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An investigation into the behavioural effects of polyamines, polyamine antagonsits and novel compounds in vivo.

Désirée H Murphy

A thesis submitted in partial fulfilment of the requirements
of the University of Dublin, Trinity College
for the degree of Doctor of Philosophy

Discipline of Pharmacology
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An investigation into the behavioural effects of polyamines and novel polyamine analogues *in vivo*. Désirée Helen Murphy.

The behavioural effects of polyamines and novel polyamine analogues were investigated in laca mice. Central administration of spermine (100μg icv) causes CNS excitation, which culminates in fatal tonic convulsions. The NMDA receptor antagonists, ifenprodil, eliprodil, arcaine, memantine and N^1-dansylspermine antagonised the effects of spermine. Two L-type calcium channel antagonists, nisoldipine and nitrendipine also were shown to inhibit spermine effects. Novel polyamine analogues, Bu 36b and Bu 43b illustrated the most inhibitory effects in the spermine CNS excitation model, while Bu 31b and Bu 40b were not as effective. Bu 33b and Bu37b did show some inhibition. These results suggest spermine effects may be mediated in part receptor through the NMDA and in part through L-type calcium channels. In the $ACTH_{1\text{-}24}$ model, behaviours including recurrent yawning, stretching, tail grooming, penile erections and excessive grooming were measured. Ifenprodil, nitrendipine, putrescine and Bu 31b reduced each behaviour. Eliprodil and Bu 33b reduced all behaviours except penile erections. Arcaine, Bu 37b and Bu 43b did not reduce yawning or penile erections, but reduced the other behaviours. Nisoldipine and Bu 40b only reduced stretching and excessive grooming. Only Memantine did not reduce yawning. DFMO did not reduce tail grooming, while N^1-dansylspermine did not reduce penile erections or grooming. The results showed that the behavioural effects of $ACTH_{1\text{-}24}$ could be attenuated through NMDA receptor and L-type calcium channel inhibition. The novel polyamine analogues also had an inhibitory effect. HPLC analysis of the polyamine levels from the two behavioural models showed that in the spermine model polyamine levels were increased with each stage from stages 1 to 4 and decreased in stage 5. In the $ACTH_{1\text{-}24}$ model, there was little increase in polyamine levels. It is likely that raised spermine levels in particular, contribute to the behavioural effects observed in the CNS excitation model, but it is unlikely that raised polyamine levels are directly involved in the behavioural effects observed in the $ACTH_{1\text{-}24}$ model. Standard behavioural techniques were employed to determine the effects of the novel compounds on their own. Bu 31b, Bu 43b and Bu 36b showed the most pronounced behavioural effects, while Bu 37b, Bu 33b and Bu 40b showed a lesser profile of effects. Polyamine-like effects such as pelvic elevation, 'tip-toe walking', hypothermia, tremor and convulsions were observed. These findings would suggest the novel polyamine analogues can inhibit spermine effects in vivo, but may also have some polyamine agonist-like effects. These compounds would need to be further investigated in other behavioural models, to assess possible therapeutic benefits in various disease states.

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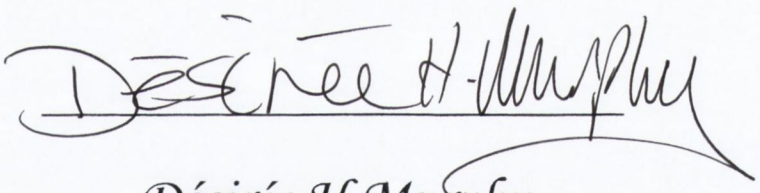

Désirée H Murphy

Summary:

The polyamines, putrescine, spermidine and spermine are ubiquitous compounents of eukaryotric cells in nature and are found in high concentrations in brain tissue. Polyamines have been implicated in cell growth, differentiation and nerve regeneration, but at high doses are neurotoxic. Administration of spermine (100μg icv) causes CNS excitation culminating in fatal tonic convulsions, within 8 hours. It is thought that spermine mediates this effect at least in part, through enhancement of activity on the NMDA receptor through positive modulatory extracellular binding site on the NMDA macrocomplex.

To further enhance understanding of the mechanism of action of polyamines the antagonist potential of NMDA receptor antagonist, calcium channel antagonists, N^1-dansylspermine and 6 novel polyamine analogues (Bu 31b, Bu 37b, Bu 33b, Bu 40b, Bu 36b and Bu 43b) from Brock University Canada, was assessed in the spermine CNS excitation model.

The investigation revealed that the NMDA antagonists ifenprodil, eliprodil, arcaine and memantine inhibited the spermine-induced CNS excitation. L-type calcium channel antagonists, nisoldipine and nitrendipine, also were shown to inhibit the development of spermine-induced convulsions. N^1-dansylspermine, a potent polyamine antagonist, also inhibited the effect of spermine. Of the novel polyamine analogues, Bu 36b and Bu 43b reduced the development of CNS excitation to the greatest degree. Bu 31b and Bu 40b did not have a pronounced inhibitory effect on spermine-induced convulsions. However, Bu 33b and Bu 37b did elicit an inhibitory effect on the development of spermine-induced CNS excitation.

The second focus this study investigated the behavioural effects of $ACTH_{1-24}$. Administration of $ACTH_{1-24}$ leads to the development of recurrent episodes of stretching and yawning, penile erections, tail grooming and excessive grooming. It is thought that

these effects could be mediated through ornithine decarboxylase activity, and therefore could be polyamine related.

This investigation examined all of the previously mentioned compounds, to see their inhibitory effect on the development of the $ACTH_{1-24}$ behaviours. The results revealed that ifenprodil at 30mg/kg reduced all the behaviours. Similarly eliprodil reduced all the behaviours except penile erections. Arcaine reduced stretching, tail grooming and grooming. Memantine reduced yawning, tail grooming, penile erections and grooming. The L-type calcium channels, nisoldipine reduced tail grooming and grooming, while nitrendipine reduced each behaviour. The polyamine putrescine also reduced all the behaviours to a very pronounced extent. DFMO reduced each behaviour except tail grooming. N^1-dansylspermine reduced yawning, stretching and tail grooming. The results for the novel compounds showed that Bu 31b produced the most inhibition by reducing each behaviour. Bu 33b was the next most effect as this inhibited each behaviour, except penile erections. Bu 37b and Bu 43b inhibited the development of stretching, tail grooming and grooming. Bu 40b reduced two behaviours tail grooming and grooming. The least effective compound was Bu 36b which only reduced grooming. It is interesting to note that the polyamine analogues did not show a similar rank order in potency in the two different behavioural models used.

There is uncertainty as to the mechanisms involved in the two behavioural models used, and the third part of this study used HPLC to investigate polyamine levels in homogenates of whole brain after spermine or $ACTH_{1-24}$ administration. In the spermine model all the polyamines were raised from stage 1 to 4 while stage 5 saw a decrease. These findings suggest that the development of spermine induced CNS excitation is caused by a cascade of events, in particular the metabolic transformation of polyamines into derivatives via the retro-conversion pathway and further polyamine synthesis and increasing overall polyamine levels. The results obtained for $ACTH_{1-24}$ showed little increase in polyamine level suggesting polyamines may not be involved in the development of $ACTH_{1-24}$ behaviours.

The final part of this study examined the behavioural effects of the novel compounds, Bu 31b, Bu 37b, Bu 33b, Bu 40b, Bu 36b and Bu 43b, on their own *in vivo*. Standard behavioural techniques included, locomotor activity, rotarod, weight, temperature and an adapted Irwin profile were used. The results showed that some of the compounds produced more behavioural effects than others. In particular, Bu 31b, Bu 43b and Bu 36b showed more behavioural effects generally, while Bu 37b, Bu 33b and Bu 40b showed least behavioural effects. The compounds did show many behavioural effects similar to the polyamines, including anorexia, hypothermia, tremor and 'tip-toe walking' behaviours.

Acknowledgements:

I would like to take this opportunity to pay a huge tribute to Dr. Karen Doyle, who was brought in under unfortunate circumstances. Without her knowledge, expertise, kindness, understanding and continuous encouragement this would not have been possible. I have enjoyed immensely working with Karen; it has been both a pleasure and honour. Not only has she been a great inspiration and mentor, but also a good friend. I know one person who is looking down on her and thanking her from the bottom of his heart.

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To my husband Mike, thank you for being so supportive and encouraging even when the monster would rise it's head on occasions. I am so looking forward to our lives and future together.

Finally, this is the most difficult part to write and to acknowledge, Professor Graham Shaw, to whom I now dedicate this work. I have been so lucky to have known you, even though it was only for a short time. On a professional level it was an honor and privilege

to have worked with you. You were a wonderful mentor, who made what we were doing exciting and fun, interesting and straightforward. You were an inspiration to me and to many others, I know Karen, Dr. Brian Kirby and Dr. Jun Lee, who too were students of yours, feel the same way. On a personal level, you became a very good friend and father figure. I remember our endless chats about the government, the state of the country, rugby, fishing and Africa. I think at times we put the world to right, certainly several political figures! My fondest memories of you are, your smiling face, endless good humour, modesty and gentleness, which are the rarest of qualities of any person, yet you possessed all of them with casual ease. To find even just one of these qualities in one person is rare, but to find all of them in a person, you Prof, is a very, very rare thing indeed, how very fortunate we are to have known you. Your passing has created a huge void that will never be replaced; you have enriched my life as well as many, many others by simply knowing you.

Thank you Prof.

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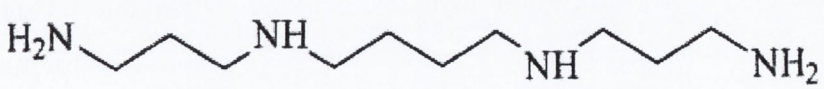
1.1 Introduction.

1.1.1 Background:

Van Lewenhoeck in 1677 discovered the existence of spermatozoa in seminal fluid and possibly the first indication of polyamines. Since then, the polyamines have been isolated from various tissues. The polyamines - putrescine, spermidine and spermine are found in tissues throughout the body (Seiler, 1991) (Figure 1.1). Polyamines found in the brain are synthesized within the brain, as there is limited exchange between the blood and the brain (Shin et al, 1985). The entire range of functions of the polyamines is not fully elucidated but they have been implicated in normal cell growth, differentiation and nerve regeneration (Dot et al, 2000).

Putrescine

Spermidine



Spermine

Figure 1.1: Structures of putrescine, spermidine & spermine, the common naturally-occurring polyamines.

Polyamines are aliphatic amines that are positively charged at physiological pH (Rock & Macdonald, 1995). The charged amine groups are distributed along the aliphatic carbon chain and the orientation and charge is important to the interaction of polyamines with nucleic acids and proteins (Rock & Macdonald, 1995). The amino groups are strongly basic, which enables the polyamines to form covalent bonds, thus modifying protein structure (Seiler, 1991). Polyamines carry a positive charge on each nitrogen atom along the entire length of the carbon chain. This positive charge enables polyamines to interact electrostatically with polyanionic macromolecules within the cell. Binding energy increases with the number of charges i.e. putrescine<spermidine<spermine (Seiler, 1991). This allows electrostatic interactions with DNA, RNA, proteins and negatively charged membrane constituents (Seiler, 1994). In addition polyamines can also interact with acidic phospholipids in membranes. Spermidine and spermine can bridge the major and minor grooves of DNA, either holding together two different molecules or two different parts of the same molecule (Mathews, 1993).

