~

MODULATION OF THE EFFECTS OF LINDANE ON COGNITIVE FUNCTION AND OXIDATIVE STRESS BY NEUROSTEROIDS IN RATS

THESIS

SUBMITTED TO THE FACULTY OF MEDICAL SCIENCES

UNIVERSITY OF DELHI

TOWARDS THE PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF MEDICINE

(PHARMACOLOGY)

Dr Kinshuk Sahaya

Department of Pharmacology

University College of Medical Sciences & G.T.B. Hospital

Delhi- 110095

April 2007

UNIVERSITY COLLEGE OF MEDICAL SCIENCES
& G.T.B. HOSPITAL
(University of Delhi)
Delhi -110095

CERTIFICATE

This is to certify that **Dr. Kinshuk Sahaya** carried out the work of this thesis entitled **"Modulation of the effects of lindane on cognitive function and oxidative stress by neurosteroids in rats"** for the partial fulfillment of the degree of doctor of medicine (Pharmacology) of the University of Delhi for the requisite period under the regulations in force. This thesis is a bona fide record of the work done by him under our direct supervision and guidance. The work was carried out in the departments of Pharmacology and Biochemistry, University College of Medical Sciences and GTB Hospital, Delhi-110095.

SUPERVISOR

P Mahajan

Dr. P Mahajan
Professor
Department of Pharmacology
University College of Medical Sciences & GTB Hospital
Delhi-110095

CO-SUPERVISORS

Pramod Kumar

Dr. P. K. Mediratta
Professor
Department of Pharmacology
University College of Medical Sciences &
GTB Hospital
Delhi-110095

Rafat S.

Dr. R. S. Ahmed
Reader
Department of Biochemistry
University College of Medical Sciences &
GTB Hospital
Delhi-110095

To my parents, for everything I am and their amaranthine optimism.
To Riya, the large bundle of joy in a small wrapping.

Contents

Acknowledgement

It is a pleasure to thank all the people who have made this thesis possible.

First and foremost, my words of thanks to Dr Prabha Mahajan, Professor, Department of Pharmacology, and my supervisor of the thesis. Without her insightful advice, calm, composed and highly understanding persona, constant encouragement and continuous positive inputs this thesis could never have been conceived or completed. For, she is not only my guide for this thesis but also of my future.

I thank Dr Pramod Mediratta, Professor, and my co-supervisor, from the bottom of my heart for painstakingly working out the finer details of this research. Her expertise in the field of neuropharmacology helped me complete this thesis with ease and in time. I am obliged to her for the hours she has spent correcting the manuscript and my grammar too.

I am indebted to Dr Rafat S. Ahmed, Reader, Department of Biochemistry, and my co-supervisor. Her knowledge of the intricacies of biochemistry and her ever approachable nature ensured the smooth conduct of my research work.

My ever gratefulness to Dr K.K. Sharma, Professor and Head, Department of Pharmacology. His affectionate encouragement, perfectionism and positive outlook are constant source of inspiration.

I thank Dr Naresh Khanna, Professor, Department of Pharmacology, for her support and invaluable suggestions that helped me in this research.

My gratitude to my colleague, Dr Krishna. She provided me with support, encouragement and immense help. Her cooperation in trying times proved to be an invaluable succor.

I would of course, like to thank Dr Govind Garg, Dr Harsh Negi, Dr Tarun Arora, Dr Saurabh Arya, Dr Sparsh Gupta, Dr Kapil Mehta, Dr Neeraj Rathi, Dr Amit Kumar, Dr Achint Kumar, Dr Rajni Mathur, Dr Reeta Kh, Dr Shalini Gupta, Dr Seema, Dr Pooja Gupta, Dr Devesh Gupta, Mrs Sonam Kalra and Ms. Nidhi Bharal for their co-operation and encouragement.

Sincerest words of thanks go out to Mrs. Vijay Gupta, Mrs. Sneh Bhatia, Mrs. Manju Bindal, Mrs. Harsh Mendiratta, Mr. Sanjay, Mr. Dharmender Arora and other able technical staff of the Department of Pharmacology and Central Animal House for their never ending help.

I am overwhelmed by the tireless efforts of my sister, Mrs. Pallabi Shubhanjlee and the precious hours she spent away from her daughter Riya, formatting my thesis, making sure it is “The Best”.

In the end I hope that probably this research, by contributing something more to the vast world of neuropharmacology can honor the lives of the rats sacrificed for it.

April 2007
New Delhi

Dr Kinshuk Sahaya
Department of Pharmacology, UCMS

Abbreviations

µl	Microlitre
3β HSD	3β-hydroxy steroid dehydrogenase
4CD	4'-chlorodiazepam
5α-R	5α-reductase
A	Absorbance
AD	Alzheimer's Disease
AEDs	Antiepileptic drugs
ALPX	Alphaxalone
ANOVA	Analysis of variance
AP	Allopregnanolone
BDNF	Brain derived neurotrophic factor
BZD	Benzodiazepines
CAT	Catalase
CBR	Central BZD receptors
CBZ	Carbamazepine
CNS	Central nervous system
Co	Concentration
CPCSEA	Committee for the purpose and control of
CT	Computed tomography
D	Dilution factor
DALYs	Disability adjusted life years
DBI	Diazepam binding inhibitor
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
DNA	Deoxyribose nucleic acid
DOC	Deoxycorticosterone
DTNB	5,5'-dithiobis-2-nitro benzoic acid
ECT	Electroconvulsive therapy
EDTA	Ethylene diamine tetra acetic acid
EEG	Electroencephalography
GABA	Gamma-amino-butyric acid
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GTCS	Generalised tonic-clonic seizures
H_2O_2	Hydrogen peroxide
HSOR	3α- hydroxysteroid oxidoreductase
i.p.	Intraperitoneally

IVIG	Intravenous immune globulin
JME	Juvenile myoclonic epilepsy
KA	Kainic acid
KCl	Potassium chloride
Kg	Kilograms
LTP	Long Term Potentiation
LTP	Long term potentiation
M	Molar
MAP2	Microtubule associated protein 2
MDA	Malondialdehyde
MDRC	Mitochondrial Diazepam Binding Inhibitor
MES	Maximal electroshock seizure
mg	Milligram
mmol	Millimole
MRI	Magnetic resonance imaging
nAchRs	nicotinic acetylcholine receptors
nm	Nanometer
NMDA	N-methyl-D-aspartate
NS	Neurosteroids
OCP	Oral contraceptive pill
OD	Optical density
OFRs	Oxygen free radical
OS	Oxidative stress
P450scc	Cytochrome P450 side-chain-cleaveage
PBR	Peripheral BZD receptors
PREG	Pregnenolone
PREG-S	Pregnenolone sulfate
PROG	Progesterone
PTZ	Pentylenetetrazole
ROS	Reactive oxygen species
rpm	Revolutions per minute
RR	Retention rate
s	Seconds
s.c.	Subcutaneous
SDL	Step-down latency
SE	Standard error
SFZ	Shock free zone
SOD	Superoxide dismutase

StAR protein	steroidogenic acute regulatory protein
SWC	Spike wave complex
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloro acetic acid
THDOC	Tetrahydrodeoxycorticosterone
TIA	Transient ischaemic attack
TL	Transfer latency
TRIS	2-Amino-2-(hydroxymethyl) propane-1,3-diol
TTN	Trikontatetraneuropeptide
U	Units
WHO	World health organisation

Introduction

Neurosteroids (NS) are steroids that are newly synthesized from cholesterol and are present in the nervous system even after removal of peripheral steroidogenic gland. Neuroactive steroids are active on neural tissue while neuroinactive steroids are synthesized in brain but are inactive on neural tissue [1-3]. Occurring as unconjugated steroids, sulfated esters and fatty acid esters of steroids, they are involved in the control of metabolic, behavioral, and psychical processes including cognition, stress, anxiety, sleep etc.[4,5,1]. Examples of known neurosteroids include progesterone (PROG), pregnenolone (PREG), pregnenolone sulfate (PREG-S), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), allopregnanolone (AP), tetrahydrodeoxycorticosterone (THDOC) etc [6,7]. 4' chlorodiazepam (4CD) not a NS in its own right, increases the production of NS by acting through mitochondrial diazepam binding inhibitor receptor complex (MDRC)[8].

In the last few decades a lot of emphasis has been laid on the role of NS in the functioning of the nervous system. They have been implicated in various functions in the brain; notable among which are their role in neuroprotection, reinforcement of long term memory, in active avoidance behavior and learning. Previous workers have noticed a general trend toward decreased levels of PREG-S, DHEA-S, PROG, and AP in Alzheimer's disease patients' brain[3,9]. DHEA, DHEA-S, androstenedione, testosterone, dihydrotestosterone and aldosterone have been correlated with improvement of memory retention in foot-shock active avoidance training. PREG-S and DHEA-S were reported to facilitate memory and AP was shown to possess rewarding properties. As little as 150 molecules of PREG-S significantly enhance post-training memory processes when injected into the amygdale of rats[1,10–14]. The effects of NS though, are not explained solely on the basis of intracellular steroid receptors, it is now well recognized that NS can mediate their effects through genomic mechanisms, through the agency of steroid receptors and through nongenomic mechanisms through $GABA_A$ and several other receptors. NS bind to a specific site on $GABA_A$ receptor complex-distinct from benzodiazepine, barbiturate or convulsant recognition site.

Besides $GABA_A$ the target receptors of NS include nicotinic and muscarinic acetylcholine, σ, NMDA, serotonergic, kainate, glycine, neuropeptide receptors, voltage gated Ca^{2+} channel, Microtubule-associated protein 2 [3,6,15-25]

NS are now being proven to have a definite role in the antioxidant defenses of the brain. PROG has been indicated to have an inhibitory action on lipid peroxidation.

DHEA and β estradiol pretreatment reduce DNA damage induced by oxidative insult. Neuroactive steroids protect retinal cells from oxidative stress, and this effect is mediated by σ_1 receptors. Probably an in-brain oxidative stress-related pathway exists, which is closely related to NS biosynthesis [26,27].

Lindane, a gamma isomer of 1,2,3,4,5,6-hexchlorocyclohexane (γ-HCH) is an organochlorine pesticide used extensively in agriculture. In acute poisoning neurobehavioural effects like memory impairment, irritability, and aggression have already been noted for lindane. Lindane has effects on long-term potentiation (LTP) in the hippocampus. It also interferes with the ability of avoidance response with a single dose in rats [28-30].

Lindane also reportedly influences the metabolism of pregnenolone (PREG) and progesterone (PROG) in mice ovaries. It inhibits the conversion of cholesterol to PREG by inhibiting the enzyme cytochrome P450 side chain cleavage (P450scc), the rate limiting step in NS biosynthesis. It has also been claimed that lindane inhibits the activity of steroidogenic acute regulatory (StAR) protein, which mediates an important step in hormone-regulated steroidogenesis, the intramitochondrial transfer of cholesterol to the P450scc enzyme[31,32]. Lindane has also been shown to be a strong oxidant causing free radical generation in tissues including brain through lipid peroxidation [33,34].

Thus, the anti-amnestic, neuroprotective and antioxidant properties of the several NS make them a strong contender to reverse the neurobehavioural effects of lindane. It is also a possibility that oxidative stress may have a significant role to play in the same. With these lacunae in the literature covering this aspect of effect of NS the present research was designed to estimate the effect of PROG, PREG-S and 4CD on lindane induced changes in cognition.

Review of Literature

Neurosteroids

Neurosteroids (NS) are defined as steroids that are newly synthesized from cholesterol or another early precursor in the nervous system. They are present in nervous system even after removal of peripheral steroidogenic glands[1,2]. NS can be active or inactive depending upon their action on the nervous system[6]. Neuroactive steroids are active on neural tissue and may be synthesized in brain or in endocrine glands. Neuroinactive steroids are synthesized in brain and are inactive on neural tissue[3]. The term NS should not include all the steroid metabolites formed from circulating hormones within the nervous system. Androgens, estrogens and glucocorticoids are classically excluded from the definition as they disappear from the brain after gonadectomy and adrenalectomy[35]. NS occur in the nervous system as unconjugated steroids, sulfated esters of steroids or fatty acid esters of steroids[4]. These various forms of steroids are involved in the control of metabolic, behavioral and psychical processes including cognition, stress, anxiety, sleep etc[5,1]. Various examples of known NS include progesterone (PROG), pregnenolone (PREG), pregnenolone sulfate (PREG-S), dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEA-S), allopregnanolone (AP), tetrahydrodeoxycorticosterone (THDOC) etc. These NS have been described to have varying effects on the nervous system and these are mediated through several mechanisms. Besides their effect at transcriptional level, NS may act through membrane receptors like gamma-aminobutyric acid ($GABA_A$), nicotininc, muscarinic, N-methyl-D-aspartate (NMDA), sigma (σ), kainate, glycine, serotonergic and neuropeptide receptors. They also afford neuroprotection and induce neurite outgrowth, dendritic spines and synaptogenesis[36,3].

Biosynthesis of neurosteroids

The first step in the biosynthesis of NS involves the conversion of cholesterol to PREG. This step is the rate limiting step and is catalysed by the enzyme cytochrome P450 side-chain-cleavage (P450scc). The enzyme P450scc is found in steroidogenic organs, placenta, primitive gut and the brain. In the brain it is involved in the regulation of NS biosynthesis. P450scc has been identified throughout the brain and especially in the white matter[37]. More specifically the enzyme has been located to the mitochondria of oligodendrocytes and the myelinating glial cells of the central nervous system[38]. It has also been located in type I astrocytes, Purkinje cells[39,40]. Besides the cells of central nervous system, P450scc has also been isolated in rat retina[41] and sensory neurons of mouse embryo[42]. Similar expression of the P450scc enzyme has been reported for the human nervous system as well[3]. NS biosynthesis has also been identified in human sciatic nerves, possibly in Schwann cells[43]. Steps involved in the biosynthesis of steroid hormones are summarized in Fig. 1

The conversion of Δ^5-3β-hydroxysteroids (PREG, 17OH-PREG) into Δ^4-3-ketosteroids (PROG, 17OH-PROG) is catalysed by the enzyme 3β-hydroxy steroid dehydrogenase (3β HSD). The enzyme has been isolated from rat amygdale and septum. At cellular level 3β HSD has been identified in Schwann cells, astrocytes and oligodendrocytes. Endozepine trikontatetraneuropeptide (TTN) stimulates the 3β HSD activity and this effect is mimicked by 4'-chlorodiazepam[36].

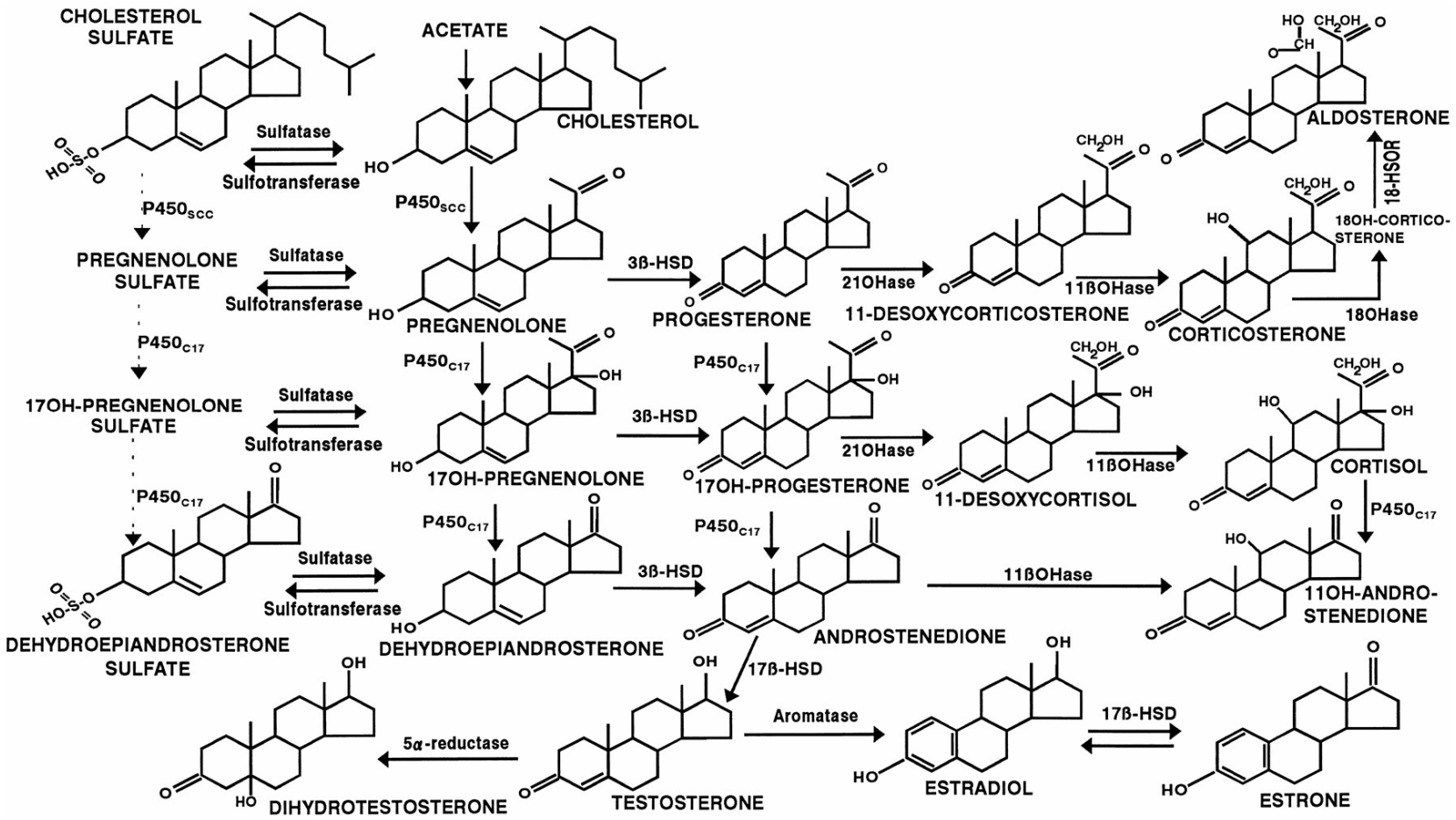

Fig. 1 Biosynthesis of steroid hormones in endocrine glands. P-450scc, P450c17, 3β-HSD, 17β-HSD, 11βOHase, 11β-hydroxylase; 18bOHase, 18-hydroxylase; 18-HSOR, 18-hydroxysteroid oxidoreductase; 21OHase, 21-hydroxylase.

The conversion of C_{21} steroids (PREG, PROG) into C_{19} steroids (DHEA, androstenedione respectively) is mediated by the enzyme system 17α-hydroxylase/17, 20 lyase (cytochrome P-450c_{17}, P-450c_{17}). Although this enzyme is well identified in the peripheral steroidogenic tissues but its presence in adult human or rat brain is still uncertain. The enzyme has been identified in embryonic rat brain, and the high concentration of DHEA suggests its presence in adult brain, but conclusive evidence is still lacking[3,36]. TTN has been shown to stimulate the conversion of PREG into 17α-hydroxy-PROG in frog brain, hinting that endozepines can increase P-450c_{17} activity in brain[44].

The enzyme 5α-reductase (5α-R) is a microsomal NADPH-dependent protein which acts specifically on steroids possessing a C_4-C_5 double bond and a ketone group at the C_3 position. This enzyme catalyzes the transfer of two hydrogens from NADPH causing the reduction of the C_4-C_5 double bond and the formation of 5α-reduced metabolites. In particular, 5α-R catalyzes the conversion of testosterone, the main circulating androgen, into dihydrotestosterone (5α-DHT) and the transformation of progesterone into dihydroprogesterone (5α-DHP). Evidence for the conversion of PROG to deoxycorticosterone (DOC) is lacking in brain, but the DOC formed in zona fasiculata of the adrenal cortex is reported to be reduced to 21-hydroxy-5α-pregnan-3, 20-dione by 5α-R in brain and then converted to allotetrahydro-DOC by 3α- hydroxysteroid oxidoreductase (HSOR) enzyme[6]. In vitro studies have shown the existence of 5α-R bioactivity in brain tissue and especially in primary cultures of nerve cells[45-49]. In the CNS of mammals, the 5α-R gene is primarily expressed in glial cells. The presence of 5α-R-like immunoreactivity has been found in astrocytes, ependymocytes and tanycytes within various brain regions including the hypothalamus, thalamus, hippocampus, cerebral cortex, and circumventricular organs[50]. In humans the enzyme activity has been localized to the frontal lobe, temporal neo-cortex, hippocampus and subcortical white matter[51-54].

Aromatase is responsible for the conversion of androgens into estrogens. The enzyme has been located in the neurons and not glial cells. In humans, the enzyme is distributed to the frontal and temporal neocortex[36,3].

Sulfotransferases mediate the sulfate conjugation of the NS. Together with sulfatases responsible for removal of the sulfate moiety, these enzymes are important regulator of NS functions. Addition/removal of the sulfate moiety can have significant changes in the effect of NS on several receptors[36].

Being lipid soluble, systemically administered NS can enter the CNS. Chronic PROG treatment has been shown to cause elevated levels of AP, and chronic AP administration mimics the effects of chronic PROG administration. Inhibition of the enzyme 3α-hydroxy steroid dehydrogenase or 5α-R, responsible for the conversion of PROG to AP, inhibits the effects of chronic PROG administration[55].

Neurosteroid metabolism and mitochondrial diazepam binding inhibitor receptor complex

Benzodiazepines (BZD) bind to two kinds of receptors, the central BZD receptors (CBR), that are a macromolecular complex contain a GABA receptor site, and a chloride ion channel and peripheral BZD receptors (PBR). The PBRs differ from the CBRs by their lack of coupling to $GABA_A$ receptors and their ligand specificity[35,56]. Although named PBRs they bind not only to non-BZD moiety but they are also located in the central nervous system, especially in the glial cells. As a result of these observations the terminology of the receptors was changed to mitochondrial diazepam binding inhibitor receptor complex (MDRC). Diazepam binding inhibitor (DBI) is an endogenous 9kDa polypeptide which has the ability to displace diazepam from the BZD recognition site of $GABA_A$ and mitochondrial BZD receptors[6]. The MDRCs are important regulator of steroidogenesis. Ro-5-4864 (4'- chlorodiazepam, 4CD) and alpidem are agonists for these receptors and PK 11195 is a partial agonist. The agonists have been shown to increase the brain PREG synthesis without any effect on the blood PREG

concentration. The ligands of these receptors facilitate the intramitochondrial flux of cholesterol thereby increasing the availability of cytochrome P450scc[8]. They may form a pore through which cholesterol molecules could be transported into the mitochondria[57]. 2-Arylindone-3-acetamides (FGIN-1), another class of ligands, bind with high affinity and specificity to MDRC and demonstrate stimulation of pregnenolone synthesis co-relating with their binding affinities. PK 11195 binds with a still higher affinity but fails to inhibit any rise in pregnenolone biosynthesis[6].

