makes a computer reconstruction of the same in axial, sagittal and coronal planes, or a 3D display (Fig. 7, 8, 9).

Tc-99m HMPAO and I-123 iodoamphetamine are lipophilic substances that can cross the blood-brain barrier and their distribution reflects the regional blood flow at the time of tracer injection.

SPECT imaging in strokes

A most dramatic difference between emission tomography (SPECT) and transmission tomography (X-ray CT) occurs within the first day or two after an acute cerebral infarction. CT and MRI images which rely on changes in anatomy morphology, remain normal for sometime after onset of symptoms while SPECT documents the changes in blood flow and metabolism that are present right from the onset. Perfusion SPECT accurately identifies chronic cerebral infarction and is more accurate that CT in the early phases of acute infarction. The extent of the defect is often greater on SPECT than on CT because alteration in perfusion may occur because of ischaemia (ischaemic penumbra), diaschisis or neuronal loss. Delayed imaging with I-123 iodoamphetamine differentiates peri-infarct ischaemia from central necrosis and may therefore be useful to predict outcome and to plan treatment. Serial SPECT scans may document "luxury perfusion" in the region which initially showed absence of perfusion.

About 50% of patients presenting with early clinical symptoms of dementia cannot be accurately diagnosed by clinical criteria. SPECT can differentiate multi-infarct dementia from Alzheimer's disease (AD). The perfusion defects in AD are almost always bilateral (although they may begin unilaterally and may be asymmetrical in intensity) and involve the association cortex, being most severe in the posterior temporoparietal lobes.

Blood flow changes in seizures

Almost half of the patients with focal epilepsy fail to respond to medical management. Neither CT nor MRI is a sensitive test for detecting the site of the epileptic focus. PET is very expensive and is not available except at a few centres. SPECT identifies unilateral temporal foci in patients with intractable partial seizures who are surgical candidates. Immediately after a seizure SPECT shows a seizure focus as a region of hyperperfusion (with sensitivity 75-93%). In between seizures, the same focus is seen to be a region of hypoperfusion.

SPECT and tumour vascularity

Highly vascular tumours like glioblastomas show up on SPECT as region of hyperperfusion. With radiotherapy or chemotherapy the regression of the tumour (or otherwise) can be documented by serial SPECT studies.

Positron emission tomography (PET)

PET imaging provides quantification of regional cerebral blood flow (CBF), oxygen extraction fraction (OEF) and cerebral metabolic rate of oxygen (CMRO2) and glucose (CMRG1). In patients with stroke, PET can distinguish reversible ischaemic from irreversible infarction, which aids in selecting patients

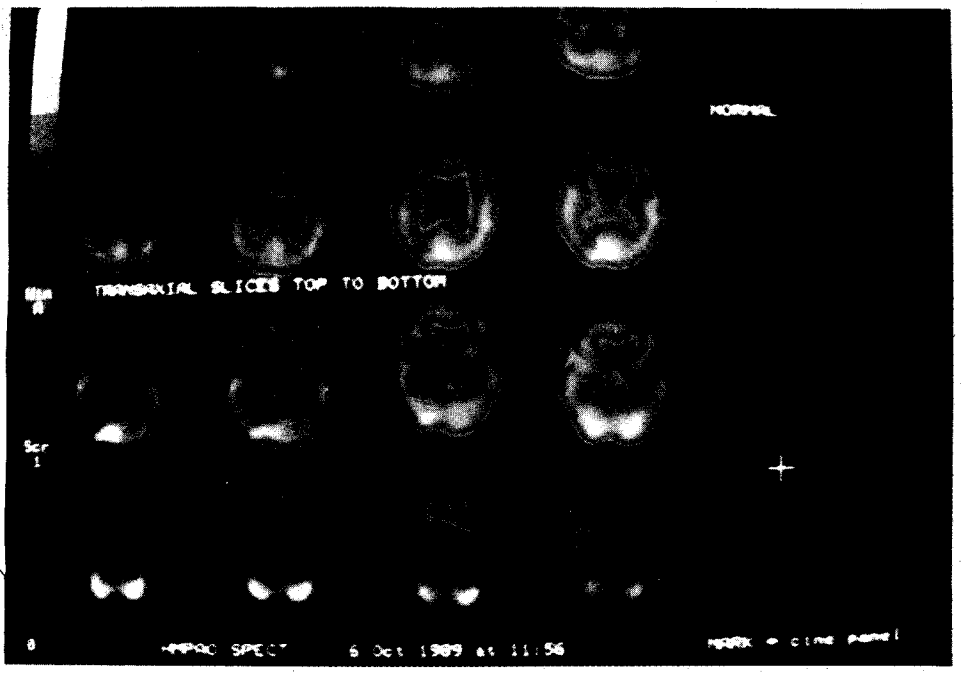

Fig. 7 Normal HMPAO-SPECT, image of brain perfusion-Cross sectional images from vertex of base.

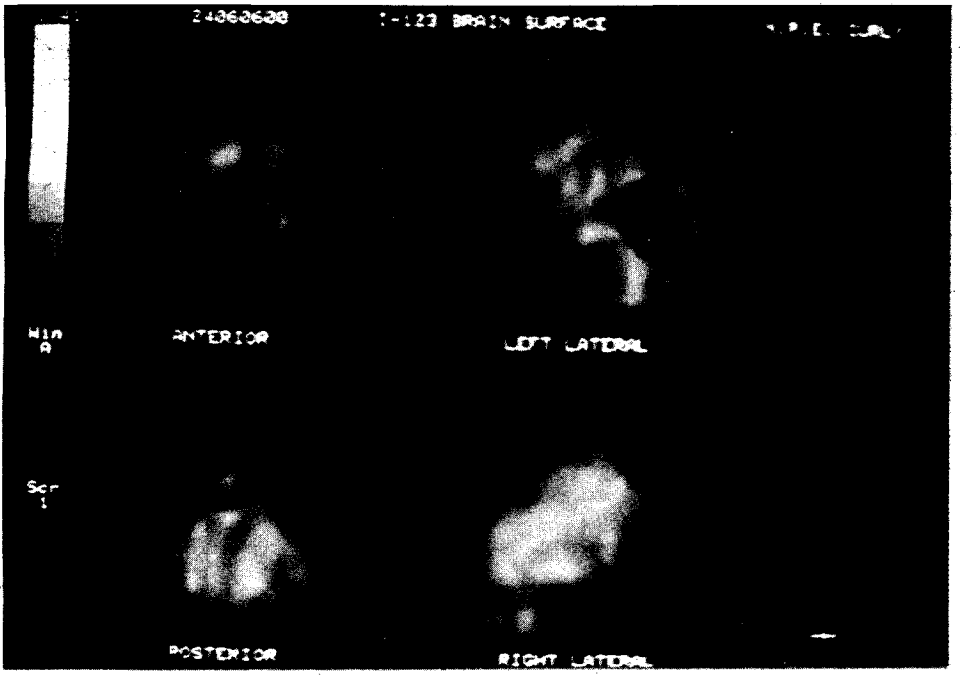

Fig. 8 3D view of SPECT brain showing large infarct in left posterior parieto occipital region.

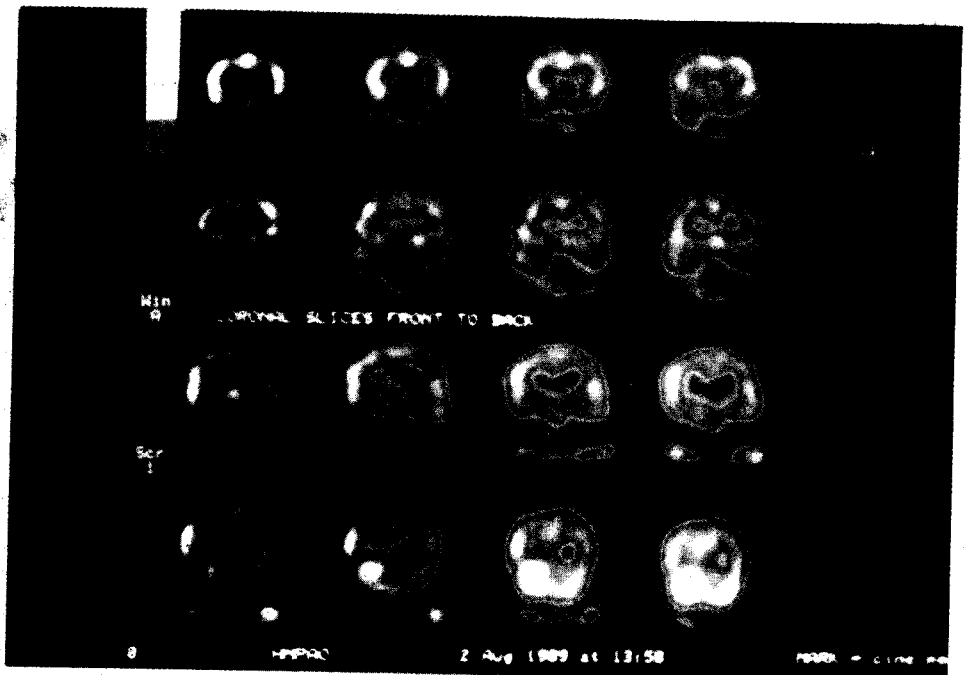

Fig. 9 Coronal views of HMPAO-SPECT of brain showing hypoperfusion of right temporal area.

for medical or surgical treatment (e.g. endarterectomy or extracranial-intracranial bypass). Measurement of regional brain blood flow and oxygen metabolism provides a quantitative assessment of the response to therapeutic interventions. In normal brain regional blood flow and metabolism are *coupled*, in patients with stroke, the two are often *dissociated.*

In patients with stroke, areas of low X-ray attenuation often displayed by CT two weeks later, are shown on early PET study as areas of low oxygen metabolism, and low blood flow. At times, reduction in oxygen metabolism is less than the reduction in the blood flow in the affected region, indicating an increased extraction of oxygen from blood. The level of oxygen metabolism within the affected region is an indication of the ultimate viability of the brain tissue and is a better predictor than regional blood flow measurement. In stroke when blood flow is reduced, vasodilatation may compensate to some degree. Further reduction in blood flow below a critical level results in a fall in oxygen metabolism, which if sufficiently severe can result in irreversible brain damage.

Oxygen metabolism is linked to glucose metabolism in the brain. In patients with stroke, the region of diminished glucose uptake as shown on F18 DG PET soon after onset of stroke is often much larger than the low attenuation zone observed on late CT. The reduced blood flow, oxygen metabolism and glucose metabolism extend out from a central core (characterized by low attenuation on late CT) and the hypometabolic regions include subcortical and cortical regions as well as the contralateral cerebellum. Such decreases of functional parameters are detectable before structural abnormalities are detected by CT and MRI.

Clinical management decisions are made easy with PET studies in stroke. Regions with low regional blood flow and only mild reduction in oxygen metabolism represent ischemic but viable tissue which deserve urgent salvage through end-arterectomy or EC-IC bypass surgery. Lesions with intermediate levels of reduced oxygen metabolism may benefit by reducing cerebral metabolic demand with anesthetics or receptor blocking drugs such as naloxone (which blocks opiate receptors). Regions with decreased oxygen metabolism below threshold levels for viability would probably not be helped by such treatment. Serial monitoring of functional parameters help assess treatment.

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Oncogenes, Anti-oncogenes and Molecular Basis of Malignant Transformation

M.R. DAS

This article essentially aims to discuss current ideas on the molecular basis of malignant transformation along with a brief description of some of the highlights of our current activities. In doing so it is proposed to trace the developments leading to the current understanding and to attempt a glimpse into future possibilities giving some examples from our own work.

An overview of our present understanding

The beginning and the models

There are a hundred or more diseases which come under the general category of cancer. However, the only common feature in all these diseases is the fact that cells divide when they should not. It was also known for the past several decades that there are essentially three aetiological agents for cancer: chemical agents, such as cigarette smoke, physical agents, such as high energy radiations, and biological agents, such as viruses. It must be emphasised at this point that viruses have been shown to be causative agents of different kinds of cancer, mostly in animal systems. They provided a very convenient system for studying basic molecular events governing cancerous transformation in comparison to cancers caused by chemical agents or physical agents. If one has to examine model systems using cancers induced by chemical or physical agents, it is necessary to overcome a major obstacle, namely, the existence of side reactions or damage that these agents caused, apart from events strictly related to malignant transformation. On the other hand, both DNA tumour viruses and RNA tumour viruses (the latter group also known as retroviruses or oncornaviruses) found to be

causative agents in a number of animal systems formed a more convenient experimental system. These viruses contain only a small number of genes. It was comparatively easier to follow the functioning of these genes after a virus infects a host. In addition, development of methodologies in growing animal cells in culture in the fifties considerably helped the design of experiments with viruses as model systems. In principle, conversion of normal cells to cancer cells using viruses in tissue culture enabled scientists to look at transformational events without the intervention of hormonal influences which are invariably present in whole animal model systems.

Although the very first virus that was suspected to be a causative agent of cancer was an RNA tumour virus (Rous 1911), in terms of understanding molecular events, greater progress was made initially with DNA viruses. It was clearly demonstrated (Sambrook *et al* 1968) that when a DNA virus enters a host, the viral DNA gets integrated with the chromosomal DNA of the host. Subsequently, viral specific mRNAs are transcribed and transformation specific proteins are made in these cells.

On the other hand, there was a conceptual difficulty in understanding the mechanisms of function of RNA tumour viruses. Retroviruses, like DNA tumour viruses, caused heritably stable transformation and this was somewhat difficult to understand although Temin, (1964a,b) had suggested the pro-virus hypothesis which invoked the conversion of information present in the genome of RNA tumour viruses to DNA. The experimental evidence provided by Temin at that time was somewhat tenuous.

In the late 60's, a number of different groups got interested in the study of retroviruses. Among these was the group of Sol Spiegelman at the Institute of Cancer Research at Columbia University, New York. He had earlier worked out the mechanism of replication of RNA bacterial viruses (bacteriophages) through RNA replicase and was interested in examining the existence of similar events in the life cycle of retroviruses. However, some of the initial experiments carried out in the second half of 1969 suggested dissimilarities in the mechanisms of replication of RNA bacterial viruses and retroviruses. A careful examination of the homology between radioactively labeled retroviral RNAs with normal and tumour DNAs from host animals, using both mice and chicken systems, demonstrated unequivocal evidence of nucleic acid homology between viral RNAs and the corresponding host DNAs (Das and Spiegelman, unpublished results). There was no indication of similar homology with RNA bacterial viruses, such as Q or MS2 with *E.coli.*, model systems that were used in studying the mechanisms of replication of bacterial RNA viruses. This was a clear indication of possible differences in the mechanism of replication of retroviruses and RNA bacteriophages and perhaps one of the first indication suggesting the existence of viral specific information in the host chromosomal DNA.

Both Temin (1970) and Baltimore (1970) demonstrated the presence of an enzyme, reverse transcriptase, in RNA tumour viruses which could use deoxynucleotides as substrates for making tri-chloro-acetic acid precipitable

polymers. These observations were confirmed and extended by or group working at Columbia (Spiegelman *et al* 1970 a,b,c). This work included the demonstration of the existence of three different types of DNA polymerising activities, RNA-dependent and DNA-dependent. In addition, the heteropolymeric nature of the DNA synthesized using reverse transcriptase reactions with endogenous templates, and the homology between the newly synthesized DNA and the template RNA were also clearly demonstrated by us (Speigelman 1970 a).

The work on structure-function relationship of reverse transcriptase yielded novel aspects about the active site of the enzyme and the template-as well as primer- binding sites (Parnaik and Das 1981, 1983). Investigations on inhibitors to reverse transcriptase by nucleotide analogues formed another aspect of the studies (Furmanski *et al* 1980). These investigations were significant and have partly formed the basis for the use of similar compounds in the treatment of diseases caused by retroviruses such as AIDS. A variety of other inhibitors were also investigated with a view to understand the mechanism of function of the enzyme (Reddy *et al* 1983, Parnaik *et al* 1983, Brahmachari *et al* 1985).

Viral and cellular oncogenes

The discovery of reverse transcriptase triggered great interest among a number of investigators and the subsequent years saw an enormous amount of activity in terms of understanding the molecular mechanism of retroviral infection and that of replication of retroviruses (Gilboa *et al* 1979, Das *et al* 1981). The intensive work in tumour virology led to another unexpected result. It was initially shown that the gene responsible for transformation in acutely transforming viruses was the oncogene appearing downstream of the *gag*, *pol* and *env* genes in the retroviral genome. But, then, it was also discovered that the oncogene is not an essential gene required for the life cycle of a retrovirus. Retroviruses which are defective in transformation do not have oncogenes and they replicate happily in host cells. However, what was surprising was that retroviruses defective in transformation, after a few cycles of replication in normal host cells became suddenly transformation competent (Bishop 1978). Furthermore, when known viral oncogenes were challenged with normal cellular DNA, they were found to be homologous to already existing sequences in the DNA (Das and Mink 1979). These observations led to the concept that the potential information for cancer or the oncogenic information is already present in the normal make up of the mammalian genome. Retroviruses, in principle, can abstract the oncogenes from normal cellular DNA and become transformation competent. When the transforming gene is present in the virus, it is known as viral oncogene (v-onc). When they are present in the normal cellular DNA, they are referred to as cellular oncogenes (c-oncs) or proto-oncogenes.

Another unexpected finding made during the course of investigations on oncogenes was that these genes were evolutionarily conserved. This observation suggested a useful role for the existence of cellular oncogenes in normal cells. In fact, there is increasing evidence that they play a role during the course of normal

development and differentiation. What is perhaps happening in the triggering of cancer is the expression of some of these genes at the wrong time either with or without mutations.

Although the major developments in our understanding of cellular and viral oncogenes initially came from the study of retroviruses, it was found soon after, that in chemically induced cancers as also in naturally occurring cancers, oncogenes could be the target site for the triggering of cancer (Shih *et al* 1979, 1981, Shilo and Weinberg 1981, Cooper *et al* 1981, Krontiris and Cooper 1981). In other words, there appears to be increasing realisation that irrespective of aetiology, the ultimate target sites for triggering transformation could be oncogenes.

Oncogenes, anti-oncogenes and co-operation among these genes

Initially the existence of proto-oncogenes was discovered through retroviruses. Using elegant molecular biological techniques, the number of oncogenes that have been discovered as of now is close to sixty (Bishop 1991). Furthermore, the definition of proto-oncogene has become more general. If manipulation of any sort generates a transforming allele (or "oncogene"), the normal counterpart of the same gene is almost automatically taken as a proto-oncogene. Generally, the cognate proteins coded by oncogenes have limited number of functions. They belong to growth factors, growth factor receptors, protein kinases, guanine binding proteins and RNA transcription factors. This observation has prompted the view that, in principle, any gene encoding a growth factor, a receptor, an intracellular signalling molecule or a protein which regulates transcription may be considered a proto-oncogene (Kahn and Graf 1986).

More recently, the role of recessive mutation in cancers has assumed significance (Sager 1989, Marshall 1991). Although no specific gene has been identified as responsible for the suppression of neoplastic growth by cell fusion, the occurrence of suppression in experiments involving fusion of normal cells and cancer cells has been taken as evidence that cancer cells contain recessive defects involved in tumorigenesis. In fact, an examination of human tumours tends to demonstrate that lesions in the proto-oncogenes and tumour suppressor genes are almost equally prevalent among human cancers (Hunter 1991).

There is thus growing evidence that cancer arises as a consequence of a number of factors and in fact, it is a multi-step process. Molecular analysis of human cancers has demonstrated the existence of multiple genetic lesions in a single cancer including chromosomal translocations, gene amplification, and point mutations, and in a number of cases, the mutational activation of an oncogene and the loss of a growth suppressor gene have been found in the same cancer cell (Hunter 1991). So, despite the fact that there has been considerable progress in our understanding of a number of genes which are involved in cancerous transformation, we are still far away in our understanding on any specific sequence of events of gene expression which may lead to a specific human cancer. Yet, there is tantalising hope that a full understanding of the functions of oncogenes and tumour suppressor genes would eventually lead to greater understanding of the

molecular events governing cancer and through this understanding to newer strategies for cancer therapies.

Some examples of our recent studies

An example of function of an oncogene

The *ras* oncogene is expressed in a number of human and animal cancers. This is one of the oncogenes that has been studied by a very large number of investigators. But the precise mechanism of its function still remains unknown. The *ras* gene codes for a 21 kDa protein which shares properties such as GTPase activity and GTP binding ability with G-proteins which play a major role in signal transduction across membranes. Because of the similarity in properties with G-proteins it has been tacitly assumed that p 21 *ras* functions in a way similar to G-proteins. However, G-proteins are generally trimeric proteins made up of alpha-, beta-, and gamma-subunits. In addition, some of the more recent results have demonstrated evidence that mutant *ras* genes which differed in their ability to bind to GTP or have altered GTPase activities were equally efficient in transformation (Finkel *et al* 1984, Lacal *et al* 1986, Der *et al* 1986). Also, protein-folding studies (McCormick *et al* 1985) showed that the GTP binding region of p21 *ras* is far away from the crucial mutations which convert c-*ras* to v-*ras*.

We have examined this problem in great detail and have discovered a new function for *ras* proteins. It has been shown that in the presence of *ras* proteins, there is a large increase in the phosphorylation of a 38-kDa protein present in plasma membrane preparation from normal liver cells (Hegde and Das 1987). Purified G-proteins like transducin, nef-HIV1 and Ef-Tu also stimulate the phosphorylation of p38 on identical residue(s) as in the case of *ras* proteins. Similar phosphorylation of p38 was observed in the presence of glucagon in a GTP dependent fashion when whole membrane preparations were used for phosphorylation reactions (Hegde and Das 1990). Phosphorylation of p38 occurs also *in vivo*. In order to check whether p38 itself is a part of a G-protein, we carried out experiments using antibodies specific to subunits of G-proteins. The results showed that p38 was immunologically identical to the beta subunit of G-proteins (Hegde and Das, unpublished data). With a view to understand the nature of the G-alpha associated with p38, we have carried out experiments using streptozotoxin and pertussis toxin. Streptozotoxin is known to deplete G_i in the liver and pertussis toxin is known to inactivate G_i. Treatment of rats with the toxins abolishes p38 phosphorylation in liver cell-membranes of the treated animals, suggesting the involvement of G_i in the phosphorylation reaction. We therefore suggest that p38 phosphorylation might play a role in modulating the activation of (a) G-protein(s) by controlling the association of beta and gamma subunits with G-alpha. Thus, *ras* proteins could influence G-protein-mediated processes by activating endogenous G-proteins through phosphorylation of the beta subunits. It has been shown that *ras* proteins can stimulate G-protein-mediated processes like inositol lipid metabolism in mammalian cells but no direct activation of phospholipase C by *ras* proteins has yet been demonstrated.

The mechanism of *ras* function suggested above could account for these observations.

There is increasing realization that the non-dividing state of cells is an actively maintained process. In this context, it may be noted that c-*ras* gene products are more efficient in p38-phosphorylation compared to products of transforming *ras*. We have also demonstrated that p38 is an evolutionarily conserved protein from yeast to man and hence it would appear that p38 and its phosphorylation is involved in the control and regulation of cell division.

Correlation of oncogene expression in chemically induced cancers

Intense search for any direct role of viruses in human cancer in the past two decades has failed to establish any such direct involvement in most human cancers. On the other hand, as most human cancers are primarily of environmental origin and could be traced to chemicals or their metabolites (ingested as trace contaminants in food or other sources), we have made concerted efforts in understanding the molecular basis of cancers induced by chemicals.

To begin with, we used a rat model system, the Zajdela Ascitic Hepatoma (ZAH), a chemically induced hepatoma adapted to grow in the peritoneal cavity of the rat. The oncogenes, *myc*, *ras* and *myb* were found to be expressed in this tumour. In ZAH, there is no evidence for translocation of *myc*, unlike in several other instances where translocation of *myc* adjacent to immunoglobulin promoter sites has been reported. Transcription of the translocated *myc* has been invoked as a possible mechanism of function of this oncogene in tumorigenesis. On the other hand, we have demonstrated that in ZAH, expression of *myc* is correlated with undermethylation. What is of added interest is that during the development of rat embryos *myc* is expressed in liver tissues and there is also concomitant undermethylation of the *myc* gene. However the undermethylation of *myc* is reversible on attaining adulthood when the cells are no longer undergoing division. On the other hand, undermethylation found in the chemically transformed tumour cell line, ZAH, is stably inherited by daughter cells (Parnaik *et al* 1987)

A tumour associated transplantation antigen from Zajdela ascitic hepatoma

The cell surface properties of cancer cells play a major role in determining several interesting properties of transformed cells. These include, their immunological properties, metastatic properties and their social interactions including properties such as invasiveness. With a view to understand some of these processes, we have also carried out studies on the cell surface proteins of ZAH. These studies have demonstrated major differences in the glycosylation of cell surface proteins of ZAH cells as compared to normal rat liver cells (Jain and Das, unpublished data).

We have also isolated and characterised for the first time a 100-kDa tumour associated transplantation antigen (TATA) from ZAH membranes. The isolation and characterisation of a TATA from a chemically induced tumour was achieved in our laboratory using elegant and novel methodologies also

developed in our laboratory (Srivastava and Das 1984). We have demonstrated use of the antigen in the partial protection of animals against tumour take following immunisation. This is an area which would require considerable future investigations.

A ribonuclease from human milk and its role in human mammary neoplasia

The Bittner Virus or the Mouse Mammary Tumour Virus (MuMTV) was one of the first oncornaviruses in which the presence of reverse transcriptase was demonstrated, following the discovery of the enzyme in 1970. By that time, it was also well established that MuMTV is propogated through the milk of the mother. Excellent classical genetics data are also available on the inheritance of MuMTV from studies using a number of high- and low- incidence strains of mice.

It is known that the age-adjusted incidence of mammary cancers in the Parsi community is about three times as much as in any other Indian community. Furthermore, among the cancers reported in Parsi women, almost 50% are breast cancer (Jussawala *et al* 1970, Paymaster and Gangadharan 1970). Because of this reason, we had in the early 70's looked for possible evidence for reverse transcriptase associated with virus like particles in human milk samples from both Parsi and non-Parsi donors. During the course of our experiments, we demonstrated the existence of a ribonuclease activity in human milk. It had also been shown that the RNAse activity present in different milk samples directly correlated with their ability to destroy the genome of RNA tumour viruses (Das *et al* 1976). This observation was followed by another study using milk samples from Parsi-, Hindu-, Tanzanian Black- and American Caucasian- women. The study essentially pointed to a reverse correlation of the level of the ribonuclease present in human milk and the incidence of mammary cancer. A striking observation was that milk samples from women belonging to the Parsi community contained only small amounts of ribonuclease or in some cases no ribonuclease at all. In order to examine the biological significance or possible role played by the ribonuclease we decided to purify and characterise the enzyme.

From non-Parsi women, a high molecular weight ribonuclease was purified (Hemavathi and Das 1985). The protein has a molecular weight of 83-kDa and it behaves like an allosteric enzyme. There is a 10-16 fold stimulation of activity with the addition of small amounts of precursors of RNA, namely, different nucleotides. It was also shown that the enzyme preferentially degrades messenger RNA or viral RNAs in comparison with rRNA's or tRNA's. Experiments are underway to examine possible protective effect of the ribonuclease and to check whether the presence of the ribonuclease in a tumour tissue could be used as a useful marker for the prognosis of human mammary cancer.

Future perspective: knowing and doing

The above account of the present concepts, of the molecular basis of carcinogenesis and the results of our more specific investigations in this area leads us to briefly discuss future prospects. To begin with, there are encouraging

attempts for testing novel types of cancer therapy on the basis of knowledge gained by the study of cancer causing viruses and oncogenes. The strategy aims at a precise attack on cancer cells to deprive them of proteins they need to divide and grow. They are in fact the first clinical fruits of intense research in the past two decades on oncogenes (Marx 1990). It has been shown that quite a few of the cancer causing genes are aberrant forms of the genes that encode growth factors and other proteins that control cell growth and differentiation. The question, then, is, could new cancer therapies be devised that work by blocking the activity of the growth factors and other proteins that oncogenes encode. Although therapies might be directed against the action of any of the oncogene products, there is some advantage at the moment to direct immediate therapies against growth factors. They usually work at the cell membrane and hence are more easily accessible. It would be harder to interfere with an oncogene product which is a nuclear protein.

There is considerable evidence available which demonstrates that many cancers including breast and lung cancers produce large quantities of growth factors (Marx 1990). Work by Marc Lippman's group at George Town University has shown for example, that breast cancer cells produce factors such as, epidermal growth factor, platelet-derived growth factor, insulin-like growth factor I and II and some of the growth factors belonging to the fibroblast growth factor family. Some of these agents are likely to contribute to the ability of the cancer cells to grow and invade new tissues. It is therefore logical to attempt locking their activity and such attempts are being made currently.

Considerable recent effort has been focussed on human mammary neoplasia. Among the oncogenes and growth factors implicated in human breast cancers, one of the most promising as a possible target for therapy, is the oncogene, *neu*. This is also known as erb B2 or HER2. The *neu* gene codes for a protein which shares several characteristics of a growth factor receptor. Scientists at the University of California, School of Medicine have demonstrated that women whose tumour cells have extra copies of the *neu* oncogene were more likely to relapse and die than patients whose tumours did not have the gene amplification. The hypothesis was that having extra copies of *neu* and therefore of the receptor it encodes, might enable tumour cells to grow and spread more aggressively than cells without the gene amplification. In addition, the UCLA group has shown that they can inhibit the growth of human breast cancer cells that have been planted into nude mice by injecting the animals with a monoclonal antibody that binds to the *neu*-receptor protein. Attempts are underway for a clinical trial of the monoclonal antibody in patients with advanced breast cancer.

What has been attempted in this article is essentially to present some of the excitements happening in the field of basic cancer research in the past couple of decades and their potential, giving some specific examples of our contribution. This account does not deal with the specific problems of neuro-oncology but reflects the general principles which may be applicable to the malignant tumors of the central nervous system. There is a whole lot that remains to be understood

and it is hoped that such understanding would lead to newer strategies for therapies, as indicated for mammary cancer.

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Growth Factors in Development of Gliomas

V.S. LALITHA

Since the work of Temin and Todaro on the reduced serum requirement of transformed cells, there is growing appreciation of the role of growth factors in the development of tumours. It has been proposed that production of growth factors by transformed cells which also possess the corresponding receptors is the cause of uncontrolled proliferation through autostimulation (Sporn and Todaro 1980, Sporn and Roberts 1985). However, the gene transfer experiments with a few growth factor gene viz. macrophage colony stimulating factor (M-CSF), platelet derived growth factor (PDGF), epidermal growth factor (EGF) demonstrate that primary cells can not be transformed though the immortalised cells turn tumorigenic, indicating the necessity for prior immortalisation. There is some recent evidence to suggest that the ability of cells to form metastases is associated with their capacity to produce autocrine growth factors (Rodeck *et al* 1987). It appears that growth factors have a causal role in the later stages of tumour progression by conferring on the tumour cells selective growth advantage (Lang and Burgess 1990). For their continuous growth , malignant tumours depend on the stroma which contains feeding blood vessels. Some of the tumour cell-derived growth factors have a stimulatory effect on the stroma, i.e. the fibroblasts and blood vessels (Neal 1987).

The growth factors belong to a family of signal molecules which play a crucial role in the regulation of normal growth and development. When they bind to their receptors on the cell surface they trigger the generation of active second messengers and intracellular enzyme cascades which are responsible for transmission of external signals from the cell surface to the nucleus for subsequent activation of genes crucial for cell proliferation and differentiation. One of the perturbations currently implicated in the tumorigenic transformation is in signal

transduction pathways at distinct levels, which are being dissected in detail over the past 10 years with a view to explore new therapeutic avenues. In a tumour cell the subversion of growth regulation may be brought about by (a) production of either abnormal growth factors stimulating aberrant growth or abnormal receptors which remain constitutively activated in the absence of their ligand, thus bypassing the need for the factors and (b) activation of post-receptor processes in the intracellular pathways of transducing and transmitting the mitogenic signal. Some of the growth factors or their receptors are encoded by oncogenes e.g v-sis for B chain of PDGF, int 2 and hst for FGF, erb B for EGF receptor and fms for receptors of colony stimulating factor (Rayter *et al* 1989).

Growth factors and abnormal glia

Several growth factors and their receptors have been demonstrated in foetal and adult glial cells. Epidermal growth factor (EGF), insulin like growth factors (IGFs), fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) are mitogens for astrocytes (Westphal and Hermann 1989). EGF and its receptors are detected on developing and reactive astrocytes. The astrocytes in the foetal and adult brain express the genes for IGFs. FGFs-acidic and basic are also produced by astrocytes. Secretion of PDGF and the presence of its receptors on astrocytes are well documented.

The role of growth factors in the development of glia has been studied elaborately in culture system (Raff 1989). It is now well established that soluble factors which are involved in controlling the schedule of appearance of oligodendroglia and astrocytes in the dissociated cultures of developing rat optic nerve have turned out to be the two previously characterised polypeptide growth factors (PDGF) and ciliary neurotrophic factor(CNTF). The adult rat optic nerve, as seen in silver impregnated preparations, contains oligodendroglia and two types of astrocytes-Type 1 astrocyte with processes terminating mainly on blood vessels or at the surface of the nerve and Type 2 astrocytes with fine longitudinal processes terminating at the nodes of Ranvier. Using antibodies against galactocerebroside (a major myelin glycolipid), glial fibrillary acidic protein (a major subunit of glial filaments) and A2B5 (an epitope present on oligodendrocytes and their precursors), it has been convincingly demonstrated that Type 1 and 2 astrocytes are derived from two different precursor cells, the former from $A2B5^-$ progenitors and the latter from $A2B5^+$progenitors which also give rise to oligodendrocytes (Miller *et al* 1985). Type 1 astrocytes appear in the rat optic nerve first on the embryonic day 16, oligodendrocytes on the day of birth and Type 2 astrocytes at the beginning of the second postnatal week. Before birth Type 1 astrocytes secrete PDGF which stimulates the proliferation of $A2B5^+$ bipotential progenitor cells (02A lineage), most of which differentiate subsequently to oligodendroglia. After birth it is believed that Type 2 astrocytes secrete CNTF-like protein which along with extracellular matrix molecules, induces the $A2B5^+$ progenitors to differentiate stably to type 2 astrocytes (Lillien *et al* 1990). 02A lineage cells are now demonstrated throughout the central

nervous system wherever the axons are myelinated, implicating the collaboration of Type 2 astrocytes and oligodendroglia in myelination of axons (Raff 1989).

Growth Factors and glioma

Gliomas are now being examined for various growth factors and their receptors.

Epidermal Growth Factor(EGF)

Originally this growth factor was isolated from the male mouse submaxillary salivary glands. A similar protein (Urogastrone) was detected in the human urine. EGF was found to cause premature eye opening and eruption of incisor teeth and also to induce maturation of foetal lung in mice, while urogastrone inhibited gastric secretion. The aminoacid composition of murine EGF and human urogastrone is similar. EGF is a 53 aminoacid peptide with Mr.6000. Though EGF has been detected in human tissues including Brunner's glands, the anterior pituitary , some cells in bone marrow , skin and its appendages, the kidney, male genital tract and milk, yet its physiological role is not understand completely (Neal 1987). EGF receptors have been identified on a variety of cells such as fibroblasts, glial cells and many types of epithelial cells. EGF receptor has three domains - an external regulatory domain responsible for binding ligands, a transmembrane portion and a cytoplasmic catalase domain with tyrosine kinase activity. The structure of the cytoplasmic and transmembrane domains of EGFr, is very similar to the protein encoded by an oncogene, v-erb-B. EGFr gene is located on the short arm of chromosome 7. The cytogenetic analysis of glial tumours has revealed in many glioblastomas overrepresentation of chromosome 7 (Bigner *et al* 1985) and amplification of EGFr gene with structural rearrangements (Bigner and Vogelstein 1990) The truncated receptor without the regulatory domain remains constitutively activated. The biochemical composition of EGFr of the tumour cells differs from the normal receptor. Recent work on glioma cell lines demonstrates that cell migration and invasion *in vitro* are related to the presence of EGF in the culture medium and EGFr on tumour cells (Lund-Johansen *et al* 1990). It appears that EGFr gene amplification is associated with advanced stage of glioma progression.