Polyamines, through electrostatic interaction with DNA, have the ability to alter DNA structure through a DNA-matrix interaction, suggesting they also influence function (Seiler, 1991 & Basu et al, 1993). In the nucleus, polyamine depletion results in partial unwinding of DNA, unmasking new sequences. These sequences are potential binding sites for factors which regulate transcription (Morgan, 1987). This may provide a mechanism whereby polyamines regulate the transcription of growth regulatory genes such as *c-myc* (Hampel, 1991). Studies have shown that polyamines interact with individual DNA molecules rather than multiple DNA molecules (Tabor and Tabor, 1984). It has been noted that polyamines interact with a high preference for pyrimidine residues such as thymidine. This may be influenced by neighboring nucleotides and the nature of secondary structures (Xaio, et al, 1991).

Spermidine and spermine increase membrane rigidity, through the formation of complexes with phospholipids and proteins. They also may prevent lipid

peroxidation, thereby having an antioxidant role (Tadolini, 1988). However polyamines have been shown to have more specific effects on neurons. Polyamines have been implicated in the regulation of membrane-bound enzymes, receptor ion channels such as, adenylate cyclase and ion channels, such as, N-methyl-D-aspartate, (NMDA), Inward rectifier potassium channels (Kir), and voltage-activated Ca^{2+} channels (Nichols et al, 1997; Johnson, 1996; Williams, 1997).

Electrostatically bound and free polyamines are in dynamic equilibrium. The true intracellular concentration of free polyamines is uncertain, but it is likely that they constitute only a very small fraction of total spermidine and spermine. More than likely the free, not total, polyamines are essential for the biological actions of the polyamines (Seiler, 1991).

1.1.2 Polyamines as Neurotransmitters & Modulators:

The accepted criteria for a substance to be established as a neurotransmitter require that the substance must be present in neurons, there must be a system for synthesis and inactivation of the compound, a specific release mechanism and evidence of biological actions (Shaw, 1994). The criteria for neuromodulation, is neurotransmission which has no measurable effect on membrane permeability per se, but modulates the responsiveness of a neuron to other inputs (Longstaff, 2000). The following sections summarise evidence supporting the possibility that polyamines may be neurotransmitters or neuromodulators in the CNS.

1.1.3 Distribution:

Polyamines are widely, but heterogeneously distributed in the brain (Shaw and Pateman, 1973). Tissue concentrations peripherally vary greatly, although the orders of magnitude are similar in all vertebrates (Seiler et al, 1994). Cells which are growing at a faster rate have higher polyamine concentrations than those which are not (Seiler et al, 1994). Putrescine levels range from 10nmol/g in the brain to

225nmol/g in the prostate. Spermidine levels range from 70nmol/g in skeletal muscle to 8890nmol/g in the prostate. Spermine levels range from 240nmol/g in skeletal muscle to 5670nmol/g in the prostate (Seiler et al, 1994). Due to the blood-brain barrier and the limited exchange of polyamines between the blood and brain, generally all of the polyamines found in the brain are synthesized in the brain itself (Russell and Meier, 1975). Within the brain, polyamine concentrations vary from location to location (Figure 1.2).

Brain area	Putrescine (nmol/g wet mass)	Spermidine (nmol/g wet mass)	Spermine (nmol/g wet mass)
Frontal cortex	9.4 ± 1.3	235 ± 22	221 ± 20
Rear cortex		368 ± 56	324 ± 50
Olfactory bulb	5.5 ± 0.4	446 ± 81	506 ± 88
Hippocampus	7.1 ± 1.2	420 ± 107	334 ± 64
Hypothalamus	22.9 ± 2.0	591 ± 109	235 ± 54
Striatum		420 ± 107	285 ± 38
Midbrain	6.2 ± 1.1	884 ± 176	237 ± 61
Medulla	3.7 ± 0.8	1016 ± 77	157 ± 33
Cerebellum	13 ± 1.3	674 ± 58	381 ± 47

Figure 1.2: The variation (mean ± SD) in the distribution of polyamines in different regions of the rat brain. Putrescine data (n=3) is adapted from Seiler and Schmidt-Glenewinkel, 1975. Spermidine and spermine data (n=40) is adapted from Al-Deen, 1991.

From Figure 1.2, it is apparent that the highest concentrations of spermidine are located in areas rich in white matter, in particular the brain stem. Due to this, spermidine was proposed to be a constituent of myelin (Seiler & Schmidt-Glenewinkel, 1975). Altered distribution of polyamines has been observed in myelin-deficient strains of mice (Russell and Meier, 1975). High spermine concentrations are found in the olfactory bulb, hippocampus, cerebellum and rear cerebral cortex (Figure 1.2). Putrescine concentrations in the mammalian brain are small by comparison (Figure 1.2), but much larger amounts of putrescine are found in fish brain and frog sciatic nerve (Seiler & Schmidt-Glenewinkel, 1975).

1.1.4 Polyamine Synthesis & Metabolism:

Synthesis:

Most cells synthesise polyamines, as they are required. Anuclear red blood cells cannot synthesise polyamines, and instead they accumulate polyamines through an uptake mechanism and participate in the transport and excretion of polyamines (Seiler, 1991). Within all other cells, putrescine is produced by the decarboxylation of ornithine in a reaction which is catalyzed by a rate-limiting enzyme ornithine decarboxylase (ODC). Therefore, polyamine levels are modulated by changes in ODC activity, which is predominantly regulated by the rate of synthesis of this enzyme, as it has a rapid turnover time of approximately 10 minutes (Rock & Macdonald, 1995). Spermidine and spermine are produced by the addition of aminopropyl residues. Spermidine is initially formed from putrescine and spermine from spermidine (Seiler, 1991). The aminopropyl residue is formed from methionine which reacts with ATP to produce S-adenosylmethionine (AdoMet), which is then decarboxylated to decarboxy-S-adenosylmethionine (dAdoMet). This compound donates the aminopropyl group to convert putrescine to spermidine, and spermidine to spermine, in reactions catalysed by spermidine synthase and spermine synthase respectively (Seiler, 1991). The second product of dAdoMet is 5'methylthioadenosine, which is produced in equimolar amounts during the

production of spermidine and spermine, and may then be used for the formation of ATP (Seiler, 1991) (see Figure 1.3).

Breakdown:

There are two pathways responsible for the breakdown of the polyamines, the retroconversion pathway and the terminal catabolic pathway (Seiler, 1991). The first step of the retroconversion pathway is the conversion of spermidine and spermine to their monoacetyl derivatives, N^1-acetylspermidine and N^1-acetylspermine respectively. Spermidine/spermine N^1-acetyltransferase (SSAT) catalyzes this reaction. The monoacetyl derivatives are substrates of polyamine oxidase (PAO). PAO splits the N^1-acetylpolyamines into putrescine (from spermidine) or spermidine (from spermine) and an aldehyde, 3-acetamidopropanal, which is subsequently oxidized to β-alanine. The polyamines produced by this process can be re-utilized for polyamine synthesis, indicating that polyamine synthesis and the retroconversion of polyamines can be a cyclic process (Seiler, 1991) (see Figure 1.3).

The terminal catabolic pathway involves an oxidative deamination reaction, whereby Cu^{2+}- containing amine oxidases are responsible for the oxidative deamination of polyamines resulting in the production of aldehydes, hydrogen peroxide and ammonia (Seiler, 1991). The aldehydes may be further metabolized by aldehyde dehydrogenase into amino acids avoiding aldehyde toxicity. In the absence of dehydrogenase, the aldehydes derived from spermidine and spermine can produce toxic acrolein through spontaneous β-elimination. Cu^{2+}- containing amine oxidizes are unevenly distributed in tissues and it is generally thought that these reactions do not participate in the regulation of cellular polyamine levels under normal conditions. However, the terminal catabolic pathway might have regulatory functions in specific tissues such as the intestine and placenta, where Cu^{2+}-containing amine oxidases have been found in high concentrations (Seiler, 1991).

A regulatory factor to polyamine breakdown is Antizyme (AZ). Antizyme (AZ), is expressed when there is an increase in intracellular concentrations of polyamines. AZ binds to ODC and the AZ-ODC complex is degraded by the 26 S proteasome, contributing to synthesis regulation. Antizyme inhibitor (AZI), liberates ODC in the presence of growth stimuli by virtue of having a higher affinity for AZ than ODC has (Nilsson et al, 2000). Additionally through down-regulating polyamine uptake, AZ can alter polyamine homeostasis. Three forms of AZ have been identified and characterized; AZ1 is strongly associated with ODC degradation; AZ2 has low capacity to induce ODC degradation while being more involved in negative regulation of polyamine transport (Zhu et al, 1999); and AZ3 is only found in testis germ cells, and only expressed at a certain stage of spermatogenesis (Ivanov et al, 2000).