Neurosteroid effector mechanisms

The sedative-anesthetic effect of PROG over CNS was first described by Selye[58]. Over the last six decades understanding of effects of NS has increased tremendously. The endocrine effects of steroids like PROG have been known to be mediated through intracellular steroid receptors. These receptors exist predominantly in the cytoplasm and are classified as Type 1 receptors. They are bound to heat shock proteins (hsp) and on binding to PROG the hsp complex is released and the receptor forms a dimer with another identical receptor. The homodimer translocates to the nucleus where it binds to a specific base sequence (AGAACA) on the DNA[59]. Many effects of NS are exerted rapidly, and thus some of their effects could not be explained solely on the basis of such intracellular steroid receptors[6]. Subsequently, the influence of NS over the $GABA_A$ receptors was demonstrated[15]. It is now well recognized that NS can mediate their effects through genomic mechanisms through the agency of steroid receptors and through nongenomic mechanisms through $GABA_A$ and several other receptors. NS bind to a specific site on $GABA_A$ receptor complex, distinct from BZD, barbiturate or convulsant recognition site. 5α-3α-pregnenolone can enhance $GABA_{A,}$ receptor mediated chloride currents even in the presence of maximal effective concentrations of pentobarbital[6]. Besides $GABA_A$ the target receptors of NS include nicotinic and muscarinic acetylcholine receptor, σ receptors, NMDA receptors, , serotonergic, kainate, glycine, neuropeptide receptors, voltage gated Ca^{2+} channel and Microtubule-associated protein 2 (MAP2)[3,16-25].

Neurosteroid and $GABA_A$ receptors

The GABA system is the major inhibitory system in the mammalian CNS. There are two distinct GABA receptors, $GABA_A$ and $GABA_B$. The inhibitory action of GABA is mediated via the activation of specific receptors the $GABA_A$ receptor which belongs to the ligand gated ion channel family, together with nicotinic acetylcholine, glycine and $5HT_3$ receptors. Each $GABA_A$ receptor

consists of five subunits forming a chloride channel (Fig 2a, 2b) and at least 18 different subunits have been described (6 α, 3 β, 3 γ, δ, ε, π, and 3 ρ)[60]. An α, β, γ subunit each is necessary to form receptor molecules that exhibit properties of native $GABA_A$ receptor[6]. Different combinations of subunits contribute to distinct pharmacological properties of $GABA_A$ receptor and the expression of subunits is heterogeneous in the brain. The function of each subunit is not perfectly clear, but several studies point to special importance of some subunits[18].

The sedative effect of benzodiazepines is mediated via α1 subunit containing $GABA_A$ receptors[61]. The α2 subunit is considered to mediate benzodiazepine induced anxiolytic effects[62]. The $GABA_A$ receptor α4 subunit is also implicated in the regulation of anxiety[63]. A concentration dependent decrease of the α4 subunit is seen after 4 days application of AP to developing neuronal cells[64], whereas in the hippocampus and cerebellum an increase in this subunit can be detected after withdrawal from chronic PROG (or AP) exposure and after short term treatment[63,65,66]. The $GABA_A$ receptor β2 subunit has been shown to be involved in mediating the effect of anesthetic drugs like etomidate, alphaxalone, pentobarbital and propofol[67,68].

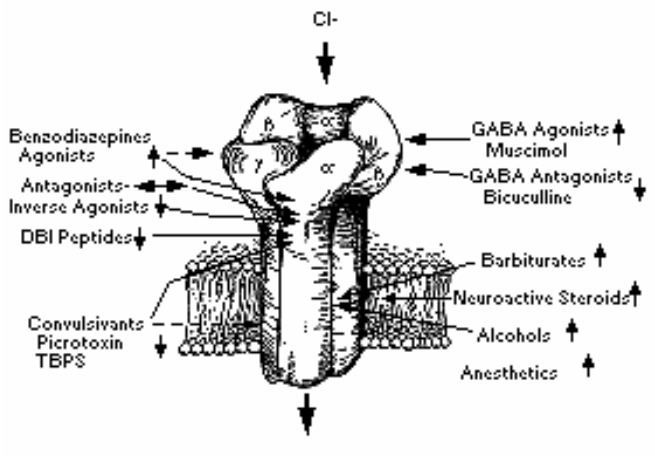

Fig 2a Structure of a $GABA_A$ receptor

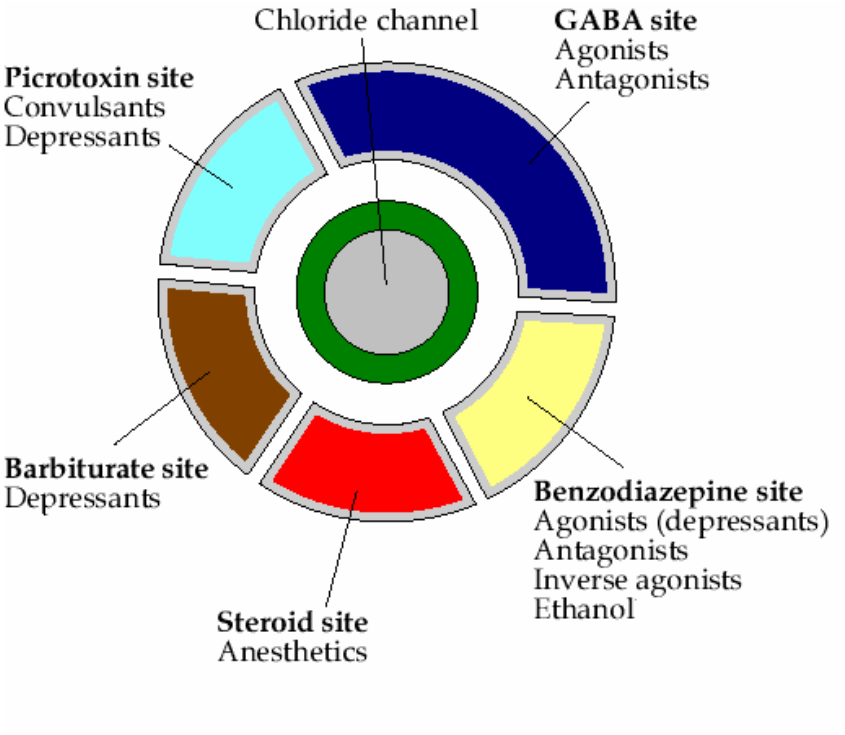

Fig 2b $GABA_A$ receptor and sites for binding of different agents
(The fig does not reflect the subunit structure of the receptor.)

The γ subunit unlike for BZD is not essential for the modulation of response to NS, nonetheless they do exert some influence. The γ1 units are highly expressed in the glial cells, which as mentioned above are the chief site of NS biosynthesis. It is a possibility that the locally produced steroids may act as a modulator of the $GABA_A$ receptors present on these cells[69]. The γ2 subunit is involved in synaptic targeting and clustering, in anxiety regulation, and is changed during hormone manipulation and pregnancy[65,70,71]. The δ subunit is important for NS effects on the $GABA_A$ receptor[72] and receptor knockout studies revealed that absence of the δ subunit decreases the sensitivity to neuroactive steroids like pregnanolone and alphaxalone, influencing the duration of anesthesia and anxiolytic effect of those steroids[73]. Some $GABA_A$ receptors identified in arthropods, are insensitive to the action of steroids. This insensitivity could be due to the presence of ρ subunit. An interesting advantage is seen in some species of water beetle (*Coleoptera*). On being attacked the beetle releases secretions rich in steroids. It is a possibility that these steroids can cause sedation in the vertebrate predator helping in the escape of beetle. Linking the final piece of the puzzle is the hypothesis that probably steroid binding sites in $GABA_A$ receptors appeared after the evolution of chordates[69].

Initial studies hinted at the action of NS at the level of GABA receptors. The evidence that intracellular steroid application failed to activate GABA receptor mediated current, as against extracellular application proved that NS act at the level of receptors and not the membrane directly[17]. Interaction of steroids with $GABA_A$ receptor is stereoselective and can be agonistic (AP, PROG) (positive modulators), or antagonistic (PREG-S, DHEAS) (negative modulators). A third type of interaction is seen with 3β-hydroxypregnane steroids. They do not have an antagonistic action with GABA but in rat cortical vesicles they act as antagonists against potentiation of $GABA_A$ receptor function by 3α- hydroxypregnane steroids[74].

The influence of steroids on channel is both in terms of opening time and frequency. 5β-pregnan-3α-ol-20-one has been shown to increase the average

channel open duration. This effect is mediated by increasing the probability that the channel will enter naturally occurring open states of relatively longer duration. The steroid also increases the frequency of single-channel opening[69]. PROG needs relatively high concentration to produce a modest enhancement of GABA evoked current, but inhibits glycine-evoked responses at site distinct from strychnine. DOC also potentiates the GABA response and inhibits the glycine responses. High concentration of PREG-S inhibits the responses of both receptors[16],[69]. PREG-S was proposed as an endogenous antagonist of the $GABA_A$ receptor after it was demonstrated that it competitively inhibited the binding of the convulsant [^{35}S]t-butyl-bicyclophosphorothionate (TBPS), antagonized pentobarbital-stimulated [^{3}H]flunitrazepam binding to synaptosomes and inhibited muscimol-stimulated $^{36}Cl^-$ uptake in brain synaptosomes[75].

AP and Allo-THDOC have also been shown to have GABA enhancing activity. Submicromolar concentration of these steroids was shown to facilitate GABA-activated Cl- conductance whereas micromolar concentrations directly stimulated Cl- conductance. These NS unlike BZD do not antagonize glutamate receptor-mediated inward currents[17]. Recent evidence has now demonstrated that AP-stimulated GABA-mediated chloride ion flux is inhibited by 3β-hydroxy-5α-pregnan-20-one (iso-AP)[74].

NS also seem to be involved in the regulation of GABA receptors. A single administration of progesterone (150 mg/kg, i.p.) significantly decreased the specific binding of [^{3}H] muscimol to the nucleus caudatus and nucleus accumbens as early as 1h after injection. A similar tendency was also present in the dentate gyrus of the hippocampus. These changes in GABA receptors in basal ganglia and hippocampus may be important for the behavioral manifestation of an interaction between the GABA receptor complex and NS. AP was hypothesized to be the most probable regulator produced by metabolism of PROG. The authors hypothesized that the higher dose of progesterone used caused excessive stimulation of GABA receptors by its metabolites and brought about rapid changes in the GABA receptor

number and/or affinity[23]. Although previous workers have reported an increase in GABA receptor binding after administration of PROG, the dose used was comparatively very low (10 mg/kg)[76]. Another regulatory factor is 3α,5α THP, local production of this NS can regulate $GABA_A$ receptor functioning. Withdrawal of 3α,5α THP causes relative insensitivity of $GABA_A$ neurosteroid neuromodulation[3].

Stell et al demonstrated that δ subunit-containing $GABA_A$ receptors are activated by ambient GABA, giving rise to a tonic conductance in cerebellar neurons. Physiological concentrations of NS selectively enhance this conductance and thereby modulate the excitability of specific neuronal populations[72].

Several effects of NS are maybe due to actions on tonic inhibition generated by δ subunit-containing $GABA_A$ receptors of cerebellum, hippocampus, thalamus, striatum, and all layers of the cortex.

Prolonged neurosteroid administration has been shown to alter the expression of $GABA_A$ receptors[77]. On subacute exposure (2 days), PROG caused increased levels of α4 and δ proteins and modest reduction in expression of a1 and g2 proteins. On exposure to high dose PROG, hippocampal neurons show increased expression of δ subunits and this has been co-related with the seizure resistance and antianxiety effects in such a scenario[55,78,79].

N-methyl-D-aspartate (NMDA) receptors

NMDA receptor is involved in long-term potentiation, memory and epilepsy. NMDA receptor requires co-assembly of the NR1 subunit with at least one NR2 subunit. Eight splice variants of the single NR1 gene have been identified, whereas four different genes encode the NR2A, NR2B, NR2C and NR2D subunits.

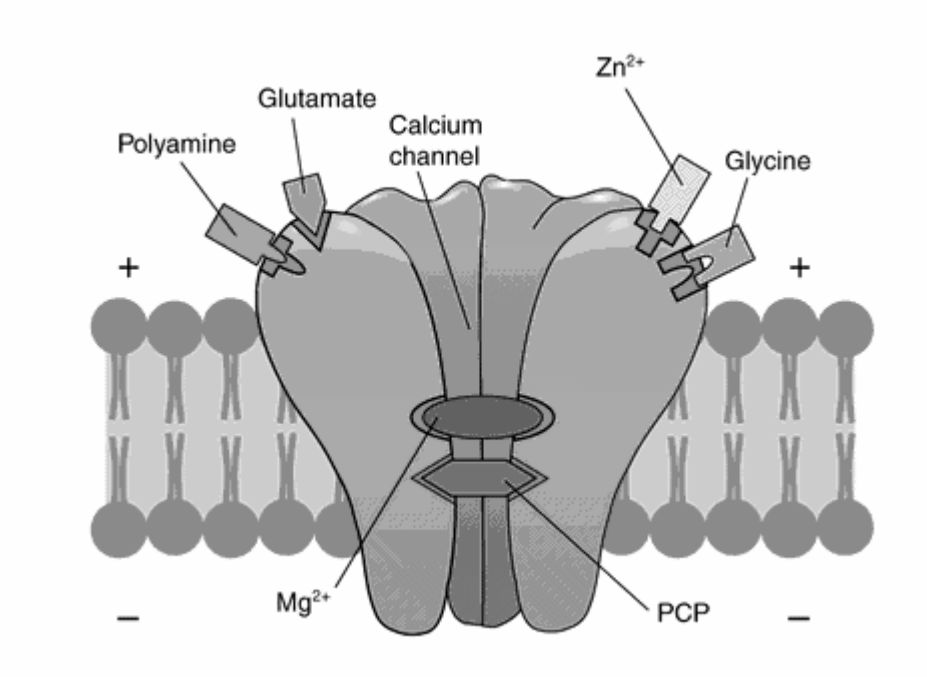

Fig 3: NMDA receptor and its binding sites. PCP: phencyclidine

The NR1 subunit is broadly expressed throughout the CNS, whereas NR2 subunits display distinct, although overlapping, expression patterns[80]. PREG-S is a positive allosteric modulator at NMDA receptor and selectively augments glutamate-induced depolarisations in spinal cord neurons. Responses elicited by kainate or AMPA receptors are inhibited by PREG-S. PREG-S augments NMDA receptor-mediated elevations in the intracellular Ca^{2+} concentration. The positive allosteric effect is achieved by increasing the open time of NMDA activated channels, by increasing the frequency of channel opening. The site of action could be a unique steroid recognition site distinct from glycine co-agonist and polyamine site[6,20]. Another recent research has indicated that the molecular mechanism of PREG-S

potentiation of NMDA receptor responses is attributable to an increase in peak channel open probability (P_o), acting through the NR1B/NR2B subunit of NMDA receptors. Responses to glutamate recorded in the continuous presence of PREG-S exhibit marked time-dependent decline and the decline is induced by a change of the NMDA receptor affinity for PS after receptor activation[81].

3α-ol-5β-pregnan-20-one sulfate (3α5βS) is a negative modulator of NMDA-induced currents and inhibits NMDA-stimulated increases in intracellular calcium. 3α-ol-5β-pregnan-20-one hemisuccinate (3α5βHS) is a synthetic analog of the 3α5βS and an inhibitor of NMDA-induced currents. 3α5βHS inhibits NMDA induced currents, protects cultured neurons against exposure to NMDA, inhibits NMDA-induced seizures, and at a nonsedating dose reduces cortical and subcortical infarct size in the middle cerebral artery occlusion model for stroke. 3α5βHS is still neuroprotective when administered 30 min after the onset of focal cerebral ischemia in rodents and represents a potentially useful compound for the treatment of stroke[82].

NR2 subunits are key determinants of modulation by PREG-S and 3α5βS. The modulatory effects of PREG-S, but not 3α5βS, on dose-response curves for NMDA, glutamate and glycine are consistent with a two-state model in which PREG-S either stabilizes or destabilizes the active state of the receptor depending upon which NR2 subunit is present. The modulatory effect of PREG-S is contingent upon the NR2 subunit composition of the NMDA receptor, and that PREG-S inhibits, rather than enhances, the function of NR1/NR2C and NR1/NR2D receptors.

PREG-S increases the efficacy of NMDA at NR1/NR2B receptors. It enhances only the potency of glutamate with no effect on the maximum glutamate response in much the same way as benzodiazepines enhance the potency of GABA at the $GABA_A$ receptor without affecting the maximum GABA response. Thus, the effect of PREG-S on the agonist concentration response curve depends upon both the subunit combination and the particular

agonist used. The PREG-S binding site responsible for potentiation may be partially or entirely located on the NR2 subunit. Probably, PREG-S enhances NMDA receptor activation at synapses containing predominantly NR2A or NR2B subunits, and decreases NMDA receptor activation at synapses containing predominantly NR2C or NR2D subunits. The NR2A subunit appears after birth and becomes highly expressed in hippocampus and cortex, with moderate expression in other fore-, mid-, and hindbrain regions. The NR2B subunit appears during embryonic development, and is expressed at high levels in the cortex, hippocampus, striatum, thalamus and olfactory bulb, and to a lesser extent in midbrain regions. NR2B subunit plays an important role in cognition .The NR2C subunit appears after birth, and is expressed primarily in the cerebellum. The NR2D subunit is strongly expressed in embryonic and neonatal stages but is present at lower levels in the adult. Thus, the inhibitory effects of PREG-S are likely to be particularly prominent in cerebellum and in the developing nervous system[80]. NMDA receptors also regulate neurosteroidogenesis through a transneuronal mechanism, which implies $GABA_A$ receptor activation. The early NMDA-mediated stimulation of neurosteroid synthesis seems to play a critical role in acute excitotoxicity; consequently, its inhibition is likely to delay neuronal cell death[83].

Other receptors/mechanisms

Low micromolar concentrations of Allo-THDOC, DHEA, and PREG block the voltage gated calcium channels in hippocampal neurons. The blockade is reversible and rapid. Similar property has been show for PREG-S as well, but lacking in PROG[6]. The peripheral analgesic activity of THDOC has also been attributed to the influence of NS over voltage-gated calcium channels[84]. The potent peripheral analgesia induced by 5α-reduced neurosteroid Alphaxalone (5α-pregnan-3α-ol-11, 20-dion, ALPX), AP, (3α, 5α, 17β)-3-hydroxyandrostane-17-carbonitrile (ACN) is mediated in part by effects on T-type Ca^{2+} channels. Also, $GABA_A$ channels do not contribute to baseline

pain transmission, but they can enhance anti-nociception mediated by blockade of T-type Ca^{2+}channels[85].

Selective serotonin reuptake inhibitors through poorly understood mechanisms are shown to increase brain NS concentration, particularly AP. Besides their antianxiety and antidepressant effects, this could be responsible for their anticonvulsant activity[24].

Shiraishi et al demonstrated that ALPX (0.1-100 micro M) inhibited nicotine-induced Ca^{2+} increases in a concentration-dependent manner. ALPX inhibited high K^+-induced Ca^{2+} increases, but the inhibition was observed only at 100 μM concentration. ALPX also inhibited nicotine-induced inward currents, and the inhibition was unaffected by picrotoxin. At anesthetic concentrations, ALPX inhibits nicotinic acetylcholine receptors (nAchRs) in adrenal chromaffin cells. The authors proposed that ALPX may affect the sympathetic and other nervous systems via inhibition of nAChRs[86].

Studies demonstrate that in concentrations similar to those endogenous in the hippocampus, PREG-S inhibit GABAergic synaptic transmission by a presynaptic effect. PREG-S causes specific activation of G protein-coupled σ_1 receptors, resulting in modulation of both action potential-dependent and independent IPSCs, and enhancement of short-term presynaptic facilitation[87,88].

PROG has already been shown to potentiate myelination of peripheral nerves. It has also been shown to stimulate neurite growth. PREG and its chemically synthesized analog 3-methoxypregnenolone (MePREG) also stimulated the polymerization of microtubules and significantly enhanced neurite outgrowth of nerve growth factor-pretreated PC12 cells. Probably, MAP2 is a specific receptor for PREG and MePREG towards this effect[25,35].

Neurosteroids- behaviour and memory

In the last few decades, a lot of emphasis has been laid on the role of NS in functioning of the nervous system. They have been implicated in various functions in the brain; notable among which are their role in neuroprotection and reinforcement of long term memory in active avoidance behavior and learning[1,13,14]. Steroids derived from PROG during menstrual cycle have been proposed to explain some differences between men and women in the incidence of anxiety and mood disorders[89]. It seems that fatigue during pregnancy may be a consequence of higher concentrations of PROG and GABA agonistic 3α-reduced neuroactive steroids like 3α,5α- TH PROG, whereas a rapid decline in these substances may lead to post partum depression[90].

A general trend toward decreased levels of PREG-S, DHEA-S, PROG and AP in Alzheimer's disease patients' brain is seen, suggesting a possible neuroprotective role of these NS in Alzheimer's disease[9]. In mice, immediate post-training intracerebroventricular administration of PREG, PREG-S, DHEA, DHEA-S, androstenedione, testosterone, dihydrotestosterone, or aldosterone has been correlated with improvement of memory retention in foot-shock active avoidance training. PREG-S and DHEA-S were reported to facilitate memory and AP was shown to possess rewarding properties[10-12]. Influence on memory by NS probably occurs by modulation of $GABA_A$ transmission. NS have a role in the development of embryonic nervous system and a decrease in their levels has been suggested to contribute to the process of aging[35].

The role of NS in anxiety has also been shown by different workers demonstrating anxiolytic and anxiogenic activity of different or even same NS in different studies[22,91,92]. Anticonvulsive, anesthetic and anxiolytic effects of neuroactive steroids are mediated by their capacity to positively modulate $GABA_A$ receptor function, i.e. these substances act to increase GABA-ergic effects by increasing the frequency and duration of chloride

channel openings. On the other hand, inhibition of $GABA_A$ receptor function which is mostly documented for the NS PREG-S and DHEA-S produces effects ranging from anxiety and excitability to seizure susceptibility[3].