Another growth factor belonging to EGF family is the transforming growth factor α(TGF α), isolated originally from the conditioned medium of transformed cells and it binds to EGFr generating stimulatory signals. It is present in glioma tissue and cell lines (Nickell *et al* 1983 and Nister *et al* 1988). TGF α contains 68 aminoacids with a molecular weight around 7000d and has 40% structural homology with EGF. It has been suggested that EGF is secreted by cells in the resting stage while TGF is secreted during development and malignant states.

TGF β,initially found to be secreted by the transformed cells, has a synergistic action with TGF α.TGFs β are of high molecular weight and one of them consists of 2 peptide chains with Mr.12500d. TGF β is a bifunctional molecule stimulating the proliferation in some cell types and inhibiting it in the others. It is secreted by both normal and neoplastic cells. It is secreted by both normal and neoplastic

cells. It seems to have an important role in embryonic development, cell differentiation, hormone secretion and immune function. It has been demonstrated in both glial and glioma cells (Takahashi *et al* 1990). TGF β, by suppressing the function of T lymphocytes may contribute to the impairment of immune surveillance of gliomas.

Platelet derived growth factor (PDGF)

Initially isolated from platelets, PDGF is secreted by a variety of cells and it acts primarily on connective tissue cells and glial cells (Heldin *et al* 1981). It is a glycoprotein with Mr.30 Kd consisting of 2 peptide subunits - chain A (17 Kd) and B (15 Kd), which bind to two distinct types of receptors. Gene encoding A chain is on chromosome 7 and B chain on chromosome 22. B chain shares homology with a transforming protein by v-sis oncogene. PDGF and its receptors are present in many glioma cell lines. In human glioma there is overexpression of PDGF genes and occasionally the genes of the receptors. In a recent report (Hermansson *et al* 1988), mRNA for PDGF A chain is found in abundance in anaplastic glioma cells and mRNA for PDGF B chain in endothelial cells indicating a possible role of PDGF in autocrine stimulation of neoplastic glial and endothelial cells. In addition to getting secreted and acting on the receptors on the cell membrane, PDGF can also bind and activate the receptors in the cytoplasm - an ultra short autocrine loop.

Insulin-like growth factors (IGFs)

Though structurally similar to insulin, IGFs have distinct receptors and different actions. While insulin is mainly concerned with glucose metabolism, IGFS stimulate cell proliferation. IGFI which is somatomedin C, is regarded as the mediator of growth hormone action. It contains 70 aminoacids with 48% sequence homology with human proinsulin. IGF II contains 67 aminoacids exhibiting 50% structural homology with IGF I. IGF II is present predominantly in the brain while IGF I at the peripheral tissues. The receptors for IGF II are found in abundance in the glioma cell membranes. IGF family could be another autocrine system operating in gliomas(Gammeltoft *et al* 1988).

Fibroblast growth factor (FGF)

It was 40 years ago that both clinical and basic researchers proposed that there exist in the tissues, molecules which stimulate the growth of fibroblasts and blood vessels helping in the process of wound healing. Subsequent attempts to purify such factors have resulted in the identification of a family of structurally related proteins which bind to heparin. The fibroblast growth factor family includes acidic (Mr. 16000 d) and basic (Mr. 17000 d) fibroblast growth factors, the basic form being much more potent than the acidic. The gene for acidic FGF is localised on chromosomes 5 and the basic FGF on chromosome 4. Originally isolated from brain and pituitary, FGFs have been found in a variety of tissues. A number of cell types in addition to fibroblasts and endothelial cells including neural and endocrine, respond to FGFs. The protein products of hst oncogene,(human stomach carcinoma) int 2 and KS gene (Kaposi's sarcoma) have

a striking sequence homology with FGF. Interleukin-I (IL-1) is also structurally similar, with 25% homology. Many of the proteins belonging to FGF family lack the signal peptide which is important for secretion by the cell. In glioblastomas and anaplastic gliomas, vascular proliferation is a prominent finding. The tumour angiogenesis factor isolated from gliomas is found to be identical to basic FGF. In glioma tissues abundant mRNAs for basic and acidic FGFs are detected in most of the samples examined (Takahashi *et al* 1990) and the expression levels of basic FGF are greater in tumours of high grade malignancy. Glioma cells *in vitro* produce FGFs and they have the receptors (Libermann *et al* 1987, Sato *et al* 1989).

Nerve growth factor (NGF)

Initially isolated from a transplantable mouse sarcoma (sarcoma 180), NGF was found subsequently in the male mouse submaxillary salivary glands in abundance. It is a homodimer of peptide chains each with a molecular weight of 13000 d. It plays an important role in the development of neural crest-derived neurons i e . sympathetic and dorsal root ganglia. It has been shown recently that NGF has a trophic effect on the cholinergic neurons of the basal forebrain (Levi Montalcini 1987). Administration of NGF before or after transplacental exposure of ethyl nitrosourea in the rats has been shown to result in the reduction of neural tumours in the offsprings (Vinores and Perez-Polo 1980). One of the cell lines derived from experimentally induced gliomas in the rat responds to NGF by retardation in the cell proliferation and enhanced differentiation (Russell and Rubinstein 1989).

Interleukins

Interleukin 1 is a glial mitogen, interleukin-3 induces microglial proliferation and interleukin 2 modulates the proliferation of oligodendroglia. In some of the glioma cell lines and the tumour tissues mRNAs of interleukins have been detected (Lichtor *et al* 1990).

We are still in the data gathering era in neurooncology with reference to understanding of the significance of growth factors in various stages of neoplastic development. Using purified growth factors and the molecular probes it is demonstrated that secretion of some of the growth factors (EGF, PDGF and FGF) by glioma cells increase with the grade of malignancy. At the moment, it appears that these growth factors act in an autocrine fashion on tumour cells during the progression phase and also in a paracrine manner on stroma stimulating angiogenesis. In some tumours multiple growth factors seem to be operating at the same time (Pollack *et al* 1990a).

Therapeutic potentials

In order to stop the growth factor-induced cell proliferation, it is essential to understand the mechanisms of their action. When the aminoacid sequence of various growth factors is compared, the extent of homology shared among some growth factors is such that they are being assigned to gene families. For instance

EGF, TGF α and protein encoded by one of the genes of vaccinia virus belong to the same family and interact with the same receptor. Similarly TGF β family, FGF family and IGF family of growth factors share the binding sites within the respective families. When the structure of the growth factors receptors are examined, the degree of diversity is found to be far less than that of growth factors because of the convergence of the post-receptor mechanisms by which the mitogenic signals cross the membranes and reach the nucleus. The receptors can be classified into 2 major groups,(1) those linked to G protein e.g. bombesin and PDGF, (2) those with tyrosine kinase activity e.g. EGFr, Neu-oncogene protein and trk gene family. The G-protein linked receptors generate second messengers, either diacyl glycerol or cAMP or both. They activate phospholipases which are responsible for the formation of inositol phosphate required for mobilisation of intracellular calcium and also generation of diacyl glycerol-a second messenger that activates protein kinase C (PKC). One of the important PKC-mediated events in fibroblasts is the phosphorylation of protein with Mr 80 Kd leading to mitosis of the cells. Alternately there is an increase in intracellular cAMP, mediated through the synthesis of E-type prostaglandins and this results in phosphorylation of a 58 Kd protein. Thus cAMP and PKC represent separate signal transduction pathways. However occasionally there is a cross-talk between these two major membrane signalling systems. Dual mechanisms operating simultaneously have an additive or synergistic effect. The receptors with tyrosine kinase activity on activation undergo autophosphorylation generating the mitogenic signal. In cells stimulated with growth factors, over 100 genes have been shown to be activated. Of these c-fos and c-myc are the most important proto-oncogenes which are the most relevant target genes responsible for cell proliferation (Muller *et al* 1984).

The growing appreciation of growth factors, their receptors and the mechanism of their action has stimulated the interest in developing novel methods to block pharmacologically the mitogenic signals at various levels starting from the level of growth factors and their receptors through various steps in the signal transduction pathways. There are a few reports on therapeutic trials in patients with gliomas using monoclonal antibodies against EGFr. It appears that it is not impossible to design specific antagonists which would act at the cell sensitive points in the pathway of the mitogenic signal from the cell membrane upto the nucleus. Attempts are being made to block the PKC mediated pathway in glioma cells *in vitro* (Pollack *et al* 1990b).

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Experimental and Clinical Drug Trials in Cerebral Gliomas

A.K.MAHAPATRA

Cerebral gliomas constitute approximately 50% of all intracranial tumours. Even 100 years after the first surgical intervention for their treatment the prognosis for patients with malignant glioma largely remains poor (Green *et al* 1978, Walker *et al* 1980. Green *et al* 1984, Shapiro and Shapiro, 1986, Stenning *et al* 1988). Hence, basic biological research and different therapeutic approaches for their management have been major areas of research interest during the last two decades (Bloom *et al* 1986, Solero *et al* 1979, Stenning *et al* 1988, Bradford *et al* 1990). Resistance to chemotherapy is a major problem (Darling and Thomas 1980, Tofilon *et al* 1985, Shapiro and Shapiro 1986). The ideal chemotherapeutic agent is yet to be found. In order to find out the drug sensitivity animal experiments and *in vitro* studies are essential. This chapter provides an overall view on current state of chemotherapy for gliomas based on information gained from *in vitro* and *in vivo* studies , animal experiments and clinical trials in patients.

Malignant gliomas are unique tumours with several unique factors influencing their growth and multiplication in the brain. Some of those factors are blood-brain barrier, tumour-blood barrier, heterogeneous malignant cell population along with normal cells, variability in karyotypes existing in various part of the same tumour and presence of large number of cells in (C) phase of cell cycle (Shapiro and Shapiro 1986, Kimmel *et al* 1987). It is therefore vital to know the limitations of *in vitro* experiments as it is difficult to provide a microenvironment exactly similar to that of human brain (Darling *et al* 1983, Kimmel *et al* 1987 and Thomas 1988).

Study of chemosensitivity in *in vitro* glioma cell culture

In the recent years there has been great interest in evaluating the chemosensitivity in *in vitro* culture (Hoshino *et al* 1985, Darling and Thomas 1980, Tofilon

et al 1985, Thomas *et al* 1985 Twentyman and Luscombe 1987). For this, it is essential to be familiar with some commonly used terms such as cell cycle, labeling index (LI), growth curve, colony forming efficiency(CEF), clonogenic assay, multi-cellular tumour sphenoid(MTS) and methods to assess viability of tumour cells (Kimmel *et al* 1987, Darling and Thomas 1988).

Table 1: Factors which influence chemosensitivity

– Age of the patient
– Nature of the tumour
– Blood brain barrier
– Time of drug therapy
– Ratio of hyperdiploid and near diploid cells.
– Variability of karyotypes
– Nature of drug and dosage
– Mode of drug administration

Labeling Index (LI)

In respect to cell cycle estimation of cells in active mitosis is important. It is the cells in growth phase which help in growth of the tumor. Thus tumour doubling time in dependent on number of cells in mitosis. Cells in mitosis produce DNA which can be labeled with either ^{3}(H) thymidine or Bromo Deoxy Uridine(BUDR). LI is defined as the percentage of cells labeled. In glioblastoma LI may vary from 5-10% and in low grade astrocytoma this may be as low as 1% (Kimmel *et al* 1987). In a malignant glioma LI may also vary from one region to another depending on malignancy and presence or absence of necrotic or viable cells.

Growth Curve

Determination of growth curve is an important step prior to *in vitro* study of drug sensitivity. This give an impression of the degree of malignancy and rate of tumour growth. Roughly a third of the viable tumour is in active growth, and is responsible for increase in tumour size. Normally one cell cycle in malignant glioma has been estimated to be 70-80 hours and in culture cell doubling time ranges from 24 to 48 hours. For growth curve tumour cells are grown in multiwell plates and cell counting is done every 24 hours. Initially there is a phase when the cells get settled and have very little mitosis. This period is called "lag phase". Immediately following this cells rapidly multiply and grow in number, the cell count increases steadily. The phase is called "exponential phase". After a period of growth the cell multiplication diminishes resulting in a very little rise in cell count, thus providing a plateau to the growth curve(Fig. 1). From this growth curve it is easy to find out the cell doubling time *in vitro*, which helps in drug assay.

In Vitro Culture

For *in vitro* drug assay one has to have basic knowledge of *in vitro* culture. Infact there are several methods of culturing a tumour. These are (i) monolayer culture in petridish or flask (ii) multicellular tumour sphenoids (MTS) (iii) organ cul-

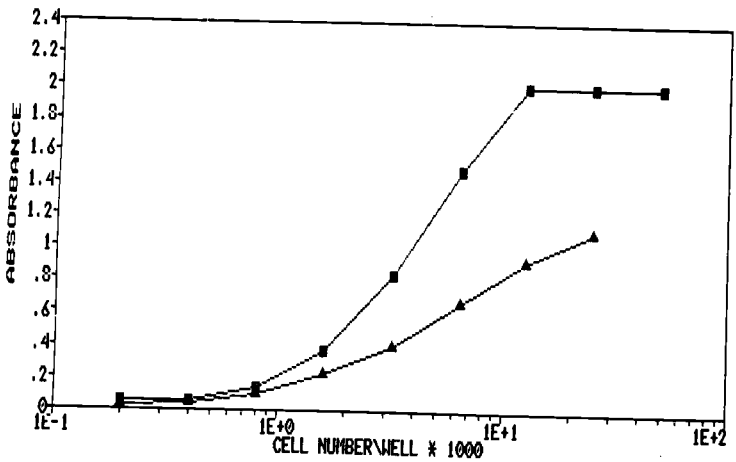

Fig. 1 Graphs showing growth curves of two glioma cell lines.

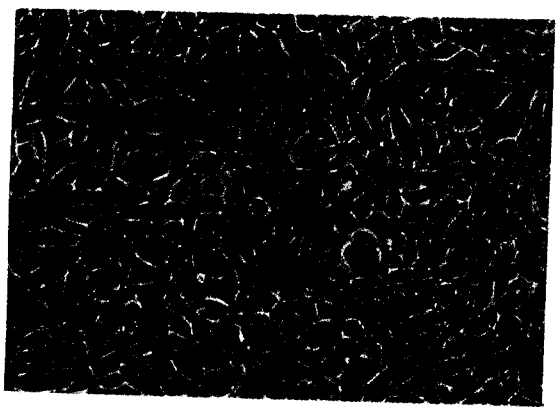

Fig. 2 High power view shows densely packed glioma monolayer grown in *in vitro* culture.

ture. Monolayer culture is by far the most commonly used method. In this method either the tumour tissue or an established cell line is allowed to grow in a flask containing culture medium enriched with calf or horse serum. Cells get attached to the bottom of the flask and grow rapidly to form a single layer of glioma cells(Fig.2). After the cell layer is densely packed cells have to be transferred to another bigger flask for further growth. This process of transfer of cells is called passage and designated (P_0, P_1, P_2) depending upon the number of times they have been transferred.

Multicellular Tumour Spheroid (MTS)

Multicellular tumour spheroids are three dimensional structures containing large number of tumour cells, which simulate the tumour growth in natural

micro-enviornment (Darling *et al* 1983, Sano *et al* 1982, Darling and Thomas, 1988). To make spheroids cells are harvested from monolayer after trypsinising the flask. The cells are inoculated into a spinner flask where cells aggregate to form a clump instead of being attached to the bottom of the flask to form a monolayer. The cell clump then proliferates to form a small tumour nodule or spheroid (Fig. 3). The alternate method of MTS culture is by Fluid overlay method. In this method tumour cells are suspended in culture medium overlay-

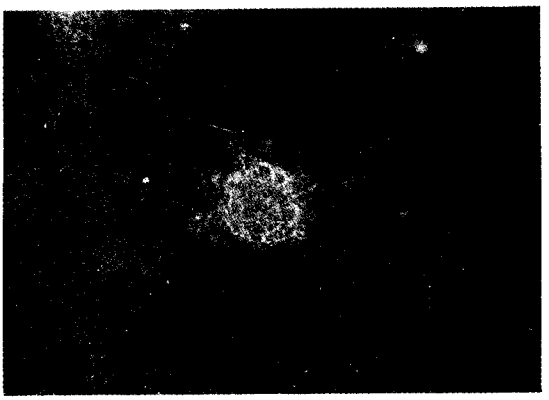

Fig. 3 High power view of a glioma spheroid (Black arrows).

ing 1% agar. As the cells do not get attached to agar they tend to aggregate and grow (Yuhas *et al* 1978). Once the spheroids reach a critical diameter a central area of hypoxia begins to develop surrounded by a rim of viable tumour cells. The viable rim of cells is constant irrespective of the size of the spheroid depending upon the distance upto which oxygen can diffuse. MTS offers several advantages over monolayer culture. It provides an intimate contact between tumour cells, cell heterogeneity, and an environment simulating tumour *in vivo*. A good correlation has also been claimed between the growth rate *in vivo* and spheroid growth rate (Stewart *et al* 1983). MTS provides an opportunity to study the effect of drug on three dimensional structure and also an unique advantage of shedding of tumour cells in different positions of the spheroid due to the drugs, which helps in assessing the chemosensitivity (Tinigawa *et al* 1982).

Effect of chemotherapeutic agents on MTS can be assessed in several ways (Darling and Thomas 1988), (i) the growth of individual spheroids is monitored by microscopy after drug treatment, (ii) drug treated spheroids are pooled and disaggregated by trypsin and plated in petridishes at low cell density and the number of colonies are counted (Yuhas *et al* 1978, Twentyman 1980). Recently chemosensitivity of cells in spheroids has been measured by sister chromatid exchange assay, which may be useful in determining the response of spheroids composed of sensitive or resistant cell population (Tofilon *et al* 1983, 1984, Doon *et al* 1986).

Assessment of viability of tumour cells

For *in vitro* chemosensitivity, assessment of cell viability is essential to find out ID 50. There are several methods to assess the viability of cells. One of them is that traditional method of counting in the haemocytometer chamber. The other method is by using electronic particle counter (Coulter Counter) or by counting colonies. Recently radionuclide assay has been used to assess cell viability using ^{35}S methionine (Thomas and Darling 1985, Darling 1985, Darling and Thomas 1988). This method is time consuming and also depends on availability of isotope and carries radiation hazards. The viability is assessed by putting ^{35}S methionine in to cells grown in multiwell plate; the viable cells take up radioactivity. Then the multiwell plate is exposed to an autoradiographic film (Fig. 4) which is later read by ELISA plate reader to find out the optical density (OD) of viable cells.

More recently 3-4, 5 dimethiazole 62, 5 diphenyl tetrazolium bromide (MTT) is used to assess the cell viability. 0.5-1% solution of this compound is added to cells grown in multiwell plate. The salt enters into the cell, gets utilized in Tri Carboxylic Acid Cycle (TCA) and is converted into an insoluble formazan crystal (Fig. 5) (Mosmann 1983, Twentyman and Luscombe 1987). Formazan crystal formed may be dissolved in a variety of chemical solvents such as dimethylsulfoxide (DMSO) and the optical density (OD) of resulting solution is measured by using a scanning spectrophotometer (ELISA plate reader) with a wave length between 540 to 570 nanometer (Twentyman and Luscombe 1987). This rapid calorimetric evaluation is a reliable method for studying cell viability (Mosmann 1983, Carmichael *et al* 1987, Denizat and Lang 1986) required for assessment of chemosensitivity. This also reduces the problem of radiation hazard and is possible even if the isotope is not available (Table 2).

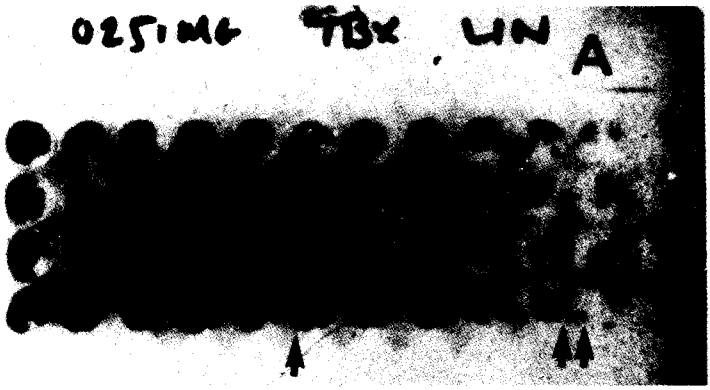

Fig. 4 Photograph of an autoradiographic plate exposed to ^{35}S methionine in a multiwell plate to assess cell viability. Single arrow shows area with more viable cells and double arrow area less dark because of less number of viable cells.

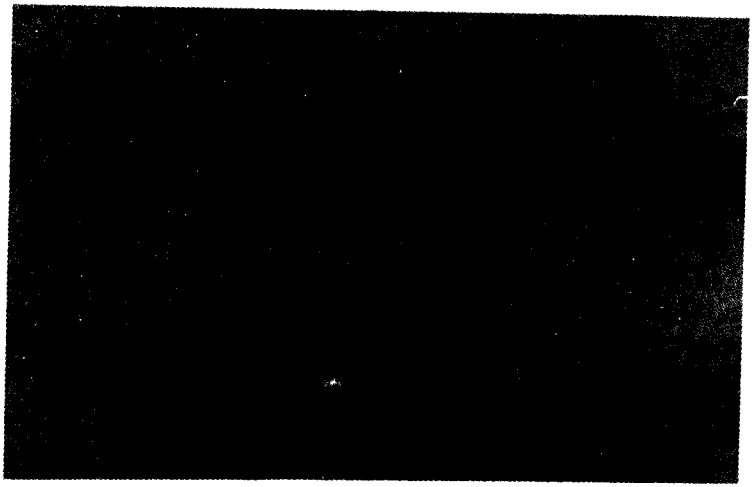

Fig. 5 High power view of glioma cells in *in vitro* culture after adding MTT solution, shows granules (a) and crystals (b) due to formation of formazan.

Table 2: Comparison of assay methodology

	MTT	^{35}S-methionine
	Cells grown in multiwell plates	
	Add MTT	Add ^{35}S-methionine
	4 hours incubation	
1st day	Add DMSO	Remove medium	Wash and fix with methanol
	Shake the plate	
	Read absorbance at 570/630 nm with densitometer	Dry the plate overnight
2nd day		Wash with TCA
		Dry the plate for five hours
		Add scintillation fluid
		Centrifuge the plate for 1 hour
		Expose to autoradiography film for 10-48 hours
3rd day		Develop the film
		Read absorbance at 430 nm with densitometer

Colony forming efficiency (CFE)

Monolayer cloning assay has been developed for human brain tumours (Rosenblum 1980, Rosenblum *et al* 1980, Rosenblum *et al* 1983). It has been claimed that 15% of the surgical specimens can give rise to colonies. However,

there is inconsistent colony forming efficiency. In a larger study it has been observed that clonogenic assay tends to overpredict clinical sensitivity (Rosenblum *et al* 1983). These authors had shown an apparent relationship between the age of the patients and *in vitro* cell death; younger patients with glioma have more *in vitro* drug sensitivity as compared to older patients. Colony assay indicated a relationship between *in vitro* and clinical resistance (Rosenblum *et al* 1980). There is also an indication that the human glioma cells grow well in soft agar and form colonies, hence can successfully be used for *in vitro* drug assay (George *et al* 1984 and Malne *et al* 1984). However, use of such methods of assay must be weighed against the considerable difficulties which they pose technically as compared to other available methods.

In vitro drug exposure, aims, principles and limitations

The aim of *in vitro* culture drug testing is to find out the ideal drug and its optimal concentration to kill either 50% or 90% of tumour cells (ID_{50} or ID_{90}). It is also aimed to compare one drug with another (Table 3) to find out most effective agent (Darling and Thomas 1980, Sichen *et al* 1985, Carmichael *et al* 1987, Kimmel *et al* 1987, Darling and Thomas, 1988, Bradford *et al* 1990). Surprisingly enough there are only a few studies which tried to correlate the *in vitro* response with *in vivo* response (Darling and Thomas 1983, Von-Hoff *et al* 1983, Thomas *et al* 1985).

Table 3: Concentration of chemotherapeutic agents used in *in vitro* sensitivity test

Drug	Abbreviation	Stock solution	Concentration range
CCNU	CCNU	4mg/ml	40-0.013 μg/ml (Etogel)
Procarbazine	PCB	5mg/ml	1000-4 μm/ml
Vincristine	VCR	100μg/ml	10-3.12$\times 10^{-6}$ μg/ml (Ham's)
Adriamycin	ADR	1mg/ml	10-10^{-4} μg/ml (Ham's)
Diasaquone	AZQ	1mg/ml	10-10^{-4} μg/ml
5 Flurouracil	5-FU	1mg/ml	10-10^{-4} μg/ml
Bleomycin	BLM	10μg/ml	10-10^{-4} μg/ml
VP 16-213	VP 16	100μg/ml	10-10^{-4} μg/ml

Drug exposure *in vitro* can be done in monolayer culture in multiwell plates (Darling and Thomas, 1980, Sichen *et al* 1985, Twentyman and Luscombe 1987), in multicellular tumour spheroids (Yuhas *et al* 1978, Darling *et al* 1983) and rarely in soft agar colony (Tanigawa *et al* 1982, Rosenblum 1980, George *et al* 1984).

Principle of drug sensitivity is to compare the growth of control tumour cells to drug treated tumour cells. A fixed number of tumour cells are grown in multi-well plate for few days till they form a healthy monolayer. These different drugs in varying concentrations are added to the multiwell plate leaving few wells

unexposed to drug which act as control. Drug exposure less than 6 hours is considered as short term exposure (Sichen *et al* 1985, Darling and Thomas 1988). Drug exposure between 6 and 24 hours is called intermediate exposure and drug exposure for more than 24 hours is considered as long term drug exposure. After the drug exposure the culture medium along with the drug is removed and fresh culture medium is added to the multiwell plate. Cells are allowed to recover from the effect of the drug, over next 4-6 hours, prior to the assessment of cell viability. Cell viability is assessed either by adding ^{35}S methionine or by adding 0.5-1% MTT solution. Then either the autoradiographic plate or multiwell plate incase of MTT study is read by ELISA plate reader and optical densities are plotted on graph and ID50 value for each drug (Fig. 6a & b) is found out comparing drug treated cells with control cells. Kornblith *et al* (1978, 1981) and Darling and Thomas (1983) reported heterogeneity of response to drugs in *in vitro* culture. Depending upon their sensitivity to a drug, patients are called *in vitro* responders or nonresponders (Darling and Thomas 1980, Thomas *et al* 1985). Thomas *et al* (1985) reported 73% of grade III astrocytoma patients as *in vitro* responders as compared to only 27% of grade IV astrocytomas. Limitations of *in vitro* drug study are many. These are (a) variability of karyotypes in tumour cells (b) dose response curve differs in different clones of cells, (c) mixed cell populations from different regions can have different chemosensitivity, (d) difficulty to derive any conclusion in case of small biopsy due to sampling error. Till date all the *in vitro* studies undertaken dealt with single drug at a time with single exposure. There is no study so far dealing with effect of chemotherapy in radiated tumour cells which is the usual feature in patients with gliomas. Thus *in vitro* studies did not take into account many parameters which complicate the management of patients with gliomas. The precise mechanism of drug resistance is not clear. Glioma cells resistant to one or a group of drugs need not be

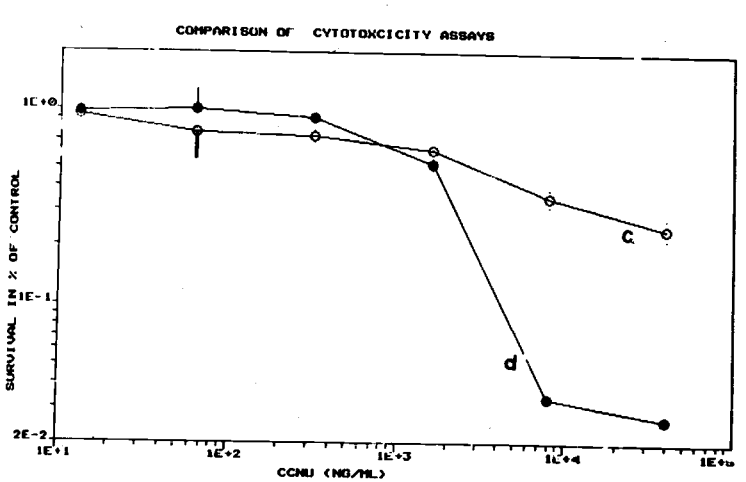

Fig. 6(a) Graph showing drug sensitivity *in vitro* culture using CCNU (d) along with control cells (c).

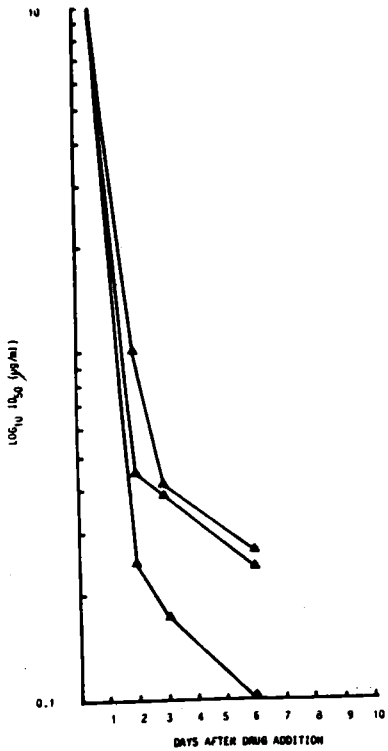

Fig. 6(b) Graph showing response to three different drugs in *in vitro* culture up to 6 days following drug addition.

resistant to other drugs (Darling and Thomas 1988). Hence, it is clear that considerable work needs to be done in relation to drug resistance in human gliomas using clinical material. It is not at all clear if the mechanism by which cells become resistant to drugs *in vitro* is relevant for clinical drug resistance.

Chemotherapy in experimental animals

There are several studies dealing with experimental gliomas in animals (Bradley *et al* 1978, Bloom *et al* 1986, Friedman *et al* 1986). All these experiments are done in immunosuppressed rats or mice. This is usually done using either subcutaneous or intracerebral glioma xenograft. Subcutaneous grafts offer some advantages over intracerebral grafts as it is (i) easy to assess their growth (ii) easy to assess the response to chemotherapy and (iii) easy to excise partially before chemotherapy. However, subcutaneous graft fails to provide the same microenvironment as that of an intracerebral graft. Bloom *et al* (1986) observed much faster doubling time in the intracerebral xenograft as compared to the subcutaneous xenograft (4-6 days vs 8.1 ± 1.5 days). In grade IV glioma subcutaneous xenograft, Bloom *et al* (1986) reported a doubling time of 10.8 days

which reduced to 3.5 days following partial excision. They also observed that the preoperative BCNU and procarbazine could significantly rise the doubling time and hence reduce the postoperative tumour growth. Surprisingly, however, same drugs (BCNU, PCB) given 10 days after surgery had no effect on tumour doubling time. In mice with grade IV glioma xenograft, BCNU and PCB reduced tumour growth, while, 5 FU and cyclophosphamide failed to do so. In intracerebral xenograft, Bloom *et al* (1986) observed weight loss in animals 7 days following graft and their median survival was 67 days (34 to 101 days). In grade IV intracerebral graft median survival was 50 days. Sixty percent animals receiving BCNU and 50% animals receiving PCB had a median survival of 125 days.

Shapiro and Shapiro (1986) studied the role of methotrexate in mice glioma by tagging the drug with radioactive carbon (C^{14} MTX) which was injected either by intravenous or intracarotid route. They found much higher drug concentration in tumour following intracarotid injection and tumour-cortex ratio of the drug was also higher (1.7 vs 2.5).

Friedman *et al* (1986) studied drug sensitivity for medulloblastoma in rats. They recorded the tumour doubling time as 3.17 days while in culture doubling time was 31 days. The cell cycle time was only 24 hours. They implanted medulloblastoma cells from a cell line subcutaneously and intracerebrally. Then they studied the effect of several drugs in *in vitro* culture and also on tumour implants. They observed that all the drugs worked well in *in vitro* cultures and some of them did work in reducing the size of tumour implanted subcutaneously, however, all these drugs failed to produce any effect on intracerebral tumour growth, thus highlighting the limitations of such animal experiments.

Chemotherapy in glioma patients

Use of chemotherapy is a major development in the management of malignant gliomas over last decade. Even though the result is not very encouraging, there is a modest, but significant improvement in survival (Stenning *et al* 1988). There are several large multicentric studies using various drugs single or in combination now available (Soloro *et al* 1979, Walker *et al* 1980, Levin *et al* 1980, Eagan *et al* 1981, Chang *et al* 1983, Green *et al* 1983, Stewart *et al* 1986, Shapiro and Shapiro 1986, Vole *et al* 1986, Stennining *et al* 1988). Chemotherapy is divided into cycle specific or cycle nonspecific drugs. Among the commonly used drugs, 5FU, MTX and vincristine are cycle specific and BCNU, CCNU, procarbazine (PCB) and cisplatin are cycle nonspecific drugs. Initial cycle specific drug followed by cycle non specific drugs have been suggested by several authors. Cycle specific drugs affect the cells in active proliferative state. However, only 5-10% of tumour cells are at any time in mitosis depending on the grade of malignancy. In glioblastoma about 10% cells are in active proliferative phase. Hence, cell cycle specific drugs can only kill or prevent the growth of a small population of tumour cells, while non-specific drug can affect cells even in resting or non proliferative state. Thus an ideal chemotherapeutic regime must have a combination of both

cell cycle specific and non-specific drugs. The commonly used chemotherapeutic agents in patients with glioma are summarized in Table 4.

Table 4: Dosage of some commonly used drugs

Name of the drug	Mode of administration	Dosage/$qm
CCNU	Oral	100 mg
BCNU	I.V.	80 mg
Vincristine	I.V.	1.4 mg
Procarbazine (PCB)	I.V.	100 mg
Cisplatin	I.V.	100 mg
Mitoxantrone (Navantrone)	I.V.	15 mg
Arabinfuranosyl Cytocine	I.V.	3000 mg
Amepthopterin	I.M	10 mg
Methyl Prednisolone	I.V.	20 mg
Tiazefurin	I.V.	500 mg
Adriamycin	I.V.	40-60 mg
Bleomycin	I.V.	20-30 mg

Most of the drugs are either used orally or intravenously. Few authors have used intracarotid injection of drugs to get a higher concentration in the tumour (Stewart *et al* 1984, Shapiro and Shapiro 1986). BCNU, AZQ and cisplatin have been tried by intra-arterial route (Greenberg *et al* 1984, Foo *et al* 1985, Safdari *et al* 1985). Not surprisingly this technique did not gain popularity mostly because of invasive nature of drug delivery and doubtful advantage over intravenous route. BCNU has been associated with significant retinal damage when injected below the ophthalmic artery and, even if injected supraselectively distal to the retinal artery, neurotoxicity has been reported. Cisplatin is also reported to have neurotoxicity (Foo *et al* 1985). Nitrosourea is the most widely used agent in gliomas. When used as an adjuvant to surgery and radiotherapy, BCNU has shown to improve median survival and also increase the number of patients who survived longer than 18 months (Walker *et al* 1978, Green *et al* 1983, Chang *et al* 1984, Shapiro and Shapiro 1986, Vole *et al* 1986). The advantage of CCNU and methyl-CCNU over BCNU is that the drugs can be given orally. Unfortunately in trials they have not been shown to be as effective as BCNU.