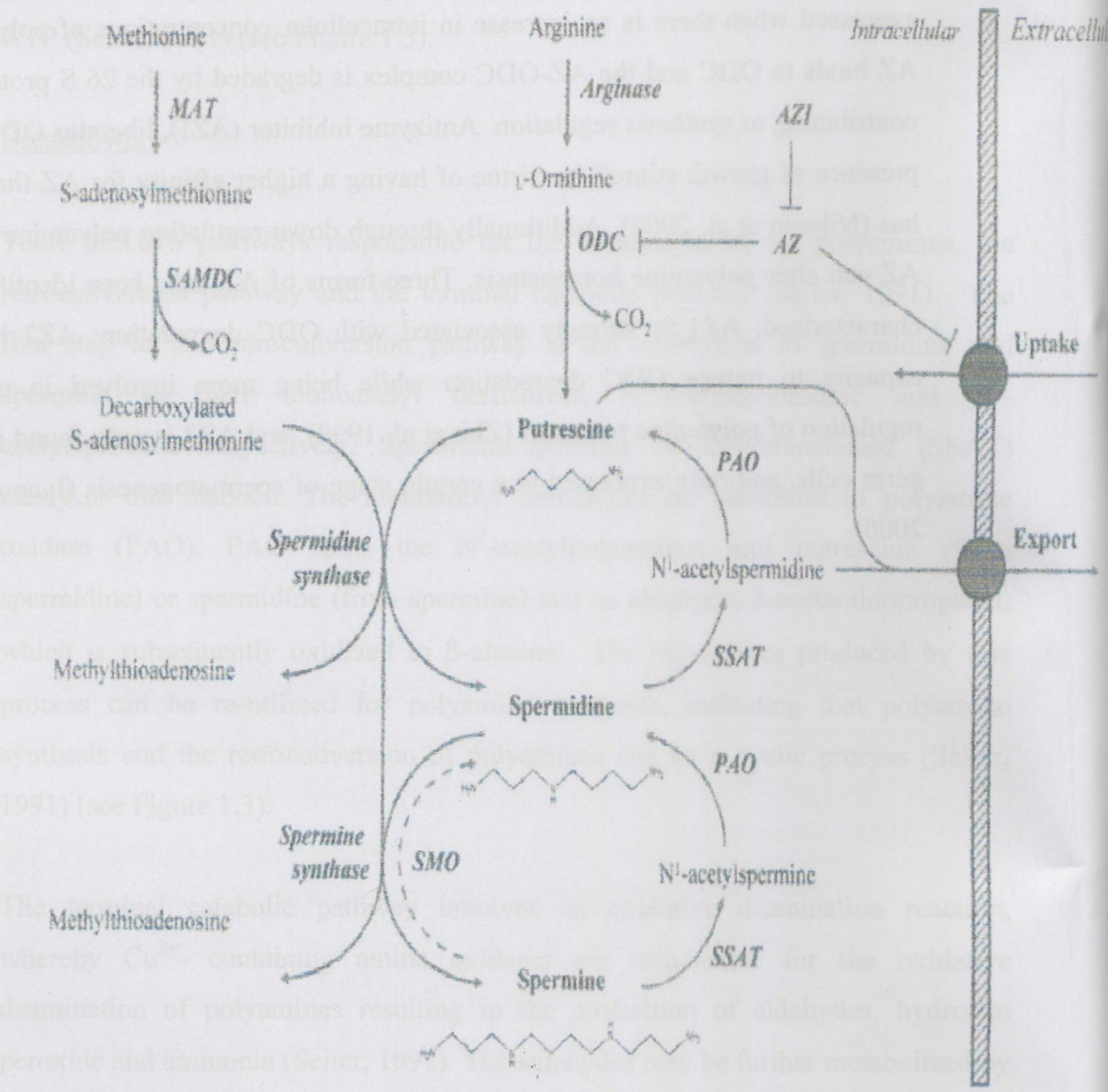


Figure 1.3 the reactions of polyamine synthesis, and breakdown through the retroconversion pathway. Also shown in this figure is the exchange of polyamines through the uptake and export channels in the membrane, between the intracellular and extracellular space of the cell. ODC: Ornithine Decarboxylase, PAO: Polyamine Oxidase, SSAT: Spermidine/spermine N^1-acetyltransferase, dcSAM: Decarboxylyated S-adenosylmethionine, SAMDC: S-adenosylmethionine Decarboxylase & SMO: Spermine Oxidase, AZ: antizyme, AZI: antizyme inhibitor, MAT: methionine adenosyltransferase. (Figure adapted from Wallace et al, 2003).

1.1.5 Polyamine Transport:

Polyamines from a dietary source absorbed by the gut contribute to the total polyamine pool and overall are major contributors to the total polyamine body content (Wallace et al, 2003). In recent years, much effort has been put into cloning the mammalian polyamine transporter, but unfortunately it has not yet being cloned. Yet it has been suggested that polyamine transport is a carrier-mediated, energy-dependent and saturable process (Seiler et al, 1990; Wallace et al, 2003). Recent evidence has led researchers to believe that some cells have a single carrier for all three main polyamines, while most cells types appear to have two classes of carriers: one with a preference for putrescine and the other for spermine and spermidine (Kashiwagi et al, 1992; Wallace et al, 2003). Selective uptake inhibitors have been found to deplete only two out of the three polyamines, i.e. spermidine and spermine (Wallace et al, 2003). Other studies have found that chemically synthesized analogues of the polyamines, such as Methylglyoxal-bis guanylhydrazone (MGBG), share the same polyamine uptake pathways in cells (Porter, 1985; Williams-Ashamn and Seidenfeld, 1986; Pegg, 1988).

Not only are polyamines taken up into cells, they also can be transported out of cells. Export is a selective process and is regulated by the cellular growth of the cell, i.e. export is switched on to decrease the cells growth rate and switched off in response to growth stimuli (Wallace and Keir, 1981). In the most part, the major polyamines exported from cells are N^1-acetylspermidine and putrescine, (see Figure 1.3). This is despite the fact that spermine is the predominant polyamine in the normal intracellular polyamine pool in human cells (Wallace et al, 1998). Therefore the evidence indicates that export is a selective and regulated process, with metabolism required before efflux (Wallace et al, 2003). Initial evidence with selective uptake inhibitors has indicated that the inward and outward transporters are separate and distinct, since the inhibitors of uptake had no effect on export (Wallace et al, 2003).

1.1.6 Polyamines & Ion Channels:

Recent recombinant receptor techniques and increased availability of polyamine analogues have provided better understanding of the mechanisms of action of the polyamines. Polyamines have been demonstrated to modulate the activity of voltage sensitive ion channels, in particular delayed rectifier K^+ and voltage-activated Ca^{2+} channels (Johnson, 1996).

Potassium channels:

Under physiological conditions, inward rectifier K^+ $(K_{ir})^{37-39}$ channels are used to maintain the resting membrane potential near the K^+ equilibrium potential (Williams, 1997). In excitable cells, neurons and muscle cells, inward rectifier K^+ channels are important for the control of excitability thresholds and shape the action potential (Nicolas et al, 1997). Intracellular magnesium ions block inward rectifier K^+ ion channels. However, block by Mg^{2+} cannot account for all gating and rectification properties (Williams, 1997). Recent studies have found intrinsic gating by, intracellular polyamines. This occurs at IRK1 and HRK1 channels, which are subunits of inward rectifier K^+ channels. Lopatin et al (1995), examined this mechanism of block of HRK1 channels. They proposed that molecules of spermine line up end-to-end to block a narrow pore that constitutes the K^+ conduction pathway. Spermine, spermidine and putrescine were active but the potency order is such that spermine is more potent that spermidine and considerably more potent than putrescine (Williams, 1997). The effect of the polyamines at blocking the inward rectifier K^+ channels is immediate and obvious, whereby they control the excitability of the resting membrane. An increase in polyamines will increase rectification of K^+ channels and increase cellular excitability, whereby reducing affinity at inward rectifier K^+ for polyamines altering membrane potentials. Increased polyamine levels have been reported in seizure activity (Doyle & Shaw et al, 1996), therefore it is conceivable that the increase in polyamines could contribute

to over-excitability in neurons through increase in the blockage of inward rectifier K^+ channels (Williams, 1997).

Calcium channels:

The increase in Ca^{2+} levels triggers a wide variety of physiological and pathophysiological events in neurons, such as vesicular neurotransmission, activation of ion channels, growth control, synapse development and expression of genes to mention a few (Scott et al, 1994). A sustained increase in intracellular Ca^{2+} will produce neuronal damage, generated by depolarization-induced Ca^{2+} influx from the extracellular environment mediated through voltage-activated Ca^{2+} channels (Scott et al, 1994). Ca^{2+} regulation dynamics in central neurons is complex. Ca^{2+} levels can rise in neuronal cytoplasm, but nerve cells are equipped to cope with excessive Ca^{2+} entry and can dispose of large quantities of Ca^{2+} (Mody et al, 1995). Interestingly, spider toxins have been used to investigate voltage Ca^{2+} channels. Polyamine toxins isolated from the venom of American spider *Agelenopsis aperta* have been found to interact with voltage-activated Ca^{2+}channels (Llinas et al, 1989). Polyamines have a modulatory action which may involve second messenger pathways rather than direct interaction with Ca^{2+}channels (Scott et al, 1994). The polyamine funnel web spider toxin (FTX) and ω-agatoxin GIVA contributed to the identification of a new calcium channel called P-type channel (P for purkinje) (Llinas et al 1989). Further to this there is strong evidence that polyamines modulate various cationic ion channels (Scott et al, 1993), including L-type calcium channels (Schoemaker, 1992).

Several investigations have examined the possibility of different calcium channel subunits. These investigations showed that calcium channels have various types of subunits, the known subunits are: α1 subunit (α1c, α1d, α1s) the L-type calcium channel has $Ca_v1.1$-1.4, $Ca_v1.2$-1.3. Dihydropyridines, nisoldipine and nitrendipine, bind to $Ca_v1.1$-1.4 prevent calcium influx. Phenylalkylamines, verapamil, bind to $Ca_v1.2$-1.3. The second L-type calcium channel is α2δ subunit. It is thought that

gabapentin may mediate its effects through binding to these $\alpha 2\delta$ subunit (Gee et al, 1996; Stefani et al, 1998). Polyamines have shown to interact with this site also (Dissanayake et al, 1997). While N-type voltage sensitive calcium channels have ($Ca_v2.2$) and P/Q-type are ($Ca_v2.1$) (Doyle et al, 2004). Gabapentin was shown to inhibit both N-type and P/Q-type presynaptic calcium channel activities (Bayer et al, 2004).

There are three main possible mechanisms of action for polyamine toxins on voltage-activated Ca^{2+} channels: 1. They bind directly to the Ca^{2+} channels: 2. They bind to lipid or other constituents of the cell membrane affecting the lipid micro-environment and altering channel properties or 3. By modulation of Ca^{2+} channels via secondary messenger pathways (Scott et al, 1994). Polyamine toxins including FTX, are thought to act directly on the calcium channels themselves (Llinas et al, 1989). Spermines action may involve its ability to cross-link phospholipids and stabilize membranes (Ballas et al, 1983). There is still a paucity of evidence that polyamines activate or inhibit secondary messenger pathways but, the possibility remains that polyamines may interact with membrane-spanning receptors or membrane-bound enzymes of G proteins (Scott et al, 1994). An increase in nitric oxide production, recently found to regulate voltage-activated Ca^{2+} currents in hippocampal neurons (Eccles et al, 1991). Adding to this Herman et al (1993), found that putrescine modulates Ca^{2+} currents via protein kinase C inhibition.

Further evidence has shown that spermine and spermidine may directly interact with Ca^{2+} channels. Spermine and spermidine allosterically inhibit the binding of the Ca^{2+} channel ligands diltiazem and nitrendipine to membranes, while putrescine is less effective (Schoemaker et al, 1992). Pullan et al (1990) further discovered that spermine and spermidine modulated ω-conotoxin GVIA binding to hippocampal synaptosomes, resulting in modulating its actions on N-type voltage sensitive calcium channels (Cav2.2) (Pullan et al, 1990).

Various calcium antagonists have been examined to determine a better understanding of the molecular mechanism of calcium channel inhibition. Dihydropyridines, nisoldipine and nitrendipine, bind to the α1subunit of the L-type calcium channel and prevent calcium influx, thus reducing excitation in cells (Shoemaker et al, 1992). More recent studies have found that nisoldipine and nitrendipine may directly block spermines action at L-type calcium channels or modulation of spermine induced effects through the NMDA receptor (Doyle et al, 2004). It has been proposed that nitrendipine directly interacts with the NMDA receptor in a similar manner to MK801 (Skeen et al, 1994). Nitrendipine has also been shown to inhibit NMDA induced and BAY K 8644 induced seizures (Palmer et al, 1993). Nisoldipine was shown to be more potent at inhibiting NMDA seizures than nitrendipine and verapamil (Palmer et al, 1993). Further studies have found a dual mechanism to spermine neurotoxicity which involved NMDA receptor activation at low concentrations and voltage sensitive calcium channel activity at high concentrations (D'Hooge et al, 2003).

It is possible that calcium channels, in particular L-type channels, may be involved in the development of spermine induced CNS excitation and seizures, although the exact mechanism is still elusive (Doyle et al, 2004).

1.2. Polyamine Interactions with Glutamate Receptors:

Glutamate is a major neurotransmitter in the mammalian nervous system. Pharmacological characterization of responses and molecular biological advances have led to the identification of different ionotropic and metabotropic glutamate receptors. Glutamate receptors mediate most of the excitatory neurotransmission in the CNS. Glutamate and related excitatory amino acids are toxic to central neurons (Ozawa, 1998). Excessive activation of glutamate receptors (GluRs), under stress to the brain, such as ischemia, head trauma and epileptic seizures leads to central neuronal death (Meldrum and Garthwaite, 1990). The ionotropic receptors include the *N*-methly-*D*-aspartate, (NMDA) receptor, α-amino-3-hydroxy-5-methyl-4-

isoxazole propionate (AMPA) receptor, kainate and quisqualate receptors (Watkins et al, 1990). Multiple subtypes have been identified for each of these receptors (Hollman and Heninemann, 1994).