PROG has been shown to have a neuroprotective role on learning and memory impairment and hippocampus damage in rats[13]. AP, PREG-S, PROG, and MDR ligand 4'-CD possess neuroprotective activity in hypoxic stress models in rats. Further, neuroprotection after injury has been demonstrated in contusion of prefrontal cortex and spinal trauma with PROG, AP and PROG respectively NS also play a role in neuroregeneration. PROG inhibits reactive astrocyte proliferation after brain injury thus facilitating axonal growth and in high doses, promotes re-myelination in mouse sciatic nerve[7,93, 94].

Cognition and neuroprotection

NS have a complex effect on memory and cognitive processes, while some NS facilitate it, others inhibit it. PREG-S, PREG, DHEA, DHEA-S have been shown to facilitate memory on intracerebroventricular injection, whereas AP disrupts memory on injection into magnocellular nucleus[6]. Although role of GABAergic transmission has been implicated in the influence over memory, but it definitely is not the sole factor as both positive and negative modulators can have stimulatory influence over memory[35].

An age-related decrease in circulating concentrations of estradiol (in menopause) and testosterone (in andropause) and a significant fall in plasma concentration in both women and men of PREG, PREG-S, DHEA, DHEA-S and 3α, 5α-THP has been observed[95]. Weil-Engerer et al demonstrated a general trend toward lower levels of NS (PREG, DHEA, PROG, PREG-S, DHEAS, 3α,5α-THP) in six brain regions (hypothalamus, striatum, frontal cortex, cerebellum, amygdale, hippocampus) of Alzheimer Disease patients compared with controls[9]. Also β-amyloid peptides (Aβ) and pathologic tau proteins (PHF-tau), the two biochemical hallmarks of AD

were correlated with the levels of the NS PREG-S and DHEA-S in distinct brain regions. PHF-tau levels were significantly and negatively correlated with DHEA-S concentration in hypothalamus. Levels of cortical Aβ were significantly and negatively correlated with PREGS levels and to some extent with DHEAS in the striatum and cerebellum. A significant reduction has also been noticed in the levels of AP in AD patients[96].

In another recent study, Malik et al demonstrated improvement of cognitive function in post-somatosensory traumatic model in rats by Fluasterone (DHEF), a DHEA analog. Cognition was assessed on the basis of beam walk performance, Morris water maze and neurological reflexes. The authors postulated that the effect of DHEF could be related to its antioxidant, anti-inflammatory, protection against NMDA induced neurotoxicity and $GABA_A$ antagonistic activity[97].

Johansson et al demonstrated that AP (2mg/kg) after intravenous injection inhibits memory in the Morris water maze model. This inhibition was found to be short lived and correlated with a higher dose of the drug. At a lower dose (1mg/kg) AP facilitated memory. The timing of inhibition of memory was co-related to the high concentration of AP in hippocampus[14].

The influence of PROG over memory and cognition is complex. At one end, authors have argued that all the effects of PROG are due to their conversion to AP in the brain[55]. AP has been proven to disrupt memory, albeit in a concentration dependent manner[14] hence PROG should also disrupt memory. The literature however suggests a confusing picture. Djebaili et al have demonstrated a decrease in cell death and cognitive deficits after experimental contusion to rat pre-frontal cortex by AP and PROG when used one day after injury. Although the effects of the two drugs were similar, there were subtle differences to indicate that the effect of PROG is not merely due to conversion to AP[7].

Compounding to the complexity is the role of neuroactive steroids (e.g. estrogen) which also have strong influences over memory. Estrogens exert neurotropic, neuroprotective effects and influence long term potentiation. Estrogen treatment has proved beneficial on verbal memory tests in surgically post-menopausal women. Higher incidence of Alzheimer's disease in women is also linked with estrogen deficiency in post-menopausal state, as in men testosterone can be converted to estradiol through aromatase throughout life whereas females develop a greater deficiency in the post-menopausal state[3]. Some workers have demonstrated a beneficial effect of hormone replacement therapy over episodic memory and verbal learning in post-menopausal women[97,98]. In a recent study, PROG failed to demonstrate any effect on NMDA receptor modulation and memory enhancement in ovarectomized rats unlike estrogen, which proved to be more efficacious in lower doses[99]. Grirorova and Sherwin failed to demonstrate any improvement in working memory and executive functioning between healthy elderly post-menopausal women using or not using hormone therapy[100]. In another study, in ovarectomized rats, tonic low-dose and cyclic estradiol treatments improved spatial performance, while the addition of progesterone reversed these beneficial cognitive effects of estradiol. PROG was able to reduce the death rate in tonic low-dose estradiol group[101]. It has been hinted that adequate dietary Ca^{2+} is required for estradiol to show a memory enhancing effect. Even for a inhibitory effect of PROG, which unmasked only in concomitant estradiol administration and not alone, adequate Ca^{2+} was deemed necessary[102]. In a study by Tanabe et al, unlike estrogen alone or in combination with PROG, PROG alone failed to demonstrate the improvement in spatial memory after experimental ovariectomy, but PROG led to the improvement of scopolamine induced spatial memory defects in the rats independent of estrogenic influences[103].

PREG-S has been described as one of the most potent memory enhancers. Immediate post-training, stereotactically guided, intrahippocampal administration of PREG-S resulted in memory enhancement at a lower dose than with DHEA-S. Fewer than 150 molecules of PREG-S significantly

enhanced post-training memory processes when injected into the amygdale. Intra-amygdally administered PREG-S was approximately 104 times more potent on a molar basis in producing ME than when PREG-S was injected into the hippocampus. This memory enhancement did not occur on injection of PREG-S into caudate nucleus over the range of doses tested in other brain structures. Memory enhancing activity was also noted for PREG and testosterone. The effects of these compounds occurred at a range far wider than what has been seen earlier for other excitatory substances[10],[104]. The influence of PREG-S on memory through hippocampus, amygdala and basal nuclei of forebrain is believed to be through trophic effects on neurons and glial cells and to modulate the activity of a variety of neurotransmitter receptors and ion channels, including type A gamma-aminobutyric acid, N-methyl-D-aspartate, sigma receptors, N- and L-type Ca^{2+} channels[105].

In a study using anti-sense sigma receptor cDNA, anti-amnesic effects of PRE-084 (selective σ_1 agonist) and DHEA-S were blocked by cDNA treatment in the short- and long-term memory tests. However, anti-amnesic effects of PREG-S remained unchanged. Thus, for PREG-S the role of σ1 receptors seems to be different than other NS[106]. The septo-hippocampal pathway has also been implicated as the region of influence of PREG-S infusion of PREG-S into the medial septum enhanced acetylcholine release by more than 50% of baseline and improved recognition memory of a familiar environment[107]. PREG-S enhances LTP in CA1 pyramidal neurons at nanomolar concentrations. The maximal effect of PREG-S on both induction and maintenance phases of LTP is observed at 300 nM and requires 10 min of superfusion. PREG-S enhances the response induced by NMDA application[108]. Also, PREG-S, DHEA-S steroids can regulate gene expression via the PROG receptor after intracellular oxidation. This too is postulated to be one mechanism of their control over memory[109].

4'-chlorodiazepam as mentioned above increases the synthesis of NS by increasing the delivery of cholesterol. The role of BZD ligand in the memory has also been explored. Endogenous benzodiazepine/GABA-A mechanisms

that down-regulate memory in amygdala, septum and hippocampus are activated in response to the anxiety and/or stress associated with each task. Immediate post-training microinjection of the benzodiazepine antagonist, flumazenil into the hippocampus enhances retention of habituation. The post-training administration of flumazenil into amygdala, septum and hippocampus enhances retention of avoidance learning. Post-training intra-amygdala administration of picrotoxin or Ro5-4864 (4CD) enhances retention[110]. Post-training i.p. (2.0 or 5.0 mg/kg), i.c.v. (2.5 micrograms/rat), or intra-amygdala (1.6-40 ng/amygdala) administration of Ro 5-4864 causes memory facilitation of step-down inhibitory avoidance in rats[111].

Another major mechanism of NS influence over memory is their neuroprotective property. They are considered important for growth and survival of neurons and provide a neurotrophic support. PROG increases the survival of motor neurons after axonotomy[112]. PROG inhibits the proliferation of astrocytes *in vitro* and this could have physiological implications in limiting the post traumatic gliosis in brain[113]. PROG has been proven to facilitate sciatic nerve regeneration, an effect blocked by PROG antagonist as well as inhibitor of the conversion of PROG to PREG[114,115]. Through its genomic action, PROG is purported to promote myelination by stimulation of Schwann cells[116]. PROG also improved the histological and clinical recovery after experimental spinal cord injury in rats. The authors hypothesized that several properties of PROG could be responsible for the effect. These included prevention of excitotoxic cell death by inhibiting NMDA receptors, potentiation of $GABA_A$ receptors, reduction of permeability of blood-brain-barrier, limiting lipid peroxidation, formation of new myelin sheaths and limiting gliosis[94]. Djebaili et al have recently demonstrated the reduction in apoptosis, cell death, and cognitive dysfunctions in rats with experimental contusion of pre-frontal cortex, following treatment with AP and PROG[7].

β-Estradiol, DHEA, DHEA-S have also been proven to protect neurons against NMDA induced neurotoxicity. This protection was only partially

reversed by rimcazole, σ1 receptor antagonist. This hints at the involvement of non σ mechanisms in protection of neurons by NS[117]. DHEA and DHEA-S have also demonstrated neuroprotection for hippocampal structures *in vivo*[118]. AP, PREG-S , PROG and MDR ligand 4CD possess neuroprotective activity in hypoxic stress models in rats[93].

Many reports have suggested that a decrease in NS levels may contribute to the process of aging and decrease in NS levels with age have now been well documented[35].

Other neurobehavioural effects of neurosteroids

Extensive research has been carried out on the role of NS in epileptogenesis. In humans, the most striking example is that of increased seizure susceptibility and menstruation. This has been correlated to the altered levels of 5α, pregnan-3α, 2α-diol in this phase of the menstrual cycle[89,119]. The effectiveness of adrenocorticotrophic hormone (ACTH) in infantile spasms is speculated to be related to its capacity to increase P450scc. In brain, this may in effect lead to increased AP levels, which have been shown to possess anticonvulsant and anesthetic properties[6]. PROG has been proven to be an effective anticonvulsant against the secondarily generalized component of amygdala-kindled seizures in male rats.. AP is an effective anticonvulsant against the secondarily generalized component of the seizure, but not against the amygdala focal discharge[120]. Synthetic neuroactive steroid ganaxolone (3α-hydroxy-3β-methyl-5α-pregnane-20-one) is an orally active analog of AP. It was found to be an effective anticonvulsant in neurosteroid withdrawal in pseudopregnancy model of rat, and this supports the use of ganaxolone as a specific treatment for perimenstrual catamenial epilepsy[77]. Negative modulators of $GABA_A$ receptors like PREG-S have been demonstrated to be proconvulsant with their activity mediated through $GABA_A$ receptors, and also possibly by effects on NMDA receptors[121].

Many NS, especially the 3α–hydroxyl ring A-reduced steroids are potent anticonvulsants. NS, like AP and 5α–3α–THDOC have been proven to have strong anxiolytic effects[92]. The anxiogenic-anxiolytic response is a complex one as PREG and PREG-S both can be anxiogenic themselves but attenuate the anxiogenic effect of ethanol in mice. This effect could be mediated through the NMDA receptors[91]. Inhibition of the hippocampus, mediated by the pregnanolone's action at the $GABA_A$ receptor, produces a general anxiolytic effect. However, similar inhibition in the lateral septum attenuates active avoidance of anxiogenic stimuli, but not passive avoidance of aversive stimuli[122].

Neurosteroids and oxidative stress

NS are now being proven to have a definite role in the antioxidant defenses of brain. PROG has been indicated to have an inhibitory action on lipid peroxidation. On comparison of repeated brain levels of 8-isoprostaglandin F2 alpha (8-isoPGF2 α), a marker of lipid peroxidation, after cortical contusion in male rats treated with either progesterone or the oil vehicle; the brains of progesterone treated rats contained approximately one-third of the 8-isoPGF2 alpha found in oil-treated rats[123]. In another study, DHEA and β estradiol pretreatment reduced DNA damage induced by oxidative insult. This effect was antagonized by pretreatment with a σ1 receptor antagonist suggesting that neuroactive steroids protect retinal cells from oxidative stress and that this effect is mediated by σ1 receptors[26].

Tunez et alshowed that DHEA reduces oxidative stress in synaptosomes isolated from the brain of 3-nitropropionic acid (3PA) induced oxidative stress in striatal and brain cortex synaptosomes. The authors have suggested that DHEA may protect mitochondrial and maintain synaptic integrity against damage induced by 3PA[124]. In a study exploring the control of DHEA secretion in the brain it was found that DHEA levels increased in response to Fe^{2+}, β amyloid induced oxidative damage, and this increase was attenuated by the use of an antioxidant like vitamin E[125]. In another study, a significant increase in brain DHEA level 24h after castration was observed, which was totally blocked by AD4 N-acetylcysteine amide (AD4) (a newly developed brain penetrating antioxidant). These data suggest that DHEA synthesis may be affected by free radicals, indicating the possible existence of an in-brain oxidative stress-related pathway leading to brain DHEA production[27]. The role of NS in antioxidant defense mechanism still needs to be explored further. Very limited data is available for other NS, including PREG-S and NS synthesis enhancer, 4CD. 4CD has been proven to have a role in mitochondrial respiration and mitochondrial membrane stabilization[126,127]. Since 4CD increases the production of NS and PREG-S can act through σ_1 receptor, indicated to have a role in antioxidant

mechanism, it is a strong possibility that the two agents can have influence over oxidative stress *in vivo*.

Pesticides, lindane, cognition and oxidative stress

Pesticides

After the green revolution, the presence of pesticides in various forms has become more or less ubiquitous in the environment surrounding us and exposure to the same is more or less inevitable. By definition, pesticide is any substance or mixture of substances intended for preventing, repelling, destroying or mitigating any pest. Depending on their use, they have been classified as insecticides, rodenticides, herbicides (weedicides), fungicides and fumigants. The important properties determining the potential of pesticide to contaminate the human environment are degradability of agent, its mobility through air, water and soil, and its capacity for bioaccumulation and bio-magnification via food chain[128].

Based on their degree of persistence, pesticides can also be classified as highly persistent, moderately persistent, and non-persistent[129]. The chlorinated hydrocarbon insecticides need a special mention in this regard as environmental pollutants because of their slow degradability by biotic pathways in animals and men (due to high lipid solubility and storage in lipid tissues) and abiotic pathways in soil and water (mainly by microorganisms and photochemical reactions).

Among insecticides there are four major groups, organophosphorus insecticides which are esters of phosphoric acid or thiophosphoric acid e.g. dichlorvos, parathion etc, Carbamate insecticides, which are esters of *N*-methylcarbamic acid like carbaryl, aldicarb, carbofuran, methomyl, and propoxur (Baygon). Organochlorine insecticides including the chlorinated ethane derivates, the cyclodienes, and the hexachlorocyclohexanes. E.g. DDT, methoxylchlor, endrin, aldrin and deildrin. Botanical insecticides including nicotine from tobacco, Pyrethrum from the flowers of

Chrysanthemum cinerariaefolium and Rotenone from roots of the plant *Derris elliptica*.

Lindane is an organochlorine and the gamma isomer of hexachlorocyclohexane (HCH).

Pesticide induced toxicity on nervous system

Over the years, extensive research has been carried out on the effects of pesticides and more and more information regarding their adverse effects is coming to the forefront. In children, pesticide exposure has been seen to be associated with decreases in stamina, gross and fine eye-hand coordination, 30 minute memory and in the ability to draw a person[130].

The organochlorine (OC) insecticides stimulate the nervous system and induce paresthesia, susceptibility to stimulation, irritability, disturbed equilibrium, tremor, and convulsions. Some, like aldrin, dieldrin and lindane induce facilitation and hyperexcitation at synaptic and neuromuscular junctions, resulting in repetitive discharge in central, sensory and motor neurons. DDT exerts its toxic effect in the nervous system by adversely affecting the axons membrane.

The organophosphate (OP) and carbamate insecticides inhibit acetylcholinesterase (AChE), resulting in an accumulation of acetylcholine (Ach). The accumulated ACh in CNS will induce tremor, incoordination, convulsion, etc. In the autonomic nervous system it will cause diarrhea and involuntary urination. At neuromuscular junction it will lead to contraction of the muscles, followed by weakness, loss of reflexes, and paralysis. The inhibition of AChE induced by carbamate is readily reversible, whereas that following exposure to OP compounds is generally less readily so.

Lindane has been found to increase the incidence of convulsions due to the induction of brain CYP450 by increasing the expression of P450 1A1/1A2; 2B1/2B2 and 2E1 isoenzymes[131]. Anticholinestrase pesticide dichlorvos

(DDVP) or methomyl (MET) show dose dependent seizure and lethality due to its action on muscuranic, nicotinic and NMDA receptors[132].

Pesticide-induced oxidative stress

Pesticides cause tissue injury, tumor promotion, apoptosis, immunosuppression, etc. by way of free radical generation and derangement of antioxidant mechanism[133]. Biological effects of pesticide are initiated through physiological interactions between a toxin and such specific necromolecules as enzymes, membranes, immunoglobulins, nucleic acid, cytokines and so on. Enzyme systems in the body can convert pesticides to highly reactive intermediates, metabolites or secondary active products.

Metabolism of certain classes of pesticide also results in the generation of oxygen free radical (OFRs) such as superoxide anion, hydrogen peroxide and the hydroxyl radical. The reaction of OFRs with polyunsaturated lipids is a particularly toxic event because it initiates the membrane damaging chain reaction process of lipid peroxidation. Many pesticides induce cytochrome P450 (CYP450) and also elicit an increase in the rate of oxygen free radical production by liver microsomes[134]. Mechanism of CYP450 induction is either at the transcriptional or at post-transcriptional level.

Several pesticides groups such as organochlorine, organophosphate, carbamates and herbicides stimulate lipid peroxidation of cellular membrane[134]. Such pesticides can contribute to the process of membrane peroxidation through several mechanisms:

- Direct initiation of a chain reaction by free radical formation during pesticide metabolism; for example, abstracting hydrogen from other molecule.

- Indirect initiation by the production of OFRs during pesticide metabolism; for example, paraquat metabolism can lead to $O_2^{-\bullet}$ formation.

- Inhibition of enzyme system that are involved in the control of reactive oxidizing entities; for example, dithiocarbamate is an inhibitor of superoxide dismutase.

- Decreasing natural antioxidant that regulate the adverse reaction of peroxidation; for example, the reduction of GSH level in blood after malathione exposure.

Lipid peroxidation has been postulated as the primary event mediating the toxicity of a broad spectrum of pesticides. Dowla et al observed that *in vitro* activities of delta-amino levulinic acid dehydratase and Cu-Zn SOD in human red blood cells were inhibited after methamidophos exposure[136]. Gromov et al found that delta-methrine and dichlorovos (DDVP) exposure results in decreased catalase (CAT) activity in brain of rats[137]. In pesticide poisoning cases, tissue glutathione reductase (GR), glutathione peroxidase, SOD and CAT activities, as well as malondialdehyde production are increased but GSH levels are decreased suggesting adaptive measure to tackle any insecticide accumulation[138]. These enzymes efficiently scavenge toxic free radicals and partly protect against lipid peroxidation from acute / chronic pesticide exposure.

Lindane

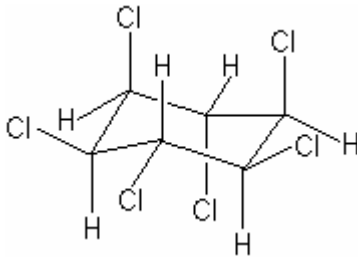

Fig 4 Lindane

Physico-chemical properties and toxicological profile [139]

Lindane is the gamma isomer of 1,2,3,4,5,6-hexchlorocyclohexane (γ-HCH) (Fig 4). It is a colourless crystalline solid, having a melting point of 112.9°C. It is very slightly soluble in water at 20°C, 10 ppm; moderately soluble in absolute alcohol, 6.7%; slightly soluble in petroleum oils; soluble in acetone, aromatic and chlorinated solvents. Lindane is stable to air, light, heat and carbon dioxide, not attacked by strong acids but in the presence of alkali it is dehydrochlorinated to trichlorobenzene. It is corrosive to aluminum at 20°C.

Single toxic dose of lindane are as follows:

Oral: LD50 rat 88-225 mg/kg

Dermal: LD50 rat (M) 1000 mg/kg

rat (F) 900 mg/kg

Dermal: LD50 rabbit 900-1000 mg/kg

Most susceptible species: Cattle, minimum toxic dose 25 mg/kg; for calf 5 mg/kg. Young animals are more sensitive than adults. Lindane is an organochlorine pesticide used extensively in agriculture. Wooldridge successfully treated human scabies (skin disease caused by mites) with 1% lindane cream, and this treatment continues to be widely used[140]. Lindane shampoo is also used for pediculosis. Lindane is also used as a general

insecticide. There have been numerous reports through the years of major toxicity and death associated with accidental or deliberate exposure to lindane[141]. In one incident, lindane intended for preservation of seed grains was instead mixed with food grains and was consumed. The onset of signs of poisoning was sudden with seizures of the mixed type, i.e., grand mal, petit mal, and myoclonus, predominating. Other effects included intention tremors, memory impairment, irritability, and aggression[28].

The data for lindane, specifically on cognitive dysfunction is limited[142]. Tilson et al demonstrated interference with the ability of avoidance response with a single dose of lindane in rats[30]. Desi found that repeated exposure to lindane increased the number of errors made in a food-reinforced maze[143]. Lindane has effects on long-term potentiation in the hippocampus, and it is possible that this effect may compete or interfere with the utilization of new information. The post-training administration of lindane did not affect retention. This suggests that the process of memory consolidation is not altered[29]. Alteration of motor and grooming activities in rats occurs on chronic administration of lindane, which has been correlated with inhibition of activities of cerebral Na+, K+- ATPase, Mg2+- ATPase and AchE[34]. It has also been postulated that lindane achieves its behavioral effects by interfering with gamma aminobutyric acid (GABA)[30]. Lindane has also been shown to interact with PBR. In a study by Griffith and Woolley, the authors when using hypothermia and anorexia as indices of lindane toxicity, demonstrated that toxicity of lindane was ameliorated by diazepam, phenytoin and exacerbated by Ro-5-4864 (4CD). The authors hypothesized that lindane acts at the picrotoxin binding site of $GABA_A$ receptor[144].

Lindane also reportedly influences the metabolism of PREG and PROG in mice ovaries. It inhibits the conversion of cholesterol to PREG by inhibiting the enzyme P450scc[145]. Contrary to this, other workers have found no effect of lindane on P450scc activity with conflicting reports regarding inhibition of 3β HSD activity. It has been further claimed that lindane inhibits the activity of steroidogenic acute regulatory (StAR) protein, which mediates an

important step in steroidogenesis, the intramitochondrial transfer of cholesterol to the P450scc enzyme[31,32].