Walker (1978) reported 14 weeks as median survival in patients with malignant gliomas managed by surgery alone. However, surgery with postoperative radiotherapy increased the median survival to 36 weeks. Green *et al* (1983) reported 18 months survival in 27% with malignant glioma who were managed by radical surgery, postoperative radiotherapy and BCNU therapy. Shapiro (1986) reported various phase III studies undertaken by U.S. Brain Tumour Study Group between 1978 and 1984, in which large number of patients were studied using a variety of drugs (Table 5). The percentage of patients surviving 18 months ranged from 16 to 29%. All of these patients received radiation and

single chemotherapeutic agent. Vole *et al* (1986) reported, tumour free period (TFP) and mean survival in 278 patients treated for grade III and IV gliomas. Sixty five patients had only surgery and their TFP was 3.5 months and mean survival was 7.2 months. Sixty nine patients had only radiation and 43 patients received only combination chemotherapy, their TPF and mean survivals were not significantly different. Hundred and one patients had surgery followed by radiotheraphy and polychemotherapy (COMP). Their TFP and mean survival were 14.5 months and 19.5 months respectively. Stewart *et al* in 1986 reported the result of cisplatin and arabino furanosyl cytosine in 25 patients with glioblastomas. Their median survival was 45 weeks. Most of the studies have shown a modest increase in survival. Combination chemotherapy is favoured. Commonly used combination is CCNU 80 mg/$qm day one, vincristine 1.4 mg IV/$qm day one and procarbazine 100 mg/$qm day one to 10, repeated every 6 weeks for 10-12 cycles (Levin *et al* 1980, Thomas and Darling 1985, Green *et al* 1983). Stewart *et al* (1983, 1984) reported the utility of combination chemotherapy using cisplatin along with some other drug. They also used cisplatin in cases of recurrent gliomas.

Table 5: Chemotherapy trials: Results of some large phase III studies in malignant glioma

Name of trial	Number of patients	RT & drug	18 months survival (%)
6901 (1978)	222	BCNU	19
7201 (1980)	358	Methyl CCNU	23
		BCNU	27
7501 (1983)	527	PCB	29
		BCNU	16
7702 (1984)	557	BCNU	16
		Streptozotocin	25

In vitro and in vivo chemosensitivity correlation

Most of the studies dealing with *in vitro* chemosensitivity did not correlate with *in vivo* observations on patients, except those by Darling and Thomas 1983, Thomas and Darling 1985, Von Hoff *et al* 1983). Von Hoff *et al* (1983) studied *in vitro* and *in vivo* response in 246 patients with glioma. In 203, *in vitro* resistance was encountered and among them 172 had *in vivo* resistance with a 85% correlation. However, it is interesting to note 15% with *in vitro* resistance did show useful response in patients. On the contrary, among the *in vitro* responders there were only 66% patients manifesting therapeutic response. Thus 34% *in vitro* responders were non-responders *in vivo*.

Thomas and Darling (1985) analysed 117 patients with grade III and IV gliomas and tried to correlate *in vitro* drug sensitivity with recurrence free interval (RFI) in patients. They divided their patients into three groups (a) *in vitro* responders, i.e. the drug was found effective in *in vitro* culture (22 patients), (B) *in vitro* non responders, when drug did not work in *in vitro* cultures, (18 patients) and (C) in

77 patients no *in vitro* testing was done. When these groups were compared for their RFI, patients in group a had significantly longer recurrence free interval than group-B and $P < 0.0001$. Surprisingly when Group-A and B put together were compared with group-C there was no significant difference. Amongst *in vitro* responders 73% patients had grade III glioma and 27% were with grade IV glioma. On the contrary among the *in vitro* non-responders only 28% belonged to grade III compared to 72% with grade IV tumours. There were two factors which correlated well in *in vitro* and *in vivo* study; these were, patients below 40 years of age and grade III glioma. Interestingly, among three drugs PCB, CCNU and VCR which were effective *in vitro* only CCNU correlated with recurrence free interval. Thus the above study brought out many limitations of *in vitro* study as a predictor of *in vivo* response.

Malignant gliomas continue to be a formidable clinical challenge, and the prognosis largely remains poor. *In vitro* culture and animal experiments have great limitations in predicting clinical response. Currently available drugs have modestly increased the survival, yet there is a vast scope of work for newer approaches to improve upon the existing results.

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Current Concepts of Pathology of Pituitary Adenomas

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Pituitary adenomas have been earlier classified into chromophobe, acidophil, basophil adenomas based purely on their tinctorial characteristics at light microscopic level. The chromophobe adenomas were considered generally as non-secretory and clinically non-functioning, while acidophil and basophil adenomas were considered to be functioning tumours associated with clinical evidence of endocrine hyper-functioning such as acromegaly, galactorrhoea, Cushing syndrome and rarely hyperthyroidism. However, it was observed that many chromophobe adenomas may be associated with evidence of excessive secretion of one or the other hormones based on clinical presentation and by using specialised techiques like immunohistochemistry, electron microscopy, serum hormone estimation by radioimmuno-assay and immunoelectron microscopy (Horvath and Kovacs 1976, 1980, Kovacs *et al* 1977 Roy 1977, 1983, Scheithauer 1984, Bassetti *et al* 1986, Kovacs 1986, Sarkar *et al* 1990). The past technique is used to localise a particular hormone using monoclonal antibody not only at cellular level but at the level of individual secretory granules (Bassetti *et al* 1986).

On the basis of use of these specialised techiques a new classification of pituitary adenomas has been put forward by Horvath and Kovacs and this is given below (Horvath and Kovacs 1980, Kovacs 1986).

1. Null cell adenomas: Non-oncocytic and oncocytic types;
2. Lactotroph adenomas: Sparsely granulated and densely granulated types;
3. Somatotroph adenomas: Sparsely granulated and densely granulated types;

4. Corticotroph adenomas: (including silent adenomas);
5. Thyrotroph adenomas;
6. Gonadotroph adenomas; and
7. Plurihormonal adenomas.

We have used the above mentioned techiques to study a series of 200 cases of pituitary adenomas and have found the above classification to be more useful in reflecting the biology of these tumours. The important characteristic features of each type of these tumours are given below and for comparison the findings in our series are provided.

Null cell adenoma

These tumours generally occur in males and are not associated with any clinical evidence of endocrine hyper-function, nor is there any increase in serum hormone level. Even on immunohistochemistry (IH) no evidence of hormone secretion can be demonstrated. However, in a small number of cases either the alpha-subunit or the beta-subunits of glycoprotein hormones can be demonstrated. It should be noted that glycoprotein hormones are present in combination of alpha and beta subunits, the alpha subunit being common to all the hormone and the beta subunits is specific for individual hormone type. The hormone is active only when there is a combination of both these subunits (Scheithauer 1984, Black *et al* 1987, Jameson *et al* 1987, Landolt *et al* 1988, Sarkar *et al* 1990). On electron microscopy the tumour cells contain varying number of secretory granules with poorly developed rough endoplasmic reticulum (RER) and Golgi apparatus (G.A.). These cells are thus considered as secretorily inactive. It should be noted that granules are always present in these non-functioning tumours and since no hormone can be demonstrated in them their significance is not known. A small number of tumours in this group consist of cells containing a large number of mitochondria, a characteristic feature of oncocyte and these tumours are known as oncocytoma (Kovacs 1986). In our own series there were 65 cases of null cell adenoma with male, female distribution being 3:1 and age ranging from 22 to 57 (median 40 years). All these tumours were chromophobe adenomas on light microscopy, serum hormone was not elevated in any one of these cases. Immunohistochemistry revealed no localisation of hormone in 46 of these tumours, but alpha or beta subunits of one or the other glycoprotein hormones were present in 19. Electron microscopically the tumours consisted of secretorily inactive cells and four were found to be oncocytomas. Even on immunoelectron microscopy, no hormone localisation could be demonstrated.

Lactotroph adenoma

They constitute from 27 to 44% of all pituitary adenomas in different series in the literature (Roberts 1979, Kovacs 1986). They occur commonly in young women and clinically manifest with galactorrhea and/or amenorrhea. The male patients

manifest clinically with impotence or decreased libido but a large number of male patients do not have any endocrine functional abnormality. Light microscopically, most of these tumours are chromophobe although a few can be acidophil or mixed acidophil and chromophobe adenomas. Serum prolactin level is generally highly elevated. Electron microscopically the tumours usually consist of sparsely granulated secretorily active cells (with prominent RER and well developed GA). Very rarely oncocytoma with excessive prolactin secretion has been reported (Chowdhury *et al* 1986). Immunohistochemically the tumour cells are strongly positive for prolactin and immunoelectron microscopy also reveals localisation of prolactin only. Ultrastructurally in some tumour cells very marked prominence of rough endoplasmic reticulum forming whorls of rough endoplasmic reticulum cisterns can be seen. Such structures are known as Nebenkern. In PRL-secreting oncocytoma, although cytoplasm of most of the cells are filled with mitochondria, 10 to 15% of tumour cells have prominent RER and GA and it is likely that these secretorily active cells are responsible for clinical hyper-function (Roy 1983, Sarkar *et al* 1990). In our series there were 47 lactotroph adenomas, all of which were chromophobe adenoma by light microscopy. Although majority presented with clinical evidence of endocrine hyperfunction a few (8) were clinically "non-functioning". Chromophobe adenomas occurred at a relatively younger age. They were observed more frequently in male in the clinically non-functioning group in female in the hyper-functioning adenomas. Serum prolactin was markedly elevated in all the cases and it was even higher in male patients. Immunhistochemistry showed strong positive reaction for PRK in all of our cases and immunoelectron microscopy localised only PRL within secretorily active tumour cells and one case in our series was an oncocytoma.

Somatotroph adenoma

This tumour constitutes 15 to 30% of all pituitary adenomas in different series and presents with either acromegaly alone or in combination with galactorrhoea (Roberts 1979, Scheithauer 1984, Kovacs 1986). Light microscopically these can be acidophil, chromophobe or mixed acidophil-chromophobe adenoma (Saeger and Ludecke 1983, Kovacs 1986, Sarkar *et al* 1990). Serum growth hormone is invariably elevated and electron microscopically the tumour consists of either densely granulated or sparsely granulated secretorily active tumour cells. In small number of cases spherical non-membrane bound structures consisting of intermediate filaments are observed. These have been described as spherical filamentous bodies and are generally present within the secretorily active, sparsely granulated tumour cells. Often a few secretory granules or other cell organelles can be seen embedded inside them (Roy 1977, 1983). In our series we had 22 cases of somatotroph adenomas, male to female distribution being 1:2 and age range 24 to 58 years (median 28 years). Light microscopically 15 of them were chromophobes and 7 were acidophil adenomas. Serum growth hormone was elevated in all the cases. Immunohistochemistry revealed the presence of growth hormone in most tumour cells in all the 22 cases. Electron microscopy

showed both densely and sparsely granulated secretorily active cells in most of the tumours and in some of them spherical filamentous body was also observed. Even in the densely granulated tumour, a small number of sparsely granulated cells were seen and they may be more important for their functional activity. Immunoelectron-microscopy revealed the presence of growth hormones within the secretory granules.

Corticotroph adenoma

These tumours constituted 7 to 15% of all the pituitary adenomas in different series (Scheithauer 1984, Kovacs 1986). These are found more commonly in women and the majority of them present with Cushing syndrome, although many can remain clinically silent (Horvath and Kovacs, 1980, Kovacs 1986). Serum ACTH level is elevated and immunohistochemically ACTH can be demonstrated with strong positive reaction in most of the tumour cells. Electron microscopically tumour cells are generally densely granulated and many tumour cells may show diffusely dispersed intermediate filaments, at times forming perinuclear aggregate, representing Crock's hyaline. In our series there were 4 patients who presented with Cushing syndrome, all being female and age ranged from 22 to 44 years (median 30 years). Light microscopically two were diagnosed as chromophobe adenoma and the other two as basophil adenoma. Immunohistochemistry revealed strong positive reaction for ACTH in all of them.

Thyrotroph adenomas

It is a very rare tumour constituting only about 1% of all pituitary adenomas. It may be associated with either hyper or hypothyroidism and is generally a chromophobe adenoma by light microscopy (Horvath and Kovacs 1980, Scheithauer 1984, Kovacs 1986). Serum TSH level is elevated and immunohistochemistry generally reveals strong positive reaction for TSH in the tumour cells. Electron microscopically, sparsely granulated tumour cells are seen.

Gonadotroph adenoma

This is also a very rare type of pituitary adenoma occurring mostly in elderly males with a large majority being functionally silent. Light microscopically these are generally chromophobe adenomas with the sparsely granulated secretorily inactive cells. Serum hormone level is not always significantly elevated and diagnosis is generally made by demonstration of FSH/LH by immunohistochemistry (Horvath and Kovacs 1980, Scheithauer 1984, Kovacs 1986).

Plurihormonal adenoma

These tumors are a relatively recently described entity based on the demonstration of more than one hormone production as revealed by immunohistochemistry and serum hormone assay (Heitz 1979, Horvath *et al* 1983, Saeger and Ludocke 1983, Sarkar *et al* 1990). These can be monomorphous, that is two or more hormones are produced by same group of tumour cells (Horvath *et al*

1981, 1983, Kovacs 1986) or plurimorphous that is different hormones are produced by different groups of tumour cells (Kovacs *et al* 1977, Hovarth and Kovacs 1980). These are more commonly found in patients presenting with acromegaly, at times associated with galactorrhoea and amenorrhoea. It may be noted that in the majority of cases, clinical manifestation is due to excessive release of only one hormone and the presence of other hormone is revealed only following investigations. Thus presence of other hormones may not be reflected in the clinical picture or even as elevated level in the serum (Sarkar *et al* 1990). In our series we had 60 cases (out of 200 cases-30%) of plurihormonal adenomas. Their age ranged from 14 to 57 years and male to female ratio was 1:4. Eight of these 60 cases had no clinical evidence of endocrine hyperfunction and 52 were hyperfunctioning; most of them presented with acromegaly. In the "non-functioning" plurihormonal adenomas PRL was found to be present in all the 8 cases in addition to one or more other hormones. In the clinically hyper-functioning group the combination of GH and PRL was the commonest finding.

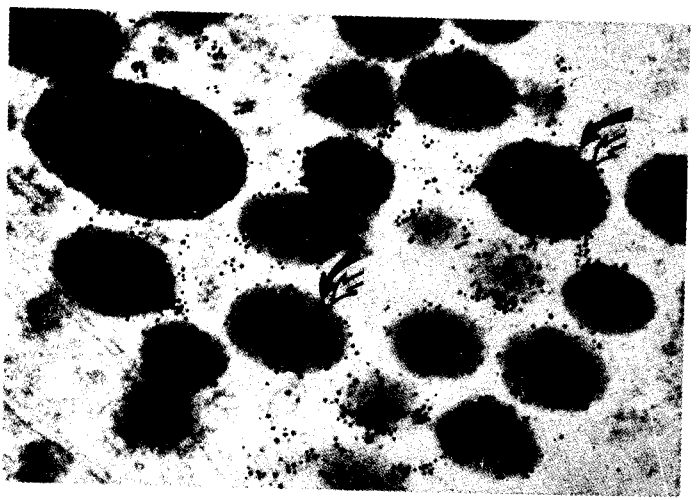

Fig. 1 Electron micrograph of double immunohistochemical labelling technique sharing simultaneous localization of 20nm gold particles for GH(open arrow head) and 10nm gold particles for PRL (arrow) in the same secretory granule X 1,10,000.

Table 1: Comparative incidence of pituitary adenomas

	Reported incidence	Incidence in our series
(1) Null cell adenomas	20-30%	33.5%
(2) Prolactinomas	27-44%	23.5%
(3) Somatotroph	15-30%	11%
(4) Plurihormonal adenomas	14-64%	30%
(5) Corticotroph adenomas	7-15%	2%
(6) Thyrotroph adenomas	1%	0
(7) Gonadotroph adenomas	5%	0

This group of GH-PRL-secreting plurihormonal adenoma is the most frequent type of all plurihormonal adenomas. These have been further subdivided by Kovacs and his group into 3 categories (Horvath *et al* 1981, 1983, Kovacs 1986). The three categories are,

1. Acidophil stem cell adenoma,
2. Mammosomatotroph adenoma
3. Mixed somatotroph-lactotroph adenoma.

Of these , first two are believed to be monomorphous in nature and the last one in polymorphous. Of the two monomorphous types , acidophil stem cell adenoma is considered to consist of undifferentiated stem cells and is associated with more aggressive clinical behaviour (Hovarth *et al* 1981). Mammosomatotroph adenoma on the other hand is considered to be a slow growing tumour consisting of well differentiated tumour cells, and is believed to be extremely rare of the three types of GH-PRL secreting adenomas (Horvath *et al* 1983, Kovacs 1986).

We analysed 15 GH-PRL secreting plurihormonal adenomas by immunoelectron microscopy and were rather surprised to find that in all the 15 cases the tumour was composed of well differentiated tumour cells and in each tumour, all the tumour cells examined at the ultrastructural level by immunoelectron microscopy were found to contain both GH and prolactin not only within the same cell but within the large majority of secretory granules (Fig. 1). Therefore, these 15 cases belong to the category of mammosomatotroph type of GH-PRL plurihormonal adenoma and hence we feel that these are not as rare as has been observed by Kovacs and his group. Somewhat similar observation has also been reported recently by other workers (Halimi 1982, Ishikawa *et al* 1983, Bassetti *et al* 1986). Table 1 shows the comparative incidence of pituitary adenomas in our series and the different series reported in literature.

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Trace Metals in Neurological Disorders

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During the recent years trace metals are being increasingly implicated in the aetiopathogenesis of a number of neurodegenerative disorders notably in motor neuron disease and dementia. The observation of clinical and pathological features in metal toxicity similar to that seen in some neurological disorders has led to intensive research both at the field level and in the laboratory. While considering a candidate metal as having a specific role in a disease, certain important issues need to be addressed which relate to the methodological problems in analysis, determination of normative data in a defined population exposed to a specific geochemical environment, the confounding factors of presence of other trace and heavy metals, influence of dietary pattern and the interaction of multiple factors some of which are known and others, still to be discovered. Awareness of these important issues is necessary for those dealing with the problem of neurotoxicity of trace metals.

In the living tissue many elements and metals are ubiquitously found, some of which are considered as macrominerals as they are present in high concentration and others are termed as micronutrients or 'trace elements', since these are present in a much lower concentration of less than μg/gm net weight as measured by available techniques. The 'essential' trace metals are those (i) which have a definite physiological role in biological systems, (ii) present in all healthy tissues of all organisms in relatively constant amounts, (iii) whose withdrawal produces similar structural and functional abnormalities indifferent species, and (iv) abnormalities produced by the deficiency prevented or reversed by the addition of the metal (Reinhold 1975). Based on this definition the trace metals and elements are classified as essential, possible essential and non-essential (Table 1).

The tissue concentration of some elements and metals may vary considerably depending on the amount in environment and the extent and duration of ex-

posure. High concentration of trace metals in neural tissue may lead to toxicity resulting in neurological syndromes. A thorough understanding of function of trace metals in biologic systems facilitates an enlightened approach to the disease states. It has been convincingly demonstrated that manganese, zinc and copper are necessary for development, and deficiency of these trace metals would result in a variety of developmental disorders, those specific to nervous system being neural tube defects, hypoplasia of cerebellum and behavioural abnormalities (Hurley 1983).

Table 1: Classification of trace metals and elements

Essential	Possible essential	Non-essential
Cobalt	Nickel	Aluminium
Copper	Silicon	Arsenic
Iodine	Tin	Boron
Iron	Vanadium	Cadmium
Manganese		Germanium
Molybdenum		
Selenium		
Zinc		

The trace elements and metals may be a part of physiological compounds (cobalt in vitamin B12), essential for enzyme function (e.g. iron and copper for cytochrome oxidase, iron for catalase and succinic dehydrogenase and zinc for carbonic anhydrase) or form an integral part of enzymes and termed as metalloenzymes (e.g. cytosolic superoxide dismutase (SOD) contains copper while zinc and manganese are constituents of mitochondrial SOD).

Normal values of trace metals

Trace metals are usually measured in body fluids and hair. It is well known that certain metals attain high concentration in tissues other than body fluids and tend to accumulate preferentially at specific sites in the tissues. Thus estimation of metals in target tissue is valuable since it may provide a more accurate measure. A comparison of the normal levels of trace metals in biological system from the published literature shows a wide variability. Further, errors in analysis may be due to wrong analytical methods used, failure to collect valid samples, presampling factors, quality control in sampling, sample preparation, and preservation and storage of specimens (Iyengar 1983). It is difficult to state what is a 'normal' or a baseline value. That it is important to establish a reference range of values taking into consideration the regional differences in diet and environmental factors has been adequately emphasised in the literature. Considering strictly, lead and cadmium should not be present in the human system. However, due to environmental factors, these metals are found in the body fluids. It has been observed that men have higher blood lead levels than women and smokers have higher values than nonsmokers. It is thus easy to imagine that high levels could

occur due to environmental influences leading to toxicity. Dietary habits can strongly affect the level of selenium which is influenced even by short term alteration in dietary pattern. Cosmetics, aerosols, detergents, shampoos can alter the concentration of various metals in hair. Diet and environment have also been demonstrated to have considerable influence on trace element distribution pattern in tissues and fluids. Thus it becomes clear that levels of trace metals would vary from country to country and there would be regional differences within the same country.

Neurotoxicity of trace elements and metals

Various mechanisms have been postulated to explain the cell damage and cell death caused by trace metals. A metal may be toxic by virtue of its effects due to selective accumulation at specific sites. With this type of toxicity, there is no specific interaction with a molecular target, but highly reactive metal cations are directed in sufficiently high concentration to a group of target cells. Another mechanism of trace metal induced damage is by specific interaction with target molecules. Arsenic reacts with the alpha lipoic acid molecule and results in its inactivation. A third mechanism is by the interaction of the metal with essential elements by competition for biochemical and transport mechanisms. Lithium for example effectively mimics sodium in transport into mammalian cells (Clarkson 1983). Metals can also induce cellular damage by uncoupling of oxidative phosphorylation. Nucleic acid metabolism may be affected by metals by alkylation of DNA and inhibition of DNA repair mechanisms may occur (Bradley and Krasin 1982). As metals form an essential component of enzymes such as superoxide dismutase, metal toxicity can also result in free radical induced oxidative damage to cell membranes, intracellular proteins and nucleic acids.

The toxic form of the metal is usually the free cation. Intracellular metal-protein complex formation thus serves as a protective or detoxification process by reducing the availability of the free metal ion. For the essential metals such as iron and zinc, there are apoproteins such as ferritin and metallothionein whose induction of synthesis involves specific genetic mechanisms. It is interesting to speculate whether nature has provided genetic information for the purpose of detoxification (Goyer 1983). There is evidence that morphologically discernible inclusion bodies are formed intracellularly with exposure to lead in the renal tubular lining cells, and mercury in the kidney and liver. These inclusion bodies are insoluble metal-protein complexes which can be demonstrated in tissue sections. When the toxic effects of the metal on the cell far outweigh the nature's protective detoxification mechanisms cell death would eventually ensue. Neurological symptoms and signs in the humans and the animals have been observed in acute and chronic neurotoxicity induced by a variety of trace metals (Anger *et al* 1985). Almost all parts of the neuraxis appear to be involved. Some of the commonly observed features in the humans are listed in Table 2. It is noteworthy that more than one metal may induce similar symptoms e.g. tremor can occur in toxicity due to manganese, zinc, aluminium and cobalt and likewise encephalopathy is seen in aluminium and cadmium toxicity (Anger *et al* 1985).

Table 2: Symptoms and signs of neurotoxicity of metals/chemicals

I.	Personality changes: (abnormal behaviour, aggressiveness, apathy, catatonia, depression, delirium, euphoria, emotional lability, decreased libido, mania, neurosis, sleep distrurbances).
II.	Cognitive functions: (agnosia, aphasia, acalculia, decreased concentration, intellectual deterioration, impaired judgement, memory disturbances).
III.	Motor system: (motor weakness, cerebellar signs, cranial nerve palsies - III, IV, VI, IX, X; extrapyramidal features, seizures).
IV.	Sensory system: (deafness, tinnitus, vertigo, taste disturbances, anosmia, paraesthesia, loss of sensations, visual loss, diplopia).

Neurodegenerative disorders and trace metals

In the relentless search for aetiology of a number of degenerative disorders multidisciplinary approach to study of trace metals has yielded promising results. Evidence for the role of trace metals in amyotrophic lateral sclerosis (ALS) in the high endemic foci in Guam as also in amyotrophic lateral sclerosis-Parkinsonism dementia complex and senile dementia of Alzheimer's type has been accumulating (Garruto 1987). However, in sporadic ALS the results reported in the literature are conflicting, at best there is only a trend and no definitive conclusions can be drawn. In this section the current status of trace metals in motor neurone disease will be discussed.

Among a large number of factors postulated in the pathogenesis of motor neuron disease (MND) namely viruses, toxins, immunological and biochemical abnormalities, trophic factors, defective DNA repair and others, trace metals have received considerable attention (Mitsumoto 1988). The neurological features of lead poisoning have long been recognised and cases with similar features but without a history of exposure to lead were also identified in the nineteenth century which later on were considered to be cases of ALS. It may be said that this was the beginning of research to explore the possibility of metals playing a role in the aetiopathogenesis of MND. It is now well known that there is a subgroup of patients resembling ALS patents with a definite evidence of lead poisoning. Chelating agents have been demonstrated to be beneficial in this group (Livesley and Sissons 1968) but not in ALS cases without exposure to lead (Campbell 1955). In some classical cases of ALS although elevated lead levels in blood, urine, CSF and spinal cord tissue had been demonstrated, the response to chelating agents was disappointing and the illness continued to progress (Conradi *et al* 1982; Conradi *et al* 1978; Petkau *et al* 1974). It must be emphasised that many studies have failed to demonstrate increased lead levels in sporadic ALS patients (House *et al* 1978, Stober *et al* 1983). Thus from the available evidence, lead cannot be assigned a definitive aetiological role in ALS. The explanation for the elevated lead levels is not clear, and it has been suggested that this may reflect the effect of the disease rather than the cause of the disease.

In the recent years, aluminium has been implicated in the formation of neurofibrillary tangles in the cortical neurons of Alzheimer's disease, spinal cord

and cortical neurons of Guam ALS-PD complex (Garruto *et al* 1984) and dialysis dementia. Based on these observations a common aetiology for these disorders has been suggested (Appel 1981, Gajdusek 1985). In sporadic ALS, with the exception of a single case, neurofibrillary tangles have not been demonstrated (Meyers *et al* 1974). Even in this single patient who was a boxer with ALS, the neurofibrillary changes could not be confidently related to ALS since boxers are known to have similar changes as a result of repeated trauma to the head (Corsellis *et al* 1973). Experimental studies have also confirmed that administration of aluminium salts induces neurofibrillary degeneration (De Bone *et al* 1976, Troncoso *et al* 1982). Although high levels of aluminium have been reported in spinal cord tissue of Guamian ALS and PD and in Japanese ALS patients, as yet, there are no studies on aluminium in spinal cord tissue of sporadic ALS patients. However, aluminium content in muscle of ALS patients was not elevated compared to control muscle (Pierce-Ruhland and Patten 1985). Determination of metals in muscle tissue in ALS has been done with the presumption that muscle could be a storage site and retrograde transport of metal to the neuron might occur. Yase has made an interesting postulation of absorption of lead and aluminium in the presence of mineral deficiency due to low intake of calcium and magnesium leading to metal-induced calcification with hydroxy-apatite formation and degeneration as seen in Guam ALS (Yase 1987). The further evidence for such a hypothesis is the decrease in the incidence of new cases of ALS in Guam after altered dietary pattern (Reed and Brody 1975).

Yase and co-workers measured manganese by neutron activation analysis in spinal cord and brain of ALS patients from Kii Peninsula and observed that there was no significant difference compared to the controls (Yase *et al* 1968). These studies later extended to include Guamian and sporadic ALS patients, confirmed the earlier observation. On the contrary, others using this technique in combination with radiochemical separation method reported elevated manganese level in the spinal cord (Miyata *et al* 1983 and Mitchell *et al* 1986).

The report of ALS cases in an environment of high selenium content in soil, has been a cogent reason for examining the possibility that selenium may be an aetiological factor in classic ALS (Kilness and Hochberg 1977). It may be recalled that wide variation in selenium in body fluids and tissues may occur even with transient alteration of dietary selenium content. Selenium poisoning is also well known in farm animals. Significant elevation of selenium in spinal cord and liver in ALS patients compared to those with non-neurological disorders has been reported by several workers (Kurlander and Pattern 1979, Mitchell *et al* 1984, 1987) and in erythrocytes by others (Nagata *et al* 1985). The important protective role of selenium in reducing or preventing the free radical induced damage of cell and its implication in ALS in the context of higher environmental concentration offer fresh approaches for further investigation.

A number of other elements and metals such as cobalt, iron, chromium, copper, zinc, magnesium, cesium, rubidium have not been found to be significantly different in ALS compared to controls.

Despite methodological problems involved in measurement of small concentration of trace metals, certain general conclusions can be drawn about the role of trace metals in aetiopathogenesis of amyotrophic lateral sclerosis. In the endemic form of ALS in Guam and Kii Peninsula, aluminium may be implicated. The deficiency of dietary calcium and the consequent abnormality of bone metabolism lead to impaired detoxification of metals as the binding to bone is affected (Barry 1975, Yanagihara *et al* 1984). In the classical ALS also a similar mechanism may exist since abnormal calcium metabolism has been described (Conradi *et al* 1978). However, in sporadic ALS the results of heavy metals and trace metals notably lead, aluminium, manganese and selenium estimation reported in the literature are at present conflicting. It is hoped that with better techniques these controversies would be resolved. The postulated mechanisms of action of the trace metals include (a) inhibiting effect on neuronal transmission leading to degenerative changes, (b) effect on nucleic acid metabolism and DNA repair mechanism and (c) effect on free radical mediated cell damage leading to cell degeneration and death (Mann and Yates 1974, Bradley and Krasin 1982).

The recognition of role of trace metals in pathogenesis of ALS has certainly led to new insights into the underlying mechanisms in ALS and is a fertile ground for further research involving a multidisciplinary approach.

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Clinical Manifestation of Environmental Toxins

DEVIKA NAG

The central nervous system (CNS) is normally protected from neurotoxic substances by the blood brain barrier. However, some substances like anesthetics, analgesics and tranquilizers penetrate readily. In the immature brain the barrier is not so effective, so that toxic doses of some compounds e.g. inorganic lead salts may accumulate in the brain tissue of children, whereas in the adult their effects are more on the peripheral nervous system. There is also a specificity or selective vulnerability of neurons to certain neurotoxic substances. This again may lead to variations of clinical response to a neurotoxin e.g. small neuron such as granule cells of cerebellum and visual cortex are preferentially affected by methyl mercury, whereas excitatory aminoacids e.g. domoic acid damages hypothalamic neurons. The globus pallidus is more sensitive to hypoxia. Manganese selectively affects the basal ganglia neurons. The foetal brain may be permanently damaged in intrauterine life if the mother is exposed to a neurotoxin.

The cells most sensitive to lack of oxygen are neurons in the cerebral cortex, cerebellar cortex and hippocampus. In the cerebral cortex, neurons of the fourth layer, having major afferent inputs from the sensory system are selectively damaged in severe anoxia. The sensitive neurons are small granule cells in layer 5, whereas large motor pyramidal cells are more resistant. In the cerebellar cortex, in decreasing order of sensitivity are the Purkinje cells, granule cells and lastly Golgi cells. In the hippocampus the pyramidal cells of field H_1 are most sensitive followed by H_2 and then granular layer of fascia dentata. Selective damage to the basal ganglia and the associated subthalamic nucleus and substantia nigra is seen secondary to toxic effects of certain chemicals.

The synaptic clefts between axons and dendrites of the neurons are considered to be especially vulnerable to exogenous chemicals carried by the bloodstream since the postsynaptic membrane is the site of receptors for chemical transmitters in the nervous system. Thus these may block the access of normal chemical transmitters to postsynaptic receptors, acting as false transmitters or affecting concentrations of transmitters through effects on synthesis, storage, release, reuptake or enzymatic inactivation mechanisms.

The clinical picture of environmental neurotoxins may, therefore, manifest itself in various ways depending on the method of exposure, dose and duration of exposure, and selective vulnerability of the nervous system.

The nervous system is also capable of developing tolerance or adapting to some types of damage hence function may return to normal during continuous exposure to a toxic substance. Damage may exist in the nervous system which may be detected only by cytologic, neurochemical or positron emission tomographic techniques even when no functional disturbance is observed. On the other hand, alterations in gait, visuo-motor performance, emotional state, and many other behavioural parameters may be the earliest and most sensitive signs of nervous system toxicity.

The limbic hippocampus has a low seizure threshold and seizures or convulsions due to toxic substances may result from effects on this system. The limbic system as defined by Papez, controls emotional behaviour. It has been observed that one of the most specific alterations of emotional behaviour by toxic substances is caused in individuals chronically exposed to low levels of inorganic mercury. the earliest sign of toxicity is a change in behaviour called "erethism" which is characterized by apprehension, emotional lability and irritability. Any of these symptoms may mimic a simple anxiety state. Early signs of involvement of motor and autonomic functions of the nervous system (fine tremors and salivation) may also occur with erethism. Sequelae to acute exposure of carbon monoxide include loss of memory, depression, and emotional instability which may persist for several weeks.

There are six types of damage to CNS produced by various neurotoxins

(1) Anoxic damage to the gray matter with variations in the pattern of damage depending on the nature of anoxia (cytotoxic/anoxic).

(2) Damage to myelin from substances affecting oligodendrocytes or Schwann cells, resulting in encephalopathy if central white matter is involved or polyneuritis if peripheral nervous system is damaged.

(3) Preferential damage of the axons of peripheral neurons.

(4) Primary damage to perikarya of peripheral neurons.

(5) Damage to synaptic junctions of the neuromuscular system.

(6) Lesions restricted to specialized CNS nuclear groups.

Environmental Neurotoxins

These may be broadly divided into the following categories:

1. Elements:

Non-Metallic – e.g. Fluoride

Metallic: *Natural compounds*: Lead, Arsenic, Manganese, Aluminium, Thallium, Mercury, Tin.

Man made compounds: Methyl mercury, Arsphenamine, Organo-manganese, Triethyltin, Trimethyltin, Barium Carbonate, Cadmium compounds, Maneb (Fungicide with manganese).

2. Chemicals:

Agricultural – Pesticides Organochlorines, Organophosphates, Carbamates, Pyrethrins.

Industrial – Solvents, Petroleum products.

Others – Acrylamide, Polyurethane foam, Carbon disulfide, Methyl phenyl tetrahydropyridine, Hexachlorophene.

3. Biological agents :

Plant – Lathyrus sativus (BOAA), Cassava, (cyanogen), Cycad.