1.2.2 AMPA Receptors:

Polyamine-like compounds can block the ion channels of some AMPA receptors (Williams, 1997). AMPA receptors have been cloned and it has been shown that the subunits include GluR1-GluR4 (also called GluRA-D). Native AMPA receptors are more than likely hetero-oligomers composed of a combination of GluR and KA subunits (Williams, 1997). Extracellular polyamines have a higher affinity at receptors containing the subunits with a glutamine at the Q/R (glutamine-arginine) site than subunits with an arginine at this site. This highlights the importance of the Q/R site in glutamate receptors for channel block and most importantly polyamine binding (Williams, 1997). The polyamines, spermine and spermidine, mediate inward rectification of Ca^{2+}-permeable AMPA receptors which have glutamine in the Q/R site (Bowie and Mayer, 1995). It is thought polyamines enter from the intracellular side at positive potentials and block ion flow when the Q/R site is occupied by glutamine (Ozawa, 1998).

The rectification of AMPA receptors is caused by intracellular factors likely to be polyamines. In one study spermine restored the intracellular surface of AMPA receptors which are Ca^{2+} permeable, while spermine has no effect on AMPA receptors which are not Ca^{2+} permeable (Williams, 1997). Spermidine can also block AMPA receptors but is much less potent than spermine whereas putrescine is virtually inactive. It has been proposed that the reduction of current flow through AMPA receptors by intracellular polyamines is due to direct blockage of the ion channel (Williams, 1997). It has been suggested that, the polyamines can actually permeate the ion channel of the receptor. Polyamines may enter the channel pore from the inside of the cell and pass through the channel to the outside of the cell (Williams, 1997).

1.2.3 Kainate Receptors:

Similarly to AMPA receptors, most native kainate receptors gate Na+ but are relatively impermeable to Ca^{2+} and have current-voltage I (isoleucine) –V (valine) site relationships that are close to linear. Kainate receptor subunits include GluR5-GluR7 and KA-1-KA2 (Bowie & Mayer, 1995). GluR5-GluR7 represent the low affinity kainate-binding site, while KA-1-KA2 correspond to the high affinity kainate-binding site (Ozawa et al, 1998). Kainate receptors expressed from the edited form of GluR6, GluR(6)R, do not show rectification and spermine had no effect on conductance through GluR(6)R channels (Bowie & Mayer, 1995). It has been suggested that Kainate receptors may be involved in epileptic seizures, due to the presynaptic action of kainate on mossy fiber terminals, which leads to a massive glutamate release (Ozawa et al, 1998). Kainate receptors in the foetal and neonatal nervous system may be very sensitive to polyamines. This in turn would have an impact on synapse formation, plasticity and neuronal loss during development (Williams, 1997).

1.2.4 Blockage of AMPA and Kainate Receptors: Physiological Relevance:

AMPA and kainate receptors that show rectification controlled by polyamines and which are Ca^{2+} permeable, are expressed on particular types of neurons and glial cells. Calcium entering cells through these channels could be involved in synaptic plasticity and neurotoxicity (Williams, 1997). Intracellullar polyamines can block AMPA and kainate channels by 18-36% at resting membrane potential (RMP). During synaptic activity, as the cell becomes depolarized, polyamine block increases and Ca^{2+} influx is reduced. A decrease in polyamine concentration would reduce the channel block, increase cell excitability and increase Ca^{2+} (Bowie & Mayer, 1995). It is thought that due to the lower affinity of polyamines for AMPA and kainite receptors rather than K^+ channels, fluctuations of polyamine concentrations in the micromolar range may have a more marked effect on activity

of AMPA/kainate receptors than strong inward rectifier K^+ channels (Williams, 1997).

1.2.5 N-methyl-D-aspartate receptor:

Many studies have been carried out to establish the role of excitatory amino acids, L-glutamate and L-aspartate, in the CNS. The discovery of excess glutamate neurotransmission during brain ischemia/hypoxia and hypoglycemia causing neuronal cell death sparked interest, particularly in the N-methyl-D-aspartate receptor. The NMDA receptor is widely distributed in the mammalian CNS, in particular in the superficial layers of the cerebral cortex, in the CA_1 field, and dentate gyrus of the hippocampus, in the granule cell layer of the cerebellum, and in the striatum, septum, thalamus and spinal cord (Scatton, 1993). The NMDA receptor is a macrocomplex with many binding sites attributed to it and also it contains an integral channel which gates Na^+, K^+ and Ca^{2+} ions (Scatton, 1993). The macrocomplex has many distinct recognition sites for endogenous and exogenous ligands:

1. a recognition site for endogenous agonists, glutamate and aspartate,
2. a co-agonist binding site for glycine,
3. a divalent cation binding site within the channel pore where Mg+ ions bind to produce a voltage-dependant channel block,
4. a divalent cation binding site near the external mouth of the channel where Zn^{2+} ions bind to produce another voltage-dependant block,
5. a binding site for dissociative anesthetics such as ketamine and phencyclidine, (PCP) and MK-801,
6. modulatory binding sites for polyamines (stimulatory and inhibitory) (Seiler, 1990; 1991; Williams 1990; 1991; Scatton 1993). See Figure 1.4.

Activation of the NMDA receptor by NMDA or glutamate (or aspartate) depends on the presence of glycine. Glycine is as necessary as glutamate for activation. Upon

activation, the ligand-gated ion channel allows the influx of calcium ions and a smaller proportion of sodium ions. Under normal physiological conditions the NMDA receptor is blocked by magnesium ions but this block is removed due to postsynaptic depolarization of the membrane, commonly by the activation of co-localised AMPA/Kainate receptors. The effect of polyamines on the NMDA receptor was first established in 1988 by Ransom and Stec, whereby they demonstrated that the polyamines, spermine and spermidine, increased the affinity of the binding site for [^{3}H] MK-801 in the presence or absence of glutamate and glycine. As MK-801 can only bind to its binding site when the ion channel is open (i.e. open channel blocker), this suggests that the polyamines are positive modulators of the NMDA receptor. It has been proposed that this enhancement of NMDA receptor activation by the polyamines is mediated through a novel site on the NMDA receptor, known as the stimulatory polyamine site (Ransom & Stec, 1988; Williams et al, 1991).

In the presence of saturated concentrations of glycine, spermine potentiates NMDA currents, increasing the frequency of channel opening. Spermine also increases the affinity of glycine for the NMDA receptor (Benviste and Mayer, 1993). Subsequently it was shown that high levels of polyamines might in fact inhibit the binding of radiolabelled channel blockers (Williams et al, 1989). Spermine also has the ability to inhibit the NMDA receptor through and action within the ion channel, which is voltage-dependant and is postulated to be due to a fast open channel block similar to a block caused by Mg^{2+} (Romano and Williams, 1994). Extracellular magnesium ions which cause a voltage-dependant block have also been shown to potentiate NMDA receptor activity similarly to spermine. Spermine has both stimulatory and inhibitory effects at the NR1A/NR2A and NR1A/NR2B receptors also (Williams 1994), whereby it has four mechanisms of action 1: glycine-independant stimulations, 2: voltage-dependent block, 3: an increase in the affinity for glycine and 4: increase in the affinity for NMDA and glutamate (Williams 1994).

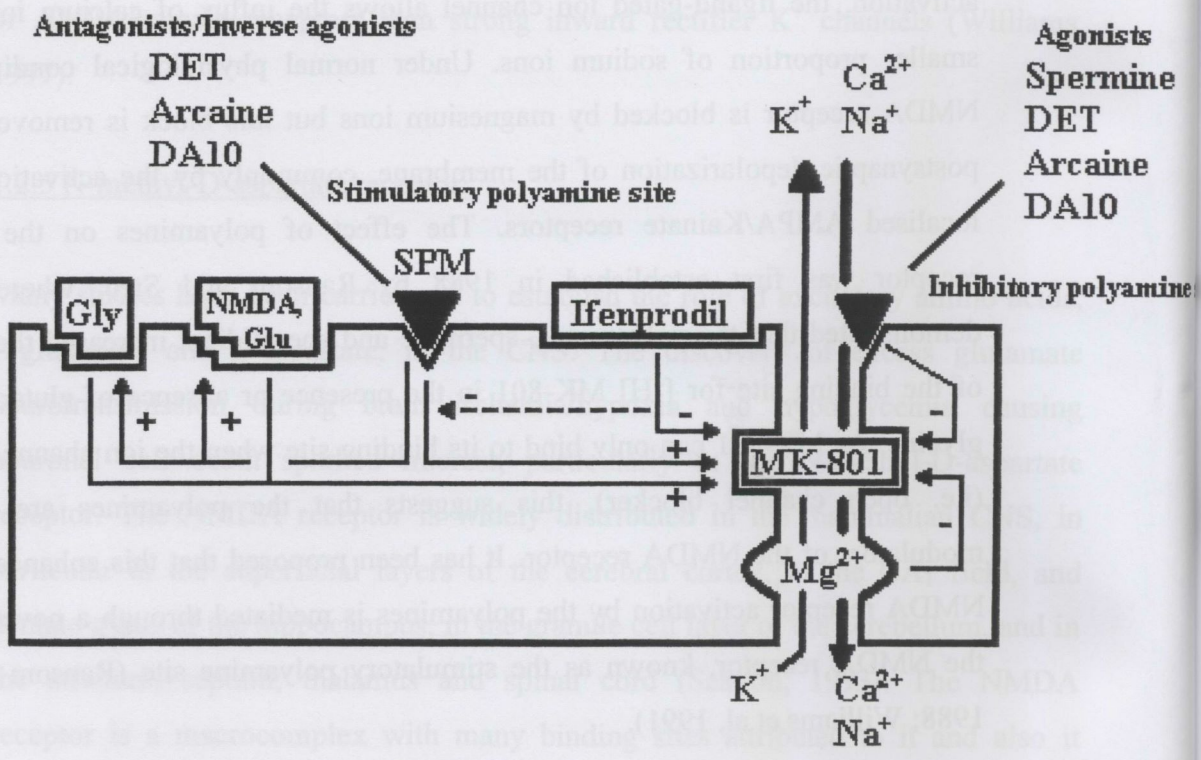


Figure 1.4 A schematic representation of the NMDA receptor. Glycine (Gly), Glutamte (Glu), Spermine (Spm), Diethylenetriamine (DET), 1,10-diaminodecane (DA10).Modified from: Romano, C. & Williams, K. (1994).

1.2.6 NMDA Receptor: Subunits and Distribution:

Several distinct NMDAR subtypes have been identified which has led to the development of determining the involvement of the specific subunits in synaptic transmission. The multiplicity of NMDAR subunits can be exploited for therapeutic advantage (Cull-Candy et al, 2001). Each NMDA receptor consists of a large extracellular N-terminal domain, three membrane spanning domains M1, M3 and M4, while M2 is a re-entrant loop (Cull-Candy et al, 2001; Stephenson, 2001). The cloning of NMDAR subunits has lead to the identification of 6 known subunits, NR1, NR2A-NR2D and NR3A. The NR1 subunit is ubiquitously expressed in

neurons throughout the brain whereas the different NR2 subunits have more restricted patterns of distribution (Cull-Candy et al, 2001; Stephenson, 2001). NR2A and NR2B mRNAs are expressed predominantly in forebrain, whereas the NR2C mRNA is the predominant NR2 subunit in cerebellum with NR2D being expressed mostly in spinal cord (Cull-Candy et al, 2001; Stephenson, 2001). Further, the glycine co-agonist binding site is localised to the NR1 subunit whereas the determinants for L-glutamate binding reside within the NR2 subunit. All this is consistent with native NMDA receptors consisting of both NR1 and NR2 subunits (Cull-Candy, et al 2001).