Lindane has been shown to be a strong oxidant, causing free radical generation in tissues including brain through lipid peroxidation[33,34]. This oxidative stress seems to be reversible by itself in acute exposure or by other known antioxidants like ascorbic[146].

Oxidative stress and cognition

Extensive research has been carried out in the role of oxidative stress and cognitive impairment. In a study by Head et al , increasing oxidative damage with increasing age was found in a canine model of human brain aging[147]. Increased malondialdehyde (MDA), which indicates increased lipid peroxidation, was observed in the prefrontal cortex and serum but not in cerebrospinal fluid (CSF). Oxidative damage to proteins (carbonyl formation) also increased in brain. An age-dependent decline in GS activity, an enzyme vulnerable to oxidative damage, and in the level of reduced glutathione (GSH) was observed in the prefrontal cortex. In the pathogenesis of AD oxidative damage appears to play an important role in the slowly progressive neuronal death. In addition to the presence of senile plaques and neurofibrillary tangles, postmortem analysis of AD brain has also identified markers of oxidative stress including protein nitrotyrosine, carbonyls in proteins, lipid oxidation products and oxidized DNA bases[148]. In another model, workers have also suggested that Amyloid beta protein 42 (Abeta42) is not sufficient alone to induce an Alzheimer's disease-like symptomatology and a decrease in the brain's antioxidant defense system leads to the Abeta42-independent oxidative stress necessary for the peptide to induce histopathological changes and memory loss[149]. In view of the putative role of oxidative stress in cognitive impairment, role of various antioxidants has been explored in reversal of experimental and clinical memory loss. In apolipoprotein E-deficient mice, mice treated with vitamin E display a significantly improved behavioural performance in the Morris water maze compared to the group on a regular diet. This improved performance has been found to be associated with preservation of the dendritic structure. In addition, whilst untreated apolipoprotein E-deficient mice display increased levels of lipid peroxidation and glutathione, vitamin E-treated mice showed near normal levels of both lipid peroxidation and glutathione[150]. Another antioxidant, trans resveratrol, a polyphenolic compound, on chronic administration has been demonstrated to prevent cognitive impairment by intracerebrovntricular injection of streptozocin (STZ) in rats. This benefit

was also associated with a decrease in the oxidative stress level induced by STZ in brain[151]. Similarly ascorbic acid, another proven antioxidant, improved learning and memory of aged mice as indicated by decreased transfer-latency and increased step-down latency. Ascorbic acid also provided protection to young animals from scopolamine- and diazepam-induced impairment of memory and was found to be more potent than piracetam[152]. Thus, oxidative stress plays a strong role in cognitive impairment, and probably factors which can effect a reversal of oxidative stress can mediate a reversal in cognitive dysfunction as well.

With the above presented review, it can be safely hypothesized that the varied mechanisms of cognitive dysfunction caused by lindane ensure that a partial reversal at the least can be effected through NS. Also, the converging influence of the two agents over oxidative stress and that of oxidative stress over cognition means that attenuation of oxidative stress by NS can be a potent mechanism of their efficacy in this case.

Aims & Objectives

- To study the modulation of cognitive dysfunction due to lindane, by neurosteroids

- To study the modulation of oxidative stress due to lindane, by neurosteroids.

- To correlate the effects of lindane and neurosteroids on oxidative stress and cognitive dysfunction.

Material & Methods

Animals

Male wistar rats, weighing between 150 to 220 g were used. The animals were procured from the Central Animal House, University College of Medical Sciences. The animals were housed in standard laboratory conditions (natural hours light and dark cycle; 23±1°C temp. and 50 ± 2% humidity) with pellet diet and water available *ad libitum*. Appropriate permissions were taken from Institutional Animal Ethics Committee and care of the animals was as per "CPCSEA Guidelines for laboratory animal facilities".

Chemicals

- Lindane (Sigma chemicals Inc).
- Progesterone (Sigma chemicals Inc).
- Pregnenolone Sulfate (Sigma chemicals Inc).
- 4'-chlordiazepam (Fluka).
- Tween 80.
- Groundnut oil.
- Laboratory chemicals required for estimation of various biochemical parameters.

Groundnut oil was used as a vehicle for lindane. To limit the weight gain due to oil consumption, the concentration of lindane was maintained so that no animal received more than 0.5 ml of oil per day, anytime during the study period.

The vehicle for NS was distilled water with two drops of tween 80 added per 10 ml of suspension of i.p. injections, the concentration was maintained so that animals received 0.5ml/100g of suspension per animal per day.

Groups

Animals were randomly divided into eight groups having 10 rats /group.

Group 1: Control/vehicle group for lindane. Groundnut oil orally for 6 weeks followed by vehicle for NS (tween 80 in distilled water) i.p. for one week.

Group 2: Lindane 15mg/kg/d in groundnut oil orally for 6 weeks followed by vehicle for NS i.p. for 1 week

Group 3: Vehicle for lindane (groundnut oil) orally for 6 weeks followed by progesterone 15mg/kg/day i.p for one week

Group 4: Vehicle for lindane orally for 6 weeks followed by pregnenolone sulfate 2 mg/kg/day i.p for one week.

Group 5: Vehicle for lindane orally for 6 weeks followed by 4' chlorodiazepam 0.5 mg/kg/day i.p for one week

Group 6: Lindane 15mg/kg/d in groundnut oil orally for 6 weeks followed by progesterone 15mg/kg/day i.p for one week

Group 7: Lindane 15mg/kg/d in groundnut oil orally for 6 weeks followed by pregnenolone sulfate 2 mg/kg/day i.p for one week

Group 8: Lindane 15mg/kg/d in groundnut oil orally for 6 weeks followed by 4' chlordiazepam 0.5 mg/kg/day i.p for one week

These groups were evaluated for cognitive function one day before the start of treatment and once weekly on the same day from the start of treatment till the end of treatment. Animals were trained on each day prior to assessment of cognition. Finally, the animals were sacrificed and brain taken out to assess the oxidative stress.

Assessment of cognition

Cognition was assessed on the basis of two separate experiments:-

Step down latency (SDL) in continuous avoidance apparatus

This apparatus consisted of a wooden block placed in the center of a grid floor of a continuous avoidance apparatus. The block served as a shock free zone (SFZ). The rat was placed on the SFZ and on stepping down was given electric shock (20V) through the grid floor. The experiment was repeated after 24 hrs without shock and the time taken for the rat to step down was measured. This is known as the step down latency. Prolongation of the step down latency is a parameter of learning.

A cutoff of 180 seconds was chosen and for the animal which did not step down in this period, the time to step down was taken as 180 seconds[153-155].

Transfer latency (TL) on elevated plus maze

The elevated plus maze consists of two open arms (50 by 10 cm) and two closed arms (50 by 10 by 40 cm) with an open roof. The maze is elevated to a height of 50cm from the floor. The animals were placed individually at either ends of the open arms and allowed to enter either of the closed arms. During the first time screening if the animal did not enter an enclosed arm within 180 sec, it was not included in the experiment. During training, if the animal did not enter an enclosed arm within 180 sec it was gently pushed in the closed arm. To become acquainted with the maze, the animals were allowed to explore the maze for 20 sec after reaching the closed arm and then returned to their home cage. The animals were retested 24h after the first day training and the time taken to enter the closed arm was taken as transfer latency (TL). A time of 180 seconds was taken as cut-off and

animals not entering the closed arm in this period were assigned the transfer latency of 180 seconds [156-158].

All tests were conducted in the Neuropharmacology laboratory, Department of Pharmacology between 0900 and 1600 Hrs.

Assessment of oxidative stress

At the end of study period, the animals were sacrificed by ether anesthesia, the brain was quickly dissected out in toto, washed with ice-cold sodium phosphate buffer, weighed and stored over ice. The brains were further processed within half hour of dissection, and the estimation of oxidative stress done in the same working day. Brain tissue was homogenized with 10 times (w/v) sodium phosphate buffer (7.4 pH, ice cold, mixture of KH_2PO_4 and Na_2HPO_4). The homogenate was centrifuged at 3000 rpm for 15 min.

The parameters of oxidative stress used were malondialdehyde (MDA) and reduced glutathione (GSH).

Measurement of lipid peroxidation

Malondialdehyde (indicator of lipid peroxidation) was estimated as described by Okhawa et al[159].

Principle

Acetic acid detaches the lipid and protein of the tissue. The protein in the reaction mixture is dissolved by the addition of lauryl sulfate. MDA reacts with the lipid peroxides, hydroperoxides and oxygen double bonds to form the color adducts with absorption maxima at 532 nm.

Reagents

- Sodium lauryl sulfate (8.1%)
- Acetic acid (20%) (pH 3.5)
- Thiobarbituric acid (0.8% w/v)
- Butanol: pyridine (15:1 v/v)

Procedure

Acetic acid (20%, pH 3.5) 1.5 ml, thiobarbituricacid (0.8%), sodium lauryl sulfate (8.1%) 0.2 ml were added to 0.5 ml of supernatant obtained above. The mixture was heated at 100^0C for 1h. the mixture was cooled with tap water and 5 ml of butanol: pyridine (15:1 % v/v) and 1 ml of distilled water were added. The mixture was vortexed vigorously and was centrifuged at 4000 rpm for 10 min. Thereafter the organic layer was withdrawn and absorbance measured at 532 nm using a spectrophotometer.

Standard curve

Various samples of external standard tetraethoxypropane (1-10 nmol) were subjected to the steps mentioned in the above procedure. The readings of absorbance were plotted against the concentration of MDA to produce a standard curve.

The concentration of MDA was determined by the linear standard curve and expressed as nmol/ g wet brain tissue.

Estimation of reduced glutathione

Reduced glutathione was estimated by the method described by Ellman[160].

Principle

Bis (p-nitrophenyl) disulphide reacts with aliphatic thiol compound at pH 8.0 to produce 1mol of p-nitrophenol anion per mol thiol. Since the anion is highly colored, it can be used to measure the thiol concentration.

Reagents

- Phosphate buffer (K2HPO4) 0.3M (pH 8.4)
- 5,5 Dithiobis 2-nitrobenzoic acid (DTNB) 0.4% w/v (in 1% trisodium citrate)
- 5% tricarboxylic acid (TCA).

Procedure

To 0.5 ml of the supernatant obtained above 1 ml TCA (5%) was added and the mixture centrifuged to remove the proteins. To 0.1 ml of this homogenate, 4 ml of phosphate buffer (pH 8.4), 0.5 ml of DTNB and 0.4 ml double distilled water were added. The mixture was vortexed and absorbance read at 412 nm within 15 min.

Standard curve

Various concentrations of standard glutathione (5-50 μg) were subjected to the steps mentioned above. The readings of absorbance were plotted against the concentration of GSH to produce standard curve.

The concentration of GSH was determined by linear standard graph and expressed as μg/g wet brain tissue.

Results

Memory parameters

Step down latency (SDL)

At day 0 no significant differences were found among the SDLs of all the groups. A significant reduction in SDL was found for the lindane treated group at week 6 and 7 as compared to both control ($p<0.001$) and day 0 of lindane treated group ($p<0.05$) (Table 1; Fig 5,6). A significant reduction in SDL was also noted for lindane+ PREG-S and lindane + 4CD group at week 5 ($p<0.05$) and week 6 ($p< 0.001$). At 7 week PREG-S and 4CD antagonized the effect of lindane on SDL (Table1,3; Fig 7,8,13). The difference between lindane alone treated groups and lindane+ PREG-S and lindane+4CD was 67.47% and 68.13% respectively. Although this difference was not statistically significant when ANOVA was applied but was found to be statistically significant ($p<0.02$) when t-test was used. PROG failed to modulate the effect of lindane on SDL.

Table 1: Effect of various treatments on SDL (Mean(s) ± SE)

Groups/ Weeks	Treatment (6 week+1 week)	0	1	2	3	4	5	6	7	Within-group variation (p, F, dF)
Control	Vehicle	97.66 ± 25.49	104.54 ± 20.38	135.1 ± 16.06	98.71 ± 20.86	110.57 ± 19.77	142.88 ± 19.51	134.38 ± 19.09	144.5 ± 15.81	0.152, 1.598, 7
Lindane	Lindane 15mg/kg/d p.o	99.65 ± 14.24	104.53 ± 13.84	101.33 ± 16.36	116.45 ± 13.4	92.49 ± 21.91	85.42 ± 20.92	32.98 ± 6.44 [b,f]	31.07 ± 5.78 [b,f]	**<0.001**, 6.32, 7
Only PROG	PROG 15mg/kg/d i.p	106.95 ± 23.54	97.48 ± 21.43	133.89 ± 18.52	117 ± 24.84	140.7 ± 13.89	148.38 ± 11.74	136.26 ± 13.91[b]	151.23 ± 17.75	0.199, 1.458, 7
Only PREG-S	PREG-S 2mg/kg/d i.p	106.44 ± 15.9	124.32 ± 13.92	133.62 ± 12.8	144.18 ± 12.94	91.76 ± 19.02	92.96 ± 15.87	136.88 ± 15.13	124.88 ± 16.49	0.06, 1.774, 7
Only 4CD	4CD 0.5mg/kg/d i.p.	101.02 ± 21.76	112.32 ± 16.26	118.08 ± 17.5	65.38 ± 21.3	107.54 ± 21.49	121.39 ± 20.35	124.26 ± 15.28	123.26 ± 14.44	0.108, 2.06, 7
Lindane+ PROG	Lindane 15mg/kg/d p.o+ PROG 15mg/kg/d i.p	115.54 ± 21.23	116.87 ± 19.72	113.64 ± 20.57	92.05 ± 20.22	86.44 ± 17.89	77.53 ± 19.42	35.14 ± 16.7 [b,c,f]	38.3 ± 16.62 [b,c,f]	**<0.01**, 3.987, 7
Lindane + PREG-S	Lindane 15mg/kg/d p.o+ PREG-S 2mg/kg/d i.p	100.24 ± 14.22	108.66 ± 11.91	96 ± 11.13	99.9 ± 20.37	79.16 ± 14.43	64.88 ± 12.43[a]	28.58 ± 7.1 [b,d,f]	95.52 ± 23.04 [g]	**<0.01**, 3.836, 7
Lindane +4CD	Lindane 15mg/kg/d p.o+ 4CD 0.5mg/kg/d i.p.	124 ± 18.32	117.96 ± 17.55	108.26 ± 13.67	107.9 ± 18.94	74.88 ± 17.51	60.06 ± 15.14 [a]	31.88 ± 5.54 [b,e,f]	97.5 ± 18.12 [g]	**<0.001**, 4.815, 7
Between-groups comparison (p, F, dF)		0.9813,0. 2133,7	0.9679,0. 2581,7	0.4986, 0.2581,7	0.2340, 1.363,7	0.2495, 1.328,7	**0.0011**, 3.946, 7	**<0.001**, 16.432, 7	**<0.001**, 7.406,7	

[a] p<0.05 as compared to control group
[b] p<0.001 as compared to control group
[c] p< 0.001 as compared to PROG only group
[d] p< 0.001 as compared to PREG-S only group
[e] p< 0.001 as compared to 4CD only group
[f] p<0.05 as compared to the day 0 of the corresponding group
[g] p< 0.02 compared to lindane alone treated group (t-test)
[g] NS compared to lindane alone treated group (ANOVA)