Mycotoxin – Ergot

Animal – reptile venom.

Aquatic – Fish (saxitoxin tetradotoxin), Mussels (Domoic Acid).

Bacteria – C1. tetanis, Diptheria, C1. botulinum

Viral – Rabies virus

4. Gases :

Natural – Carbondioxide, Methane

Man made – Carbon monoxide, Methyl-iso-cyanate (MIC), Phosgene, 'Tabun', 'Sarin', 'Soman' (Nerve gas).

5. Radiation :

Natural: Solar, Radon, Thorium.

Man made: Microwave, Nuclear bomb

6. Physical agents :

Noise, Heat, Solar light, Electricity, Magnetic fields, Ultrasound, Vibration.

Clinical manifestations of some environmental neurotoxins:

1. *Behaviour Disorders Psychosis/Affective disorder* :

Thallium, Manganese, Ergot, Organo-phosphate pesticides, Mercury compounds, Carbondisulfide, Mucunia pruriens (Kavach).

2. Dementia/Cognitive dysfunction :

Mercury compounds, Domoic acid (in mussel poisoning), Lead, Carbondisulfide, Pesticides, Manganese, Aluminium.

3. Headache:

Lead

4. Mental clouding/Encephalopathy with raised intra-cranial tension:

Lead, Mercury, Pesticides
- Organochlorines
- Organotin compounds
- Hexachlorophene
- Triethyltin

5. Visuomotor impairment:

Pesticides, toluene, tellurium.

6. Seizures/Myoclonus:

Pesticide – Organochlorines
Lead, Organomercurials
Organotin, Benzene Hexachloride
Mercury compounds.

7. Auditory loss/Anosmia :

Noise/Cadmium, toluene

8. Visual loss :

Mercury, n-Hexane, Pesticides, Triethyltin.

9. Cerebellar Ataxia :

Mercury methyl compounds, Kepone, DDT, n-Hexane.

10. Sensory Ataxia :

Thallium, Arsenic, Triorthocresyl phosphate (TOCP), Acrylamide.

11. Extrapyramidal syndrome (Parkinson's syndrome) :

Manganese, Carbonmonoxide, Methylphenyl tetrahydropyridine (MPTP), Lithium

12. Speech Impairment/dysphagia :

Petroleum solvents, Mercury

13. Motor Neuropathy :

Lead compounds.

14. Sensory-Motor Neuropathy :

Acrylamide, Thallium, Methyl-n-Butyl Ketone (MBK).

15. Sensory Neuropathy :

Cranial – Trichlorethylene (TCE)

Spinal – Diaminoproprionitirle

(U. Foam = polyurethane compound).

16. Muscle weakness/Myoneural junction :

Nerve gas

Petroleum solvents, tetrodotoxin,

Saxitoxin.

17. Anterior Horn cell degeneration :

? Cycad

18. Spastic Paraplegia:

Lathyrus sativa, Cassava

19. Autonomic dysfunction sacral neuropathy :

Diaminoproprionitrile (DMAPN)

20. CNS Tumours ??:

Petrochemical industry, Anesthetics

21. Foetal malformation (anencephaly):

Spoiled potato (glykoalkaloids)

The following description will deal with some of our observations on neurotoxic effects in humans exposed to pesticides commonly used in India.

There are 5 types of pesticides in large scale used both in rural and urban areas.

(1) Organochlorines e.g. DDT, BHC, Lindane, Endosulfan
(2) Organophosphates e.g. Malathion, Parathion, Fenthion
(3) Carbaryl
(4) Aluminium Phosphate
(5) Pyrethrins
(6) Naphthalene

Organochlorines

These persist in the environment for a very long time and are widely used in India. The commonest in use are DDT, BHC, Lindane and Endosulfan. The estimated annual consumption on a national level has been 6500 tons DDT, 25,800 tons BHC, 600 tons Lindane and 3000 tons endosulfan (1986-87). These

pesticides may persist in soil and water from a few weeks to 40 years. The second major group are the *Organophosphates*, which have a greater acute toxicity than organochlorines but have a short life 4-8 hours to 7 days. The third are the carbamates with a half life of 6.6 days and lastly pyrethirns which have a half life of a few days to a week.

Naphthalene is not thought of in an agricultural context as a pesticide but has widespread household use and accidental/chronic clinical problems have been encountered in urban areas.

Human neurotoxic syndromes after exposure to pesticides have been classified as follows:

(i) *Acute syndrome*	(a)	such as in accidental ingestion, suicidal or homicidal attempts.
(ii) *Chronic syndromes*	(b)	While in use, in pesticide sprayers in farms, national malaria control programmes, pest control, in house construction, in aeroplanes and public/recreational areas.
	(c)	During manufacture of pesticide in factory formulating plant.
	(d)	Via food chain indirectly by consuming food with high pesticide residue: water, fowl, animal products, fruits and vegetables.
	(e)	Placental route to foetus via maternal circulation.
(iii) *Delayed neural toxicity*		with symptoms coming on several years after exposure even though the individual is no longer in contact with the toxin.

In Uttar Pradesh, aldrin, dieldrin, BHC, DDT, Malathion, Parathion, Fenthion, Carbaryl and Pyrethrisn as well as napthalene are extensively used.

In 1975 eight cases of epileptic fits in one family were traced to accidental ingestion of BHC in Surayarb village, Sitapur. The amount ingested was not lethal and the subjects responded to therapy. The electroencephalogram showed abnormalities suggestive of cortical dysrhythmia.

In the 1977 tragedy of Lakhimpur Kheri, 256 cases of acute and chronic ingestion were reported. Organochlorine pesticide was implicated in 56 deaths. The survivors showed evidence of–

(i) Cerebral cortical hyperexictability with convulsions that simulated epilepsy and status epilepticus.

(ii) Behavioural changes in the form of extreme apprehension and exaggerated 'startle' reaction.

(iii) Sensory paresthesias and tingling over circumoral area and entire face.

(iv) Cranial nerve palsies.

(v) Papilloedema

(vi) Coarse static tremors, irregular 6-8/min, and intention tremors.

(vii) Ataxia and staggering gait.

(viii) Pyramidal signs (reversible).

Chronic effects of this group of pesticides were then studies in collaboration with ITRC and Neurology department KGMC. 29 workers engaged in organochlorine spraying (DDT) were subjected to detailed Bhatia Battery tests, Weschler memory scale, and Bender Visuomotor Gestalt test plus electrophysiological tests. DDT levels in sprayers were recorded to be 8.5 times higher than those of controls.

The findings suggested that there was:

(1) Statistically significant impairment of visuo-motor function (55.5% had poor performance on BGT).

(2) Subclinical sensory motor neuropathy in workers (18%) and clinical signs in 32.8% (mean age 30.14 years).

(3) Behavioural changes

(4) Electrophysiological changes consisted of prologed terminal motor latency, F and H latency and repetitive muscle activity.

(5) 50% of these also had non specific abnormalities in EEG (slowing and absence of alpha activity).

These findings could be due to impurities in the organochlorines or the pesticide itself. Impurities are known to give rise to greater toxicity than the original pesticide. The climatic conditions in our country increase the load of toxic DDT/organochlorine residues and lead to chronic toxic syndromes. Other workers using aldrin, dieldrin were reported to have behavioural changes such as:

- Depression, insomnia
- Oral automatism
- Myoclonus
- Ptosis and
- Hyper-reflexia

These clinical features last from 2 days to 3 months after withdrawal from exposure.

Organophosphates

The organophosphates used in the country are parathion, malathion, diazion and fenthion. They have a similar method of absorption i.e. ingestion, inhalation and

dermal absorption. Their mode of action is by phosphorylation of acetylcholinesterase causing excessive accumulation of Ach at cholinergic junction (myoneural junction) causing muscarinic effects and at autonomic ganglia causing nicotinic effects.

Table 1

Nicotinic	Muscarinic
1. Fasciculations	1. Wheezing, salivation, lacrimation
2. Cramps	2. Pulmonary oedema
3. Muscle paresis	3. Anorexia, nausea, vomiting
4. Hypotension	4. Abdominal cramps, frequency of urination
	5. Blurring of vision

In a study involving 97 sprayers of organophosphates for at least one year we observed neurotoxic syndromes involving –

(1) Difficulty in concentration and poor short term memory (16)

(2) Macular degeneration in 11, choroiditis in 1, pseudopapilloedema in 2.

(3) Slowing of motor nerve conduction in 29.

Wadia from Pune, in a study of 200 cases of acute and subacute organo-phosphate poisoning has reported 5 clinical presentations.

(a) Muscarinic syndrome leading to death

(b) Proximal limb weakness

(c) Cranial nerve paralysis

(d) Guillain Barre like syndrome

(e) Delayed neurotoxicity with wasting of limbs and lower motor neuron paresis.

In an epidemiological study of 100 matched pairs of individuals with history of organophosphate toxicity from Colorado, USA (1988), there was no significant clinical difference in ophthalmic, audiometric or electroencephalographic tests. Tests for cognitive functions however showed abnormalities in short term memory, abstract thinking and mood affective behaviour. There was also a significant hyper-reflexia.

The Environmental Protection Agency, USA collected clinical data on 583 persons working in farms and pest control business. Their analysis showed that 200 had to be hospitalized for toxic syndromes and of these 13% showed slowing in nerve conduction velocity The pesticides involved were organophosphates, namely diazinon, malathion and chlorpyrifos.

Carbaryl is a contact nerve toxin and was marketed under the name of Sevin in India by Union Carbide Co., USA. Symptoms of acute carbaryl toxicity cause acute abdominal pain, blurred vision, lacrimation, sweating, light headedness and

general weakness. Results of chronic toxicity have not been documented so far. The product has been withdrawn from the market following Union Carbide's Bhopal gas disaster.

Pyrethrin is considered to be a safe pesticide. It is moderately persistent in food. Contact with skin can cause dermatitis, rhinitis and asthma. In higher concentration the follows effects on CNS are observed:

(a) Hyperexicitability and headache

(b) Tremulousness

(c) Corneal ulcer

(d) Ataxia

(e) Paresis of extremities.

These were reported in 1988-89 by Chinese scientists in workers in Pyrethrin manufacturing factories. There have been no recorded reports of neurotoxicity in India due to this compound so far.

Aluminium Phosphide is used as a fumigant for stored grain. However, when it comes in contact with damp air toxic phosphine gas is released. Over 452 cases of toxicity have been reported from India in one year. The mortality is from 56-62%. Suicide attempts are common with this chemical.

Clinical features are: (1) Delirium, acute encephalopathy and convulsions; (2) Cardiac dysarhythmias and pulmonary edema. Other symptoms are abdominal spasms, swollen jaw, anemia and spontaneous bone fracture. Several deaths and clinical reports have been documented from Rajasthan, Gujarat, Maharashtra, Chandigarh, Uttar Pradesh and New Delhi.

Napthalene is widely used in Indian homes as a moth, silver fish repellent and wool preservative. This compound is a hydrocarbon from middle oil distillate of coal tar. It is available in the form of small white balls. Accidental ingestion causes:

(1) Headache, confusion, disorientation, ataxia.

(2) Convulsions and coma leading to death in susceptible children.

(3) Gastro-intestinal symptoms like nausea, vomiting, diarrhoea and dysuria.

(4) Jaundice, acute nephritis and hemolytic anemia in persons with glucose 6-phosphate dehydrogenase deficiency.

In case of inhalation as a vapour napthalene may cause malaise, headache, disorientation, dermatitis, conjunctivitis and retinal injury. Continuous long term inhalation of the vapour due to naphthalene being sprinkled on bed clothes may produce these toxic symptoms. The fatal dose is 2 to 2.6 g in children and 5-15 g in adults. 3 cases of naphthalene toxicity have been observed by us. Two of these were due to acute accidental poisoning and one was due to chronic inhalation. All responded to therapy.

Neuropathy target esterase (NTE) is a membrane bound protein with high esterase catalytic activity. The physiological function of NTE is not know. There is, however, overwhelming evidence of modification of the structure of NTE by covalent binding of some organophosphate esters initiating an irreversible polyneuropathy. Studies of NTE can generate successful prediction concerning (a) prophylaxis and (b) effects of low level administration of neurotoxicants like pesticides. Purification of NTE is reaching a point where antibodies may be obtained for NTE assays in toxicology research on pesticides in human subjects.

Evidence elsewhere suggests that pesticides may be involved in :

(1) Azospermia
(2) Teratogenicity and birth defects
(3) Foetal stillbirth (?)
(4) Visual:Macula/optic pathway defect
(5) Some types of carcinomas (soft tissue sarcoma)
(6) Sensory neuropathy
(7) Depressive affective disorder
(8) Encephalopathy with radiculopathy
(9) Degenerative neurological disorders such as Parkinson's syndrome or motor neuron disease like presentation
(10) Cardiac conduction defects.

Whether this is a toxin which could effect all individuals or only the susceptible ones due to change in neurotransmitter status, is still unclear and needs further scientific work.

It is of concern to us as a nation, as scientists to create public awareness, strict implementation of precautions when these toxicants are used, constant monitoring by enforcement agencies, destruction of all containers used in storage of pesticide or having them recycled by manufacturers and most important, to monitor the health of workers, rural/urban populations exposed to these chemicals.

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The Neurotoxicity of 1-Methyl-4-Phenyl-1, 2, 3, 6-Tetrahydropyridine

R.C. SRIMAL AND C. NATH

Among the neurotoxins, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is perhaps the most interesting and unique due to its property of inducing parkinsonism in human as well as non-human primates by selective degeneration of the nigral dopaminergic neurons. The properties of MPTP began unfolding in 1982 with a surprise clinical observation that some young adults n California (USA) developed classical features of Parkinson's disease which otherwise afflicted only the elderly persons. The only common link among these young parkinsonian patients was the recent use of a 'new synthetic heroin'. Analysis of the sample of this heroin revealed the presence of a meperidine analogue 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) and sufficient amount of a byproduct -MPTP. Ultimately, MPTP was identified as the offending agent which caused parkinsonism (Langston *et al* 1983). The discovery of MPTP also solved the mystery of an earlier case of Parkinson's disease in a young chemist who used to self administer a meperidine analogue, synthesized by himself (Davis *et al* 1979). These unfortunate incidents provided a new direction to the research related to the development of an animal model for the study of Parkinson's disease. This was also one of the rare instances where the clinical observations were followed by laboratory investigations in animals.

Chemistry

MPTP is a highly lipophilic tetrahydropyridine derivative whose structure is shown in Fig. 1. Several investigators have studied the structure activity relationship of this toxin in an effort to understand its mechanism of action and to identify

the active pharmacophore. In brief, the conclusions are that the toxicity of MPTP is lost by (1) removal or substitution of CH_3 at N, (2) alteration in double bond in N containing ring, (3) removal of phenyl ring or (4) substitution on the phenyl ring (Heikkila *et al* 1985).

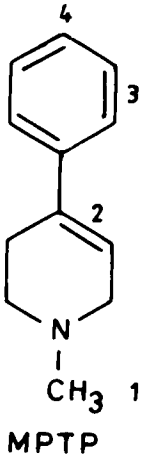

Fig. 1.

Neurological effects

MPTP has been studied in a wide range of animal species, from primates to leeches. However, the characteristic parkinsonian effects of MPTP have been found only in the primates. In other species the behavioral effects were either non-existent or were observed for a brief period even though depletion and degeneration of dopaminergic neurones was observed. The results of study in different species are summarised below:

Human

MPTP induces parkinsonism in humans which is clinically very much like idiopathic Parkinson's disease except for the age of the patient and the onset of the symptoms. The patients of MPTP-induced parkinsonism were all young (mid 20's) and they developed classical symptoms like generalised slowing, difficulty in movements, fixed stare, cog wheel rigidity, pill rolling tremors and micrographia within 4-5 days after consuming contaminated synthetic heroin over a period of 5-8 days (Langston *et al* 1983). In contrast, idiopathic parkinsonism develops slowly over a period of years in elderly patients. The antiparkinsonian drugs are, however, effective against MPTP-induced parkinsonism (Langston *et al* 1983).

Similar to the idiopathic parkinsonian patients, in the MPTP patients also, turnover of nigral dopamine is markedly decreased. Conversion of 18F-6-Flurodopa into 18F-6-Flurodopamine in nigral neurons is reduced (> 70% in symptomatic

patients) as revealed by PET studies (Calne, *et al* 1985). Cerebrospinal fluid (CSF) level of homovanillic acid (HVA), a major metabolite of dopamine is low (34% of healthy controls). In MPTP exposed patients CSF level of 3-methoxy phenylethyleneglycol (3-MHPG), a metabolite of norepinephrine is high, while in idiopathic parkinsonism it is low. CSF level of 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, remains unaffected in both the types of parkinsonism (Burns *et al* 1984). Histopathological changes in brain have been studied in one patient of MPTP. There was marked loss of pigmented neurons with eosinophilic inclusion bodies in the substantia nigra. Other brain areas were unaffected. In idiopathic parkinsonism, neurodegeneration occurs in the substantia nigra and also in other areas like basal nucleus of Myenert, locus coeruleus and the dorsal nucleus of vagus (Kopin and Markey 1988). However, it is not certain that neuronal degeneration found in the brain areas other than the substantia nigra in idiopathic parkinsonism is part of age-related neuronal destruction or specifically due to Parkinson's disease.

Non-human primates

MPTP produces parkinsonian effects in all the species of non-human primates investigated so far (rhesus, squirrel and cyanomologus monkeys, marmosets and baboons). Following MPTP (cummulative dose range 1-10 mg/kg given in different schedules by iv, ip, im or sc routes), the effects (hypokinesia, muscular rigidity, hunched posture, intermittent eye lid closure, difficulty in picking up food, freezing of movements and postural tremors) develop within a week and gradually increase in severity (Burns *et al* 1984, Langston *et al* 1983, Gopinath *et al* 1990). However, the degree of motor impairement is highly variable and difficult to predict. Survival of monkeys ranges from one to several weeks. Some monkeys recover spontaneously which has not been observed in human parkinsonism. By using ^{76}Br-spiperone as ligand for D_2-receptors in PET studies in baboons, the spontaneous recovery has been linked to the increase in striatal D_2-receptors (Hantraye *et al* 1986). The involvement of other factors like age and sex in the variability of parkinsonian effects in the monkeys are not conclusive. With the aim of reducing mortality, some investigators have tried to produce hemiparkinsonism in monkeys by intracarotid injection of MPTP (Guttman *et al* 1990). In such a model, one half of the body serves as control for comparison, if a proper dose has been selected for unilateral effect.

MPTP causes marked decrease in the level of tyrosine hydroxylase, dopa-decarboxylase and dopamine, selectively in the striatum. During acute phase, HVA, MHPG and 5-HIAA are decreased in CSF, while in chronic phase (after 3 months) only HVA remains low (Burns *et al* 1983).

Histologically, MPTP produces neuronal cell loss in the substantia nigra. The nigral dopamine neurons show lesser fluorescence and distortion of axons. The other brain areas are spared by MPTP (Burns *et al* 1983, Jacobowitz *et al* 1984). However, Forno *et al* (1986) reported eosinophilic inclusion bodies in substantia nigra, dorsal motor nucleus of vagus, dorsal raphe nucleus and nucleus of Meynert in squirrel monkeys. It has also been reported that amongst primates,

cynomolgus monkeys are more sensitive to MPTP than rhesus. A comparison of the idiopathic and MPTP-induced parkinsonism has been made in Table 1.

Table 1: Comparison of Idiopathic and MPTP Parkinsonism

Parameter	Idiopathic parkinsonism	MPTP induced parkinsonism	
		Human	Monkey
(A) Clinical features			
Classical sign and symptoms	Present	Present	Present
Age	Elderly	Any age	Ambiguous
Onset	Slow	Rapid	Rapid
Reversibility	No	No	In some cases
Anti-parkinsonian drugs	Effective	Effective	Effective (inconsistent)
(B) Neurochemical changes			
Dopamine (Nigrostriatal)	Decreased (more in putamen)	Decreased (more in caudate)	Decreased
Norepinephrine	Decreased	Increased	Decreased (acute phase)
Serotonin	No change	No change	Decreased (acute phase)
(c) Neuronal degeneration			
Nigral dopamine neurons	Cell loss	Cell loss	Cell loss
Other areas	Affected	Spared	Spared

Rodents

Rodents in general are refractory to nigrotoxic effects of MPTP. Acute transient behavioural effects like clonic movement of front paws, immobility, Straub reaction and piloerection have been reported in rodents, following MPTP (Chiued *et al* 1984). It also depletes dopamine persistently and norepinephrine only in acute phase without affecting 5-HT in mice (Hallman *et al* 1985). Sensitivity of mice of MPTP depends upon their strain. Sonsalla and Heikkila (1986) reported that dopamine depletion in neostriatum by MPTP was more marked in C-57 black mice in comparison to albino mice. Neuronal damage by MPTP has been demonstrated to be more wide spread in older mice than in younger animals (Gupta *et al* 1986, Ricaurte *et al* 1987). In rats, depletion of dopamine and rise in 5-HT in substantia nigra and raphe have been shown (Chieuh *et al* 1984). Fredriksson *et al* (1990) demonstrated that l-dopa could reverse the MPTP-induced hypoactivity in mice as is observed clinically, thus providing evidence for the use of black mice as a suitable animal model for the study of parkinsonism. Inspite of these changes MPTP does not produce consistent classical parkinsonian effects-hypokinesia, muscular rigidity, catatonic posture and tremor-in rodents, similar to those observed in primates (Kaakkola and Terravainen 1990).

Other species

The effect of MPTP in other animals species e.g. beagle dog, cat, rabbit, guinea pig and frog have also been explored but they are much less susceptible except

the beagle dog (Kopin and Markey 1988). Beagle dogs, however, exhibit similar extensive and specific loss of nigral neurons following MPTP as in primates (Parisi and Burns 1986).

Mechanism of MPTP toxicity

Extensive studies have been conducted to determine the mechanism of MPTP induced nigrotoxicity. Though the final answer is still not available the whole process can be divided into following steps:

1. Bioactivation
2. Intraneuronal uptake
3. Intraneuronal accumulation
4. Effect on intracellular enzymes

(1) Bioactivation

MPTP is rapidly oxidized into a charged pyridinium derivative MPP + (1-methyl-4-phenylpyridinium) in two steps (Chiba *et al* 1984). This biotransformation takes place in virtually all the organs except eye (Langston *et al* 1983). *In vitro* and *in vivo* studies have provided evidence for the involvement of monoamine oxidase (MAO-B) enzyme at first step in which MPTP is oxidised to $MPDP^+$ (1-methyl-4-phenyl-2, 3-dihydropyridinium) which may be further oxidised by MAO or non-enzymically. However, the exact mechanism at step II is not known. It may also be auto-oxidation (Langston 1985). In rodent brain crude mitochondrial preparation MPTP is transformed into MPP^+ which is blocked by pargyline (a non-selective MAO inhibitor) and deprenil (MAO-B inhibitor) but not by clorgyline (MAO-A inhibitor). However, the kinetic constants, of MPTP are similar to those of benzylamine, a MAO-B substrate (Chiba *et al* 1984, Heikkila *et al* 1984, 1985). Autoradiographic studies have shown that MPTP binding sites in the brain correspond to MAO distribution (Parson and Rainbow 1984). *In vivo* pretreatment with pargyline or deprenil, but not clorgyline, prevents MPTP-induced dopamine depletion in mice (Heikkila *et al* 1984) and parkinsonian toxicity in monkeys (Cohen *et al* 1984). Deprenil increases dopamine level in parkinsonian patients and in combination with l-dopa increases the life expectancy of the patients in comparison to patients being treated only with l-dopa (Kopin and Markey 1988).

On the basis of these evidences it was concluded that instead of MPTP its active metabolite MPP + is neurotoxic. It has been further supported by direct injection of MPP + in the substantia nigra which decreased dopamine and produced motor deficit, more pronounced than MPTP, in rats (Bradbury *et al* 1986). Systemically, administered MPP + is not neurotoxic since it cannot cross the blood-brain barrier easily.

(2) Intraneuronal uptake

MPP + has high affinity for dopamine uptake sites. In rat striatal synaptosomal preparation, MPP + but not MPTP, is taken up at a rate similar to dopamine. In mouse striatal slice preparation, uptake of MPP + is 25 times greater than

MPTP. MPP + is also taken up by noradrenergic and serotonergic neurons but at 10 times lesser rate than that in dopamine neuron (Javitch and Snyder 1984). These observations suggest that MPP + is formed outside the dopamine neuron. The non-dopamine neuronal sites proposed for oxidation of MPTP are glial cells or 5-HT neuron (Langston 1985). *In vivo* studies also confirm these *in vitro* observations. Specific dopamine uptake blockers, mazindol and nomifensine, prevent the MPTP induced dopamine depletion in mice (Sundstorm *et al* 1986) and parkinsonism in primates (Schultz *et al* 1986). The selective inhibitors of noradrenergic and serotonergic uptake mechanisms do not prevent neurotoxic effects of MPTP (Singer *et al* 1987). The higher uptake of MPP + in dopamine neurons also explains why among the neurotransmitters, dopamine is most affected by MPTP.

(3) Intraneuronal accumulation

MPP + selectively accumulates in the substantia nigra (half life: 5 days) in primates but not in rodents (Langston 1985). One striking difference between the substantia nigra of these two species is the neuromelanin, which is present in primates but absent in rodents. Taking this clue, studies were carried out on neuromelanin. It has been demonstrated by *in vitro* studies that MPP + binds to neuromelanin obtained from the autopsied human substantia nigra and the binding is inhibited by chloroquin. Chloroquin pretreatment prevented MPTP induced parkinsonism in monkeys without affecting MAO and dopamine uptake (D'-Amato *et al* 1986). The binding of MPP + with neuromelanin explains the refractoriness of rodents and anatomical selectivity (substantia nigra) for MPTP neurotoxicity. The precise role of neuromelanin in MPTP toxicity is not known, but possibilities are: (i) neuromelanin being redox polymer containing free radicals, may make cells more vulnerable to MPP +, (ii) neuromelanin and MPP + complex itself is toxic, (iii) neuromelanin acts as a storage site for MPP + or (iv) neuromelanin promotes formation of MPP + from MPDP + (Kopin and Markey 1988, McCrodden *et al* 1990). MPP + may be stored in the vesicles besides neuromelanin (McCrodden *et al* 1990). Release of MPP + from dopaminergic nerve terminals has been shown on stimulation (Keller and Da Prada 1985).

(4) Intracellular enzymes

Two main mechanisms have been suggested for the neurotoxicity of MPP^+, either or both of which may be responsible for cell death.

(i) Free radicals are known to cause cellular toxicity by membrane lipid peroxidation, oxidative damage to nucleic acids, or inactivation of vital enzymes. By spin trapping technique it has been shown that MPP^+ increases formation of superoxide and hydroxyl free radicals during incubation with NADPH cytochrome P-450 reductase in the presence of oxygen and ethanol. A hydroxyl radical scavenger inhibits MPP^+ potentiation of free radical formation *in vitro* (Sinha *et al* 1986). Inhibition of superoxide dismutase (an inhibitor of free radicals) by diethyldithiocarbamate (DDC) potentiates MPTP toxicity in mice (Corsini *et al* 1985).

(ii) MPP^+ binds with mitochondria by an energy-dependent uptake mechanism and inhibits their energy metabolism by inhibiting the oxidation of NAD-linked substrates (Nicklas *et al* 1985). It is actively accumulated by mitrochondria in such a way that 50-100 times the external concentration can be achieved (Ramsey and Singer 1986). The cellular ATP is depleted and alterations in cellular calcium content can by observed (Di Monte *et al* 1986, Kass *et al* 1988).

Inspite of the progress made in understanding of the biochemical mechanisms of MPTP selective nigro-toxicity, it is not clear why the ventral tegmental area which also contains pigmented dopamine neurons is not degenerated by MPTP. Knowledge is also incomplete on some other aspects of MPTP such as-effects on neurotransmitters other than dopamine, nonparkinsonian effects, and ultimate fate of MPP^+.

It is clear that MPTP has emerged as a very interesting and important neuropharmacological research tool to study Parkinson's disease and neurodegenerative process. It has provided important lead in the research areas of parkinsonism, specific neurochemical and anatomical neurotoxicity and neurotoxicity of free radicals. A more complete knowledge of MPTP may provide better understanding of neurodegenerative processes.

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Pathology and Probable Pathogenesis of Dementia

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Dementia is a symptom complex (not a disease by itself) characterised by acquired global impairment of intellect, memory and personality, without impairment of consciousness. Though it has some commonality to the pathological features seen in aging and is common in elderly population, it is not an exaggeration or acceleration of the natural aging process. The definition of this entity according to Diagnostic and Statistical Manual of Mental Disorders (DSM III) and American Psychiatric Association is, "cognitive impairment of sufficient severity to interfere with work or activity of daily living, incorporating memory loss plus atleast one other cognitive deficit like apraxia, agnosia, visuospatial impairment". The memory and other cognitive functions show a decline in comparison with the patient's previous level of functioning as determined by (a) decline in performance, (b) clinical examination, and (c) neuropsychiatric testing (Mekhann *et al* 1984).

The dementing illnesses in a sense, are all secondary, but at the present state of knowledge, in a large number of patients no definite cause can be identified and hence are labelled primary or idiopathic dementia, a group of degenerative diseases of the nervous system. Depending on the predominant topographic distribution of the lesions, they can be classified as cortical and subcortical dementias. Cortical dementias are characterised by prominent cortical dysfunction like aphasia, apraxia, agnosia and acalculia, poor registration of memory, relatively normal cognitive processing speed despite the inaccuracies, with less disturbances of mood, personality and motor control. The subcortical dementia, on the other hand, have prominent personality and mood disturbances, motor deficits, associated with poor memory recall, slowness of cognition and preserved language. These diseases appear in previously normal individual and follow a

subacute or slow unremitting course. The clinical syndromes vary according to the group of neurons affected. Although, at times the expression of the disease may be asymmetrical, involvement is usually bilateral. However, the exact basis for the selective vulnerability of specific topographic areas/groups of neurons is unknown.

Alzheimer's disease and Pick's disease are the examples of cortical dementia. Dementia associated with Huntington's chorea, Parkinson's disease, progressive supranuclear palsy, hereditary olivopontine cerebellar degeneration are the examples of subcortical type. Multi-infarct dementia, Binswanger's disease and to a limited extent, dementia pugilistica are caused by deranged/altered cerebrovascular dynamics and vasculopathies, damaging the associative and integrating fibre tracts by the ischemic process. Creutzfeldt Jakob disease and Gerstmann Straussler syndrome with rapidly progressive dementia, have been shown to be caused by a transmissible agent. Similar cognitive dysfunction is caused by many other conditions like subdural haematoma, meningioma and other tumours, normal pressure hydrocephalus, nutritional deficiencies, endocrinopathies and these are referred to as secondary dementias. By timely therapeutic intervention, the neuropsychological abnormalities are reversible in the secondary form.

The pathological aspects of dementia can be analysed at several levels (a) regional distribution of disease process (b) neuronal population/systems at risk (c) abnormalities occurring in individual nerve cells (d) molecular pathology of the cellular constituents. The pathological aspects of some of the important primary dementias are discussed here. Generalities in pathological and neurochemical features are highlighted to get some insight into structure and function correlation.

Alzheimer's Disease

Alzheimer's disease (AD) is the most frequent type, constituting 50-60% of all dementias in the West. It is generally felt that in India, this disease is rare. however, in an unpublished prospective study at National Institute of Mental Health and Neurosciences, Bangalore, during the period 1980-1985, nearly 30% of cases of dementia were found to belong to the group of senile/presenile dementia of Alzheimer's type as per the DSM III criteria (D. Nagaraja, personal communication). Only two pathologically and immunochemically confirmed cases of AD, one based on brain biopsy and the other on autopsy have been recorded from India (Shankar *et al* 1988). One more case was reported purely based on histological grounds (Somasundaram and Menon 1975).

At present there are no laboratory investigations specific for AD. The diagnosis is one of exclusion and the definitive diagnosis can only be made by examination of the brain tissue at biopsy or autopsy. In near future, new imaging techniques like brain mapping, MRI, PET, etc. which permit evaluation of regional changes in the brain structure, mechanism and transmitter specific circuits should provide new insights into the relationship between the clinical symptoms and brain abnormalities.

In AD, there is selective involvement, dysfuction/death of population of neurons in basal forebrain, amygdala, hippocampus, neocortex and brain stem, leading to impairment of memory, language and visual perception (Perry 1986, Perri 1988). Alterations were noted in a variety of neurotransmitter circuits-cholinergic, peptidergic and monoaminergic systems (Davis 1988). Neurons within basal forebrain provide the major cholinergic innervation of cortex and in AD dysfunction and death of these neurons appear to be responsible for a reduction in forebrain cholinergic markers, viz., the activity of choline acetyltransferase, acetylcholine esterase, uptake of ^{3}H-Choline and the synthesis of acetylcholine from labelled glucose. The number of M_2 muscarinic and nicotinic receptors appear to be reduced, but M_1 receptors are not appreciably altered. Many patients with AD show changes in monoaminergic neurons including noradrenergic and serotonergic nerve cells of locus ceruleus and raphe respectively. In addition alterations in serotonergic receptors have been described. Somatostatin neurons show neurofibrillary tangles (NFT) and density of somatostain receptors in cortex is reduced; however, stimulated release of somatostatin does not appear to be altered. Corticotrophin releasing factor (CRF) immunoreactivity is also reduced in the cortex with a reciprocal upregulation of CRF receptors. On the basis of neurochemical studies a variety of peptidergic neurons intrinsic to cortex appear to be spared in AD (Emson and Lindvall 1986). Degenerative changes also occur in some nerve cells in amygdala, hippocampus and associated circuits and neocortex. However, in these instances, the transmitter specificities of these vulnerable neurons have not yet been clarified. Some of these neurons are thought to use excitatory aminoacids as neurotransmitters.

In Alzheimer's disease, affected neurons reveal several types of cytoskeletal pathology. The classical triad described includes neurofibrillary tangles (NFT), senile plaques (SP) and granulovacuolar degeneration (GVD) (Perri 1986). The NFT are intracellular aberrant aggregates of native neurofilaments in neurons located in amygdala, hippocampus, neocortex, basal nucleus of Myenert and several brainstem nuclei like locus ceruleus and raphe. These NFT are made up of highly insoluble, cross linked neurofilaments of 10 nm diameter arranged singly or as paired helical filaments (PHF). These are demonstrated easily by silver impregnation. Immunochemically, the NFT react with antibodies to neurofilaments, microtubule associated protein (MAP 2), 'tau', and ubiquitin (Iqbal *et al* 1986, Cole and Timiras 1987, Goldman *et al* 1986). These filaments show high degree of phosphorylation, thus imparting rigidity and insolubility. The presence of dementia in AD appears to correlate with the number of NFT in the cortex, suggesting that this cytoskeletal pathology may be associated with changes in dynamic properties of the neurons, whose dysfunction and elimination are important in clinical expression of the disease. Available evidence suggests that some NFT represent tombstones, marking the sites of 'once alive' cells. In experimental studies, the electrical activity could be recorded in the cerebral neurons of rabbits, even in cells bearing the NFT. Similar evidence is lacking in human system.