Imunoprecipitation with NR2 antibodies established the presence of NR1/NR2A in forebrain and cerebellum, NR1/NR2B was found predominantly in the forebrain, NR1/NR2C in the cerebellum and NR1/NR2D in the forebrain and the thalamus (Sheng et al, 1994; Blahos et al, 1996). Sheng et al (1994) showed that anti-NR2A antibodies resulted in precipitation of NR2B subunit immunoreactivity and vice versa thus suggesting the existence of NR1/NR2A/NR2B receptors. NR1, NR2B and NR2A subunits were all co-associated in the final immune pellet proving directly the existence of a NR1/NR2A/NR2B receptor (Chenard et al, 1999; Stephenson, 2001). When NR1, NR2A and NR2B subunits are co-expressed in mammalian cells, the result is a heterogeneous mix of NR1/NR2A, NR1/NR2B and NR1/NR2A/NR2B receptors (Stephenson, 2001). A summary of the distribution is in Figure 1.5.

Brain Region	Subunit complements
Forebrain	NR1/NR2A; NR1/NR2B; NR1/NR2D; NR1/NR2A/NR2B; NR1/NR2A/NR2D NR1/NR2A/NR2D
Cerebellum	NR1/NR2A;NR1/NR2C; NR1/NR2A/NR2C NR1/NR2A/NR2B
Thalamus	NR1/NR2D
Spinal Cord	NR1/NR2D

Figure 1.5: A summary of the subunit complements of native NMDA receptors (Adapted from Stephenson, 2001).

Further studies have found that the NR2 subunit influences the stimulatory effects of polyamines. There is no positive modulatory site for the polyamines on the NR1:NR2A subunit (Sharma and Reynolds 1999). Spermine did not block activity at the NR1/NR2A receptor subunit (Igarashi et al, 1997). This is due to the presence of the polyamine site on the NR2B receptor subunit (Williams, 1994; Chenard et al, 1999). Site directed mutogenesis has shown that in the M1 and M3 transmembrane domains are affectively blocked by spermine (Kashiwagi et al, 1997). Further studies indicated the ability of spermine to block NMDA receptors of up to potentials of -60mV (Araneda et al, 1999). However, the more negative potentials, the block was relieved, providing evidence of voltage dependant channel block by spermine, but which is weak and slow to develop in-comparison to extracellular Mg^{2+} (Williams et al, 1994). Figure 1.6 summarises the effects of spermine which

are controlled by the subunit composition in the NMDA receptor complex and pH (Johnson, 1996). Different subunit combinations are more than likely responsible for the different sensitivities to polyamines. Therefore the modulatory, either stimulatory or inhibitory, actions of spermine, will depend on different receptor subtype composition (Johnson, 1996).

NMDA receptor subunit combinations

Characteristic Effects of spermine	NR1A/ NR1A	NR1A/ NR2A	NR1A/ NR2B	NR1A/ NR2C	NR1A/ NR2D	NR1B / NR1B	NR1B/ NR2B
Glycine independent Stimulation	+	-	+	-	-	-	-
Glycine dependent Stimulation	+	+	+	-	-	+	+
Voltage dependent Inhibition	+	+	+	-	-	+	+
Decreased affinity for agonist	+	-	+	-	-	+	-
pH sensitivity	+	+	+	+	+	+	-

Figure 1.6: The subunit-specific effects of spermine and pH on recombinant NMDA receptors (Adapted from Johnson, 1996).

1.2.7 NMDA receptors in multiple pathologies:

Recent studies have investigated the benefit of using analogues in interactions with the subunits of the NMDA receptor. These findings may provide a platform in therapeutic intervention of chronic diseases. In particular the receptor subunit

which is providing much interest is the NR2-B-containing receptors. By using ifenprodil congeners, now second generation NR2B antagonists, CP-101,606 (Pfizer) and Ro 25,6981 (Roche) have been developed which have high affinity for the NR2-B receptors (McCauley, 2005 & Chazot, 2004).

NR2B is associated with pain both acute and chronic. Distribution of this subunit is found in the dorsal horn of the spinal cord. Nerve lesions significantly reduce NR2B expression, while NR2B knockout mice display hypersensitivity to acute noxious stimuli illustrating importance of the NR2B in acute nociception (Chazot, 2004). In a chronic pain model, examining the forebrain found that there was a selective over-expression of NR2B subunit protein which enhanced responsiveness to peripheral injection of inflammatory stimuli, indicating strong evidence of the NR2B-subunits playing a role in chronic pain (McCauley, 2005 & Chazot, 2004).

In Huntington's disease (HD) evidence has implicated NR2B activation as a trigger for degeneration of the medium spinal striatal neurons. Polyglutamine expansion in huntingtin genes impairs the ability of huntingtin protein to bind to its neuron element which elicits sensitization of NMDA receptors and potentiates glutamate-induced toxicity (Sun et al, 2001).

In schizophrenia the mRNA encoded NR2B subunit and NR1/NR2B-type binding sites are increased in hippocampal and cortical regions (Gao et al, 2000). More interestingly is the insight into bipolar disorder and the association with lithium, the neuroprotective mechanism of which has been illusive up to now. Concomittant with lithium neuroprotection in cortical neurons, NR2Btyr phosphorylation is greatly reduced, indicating this selective protein modification may be responsible for lithium protection in bipolar disorders (Hashimoto et al, 2002).

There is also evidence of a role for NR2B in further disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD). The synergistic evidence of a role for NR2B subunits in Parkinson's disease (PD) has been shown through a MPTP-

treated primate model, whereby using NMDA receptor antagonists prevented motor abnormalities elicited by nigrostriatal lesions and ameliorated nigral cell loss, indicating that manipulation of the glutamate system may produce anti-parkinsonian effects (Nash et al, 2004). This has been further emphasized with evidence that NR2B compounds may be synergistic with L-DOPA (Nash et al, 2004). Further to this NR1/NR2B receptors in the striatum, have been reported to be reduced in animal models of Parkinson's disease (Chazot, 2004; McCauley, 2005). In relation to Alzheimer's disease, recent approved therapies have allowed for a low affinity noncompetitive NMDA channel blocked namely memantine, to be used in this disease. Memantine exhibits different kinetic properties in comparison to other non-selective channel blockers which is why it is well tolerated (Lipton et al, 2004). Further to NMDA receptor subunits and disease, it has been shown that there is a lower level of NR2A and NR2B expression in affected areas within the brain in Alzheimer's disease, indicating NR2 subunit neuronal loss is associated with AD (Hynd et al, 2004).

NR2B subunit expression decreases with age correlating with reduced Long Term Potentiation (LTP) and inferior cognitive performance. NMDA receptors in the frontal cortex of aged humans are known to decrease, and the pharmacological properties indicate a loss of the NR1/NR2B subtypes as opposed to other receptor subtypes (Piggot et al, 1992). Over-expression of NR2B has profound benefits on cognitive performance. Increased synaptic strength via tetanic-induced LTP has been observed which selectively increases in NR2B try phosphorylation (Nakazawa et al, 2001).

There is strong evidence to support the notion of targeting NR2B subunit antagonists as potential therapeutic interventions, as there is such a strong correlation between the NR2B subunits in many diseases. Previous polyamine receptor antagonists such as ifenprodil and eliprodil have shown side-effects, which has hindered their full therapeutic development. Newer antagonists, CP-101, 606 (Pfizer) and Ro-265981(Roche) which are ifenprodil congeners, have shown similar

antagonist activity at the NR2B subunits receptor site to ifenprodil, with the added advantage of limited side-effects (Chazot, 2004; McCauley, 2005). The therapeutic promise of the NR2B subunit is due to a combination of; (1) the mode of action and (2) targeted molecular properties. These are summarized below (Chazot, 2004):

(1) Targeted mode of action of NR2B- directed compounds:
    a. Selective subpopulation of NMDA receptors
    b. Efficiency action at high agonist concentrations
    c. Efficiency action at low pH
    d. Potency high- even at depolarisation
(2) Targeted molecular properties:
    a. Distinct anatomical location on the NR2B
    b. Extrasynaptic location of NR2B in the neuron
    c. Importance of protein modification in CNS pathophysiology- NR2B tyrosine phosphorylation.

1.3 Behavioural activities of polyamines alone:

Polyamines are active compounds, which play significant roles in physiological and pathological conditions (Gimenez-Llort et al, 1996). Previous studies have found that polyamines produce a dose-dependant motor depression and toxicity (Gimenez-Llort et al, 1996). The assessment of motor activity consisted of measuring locomotion, rearing and intense grooming, thought to be mediated through the NMDA receptor (Gimenez-Llort et al, 1996), this suggests that the NMDA receptor is a target site for the pharmacological effects of polyamines (Gimerez-Llort et al, 1996). Other studies have also shown that the actions of polyamines on the central nervous system can cause sedation and hypothermia in both mice and rats (Anderson et al, 1975). Furthermore, administration of the polyamines, putrescine, spermidine or spermine, causes CNS excitation resulting in convulsions within a few hours after injection (Anderson et al, 1975).

Intracerebroventricular injection of spermidine and spermine initially causes sedation, hypomotility, tachypnoea, anorexia and adipsia in mice and intraventricular injection in rabbits produce the same effects. These effects may be reproduced through intraperitoneal doses of polyamines at much larger doses, confirming the limited penetration of the blood-brain barrier (Shaw, 1972; Anderson & Shaw, 1974; Anderson et al, 1975; Kohan et al, 1981). Spermidine and spermine have been shown to inhibit spontaneous climbing and wheel running, an action which is interpreted as modulation of limbic dopamine function (Hirsch et al, 1987). Apomorphine-induced stereotypy is not antagonised by polyamines; neither did polyamines produce asymmetries or circling (Hirsch et al, 1987). Following spermidine and spermine administration in mice, over a period of days there is development of quadriplegic paralysis (Anderson et al, 1975). This is shown to be a neurotoxic effect with its origin in bilateral lesions of the ventral medulla involving the pyramidal tracts and extending into the spinal cord (Anderson et al, 1975).

Further studies have examined the behavioural effects of polyamines individually. Putrescine was given to mice by intracerebroventricular injection (140μg). The onset of putrescines effects were slow to manifest, beginning 1-4 days after administration. These effects consisted of paralysis and fatal clonic convulsions (Anderson et al, 1975). Interestingly there was an increase in spermidine and spermine concentrations in the mice brains 24 hours after putrescine injection. The behaviours seen are similar to the behaviours when spermidine and spermine are administered. It is not unreasonable to suggest that these behaviours could be attributed to the increases in spermidine and spermine (Anderson et al, 1975).