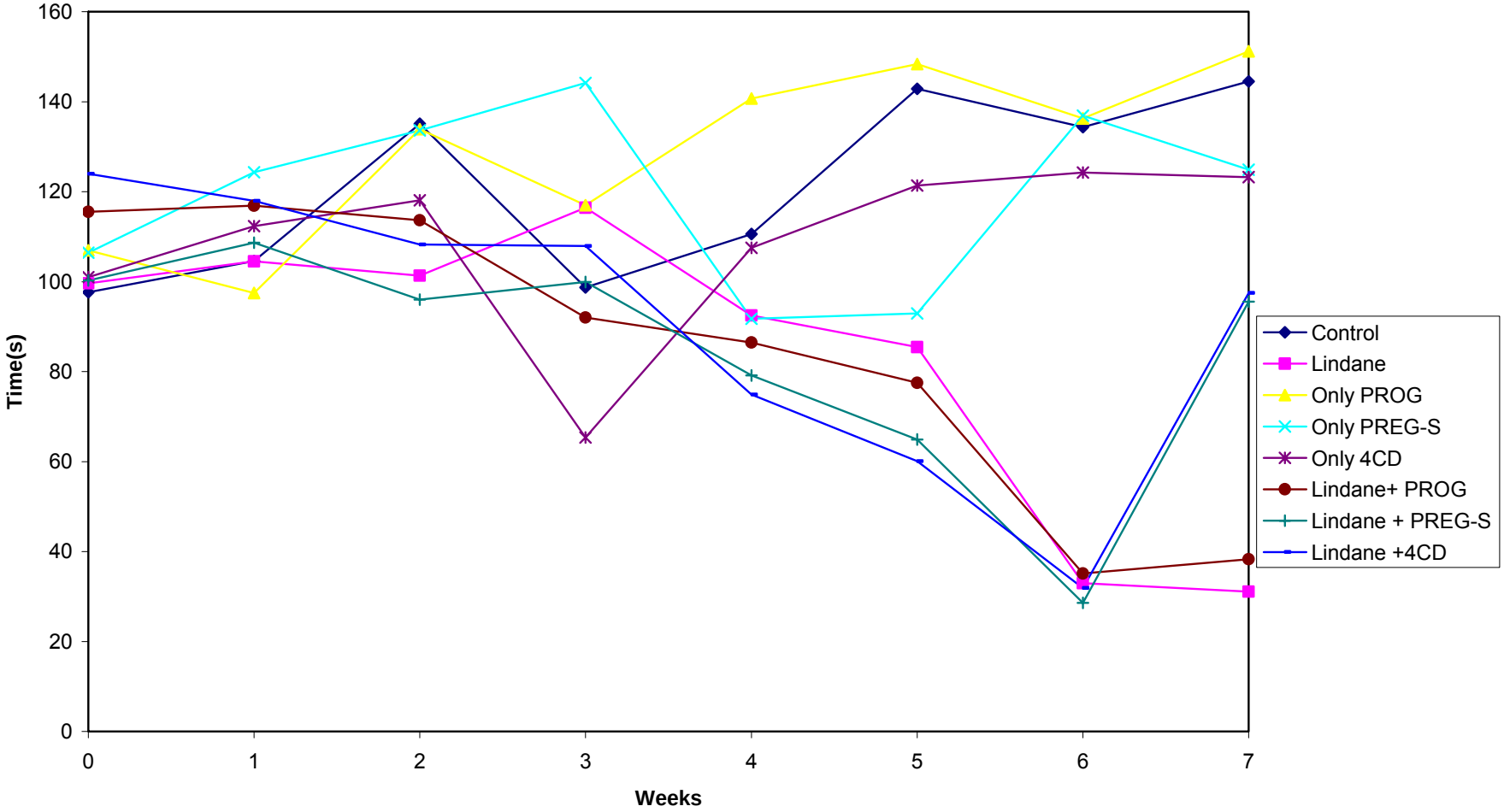

Fig 5: Effect of various treatments on SDL over seven weeks

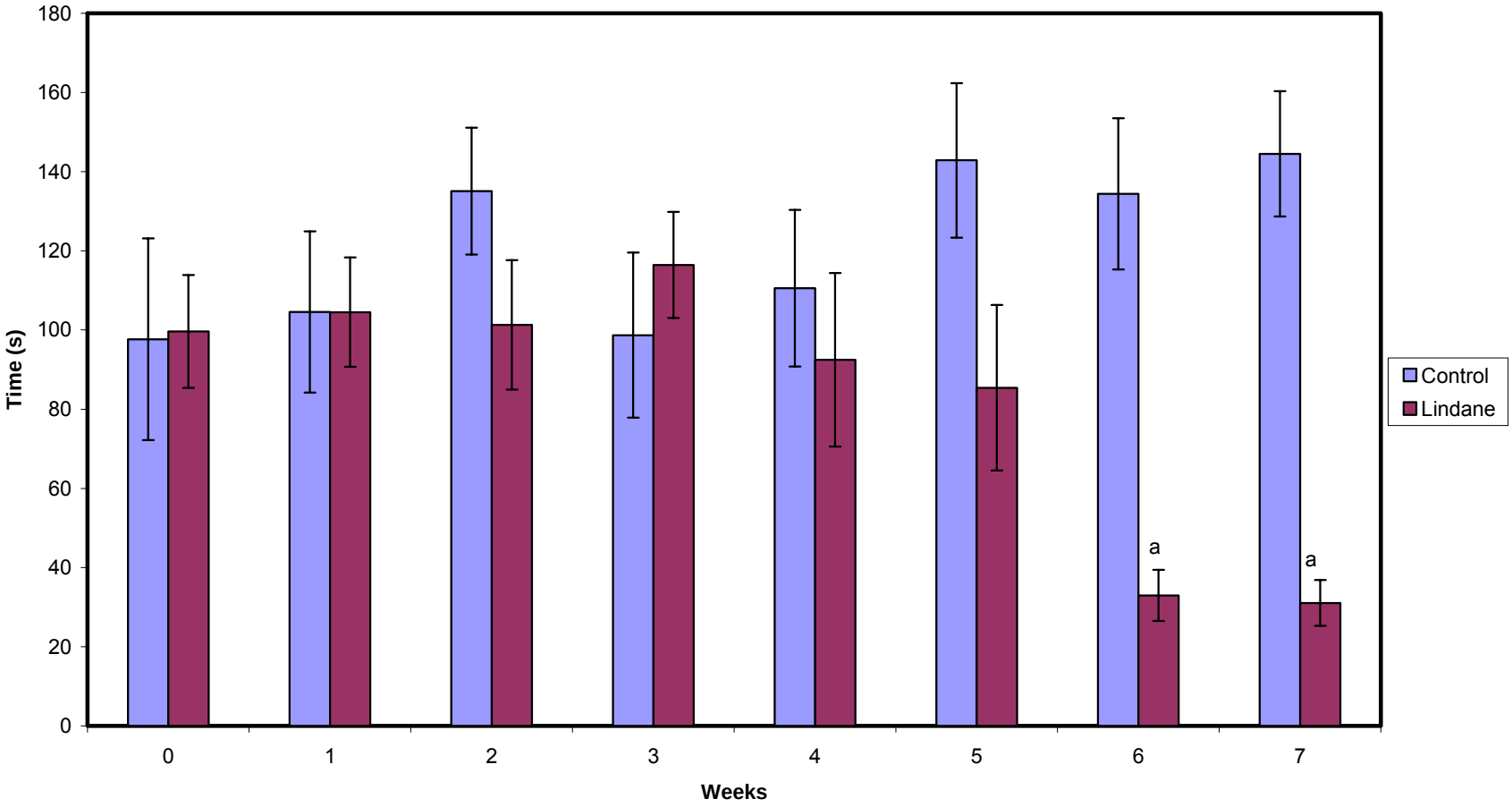

Fig 6: Effect of lindane over SDL

a p< 0.001 as compared to control

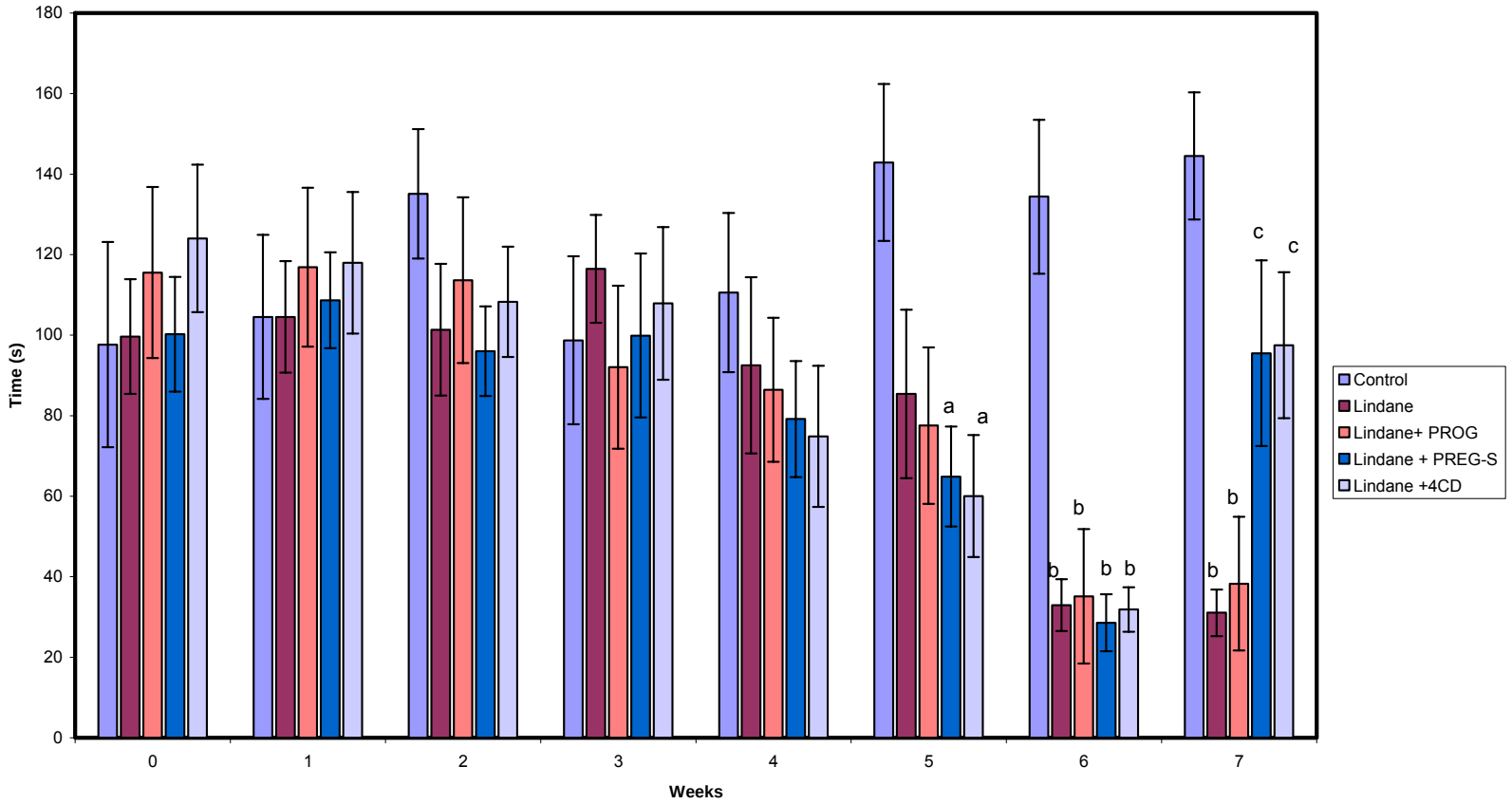

Fig 7: Effect of various treatments on SDL

a $p<0.05$ as compared to control

[b] $p<0.001$ as compared to control

c $p< 0.02$ compared to lindane alone treated group (t-test) as compared to PROG only group

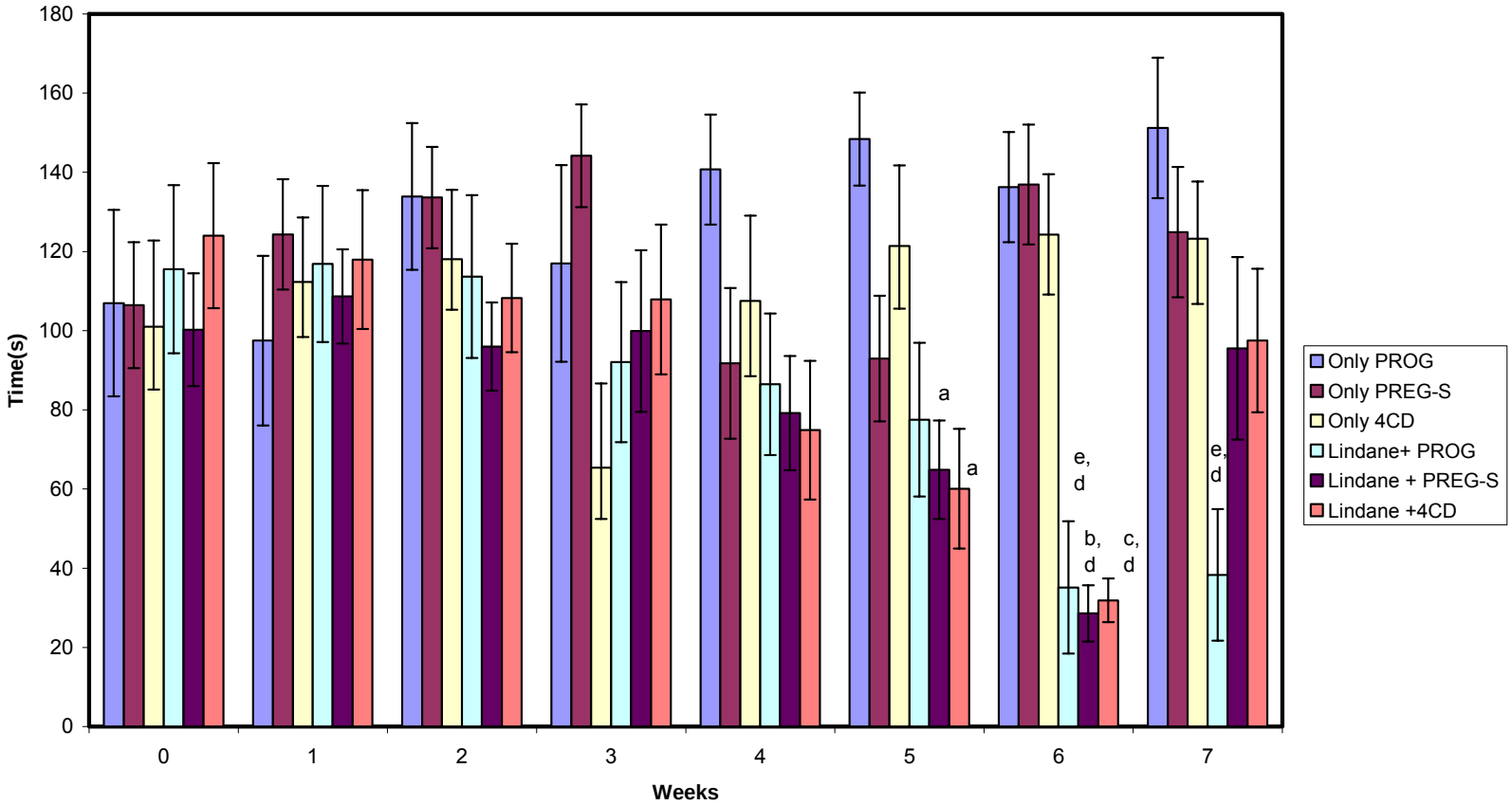

Fig 8: SDL in various drug treated groups

a p<0.05 as compared to control groups
b p<0.001 as compared to PREG-S only group
c p<0.001 as compared to 4CD only group
d p<0.001 as compared to control group
e p<0.05 as compared to day 0 of the corresponding group

Transfer latency (TL)

At day 0 no significant difference was found among the TLs of all the groups. In the lindane treated group a significant prolongation as compared to control ($p<0.001$) was noticed from 2nd week onwards (table 2, Fig 9, 10). A similar trend was also noted in the lindane+PROG, lindane+PREG-S and lindane+4CD groups, between week 2 to 6 as compared to control and PROG only, PREG-S only and 4CD only respectively($p<0.001$). Significant prolongation was also noted at 7th week for the lindane +PROG group($p<0.001$) and the lindane+PREG-S, lindane+4CD groups ($p<0.05$). PROG, PREG-S and 4CD failed to modulate the effect of lindane on TL (Table 2,3; Fig 11,12,13) .

Table 2: Effect of various treatments on TL (Mean(s) ± SE)

Groups/ Weeks	Treatment (6 week+1 week)	0	1	2	3	4	5	6	7	Within group variation (p, F, dF)
Control	Vehicle	5.3 ± 1.05	5.07 ± 1.35	4.35 ± 0.94	4.93 ± 0.95	4.62 ± 1.05	4.67 ± 0.96	3.95 ± 0.63	3.25 ± 0.46	0.651, 0.725, 7
Lindane	Lindane 15mg/kg/d p.o	10.73 ± 1.05	10.99 ± 0.96	19.66 ± 1.11[a]	33.44 ± 4.14[a]	36.17 ± 3.99[a]	41.38 ± 4.60[a]	51.63 ± 14.52[a]	51.53 ± 14.79[a]	**<0.001**, 6.025, 7
Only PROG	PROG 15mg/kg/d i.p	10.53 ± 5.29	9.23 ± 4.30	5.14 ± 0.98	3.84 ± 0.72	5.49 ± 0.84	4.93 ± 0.60	4.96 ± 0.84	6.11 ± 1.47	0.406, 1.05, 7
Only PREG-S	PREG-S 2mg/kg/d i.p	7.18 ± 1.17	5.82 ± 1.02	5.2 ± 0.45	4.48 ± 0.87	5.52 ± 0.90	4.98 ± 0.31	5.22 ± 0.59	6.22 ± 0.99	0.316, 1.199, 7
Only 4CD	4CD 0.5mg/kg/d i.p.	5.24 ± 0.6	6.16 ± 1.15	3.94 ± 0.66	4.58 ± 0.83	6.4 ± 0.79	5.28 ± 0.51	5.68 ± 0.76	5.62 ± 0.53	0.186, 1.494, 7
Lindane+ PROG	Lindane 15mg/kg/d p.o+ PROG 15mg/kg/d i.p	10.66 ± 1.94	11.15 ± 1.78	18.51 ± 3.68[a,b]	28.14 ± 3.27[a,b]	30.93 ± 2.92[a,b]	38.89 ± 5.45[a,b]	50.78 ± 9.72[a,b]	46.42 ± 7.88[a]	**<0.001**, 11.025, 7
Lindane + PREG-S	Lindane 15mg/kg/d p.o+ PREG-S 2mg/kg/d i.p	11.02 ± 1.49	11.88 ± 1.07	16.96 ± 0.94[a,c]	25.12 ± 2.47[a,c]	26.36 ± 3.72[a,c]	38.02 ± 5.91[a,c]	47.84 ± 6.39[a,c]	33.26 ± 6.92[e]	**<0.001**, 15.048, 7
Lindane +4CD	Lindane 15mg/kg/d p.o+ 4CD 0.5mg/kg/d i.p.	10.86 ± 0.89	14.28 ± 1.00	17.36 ± 1.83[a,d]	25 ± 3.74[a,d]	24.38 ± 3.03[a,d]	36.24 ± 4.59[a,d]	47.17 ± 3.99[a,d]	39.9 ± 4.12[e]	**<0.001**, 25.804, 7
Between-groups comparison (p, F, dF)		0.2275, 1.379, 7	0.0350, 2.438,7	**<0.001**, 19.53,7	**<0.001**, 25.775,7	**<0.001**, 27.648,7	**<0.001**, 24.001, 7	**<0.001**, 12.419,7	**<0.001**, 9.833, 7	

[a] p<0.001 as compared to control
[b] p<0.001 as compared to PROG only
[c] p<0.001 as compared to PREG-S only
[d] p<0.001 as compare to 4CD only
[e] p< 0.05 as compared to control