In AD, the senile plaques (extracellular lesions) similar to NFT are abundant, quantitatively much above the level noted in normal aging. The plaques are noted in amygdala, hippocampus and neocortex. They are composed of aggregates of abnormal axons/nerve terminals (neurites), abnormal dendritic processes, admixed with astrocytic and microglial elements and extracellular amyloid (highly insoluble, congophillic, polypetide fibrils in B-pleated sheet configuration). The neurites are enlarged and dystrophic axon terminals filled with filaments (including PHF) and membrane bound dark bodies. Majority of them represent degenerative neuronal elements, while others represent abortive regenerates. Recent studies indicate that cholinergic, monoaminergic and peptidergic systems participate in the formation of senile plaques. Immunochemically, the dystrophic neurites and the central core show reactions similar to NFT with various antiboies. The number of plaques per millimeter of cortex show correlation with the reduction in cholinergic and somatostatinergic markers and the degree of cognitive dysfunction. However, Tomlinson and his colleagues, basing on large number of autopsies studied, noted that in the brains of some dementics, no or minimal number of NFT and SP were noted (Tomlinson *et al* 1970). This finding casts doubt on the hypothesis that there is a linear correlation between quantum of NFT and SP in the brain, and the degree of dementia.

The granulovacuolar degeneration (GVD-an electron dense granule within membrane bound vacuole in the cell) is frequent in the cytoplasm of pyramidal neurons of hippocampus of AD cases. Immunocytochemical studies have revealed that the granules show 'tubulin' like and 'unbiquitin' like immunoreactivity. This cytoskeletal pathology is not seen in the neocortex. The observation that the presence of GVD in pyramidal neurons of the hippocampus correlated with the loss of these nerve cells and the degree of dementia, suggest that GVD may be a unique type of cytoskeletal pathology affecting the function (Blessed *et al* 1968).

Hirano bodies, rod shaped or oval, eosinophilic, cytoplasmic inclusions (originally described in the hippocampus of Guamanian patients with amyotrophic lateral sclerosis-Parkinsonism dementia complex) are commonly seen in the pyramidal cell layer of hippocampus of AD patients. Electron microscopically these structures have a filamentous structure in a paracrystalline form and show actin like immunoreactivity. Hirano bodies unlike the other cytoskeletal forms are not specific for any disease, and are seen even in normal states.

Some of the patients with AD have deposits of amyloid along the walls of leptomeningeal and intracortical vessels. Some disagreement exists regarding the frequency of congophilic angiopathy in AD. The chemical nature of the vascular amyloid and the plaque core amyloid was found to be essentially similar. It has been suggested that the amyloidogenic serum proteins damage cerebral vessels and on reaching the neuropil, form the palque core amyloid. In some plaques, association of vascular elements to the amyloid core have been shown. By high voltage electron microscopy and serial sectioning, the intraneuronal NFT also show the characteristics of amyloid; the exact origin of which is not clear, though it is ascribed to the altered neurofilament material.

Co-localisation of calcium, aluminium (Perl and Brody 1980, Garruto *et al* 1984) and silicon (Rees and Cragg 1983) in the palque core, the former two in the NFT and the association of Guamanian Parkinsion dementia complex with low dietary calcium and magnesium and high aluminium has raised the tantalising questions regarding the possible role of some environmental factors in the development of AD. No definite answer has been found.

Pick's Disease

This type of dementia, described by Pick and Chian, and again by Alzheimer (1911) is much less common. the disease seems to be familial in some cases, but mostly sporadic. Seizures and abnormal movements are rare. At times it is difficult to clinically distinguish dementia of Pick's disease from AD. Characteristically, insight is lost early leading to disturbed behaviour and a fatuous affect, while memory is relatively preserved. Dysphasic phenomenon, dyspraxia and visuospatial disorders occur much less commonly and with a later onset than AD. As with AD, a confident diagnosis can be made only on postmortem. Grossly there is severe, focal or lobar atrophy with particular involvement of frontal and temporal lobes, usually sparing the posterior one third of superior temporal gyrus. The brain weight is reduced (often less than 1000 gms), the ventricular system is symmetrically dilated and both the cortical grey and white matter are reduced.

Microscopic changes consist of severe loss of cortical neurons, with reactive gliosis, that extends to the subcortical white matter. Some neurons contain a discrete, oval intracytoplasmic intensely argentophilic body, the Pick's body. Ultrastructurally it consists of granular and filamentous material, occasionally twisted tubules. Immunochemically these bodies react similar to NFT (Goldman and Yen Shu-Hui 1986). The topographic and density distribution of Pick's bodies in hippocampus is found to be similar to those already demonstrated for Hirano bodies, granulovascular degeneration and NFT in AD, thus suggesting a common neurotransmitter defect that may underlie the dementia of Pick's disease as well (Ball 1979). Not a single pathologically confirmed case of this disease has been reported from India. However, recently a probably case has been noted in Bombay.

Parkinson's Disease and other Extrapyramidal Disorders

Parkinsonian symptoms occur during the course of a number of dementing syndromes including AD and dementia may occur in Parkinson's disease as a late phenomenon (Quinn *et al* 1988). The pathology of Parkinson's disease (PD) itself involving the dopaminergic and serotonergic systems can cause cognitive dysfunction, but by itself it seems unlikely to be responsible for excessive prevalence of dementia. In AD neurofibrillary tangles are found in hippocampus and nuclei involved in cholinergic projections to neocortex. In PD, Lewy bodies are distributed in the same nuclei, in addition to substantia nigra. Loss of neurons and shrinkage are noted in nucleus basalis, both in AD and PD (Price *et*

al 1986). This loss has been greater in demented than in non-demented Parkinsonians. Other brain stem nuclei affected both in AD and PD include locus ceruleus, dorsal raphe, both of which project to the cerebral cortex. Somatostatin like immunoreactivity is reduced in neocortex of AD, so also in the frontal cortex of PD, with dementia. Some of these cases of PD with dementia, in addition to Lewy bodies, also show NFT and plaques in hippocampus, similar to AD. These features support the hypothesis that a summated pathological substrate of subclinical AD with PD in at-risk neuronal population leads to dementia. Further work in this line is necessary.

Huntington's Chorea

This autosomal dominant disorder, with a gene called 'G 8' located on the short arm of the 4th chromosome controlling the disease process, manifests usually during 3rd to 5th decade, with chorea. It progresses relentlessly to manifest, only in some cases, dementia, personality changes, memory loss, and agnosia and apraxia, a few years later. Occasionally, dementia alone constitutes the obvious clinical picture! (Jervis 1963, Hayden 1981).

Pathologically, the brain is atrophic. The cardinal feature is the striking, selective symmetrical atrophy of caudate nucleus and putamen, more severe anteriorly than posteriorly. Histologically, there is loss of small efferent neurons and gliosis, the large interneurons being relatively spared. In spite of generalised atrophy, definite neuronal loss and reactive gliosis, are not consistently noted in the neocortex. In addition, unlike AD, nucleus basalis of Meynert is spared in these cases. Neurochemically, there is decrease in choline acetyl transferase (CAT), glutamic acid decarboxylase (GAD) and gamma aminobutyric acid (GABA) in the striatum. The dementia appears to be secondary to retrograde degeneration in the areas receiving projections from striatum and related structures (Earle 1973).

Progressive Supranuclear Palsy

This rare disorder of unknown aetiology was defined in 1963 by Richardson, Steele and Olszewski (1964). The disease usually begins in the 50-60 yrs. age group and death occurs in 5-7 years. Cognitive and personality changes leading to dementia are early features, that are soon associated with loss of eye movements, particularly of inferior and superior conjugate deviation, Parkinsonian symptoms without tremor and dysarthria. The cognitive function abnormality arises from marked psychomotor retardation, rather than the kind of impairment associated with true dementia. The pathological changes consist of symmetrical neuronal loss and gliosis in the globus pallidus, subthalamic nucleus, red nucleus, substantia nigra, tectum, locus ceruleus, periaqueductal grey matter and dentate nucleus. Many of the remaining neurons contain 'globose' type of neurofibrillary tangles, (unlike the elongated, flame shaped ones in AD). Immunochemically these tangles show same characteristics as noted in AD. The areas of cholinergic projection are well preserved.

Progressive Primary Subcortical Gliosis

These rare cases described by Newmann and Cohn (1967) resemble AD and Pick's disease clinically. Pathologically however, mild lobar atrophy, significant subcortical gliosis with sparing of the cortex are noted. No NFT or senile plaques are noted. The gliosis is marked in the thalamic nuclei. No data on neurochemical studies is available.

Dementia and Cognitive Impairment in Psychiatric illness

Some of the psychiatric disorders present with/or progress to dementia or pseudodementia. Clinically, depression, an affective illness, is at times misdiagnosed as dementia. Physiological studies, in some have shown a reduction in blood flow to the grey matter and increased latency of auditory evoked potentials, suggesting a possible subcortical derangement. These patients, however, recover following therapy. Recent MRI studies on aged patients with depression, have revealed increased prevalence of cortical infarcts and leucoencephalopathy in comparison to age matched controls and dementia cases. These findings indicate that major depression in late life is not functional and like dementia is associated with remarkable increase in overt pathological changes in the brain (Zubenko *et al* 1990).

In type II schizophrenia, which also has dementia as a clinical component, structural damage like reduction in volume of hippocampus, amygdala and gliosis of basal forebrain have been noted (Young *et al* 1991). These two conditions could represent two time points in the temporal spectrum of dementia during the evolution (pseudodementia and dementia). Further studies are likely to provide insight into the structure and functional relation of the brain to the cognitive functional parameters.

Infections and dementia

Cruetzfeldt Jakob disease (CJD), a transmissible, unconventional, slow virus disease, is known to have dementia as the main clinical feature. Cases of CJD and Gerstmann-Strauster syndrome (variant of CJD, associated with dementia) are known to show plaque like intracerebral deposit of amyloid, without the participation of the neuritic processes (unlike AD) (Manuelidis 1985, Masters *et al* 1981). This amyloid material is known to have molecular similarities to the one noted in senile plaque of AD. In addition, the topographic distribution of characteristic spongiform encephalopathy has some resemblance to that noted in AD and other neurodegenerative diseases associated with dementia (Mizusawa *et al* 1987).

Some cases of herpes simplex type I encephalitis, at a late stage are known to progress to dementia. In these cases, the topographic involvement by the necrotising viral encephalitis is in the hippocampus and the related structures.

Progressive multifocal leucoencephalopathy, a viral infection of opportunistic nature, involving specifically the white matter (viral inclusions in

oligodendroglia) is known to present with dementia. Wide spread demyelination and deafferentation of the long fibre tracts and association bundles, like in multi-infarct dementia is the probable pathological basis for the evolution of cognitive impairment.

The acquired immunodeficiency disease (AIDS) a retroviral infection, has a devastating effect on the nervous system, though the primary target cell infected is macrophage/microglia, and not the neuron (unlike other neurotropic viral infections). Most clinical studies indicate that more than 60% of patients of AIDS eventually develop cognitive dysfunction and dementia. At autopsy, the brain of these patients show cerebral atrophy, white matter pallor and moderate loss of neurons. The cellular pathology include invasions of macrophages to form microglial nodules and giant cells. The pathological features are usually sparse and mild, despite the fact that the patients have profound cognitive impairment. The exact mechanism responsible for the impairment of neuronal function is not clear. The following are some of the proposed aetiopathogenetic mechanisms for AIDS associated dementia.

(a) co-existing opportunistic fungal and viral infections.

(b) demyelination and white matter destruction by the cytokines secreted by the infected/activated lympho monocytoid cells altering cellular function in CNS.

(c) direct neuronal killing by gp-120 protein (the HIV-I envelop glycoprotein).

(d) viral proteins causing cytotoxicity to cells either by blocking neurotransmitter or neurotrophic factors.

(e) autoimmune mechanism.

Recently, Guilian *et al* (1990) have characterised a small molecular weight (less than 2 KD), heat stable, protease resistant neurotoxic molecules, secreted by HIV infected monocytes and promonocytoid cells, but not infected lymphcytes. This substance has been found to mediate neurotoxicity via N-methyl-D-aspartate receptor (as agonist). This toxicity for chick and rat neuronal culture cells could be abolished by and NMDA receptor antagonist. To further support the NMDA receptor mediated neurotoxicity, in AIDS, Heyes *et al* (1991) have shown increase in quinolinic acid, an excitotoxic metabolite and an agonist of NMDA receptors, in CSF of patients with AIDS dementia complex. The CSF levels of quinolinic acid was found to parallel the severity of cognitive dysfunction and neurospychological deficits. After treatment of AIDS dementia with zidovudine and the treatment of opportunistic infectious specifically, CSF levels of quinolinic acid decreased parallel with clinical and neurological improvement. It is possible that quinolinic acid has a direct role in the pathogensis of brain dysfunction, in addition to being a marker for host-virus mediated events in the brain.

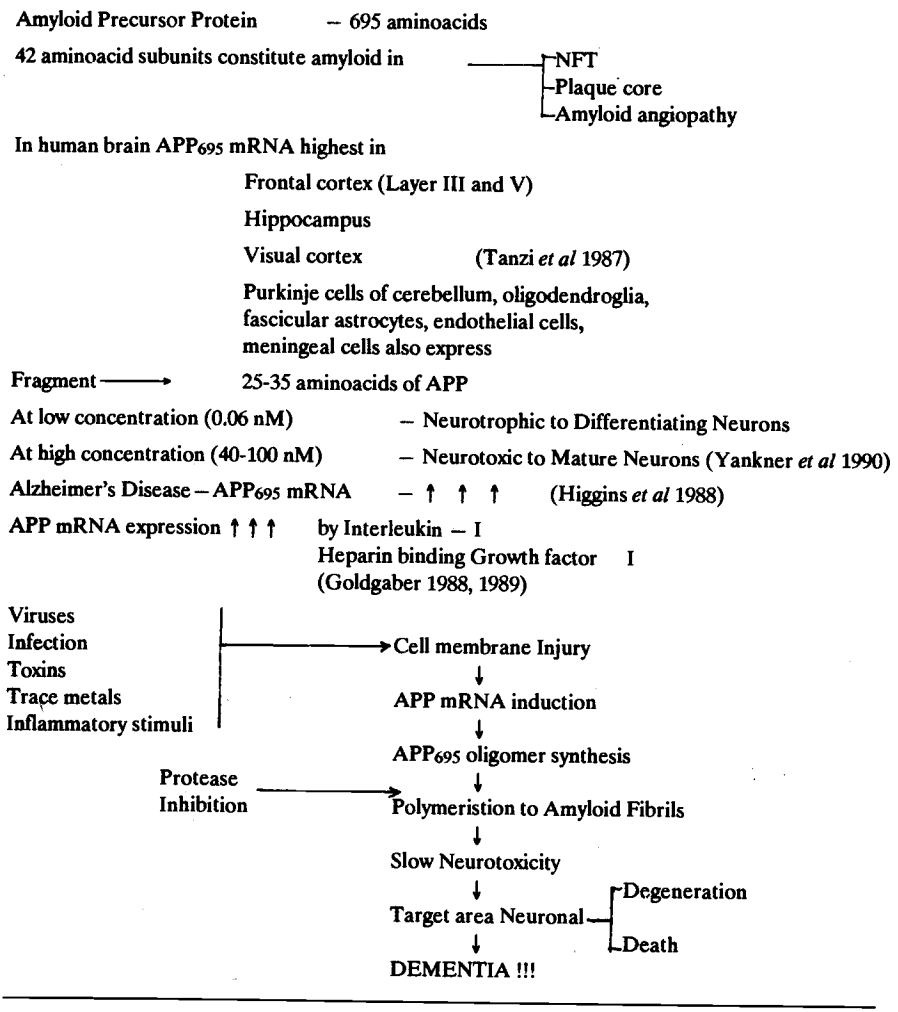
Among the bacterial infections, neurosyphilis is well known to be associated with dementia, probably secondary to vascular changes and ischemic process. Menin-

geal thickening with inflammation, neuronal loss, gliosis and rod cell proliferation are the usual microscopic features. In endemic zones, cerebral cysticerosis (infestation by larval form of tenia solium), has been noted to present with various psychiatric manifestations and dementia. Cognitive impairment is more common in cases of diffuse cerebral cysticercosis (behaving like mass lesions) or intraventricular cysticercosis (acting like a ball valve and causing hydrocephalus).

A perusal of literature on the regional and cellular pathology and neurochemical studies in dementias, brings out some commonalities in cell groups at risk and the neurochemical defects. The cholinergic defect (both the cells involved and the enzyme systems) has been considered to be responsible for the cognitive impairment in Alzheimer's disease. Now a growing list of conditions have been identified with similar cholinergic deficiency and the associated dementia, viz., Parkinsonism (especially with dementia), Parkinsonism dementia complex of Guam, Down's syndrome, Gerstmann-Straussler syndrome, hereditary olivopontine cerebellar degeneration, progressive supranuclear palsy dementia pugilistica and Korsakoff psychosis. However, this hypothesis runs into problem, by the example of Parkinsonian patients, who inspite of degeneration of more than 70% of the cholinergic system show only mild cognitive defects. This suggests that in addition to the cholinergic defect, other factors modulate the expression of the dementing process and associated psychiatric and psychological abnormalities.

Recent molecular biological studies have helped to identify the gene encoding the amyloid protein precursor (APP), made up of 695 aminoacids. The gene has been localised to the proximal part of long arm of the chromosome 21 (Goldgäber *et al* 1987). This gene encoding the APP is expressed in various human and animal tissues and is highly conserved in evolution. The cerebral amyloid deposits in the senile plaque core and on the vessels (congophilic angiopathy), to which a vital role in pathogenesis has been ascribed in AD and Down's syndrome are composed of 42 aminoacid (4.2 KD), hydrophobic fragment of the precursor protein. It is suggested that an aberrant proteolytic processing of precursor may result from an inhibition or lack of inhibition of proteases and subsequent confirmational changes/polymerisation to B pleated, rigid, insoluble sheet of amyloid in neurons and other tissues (Guiroy and Gajdusek 1990). Though this protein has been well characterised biochemically, its primary biological function and role in pathogenesis of AD is not known. Similar to the cholinergic defect and dementia the long list of diseases having amyloid in the brain, include all mentioned earlier, except hereditary olivopontine cerebellar degeneration, Korsakoff's psychosis and Parkinsonism dementia of Guam. This suggests that this amyloid protein has a modulatory role in 'specific target area' neuronal death. Recent studies of Yankner *et al* (1990) suggest that *in vitro* 25-35 aminoacid fragment of amyloid B protein is neutrophic to embryonal hippocampal neurons at low concentration and neurotoxic to mature neurons at higher concentration. This differential response of neurons to this protein and variation in gene expression and synthesis of APP protein under the influence of viruses, infection, toxins, trace metals and other unknown environmental factors could be one of the important modulating factors for the disease to manifest.

Table 1: Amyloid Protein and Dementia (possible role/mechanism)



Among the various drug therapies tried in dementia, dihydroergotamine is one of the important ones, aimed at improving the cerebral blood flow and neuronal metabolism (Klawans and Genovese 1986). This at least achieves a subjective improvement, even though cognitive function improvement has never been proved. Multi-infarct dementia, the second most common one, can be treated and its course altered by controlling hypertension and improving cerebral circulation.

The hypothesis that the cognitive impairment in AD is related to cholinergic degeneration has prompted clinical trials with choline and lecithin, acetylcholine precursors, and other cholinergic drugs, but has not yielded expected results (Wettstein 1984, Spiegel 1984, Harbaugh *et al* 1984, Cohen and Wurtman 1975).

In experimental animals nerve growth factor (NGF) a well characterised protein was found to act as a neutropic factor for the cholinergic neurons of the basal forebrain. NGF is found to be able to prevent the degeneration of cholinergic neurons in adult rats with experimental lesions, similar to cholinergic deficit in AD. These findings suggest that increasing the availability of NGF to human cholinergic cells might promote the survival in certain degenerative disease processes (Hefti and Weiner 1986). But no human NGF enough to undertake the long term drug trial and safe for human use, is available. Secondly, no beneficial treatment can be expected with NGF, if the degeneration of the cholinergic neurons in the basal forebrain represent an epiphenomenon of other primary degenerations.

Table 2: CNS Disease with Cortical Cholinergic Deficits and Expression of amyloid B-Protein

1. Choline acetyl transferase ↓↓↓
2. Meynert nucleus neuronal number ↓↓↓
3. Hippocampus involvement

1.	Alzheimer's Disease (AD)	– A4+
2.	Down's Syndrome with AD	– A4+
3.	Parkinsonism Dementia of Guam	– A4+
4.	Parkinson's Disease with Dementia	– A4+
5.	Gerstmann Straussler Syndrome	– A4+
6.	CJD (?) Kuru (?)	– A4+
7.	Hereditary olivopontine Cerebellar degeneration	– A4?
8.	Progressive Supranuclear Palsy (?)	– A4+
9.	Dementia Pugilistica	– A4+
10.	Korsakoff's Psychosis	– A4?
11.	Schizophrenia with Cognitive deficits	– A4?

A4+ – Amyloid B Protein presence in the brain

A4? – Amyloid B protein presence is not definite or recorded

If the amyloid B protein or small fragments of it are proved neurotoxic and the initiator factors for neurodegeneration, search has to be made for substances which can reverse this effect by a specific receptor mediated action. A search on these lines for peptides with sequence homologous to 25-35 aminoacid stretch of amyloid B protein (which was found to be neurotoxic to mature neurons) revealed similarity to the tachykinin family of neuropeptides. In hippocampal neuronal culture system, tachykinin was found to act as antagonist to the B 1-40 aminoacid fragment. It is possible that the endogenous tachykinins or exogenously supplemented ones may help to reverse the toxic effect of amyloid B protein (Yankner *et al* 1990). Perhaps the definitive treatment of AD and related dementias still awaits clarification of the multiple neurochemical variables, delineation of parameters that can predict the clinical course, development of appropriate, safe, long acting pharmacological agents and possibly the use of

methods to enhance the endogenous neurotransmitters and neurotrophic factors.

Table 3: Nerve Growth Factor – Alzheimer's Disease

1.	NGF-Acts as neurotrophic factor for cholinergic neurons
2.	NGF and mRNA for NGF present in human brain
3.	NGF levels highest in hippocampus, cortex, septum, Nu. Basalis (Origin of ascending cholinergic system from basal forebrain)
4.	NGF produced by target cells of forebrain cholinergic neurons
5.	Receptors of NGF-present in central cholinergic neurons
6.	NGF receptors localised in Nu. Basalis present on cell membrane of soma and dendrites
7.	NGF injected into brain of new born rat-increase in CAT activity in septal area, hippocampus, cortex
8.	NGF-seems to affect survival, fibre growth and expression of transmitter specific enzyme of cholinergic neuron
9.	Lack of endogenous NGF or reduced response to NGF might cause degeneration in Alzheimer's disease

To summarise, dementia is a psychiatric syndrome of diverse aetiologies, probably caused by a topographic pathology rather than by a characteristic cellular, chemical and/or molecular pathology, in the central nervous system. In the specified 'at risk areas', a critical mass of neurons have to be affected/depleted for the disease to manifest to a level clinically detectable. The search for various strategies to reverse this relentlessly progressive (though the times slow), degenerative process, still continues.

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Neurophysiological Correlates of Learning and Memory

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One of the most important mechanisms of adaptation is the capacity of the individual organism to learn. Such adaptation as a result of environmental encounters require that an animal be able to store experiences, retrieve the stored information and perform responses commensurating with the experience. Unfortunately, in spite of enormous experimental studies and numerous theories we are as yet far removed from knowing much about the processes underlying the control of performance of learned responses.

Our theoretical conceptions of learning and memory have been particularly weak on the question of performance of memory. This state of affair seems to stem directly from the vagueness of our definitions of learning. Let us take the concept of "a relatively permanent change" which is included in most definitions of learning. Does this requirement imply that all memories are permanent or that some are transient while others are not? On this question, the theories of learning are usually silent. There is, of course, no reason to assume that all consequences of experience are permanent. In fact, there appear to be many reasons for this not being the case. Many of the stimuli surrounding an animal have little or no adaptive significance. It makes little sense in terms of economy or adaptation that all stimuli should produce enduring consequences in the brain. On the other hand, it is of adaptive value to be able to store long term representation of experiences that are either particularly meaningful (in terms of their consequence) or frequently repeated. We will need to understand the conditions under which memories are transient, as well as those leading to the formation of lasting or long term memory, if we are to have a complete understanding of the neurophysiological correlates of learning and memory.

Memory is encoded in the brain. There is changed connectivity in particular neuronal circuits as a consequence of synaptic remodelling, and this modified connectivity, which underlies altered electrical and biochemical properties of the circuit, forms the memory trace or engram. *Engram* consists of making the circuit modification, while *recall* (retrieval or ecphory) consists of reactivating the circuit. Learning, on the other hand, consists of acquiring information about "what-leads-to-what". Therefore, learning is regarded to be cognitive. Broadly speaking, living beings acquire new information about the environment or milieu through mechanism of *learning* and they retain this information through mechanism of *memory* (Kandel 1990).

Learning

Learning is essentially of two types:

(a) Procedural Learning: In this type of learning, the organisms learn that the unconditioned stimulus (US) is preceded by conditioned stimulus (CS). In other words, they learn that a particular response or an event is reinforced when a discriminative signal is given (Mackintosh 1985).

(b) Declarative Learning: This is the next hierarchy of learning and in this the organisms learn to react to discriminative signal (CS) to avoid, postpone or modify the succeeding reinforcement or event (Mackintosh 1985).

In few cases, there can be a third type of learning known as *spontaneous learning*, where the organisms learn to avoid the succeeding reinforcement without the aid of discriminative signal (CS). The organisms attain a very high level of training and respond to the milieu or environment (secondary conditioned stimulus: SCS) without waiting for the CS or US (unpublished observation). The succession of events leading to these three types of learning is shown in Fig. 1.

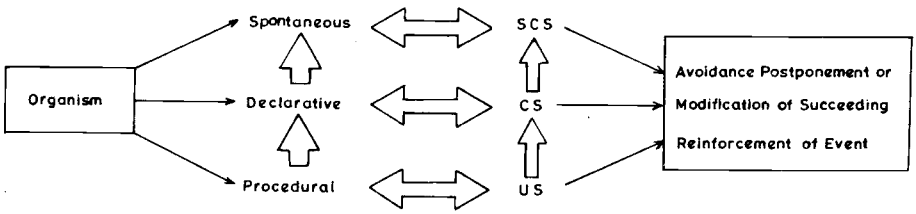

Fig. 1. Organisms learn to respond to a discriminative signal to modify succeeding event at different hierarchy of learning through inter-connected stimuli.

Memory

Retention of the information thus acquired by organisms leads to the formation of memory. There are two types of memory:

1. Episodic Memory: The brain receives and stores information about temporally dated episodes or events, and temporal-spatial relations among these events.

Episodic memory is thus, repository of personal, unique, concrete experiences which are stored in the subjects past (Tulving 1990).

2. *Semantic Memory:* This is essential for the use of language. It is a mental thesaurus. This represents an organized knowledge a person must possess about words and other verbal symbols, their meanings and referents, about relations among them; and about rules, formulae, and algorithms for the manipulations of these symbols, concepts and relations. This memory may be contrasted with episodic memory, which is autobiographical and time-and-space-tagged (Tulving 1990).

Clinical and experimental observations indicate that there are at least two forms of memory: (a) *short-term*: lasting for minutes to hours and (b) *long-term:* lasting for days, weeks or years (Kandel 1990). The central theme of consolidation process as postulated earlier by Mueller and Pilzecker (1900) was that memory trace of a recently acquired experience increases in strength after the acquisition of experience. There is, in fact, evidence that, following training, retention increases with time (Deutsch 1971). However, earlier Kamin (1957) had reported a V-shaped retention curve for subjects retested on a shuttle avoidance retention at different time intervals after training. The deficit is called the "Kamin effect" or "Kamin deficit". Ott and Matthies (1978) have offered experimental evidence to show that the short-term and long-term forms of memory start simultaneously and the deficit in retention curve is obtained at a point where these two forms overlap (Fig. 2).

What are the mechanisms for each of these two forms, i.e. STM and LTM and how are they related? Acquisition and retention of information for STM (and perhaps also for IM) depends upon the synthesis of first and second messengers (5-HT and c-AMP respectively). STM does not require protein synthesis. LTM not only requires protein synthesis but also growth of synapses and expressions of genes (Kandel 1990).

The memory has essentially three phases: acquisition, consolidation and retention. Recall and extinction are the other two phases found in LTM. The functional relationship between these phases has been schematically shown in Fig. 3.

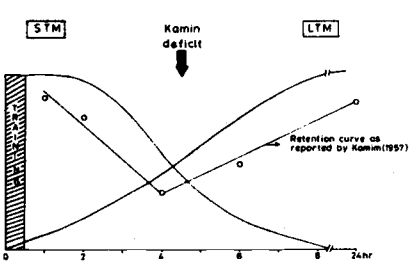

Fig. 2. The hypothesis of Ott and Matthies (1978) on the functional relation between short-term-memory (STM) and long-term-memory (LTM).
: Kamin-deficit occurring as a result of overlapping between two labile forms of the same memory.

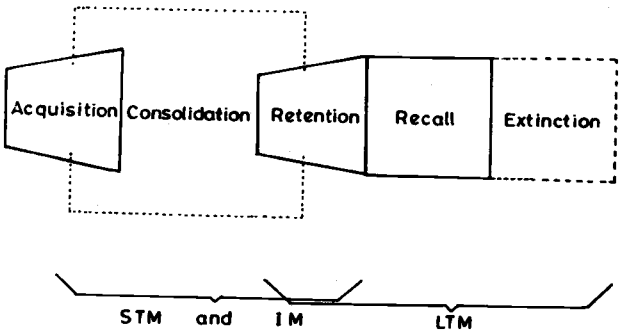

Fig. 3: Functional relationship between acquisition, consolidation and retention

Involvement in limbic system in learning and memory

Various clinical, behavioral, electrophysiological, psychological and biochemical investigations suggest that following limbic structures are important in the various processes underlying learning and memory:

1. Hippocampus
2. Amygdala
3. Olfactory system

1. Hippocampus: The hippocampus has been the focal point of memory studies in the mammalian brain ever since the observations made by Penfield and Milner (Penfield and Milner 1958, Scovile and Milner 1957) that bilateral lesions of hippocampus produce a loss of recent memory. Subsequent electrophysiological studies by Olds *et al.* (1972), Thompson (1976, 1983) and Vinogradova (1975) have done much to further implicate the involvement of hippocampus. On the phylogenetic level, the hippocampus appears only clearly in the mammalian brain, although hippocampal homologues can be found in the brains of the amphibians, reptiles and birds (Angevine, 1975). The most striking feature of hippocampal circuitry is the pattern of afferent termination. Major hippocampal afferents originating from the entorhinal cortex and contralateral and ipsilateral hippocampal subfields synapse on the dendrites of the principal cells in a laminated pattern (Raisman *et al.* 1965). The second important feature of the hippocampus is the manner in which units within the sheets of cells are functionally connected-their intrinsic circuitry. Contained within the sheets of cells is a functional circuit, oriented transverse to the longitudinal (or speto-temporal) axis of the hippocampus. This is known as the principal trisynaptic circuit (Andersen *et al.* 1971). The transverse system may be thought of as strips of cells

that form a functional circuit. The transverse system emerges as a function of the connectivity patterns of the trisynaptic circuitry.

The critical role of the hippocampus in the consolidation of learning and memory was re-emphasized when, in one of the amnestic patients with unilateral left temporal lobectomy, autopsy revealed an extensive atrophy of the right hippocampus (Milner *et al.* 1968). Also, the severity of the memory deficit for visual or verbal material was found to be correlated with the amount of hippocampal tissue included in the right or left lobectomies respectively (Corsi 1969, Milner and Teuber 1968). *Mahut* and his coauthors (1971, 1981) and Moss *et al.* (1981) dissected the mnemonic functions of medial temporal lobe structures by using two approaches. In one, they varied the site of lesion and tested the effects of ablations in both visual and tactual modalities. In the other, they varied the age at which damage to the hippocampal system was inflicted. The salient features of the results obtained from these studies are:

(a) With a task that required either retention or reversal of an object discrimination on alternate days, monkeys with amygdalo-hippocampal, hippocampal, or entorhinal, but not those with inferotemporal cortical, ablations were impaired on retention days, but not on reversal days. Hippocampal removals were also followed by a similar impairment when the task was given in the tactual modality (Mahut *et al.* 1981).

(b) Hippocampal and entorhinal ablations resulted in an impairment in retention of simple object discriminations after 48 h intervals. Monkeys with inferotemporal cortical ablations were impaired at the shortest of the three intervals (Mahut *et al.* 1981).

(c) As expected, monkeys with inferotemporal cortical ablations were impaired on the concurrent object-discrimination task, but so were those with hippocampal or entorhinal ablations (Moss *et al.* 1981). Conceivably, therefore, monkeys with neocortical ablations were impaired because of a primary defect in recognizing the physical attributes of several pairs of objects presented concurrently in the *visual modality*, whereas the impairment of monkeys with hippocampal ablations may have been due primarily to a deficit in retention of object-reward associations that was *independent of modality*.

Consensus on the function of the hippocampal system can perhaps be reached by developing measures of declarative memory that are applicable to studies on animals. Eichenbaum (1990) defined declarative memory as a representation of significant relations among items in memory that supports a capacity for their flexible use in novel situations, and assesses whether this characterization accounts for the data in both odor discrimination and place learning in rats. On the strength of his investigations, Eichenbaum drew following conclusions:

(i) Hippocampal processing is not selective to any particular behavioral paradigm.

(ii) The hippocampal system supports a relational memory representation; individual associations are supported outside this system.

(iii) Hippocampal representations are concerned with memory for specific items and events in any particular task, not for the procedures by which those memories are required.

(iv) Hippocampal representations involve the organization of memories and support their flexible use in novel situations.

2. Amygdala:

There is extensive evidence that amygdala plays an important role in memory storage. It is now well-known that in moneys as well as in humans,lesions of amygdala produce a form of antegrade amnesia-impairment in learning new information (Mishkin *et al.* 1984, Squire and Cohen 1984). Lesions or electrical stimulation of the amygdala impair retention of a variety of recently learned responses (Sarter and Markowitsch 1985, Squire 1986). In view of the findings that damage of the amygdaloid complex (AC) in rats or human patients results in retrograde amnesia which is temporally limited (Liang *et al.* 1982, Squire and Cohen 1984), it seems unlikely that the AC is a site of permanent memory storage. There is, however, considerable evidence that does not fit well with this hypothesis of AC function. Many studies have reported findings that AC manipulations (usually lesions or electrical stimulation) have no, or at best little, effect on learning in appetitively motivated tasks that are based on the formation of stimulus-reinforcement associations (Becker *et al.*1980, Berman and Kesner 1976, Eichenbaum *et al.* 1986, Goddard 1964, Olton and Wolf 1981, Schuckman *et al.* 1969, Slotnick 1985, Slotnick and Kanecko 1981). Recently, Zola-Morgan *et al.* (1989) have provided strong evidence that, in contrast to earlier reports (e.g. Murray and Mishkin 1984), lesions of the AC do not affect monkey's performance in a variety of appetitively awarded tasks. Results from a number of studies suggest that amygdala influences on memory storage may involve its two major input-output pathways – the stria terminals (ST) and the ventral amygdalofugal pathways (VAF) (Davis *et al.* 1987, Kapp *et al.* 1973, LeDoux *et al.* 1988, Liang and McGaugh, 1983a, 1983b, McGaugh *et al.* 1986, Mishkin *et al.* 1984, Ross and Grossman 1977). The findings that ST lesions significantly attenuate the amnestic effect of post-training electrical stimulation of the amygdala argue that the amygdala may be involved in modulating memory storage processing through its influences on neural processes in other brain regions (Liang and McGaugh 1983a).