Apart from the behaviours already mentioned, spermidine also produced several other behavioural effects. In mice, it was shown that spermidine (50μg) on day 2, developed ataxia in the form of abnormal high carriage. By day 3 and 4 a flaccid paralysis developed resulting in quadriplegia. The animals eventually became moribund and died (Anderson et al, 1975). Similar behaviours were reported in mice by Doyle and Shaw (1994), with the development of a high carriage or abnormal

gait with splayed digits, leading to a "tip-toe walking" behavior, which involves an arched back and severe splaying of digits when walking. In rabbits, spermidine i.c.v. administration (250μg or more), caused sedation and rapid and shallow respiration and a temperature drop. After 1-2 days, muscle weakness was seen whereby the animal's hind limbs could no longer move. Over a week, animals became emaciated but remained alert until near death. Spermidine at doses 1mg i.p or higher produced convulsions in rabbits (Anderson et al, 1975).

Spermines behavioural effects were more pronounced than putrescines and spermidines. In mice, spermine at doses as low as 10μg, caused hyperexcitability, appearing in the form of a tremor, and tonic convulsions which gradually increased in frequency and severity resulting in a fatal tonic spasm (Anderson et al, 1975). During interseizure periods a lethal tonic spasm could be precipitated by a sudden noise or picking the animal up by the tail. Doyle et al (1996), illustrated that the injection of 100μg of spermine i.c.v results in two distinct phases of behaviours. Phase one occurs within minutes of injection, which consists of scratching of the upper body, frequent face washing and some clonic convulsions which lasted for about 1 hour. The second phase began after 2 hours post injection and consisted of the development of a body tremor which worsened with time and culminated in a fatal tonic convulsion within 8 hours (Doyle and Shaw, 1996; 1998). It is thought that these two phases are mediated, at least in part, through the stimulation of the NMDA receptor macrocomplex, in particular the interaction with the polyamine binding sites, especially the positive polyamine modualtory site on the NMDA receptor (Doyle et al, 1996; 1998; Kirby et al, 2004). Mentioned already Ransom and Stec (1988) were the first to demonstrate a positive modulatory binding site for polyamines on the NMDA receptor. Subsequently, Sacaan and Johnson (1990) found a second polyamine site, a negative modulatory site. It is accepted that polyamines interact directly with the NMDA receptor at these binding sites, enhancing the effects induced by NMDA receptor stimulation and potentiating the convulsant properties of NMDA (Singh et al, 1990). Spermine very rarely produces

paralysis in mice, (1 in 300 animals). In contrast, in rabbits, spermine did produce paralysis (Anderson et al, 1975).

1.4 AdrenoCorticoTropic Hormone (ACTH):

ACTH is a single chain 39-amino acid containing peptide. The first 23 amino acids of the peptide contain the biological active residues, and the synthetic 1-23 amino acid peptide (Cortrosyn) has full activity. The structure of ACTH has species differences in the positions 25, 31 and 33, (human, bovine and ovine respectively) (www.nicholsdiag.com). Pro-opiomelanocortin (POMC) gives rise to 4 members of a family of anterior pituitary hormones (Magiakou et al, 1996). Each of these hormones are formed by the proteolytic processing of the proopiomelanocortin (POMC) molecule: producing, ACTH, ß-lipotropin, ß-Melanocyte Stimulating Hormone (ß-MSH) and ß-endorphin (Boron & Boulpaep, 2003). POMC is hydrolyzed to ACTH and ß-lipotropin (ß-LPH) which are then released into circulation. It is possible that ACTH and ß-LPH may in turn be cleaved to form a variety of peptides including ßendorphin (an endogenous opioid), and alpha- and ß-melanocyte stimulating hormone (alpha- and ß-MSH) which stimulate melanin synthesis causing darkening of the skin (Boron & Boulpaep, 2003). (Figure 1.7).

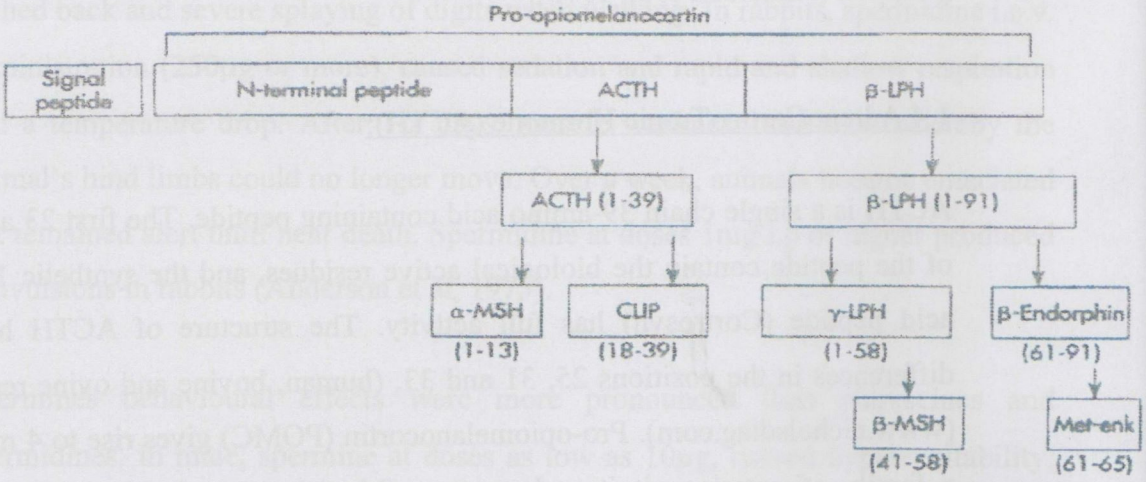


Figure 1.7 Synthesis of ACTH and the related peptides in the anterior pituitary.(Figure taken from Boron and Boulpaep, 2003).

ACTH is secreted in irregular pulses throughout the day which cause parallel increases in plasma cortisol. These small pulses are superimposed on a characteristic, diurnal fluctuation of greater amplitude (Magiakou et al, 1996). In normal individuals, ACTH reaches a peak in the early morning (6-8am) and levels become lowest late in the day and near the beginning of the sleep period (Magiakou et al, 1996). The adrenal cortisol secretion rate under basal conditions is 12-15mg/m^2 per day (Magiakou et al, 1996).This early morning increase in ACTH release is initiated by the release of CRH (corticotropin releasing hormone) and starts approximately a couple of hours before waking. The lowest levels of ACTH in blood occur just before or after falling asleep (Magiakou et al, 1996). This results in the characteristic diurnal rhythm in ACTH and cortisol secretion. The synthesis of cortisol and other steroids by the zona fasciculata and reticularis of the adrenal gland is controlled by adrenocorticotropic hormone (ACTH) (Magiakou et al, 1996).

Abnormal changes in cortisol levels occur due to hypothalamic, pituitary or adrenal malfunction. Disorders such as Cushing's Syndrome (hypercortisolemia) and

Addison's Disease (primary adrenal insufficiency) can lead to severe metabolic imbalance, which could be life-threatening (Hsu, 1983). The measurement of urinary serum or plasma cortisol, utilizing morning and evening samples and/or stress tests such as ACTH stimulation or dexamethasone suppression aids in the diagnosis of adrenal related disease (Hsu, 1983).

1.4.1 Control and regulation of ACTH secretion:

Adrenocorticotropic hormone (ACTH) or corticotropin is secreted by corticotropes in the pituitary gland, to regulate the production of steroid hormones by the adrenal cortex (Marieb, 1995). In the adrenal cortex, ACTH promotes the secretion of glucocorticoids and androgens, and mineralocorticoids to a lesser extent (Webster and Sternberg, 2004). ACTH secretion is under stimulatory control by corticotropin releasing hormone (CRH). Control of ACTH secretion from the anterior pituitary involves both a negative feedback mechanism and a CNS-stress mediated control system (Webster and Sternberg, 2004). Stress may include trauma, infection, intense heat or cold, or mental effort. Within the anterior pituitary there are six types of cells, which are named after the primary peptide/protein hormones they produce: ACTH (adrenocorticotropin), TSH (thyroid stimulating hormone; thyrotropin), FSH and LH (follicle stimulating and luteinizing hormone or gonadotropins), GH (growth hormone or somatotropin) and prolactin (lactotropin) (Marieb, 1995). Upon stimulation of the anterior pituitary by hypothalamic hormones, these adrenal hormones are released and diffuse into the portal capillary bed (Adinoff et al, 1998).

The hypothalamus-pituitary-axis (HPA) axis is stimulated by bidirectional communications from various stimulants (Webster et al, 2004). Some CNS responses which in turn stimulate CRF secretion are promoted from substances such as stimulatory neurotransmitters i.e. glutamate, mediators of inflammation i.e. cytokines (Mulla & Buckingham 1999), and CRH itself (Adinoff et al, 1998). In addition, cortisol modulates its own release i.e. feedback inhibition, which dampens potentially excessive HPA response. A negative feedback system controls circulating cortisol which feeds back to the hypothalamus and pituitary to inhibit the

secretion of CRH and ACTH. CRH secretion is decreased by inhibitory neurotransmitters e.g., gamma-aminobutyric acid [GABA]. In addition, endogenous opioids provide continuous i.e. tonic, background inhibition of CRH secretion. Naloxone suppresses inhibition of CRH secretion by opioids; insulin administration lowers blood sugar, activating ACTH and cortisol secretion (Adinoff et al, 1998) (Figure 1.9). ACTH is involved in steroidogenesis. The stimulation is mediated by activation of adenylyl cyclase. Cyclic AMP activates protein kinase A, which in turn activates cholesterol ester hydrolase. More free cholesterol is formed and converted to pregnenolone in the mitochondria (Boron & Boulpaep, 2003).

In response to stimulation from the brain, the cells of the paraventricular nucleus of the hypothalamus produce corticotropin releasing hormone (CRH) and/or arginine vasopressin (AVP) (Rivier, 1996; Webster, 2002). CRH and AVP are then channeled directly in the hypophyseal blood supply, to the pituitary gland situated just beneath the hypothalamus (Webster et al, 2004). Within the pituitary, these two hormones act together to stimulate the release of adrenocorticotropic hormone (ACTH). ACTH arrives at the adrenal cortex via the bloodstream, where it stimulates the secretion of cortisol (Adinoff et al, 1998). Cortisol then travels through the bloodstream, exerting effects on multiple organs and tissues. The HPA axis incorporates a system of controls that dampen its own activation (i.e. a negative feedback system). As previously mentioned, the hypothalamus and the pituitary gland are sensitive to inhibition by cortisol. Thus, when activation of the stress response produces increases in CRH and ACTH, the resultant elevation in cortisol, after a time delay, suppresses further CRH and ACTH production (Adinoff et al, 1998). ACTH increases the synthesis and release of all adrenal steroids, aldosterone, cortisol and adrenal androgens. It is the principal modulator of cortisol, the primary glucocorticoid in man. As the effective level of cortisol in the circulation rises, release of ACTH is inhibited directly at a pituitary level. Through this same mechanism, falling cortisol levels lead to elevated ACTH levels *(www.nicholsdiag.com)* see Figure 1.8.