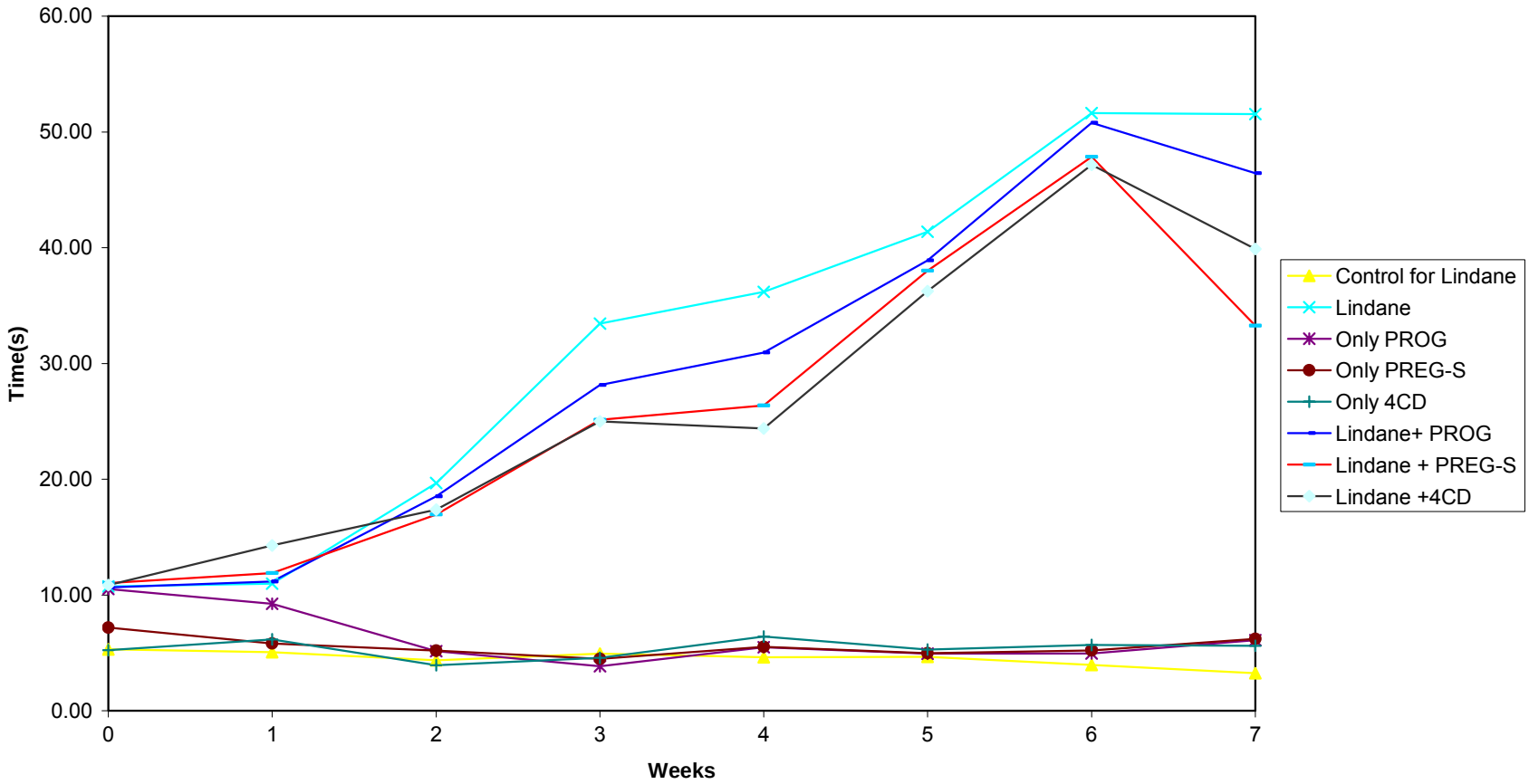

Fig 9: Effect of various treatments on transfer latency

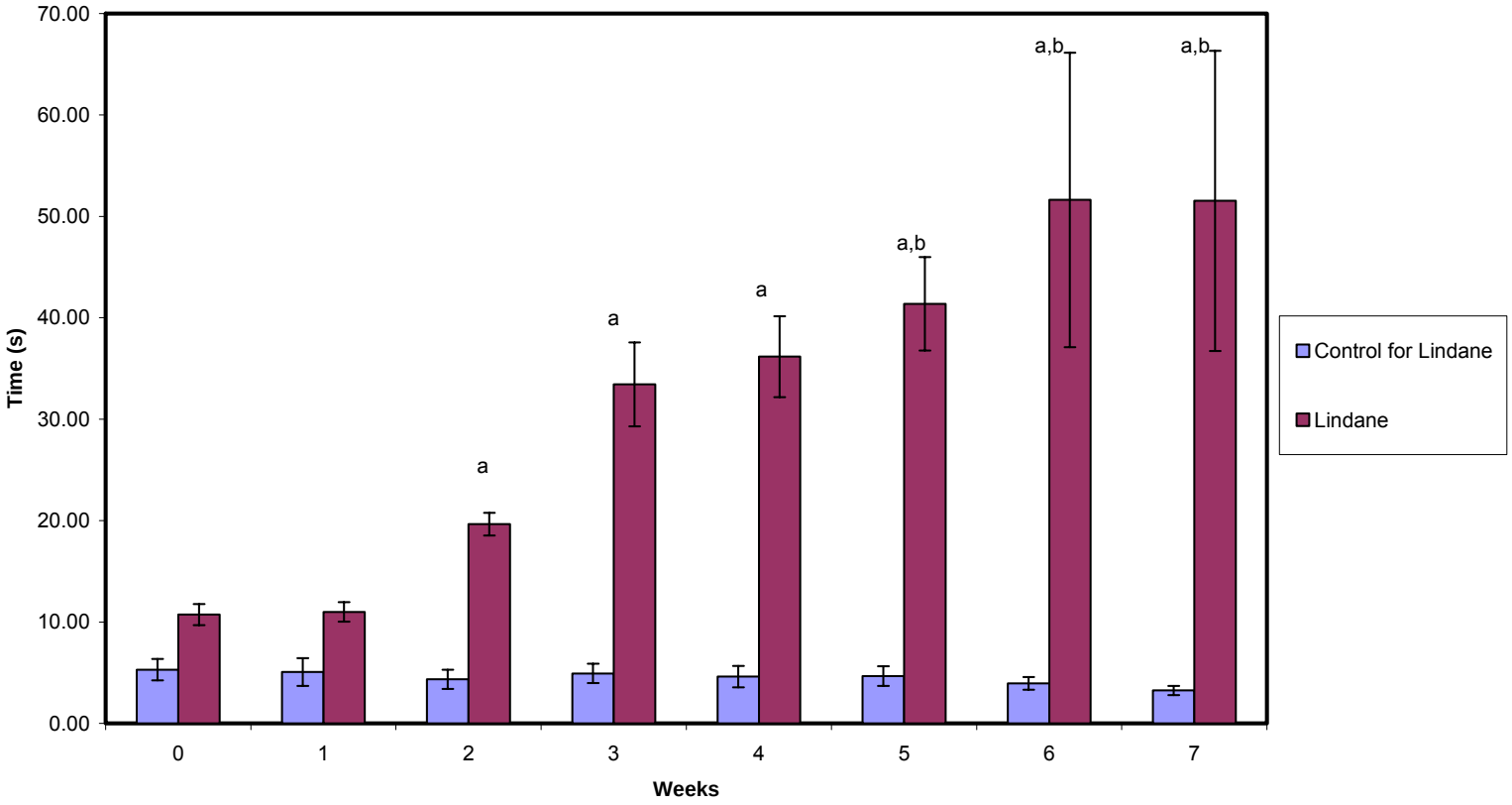

Fig 10: Effect of lindane on transfer latency

a $p<0.001$ as compared to control
b $p<0.05$ as compared to day 0 of treatment

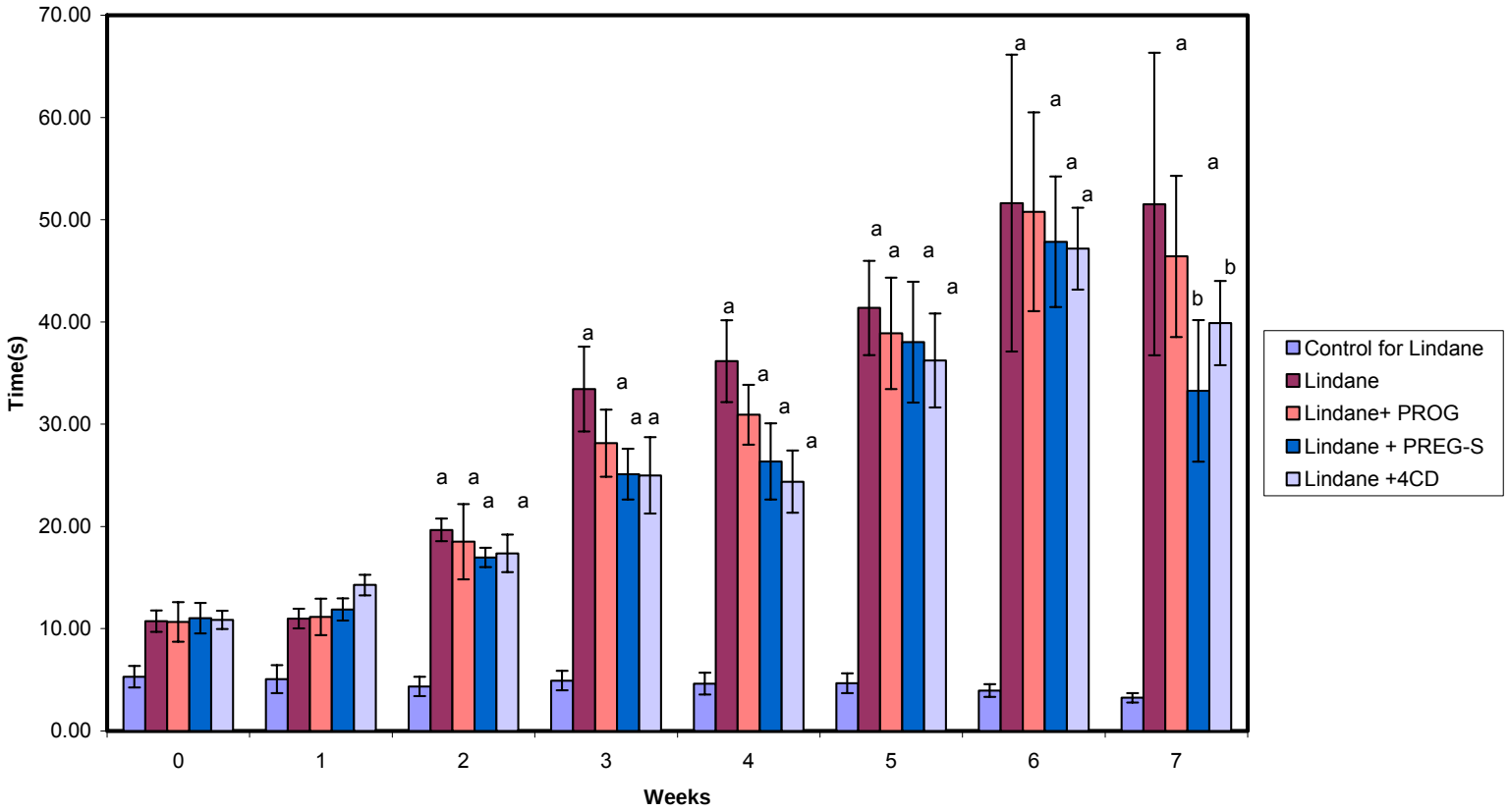

Fig 11: Effect of various treatments on TL

a $p<0.001$ as compared to control
b $p<0.05$ as compared to control

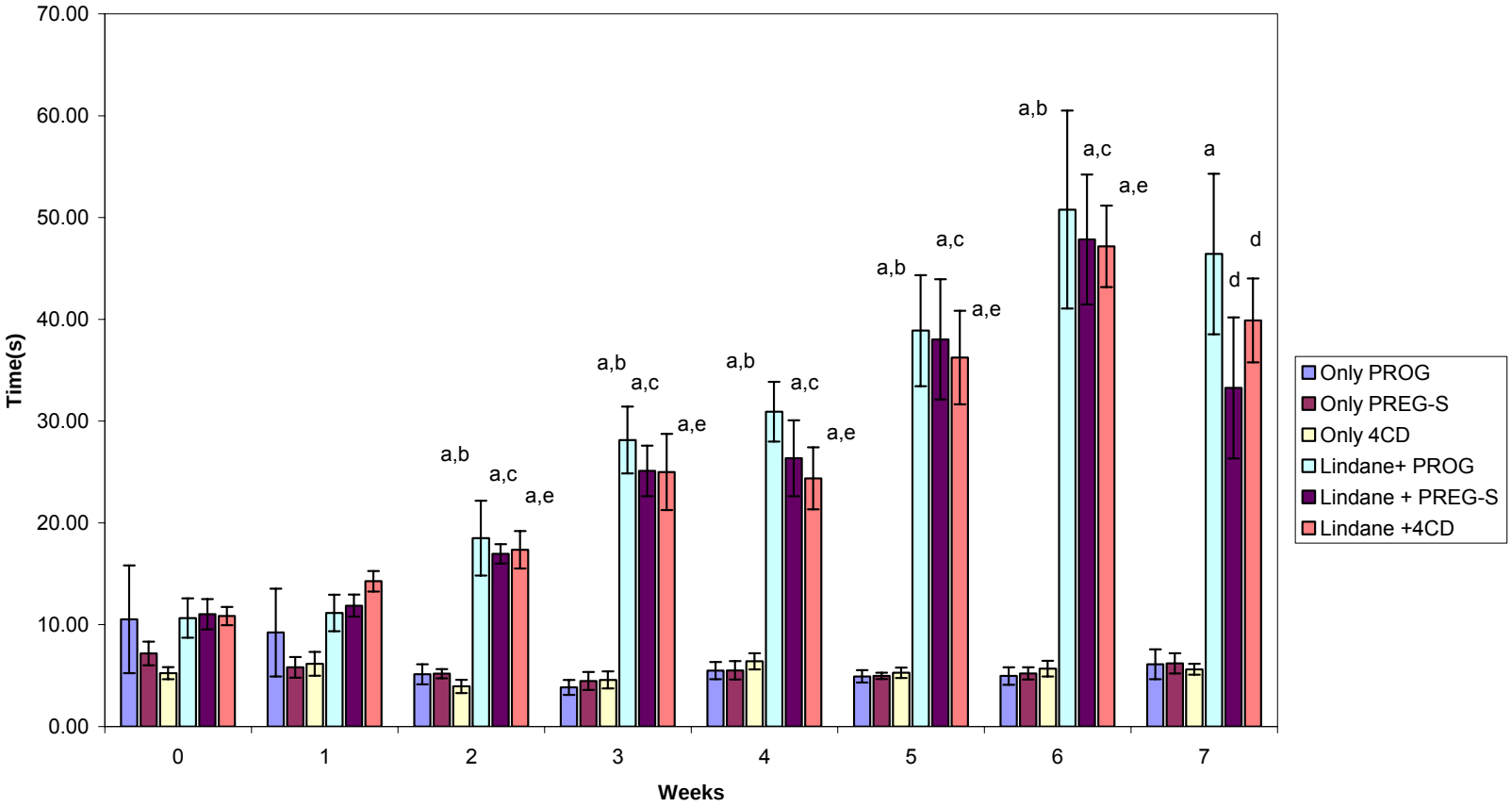

Fig 12: Effect of various treatments on TL

[a] p<0.001 as compared to control
[b] p<0.001 as compared to PROG only
[d] p< 0.05 as compared to control
[e] p<0.001 as compare to 4CD only

[c] $p<0.001$ as compared to PREG-S only

Table 3: Effect of NS on lindane induced cognitive impairment

Groups/Weeks	Treatment (6week+ 1week)	SDL (7^{th} week, mean(s) ± SE)	TL (7^{th} week, mean(s) ± SE)
Control	Vehicle	144.5 ± 15.81	3.25 ± 0.46
Lindane	Lindane 15mg/kg/d p.o	31.07 ± 5.78 [a]	51.53 ± 14.79 [a]
Only PROG	PROG 15mg/kg/d i.p	151.23 ± 17.75	6.11 ± 1.47
Only PREG-S	PREG-S 2mg/kg/d i.p	124.88 ± 16.49	6.22 ± 0.99
Only 4CD	4CD 0.5mg/kg/d i.p.	123.26 ± 14.44	5.62 ± 0.53
Lindane+ PROG	Lindane 15mg/kg/d p.o+ PROG 15mg/kg/d i.p	38.3 ± 16.62[a,b,c]	46.42 ± 7.88 [a,c]
Lindane + PREG-S	Lindane 15mg/kg/d p.o+ PREG-S 2mg/kg/d i.p	95.52 ± 23.04 [e]	33.26 ± 6.92[d]
Lindane +4CD	Lindane 15mg/kg/d p.o+ 4CD 0.5mg/kg/d i.p.	97.5 ± 18.12 [e]	39.9 ± 4.12 [c,d]

[a] $p<0.001$ as compared to control
[b] $p< 0.001$ as compared to PROG only group
[e] $p< 0.02$ compared to lindane alone treated group (t-test)
[c] $p<0.05$ as compared to the day 0 of the corresponding group
[d] $p< 0.05$ as compared to control

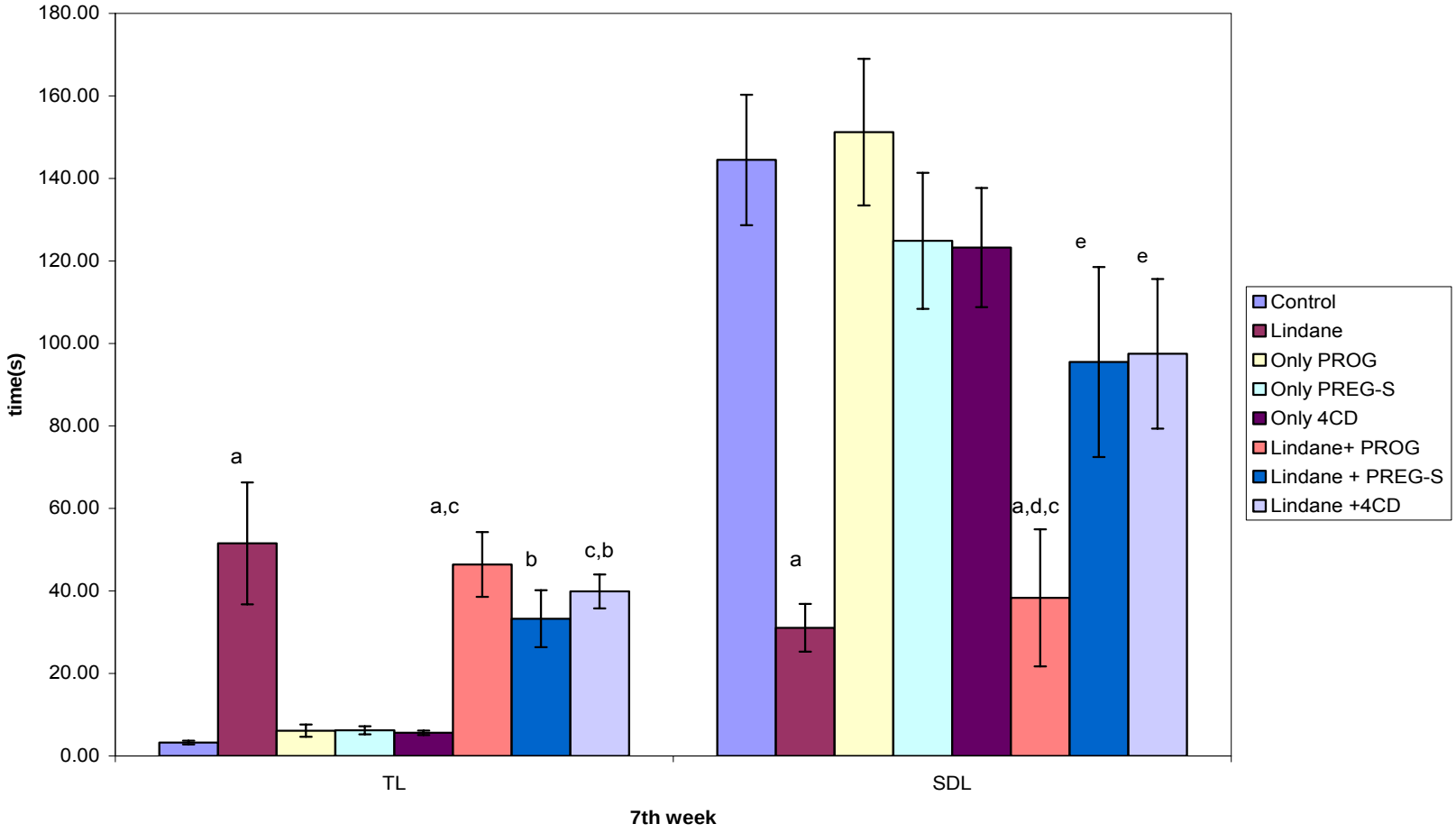

Fig 13: Effect of various treatments on SDL and TL

[a] p<0.001 as [c] a p<0.05 compared to control
[b] p< 0.05 as compared to control
[c] p<0.05 as compared to the day 0 of the corresponding group
[d] p< 0.001 as compared to PROG only group
[e] p< 0.02 compared to lindane alone treated group (t-test)

Oxidative stress parameters

Malondialdehyde (MDA)

Effect of lindane and neurosteroids on brain MDA level

There was a marked and statistically significant increase in the brain MDA levels of group treated with only lindane ($p < 0.001$). Treatment with PREG-S and 4CD attenuated the effect of lindane on MDA level, the difference between lindane alone and lindane + PREG-S or lindane+4CD was found to be significant ($p<0.001$). In contrast to PREG-S and 4CD PROG failed to modulate the effect of lindane on MDA levels. No difference was in the brain MDA levels of only PROG, only PREG-S and only 4CD and control (Table 4; Fig 14).

Table 4: Effect of lindane and NS on brain MDA levels

Group	Treatment (6week+1week)	MDA (nmol/g wet brain tissue) ± SE
Control	Vehicle for lindane+ vehicle for NS	178.57±19.43
Lindane	Lindane 15mg/kg/d p.o+ vehicle for NS	519.11±35.41[a]
Only PROG	Vehicle for lindane +PROG 15mg/kg/d i.p	181.3±23.72
Only PREG-S	Vehicle for lindane +PREG-S 2mg/kg/d i.p	116.23±5.3
Only 4CD	Vehicle for lindane +4CD 0.5mg/kg/d i.p.	125.66±13.95
Lindane+ PROG	Lindane 15mg/kg/d p.o+ PROG 15mg/kg/d i.p	428.7±37.82[a,b]
Lindane + PREG-S	Lindane 15mg/kg/d p.o+ PREG-S 2mg/kg/d i.p	185.48±11.49 [c]
Lindane +4CD	Lindane 15mg/kg/d p.o+ 4CD 0.5mg/kg/d i.p.	172.21±12.51 [c]

Intergroup variaton found statistically significant, $p < 0.001$, df=7, F=43.280

[a] $p<0.001$ as compared to control group for lindane (ANOVA followed by Tukey's test)

[b] $p<0.001$ as compared to only PROG group (ANOVA followed by Tukey's test)

[c] $p<0.001$ as compared to lindane alone treated group.

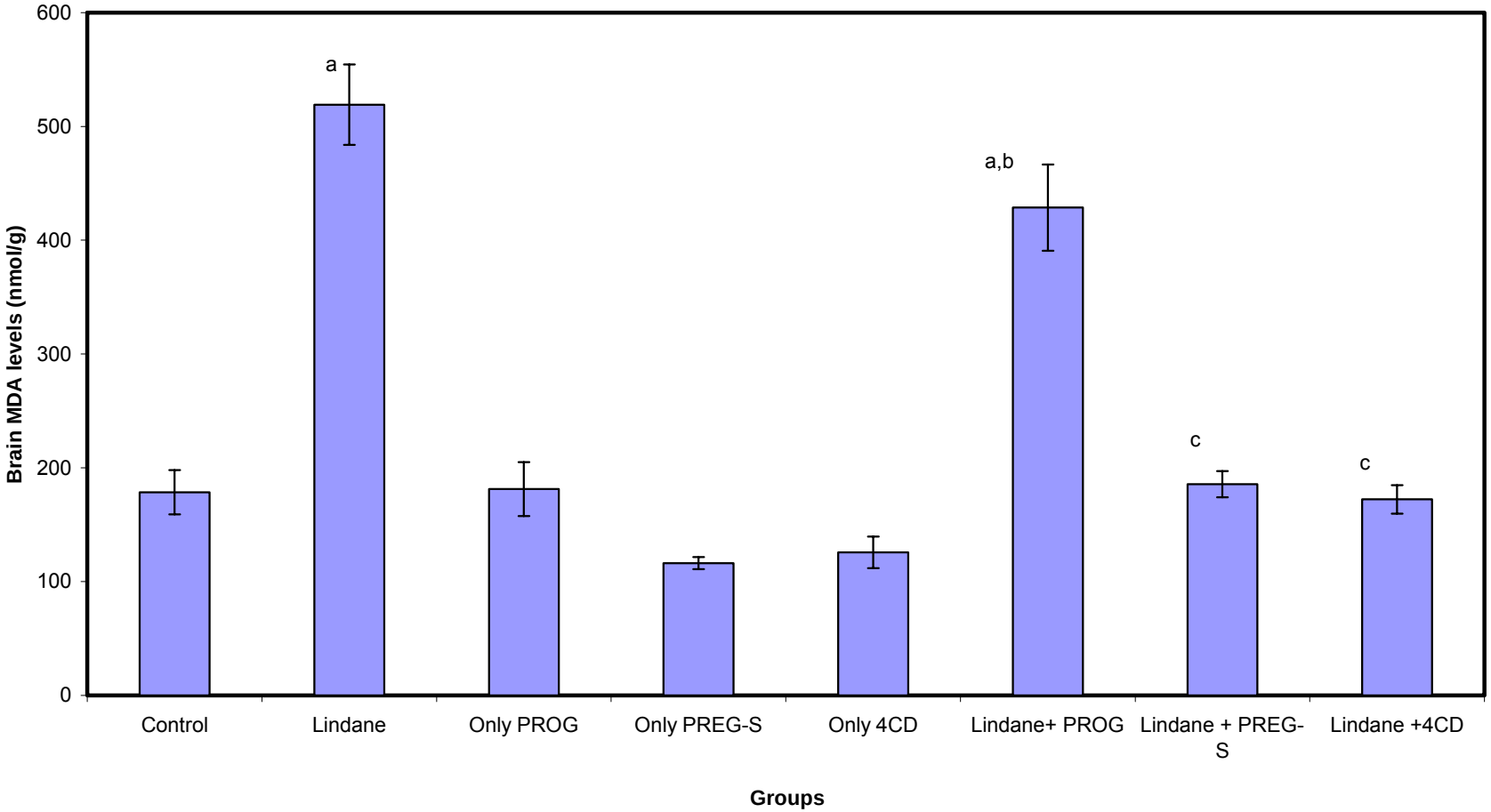

Fig 14 : Effect of lindane and NS on brain MDA levels

Intergroup variation found statistically significant, p< 0.001, df=7, F=43.280

[a] p<0.001 as compared to control group for lindane (repeated ANOVA followed by Tukey's test)

[b] p<0.001 as compared to only PROG group (repeated ANOVA followed by Tukey's test)

[c] p<0.001 as compared to lindane alone treated group (repeated ANOVA followed by Tukey's test)

Reduced glutathione (GSH)

Effect of lindane and neurosteroids on brain GSH level

A significant decrease was found in the brain GSH levels of lindane treated group as compared to control (p< 0.001). A significant increase was noted for PREG-S(p<0.001), 4CD(p<0.001), lindane+ PREG-S(p<0.01) and lindane + 4CD(p<0.001) treated groups vs. the control group for lindane as well. PREG-S and 4CD antagonized the effect of lindane on GSH. The difference between lindane alone treated group and lindane+ PREG-S and lindane+4CD group was found to be significant (p<0.001).No significant change was noted for only PROG treated group vs. the control for lindane as well as PROG+lindane vs. lindane alone treated group (Table 5; Fig 15).

Table 5: Effect of lindane and NS on brain GSH levels

Group	Treatment (6week+1week)	GSH (µg/g wet brain tissue) ± SE
Control	Vehicle for lindane+ vehicle for NS	413.6±27.75
Lindane	Lindane 15mg/kg/d p.o+ vehicle for NS	202.96±25.52[a]
Only PROG	Vehicle for lindane +PROG 15mg/kg/d i.p	457.13±35.37
Only PREG-S	Vehicle for lindane +PREG-S 2mg/kg/d i.p	648.42±31.87[a]
Only 4CD	Vehicle for lindane +4CD 0.5mg/kg/d i.p.	662.08±33.7[a]
Lindane+ PROG	Lindane 15mg/kg/d p.o+ PROG 15mg/kg/d i.p	264.41±29.2[a]
Lindane + PREG-S	Lindane 15mg/kg/d p.o+ PREG-S 2mg/kg/d i.p	610.55±29.04[b,c]
Lindane +4CD	Lindane 15mg/kg/d p.o+ 4CD 0.5mg/kg/d i.p.	627.55±33.94[a,c]

Intergroup variation found statistically significant p< 0.001, df=7, F=33.991

[a] p<0.001 as compared to control group for lindane (ANOVA followed by Tukey's test)

b p<0.01 as compared to control group for lindane (ANOVA followed by Tukey's test)

c p<0.001 as compared to lindane alone treated group

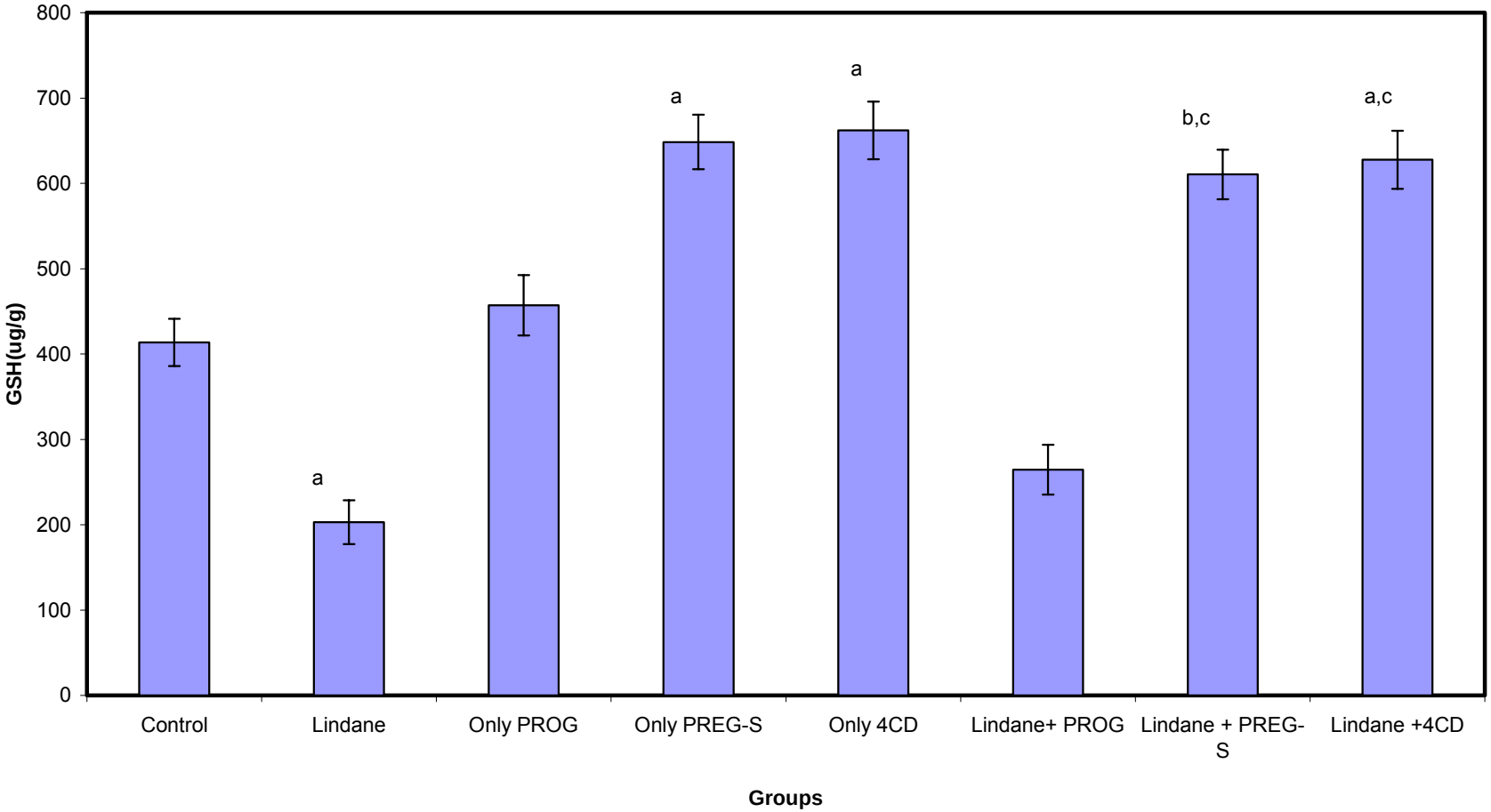

Fig 15 : Effect of lindane and NS on brain GSH levels

Intergroup variation found statistically significant p< 0.001, df=7, F=33.991

[a] p<0.001 as compared to control group for lindane (ANOVA followed by Tukey's test)

b p<0.01 as compared to control group for lindane (ANOVA followed by Tukey's test)

c p<0.001 as compared to lindane alone treated group(ANOVA followed by Tukey's test)

Discussion

Neurosteroids (NS) over the last few decades have been recognized as important modulators of neurological functioning. The influence of NS has been observed on several aspects of the functioning of the nervous system ranging from cognition to epileptogenesis and neuroprotection to embryogenesis[3,36]. NS such as PROG and AP have been shown to have a neuroprotective role on learning and memory impairment and promote neuroregeneration[7,13,93,94]. PREG-S has been described as one of the most potent memory enhancers. Fewer than 150 molecules of PREG-S have been shown to significantly enhance post-training memory processes when injected into the amygdale of rats[10,104].