3. Olfactory System

The smell system is unique among the senses in having virtually direct connections with dorsomedial thalamic nucleus (DMN), amygdala and hippocampus. All these structures are involved in the processing of certain kinds of memory in mammals, including humans. This may explain why odors are so potent in evoking memories and may also explain the findings that olfactory problems are common in human amnestics (Eichenabum *et al.* 1983; Potter and Butters 1980).

It is also interesting that smell memory in rodents displays a number of striking similarities to everyday memory in humans.

The reason that olfaction is not widely used in learning experiments is that odors cannot be used as punctuate, well-defined (temporal or spatial) cues; this vastly complicates the job of measuring physiological responses in the brain. However, some studies suggest that it may be possible to substitute electrical stimulation of the olfactory bulb for odors as cues in rats trained on a series of olfactory discriminations (Staubli *et al.* 1987).

Age associated memory impairment (AAMI)

The problem of impaired intellectual function in elderly persons has generated a great deal of research towards development of drugs which might enhance cognitive function. In particular, a need has arisen for appropriate animal models for use in preclinical evaluation of new drugs or drug combinations. A variety of procedures have been used for this purpose (Gamzu 1985, Gamzu *et al.* 1983, Giurgea 1976, Platel and Prosolt 1982, Schindler *et al.* 1984) all employing tests of animal learning or memory as analogues of human cognitive processes. Most often, drugs, brain lesions, or direct brain insult are used to induce deficits in young, otherwise normal animals (Gamzu 1985) and pharmacologic treatments are then assessed for their ability to reverse the model deficit.

When compared with induction of memory deficits in young normal animals, the testing of drugs against spontaneous memory deficits of aged animals would be expected to have greater capacity to predict successful clinical application. This expectation is based on numerous parallels between memory deficits displayed by aged animals and humans (Campbell *et al.* 1980, Dean *et al.* 1981, Kubanis and Zornetzer 1981). One reliable experimental parallel is that performance on recent memory tasks is preserved for longer periods in young experimental subjects than in good subjects (Kubanis and Zornetzer 1981). The more rapid forgetting by aged rodents is obtained after training for simple responses, such as passive avoidance (Bartus *et al.* 1980, Dean *et al.* 1981, Gold *et al.* 1981, Kubanis *et al.* 1981, Leslie *et al.* 1985, Zornetzer *et al.* 1982) and discriminated active avoidance (Gold *et al.* 1981) which can be easily studied in the contest of various pharmacologic treatments (Bartus *et al.* 1981, Sternberg *et al.* 1985).

Unfortunately, large scale pharmacologic studies of amnesia in aged mice have been limited by the cost and availability of experimental subjects. An alternative approach has been to study the effects of drugs against amnesia in young rodents that receive minimal training and are retested after long retention intervals (De Noble 1986, Flood *et al.* 1981, 1983, 1985, 1986). The assumption is that amnesia under these conditions is qualitatively similar to the more rapid forgetting of aged rodents. Both active (Flood *et al.* 1985) and passive (De Noble 1986) avoidance paradigms have previously been successfully employed to compare efficacy of various pharmacologic treatments against forgetting in young mice.

LTP and memory

The phenomenon of long-term potentiation (LTP) or synaptic enhancement refers to a substantial increase in synaptic efficacy brought about by tetanizing afferent fibres at high frequency. Bliss and Lomo's (1973) original experiments involved preparing anesthetised rabbits with two pairs of stimulating and recording electrodes. A stimulating electrode was placed in the perforant path coming from the entorhinal cortex, and a recording electrode was placed near the granule cells of the dentate gyrus on both sides of the brain. Extracellular field potentials were recorded in response to low-frequency (one per 3 sec) stimuli delivered to either perforant path. Higher-frequency stimuli (15 per sec) were then delivered briefly to one side of the brain only. They found that the size of the field potentials recorded in response to subsequent low-frequency was larger on the side of the brain that had received tetanization than on the control side, and that the increased response was sustained for several hours. They called this phenomenon "long-lasting potentiation". It was soon replicated in the rat both *in vivo* (Douglas and Goddard 1975) and *in vitro* (Schwartzkroin and Wester 1975) and has since been observed in other species, including mice, cats, and squirrel monkeys. Now called either LTP or enhancement, the phenomenon is widely studied.

The investigations of Bliss and associates (Bliss and Gardner-Medwin 1973, Bliss and Lomo 1973) marked a watershed in the research strategy of memory modulation. The fact that a few seconds of high frequency stimulation of the major inputs to the hippocampus, produced an increase in the strength of the connections formed by the input and that this could persist for weeks and months, shifted the focus of attention very substantially on the study of physiologically induced synaptic plasticity. The idea here was that if memory involves long-lasting synaptic modification elicited by neuronal impulses, then one should be able to produce some effects (but on a massive scale) by stimulating brain circuitries with appropriate patterns of activity.

This suggestion was followed by an intense search for the types of synaptic changes associated with LTP, resulting in the discovery that the stimulation caused changes in both the shapes of synapses and the formation of new contracts (Lee *et al* 1980, 1981). Other experiments have confirmed and extended these observations (Chang and Greenough 1984, Wenzel and Matthies 1985).

Alterations of these types have given new hindsight to the chemistry of synapses, and now we know that LTP is also correlated with an increase in the number of receptors for glutamate, one of the chemicals used as a transmitter in hippocampus (Lynch *et al* 1982). Since LTP is formed quickly, persists for a very long period of time, and is synapses-specific, it is suggested that it might be involved in certain types of memory. Rapid induction and persistence of LTP point to learning that occurs in a few trials and is retained (without learning) for a very long time (McGaugh *et al* 1985).

Starting with the idea generated by McGaugh *et al* (1985), Staubli (1985, 1989) used two types of drugs known to block LTP and tested them in a learning

paradigm that involved the rapid acquisition of many similar cues. They examined the effect of protease inhibitor (Calpain) and an N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-amino-S-phosphovanelic acid (APV) in a task in which rats were required to learn a long-series of two-odor discriminations, with each discrimination being acquired by training animals in about 10 trials. Thus, the characteristics of the tested memory (i.e., rapid acquisition and stable encoding) correspond somewhat to those expected of an LTP-based system. Both the drugs blocked LTP and also impaired acquisition but the retention of the olfactory memory remained intact (Staubli 1990). Qualitatively similar results were found by Staubli *et al.* (1990) with RGS tetrapeptide (Arg-Gly-Asp-Ser), a compound that acts as a partial inhibitor of the interactions between integrins and their endogenous ligands.

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Drugs Affecting Learning and Memory

H.K. SINGH AND B.N. DHAWAN

The first scientific investigation on drug affecting learning and memory was reported by Lashley (1917). He found that the maze learning of rats was enhanced by low doses of strychnine sulphate. This observation was ignored for several decades and it was only in the sixties that several investigations confirming Lashley's findings were reported. Several analeptics, including picrotoxin, pentylene tetrazol and diazamantanol were found to be facilitating learning tasks in a wide variety of tasks including maze learning, discrimination learning, escape and avoidance learning and classical conditioning. Gradually the horizon widened and as the conceptual framework became more refined, drugs facilitating, impairing and neuromodulating learning and memory were studied in greater depth and detail.

Evaluation of Drug Effects on Learning and Memory

The discovery and preparation of drugs with psychological effects has occupied the interest and energy of humans since the beginning of recorded history. The evaluation of drug effects on learning and memory is beset with many problems. Major among these are:

1. Effect on performance vs. learning and memory
2. Multiplicity of drug effects
3. Problems of behavioral taxonomy
4. Co-existence of different forms of memory.

1. Effect on performance vs. learning and memory

The most crucial problem in research concerning drug effects on learning and memory is that of distinguishing drug effects in learning from other effects of

drugs on performance. The distinction between learning and performance was brought into sharp relief by experiments on latent learning. In an early latent learning experiment Blodgett (1929) gave three groups of rats one trial each day in a simple maze. One group was rewarded in the goal box on all trials, a second group was not rewarded until the seventh day and then on each subsequent day, and a third group was only rewarded beginning on the third day and they daily. The animals in the first group showed a gradual drop in errors at the outset, whereas the error scores for the other two groups remained high until the day after the first time they found food. At that point the error scores dropped very suddenly and were almost immediately comparable to the scores of the first group. Therefore, it appears that the animals in the second and third groups had been learning much more than their performances had indicated during the nonreward trials, but that the learning had remained "latent" (i.e., not indicated by performance) until the reward was introduced.

2. Multiplicity of drug effects

(a) Dissociation: The dissociation effect refers to the condition in which habits learned by animals in a drugged state do not transfer to the normal state, but can be evoked again whenever the animal is drugged. Dissociation can also occur from the normal to the drugged state. Dissociation was demonstrated using crude curare and erythroidine (McGaugh and Petrinovich 1965) chlordiazepoxide (Sachs 1962), chlorpromazine (McGaugh 1973), pentobarbital (Overton 1964).

(b) Peripheral and central effects: The theoretical significance of the effects of a given drug on learning and memory depends on whether the primary action of the drug is on the receptor or effector systems or on the central nervous system. Any drug that has only peripheral effects can still cause an indirect alteration in CNS function by modifying the input to the CNS. Conversely, any drug which has only central effects can produce alterations in the peripheral nervous system function because of the general dominance of the CNS.

(c) Dose-response effects: Drug studies frequently assess the effect of an arbitrarily chosen single dosage on behavior and then offer generalizations about the nature of its effect on behavior. Stratton and Petrinovich (1963) have reported that the effect of physostigmine, an anticholinesterase agent, on the rate of alley-maze learning depends on the dosage level. Small doses of the drug have no effect on learning, larger doses enhance learning, and still larger doses disrupt learning. In view of the above, it is mandatory that a broad range of dosages be employed in any study in which the aim is to gain an over-all understanding of the effects of the drug on a given behavioral system.

(d) Tolerance and sensitization: Another troublesome factor with some drugs is that the effects change with repeated administration. Either increasing tolerance or increasing sensitivity may be encountered.

(e) Strain and species differences: It is now widely recognized that great caution must be exercised when generalizing from either pharmacological or behavioral

results obtained from only one species. Differences in the basic neurochemical substrates, differences in neural and anatomical structure, and differences in receptor and effector capabilities are evident between species, and all these make generalizations hazardous. Less widely recognized, however, are the less obvious, but equally important differences which exist between strains of the same species.

3. Problems of behavioral taxonomy

(a) Side effects: The side effects of drugs must be considered carefully while interpreting results on the effects of drugs on any aspect of behavior. For instance, drugs are known to enhance the performance of responses rather than affect the retention mechanism. Milner and Teuber (1968) argue that it is important to use a battery of diversified tests whenever attempting to measure the effect of a drug on level of motivation or emotionality.

(b) Apparatus factor: The type of apparatus chosen to study the effect of a drug on behavior can also influence the results. This is a serious consideration, since usually only one type of problem and apparatus is used to measure learning, and generalizations are based on the results thus obtained. Small differences in the design of a maze can produce discrepant results in studies of the effects of drugs on learning. For example, since the addition of retracing doors has been shown to increase the reliability of mazes (Silverman 1978), it should be easier to detect drug-induced differences in performance in a maze with retracing doors.

(c) Procedural factor: Strategies have been employed to overcome the difficulties created by differential motivational states induced by drugs. Motivation may be based on approach or avoidance; if a drug is known to influence thirst, and not hunger, then hunger motivation might seem to be appropriate for studying the effect of that drug on learning. The most satisfactory solution to this problem seems to be to use classical conditioned responses in an attempt to minimize the effect of most motivational influences. Drug effects on performance have been shown to be influenced by the specific training procedure used (Holland and Skinner 1961).

(d) Time of drug application: Drug application is generally done immediately after training. But pretraining application is also known to selectively improve consolidation. For instance, Decker and McGaugh (1989) have found that naloxone enhances consolidation with pre-training but not post-training administration in a Morris water maze learning. On the other hand, a new "nootropic", CGS 5649B, when administered immediately after the training failed to improve the task performance in maze learning but it significantly enhanced task performance when administered 8 h or 24 h after training (Mondadodri 1990).

4. Co-existence of different forms of memory

This phenomenon can be demonstrated by using the calcium-activated proteases ("calpain"), which degrade proteins involved in forming the cytoskeleton. Proteases have the unique property of breaking their substrate proteins, an effect

that can only be counteracted by replacing the protein. This irreversible effect of proteases makes them interesting tools for study of long-lasting phenomena such as memory. Limited experimental results to date (McGaugh *et al* 1985) indicate that calpain provides an excellent means for selectively and irreversibly changing synapses. However, it is very unlikely that calpain is involved in the production of all variants of information storage. For instance, this enzyme is not present in fish brain (Baudry *et al* quoted in McGaugh *et al* 1985) and fish certainly learn. Staubli *et al* (1984a, b) injected an inhibitor of calpain, leupeptin, into the cerebral ventricles of rats. The drug caused no observable effects on feeding, drinking, body temperature, or exploratory behaviour, but it produced a substantial impairment of the rat's ability to remember which of the two odors led to a water reward. Staubli *et al* (1984a) measured spatial memory using an 8-arm radial maze. Leupeptin produced a sizeable impairment in rat's performance in this test. In striking contrast, it had virtually no effect on shock avoidance learnings. This dissociation raises the possibility that different forms of memory with different chemical substrates co-exist. This is also supported by the experimental evidence that drugs that do block shock-avoidance memory have no effect on olfactory memory (Staubli *et al* 1985).

5. Experimental schedules

There are various types of experimental schedules to test the effect of drug on memory and learning. These schedules vary according to the reinforcement (positive vs. negative) used and type of memory (labile vs. stable) induced. The training schedules involving stable memory, as a rule, stretches over days and often weeks and are hence known as interval training. The procedures involving labile memory can be completed in one session, e.g., shock motivated brightness discrimination reaction or in several sessions, e.g., conditioned taste aversion response or radial maze training. There are different types of cues used in the different schedules (e.g., footshock, food, water, taste aversion, smell, visual). Some of the commonly used methods are summarised in Table 1.

Drugs Impairing Learning and Memory

1. CNS Depressants

(a) Barbiturates: Experiments on the effect of barbiturates on learning indicate that they impede learning. McGaugh (1972) found that the learning of food-motivated rats was retarded by injections of phenobarbital administered prior to each training session. However, he did not find any effect on rats' discrimination, learning, retention (1 month later), or reversal training. McGaugh (1972) also found that it has a direct effect on food and water intake and on eating habits. Drugged male rats ate less food and ingested more water.

(b) Anaesthetics: A large number of studies have shown that certain class of anaesthetics impair performance in acquisition tasks. McGaugh (1972) demonstrated that small doses of pentobarbital (10 mg/kg) retard problem-solving behavior in rats without seriously affecting the performance of learned

Table 1. Summary of commonly used training schedules

	Method	Reinforcement	Cue	Type of training	Type of memory	References
1.	Magazine training	Positive	Food	Interval	Stable	Ferster and Skinner (1957)
2.	Ratio schedule	Positive	Food	Interval	Stable	Holland and Skinner (1960)
3.	Interval schedule	Positive	Food	Interval	Stable	*Ibid*
4.	Differential reinforcement of low rate	Positive	Food	Interval	Stable	Oakley and Schaffer (1978)
5.	Sidman continuous avoidance response	Negative	Foot-shock	Interval	Stable	Sidman (1953)
6.	Spatial learning	Positive	Food	One-session	Labile	Poucent (1985)
7.	Radial maze task	Positive	Food	Interval	Labile	Burešova and Bureš (1982)
8.	Olfactory discrimination learning	Positive	Smell	Interval	Labile	Staubli *et al* (1989)
9.	Shock-motivated brightness discrimination reaction	Negative	Foot-shock	One-session	Labile	Ott *et al.* (1972 a)
10.	Conditioned taste aversion response	Positive	Taste aversion (LiCl)	One-session	Labile	Bureš and Burešova (1979)
11.	Morris water maze	Positive	Platform	One-session	Labile	Morris (1984)
12.	Visual discrimination task	Negative	Color	One-session	Labile	Luttges and McGaugh (1970)

responses. Possibly, however, the impairment observed is due to depression of sensory processes rather than (or in addition to) impairment of memory storage processes.

2. *Anticholinergics*

There is evidence (Pradhan and Dutta 1973) that the acetylcholine-acetylcholinesterase system is critically involved in learning and memory. A number of studies have shown that atropine impairs learning and performance in experimental animals. Buresova *et al* (1964) have presented additional evidence of learning impairment with atropine in rats. Effect of atropine on human memory also appears to be similar in that it impairs the acquisition but not the retrieval of information (Ghoneim and Mewaldt 1975, 1977).

The other anticholinergic hyoscine (scopolamine) affects declarative memory, such as recall of specific factual information, but does not disrupt procedural

memory, as is required for the retention of skill learning (Fang *et al* 1987, Nissen *et al* 1987).

A lesion of nucleus basalis magnocellularis (NBM) by ibotenic acid (IBO) (reviewed by Olton and Wenk 1987, Wenk and Olton 1987) produces a pattern of behavioral changes that is similar to that following administration of anticholinergic drugs (Murray and Fibiger 1985, Whishaw *et al* 1985). The behavioral changes following NBM lesions may, therefore, be due mainly to the loss of cholinergic cells. The behavioral effects of another neurotoxin, quisqualic acid (QUIS), injected into the NBM, suggest noncholinergic mechanisms may also be involved (Dunnett *et al* 1987, Etherington *et al* 1987, Wenk *et al* 1989). QUIS destroys more cholinergic cells than IBO, but disrupts performance less than IBO. Current studies of McGaugh and Morris suggest that the anticholinergic effect largely depends upon dose, time of application and method used (personal communication).

3. Agents Inhibiting Protein or RNA Synthesis

A number of investigators have proposed that memory storage may involve ribonucelic acid (RNA) and protein synthesis which are known to vary with neuronal activity. Therefore, several investigators have studied the effect of compounds known to interfere with RNA synthesis on learning.

(a) Antimetabolites: Dingman and Sporn (1964) reported that intracerebral injections of 8-azaguanine, which is readily incorporated into the brain RNA, impaired the ability of rats to learn a maze without significantly affecting the rats' performance of a previously well-learned maze schedule. These findings are consistent with the hypothesis that 8-azaguanine interfered with learning by interfering with the brain RNA metabolites.

(b) Actinomycin-D: Landaur (1964) reported that intracerebral injections of actinomycin-D did not affect one-trial learning in mice. The animals were injected with a dose of actinomycin-D sufficient enough to produce an 83% inhibition of brain RNA synthesis. These data are, however, not completely inconsistent with the hypothesis that memory storage involves RNA synthesis, because the residual RNA synthesis (17%) might have been sufficient to mediate memory storage.

(c) Cycloheximide and Puromycin: Squire and Barondes (1972) have found that the decay in memory following cycloheximide administration is variable depending upon the dose, task and level of training but they have consistently found disruption with memory storage. Flexner and Flexner (1969) have found that puromycin disrupts recent memory. Flood *et al* (1973) have shown that anisomycin disrupts memory across six strains of mice. In an earlier study, Flexner *et al* (1962) reported that whereas 83% inhibition of protein synthesis failed to affect either avoidance learning or simple maze learning, more complete inhibition (95%) by a combination of subcutaneous and intraventricular injections of puromycin produced severe behavioral disorientation. In another

study, Flexner *et al* (1963) reported evidence that memory storage was impaired by intracerebral injections of small doses of puromycin (0.03-0.09 mg).

4. GABA and agonists

γ-Aminobutyric acid (GABA) is one of the main neurotransmitters in the mammalian brain and is known to be involved in a variety of physiological functions (Grandison and Guidotti 1979, Krnjevic 1976, Krnjevic and Schwarz 1967, Roberts 1986). The GABA receptor is a protein complex that also contains receptor sites for benzodiazepines, as well as picrotoxin/barbiturates, which are associated with chloride channel ionophore (Costa and Guidotti 1971, Olsen 1981). At the behavioral level, there is extensive evidence indicating that retention of recently acquired information is influenced by post-training systemic injections of GABA antagonists and agonists. For instance, retention is impaired by post-training systemic injections of either GABA agonist muscimol or GABA-transaminase inhibitor amino-oxyacetic acid (Castellano and Pavone 1988, Katz and Liebler 1978).

5. Miscellaneous procedures

Evidence, as reviewed by Glickman (1961), indicates that retention of learned responses is impaired if animals are subjected shortly after training to treatment interfering with CNS activity. Amnesia produced in this way (termed retrograde amnesia) is greatest if short intervals (usually within a minute or so) elapse between training and treatment. Such amnesia can be produced by concussion, temperature changes, electro-convulsive shock (ECS or ECT), subcortical brain stimulation, hypoxia, spreading depression or topical application of certain drugs like aluminium hydroxide.

Drugs Facilitating Learning and Memory

1. Convulsants

(a) Strychnine: Studies indicate that post-training injections of strychnine enhance the learning of an association between two stimuli. Humphrey (1968) found that post-training strychnine sulphate (1.0 mg/kg) facilitated sensory preconditioning. Oliverio (1968) independently produced data supporting a similar contention. The facilitating effect of strychnine on learning has also been interpreted as indicating that the drug enhances storage processes (McGaugh and Herz 1972). This hypothesis is supported by the evidence that strychnine attenuates retrograde amnesia produced by electroconvulsive shock (ECS) if the drug is administered either before training or within a few hours after ECS treatment (McGaugh 1968).

(b) Picrotoxin: Garg and Holland (1968) have found that maze learning is enhanced by post-training administration of low doses of picrotoxin. Although the effect has not been studied in a wide range of tasks, facilitation has been found with active avoidance tasks, as well as with mazes. There have, as yet, not

been any studies in which the time of post-training administration has been varied.

(c) Metrazol: Learning is facilitated by either pre-or post-training application of metrazol. Krivanek and McGaugh (1968) found that in mice, the degree of facilitation of discrimination learning (in an appetitively motivated task) produced by post-training injections increased directly with the dose of metrazol. However, in a study using rats, Krivanek and Hunt (1967) found that the degree of facilitation of discrimination learning varied in a somewhat more complex way and also depended on time of drug administration.

(d) Bemergide (β-ethyl-β-methyl glutarimide): Post-training injections of bemergide enhance learning of rats and mice in tasks using shock motivation (Luttges and McGaugh 1970, 1971). The generality of the effects of bemergide on learning has not yet been studied.

(2) Other CNS stimulants

Amphetamine: Some early studies indicated that amphetamine impairs rats' learning, for instance, 0.5 mg/kg of amphetamine sulphate impaired rats' learning in a water maze. In a multiple U-maze schedule, Westbrook and McGaugh (1964) found no differences between performance of control groups and experimental groups given either 1.0 or 0.5 mg/kg of d-amphetamine, or 8.00 or 4.0 mg/kg of l-amphetamine. However, when larger doses were given to animals already trained, the time to run the maze and the number of errors required to attain the criterion increased with increasing dosage. Amphetamine, however, was found to enhance the learning in rodents in most situations. Low doses (0.5-2.0 mg/kg) facilitated learning in a variety of active avoidance tasks (McGaugh 1969). Since amphetamine enhances motor activity, it is important to consider whether the enhanced acquisition might be due to a direct effect of the drug on activity. His findings indicate that amphetamine administered prior to training enhances both acuisition and retention performance. Evidence from studies in which amphetamine is administered shortly after training clearly indicate that learning facilitation cannot be due solely to effects on locomotor activity. McGaugh (1969) also found that post-training administration of amphetamine facilitates discrimination learning in rats. The degree of facilitation varied with the dose, task, and age of the subjects. There are only quantitative differences between the stereoisomers or racemic forms of amphetamine and with metamphetamine.

3. Cholinergic stimulants

(a) Nicotine: In rats, nicotine facilitated acquisition in a wide variety of tasks. There have, however, been some failures to find facilitation of learning with nicotine (0.25-1.0 mg/kg) administered either before or after training (McGaugh 1965) on aversively motivated tasks. Learning was not affected by the quarternary compound nicotine bismethiodide. This suggests that the effects of nicotine on learning are probably due to central rather than peripheral actions of the drug.

(b) Physostigmine: There are many, but not all (Miller *et al* 1971) experiments (McKim 1974) confirming that learning is enhanced by post-training administration of low doses (0.25-1.5 mg/kg) of physostigmine. Since the facilitation can be obtained with post-training injections, the findings suggest that physostigmine potentiates cholinergic mechanisms involved in memory storage. Facilitation of inhibitory avoidance with pre-training injections of physostigmine (0.25 and 0.5 mg/kg s.c.) is attributed to an enhancement of response suppression, since these doses impair active avoidance (Rosic and Bignami 1971). The interpretation of the facilitatory effect of post-training physostigmine is complicated by the finding (Evangelisto and Izquierdo 1971) that post-training application of atropine (2.0 or 10.0 mg/kg) also facilitates learning.

(c) DEP: In a series of experiments, Deutsch and associates (1966, 1967, 1971, 1972) have reported that retention performance is enhanced by physostigmine or DFP if the responses are recently acquired or are almost forgotten. Deutsch interprets these findings as suggesting that learning and forgetting are based on time-dependent changes in cholinergic synapses, possibly at the postsynaptic membrane.

4. Nootropics

Nootropics are new type of compounds affecting memory and learning but are pharmacologically neither CNS stimulant nor depressant. The term was first used by Giurgea and Salama (1977) and indicates that they act ("noo") on brain ("tropic").

(a) Pemoline: The effects of pemoline on learning and memory (Plotnikoff 1971) indicate that the effects are, in general, similar to amphetamine. Facilitation has been obtained in a variety of tasks and with both pre- and post-training administration (Doty and Howard 1967). Pemoline has also been found to attenuate ECS-induced amnesia (Stein and Brink 1969).

(b) Piracetam: Piracetam (UCB 6215) has been reported by Giurgea (1972) to enhance learning. Piracetam improved the retention of different conditioned avoidance tasks when administered pre- or post-training. The amnestic effect of a ECS or hypoxia can be abolished by pre-treatment with piracetam. Wolthius (1971), in an earlier study has suggested that piracetam affects learning by acting on sensory processes rather than by influencing memory storage. This aspect merits a planned and careful investigation.

(c) Bacosides: In our laboratory, a mixture of bacosides A and B (the active constituent of the plant *Bacopa monniera* L., Hindi: *brahmi*, family: Scrophulariacae, Fig. 1) in a dose of 10 mg/kg p.o. given every alternate day facilitated the three types of learning, viz. procedural, declarative and spontaneous (Singh *et al* 1988). The rats had to learn to jump on a pole to avoid a foot-shock. The trial ended either after the animal responded by jumping on the pole or after 30s, whichever occurred earlier. The bacosides treated animals evidenced procedural learning (responding to US) from day 1 (as compared to day 3 for control group), declarative learning responding to CS) from day 6 (as

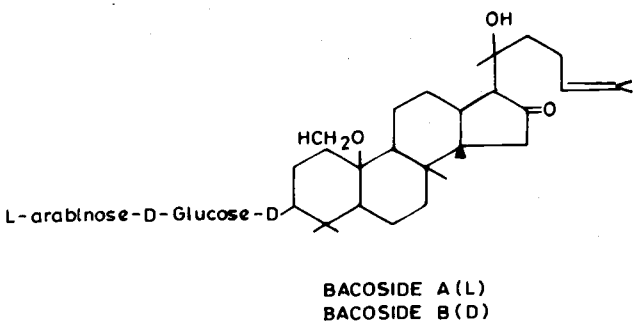

Fig. 1 Chemical structure of bacosides.

compared to day 9 for control group), and spontaneous learning (responding to SCS) almost from day 11 onwards (the control group showed no spontaneous learning at all). The reaction time of the drug treated animals was significantly lower from day 4 onwards.

We also found that bacosides possess the ability to significantly improve episodic memory in the animal experimental model (Singh *et al* 1988). Rats were trained for a foot-shock motivated brightness discrimination reaction in a Y-maze. Bacosides were administered 90 min prior to training (10 mg/kg p.o) in the experimental group and a second dose of 10 mg/kg p.o. 90 min prior to relearning. The control group received a corresponding volume of vehicle (1% gum acacia suspension). To evaluate the effect of bacosides on episodic memory, we took the following temporospatial paradigms: (a) time per trial, (b) number of negative trials and (c) number of positive responses both during training and relearning. The bacosides significantly enhanced these three paradigms of episodic memory. This facilitatory effect of bacosides on episodic memory was further evidenced both from significantly enhanced relearning index and positive responses.

Based on their experimental investigations, Ott and Matthics (1978) have postulated that memory exists in two forms, viz. short-term and long-term. Both these forms start simultaneously and a deficit in retention curve is observed at a point where these two forms overlap. We tested this hypothesis in our behavioral model, i.e., foot-shock motivated brightness discrimination reaction in the Y-maze and found that the retention curve instead of being V-shaped was W-shaped, i.e., there were deficits occurring at two points, viz. 1.5 h and 4.0 h training relearning interval. Therefore, we presumed that, at least, for our experimental models, there are perhaps three forms of memory i.e. short-term (few seconds to minutes) and long-term form(few hours to days) and in between there exists an intermediate form of memory (few minutes to hours). The two deficits occur at those points where one labile phase of the same memory

overlaps with the other labile phase (Fig. 2). The bacosides in a dose of 20 mg/kg p.o. when given 30 min prior to training abolished these deficits when the relearning was done at various time intervals after training. As was to be expected, a support dose of 10 mg/kg p.o. given 30 min prior to the 24 h relearning test also produced a significant enhancement in the relearning-index (Fig. 3). These results suggest that the facilitatory effect of bacosides is due to their ability to consolidate the retention at the earliest forms i.e. short-term memory. The facilitatory effect of bacosides persists when the other two forms, i.e. intermediate memory and long-term memory occur. This is also confirmed by the results of the preliminary biochemical investigations that bacosides treatment leads to significantly enhanced protein synthesis in certain regions of brain, i.e. hippocampus, striatum, cortex and hypothalamus. If the bacosides treated rats are subjected to the foot-shock motivated brightness discrimination test then the enhanced protein synthesis is significantly increased in hippocampus and striatum (Singh *et al* 1990).

(d) Other Pyrrolidines and Alkaloids: Several other pyrrolidine derivatives besides piracetam (like etiracetam and amiracetam) and several other representative compounds of the nootropic class, such as ergot alkaloids nicergoline and dihydroergotoxine, vincamine alkaloids and analogues, phenyl ethanolamines, deproteinized homoderivatives, pyridoxine derivatives etc. (Bente *et al* 1979, Saletu and Grunberger 1983) have qualitatively similar effects.

These compounds also decrease the delta and theta activities and increase the alpha and/or beta activities (Saletu *et al* 1984a, b). Since attenuation of slow activity and the elevation of alpha and/or slow beta activity reflect CNS changes that are just opposite to those seen in normal and pathological ageing subjects, these compounds have been used in the treatment of senile dementia of Alzheimer type in the neuropsychiatric clinic of Saletu in Vienna. However, the clinical studies are so limited that it is essential to clarify this aspect with more extensive trials.

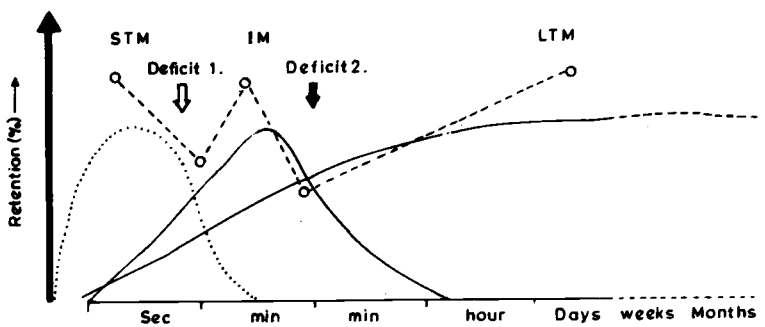

Fig. 2 The results of retention testing (0-0) obtained at various time intervals after training in our experimental model show deficit at two points (see text for detailed explanation).

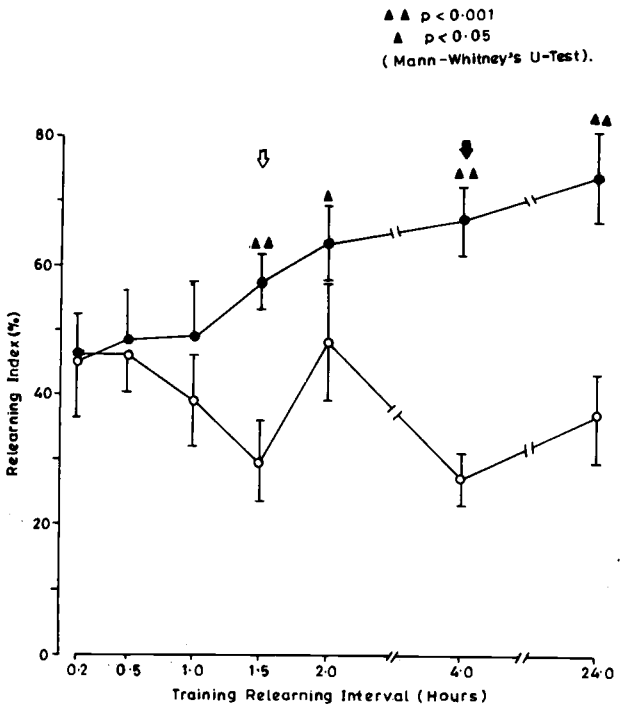

Fig. 3 The bacosides pretreatment leads to the abolition of the two deficits in the learning curve observed when STM overlaps with IS and IS overlaps with the LTM.

5. RNA and RNA Precursors

The most extensive and carefully conducted series of studies on this aspect have perhaps been done by Matthies and his co-workers (Ott and Matthies 1971a,b, 1972, 1973a,b; Ott *et al* 1972b). They have reported that, in rats, repeated administration of orotic acid prior to training delays the extinction of a brightness discrimination response in a Y-maze. Comparable effects were obtained by a single intraventricular injection of uridine-5-monophosphate (UMP) administered shortly before or after the training session. The latter effect can be blocked by cycloheximide (5 mg/kg i.p.). Pretreatment with UMP was also found to attenuate ECG-induced amnesia. Matthies and co-workers have suggested that the effects are due to an enhancement of memory storage processes, possibly by affecting protein synthesis.

6. Hormones

Hormones have a variety of influences on physiological systems involved in adaptation to environmental changes. Most studies on the influence of hormones on memory have focussed on following hormones:

(i) *Hypothalamic-Pituitary Hormones*:
ACTH, Vasopressin, Oxytocin and β-endorphin.

(ii) *Adrenal Hormones:*
(a) Medullary: Catecholamines and enkephalin
(b) Cortical: glucocorticoids and corticosterone.

There are two reasons for this interest. Firstly, there has been for many years considerable interest in stress-related hormones and, as a consequence, this area of neuroendocrinology and neuropharmacology has a long history (see review, Dunn and Kramarcy 1984). The development of new techniques and drugs has greatly accelerated research in these areas in recent years. Secondly, the fact that stress-related hormones are released by even relatively mildly stimulating experiences has suggested that these hormones may have an important adaptive role in the regulation of memory just as they do in the regulation of other adaptive physiological processes. It seems reasonable to suggest that experiences which are sufficiently exciting to elicit the release of glucose are likely to be worth remembering and that hormones which are involved in reacting to exciting events may also influence the storage of memory (Gold and McGaugh 1975 1978, Ketty 1972).