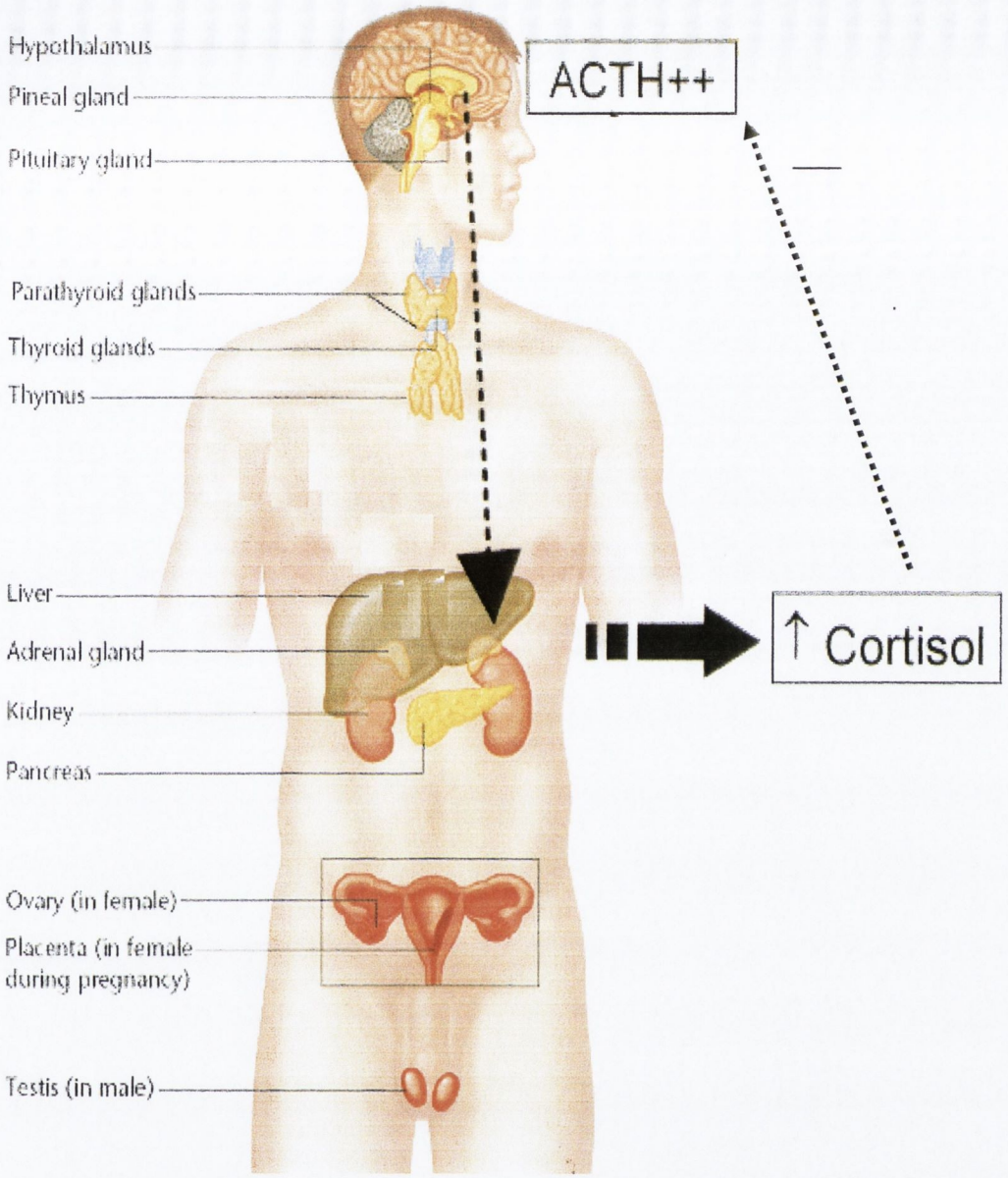


Figure 1.8 The Endocrine system: ACTH location and action. (Figure adapted from www.nicholsdiag.com).

1.4.2 Regulation of the hypothalamic-pituitary-adrenal (HPA) axis:

Almost any type of stress, mentioned already, is followed within minutes by increased secretion of cortisol. The occurrence of stress is communicated to the HPA axis by various chemical messengers which in turn activates secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus. These hormones stimulate the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to secrete cortisol. Mentioned already are substances that promote CRH secretion include glutamate, cytokines and CRH (Adinoff et al, 1998).

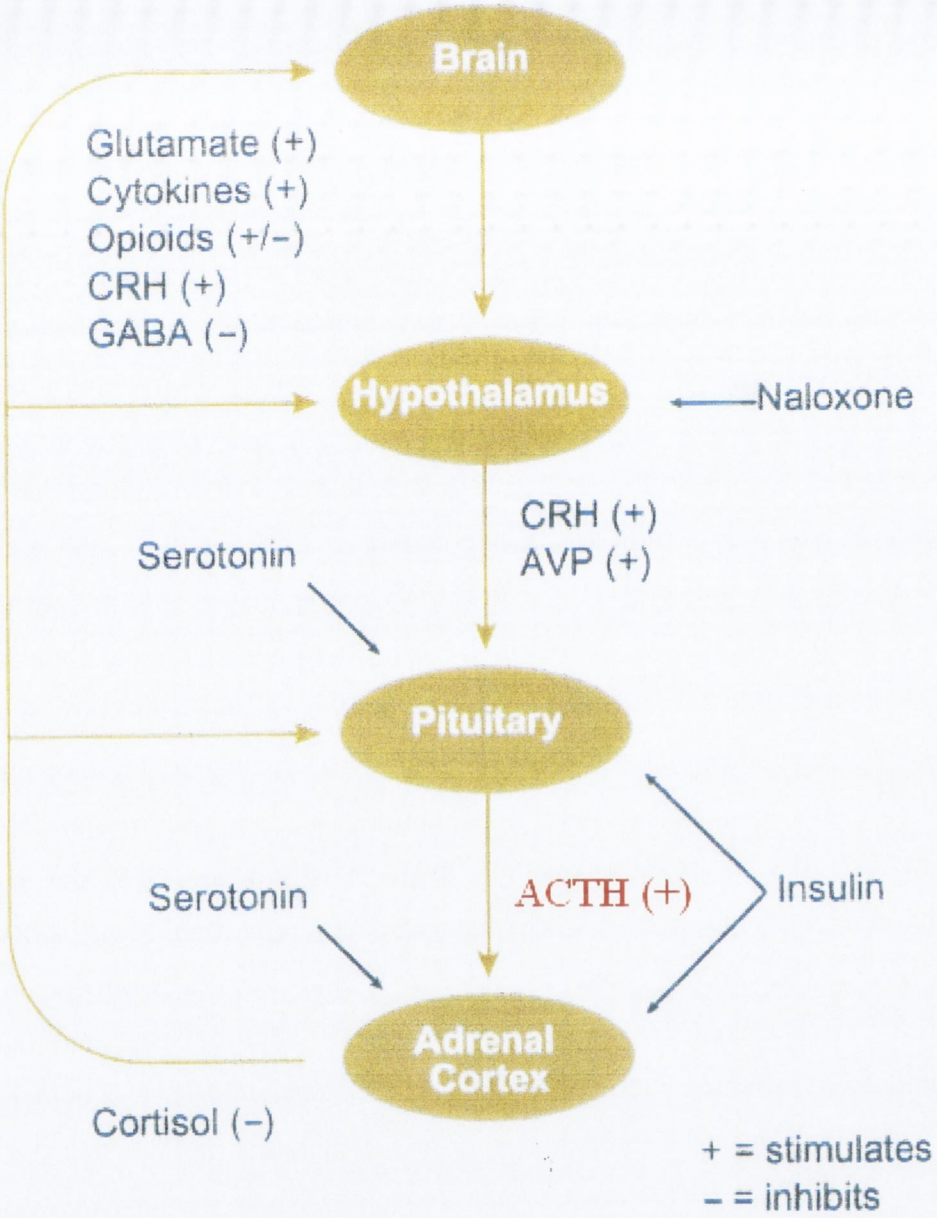


Figure 1.9. Regulation of synthesis of ACTH. (Figure adapted from Adinoff et al, 1998).

1.4.3 ACTH Induced Behavioural Observations:

Adrenocorticotrophic hormone ($ACTH_{1-24}$) is involved in biochemical functions both in nervous and adrenal tissues (Dunn & Gispen, 1977). Ferrari *et al.* in 1963 observed a change in behavior after the intracisternal administration of Adrenocorticotrophic hormone ($ACTH_{1-24}$). These behaviours are characterised by recurrent episodes of stretching yawning syndrome (SYS), penile erection (PE) and by excessive grooming (G). The effects begin to show 15-30 minutes after treatment with ACTH, and last for several hours (Ferrari et al, 1963; Bertolini et al, 1969; Gispen et al, 1975; Bertolini et al, 1981). Levine et al, (1975) showed that $ACTH_{1-24}$ stimulates adrenal Ornithine Decarboxylase (ODC) activity, the rate-limiting enzyme for the production of polyamines. As previous studies postulated the involvement of the ODC system in $ACTH_{1-24}$ behavioral effects Genedani et al., (1984) looked into the effect of α-difluoromethylornithine (DFMO) on $ACTH_{1-24}$ induced behavioural syndrome. DFMO is an irreversible inhibitor of mammalian ODC. It was hypothesised that if polyamines were in fact responsible for the syndrome, DFMO should prevent the build up of polyamines in the mammalian brain, and thus inhibit the onset of the symptoms (Genedani *et al.,* 1984). It was discovered that DFMO inhibited the SYS and the PE brought on by the intracerebroventricular injection of $ACTH_{1-24}$ in the adult male rat, and that PE was inhibited at a lower dose than that required for SYS inhibition. This evidence, along with similar results from previous experiments, supports the idea that the different components of the $ACTH_{1-24}$-induced behavioural syndrome may involve different neural pathways, but also, more importantly, that ODC and polyamines may have a prominant role in the onset of at least some of the $ACTH_{1-24}$-induced behaviours (Bertolini et al, 1968; Poggioli et al, 1984).

Genedani et al, (1994), looked at the effects of Ifenprodil on $ACTH_{1-24}$-induced behavioural syndrome. Ifenprodil is a non-competitive polyamine antagonist at the stimulatory site of the NMDA receptor complex, which is sensitive to polyamines (Schoemaker et al, 1990). Ifenprodil significantly reduced SYS and PE induced by

i.c.v. injection of $ACTH_{1-24}$. Ifenprodil had no direct behavioural effects on control animals. This finding supports the idea that polyamines may interact with the NMDA receptor to cause some aspects of the $ACTH_{1-24}$-induced behavioural syndrome (Schoemaker et al, 1990).

Further evidence to support this was found by Tintner et al. (1979) whereby they performed an experiment in which cerebral polyamine levels in mice were examined following s.c treatment daily for 3 days or as a single injection with $ACTH_{1-24}$. Animals were killed either 6 or 24 hours after completion of treatment. It was found that putrescine content was significantly increased in all brain regions in response to $ACTH_{1-24}$ (only in the telencephalon by $ACTH_{4-10}$). Telencephalic spermidine was also elevated by $ACTH_{1-24}$, but spermine content was not altered in any brain region 24 hours after treatment. However, $ACTH_{1-24}$ produced a peak elevation of all three polyamines 6hrs post injection in the telencephalic region (Tintner et al, 1979).

1.4.4 ODC Interaction, Second Messenger Systems, Protein mRNA Involvement:

As stated previously studies have indicated the involvement of the ODC system in the $ACTH_{1-24}$ induced behaviours. The mechanism of action has not been elucidated, but it has been suggested that polyamines may act as a second messenger in mediating the $ACTH_{1-24}$ behavioural effects. Polyamines may serve as a second messenger in stimulus-response coupling, in a rapid plasma membrane response (Genedani et al, 1984). Secondly, polyamines have the ability to stabilize membrane structure, which might enhance $ACTH_{1-24}$ interaction with its receptor. The third postulation of the interaction of $ACTH_{1-24}$ and polyamines is the involvement in protein synthesis. It is widely known that polyamines are involved in the synthesis of new proteins as they have charged amine groups distributed along the aliphatic carbon chain. This charge is important to the interaction of polyamines with nucleic acids and proteins. $ACTH_{1-24}$ induces the synthesis of new proteins, which may mediate some of the behavioural effects; polyamine induced synthesis might form

part of the mechanism of action (Genedani et al, 1984). However, the behavioural effects are within minutes, therefore it is not likely that protein synthesis plays a major role.