The influence of NS over cognitive processes is mediated through several mechanisms. Dominant among the putative mechanisms are influence of NS over membrane receptors like NMDA, $GABA_A$, sigma (σ), kainate, glycine, serotonergic and neuropeptide receptors. Agonist of mitochondrial diazepam binding inhibitor receptor complex, Ro-5-4864 (4'-chlorodiazepam, 4CD) has been shown to increase the brain PREG synthesis without any effect on the blood PREG concentration. The ligands of this receptor facilitate the intramitochondrial flux of cholesterol thereby increasing the availability of cholesterol to cytochrome P450scc leading to an increased NS biosynthesis[8].

Lindane has been implicated to adversely affect memory and cognition. It has been demonstrated that it interferes with the ability of avoidance response with a single dose and increases the number of errors made in a food-reinforced maze on repeated exposure [30,143]. Lindane has effect on long-term potentiation (LTP) in the hippocampus, and it is possible that this effect may compete or interfere with the utilization of new information. It also inhibits activities of cerebral Na+, K^+- ATPase, Mg^{2+}- ATPase and AchE[142] and has been postulated to achieve its behavioral effects by acting on $GABA_A$ receptors. Lindane reportedly inhibits the conversion of cholesterol to PREG by inhibiting the enzyme P450scc[145]. It has been further claimed that lindane inhibits the activity of steroidogenic acute regulatory (StAR) protein, which mediates the rate-limiting and acutely regulated step

in hormone-regulated steroidogenesis, the intramitochondrial transfer of cholesterol to the P450scc enzyme[31,32].

The present study was therefore, designed to explore the influence of NS on cognitive dysfunction caused by lindane by measuring the step-down latency (SDL) in continuous avoidance paradigm and transfer latency (TL) in the plus-maze apparatus, a model of measuring the long-term memory. In view of the influence of oxidative stress over memory and the modulatory effects of both NS and lindane on the oxidative stress status *in vivo,* malondialdehyde (MDA) and reduced glutathione (GSH), parameters of oxidative stress were also assessed in the animals' brain at the end of the study period.

The results of this study demonstrate that SDL was reduced and TL prolonged on long-term (6 week) administration of lindane. This is in accordance with the data reported by previous workers demonstrating interference with memory and LTP on acute and chronic exposure to lindane[29,30,143]. Although a decrease in SDL was noted from week 4 onwards of lindane administration but it assumed statistical significance only in the week 6. This significant decrease was maintained in the 7th week i.e., 1 week after the discontinuation of lindane and administration of vehicle for NS. PREG-S and 4CD were able to reverse the impairment in memory caused by lindane when administered for 1 week following pretreatment with lindane for 6 weeks. However, PROG failed to reverse the memory dysfunction caused by lindane.

A decrease in brain PREG-S levels has been noted in AD patients and PREG-S has been described to be the most potent memory enhancer with capacity to enhance LTP. The putative mechanisms operating have been described to be trophic effects of PREG-S on neurons, glial cells and modulation of activity of a variety of neurotransmitter receptors and ion channels, including $GABA_A$, NMDA, σ receptors, N- and L-type Ca^{2+} channels and regulation of gene expression [9,10,95,104-106,108,109]. Enhancer of

steroidogenesis, 4CD, has also been shown to improve memory in various animal models. In one study it has been shown to facilitate the step down inhibitory avoidance on post-training administration. Although the dosage used was higher in their study (2mg/kg i.p) as compared to the doses in the present study (0.5mg/kg). However, it was given as a single dose in contrast to one week's administration in our study[110,111].

Influence of PROG over memory presents a confounding picture. Djebaili et al have demonstrated a decrease in cell death and cognitive deficits after experimental contusion to rat pre-frontal cortex by AP and PROG when used one day after injury[7]. Other workers have demonstrated a beneficial effect of hormone replacement therapy with estrogen and progesterone over episodic memory and verbal learning in post-menopausal women [98,161]. PROG inhibits the proliferation of astrocytes *in vitro* and this could have physiological implications in limiting the post traumatic gliosis in brain [113]. In contrast to the above observation in a recent study by El-Bakri et al, PROG failed to demonstrate any effect on NMDA receptor modulation and memory enhancement in ovarectomized rats[99]. Further, Grigorova and Sherwin failed to demonstrate any improvement in working memory and executive functioning of healthy elderly post-menopausal women taking hormone replacement therapy[100]. In ovarectomized rats, PROG reversed the beneficial cognitive effects of estradiol over spatial performance [101]. Lagrange has suggested that all the effects of PROG are due to its conversion to AP in the brain[55] and AP has been shown to disrupt memory, albeit in a concentration dependent manner [14]. In the present study, absence of any effect of PROG over memory could be due to several factors such as the dose used, duration for which it was administered, opposing effects on memory in different paradigms and also possibly failure to influence cognitive dysfunction caused by lindane due to pharmacodynamic influences.

A significant decrease in TL by lindane was evident from week 2 onwards, with a gradual progressive deterioration peaking at 6 weeks of administration. PROG, PREG-S and 4CD led to an improvement in the

scores for TL after 1 week of administration but it was not statistically significant. The probable reason for lack of effect of any test drug over TL could be the early onset of cognitive dysfunction detected by the test, which could not be reversed on 1 week of test drug administration. Possibly, a longer duration of administration and/or higher doses of these drugs may achieve an improvement in TL.

Lindane demonstrated induction of oxidative stress in brain, witnessed as reduction in brain GSH and increase in brain MDA levels. This result is in strong agreement with the findings of previous researchers exploring oxidative potential of lindane [33,34,146]. PREG-S and 4CD were able to reverse the effects of lindane over these two parameters while PROG failed to demonstrate any effect over the same. Neuroactive steroids protect retinal cells from oxidative stress and this effect is mediated by σ_1 receptors[26] PREG-S has been reported to influence σ_1 receptors, hence it is a possibility that anti-oxidant effects of PREG-S demonstrated in this study could, at least in part, be mediated through these receptors. Data on 4CD's role in antioxidant-oxidative stress specifically, is lacking but since 4CD can increase the production of several NS, many of which like DHEA have proven antioxidant capacities, the decrease in MDA and increase in GSH observed with 4CD administration in this study could be because of production of these NS. Griifith and Woolley demonstrated the exacerbation of toxicity of lindane and proconvulsant effect on combination of 4CD and lindane in rats[144] but in their study the dose of both lindane and 4CD used were several fold (2.6 and 20 times respectively) higher as compared to this study. Although PROG has been shown to inhibit lipid peroxidation, the lack of effect of PROG on MDA and GSH changes induced by lindane in this study could be due to the inadequacy of dosage used in this study to modulate lindane toxicity.

Oxidative stress has been closely related to cognitive dysfunction. Possible role of oxidative stress in pathogenesis of AD in humans as well as several animal models of AD and cognitive impairment has also been recognized

[148,150-152]. In the present study, lindane affected both the parameters of memory and oxidative stress adversely. Both PREG-S and 4CD demonstrated favorable effects on the two parameters of oxidative stress and SDL, a parameter of cognitive functioning. Hence, a co-relation between oxidative stress and memory impairment could be a possibility.

Summary & conclusion

Summary

Neurosteroids are recognized as important modulators of functioning of the nervous system. They have been recognized to influence several neurobehavioral processes including memory and cognition. Lindane, an organochlorine pesticide has been shown to adversely affect memory and induce oxidative stress on both acute and chronic exposure. The present study was thus designed to explore the modulation of effects of lindane over cognitive function by progesterone (PROG), pregnenolone sulfate (PREG-S) and 4'-chlorodiazepam (4CD).

Male Wistar rats were used for the study. The control groups were administered a) the vehicle in which the drugs were dissolved b) lindane and c) PROG, PREG-S or 4CD alone. The other three groups received PROG, PREG-S or 4CD following pre treatment with lindane for a period of 6 weeks. Cognitive function was assessed using step-down latency (SDL) on a passive avoidance apparatus and transfer latency (TL) on a plus maze weekly starting from one day before beginning of any treatment till the end of study period. Oxidative stress was assessed at the end of the study period by examining brain malondialdehyde (MDA) and reduced glutathione (GSH) levels.

1. Control group showed no change in cognitive function during the study period.

2. The group treated with lindane showed a decline in cognitive function as measured by increased TL and decreased SDL. This group also showed significant a increase in oxidative stress as witnessed by an increase in brain MDA and decrease in brain GSH levels.

3. PROG), PREG-S and 4CD treatment without lindane did not demonstrate any change in cognitive function over the study period. No change in MDA levels were observed as compared to the control group. However,

PREG-S and 4CD treatment group demonstrated a significant increase in brain GSH levels while PROG alone treated group did not show any significant change in the brain GSH levels.

4. PROG failed to alter the lindane induced changes in TL, SDL, MDA, GSH.

5. PREG-S or 4CD treatment following lindane administration for 6 weeks was able to reverse the cognitive impairment induced by lindane as shown by improvement in SDL. The two drugs also reversed lindane induced oxidative stress as demonstrated by the decreased MDA and increased GSH levels in these groups compared to lindane only treated groups.

6. Lindane induced impairment of TL was not modified PREG-S or 4CD treatment.

Conclusion

PREG-S and 4CD were able to reverse the lindane induced cognitive impairment at least in the Step down latency paradigm. The two drugs also reversed the derangement in oxidative stress parameters of MDA and GSH produced by lindane. PROG failed to influence memory impairment and oxidative stress induced by lindane. None of the drugs were able to modulate the changes induced by Lindane in the transfer latency paradigm. Our study reveals a possible correlation between memory impairment and oxidative stress in brain signifying yet another potential role of neurosteroids in the functioning of nervous system and possible use of this group of chemicals in reversing the damage induced by toxicants like lindane in the brain.

References

1. Baulieu EE, Robel P. Neurosteroids: a new brain function. J Steroid Biochem. Molec. Biol. 1990; 37: 395-403

2. Robel P, Baulieu EE. Neurosteroids: Biosynthesis and function. Trends Endocrinol Metab 1994; 5: 1-8.

3. Stoffel-Wagner B. Neurosteroid metabolism in the human brain. Eurpoean journal of endocrinology. 2001; 145: 669-79

4. Jo DH, Abdallah MA, Young J, Baulieu EE and Robel P. Pregnenolone, dehydroepiandrosterone and their sulfate and fatty acid esters in the rat brain. Steroids 1989; 54: 287-297

5. Majewska MD. Neurosteroids: endogenous bimodal modulators of the GABAA receptor. Mechanism of action and physiological significance. Prog Neurobiol 1992; 38: 379-395

6. Kulkarni SK, Reddy DS. Neurosteroids : a new class of neuromodulators. Drugs of today. 1995; 31: 433-55

7. Djebaili M, Hoffman SW, Stein DG.Allopregnanolone and progesterone decrease cell death and cognitive deficits after a contusion of the rat pre-frontal cortex. Neuroscience. 2004;123:349-59

8. Korneyev A, Pan BS, Romeo PE, Guidotti A, Costa E. Stimulation on brain pregnenolone synthesis by mitochondrial diazepam binding inhibitor receptor ligands in vivo. J Neurochem. 1993; 61: 1515-24

9. Weill-Engerer S B, David J-P, Sazdovitch V R, Liere P, Eychenne B, Pianos A et al. Neurosteroid Quantification in Human Brain Regions: Comparison between Alzheimer's and Nondemented Patients. J Clin Endocrinol Metab 2002; 87: 5138–43

10. Flood J F, Morley J E, Roberts E. Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. Proc Nati Acad Sci. 1992; 89:1567-71

11. Finn D A, Phillips T J, Okorn D M, Chester J A, Cunnigham C L. Rewarding effect of the neurosteroid 3α hydroxy 5α-pregnan-20-one in mice. Pharmacol Biochem Behav. 1997; 56: 261-64

12. Reddy DS, Kulkarni SK The effects of neurosteroids on acquisition and retention of a modified passive-avoidance learning task in mice. Brain Res. 1998 ;791:108-16

13. Vongher J M, Frye C A. Progesterone in conjunction with estradiol has neuroprotective effects in an animal model of neurodegeneration. Pharmacol. Biochem Behav. 1999;64:777-85

14. Johansson I M Birznievce V, Lindblad C, Olsson T, Backstom T. Allopregnanolone inhibits learning in the morris water maze . Brain Res 2002; 934: 125-31

15. Harrison NL, Simmonds MA. Modulation of the GABA receptor complex by a steroid anaesthetic. Brain Res. 1984 ;323:287-92

16. Wu FS, Gibbs TT, Farb DH. Inverse modulation of g-aminobutyric acid and glycine-induced currents by progesterone. Mol. Pharmacol. 1990; 41: 597-602

17. Paul SM, Purdy RH. Neuroactive steroids. FASEB J. 1992; 6: 2311-22

18. Birzniece V. Neuroactive steroids and rat CNS. Doctoral thesis. Umeå University, Umeå, Sweden. 2004

19. Valera S, Ballivet M, Bertrand D. Progesterone modulates a neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci U S A. 1992 ;89:9949-53

20. Fahey JM, Lindquist DG, Pritchard GA, Miller LG. Pregnenolone sulfate potentiation of NMDA-mediated increases in intracellular calcium in cultured chick cortical neurons. Brain Res. 1995 16;669:183-8.

21. Monnet FP, Mahe V, Robel P, Baulieu EE. Neurosteroids, via sigma receptors, modulate the [3H]norepinephrine release evoked by N-methyl-D-aspartate in the rat hippocampus. Proc Natl Acad Sci U S A. 1995 ;92:3774-8

22. Czlonkowska AI, Sienkiewicz-Jarosz H, Siemiatkowski M, Bidzinski A, Plaznik A. The effects of neurosteroids on rat behavior and 3H-muscimol binding in the brain. Pharmacol Biochem Behav. 1999 ;63:639-46

23. Czlonkowska AI, Krzascik P, Sienkiewicz-Jaros H, Siemiqtkowski M, Szyndler J, Maciejak P, Bidzinski A, Plaznik A. Rapid down-regulation of GABA-A receptors after pretreatment of mice with progesterone. Pol J Pharmacol. 2001;53:385-8.

24. Czlonkowska AI, Zienowicz M, Bidzinski A, Maciejak P, Lehner M, Taracha E, Wislowska A, Plaznik A. The role of neurosteroids in the anxiolytic,antidepressive- and anticonvulsive effects of selective serotonin reuptake inhibitors. Med Sci Monit. 2003 ;9:RA270-5.

25. Fontaine-Lenoir V, Chambraud B, Fellous A, David S, Duchossoy Y, Baulieu EE, Robel P. Microtubule-associated protein 2 (MAP2) is a neurosteroid receptor. Proc Natl Acad Sci. 2006;103:4711-6.

26. Bucolo C, Drago F, Lin LR, Reddy VN. Neuroactive steroids protect retinal pigment epithelium against oxidative stress. Neuroreport. 2005 ;16:1203-7.

27. Maayan R, Touati-Werner D, Ram E, Galdor M, Weizman A. Is brain dehydroepiandrosterone synthesis modulated by free radicals in mice? Neurosci Lett. 2005 ;377:130-5.

28. Khare SB, Rizvi AG, Shukla OP, Singh RP, Perkash O, Misra VD, Gupta JP, Seth PK. Epidemic outbreak of neuro-ocular manifestations due to chronic BHC poisoning. J Assoc Physicians India 1977; 25:215-222

29. Woolley D, Zimmer L, Zuheir H, Swanson K. Do some insecticides and heavy metals produce long-term potentiation in the limbic system? In: Cellular and Molecular Neurotoxicology (Narahashi T, ed). New York:Raven Press, 1984;45-69

30. Tilson H A, Shaw S, Mclamb R L. The effects of lindane and chlordecone on avoidance responding and seizure activity. Toxicology and Applied Pharmacology. 1987; 88: 57-65

31. Walsh LP, Stocco DM. Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol Reprod. 2000 ;63:1024-33.

32. Sujatha R, Chitra KC, Latchoumycandane C, Mathur PP. Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. Asian J Androl. 2001 ;3:135-8.

33. Koner B C, Banerjee B D, Ray A. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Indian J Exp Biol 1998 ; 36: 395-98

34. Sahoo A, Samanata L, Chainy G B N. Mediation Of oxidative stress in HCH- induced neurotoxicity in rat. Arch Environ Contam Toxicol 2000; 39: 7-12

35. Schumacher M, Robel P, Baulieu EE. Development and regeneration of the nervous system: a role for neurosteroids. Dev Neurosci. 1996; 18: 6-21

36. Mensah-Nyagan AG, Do-Rego JL, Beaujean D, Luu-The V, Pelletier G, Vaudry H. Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. Pharmacol Rev. 1999;51:63-81

37. Le Goascogne C, Robel P, Gouezou M, Sananes N, Baulieu EE, Waterman M. Neurosteroids: cytochrome P-450scc in rat brain. Science. 1987; 237: 1212-5

38. Jung-Testas I, Hu ZY, Baulieu EE, Robel P. Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. Endocrinology. 1989; 125:2083-91.

39. Mellon SH. Neurosteroids: biochemistry, modes of action, and clinical relevance. J Clin Endocrinol Metab. 1994 ;78:1003-8.

40. Ukena K, Usui M, Kohchi C, Tsutsui K. Cytochrome P450 side-chain cleavage enzyme in the cerebellar Purkinje neuron and its neonatal change in rats. Endocrinology. 1998 ;139: 137-47

41. Guarneri P, Guarneri R, Casico C, Pavasant P, Picoli F, Papadopoulous. Neurostroidogenesis in rat retinas. J Neurochem 1994; 63: 86-96

42. Compagnone NA, Bulfone A, Rubenstein JL, Mellon SH. Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. Endocrinology. 1995 ; 136:2689-96

43. Morfin R, Young J, Corpechot C, Egestad B, Sjovall j, Baulieu EE. Neurosteroids: Pregnenolone in human sciatic nerves. Proc Natl Acad Sci. 1992; 89: 6790-3

44. Do-Rego JL, Mensah-Nyagan AG, Feuilloley M, Ferrara P, Pelletier G, Vaudry H. The endozepine triakontatetraneuropeptide diazepam-binding inhibitor [17-50] stimulates neurosteroid biosynthesis in the frog hypothalamus. Neuroscience. 1998 ;83:555-70.

45. Saitoh H, Hirato K, Yanaihara T and Nakayama T. A study of 5a-reductase in human fetal brain. Endocrinol Jpn 1982; 29:461–467.

46. Melcangi RC, Celotti P, Castano P and Martini L. Differential localization of the 5a-reductase and the 3□-hydroxysteroid dehydrogenase in neuronal and glial cultures. Endocrinology 1993; 132:1252–1259.

47. Martini L, Celotti F and Melcangi RC. Testosterone and progesterone metabolism in the central nervous system: cellular localization and mechanism of control of the enzymes involved. Cell Mol Neurobiol. 1996; 16:271–282.

48. Negri-Cesi P, Colciago A and Celotti F. The role of aromatase in the brain, in Gennazzani AR, Petraglia AF and Purdy RH eds. The Brain: Source and Target for Sex Steroid Hormones. The Parthenon Publishing Group, London.1996; 135–49

49. Negri-Cesi P, Poletti A and Celotti F. Metabolism of steroids in the brain: a new insight into the role of 5a-reductase and aromatase in brain differentiation and functions. J Steroid Biochem Mol Biol 1996; 58:455–466.

50. Pelletier G, Luu-The V, Labrie F. Immunocytochemical localization of 5 alpha-reductase in rat brain. Mol Cell Neurosci. 1994 ;5:394-9.

51. Jenkins JS, Hall CJ. Metabolism of [14C]testosterone by human foetal and adult brain tissue. Journal of Endocrinology 1977; 74: 425-9.

52. Celotti F, Melcangi RC, Negri-Cesi P, Ballabio M, Martini L. A comparative study of the metabolism of testosterone in the neuroendocrine structures of several animal species. Neuroendocrinology Letters. 1986; 5: 227-36.

53. Stoffel-Wagner B, Watzka M, Steckelbroeck S, Wickert L, Schramm J, Romalo G, et al. Expression of 5□-reductase in the human temporal lobe of children and adults. Journal of Clinical Endocrinology and Metabolism 1998; 83: 3636-42.

54. Stoffel-Wagner B, Beyenburg S, Watzka M, BluÈmcke I, Bauer J, Schramm J, et al. Expression of 5□-reductase and 3□hydroxysteroid oxidoreductase in the hippocampus of patients with chronic temporal lobe epilepsy. Epilepsia 2000; 41: 140-7.