In several reviews McGaugh (McGaugh and Gold 1989, McGaugh 1989b, 1990) has presented evidence of his extensive work on hormonal modulation of memory. His findings are that endogenous hormones are involved in memory storage, because: (1) training releases the hormone in question; (2) retention is affected by treatment affecting the release of hormones or activation of receptors; (3) the effects of treatments interfering with the release of hormones is attenuated by administration of the hormones; (4) exogenously administered hormones have greatest effects when administered shortly after training (at the time at which the training releases the hormones); (5) effects of exogenously administered hormones depend upon the levels of endogenously released hormones.

7. GABA Antagonists

Post-training systemic injection of bicuculline, a GABA receptor antagonist with central actions (Curtis *et al* 1970 1974, Olsen *et al* 1978, Zuckir *et al* 1974), has been reported to enhance retention both in rats and mice (Brioni and McGaugh 1988, Castellano and McGaugh 1989, Yonkov and Georgiev 1985). A number of studies using a variety of training tasks have reported that memory is enhanced by post-training systemic injections of chloride channel blocker, picrotoxin (Bovet *et al* 1966, Breen and McGaugh 1961, Brioni and McGaugh 1988, Castellano and Pavone 1988, Grecksh and Matthies 1981).

8. Miscellaneous

(a) Thiamine: Research with thiamine was based on an interest in the possible enhancing effect of supplementary thiamine on the intellectual functioning of retarded children, as well as on a more general interest in the effects on learning of a substance known to be essential to cerebral metabolism. Several studies

have indicated that young rats deficient in thiamine are inferior to normal rats in maze learning as well as in classical conditioning. It has also been found that thiamine deficiency occurred before the animals were 30 days old. Dietary replacement of thiamine was found to improve the learning of thiamine deficient rats (see review McGaugh and Petrinovich 1965).

(b) Glutamic acid: Glutamic acid has been the subject of a great deal of experimentation since the finding that rats given glutamic acid supplement learned a maze faster. It has also been reported that glutamic acid facilitates a pedal-pushing problem. These findings led to a flury of animal studies (McGaugh and Petrinovich 1965) all of which reported negative results. Contrary to the negative evidence just cited there were a few positive studies on human mental defectives. All these, however, can be questioned on methodological groups. In retrospect, it is not clear why glutamic acid was expected to affect learning. It is one of the nonessential amino acids and in readily synthesized by mammals in amounts large enough to make the organism independent of an outside source. Ingested glutamic acid does not enter the brain in normal animals although it may do so in some pathological states such as deep insulin hypoglycemia (McGaugh 1973).

(c) Opioid peptides: It is now well known that there are at least three families of opioid peptides: (1) the prepomelanocortins (β-, α, and γ-endorphins), (2) prepoenkephalin A (Met-enkephalin, Met-enkephalin-Arg-Phe, Leu-enkephalin) and (3) prepoenkephalin B (dynorphin A, dynorphin B, α- and β-neoendorphin) (Bloom 1983). Identification of new opioid peptides has outstripped the ability of behavioral scientists to characterize the actions of each, particularly as these peptides may have a relationship to learning and memory (Martinez *et al* 1981).

It is likely that enkephalins affect avoidance conditioning through a peripheral mechanism when they are injected systematically, because they probably do not cross the blood-brain barrier (Pardridge 1983). The adrenal medullary integrity is perhaps necessary for enkephalin effects to be evident on avoidance conditioning (Martinez and Rigter 1982). Naloxone, which only acts on peripheral opioid receptors, attenuates the actions of enkephalin on conditioning (Martinez *et al* 1984). Thus, even though it is likely that enkephalins exert their primary action to influence aversive conditioning through a receptive site somewhere in the periphery, the location and nature of this receptor is still unknown. Generally, when administered post-training, retention is enhanced by opiate receptor antagonist (e.g., naloxone and naltrexone) and impaired by agonists (e.g., morphine, β-endorphin, enkephalins) (Castellano 1975, Gallagher and Kapp 1978, Introini-Collison *et al* 1985, Izquierdo 1979, Martinez *et al* 1981, McGaugh 1989a, McGaugh and Gold 1989, Zhang *et al* 1987). Most previous studies investigating the possible role of the enkephalins in memory storage have examined the effects produced by systemic injections of enkephalins and opiate antagonists. The involvement of enkephalins in memory has also been addressed by using drugs that affect the metabolism of enkephalin (McGaugh 1987). Two enzymes, aminopeptidase and dipeptidylcarboxypeptidase, seem to play a predominant role in the inactivation of enkephalins. It has been found that

enkephalin-hydrolysing aminopeptidase is sensitive to bestatin, a potent aminopeptidase inhibitor. Enkephalin hydrolysis is inhibited by bestatin *in vivo* and *in vitro*. Memory is impaired by enkephalins as well as bestatin, which inhibits metabolism of enkephalin (Zhang *et al* 1989). The obvious inference can be that increased enkephalin level in brain leads to an impairment of memory.

E. Therapeutic use

The vast amount of data available on the neuroanatomical, biochemical, electrophysiological and neuromodulatory correlates of learning and memory suggest that a drug affecting learning and memory therapeutically is on the anvil. The RNA precursor, orotic acid, has been successfully used in neuropsychiatric clinics of Germany to rehabilitate mentally retarded children. As reported by Clarke (1991), now it may be possible to treat amnestic patients through selective neuromodulatory agents. The pre-clinical neuropsychopharmacological investigations in our laboratory on the medicinal plant *Bacopa monniera* have provided some encouraging results in this direction. The active constituents of this plant, Bacosides A and B are effective in a single dose of 10 mg/kg p.o. given every alternate days in interval training and in a single dose of 20 mg/kg p.o. in one session training. The onset of action with both the doses takes place after 1 h. They are effective in facilitating effects on mental retention capacity and increase the probabilities in a variety of responses. The effect of bacosides is manifest both in negative reinforcement as well as positive reinforcement. In animal experimental models, bacosides are effective in improving symptoms which are clinically related to age dependent EEG alterations in elderly patients.

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Recent Trends in Neurohumoral Basis of Behavioural Psychopharmacology

GAUTAM PALIT

Psychopharmacology is a rather recently developed discipline, which is concerned with two types of phenomenon. Firstly the effect of drugs on psychological processes and secondly, the effect of psychological factors in determining the response to drugs. The subject in its clinical form is still less than 30 years old dating from the introduction of the antipsychotic medication-chlorpromazine in the early 1950's. After that, revolutionary advances in the understanding of psychopharmacology have resulted in the development of a more scientific basis for treating mental illness. However, our knowledge is limited, since so little is known about the neurophysiological differences between normal individuals and mentally ill patients, nor of the biochemical basis for various neuro-psychiatric disorders. Therefore, the exact site and mode of action of various psychotherapeutic agents remain unidentified.

Behaviour is a highly complex brain function which appears to be regulated by neurotransmitters (neurohumoral) in the brain. The drugs which selectively modify the behaviour patterns are known as "psychotropic" or "psychoactive" drugs.

Neurotransmitter Life Cycle

The identification of monoamines and amino acid neurotransmitters in brain ushered in the modern era of psychopharmacology. Classic research in this field has elucidated the life cycle of these neurotransmitters and has demonstrated that apart from neurotransmitter-receptor interactions many factors determine the psychotropic action of drugs. For this class of neurotransmitters, synthesis

proceeds by sequential enzymatic modification of simple, abundant precursor molecules, such as choline, tyrosine or tryptophan. The synthesized neurotransmitters stored within vesicles in the nerve terminals, release into the synaptic cleft, and act upon receptors located post-synaptically to transfer information across the synapse. In addition, in many cases they also may interact with receptors located presynaptically on the nerve terminals to regulate the release process. An intriguing aspect of the inactivation of this group of neurotransmitters is that it involves primarily a reuptake process that recycles the neurotransmitter or its immediate precursor.

Any impairment or alterations in the neurotransmitter life-cycle may lead to hypo or hyperactivity of a neurotransmitter which may change the behavioural responses leading to a psychiatric disease. This constitutes the neurohumoral basis of psychiatric disorders. The knowledge of it is essentially required for the rational and effective therapy of these disorders. There are drugs known to modulate specially different steps in the life-cycle of a neurotransmitter and the correction in the altered neurotransmitter activity by drugs can improve the diseased state.

In recent years, emphasis has centered on biogenic amines and their receptors in the CNS, their possible casual involvement in mental illness, their probable mediation of many effects of psychotropic drugs, and sometimes the risk of serious toxic effects.

The involvement of neurotransmitters in behavioural disorders has been elucidated by experimental studies on animals and by clinical studies on human behaviour.

Animal Behaviour

Studies on animals mainly involve the effect of drugs known to act specifically through a particular neurotransmitter system on normal behaviour and induced responses mimicking human mental illness. These type of studies have provided important information about:

1. Neurohumoral mechanisms in behavioural responses and disorders.
2. Mechanism of action of psychoactive drugs.
3. The discovery of new drugs of potential value.

Human Behaviour

The involvement of neurotransmitters in psychoactive disorders is ascertained by comparing pharmacokinetic (turnover) and pharmacodynamic (clinical response) parameter before and after drug treatment of patients.

The involvement of neurotransmitters has been studied extensively in the following neuropsychiatric disorders:

1. Schizophrenia
2. Depression

3. Mania
4. Anxiety

Schizophrenia

Schizophrenia is one of the most disruptive mental illnesses. The breakthrough for psychopharmacology in psychiatry came when the phenothiazine derivative-chlorpromazine was introduced in the treatment of psychosis by Delay *et al* (1952). The revolutionary idea that drugs could exert a specific antipsychotic effect led to a search for other psychoactive substances with similar antipsychotic properties resulting in the discovery and use of a wide variety of substances that are effective in the treatment of schizophrenia and other major psychotic illnesses. These are the phenothiazine derivatives, the thioxanthene derivatives, the butyrophenones, the indoles, the dibenzoxazepines, reserpine etc. These psychoactive drugs have been termed neuroleptics and have a beneficial action on the typical symptoms of schizophrenia, e.g. delusions, hallucinations and thought disturbances. On the other hand they affect the extrapyramidal system to such an extent that muscular rigidity, tremors and other Parkinson-like symptoms such as dyskinesia occur frequently, often associated with autonomic blockade and excessive sedation. During the last decade neuroleptics have been introduced into clinical use presumably with lower incidence of extrapyramidal symptoms. Sulpiride, clozapine and thioridazine are examples of these drugs, designated as atypical neuroleptics.

Dopamine Hypothesis of Schizophrenia

The most widely accepted neurotransmitter hypothesis of schizophrenia relates to an involvement of cerebral dopamine (DA). The DA hypothesis in its simple form states that DA-ergic hyperactivity in some brain systems plays a key role in the pathogensis of schizophrenia and neuroleptics counteract this hyperactivity (Van Kammen 1979). Neuroleptics are indeed potent antidopaminergic drugs (Niemegeersand Janssen 1979). This hypothesis is based on a wealth of neuropharmacological data and inferences and on restricted biochemical and clinical findings.

Several distinct DA-ergic systems are present in the brain e.g. the mesolimbic, the mesocortical, the nigrostriatal, the tuberoinfundibulum and the intra-diencephalic systems. The mesolimbic and mesocortical DA systems with cell bodies in the ventral tegmental area and terminals in several nuclei of the limbic forebrain e.g. nucleus accumbens and amygdala and in cortical regions e.g. prefrontal cortex and pyriform cortex have been specially implicated in schizophrenia than the other DA systems (nigrostriatal and tuberoinfundibular systems) (Stevens 1979, Baldessarini 1990). The nuclei of the limbic forebrain have been associated with perceptual, cognitive attentional and affective processes which are commonly disturbed in schizophrenia. The antipsychotic potency of several neuroleptics seems to be related to their influence on DA activity in the nucleus accumbens rather than in the neostriatum (VanRee and de Wied

1982, VanRee *et al* 1987), while the nigrostriatal system is connected more with the extrapyramidal side-effects of neuroleptics.

A. Biochemical Studies

No convincing evidence for the DA hypothesis is yet available as far as biochemical evaluation of schizophrenic patients are concerned. Most but not all, postmortem studies of brains from schizophrenics and controls have shown increased number of DA receptors in the nucleus accumbens and caudate nucleus of chronic schizophrenic patients (Lee *et al* 1978). However, this may be the result of neuroleptic treatment rather than a cause of the illness. Increased receptors in both the nucleus accumbens and the caudate nucleus indicate that both mesolimbic and the nigrostriatal dopaminergic systems are involved in schizophrenia (Carlsson 1978, Melizer 1979).

Most of the neuroleptics have been found to stimulate the turnover of dopamine in striatum and limbic structures (Anden *et al* 1972). The maximum response produced in the striatum and limbic system differs (Carlsson 1978), classical neuroleptics (chlorpromazine, haloperidol) stimulate dopamine turnover more in the nigrostriatal than in mesolimbic and mesocortical system (Carlsson 1975, Westerink *et al* 1977). In contrast, 'atypical' neuroleptics (clozapine and sulpiride) with low incidence of extrapyramidal manifestations have greater effect on dopamine turnover in limbic areas than in the striatum (Zivkovic *et al* 1975, Westerink *et al* 1977). The differential effect of neuroleptics on dopamine turnover in the various brain areas might reflect different degrees of blockade of dopamine receptors (Bartholini *et al* 1976). Recent data showing time dependent changes in plasma homovanillic acid (HVA) after antipsychotic treatment, are consistent with the hypothesis that the mechanism of antipsychotic drug effect involves a slowly developing decrease in presynaptic dopamine activity (Davis *et al* 1989).

B. Neuropharmacological Studies

Indirect support for the role of DA in schizophrenia comes from the studies of CNS stimulants such as amphetamine, phencyclidine (PCP) and cocaine. Amphetamine and cocaine abuse by humans may lead to a psychotic state closely resembling paranoid schizophrenia. A major component of the action of amphetamine is a potent release of dopamine and cocaine-blocks the reuptake of DA, thus increasing its concentration at synaptic sites. These observations tend to support the idea that increased dopaminergic activity leads to psychosis, and neuroleptic drugs can diminish or block this effect.

Our study on the amphetamine induced behavioural changes in non-human primates (Rhesus monkey) suggest that dopamine may be playing an important role (Palit *et al* 1986). Additional evidence is offered by the effect of increasing dopaminergic activity in patients with active psychosis. Furthermore, DOPA (3, 4-Dihydroxyphenylalanine) which is converted to dopamine in the body can produce psychosis as a side effect.

Further support for the importance of DA in psychosis comes from studies on the synergistic antipsychotic action of O-methylparatyrosine (AMPT) and neuroleptics. AMPT is an inhibitor of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis; combined inhibition of DA synthesis and DA receptor blockade leads to successful treatment of some schizophrenics who do not respond to neuroleptics alone.

The current dopamine theory of schizophrenia suggests that certain patients with schizophrenia either have high dopamine levels or supersensitive dopamine receptors but this theory remains unproved. There is preliminary evidence from positron emission tomography (PET) studies that schizophrenia involves an increase in D_2 dopamine receptors (Davis *et al* 1989).

Other Neurotransmitters or Neuromodulators Implicated

There is some evidence for primary role of serotonin (5-HT), norepinephrine (NE), γ-aminobutyric acid (GABA), acetylcholine, histamine, prostaglandins, phenethylamine and β-endorphin in schizophrenia (Lucas 1979). The most salient findings are of elevated levels of the indole hallucinogen N, N-dimethyltryptamine in the urine or blood of schizophrenics and increased GABA in CSF. It is likely that high anticholinergic activity of atypical antipsychotic agent-clozapine is responsible for its improved efficacy and reduced EPS liability. Abnormal responses to injected histamine, elevated CSF prostaglandins, increased urinary phenethylamine, increased plasma and CSF β-endorphin, and clinical improvement of chronic auditory hallucinations with naloxone, an antagonist of the endogenous opiates suggest the involvement of these other neurotransmitters and modulators in the pathogenesis of schizophrenia (Horny Kiweicz 1982, Van Kammen and Antelman 1984, Korsgaard *et al* 1985, Dubey and Dhawan 1989, Reubi *et al* 1978).

Affective Disorders

The affective disorders are those conditions in which there is alteration of mood to such a degree as to cause serious distress or disruption of normal life. The mood may be abnormally lowered as in depression or elevated as in mania. Recently the nomenclature "affective disorders" has been replaced by the term 'mood disorder'.

Depression is very likely a heterogenous disorder. The successful search for new and better antidepressant medications has resulted in the two generations of anti-depressants. The first generation antidepressants include the tricyclic (TCA) and the monoamine oxidase (MAO) inhibitor antidepressants. During the past few years, a number of other drugs, usually chemically and sometimes pharmacologically different from these have been introduced. These drugs are often called "Second Generation" antidepressants e.g. Trazodene, Fluoxetine, Maprotiline, Alprazolam, Clomipramine etc. The second generation antidepressants have more rapid onset of action, more tolerable side effects and greater safety.

Neurohumoral basis of mood disorders

Both depressive illness and mania are associated with biochemical changes in the brain. The original biogenic amine hypothesis focussed primarily on the CNS neurotransmitters, noradrenaline (NA), serotonin (5-HT) and dopamine (DA), attributing depression and mania to be due to a relative decrease or increase of these neurotransmitters, in critical areas of the brain that regulate mood, motor activity and vegetative functions.

The original basis for this hypothesis in depression was the pharmacological evidence that reserpine which depletes, stores of biogenic amines (e.g. NA, 5-HT and DA) from nerve endings, precipitated depressive episode in about 15% of the patients who received this drug for the treatment of hypertension (Garver and Davis 1979). Whereas monoamine oxidase inhibitors, the first effective antidepressant drugs, block a major degradative pathway for amine neurotransmittes which presumably permits more amines to accumulate presynaptically and more to be released. Subsequent studies demonstrated, that, at least acutely, tricyclic antidepressants such as imipramine and amitriptyline could potentiate the activity of brain NE or 5-HT by inhibiting their uptake into presynaptic neurons, the principal means of their inactivation (Potter 1984, Hollister 1986).

The direct evidence to support the monoamine hypothesis is relatively slim. Urinary and cerebrospinal fluid (CSF) studies of NA, its metabolites 3-methoxy-4-hydroxyphenyleneglycol (MHPG), and the catalytic enzyme dopamine β-hydroxylase have been reported as being increased or decreased in the predictable direction during depressive and manic episodes. Recently, there is more direct evidence for an abnormality of 5-HT in depression than for any other neurotransmitter. Thus, depressed patients, especially those with history of nearly successful suicide attempts have been reported to have decreased levels of 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of 5-HT, in the CSF. Decreased levels of 5-HT and 5-HIAA have also been found in the brains of suicidal depressed patients (Judd 1991). Some of the newer antidepressant drugs such as fluoxetine and zimelidine have been shown to have relatively specific inhibitory effects on the uptake of 5-HT by neurons. Furthermore, there are numerous studies, shown that the precursors of 5-HT, tryptophan and 5-hydroxytryptophan, have antidepressant effects in their own right and when administered together with MAO inhibitors or 5-HT uptake blockers, potentiate the antidepressant effects of these agents. Deficits in other neurotransmitters such as dopamine and GABA have also been observed in some patients with major depression. The role of dopamine in depression has been recently reviewed by Willner (1983). There is as yet no strong and consistent evidence of the primary involvement of the dopaminergic system in the pathogenesis of depression and the mechanism of action of antidepressant treatment. However, one of the new antidepressant drugs-Bupropion, has been shown to enhance dopamine release without any effect on 5-HT or NA, suggesting that increasing dopaminergic activity may have an antidepressant effect in human.

Finally, another neurotransmitter hypothesis that has directed research in the mood disorders is the cholinergic hypothesis, which postulates increased and decreased central cholinergic tone in depression and in mania, and an imbalance between the cholinergic and adrenergic neurotransmitter systems as being a central pathophysiologic mechanism in affective disorders. Many of the tricyclic antidepressant drugs have potent anticholinergic properties. It has been proposed that the anticholinergic effects of these agents contribute to their antidepressant properties.

Recent investigations on the mechanism of action of antidepressant drugs has led to major revision of the earlier concepts of the pathogenesis of depression and the mechanism of action of these drugs. The focus of research has shifted from the neurotransmitter biosynthesis, storage and release mechanisms in the presynaptic neurons to the study of receptors on postsynaptic neurons, especially the cascade of intraneuronal molecular and biochemical events that occur in the postsynaptic neurons following the binding of the neurotransmitter to the receptor. In summary, there is general agreement that the relative functional underactivity of neurotransmitter and/or the down regulation of postsynaptic receptors have often been correlated with depressive episodes. Down regulation can be defined as a receptor subsensitivity to NE and consists of a reduction in the density of post synaptic β-adrenoceptors and is an indication of a functional increase in NE available at the receptor site. As both 5-HT and NA seem to be involved in depression, an attempt has been made to reconcile the two neurotransmitter theories of depression with the decreased sensitivity of β-receptors. It is assumed that the final common denominator of antidepressant action is an augmentation of NE release with consequent down-regulation of β-receptors. Serotonin, however, plays a permissive role in this process, acting at the level of the receptor. Block of serotonin synthesis in animal pretreated with parachlorphenylalanine (PCPA), a tryptophan hydroxylase inhibitor, negates the down regulation of receptors that normally follows treatment with desipramine. PCPA also blocks the clinical effects of antidepressants. A similar distribution of NE and 5-HT terminals in the cortex provides an anatomical basis for the interdependence of the two aminergic systems. Thus, the debate over which neurotransmitter is most involved in depression seems to have been resolved, both are involved, but NE represents the final common pathway (Sulser 1984). However, a full and precise understanding of the pathophysiology is not yet available.

Recent advances in basic neuroscience research have led to a vastly improved understanding of the possible neurobiologic mechanisms that could be implicated in long term effects of antidepression treatment (Heninger and Charney 1987). In the animal studies, the most consistent finding after chronic administration of monoamine reuptake inhibiting drugs (MARI) (tricyclic antidepressant) as well as of MAO inhibitors and of electroconvulsive therapy, is reduction of activity of the noradrenergic cyclic AMP generating system in the limbic forebrain. It may be that it is this reduced cyclic AMP response to NE

which underlies the therapeutic activity of MARI in man and which does not usually begin until several days after administration.

Even though at the present time, it is not possible to clearly document abnormalities in the regulations of monoamine receptor systems as the cause of depressive illness, it has been suggested that their dysregulation may play a critical role in the pathogenesis of depression.

The study of the metabolites of catecholamines and indoleamines in mania has not been as informative as in depression. Lithium (Li^+) is the drug of choice for treating mania. The mechanism of action of Li^+ as a mood-stabilizing agent remains unknown, although effects on biological membranes and synaptic neurotransmission are suspected. Li^+ inhibits the depolarization provoked and Ca^+ dependent release of NA and dopamine but not 5-HT from nerve terminals (Baldesarini and Vogt 1988). Li^+ may even enhance the release of 5-HT, especially in the hippocampus (Treiser *et al* 1981). It may also slightly alter the reuptake and presynaptic storage of catecholamines in directions consistent with increased inactivation of the amines. The ion has little effect on catecholamine-sensitive adenyl cyclase activity or on the binding of ligands to putative adrenergic receptors in brain tissue, although there is some inconsistent evidence that Li^+ can inhibit the effects of receptor blocking agents to cause supersensitivity in such systems (Bloom *et al* 1983). Hypothetically, treatment with Li^+ could selectively modulate the function of hyperactive neurons that contribute to the manic state (Casebolt and Jope 1989). The importance of these effects for clinical efficacy in mania is still unknown.

Anxiety Disorders

Anxiety, unlike other psychiatric conditions-such as schizophrenia and depression is both a normal emotional and psychiatric disorder. Since the discovery and extensive use of the benzodiazepines (BZs), and the more recent delineation of their receptors in human brain, there has been an assumption that the pharmacology of the benzodiazepines equates with that of anxiety.

BZs owe their success to their uncontested efficacy, rapid onset of action, and lack of relevant toxicity and replace the barbiturates and meprobamate. However, classic BZs do have certain side effects which include over sedation, potentiation of ethanol effects, motor uncoordination at high doses and development of physical dependence on long term use. A recently introduced anti-anxiety drug-Buspirone with no potential for producing tolerance-will present a new challenge to the clinician in selecting the best agent for anxious patient.

Neurohumoral Hypothesis

There is growing evidence that GABA, an inhibitory amino acid neurotransmitter may play a central role in the brain mechanisms of anxiety. There is also much interest in the effects of benzodiazepines on neurotransmission in the CNS that is mediated by GABA. This research has been stimulated by electrophysiological observation of the BZs, potentiation of the inhibitory effects of GABA,

possible by increasing neuronal receptor sensitivity to GABA, as well as by the discovery of specific binding sites for BZs in various brain regions. There appear to be many such receptors in the limbic system (especially the hippocampus, the olfactory bulb), thalamic nuclei and cortex, and there is much evidence that BZs receptors are widely distributed in nature. These sites are believed to occur in a macromolecular complex that includes GABA receptors and a chloride channel. A close interaction between GABA and BZs receptor binding leads to an increase in neuronal chloride conductance, so the final common path at the GABA-BZs complex is the chloride channel. Despite these observations the specific mechanisms by which BZs mediate their clinical effect is not completely understood. In concentrations in the therapeutic range, BZs can also reduce the excitability of some neurons by action that involves neither GABA nor alterations in membrane permeability to chloride. Thus cellular mechanisms in addition to the facilitation of GABA mediated chloride conductance may contribute to the behavioural effects of BZs (Skolnick and Paul 1982, Polc 1988, Baldessarini 1990).

Recently, noradrenergic, serotonergic, mesocortical dopaminergic systems and adenosine particularly in the limbic system have been implicated in the pathogenesis of anxiety. There are elevated plasma and CSF levels of NA and MHPG in anxiety patients. The only other monoamine to date to be seriously implicated in anxiety is 5-HT. Early animal work suggested that the anxiolytic effect of the BZs might be mediated via a reduction of 5-HT transmission. Ritanserin a 5-HT_2 receptor antagonist appears promising and 5-HT_3 receptor antagonists have shown anxiolytic activity in animals (Null 1990).

In the process of the search for an endogenous benzodiazepine-like substance, three agents that antagonize the benzodiazepine receptor have been discovered. One, a β-carboline, has been shown to provoke intense anxiety in animals and humans. Such drugs with effects opposite to those of BZs have been called inverse agonists. A second called Ro-15-1788, blocks the binding of BZs to the receptor but does not itself cause symptoms of anxiety. This agent may be useful in reversing the effects of BZs and possibly also alcohol-in cases of overdose or after anaesthesia. Finally, Costa and colleagues have isolated a peptide, called diazepam-binding inhibitor (DBI), which may be a naturally secreted substance (Gorman and Davis 1989).

Recently, novel anti-anxiety agent-buspirone that is unrelated to benzodiazepine has been released. It has pharmacological properties that are substantially different from the properties of BZs. It appears to enhance dopaminergic transmission and to antagonize serotonergic and GABA transmission (Gorman and Davis 1989). It is unlikely that any one neurotransmitter system alone is either responsible for the neuropathology of anxiety or mediates medication induced therapeutic changes.

Rapid progress in recent years has greatly expanded the understanding of neurotransmitter and drug action in the brain. Ongoing research on the well-characterized monoamine and inhibitory amino acid neurotransmitter systems

has generated new insights into the actions of many psychotropic drugs, their possible causal involvement in mental illness and their risk of sometimes serious toxic effects. Many of these recent developments stem from advances in neurotransmitter receptor research and underscore the importance of familiarity with fundamental concepts of receptor function for understanding key aspects of psychotropic drug action.

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Psycho-Neuroimmunology

A. VENKOBA RAO

There have been behavioural and experimental paradigms for psycho-neuroimmunology. They pertain to the influence of the central nervous system over the immune system and to the question of 'mind over body'.

Paradigms

Schmeck (1987) refers to the medical history of a 28 year old Fillipino woman who gave birth to a healthy female baby following an uneventful pregnancy. Two years earlier she was diagnosed as suffering from systemic lupus erythematosus with symptoms of rash, aches and pains, anaemia, liver dysfunction, renal complications and thyroid problems. Any hope of her recovery was given up. All of a sudden, she left her home in the State of Washington US. It was assumed she was not alive. A few weeks later, she returned to US, a picture of health. She narrated how a witch doctor in her native Phillipine village removed the curse pronounced on her by her former suitor. Such an instance would have been dismissed as a paradox of coincidence, but now with our knowledge of psycho-neuroimmunology, these so called 'triumphs of magic over medicine' have assumed importance and have lent themselves to scientific study. This illustrates the influence of the nervous system over the immune mechanisms in overcoming an autoimmune disorder.

A group of scientists in the University of California, San Fransisco, discovered that T lymphocytes have receptors for substance P, elaborated by the brain. The substance P is associated with pain perception and pain is an indicator of injury which calls forth for an immune mechanism. Hence, it is conceivable that substance P has a role in immunological mechanism-another link between CNS and immune system (quoted by Schmeck 1987).

Another group of scientists from NIH, Bethesda, (Md) and University of Alabama in Birmingham induced a "conditioned response" from immune system

experimentally. They exposed a set of rats to an unusual odour and later they were injected with an immunostimulant. In course of time, the rats responded to immune stimulation (evidenced by natural killer cell increase) on the exposure of odour alone. This is a replication of the classic Pavlovian conditioning of salivation in dogs in response to the bell. In a similar experiment conducted at Rochester college of Medicine and Dentistry, New York State, rats were administered liquid saccharine and also an immune depressant cyclophosphamide. Later the immune suppression took place with saccharine alone without cyclophosphamide (quoted by Schmeck 1987).

Historical

The bringing together of a group with expertise and motivation on all the problems of psychosis, dates back to Emil Kraepelin who established institutions for psychiatric research in Germany, first in Heidelberg and later in Munich. To quote Kraepelin's description of bringing psychiatry into the main stream of neurosciences: "Unfortunately we were not able to install all the departments we had intended; it seemed doubtful whether we would succeed in finding a suitable chemist under the present pitiful working conditions. We therefore decided to satisfy ourselves with an installation of two histopathological departments under Nissl and Spielmeyer, topographic histology under Brodmann, a serological one under Plaut and geneological one under Reudin and to leave further development for the future" (Hippius *et al* 1987). Later Kraepelin wrote about the need for a nerve physiologist and a statistician. The brain changes of neurosyphilis demonstrated by Kraepelin, Alzheimer and Nissl group had the status that HIV encephalopathy has now acquired. The German School demonstrated that CNS disease processes causing psychiatric symptoms existed and their pathogenesis was susceptible to elucidation. The current research in neurobiology is a resurrection of the earlier German concept.

This presentation makes an attempt to discuss psychoneuro-immunology after a brief introduction, with reference mainly to schizophrenia, depression and other psychological states in the context of immune changes. The role of viruses and factors that affect immunity like hormones and immune changes both humoral and cell mediated will be discussed. The presentation will include cancer and depression in immunological context and will end up with recent ideas on chronic fatigue syndrome.

Early studies

Are viruses the cause of mental illness or that stress and mental disorder produce hypoimmunity with increased successibility to viral infection-these interrelated questions have been debated periodically and there has been a renewal of interest with increased sophistication in immunology and information on immune deficiency states and neuro-imaging techniques.

The role of infection in mental disorders dates to 1920's when Karl Menninger (1928) observed the frequent occurrence of 'dementia praecox' picture in post

influenzal psychosis following 1918 epidemic. Menninger proposed that schizophrenia resulted from acquired infections. Subsequent viral hypotheses of schizophrenia were stimulated by the observations that slow and latent viruses would cause the disease of the nervous system after a gap of many years without antibody formation (Torry and Peterson 1973, Crow 1978) and without the characteristic gliosis (Johnson 1980).

Early studies reported increased prevalence of titres of herpes simplex virus (HSV) in persons suffering from psychotic depression and aggressive psychopathy (Rimon 1969, Cleobury *et al* 1971). Subsequent studies failed to confirm these observations, when it became known that the antibody levels for both HSV and Cytomegalo-Virus (CMV) are related to age and no differences were found in either of these antibody titres in any group of psychiatric inpatients when the factor of age was controlled. There were other suggestions that in addition to the mean titres of HSV antibody in psychotic depression, the cell mediated immunity to HSV (as measured by lymphocyte stimulation tests for p-hydroxyamphetamine and viral antigens) was similar to the inpatients who have had acute HSV infection one or two weeks previously and different from those who had suffered from HSV 8 weeks earlier or controls (Cappel *et al* 1978). It was suggested that these depressives had suffered HSV infection or its recurrence prior to the current depression. These findings have however not been reproduced.

Schizophrenia

Increased titres for a number of viral antibodies have been reported in both serum and CSF of schizophrenic patients (Albrech 1980). An increased prevalence of IgM antibodies to cytomegalovirus (CMV) in the CSF of patients with schizophrenia was found. This suggested active infection, reactivation of earlier infection or abnormal persistence of antibody from past infection (Torrey *et al* 1982). Other workers failed to find any increase in HSV, rubella, measles antibodies in the CSF of schizophrenic patients (King *et al* 1985). Yet, others found significant decrease in the titres of antibodies for measles, mumps and rubella and no change in HSV, CMV and VZV adenoviruses in schizophrenia (Gotlieb *et al* 1981). It was suggested that low antibody levels of HSV, CMV, VZV and EBV in the maternal and cord blood of infants with one or more risk factors at birth (low birth weight etc.), correlated with impaired immunity and with an increased perinatal susceptibility to viral infection ultimately leading to schizophrenia in later life. Fine *et al* (1985) reported no increase in neurological diseases in 2570 subjects known to have been exposed to a variety of infections *in utero* and followed up for 40 years. Schizophrenia, however, was not looked for in them. An increased risk for schizophrenia was reported in those exposed to 1957 influenza epidemic during their second trimester of fetal life (Mednick *et al* 1987).

An increased proportion of circulating suppressor/cytotoxic T lymphocytes has been found in schizophrenia and a decrease in the number and proportion of

circulating T lymphocytes on treatment including the drug free patients has been reported (DeLisi 1981). Antibody levels are also influenced by the hormonal effects of cortisol, androgens and oestrogens. Impaired immunity in schizophrenia is also suggested by generalised reduction of between 25% and 50% in sera and CSF levels of IgG, IgA and IgM immune globulin in chronic institutionalized patients (King *et al* 1985, DeLisi 1981). This was not specific to schizophrenia since low IgM levels occurred in about 10% of acute psychiatric patients also. IgG level was low by 30% in CSF of schizophrenics but not in the sera of other psychiatric patients.

It was hypothesised that schizophrenia could be an autoimmune disease (Burch 1964). Antibrain antibodies were detected in sera and CSF of schizophrenics but not in controls (DeLisi 1985). However, antibrain antibodies were found in 18% other psychotic patients and hence this was not specific for schizophrenia. No differences in the antibrain antibodies in serum to a variety of areas in the brain were found between patients and control subjects (Roos *et al* 1985). Antibrain antibodies in apparently healthy subjects have also been detected.

Attempts to identify virus like particles in CSF or the virus in the autopsied brains have yielded contradictory results (Mered 1983). CMV virus has not been detected on autospy in the brain of schizophrenics by either immune histochemistry or DNA hybridization. HSV virus and Paramyxo-virus have been isolated from the autopsy brains of schizophrenics. The latter report is from Russian work and the diagnosis of febrile schizophrenia was untenable. Overall, it would seem that immune function may be slightly reduced in schizophrenia but there is no clear evidence for this to be of etiological significance. There is also no direct evidence for any particular virus being involved in causation. There is evidence that obstetric complication becomes operative only in already genetically vulnerable subjects (Lewis and Murray 1987). A similar situation may be applicable regarding viral infection causing schizophrenia-during perinatal period or during pregnancy in genetically vulnerable people. Nevertheless, it is likely that some cases of schizophrenia though not all may have a viral etiology, considering the prevailing view that schizophrenias are a group of etiologically heterogeneous disorders with different subgroups.

"Season of Birth" effect

An important element in the viral hypothesis for schizophrenia has been the epidemiological finding of 'season of birth' effect. Hare and Price (1968) first observed an excess of winter births of schizophrenic patients, as against neurotic patients. Compared to the national sample of schizophrenic patients with a general population of the UK, a 7% increase in births of schizophrenics in winter months was noted (Hare *et al* 1973). Similar results have been obtained in many countries including those from Southern hemisphere (Parker and Nielson 1976). The winter birth excess has varied from 5-15%. It is suggested that many viruses are more prevalent in winter and early spring and a winter born child runs a higher risk of infection. But the present evidence tends to show seasonal infec-

tious diseases to be in excess in the year *prior* to the schizophrenic births suggesting a prenatal rather than a postnatal infection.

That schizophrenia is an infectious disease has been adduced from the family history studies and the occurrence of new cases of schizophrenia in Apartment Blocks as reported from Moscow (Kazanetz 1979). A higher concordance for same sex or siblings than different sex pairs is also in keeping with an infectious etiology. The 'contagioun' effect has been disproved by a study of the institutionalised schizophrenics and in psychiatric nurses which was found to be no different from that of General Hospital Nurses (Cooper *et al* 1987). Finally attempts to transmit infective agent from the brain tissue of the schizophrenia patients to laboratory animals have so far been fruitless (Barker *et al* 1983). The current epidemiological and family history data seem to argue against an environmental event acting postpartum.

Development disorder

Recent neuropathological findings are suggestive of events in the first and second trimester of pregnancy to be important. The newer neuro-imaging techniques have revealed ventricular enlargement (third and lateral) in a group of schizophrenic patients. The ventricular enlargement is attributed to pathological changes in the medial temporal lobe structures like hippocampus, para hippocampal gyrus and ventral insular cortex. The pathological examination has revealed atrophy or aplasia without gliosis indicating its developmental nature. The assault on the nervous system has taken place in the embryonic period when the brain is immature and hence absence of gliosis in response to damage. These remain stationary without progress long before the emergence of symptoms suggesting a similarity to neurodevelopmental disorders. The question is what it the nature of the assault-traumatic, viral, immunological or metabolic? (Roberts and Crow 1987).

Freeman (1989) in summing up of the venture for the search of causes for schizophrenia suggested that competing theories of schizophrenia need not be mutually exclusive but could represent the different facets of the same central function. Although advances in genetics and biological research including immunology seem to be rapid and are securing most attention, it might in fact be epidemiology-'a tortoise rather than the hare' of Aesop's fable-which really may illuminate the fundamental nature of the condition.

Depression

A decrease in immune responsiveness has been reported in depression as well as closely related psychological states like bereavement (Bartrop *et al* 1977) and loneliness (Kiecolt-glaser *et al* 1984). However, control studies have not lent support for these observations.

A decreased lymphocyte response to T cell mitogens, a decreased total T and B lymphocytes and no change in proportions of lymphocyte subsets were reported in severely depressed inpatients (Kronfol *et al* 1983, Schleifer *et al* 1984). An-

tibodies to Borna virus have been demonstrated in a small group of affective disorders. Injection of the virus into the tree shrews has induced cyclical behaviour change, a model of bipolar disorder. There was linking of severity of recent life events with a decrease in the activity of the natural killer cells in women whose husbands were seriously ill or had died during the previous 6 months, compared with women with husbands in good health (Irwin *et al* 1987). However, it was the severity of depression rather than the severity of life events that bore a strong association with T cell lymphocyte subsets. It is known that in states of depression, there is an increased vulnerability to infection, tumour growth and autoimmunity. An attempt to link changes in total lymphocyte numbers to cortisol hypersecretion was attempted utilising the DST. A reduced lymphocyte number was common in unipolar than bipolar patients and an abnormal DST correlated with lower lymphocytic count in unipolar but to higher count in the bipolars. The evidence at present indicates cortisol to be associated with a decreased immune response. Its clinical significance for depression or autoimmune disease is not clear. The changes in lymphocyte responsiveness is quite variable and changes reported in depression are marginal. The relationship between plasma cortisol and T and B lymphocyte numbers appears to be an inverse one. Moreover a circadian variation in the level of lymphocytes has been identified.

Lymphocytes carry beta adrenoreceptors and there is some evidence for inputs into lymph nodes. There are other CNS inputs into the immune system. Natural killer cell activity can also be mediated by beta adrenoreceptors. In depression marked alterations in non adrenergic functions occurs. Particularly elevation of plasma noradrenalin levels may have a depressing effect on the function of the immune system. It seems likely therefore that the changes in immune functioning in affective disorder may be the result of the disease rather than its cause and immunological changes are uncertain and marginal (King and Cooper 1989).

The association between cancer and depression has a long history. Galen believed that a melancholic state increased the proneness to cancer. A recent study, however, indicated that the prevalence of depression in cancer patients was 6%-a rate which is not disparate from that from the samples of patients with medical disorders (Levine *et al* 1978). It can be said that some cancer patients are significantly depressed but it is wrong to believe that cancer patients are generally depressed. However, some studies link the attitude towards cancer and the rate of mortality from cancer (Keith *et al* 1985). The doubling of risk of death from cancer in a group of men who had secured highest on depression scale has been reported. This risk persisted when controlled for other risk factors like age, smoking and alcohol use. Later it was shown that the patients were not in clinical depression but they were in a state of chronic mild distress. Currently it is held that chronic mild distress may not only be a risk factor for higher mortality but also a factor for the presenting features in a significant proportion of cancer. A direct mediation between central nervous system and immune activity is indicated by the presence of receptors on lymphocytes for ACTH. It has been postulated that beta endorphin receptors on lymphocytes may be the mechanism

whereby anger and aggressiveness combat cancer and compensate for steroid induced suppression of immune system.

There seem to be separate mechanisms for immune and endocrine responses. A strong correlation between depression and decreased immunological capacity but not adrenal function has been demonstrated, suggesting a direct communication between brain and immune system.

Seasonal affective disorders

The relationship between hyper-corticolism and immune system has been brought out in cases of seasonal affective disorders in many studies.

Studies that investigated the circadian profile of cortisol indicate that the rhythm is delayed in winter. In a study in Antarctica, the circadian rhythm of cortisol showed a low amplitude and phase advance in summer compared with other seasons (Ritchie *et al* 1983). The DST consisting of ACTH suppression by a synthetic corticosteroid has not been applied on a large scale to normal subjects. However, the World Health Organisation (1987) multicenteric diagnostic study of usefulness of the DST in depressive patients prompted further analysis of the DST result with respect to seasonality and latitude. Highest percentage of non suppression occurred in summer and the lowest in winter. Further, a meta analysis of all the data from the WHO study showed a noticeable 'cline' with latitude in northern hemisphere only. The highest percentage of non suppression occurred in the north and lowest in the south (Richmer 1987). This caution is required in defining a normal rate of percentage of non suppression. Since, findings may be asked by the time of the year or geographical location of the study independent of differences in clinical populations investigated.

Adrenocortical activity is known to suppress cellular immune function and seems to be more effective against suppressor T cells. One of the consequences of seasonal variation in corticosteroid might be a seasonal pattern in immune function. There is a support for a winter trough in immune function as indicated by a maximal depression of T cell function accompanied by an elevated B cell function in winter with a reverse pattern in summer (MacMurray *et al* 1983). Among the T cells, the highest inducer to suppressor ratio was obtained in summer. Cellular immune function has been shown to be affected in depression. For example, the response of PBL to mitogen stimulation is reduced in some depressed patients compared to normal subjects. In addition, it has been shown that the treatment of unipolar and bipolar depressed patients with tricyclic antidepressants, lithium or ECT seems to reduce the blastogenic response of their peripheral blood lymphocytes to mitogen stimulation. Hence SAD patients provide an ideal cohort in which to assess cellular immune functions before and after antidepressant therapy without the confounding variable of pharmacological treatment.

Using mitogen induced lymphocyte blastogenesis of PBL as an *in vitro* measure of cellular immunity, the immune function of both 9 untreated depressed and 9 light treated SAD patients were compared with the immune function of 9 normal

healthy volunteers. At base line the PBLs of the depressed patients had significantly greater response to the mitogen. After approximately one week of light treatment, the mitogen stimulated response of the patient's cell were significantly reduced in amplitude with a flattening of the dose response curves compared to pretreatment curves. These values were comparable to those of untreated controls. This finding was replicated the following winter in a study, which was identical except for the use of additional mitogen CON A.

Immune function of 9 untreated euthymic SAD patients was compared with that of 9 normal controls in summer. As in winter, the mitogen stimulated response of the patients lymphocytes to CON A was greater than those of normal controls. However, the PBL response to PHA did not differ between the groups. In summer the mitogen induced lymphocyte blastogenes of the PBL of normal subjects exposed to bright light was significantly increased compared to the base line. The base line difference between SAD patients and normal volunteers noted for PHA and CON A in winter and for CON A in summer are opposite to those that some researchers have observed in non seasonal depression. The difference in the response to PHA and CON A in summer may be related to the ability of PHA to stimulate the helper cells more than suppressor cells. The opposite is true for CON A. The persistence regardless of mood of the base line difference between patients and normals in mitogen response to CON A and opposite response of the patients and normal volunteers to light exposure suggest that this test of cellular immune function may be a trait marker for SAD (Skewrer *et al* 1987). If this is true, the study of immune function in SAD may provide valuable insights into the pathophysiology of the disorder, since abnormalities in the direction of enhanced PBL response has not been documented in non seasonal depression. However, no conclusions about clinical implications of immune function in SAD can be drawn, since mitogen stimulation is not necessarily a good reflection of *in vivo* immune function. The immunological abnormalities in SAD may have clinical significance.

Chronic fatigue syndrome

Chronic fatigue syndrome (CFS) is the term most recently applied to a group of symptoms which have been known by a variety of names including-'myalgic encephalomyelitis' (ME), "chronic mononucleosis syndrome", "chronic mononucleosis-like syndrome", "post-viral asthenia syndrome", "post-viral fatigue syndrome", "post-infectious neuromyasthenia", "chronic active Epstein-Barr Virus infection", "chronic Epstein-Barr Virus disease (CEBE)" as well as a number of names which were applied to specific epidemics including "Royal Free Disease" and "Iceland Disease". CFS is characterised by a multiplicity of non specific features including incapacitating exhaustion, post exertional fatigue, malaise, myalgia, weakness, subjective feverishness, sorethroat, painful lymph nodes, headaches, dizziness, light headedness, depression, impaired memory, confusion and difficulties in concentration. The illness follows two patterns: (1) relapsing and remitting and (2) continuous. The estimate of its prevalence has been around 21% to 50% (Jones and Miller 1987).

In recent years, a search for biological basis for CFS has been on and has been focused on abnormalities of immune functioning and the role of chronic or relapsing viral illness (Salit 1985). Special focus has been on abnormal Epstein-Barr serology. Earlier findings have not been reduplicated and the relationship between EBV antibody titres and clinical status has not been established (Straus 1988). Other viruses have been implicated on the basis of small groups of patients, but no consistent findings have emerged (Borysiewicz *et al* 1986). Recent attention has focussed on entero-viruses and the use of VP I antigen to detect chronic entero virus infection (Lynch and Seth 1989). Some researchers were unable to distinguish between groups of postviral fatigue patients and psychiatric patients with major depression. There is no difference between postviral fatigue patients who tested positive and those who tested negative for the VP I antigen and 12% of patients with a variety of neurological disorders and VP I antigen test positive (Halpin and Wessly 1989).

Several non specific immunological abnormalities have been reported including the reduction of absolute number of T lymphocytes, including CD 2, CD 4, and CD 8 subsets; increased levels of T cell mediated suppression; decreased natural killer cell activity, decreased *in vitro* interferon gamma production, decreased interleukin 2 production, increased levels of 2-5 oligoadenylate synthetase, a marker for interferon production, decreased *in vitro* antibody synthesis following mitogen stimulation, partial hypogammaglobulinemia, elevated circulating immune complexes and other autoimmune phenomena. These have been summarised by Abbey and Garfinkel (1990). The prevalence of abnormal findings has varied widely and does not appear to correlate with symptom severity. It has been suggested that more carefully designed studies involving comparison with major depression are to be undertaken in the light of findings of altered immunity in major depression.

One of the important findings of the syndrome is cognitive abnormalities that lead to social and occupational malfunctioning-poor concentration, dyslogia and frequency memory difficulties. The cognitive efficiency affects problem solving and findings with more sophisticated investigations like MRI, SPECT, PET have revealed demyelination and focal edema. Abnormal scans were found in 70% of cases (Abbey and Garfinkel 1990). The most common abnormality is decreased cerebral blood flow to the basal ganglia (Cummins and Benson 1984). Other findings include decreased perfusion to the frontal lobe, posterior parieto-occipital regions and diffuse hypoperfusion of the cerebral hemispheres. (Silverskioed and Risberg 1989). The significance of these findings is not clear. They may signify the unique neurobiological basis for the psychiatric symptoms of CFS. This is consistent with the prevalence of psychiatric symptoms, including depression in subcortical disorders like Parkinson's disease and Huntington's disease. Alternatively, the basal ganglia hypoperfusion may be related to more depression, as suggested by PET studies, which have shown a significantly lower rate of glucose metabolism in the head of the caudate nucleus in unipolar depressed patients than in bipolar.

CFS is most likely a heterogenous disorder and there may be atleast 4 major subgroups of patients with this diagnostic label.

(1) The first comprises the smallest group of individuals whose disability is rooted in a post-viral aesthenia syndrome. This group is characterised by a well documented infection with severe disability occurring at the time of acute infection and during the first few months that follow and the recovery, occurring gradually over approximately a year from the onset of the illness.

(2) The second group of patients seem to have an initial viral illness, but their protracted disability is the result of secondary depression and physical 'deconditioning' associated with inactivity. These individuals are prone to protracted dysfunction due to depressive vulnerability related to personal or family history of affective disorder.

(3) The third group of patients are those with primary psychopathology, typical affective disorders, anxiety disorders or somatisatin disorders. The psychiatric disorders may have escaped detection owing to the predominant somatic presentation.

(4) This group consists of "worried well" who are significantly preoccupied with the soma and have general sense of ill-health or fatigue.

We have moved quite far from the days of absolute helplessness in the understanding of psychiatric illness through demonology, teology, various psychological and psychoanalytical schools and social models. A major step has been taken with the availability of modern neuro-imaging techniques. Immunology has begun to include in its research several psychiatric disorders. However, our "rush to go molecular" should not make us ignore the other aspects of diseases. A day may come when as in physics "boot strap" theory putting together the 'wave' and 'particle' theories will emerge and what different neuroscientists see may be woven into a holistic model. It will be appropriate to conclude with the words of Charcot "Disease is ancient and nothing about it has changed; it is we who change as we learn to recognise what was formerly imperceptible".

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Neurobiology of Affective Disorders

SUMANT KHANNA

The primary disturbances encountered in psychiatry can grossly be divided into dysfunctions of thought, affect and perception. However there is a great degree of overlap in psychiatric syndromes, i.e. delusions and depression can co-exist, and often the diagnosis is based on the presumed primary or major disturbance. Affect is the cross-sectional observation of the emotional tone of an individual. Very often affective disturbances are regarded as "bipolar" involving happiness or sadness; however, affect can also include anger, irritability, bliss, etc. Current classification systems do not give due importance to such concepts. Within currently recognised affective disorders, the ones listed by the American Psychiatric Association (1987) are given in Table 1. Not much is known about the neurobiology of some of these; observations are often contradictory and confusing. Thus the current presentation will focus on Major Depression.

Table 1

Affective disorders
Depression
Mania, Hypomania
Major depressive episode
Chronic, Melancholic, Seasonal
Bipolar disorders
Cyclothymia
Dysthymia (Depressive Neurosis)

Depression is a common everyday symptom. However, there are various suggestions as to when it becomes syndromal. The criteria for Major Depression as

defined by American Psychiatric Association (1987) or what was earlier referred to as Manic Depressive Psychosis, Depressed Type (Sapolsky 1990) are more rigid. These include the presence of vegetative symptoms, a sustained profile and the optional presence of melancholic features (Table 2).

Table 2: Criteria for major depressive episode

(1)	Depressed mood, most of the day
(2)	Markedly diminished interest/pleasure in almost all activities of daily life
(3)	Significant weight loss/gain (>5%) or appetite loss
(4)	Insomnia or Hypersomnia
(5)	Psychomotor agitation or retardation
(6)	Fatigue or loss of energy
(7)	Worthlessness or excessive inappropriate guilt
(8)	Decreased concentration or indecisiveness
(9)	Recurrent thought of death, suicidal ideas, plans or attempts

Depression in Medical Disorders

Depression is recognised as a symptom of various diseases. It occurs in a wide range of medical and neurological disorders (Table 3). Although in some of them the depression may have been reactive, in others it is likely that it was due to an underlying biological substrate. Such substrate could be either biochemical or structural. Examples of biochemical abnormalities which can present as depression include hypothyriodism and diabetes mellitus. Frontal lobe tumours and other structural lesions can masquerade as depression. Depression is also a frequent concomitant of certain degenerative diseases such as Parkinson's disease, Huntington's disease and other dementias. Some workers believe that the cognitive dysfunctions observed in depression probably share similar biochemical and structural changes vis a vis depression in known organic illnesses (Cassemm 1988).

Post-stroke depression is another well recognised entity. There is no marked clarity about which side lesions are more likely to produce depression, but there seems to be a consensus that more anterior and larger lesions are more likely to produce depressive symptoms. However, a large scale epidemiological study conducted in UK failed to find significant depression in the majority of subjects who had strokes. This raises the possibility that such depressions are most probably more likely to be associated with more serious strokes, probably with greater neurological deficits and larger cortical involvement.

Epilepsy formed the model for inter-ictal psychosis based on the work of Flor-Henry(1966). According to him non-dominant lesions were more likely to present with affective changes, predominantly depression. However, most recent studies have not been able to substantiate these observations (Venkoba Rao 1989), and the effect of laterality of complex partial seizures and affective profile is not as clear as thought earlier.

Table 3: Medical illnesses causing organic depressive disorder

General etiology	Specific etiology
Infection	Encephalitis, meningitis, general paresis
Neoplastic	Gliomas, meningiomas, abscesses
Degenerative	Senile and presenile dementias such as Alzheimer's or Pick's disease, Huntington's chorea
Intraventricular	Normal Pressure Hydrocephalus
Vascular	Hypertensive encephalopathy, cerebral atherosclerosis, intracranial hemmorhage or thrombosis, systemic lupus erythematosis, polyarteritis nodosa, thrombotic thrombocytopenic purpura
Drugs	Anti-hypertensives, oral contraceptives, steroids, barbiturates, cimetidine, beta-blockers, cocaine, amphetamine
Endocrine/metabolic	Diabetic coma/shock, uremia, myxedema, hyperthyroidism, parathyroid dysfunction, hypoglycemia, hepatic failure, porphyria, electrolyte or acid-base disturbance, remote side effect of carcinoma, Cushing's or Addison's disease, sleep apnea
Anoxia	Pulmonary or cardiac failure, anaesthesia, anemia
Metals	Heavy metals, Carbon monoxide, toxins
Congenital	Epilepsy, Post-ictal states, aneurysms
Traumatic	Subdural and epidural hematomas, contusion, laceration, postoperative trauma, heat stroke
Intoxications/ withdrawal states	Bromides, opiates, tranquilisers, anticholinergics, dissociative anaesthetics, anticonvulsants
Vitamin deficiency	Thiamine, Niacin, B^{12}

Brain Imaging Techniques

CT scan studies in depression have documented increased ventricular size, enlargement of the bifrontal and bicaudate distance and dilatation of the third ventricle (Schlegel and Kretzschmer 1987). Similar observations have been observed with the MRI scan. Our own data also seems to suggest frontal and sub-cortical involvement in depression.

On PET scan, major abnormalities have been localised to the frontal lobes and caudate nuclei (Bayter *et al* 1989). There is also a suggestion of a decreased antero-posterior gradient similar to that observed in schizophrenia. Subjects with Parkinson's disease and Huntington's disease show hyperfrontality when there is co-existing depression. However due to variables such as time resolution and diagnostic issues, it is difficult to comment upon the exact implications of these findings.

Electrophysiology has also helped in understanding brain pathophysiology in depression. The resting EEG on visual analysis has mainly revealed changes in alpha activity. Computerised EEG (Table 4) has tended to implicate the non-dominant frontal regions (Khanna *et al* 1987). Middle latency evoked potentials, contingent negative variation, P300 and the beritschafts potential have consistently been found abnormal in depression (Khanna *et al* 1989). The use of more

sophisticated techniques such as Brain Electrical Activity Mapping has also shown a limitation of spread to more anterior regions of the cerebral cortex on stimulation by a vast variety of meaningless or meaningful tasks, supporting a strong information processing model for depression.

Table 4: Resting computed electroencephalographic studies in depression

	Alpha	Beta	Slow
Flor-Henry 1979	Temporal increase right only 13-20 Hz		
Kemali 1981	Decrease	Increase	
Schaffer 1983	Increase		
von Knorring 1983	Increase		
Brenner 1986	Increase	Decrease	Decrease
Knott & Lapierre 1987		Increase	Increase
John 1988	Increase	Decrease	Increase

Responses to meta-chlorophenylpiperazine in depression

	Behavioural	Cortisol	Prolactin
Maj 1989			Increased
Khanna 1991	Decreased suicidal	Augmented	Augmented
Jacobsen 1991	Euphoria		
Kahn 1988		Normal	
Kahn 1988	Normal		
Kahn 1990			Normal
Lithium Aulakh 1990		Reduced	Reduced (long-term)

The major group of post-mortem studies in depression have focussed on suicidal subjects and have found most of their abnormalities on the region of the frontal cortex. Receptor binding sites have suggested a change in serotonin receptors and uptake, although recent reports suggest that other receptor sites, eg. benzodiazepines, alpha-2 adrenergic, GABA, etc. may also be involved. This is probably more a function of high suicidality/impulsivity than of depression per se and thus difficult to comment upon.

Thus electrophysiological and other brain imaging data stresses the role of the frontal (and probably the prefrontal) cortex and the possible involvement of sub-cortical structures such as the basal ganglia. In general the work on brain imaging has been far more intensive in schizophrenia than in depression. More consistent results are required before definitive conclusions can be drawn.

Neurochemistry

Perhaps the major focus in the study of depression has been on neurotransmitters (Table 5) mostly noradrenaline, serotonin and dopamine. There have been few studies focussing on neuropeptides. Surprisingly whenever studied these

neuromodulators have been found to have altered concentration, at times conflicting with reports, making an integrative model difficult.

Table 5

	Depression	Mania
Neurotransmitters		
5-HT	–	?
ACh	+	–
NA	–	?+
DA	–	+
Related enzymes		
Platelet MAO	+	–
Plasma DBH	?–	?

The catecholamine hypothesis of depression basically pointed to a deficiency of noradrenaline in depression (Bunney and Davis 1965). Earlier data was based predominantly on tricyclic response. Subsequently various other variables such as low CSF NA and MHPG, increased urinary MHPG, blunted responses to challenges such as clonidine (growth hormone for post-synaptic and MHPG for pre-synaptic) and yohimbine increased receptor binding to H^3 clonidine receptor and yohimbine sites in the peripheral lymphocytes, have all tended to support a noradrenergic deficit state in depression. Challenges with desmethylimipramine, insulin and L-DOPA have also been confirmatory. Earlier reports suggested increased levels of peripheral epinephrine and metanephrine, but this is no longer held valid. Recent reports do not support the validity of urinary MPPG as a predictor of response to imipramine. From a psychopharmacological perspective, many psychotropic agents such as trazodone down-regulate the beta receptor.

The two disease biological model of depression involves the two systems: primarily serotonin and norepinephrine. The serotonergic hypothesis (Van Praag *et al* 1981) has also got a big boost from studies of lowered CSF 5-HIAA in suicidal risk and completed suicide subjects. There is a reported decrease in specific high affinity binding sites for imipramine in platelets in depression suggesting a problem in serotonin uptake. Completed suicide patients display an increased number of serotonin receptors although the total brain serotonin is decreased. This would implicate a functional up-regulation of the serotonergic system. Recent studies using specific probes have found blunted hormonal responses to various agents such as ipsapirone (specific 5-HT_{1A}, fenfluramine, L-tryptophan, etc. although the data with mCPP is not so conclusive (Table 6, see Khanna *et al.* 1991 for details). It is probable that different sub-systems of serotonin are involved in the pathophysiology of depression, as has been observed with Obsessive Compulsive Disorder.

Table 6: Sleep EEG in depression

1.	Sleep continuity disturbance
2.	Decreased slow wave sleep
3.	Decreased rem latency
4.	Shift of delta activity from 1st to 2nd nrem period
5.	Prolonged first rem period
6.	Increased rem density

Cholinergic involvement in depression is also currently under study. One of the major markers is the physostigmine induced REM onset, which has even been postulated as a vulnerability marker. Many of the anti-cholinergic side effects of tricyclic anti-depressants are also clinical presentations of depression. Increased acetylcholine can also influence mania, and affect sleep and cortisol patterns in normals in a way resembling the depressed state.

Evidence for involvement of dopaminergic system is much more sparse. The amelioration of depressive systems with amoxapine and the causation of such symptoms by L-DOPA are perhaps the strongest pharmacological clues. CSF HVA has been found lowered in isolated reports, but whether it is a trait marker or associated with delusional or melancholic profiles is not clear. The data from neuroendocrine challenge tests is conflicting.

It must be recognised that simple models involving single neurotransmitters have been discarded with the recognition that Dale's hypothesis is no longer valid. Functional interactions between serotonin and noradrenaline are now well recognised. A tilt in dysfunction towards one or the other probably has marked pathoplastic effects in the presentation of specific depressive symptomatology. There is thus a felt need to look at this area in greater detail.

Psycho-Endocrinology

Perhaps one of the most studied neuroendocrine dysfunctions in depression is the dexamethasone suppression test (Carroll *et al* 1981). Although this was taken to be initially a very good marker for endogenous depression, and found to correlate with a hyper-cortisolemic state, it has been found to be non-specific for various disorders, and thereby has a lower specificity than desirable. Confounding variables include stress, age, pregnancy hepatic or renal impairment, hypertension, obesity, dementia and uncontrolled diabetes mellitus. Thus a test which at one time was almost thought to be diagnostic was later recognised to have many limitations.

Other studies have also focussed on the limbic-hypothalmopituitary-adrental axis. Cortisol response to ACTH has largely been found to be inconsistent. However, corticotropin releasing factor (CRF) challenges have been found to be relatively more specific. Although the ACTH response to CRF is blunted, it does not seem to be under adequate feedback control, as the additional intake of dexamethasone is unable to obliterate the response. This suggests a central

dysfunction which probably is responsible for the dysfunction of this axis. Various behavioural responses to CRF have been postulated and specific CRF receptor sites have even been documented (Holsboer *et al* 1984). It has even been suggested that the genetic transmission of depression is related to the functional status of these receptor sites. Recent studies on wild baboons in the Transkei region in Africa have suggested that this axis is a function of both personality variables and social dominance (Sapolsky 1991). Additional work on the effect of separation of infants in infancy and just after birth resulting in persistent hyper-cortisolemic states probably raises the possibility of this being a vulnerability marker for depression. Another report also found increased volume of the adrenal glands, suggesting that the hyper-cortisolemic state was a function of pituitary over-drive or adrenal hyper-sensitivity. In conclusion the limbic-hypothalamo-pituitary-adrenal dysfunction observed in depression is probably multi-factorial and non-specific.

The Thyrotropin Releasing Hormone Test has also been consistently found abnormal in some patients with depression (Loosen *et al* 1977). The stimulation of Thyroid Stimulating Hormone is found to be abnormally low in both unipolar and bipolar depression, mania, alcoholism and various other psychiatric disorders. It thus also seems to have a low specificity for depression like the previous dexamethasone suppression test. Antibodies to thyroid hormones, and antibodies to antibodies of such hormones are found at higher levels in some cases of depression. This has been postulated to be due to either a sub-clinical autoimmune disorder (like Hashimoto's disease) or secondary to functional hypothyroidism because of their binding to free thyroxine.

Growth hormone secretion in response to insulin induced hypoglycemia and clonidine have been found to be blunted in a majority of subjects with depression. This has largely been accepted as a marker of down-regulation of the alpha-2 adrenoreceptor system which will be referred to later. A mild insulin resistance has also been documented on the Insulin Tolerance Test. It is probably a manifestation of the hypercortisolemic state of depression.

Thus there is quite consistent evidence for perturbation of function of various neuro-endocrine axes in depression. The main problem in the clinical interpretation of these tests, however, is their low specificity. The term hypothalamo-pituitary-adrenal axis has now got a limbic-prefix, suggesting that there may even be other "higher" centres which have interactional roles in the production of the observed neuroendocrine dysfunctions.

Psycho-Immunology

The CNS and the immune system are major integrative networks involved in biological adaptation. Converging knowledge utilising findings from basic neurosciences and immunology provide evidence for reciprocal interaction. These include the effect of hypothalamic lesions on immune responses, presence of neurotransmitter receptors on B and T cells, and neuroanatomical and chemical evidence of direct noradrenergic innervation of lymphoid tissue. The

peripheral lymphocyte has even been postulated as an independent endocrine system. This association is further suggested by behavioural conditioning of immune responses, the effects of stress and bereavement. The demonstration that behavioural states and perturbations of the CNS are associated with altered immune functioning suggests that alteration in immunity may be found in depression-related disorders. The humoral response to depression is probably abnormal, but has not been adequately studied.

Several studies have assessed immunity in depression through measurement of cellular responses (Schleifer *et al* 1985). Endogenous depressed patients have lower lymphocyte responses to PHA, ConA and PWM than non-endogenous patients or normal controls. Hospitalised and therefore more severely depressed patients have significantly decreased T and B cell populations and reduced lymphocyte stimulation suggesting that alteration is related to the severity of depression and is not an artefact of age or sex. This has been postulated as a dysfunction of regulatory T cells. Another persistent observation has been the consistent decrease observed in natural killer cell activity.

Altered immunity appears to be a component of the psychobiology of depression but no causal attribution is possible with the current state of the art, and its interaction with other systems such as the endocrine system are also important.

Sleep

Sleep serves not only as an indicator of neuro-anatomical integrity but also as a chemical window to the brain (Table 6). Two major themes have emerged from sleep research in depression. Firstly, subjects with a moderate to severe depression normally display a state related constellation of decreased REM latency, increased REM density, increased duration and REM density of first REM period, and decreased stage 3 and 4 nREM sleep (Gillin 1983). Secondly various sleep manipulations can ameliorate depression, eg. selective REM deprivation and partial or total sleep deprivation.

It has been hypothesised that these changes in sleep architecture may reflect an increase in cholinergic to aminergic neurotransmission in critical central synapses. Cholinergic synapses are thought to trigger the onset of sleep. This is also consistent with the cholinergic aminergic hypothesis for affective disorder (Janowsky *et al* 1972). A phase advance of the strong oscillator controlling the circadian appearance of REM sleep may be present in depression. Thirdly, it has been proposed that wakefulness-dependent homeostatic processes promoting sleep are deficient in depression. This accounts for the response to sleep deprivation and the absence of stage 3 and 4 nREM.

Thus although sleep EEG changes are consistently observed in depression, and probably correlate with the more serious disorder, the exact neuroanatomical-neurochemical substrate still needs to be elucidated.

Bio-Rhythms

Certain features of depression suggest a dysfunction of bio-rhythms. One of the most robust observations of loss of such a rhythm is in plasma cortisol, where a persistent hypercortisolemic state replaces the usual cyclical course. Similar dysfunction has also been noted with melatonin, probably implicating the noradrenergic system. Clinically itself, diurnal mood variations are recognised as melancholic features. Recent observations suggest that various drugs such a MAO inhibitors and lithium can in themselves induce rapid cycling. Shifting the time of getting up earlier can result in ameliorating depressive symptoms and even producing shifts into mania. Another observation has been the recognition of Seasonal Affective Disorder (Rosenthal *et al* 1985) characterised by winter depressions. This has led to the advent of phototherapy with bright light in these patients.

Genetics

The search for genetic clues in depression has been beset with problems of definition and measurement. However, there is convincing evidence from epidemiological and more recent laboratory work for the heritability of affective disorders. Epidemiological studies provide an estimate of life time risk of developing depression, which for women ranges between 4.9 to 8.7% and from 2.3 to 4.4.% in men (Robins *et al* 1984). This increased prevalence in women is a consistent observation suggesting enhanced vulnerability to develop depression due to underlying biological causes. Studies also suggest that with successive generations depression seems to become more common (the birth cohort effect) due to unclear reasons.

Family studies are the first used to confirm the existence of any disease clusters and therefore a genetic component. A compilation of such studies (McCruffin and Katz 1989) shows that this is indeed the case, with risk of developing depression being 3 to 6 times higher among the family members than the general population. Since family studies do not rule out environmental causes, twin and adoption studies have to be looked at for separating the genetic component. Winokur *et al* (1978) have suggested that unipolar depressions have two major genetic variants: pure depressive disorders (with family histories of only depression) and Depressive Spectrum Disorders (with family histories of alcohol dependence, hysteria in women and psychopathy in men). There have been some attempts made to determine their biological concomitants.

Twin studies have shown monozygotic concordance to be 2 to 5 times more than in dizygotic twins, strongly suggesting an important role for genetic factors. Adoption studies also support these observation (Mendelwicz and Rainer 1977). There is stronger evidence for transmission of bipolar than unipolar disorders. The prevalence of affected individuals with unipolar disorders is almost the same for both groups; however bipolar probands are more likely to have subjects affected with bipolar disorders than is the case with unipolar probands.

The next issue is the nature of what is inherited and the manner in which this happens. What is inherited in depression is not the certainty of becoming ill but rather a vulnerability for developing the disorder. Of the several attempts to integrate data into explanatory models of inheritance, the mixed model (Rice *et al* 1987) has provided the better fit than a single major locus or multi-factorial polygenic inheritance but no conclusive results can be drawn.

Linkage studies have attempted to provide more definitive evidence for localising major genes responsible. The tasks of linkage studies are two fold: to detect linkage and estimate there combination fraction, which is accomplished by analysis of log scores. Linkage studies red cell antigens, HLA, red cell enzymes and other protein polymorphisms (eg. secretor locus) have not yielded any consistent finding (McGuffin and Katz 1989).

The scope of recombinant genetic analysis has been revolutionised by application of Restriction Fragment Length Polymorphism techniques where either a "candidate gene" of some relevance to the disorder or the "shotgun method" have been used. With these approaches there have been reports of localisation to the H-ras locus on 11_p and 6_q, but these have subsequently been refuted. A strong case also exists for linkage to X-chromosome in some pedigrees studies.

Biological markers like CRF challenge, platelet MAO, CSF amines and cholinergic sleep induction are currently being studied as putative trait markers in vulnerable individuals.

Major depression appears to be a heritable illness. Preliminary attempts have been made to calculate heritability and environmental causation with genes contributing 80% of liability. More definitive and replicable proof is required.

There is convincing evidence that there are various biological markers which are associated with Major Depression. There are suggestions that some of these may be trait or vulnerability markers. There are suggestions about the neuroanatomy and neuro-chemistry of this disorder. However, these are all promising leads which have to be followed to their logical conclusions. Cause and effect relationships are not clear. Confounding variables like different methodologies, diagnostic practices, drug intake, etc. make comparisons of observations difficult.

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