55. Lagrange A. Dancing the delta shuttle: neurosteroids regulate GABAA receptor expression. Epilepsy Currents. 2006; 6: 14-17

56. Gavish M, Bachman I, Shoukrun R, Katz Y, Veenman L, Weisinger G, Weizman A. Enigma of the peripheral benzodiazepine receptor. Pharmacol Rev. 1999 ;51:629-50

57. Papadoupolos V. Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: Biological role in steroidogenic cell function. Endocr Rev. 1993; 14: 222-40

58. Selye H. The anesthetic effect of steroid hormones. Proc Soc Exp Biol Med. 1941; 46: 116-21

59. Ganong WF. The gonads: development & function of the reproductive system In Review of Medical Physiology. Appleton & Lange. Stamford. 1997: 416

60. Mehta AK, Ticku MK. An update on GABAA receptors. Brain Res Brain Res Rev. 1999; 29:196-217

61. Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature. 1999; 401: 796-800

62. Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U. Molecular and neuronal substrate for the selective attenuation of anxiety. Science 2000; 290: 131-134.

63. Gulinello M, Gong QH, Li X, Smith SS. Short-term exposure to a neuroactive steroid increases alpha4 GABA(A) receptor subunit levels in association with increased anxiety in the female rat. Brain Res 2000; 910: 55-66.

64. Grobin AC, Morrow AL. 3alpha-hydroxy-5alpha-pregnan-20-one exposure reduces GABA(A) receptor alpha4 subunit mRNA levels. Eur J Pharmacol 2000; 409: R1-R2.

65. Concas A, Follesa P, Barbaccia ML, Purdy RH, Biggio G. Physiological modulation of GABA(A) receptor plasticity by progesterone metabolites. Eur J Pharmacol 1999; 375: 225- 235

66. Follesa P, Serra M, Cagetti E, Pisu MG, Porta S, Floris S, et al. Allopregnanolone synthesis in cerebellar granule cells: roles in regulation of GABA(A) receptor expression and function during progesterone treatment and withdrawal. Mol Pharmacol 2000; 57: 1262-1270.

67. Belelli, D., Lambert, J.J., Peters, J.A., Wafford, K., Whiting, P.J.,. The interaction of the general anesthetic etomidate with the gamma-aminobutyric acid type A receptor is influenced by a single amino acid. Proc Natl Acad Sci U S A1997; 94, 11031-11036.

68. Carlson BX, Engblom AC, Kristiansen U, Schousboe A, Olsen RW. A single glycine residue at the entrance to the first membrane-spanning domain of the gammaaminobutyric acid type A receptor beta(2) subunit affects allosteric sensitivity to GABA and anesthetics. Mol Pharmacol 2000; 57: 474-484.

69. Lambert JL, Belelli D, Hill-Venning C, Peters JA. Neurosteroids and GABAA receptor function. Trends Pharmacol Sci. 1995; 16: 295-303

70. Essrich, C., Lorez, M., Benson, J.A., Fritschy, J.M., Luscher, B.,. Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. Nat Neurosci 1998; 1, 563-571.

71. Follesa, P., Floris, S., Tuligi, G., Mostallino, M.C., Concas, A., Biggio, G.,. Molecular and functional adaptation of the GABA(A) receptor complex during pregnancy and after delivery in the rat brain. Eur J Neurosci 1998; 10, 2905-2912

72. Stell BM, Brickley SG, Tang CY, Farrant M, Mody I. Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated by delta subunit-containing GABAA receptors. Proc Natl Acad Sci U S A. 2003 25;100:14439-44

73. Mihalek, R.M., Banerjee, P.K., Korpi, E.R., Quinlan, J.J., Firestone, L.L., Mi, Z.P., et al. Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice. Proc Natl Acad Sci U S A 1999; 96, 12905-12910

74. Lundgren P, Stromberg J, Backstrom T, Wang M. Allopregnanolone-stimulated GABA-mediated chloride ion flux is inhibited by 3beta-hydroxy-5alpha-pregnan-20-one (isoallopregnanolone). Brain Res. 2003 22;982:45-53

75. Majewska MD, Schwartz RD. Pregnenolone-sulfate: an endogenous antagonist of the gamma-aminobutyric acid receptor complex in brain? Brain Res. 1987 24;404:355-60.

76. Jussofie A.: Brain area specific differences in the effects of neuroactive steroids on the GABA_ receptor complexes following acute treatment with anaesthetically active steroids. Acta Endocrinol., 1993, 129, 480–485.

77. Reddy DS, Rogawski MA. Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. J Pharmacol Exp Ther 2000; 295: 1241-48

78. Maguire JL, Stell BM, Rafizadeh M, Mody I. Ovarian cycle-linked changes in GABA(A) receptors mediating tonic inhibition alter seizure susceptibility and anxiety. Nat Neurosci. 2005 ;8:797-804

79. Shen H, Gong QH, Yuan M, Smith SS. Short-term steroid treatment increases delta GABAA receptor subunit expression in rat CA1 hippocampus: pharmacological and behavioral effects. Neuropharmacology. 2005 ;49:573-86

80. Malayev A, Gibbs TT, Farb DH. Inhibition of the NMDA response by pregnenolone sulphate reveals subtype selective modulation of NMDA receptors by sulphated steroids. Br J Pharmacol. 2002 ;135:901-9.

81. Horak M, Vlcek K, Petrovic M, Chodounska H, Vyklicky L Jr. Molecular mechanism of pregnenolone sulfate action at NR1/NR2B receptors. J Neurosci. 2004;24:10318-25

82. Weaver CE Jr, Marek P, Park-Chung M, Tam SW, Farb DH. Neuroprotective activity of a new class of steroidal inhibitors of the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A. 1997 ;94:10450-4.

83. Guarneri P, Russo D, Cascio C, De Leo G, Piccoli F, Guarneri R. Induction of neurosteroid synthesis by NMDA receptors in isolated rat retina: a potential early event in excitotoxicity. Eur J Neurosci. 1998 ;10:1752-63.

84. Mediratta PK, Gambhir M, Sharma KK, Ray M. Antinociceptive activity of a neurosteroid tetrahydrodeoxycorticosterone (5alpha-pregnan-3alpha-21-diol-20-one) and its possible mechanism(s) of action. Indian J Exp Biol. 2001 ;39:1299-301

85. Pathirathna S, Brimelow BC, Jagodic MM, Krishnan K, Jiang X, Zorumski CF, et al. New evidence that both T-type calcium channels and GABAA channels are responsible for the potent peripheral analgesic effects of 5alpha-reduced neuroactive steroids. Pain. 2005 ;114:429-43

86. Shiraishi M, Shibuya I, Minami K, Uezono Y, Okamoto T, Yanagihara N, Ueno S, Ueta Y, Shigematsu A. A neurosteroid anesthetic,

alphaxalone, inhibits nicotinic acetylcholine receptors in cultured bovine adrenal chromaffin cells. Anesth Analg. 2002;95:900-6

87. Mtchedlishvili Z, Kapur J. A presynaptic action of the neurosteroid pregnenolone sulfate on GABAergic synaptic transmission. Mol Pharmacol. 2003 ;64:857-64

88. Schiess AR, Partridge LD. Pregnenolone sulfate acts through a G-protein-coupled sigma1-like receptor to enhance short term facilitation in adult hippocampal neurons. Eur J Pharmacol. 2005 ;518:22-9

89. Britton KT, Koob GF . Neuropharmacology. Premenstrual steroids? Nature. 1998 ;392:926-30

90. Reddy DS, Kulkarni S K. Role of GABA-A and mitochondrial diazepam binding inhibitor receptors in the anti-stress activity of neurosteroids in mice. Psychopharmacology (Berl). 1996 ;128:280-92

91. Melchior C L, Ritzmann R F. Pregnonolone and Pregnonlone sulfate, alone and with ethanol, in mice on the plus maze . Pharmacol Biochem Behav. 1994; 48: 893-97

92. Rodgers R J, Johnson N J T. Behaviourally selective effects of Neuroactive steroids on plus maze anxiety in mice. Pharmacol Biochem Behav. 1998; 59: 221-32

93. Reddy D S, Kulkarni S K . Neuroprotective effects of neurosteroids against hypoxic neurotoxicity in naive and benzodiazepine inverse agonist FG 7142-treated mice. Indian J Pharmacol 1997; 29: 381-92

94. Thomas AJ, Nockels RP, Pan HQ, Shaffrey CI, Chopp M. Progesterone is neuroprotective after acute experimental spinal cord trauma in Rats. Spine. 1999 ;24:2134-8

95. Morley JE, Kaiser F, Raum WJ, Perry HM 3rd, Flood JF, Jensen J, Silver AJ, Roberts E. Potentially predictive and manipulable blood

serum correlates of aging in the healthy human male: progressive decreases in bioavailable testosterone, dehydroepiandrosterone sulfate, and the ratio of insulin-like growth factor 1 to growth hormone. Proc Natl Acad Sci U S A. 1997;94:7537-42

96. Marx CE, Trost WT, Shampine LJ, Stevens RD, Hulette CM, Steffens DC, et al. The Neurosteroid Allopregnanolone Is Reduced in Prefrontal Cortex in Alzheimer's Disease. Biol Psychiatry. 2006 Sep 22; [Epub ahead of print]

97. Malik AS, Narayan RK, Wendling WW, Cole RW, Pashko LL, Schwartz AG, Strauss KI. A novel dehydroepiandrosterone analog improves functional recovery in a rat traumatic brain injury model. J Neurotrauma. 2003 ;20:463-76

98. Yonker JE, Adolfsson R, Eriksson E, Hellstrand M, Nilsson LG, Herlitz A. Verified hormone therapy improves episodic memory performance in healthy postmenopausal women. Neuropsychol Dev Cogn B Aging Neuropsychol Cogn. 2006 ;13:291-307

99. El-Bakri NK, Islam A, Zhu S, Elhassan A, Mohammed A, Winblad B, Adem A. Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: relationship to Morris water maze performance. J Cell Mol Med. 2004 ;8:537-44

100. Grigorova M, Sherwin BB. No differences in performance on test of working memory and executive functioning between healthy elderly postmenopausal women using or not using hormone therapy. Climacteric. 2006 ;9:181-94

101. Bimonte-Nelson HA, Francis KR, Umphlet CD, Granholm AC. Progesterone reverses the spatial memory enhancements initiated by tonic and cyclic oestrogen therapy in middle-aged ovariectomized female rats. Eur J Neurosci. 2006 ;24:229-42.

102. Sato T, Tanaka K, Ohnishi Y, Teramoto T, Irifune M, Nishikawa T. Effects of estradiol and progesterone on radial maze performance in middle-agedfemale rats fed a low-calcium diet. Behav Brain Res. 2004 ;150:33-42.

103. Tanabe F, Miyasaka N, Kubota T, Aso T. Estrogen and progesterone improve scopolamine-induced impairment of spatial memory. J Med Dent Sci. 2004 ;51:89-98

104. Flood JF, Morley JE, Roberts E. Pregnenolone sulfate enhances post-training memory processes when injected in very low doses into limbic system structures: the amygdala is by far the most sensitive. Proc Natl Acad Sci U S A. 1995 ;92:10806-10

105. Schumacher M, Guennoun R, Robel P, Baulieu EE. Neurosteroids in the Hippocampus: Neuronal Plasticity and Memory. Stress. 1997 ;2:65-78

106. Maurice T, Phan VL, Urani A, Guillemain I. Differential involvement of the sigma(1) (sigma(1)) receptor in the anti-amnesic effect of neuroactive steroids, as demonstrated using an in vivo antisense strategy in the mouse. Br J Pharmacol. 2001 ;134:1731-41.

107. Darnaudery M, Pallares M, Piazza PV, Le Moal M, Mayo W. The neurosteroid pregnenolone sulfate infused into the medial septum nucleus increases hippocampal acetylcholine and spatial memory in rats. Brain Res. 2002;951:237-42.

108. Sliwinski A, Monnet FP, Schumacher M, Morin-Surun MP. Pregnenolone sulfate enhances long-term potentiation in CA1 in rat hippocampus slices through the modulation of N-methyl-D-aspartate receptors. J Neurosci Res. 2004 1;78:691-701.

109. Rupprecht R, Holsboer F. Neuropsychopharmacological properties of neuroactive steroids. Steroids. 1999;64:83-91.

110. Izquierdo I, Medina JH, Da-Cunha C, Wolfman C, Jerusalinsky D, Ferreira MB. Memory modulation by brain benzodiazepines. Braz J Med Biol Res. 1991;24:865-81

111. Da Cunha C, Huang CH, Walz R, Dias M, Koya R, Bianchin M, Pereira ME, Izquierdo I, Medina JH. Memory facilitation by post-training intraperitoneal, intracerebroventricular and intra-amygdala injection of Ro 5-4864. Brain Res. 1991 ;544:133-6.

112. Yu WH. Survival of motorneurons following axotomy is enhanced by lactation or progesterone treatment. Brain Res 1989; 491: 379-82

113. Jung-Testas I, Renoir JM, Bugnard H, Greene GL, Baulieu EE. Demonstration of steroid hormone receptors and steroid action in primary cultures of rat glial cells. J steroid Biochem Mol Biol 1992; 41: 621-31

114. Koenig N, Schumacher M, Ferzaz B, Do Thi AN, Ressouches A, Guennoun R, et al., Progesterone synthesis and myelin formation by schwann celss. Sci 1995; 268: 1500-3

115. Schumacher M, Guennoun R, Robert F, Carellu C, Gago NGhoumari A, et al. Local synthesis and dual action of progesterone in the nervous system: neuroprotection and myelination. Growth Horm IGF Res. 2004; 14: S18-33

116. Jung-Testas I, Schumacher M, Robel P, Baulieu EE. Demonstration of progesterone receptors in rat Schwann cells. J Steroid Biochem Mol Biol. 1996 ;58:77-82.

117. Kurata K, Takebayashi M, Morinobu S, Yamawaki S. beta-estradiol, dehydroepiandrosterone, and dehydroepiandrosterone sulfate protect against N-methyl-D-aspartate-induced neurotoxicity in rat hippocampal neurons by different mechanisms. J Pharmacol Exp Ther. 2004; 311: 237-45

118. Kimonides VG, Khatibi NH, Svendsen CS, Sofroniew Mv, Herbert J. Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotovxicity. Proc Natl Acad Sci U S A. 1998; 95: 1852-7

119. Rosciszewska D, Buntner B, Guz I, Zawisza L. Ovarian hormones, anticonvulsant drugs, and seizures during the menstrual cycle in women with epilepsy. J Neurol Neurosurg Psychiatry. 1986 ;49:47-51

120. Lonsdale D, Nylen K, McIntyre Burnham W. The anticonvulsant effects of progesterone and its metabolites on amygdala-kindled seizures in male rats. Brain Res. 2006 ;1101:110-6.

121. Kokate TG, Juhng KN, Kirkby RD, Llamas J, Yamaguchi S, Rogawski MA. Convulsant actions of the neurosteroid pregnenolone sulfate in mice. Brain Res. 1999 ;831:119-24.

122. Bitran D, Dugan M, Renda P, Ellis R, Foley M. Anxiolytic effects of the neuroactive steroid pregnanolone (3 alpha-OH-5 beta-pregnan-20-one) after microinjection in the dorsal hippocampus and lateral septum. Brain Res. 1999 ;850:217-24.

123. Roof RL, Hoffman SW, Stein DG. Progesterone protects against lipid peroxidation following traumatic brain injury in rats. Mol Chem Neuropathol. 1997 ;31:1-11

124. Tunez I, Munoz MC, Montilla P. Treatment with dehydroepiandrosterone prevents oxidative stress induced by 3-nitropropionic acid in synaptosomes. Pharmacology. 2005 ;74:113-8.

125. Brown RC, Cascio C, Papadopoulos V. Pathways of neurosteroid biosynthesis in cell lines from human brain: regulation of dehydroepiandrosterone formation by oxidative stress and beta-amyloid peptide. J Neurochem. 2000 ;74:847-59

126. Zisterer DM, Gorman AM, Williams DC, Murphy MP. The effects of the peripheral-type benzodiazepine acceptor ligands, Ro 5-4864 and PK 11195, on mitochondrial respiration. Methods Find Exp Clin Pharmacol. 1992 ;14:85-90.

127. Casellas P, Galiegue S, Basile AS. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem Int. 2002 ;40:475-86.

128. Ramade F. Ecotoxicologic. Paris: Masson, 1979

129. Menzer RE. Water and soil pollutants. In: Amdur MO, Doull J, Klaassen CO, eds. Casarett and Doulls Toxicology: The basic science of poisons, 4th ed. New York: Pergamon Press, 1991; 872-902.

130. Porter W. Do pesticides affect learning and behavior? The Neuro-endocrine-immune connection. Pesticides and You. 2004; 24: 11-5

131. Parmar D, Yadav S, Dayal M, Johri A, Dhawan A, Seth PK. Effect of lindane on hepatic and brain cytochrome P450 and influence of P450 modulation in lindane induced neurotoxicity. Food Chem Toxicol 2003; 41: 1077-87

132. Dekundy A, Kaminski RM, Turski WA. Dizocilpine improves beneficial effects of cholinergic antagonist in anticholinestrase-treated mice. Toxicol Sci 2003; 72: 289-95

133. Gupta RC, Milatovic D, Dettbarn WD. Nitric oxide modulates high energy phosphates in brain region of rat intoxicated with diisopropylphosphosfluoridate or carbofuran: Prevention by N-tert-butyl-alpha-phenylnitrone or vitamin E. Arch Toxicol 2001; 75: 346-56.

134. Lu AYH, West SB. Multiplicity of mammalian microsomal cytochrome P450. Pharmacol Rev 1979; 31: 277-295

135. Vidala LA, Barross BM, Junqueira VBC. Lindane induced liver oxidative stress. Free Radic Biol Med 1990; 9: 169-179

136. Dowla HA, Panemanglore M, Byers ME. Comparative inhibition of enzymes of human erythrocytes and plasma in vitro by agricultural chemicals. Arch Enviorn Contam Toxicol 1996; 31: 107-114.

137. Gromov LA, Seredi PI, Syrovatskal LP, Orinova GV, Filenenko MA. Free radical mechanism of memory disorder of toxic origin and experimental therapy of the conditions. Patol Fizol Ter 1993; 4: 24-26.

138. Banerjee BD, Seth V, Bhattacharya A, Pasha ST, Chakrabory AK. Biochemical effects of some pesticides on lipid peroxidation and free radical scavengers. Toxicol Lett 1999; 107: 33-47.

139. Anonymous. Lindane (Pesticide data sheet). Available online from < http://www.inchem.org/documents/pds/pds/pest12_e.htm> last accessed on 05/10/2006

140. Wooldridge, WF. The gamma isomer of hexachlorocyclohexane in the treatment of scabies. J Invest Dermatol 1948; 10:363-366 .

141. Woolley D, Zimmer L, Dodge D, Swanson K. Effects of lindane-type insecticides in mammals: unsolved problems. Neurotoxicology 1985; 6:165-192

142. Sahoo A, Samata L, Das A, Patra S K, Chainy G B N. Hexachlorhexane- induced behavioral and neurochemical changes in rats. J Appl Toxicol 1999; 19: 13-8.

143. Desi I. Neurotoxicological effects of small quantities of lindane: animal studies. Int Arch Arbeitsmed 1974; 33:153-162.

144. Griffith JA, Woolley DE. "Central" and "peripheral" benzodiazepines and kinetics of lindane-induced toxicity. Pharmacol Biochem Behav. 1989 ;32:367-76.

145. Sircar S, Lahiri P. Effect of lindane on mitochondrial side-chain cleavage of cholesterol in mice. Toxicology 1990; 61: 41-6

146. Junquiera V BC, Koch O R, Arisi A C M, Fuzaro A P, Azzalis L A, Barros S B M et al. Regression of morphological alterations and oxidative stress-related parameters after acute lindane-induced hepatotoxicity in rats. Toxicology 1997; 117: 199-205

147. Head E, Liu J, Hagen TM, Muggenburg BA, Milgram NW, Ames AB et al. Oxidative damage increases with age in a canine model of human brain aging . Journal of Neurochemistry. 2002; 82: 375

148. Aslan M, Ozben T. Reactive oxygen and nitrogen species in Alzheimer's disease. Curr Alzheimer Res. 2004 ;1:111-9

149. Lecanu L, Greeson J, Papadopoulos V. Beta-amyloid and oxidative stress jointly induce neuronal death, amyloid deposits, gliosis, and memory impairment in the rat brain. Pharmacology. 2006;76:19-33

150. Veinbergs I, Mallory M, Sagara Y, Masliah E. Vitamin E supplementation prevents spatial learning deficits and dendritic alterations in aged apolipoprotein E-deficient mice. Eur J Neurosci. 2000; 12:4541-6.

151. Sharma M, Gupta YK. Chronic treatment with trans resveratrol prevents intracerebroventricular streptozotocin induced cognitive impairment and oxidative stress in rats. Life Sci. 2002; 71:2489-98.

152. Parle M, Dhingra D. Ascorbic Acid: a promising memory-enhancer in mice. J Pharmacol Sci. 2003; 93:129-35.

153. Izquierdo I, Fin C, Schmitz PK, Da Silva RC, Jerusalinsky D, Quillfeldt JA, et al. Memory enhancement by intrahippocampal, intraamygdala, orintraentorhinal infusion of platelet-activating factor measured in an inhibitory avoidance task. Proc. Natl. Acad. Sci. 1995; 92: 5041-57

154. Mondadori C, Hengerer B, Ducret T, Borkowoski J. Delayed emergence of effects of memory-enhancing drugs: Implications for the

dynamics of long-term memory. Proc. Natl. Acad. Sci. 1994; 91: 2041-5

155. Joshi H, Parle M. Brahmi rasayana Improves Learning and Memory in Mice. eCAM 2006;3: 79–85

156. Itoh J, Nabeshima T, Kameyama T. Utility of an elevated plus-maze for the evaluation of memory in mice: Effect of nootropics, scopolamine and electroconvulsive shock. Psychopharmacology. 1990; 101: 27-33

157. Dhingra D, Parle M, Kulkarni SK. Effect of combination of insulin with dextrose, D(-) fructose and diet on learning and memory in mice. Ind J Pharmacol. 2003: 35: 151-56

158. Naidu PS, Singh A, Kulkarni KS. Quercitin and reserpine induced orofacial dyskinesia. Pharmacology 2004; 70: 59-67.

159. Okhawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 1979; 95: 351-8

160. Ellman GL. Tissue sulphydryl groups. Archv. Biochem. Biophy. 1959; 82: 70-7

161. Maki PM, Zonderman AB, Resnick SM. Enhanced verbal memory in nondemented elderly women receiving hormone-replacement therapy. Am J Psychiatry. 2001 ;158:227-33