As previously mentioned, $ACTH_{1-24}$ when injected into the brain causes a collection of behavioural effects. The mechanism of this action is still not fully understood. Cloning studies have indicated that there are 5 receptor subtypes for melanocortin, MC1 -MC5. These are distributed at different levels in peripheral and brain tissues. MC2 is found in the $ACTH_{1-24}$ receptor found in adrenal tissues while, MC3 and MC4 are found in the CNS, particularly the hypothalamus (Argioloas et al, 2000). Previous studies have indicated that the MC4 melanocortin receptor mediates grooming, yawning and stretching but not penile erections in the periventriclular region of the hypothalamus, paraventricular nucleus and surrounding areas. Melanocortin receptors have been found to be coupled to adenylate cyclase or to phosphatidyl-inositol/ Ca^{2+} mediated signaling systems in the CNS (Argioloas et al, 2000). The $ACTH_{1-24}$ induced behavioural syndrome is distinct from the syndrome induced by dopamine, oxytocin agonists and NMDA, although each of these agents induce penile erections and yawning (Argiolas et al, 2000; Melis et al, 2000).

The polyamine spermine, given orally to young rats stimulated $ACTH_{1-24}$ and corticosterone secretion, which were observed 4-6hours after ingestion of spermine. Intraperitoneal injection had no effect, suggesting that dietary spermine may affect $ACTH_{1-24}$ and cortisone (Kaouass et al, 1994). Increased cortisol levels increase ODC activity by 230%, polyamine synthesis from ornithine by 5-180%, and intracellular polyamine concentrations 45-83% over 2 days in intestinal suckling piglets (Wu et al, 2000). $ACTH_{1-24}$ administered to twelve normal human volunteers caused a rapid increase in cortisol and aldosterone levels peaking at 30 minutes and returning to basal level within 120 minutes after $ACTH_{1-24}$ injection (Hiroi et al, 2002).

$ACTH_{1-24}$ stimulation of ornithine decarboxylase has been investigated. It was found that $ACTH_{1-24}$ could cause stimulation of ornithine decarboxylase activity in the adrenal cortex, liver and kidney (Raina et al, 1975; Almazan et al, 1983). Levine et al (1975) investigated $ACTH_{1-24}$ and the influence of cyclic AMP on ornithine decarboxylase. They found that stimulation of ornithine decarboxylase was not dependant on the early peak of cAMP but may be influenced following sustained levels of $ACTH_{1-24}$, hence there might be an alternative pathway for ornithine decarboxylase activation independent of cAMP. $ACTH_{1-24}$ stimulation and modulation of known physiological activites of the natural hormone, is dependant on Ca^{2+} and Mg^{2+} (Schotman et al, 1980). Both calcium and cyclic AMP have been shown to be mediators of adrenocorticotropic activity on protein and corticosteroid synthesis in the adrenal cortex (Schotman et al, 1980).

As previously mentioned, other studies have investigated cerebral levels of polyamines following $ACTH_{1-24}$ treatment injected daily over 3 days and examined using high pressure liquid chromatography. $ACTH_{1-24}$ acts directly in the brain to increase cerebral polyamine levels and may be linked to increased RNA and protein synthetic activities (Tintner et al, 1979). Ornithine decarboxylase has a rapid half-life (10-20minutes) (Russell et al, 1980). The induction of ODC is regulated at the transcriptional level and is proportional to the extent of stimulation (Russell et al, 1980). In adrenocortical cells exposed to $ACTH_{1-24}$, casein kinase II (CKII) has been shown to accumulate in the nuclear compartment. CKII nuclear translocation is concomitant with an increase in nuclear polyamine content resulting from $ACTH_{1-24}$-induced polyamine syntheses (Filhol et al, 1991). DFMO selectively inhibits both the $ACTH_{1-24}$-induced polyamine increase and CKII nuclear accumulation (Filhol et al, 1991). This suggests a link between intracellular polyamines and nuclear CKII (Filhol et al, 1991). $ACTH_{1-24}$ induces a rapid activation of the uptake of polyamines resulting in a 2-3-fold increase in intracellular polyamine content over 1hour in bovine adrenocortical cells, which plateaued after 2 hours (Feige et al, 1986). It has been suggested this uptake is modulated by protein kinase C and tyrosine kinase activity (Dot et al, 2000).

An interesting fact to the $ACTH_{1-24}$ induced behaviours is the ability of a wide variety of compounds to inhibit the development of the syndrome. Previous studies have implicated the involvement of nitric oxide in the $ACTH_{1-24}$ behaviours. Poggioli et al (1995), found that NO sythase was involved in the development of these behaviours. They also concluded that the induction of yawning and penile erections involves brain nitrogenergic pathways, while stretching and grooming involves peripheral nitrogenergic pathways (Poggioli et al, 1995). Interestingly, the involvement of NO has also been implicated in the induction of penile erections and yawning produced from NMDA (Melis et al, 1997a; Melis et al 1997b). Furthermore NO synthases have been demonstrated to be involved in male sexual behaviours (Benelli et al, 1995).

In addition, the Ca^{2+} channel antagonist, Nicardipine, blocked the $ACTH_{1-24}$ induced behaviours, indicting the importance of Ca^{2+} influx into the target neuron in the occurrence of the $ACTH_{1-24}$ induced behaviours (Poggioli et al, 1993; 1995; Vergoni et al, 1995).

Finally, to understanding the involvement of polyamines in $ACTH_{1-24}$ induced behaviours, it has been suggested that excitatory amino acids, in particular those acting at the NMDA receptor are involved in the central actions of $ACTH_{1-24}$ (Genedani et al, 1994). It has been hypothesize that ACTH could exert its effects by modulation of NMDA receptor activation (Spruijt et al, 1994). The study for this found that chronic administration of $ACTH_{4-29}$ did improve behavioural performance in the form of extreme locomotor activity and spatial orientation. In acute treatment $ACTH_{4-29}$ led to enhanced neuronal excitability of limbic structures (Spruijt et al, 1994) after treatment with the NMDA receptor antagonist AP5. Further evidence to illustrate that ACTH exerts it's central effects, at least in part through NMDA receptor activation in the same study, it was found that $ACTH_{4-29}$ strongly suppressed NMDA-induced enhanced locomotor activity and normalized exploratory behaviour by inhibiting the NMDA receptor antagonist AP5 (Spruijt et al, 1994). DFMO in a previous study inhibited the effects of $ACTH_{1-24}$; on the basis

of this it would seem that polyamines may influence $ACTH_{1-24}$ through interaction and modulation of NMDA receptors (Genedani et al, 1984; 1994). Pinacidil, a potassium channel opener, dose-dependently inhibited the behavioral effects of $ACTH_{1-24}$ and the complex mechanism of this behavior involves closure of potassium channels in target neurons (Vergoni et al, 1995). Therefore it is possible, polyamines may mediate some $ACTH_{1-24}$ induced effects through a K^+ channel interaction.

1.5.Polyamines Involvement in Disease states:

Polyamines have been implicated in many disease states. It is widely known that polyamines have been implicated in normal growth and differentiation of many cells types (Schuber, 1989). Stimulation of ornithine decarboxylase is involved in the growth process (Janne, 1991). Polyamines are found at greatly elevated levels in malignant cells. Moulinoux et al (1991) investigated this by radiolabelling putrescine in Lewis lung carcinoma cells, and found that spermidine synthesis was increased concurrently with tumor volume and progression.

Polyamines also exert an effect on the level of blood glucose. It has been demonstrated that icv injection of spermidine and spermine in rabbits casued a significant increase in blood glucose levels, with recovery to normal levels after 3 hours. This hyperglycaemic effect could relate to diabetes (Anderson & Shaw, 1974).

Administration of putrescine in dogs, through the intravenous route or by microinjection into the third cerebral ventricle, produced a hypotensive effect. In animals pre-treated with a H1 receptor antagonist, there was no hypotensive effect observed following putrescine adminsitration (Rossi et al, 1984). From this the hypothesis made was that a cardiovascular effect was exerted through histamine release (Rossi et al, 1984). It has been previously shown that polyamines may influence histamine release (Shaw, 1972).

Polyamines have been implicated in cerebral ischaemia. During postischaemic recirculation the only polyamine which consistently has altered concentration levels is putrescine, whereby it is dramatically increased. This increase is thought to be due to the increase in ODC activity and inhibition of S-adenosylmethionine decarboxylase (Paschen, 1992). In addition putrescine is increased in the area of incomplete ischaemia and brain oedema (Paschen, 1992). Zoli et al (1996), demonstrated increased levels in spermidine/spermine N^1-acetyltransferase in rat CNS following ischaemia, suggesting that the polyamine interconversion pathway is involved in altered polyamine levels. Hydrogen peroxide, a by-product of polyamine metabolism along the terminal catabolic pathway has been implicated in neuronal or cell damage (Seiler, 1995). Other investigations have examined NMDA antagonists and their efficacy and side effects liability for neuroprotection in experimental stroke (Dawson et al, 2001). The polyamine site antagonists used were eliprodil and ifenprodil. Ifenprodil was shown to be neuroprotective and additionally lack the motor and behavioural side effects associated with other NMDA antagonists (Carter et al, 1990). Eliprodil has been shown to have neuroprotective activity in experimental brain trauma (Toulmond et al, 1993).

1.5.1 Polyamine involvement in Epilepsy:

Epilepsy affects approximately 0.5-0.7% of the population worldwide. It is a common neurological disorder characterised by recurrent spontaneous seizures, attributed to some form of brain damage resulting from an insult such as infection, trauma or tumour growth. A seizure is an episodic high frequency discharge of impulses from neurons, which may spread across several regions of the brain. There are different subtypes of epilepsy which are classified dependant on site of origin.

Partical seizures are localised in origin, usually arising in the temporal or frontal lobe. In simple, partial seizures, consciousness remains, whereas in complex partial seizures consciousness may be affected or lost. Generalised seizures are widespread, arising simultaneously in both hemispheres, they include tonic-clonic

seizures (grand mal) whereby there is a loss of consciousness and convulsions ensue. In absence seizures (petit mal) there is a loss of consciousness for only a few seconds, and this is accompanied by a 3Hz EEG signal originating from the thalamus. Patients may present with more than one type of seizure, such as a grand mal associated with a petit mal, subsequently followed by psychomotor epilepsy (Longstaff, 2000).

Many NMDA agonists have been used to provoke epileptiform activity. NMDA receptor anatagonsits have been shown to possess anticonvulsant properties (Dingledine et al, 1999; Menendez de la Prida, 2002). Further studies have found that the excitatory amino acid, glutamate is elevated in human epileptic foci (Perry & Hensen, 1981). The activation of NMDA receptors depends on the relief of voltage-dependant Mg^{2+} blockage and the subsequent influx of Ca^{2+} and Na^{+} and efflux of K^{+} (Seiler et al, 1998). Seizures are influenced by the change in ion concentrations. AMPA receptor activity may generate individual seizures, which may lead to NMDA receptor and L-type Ca^{2+} channel recruitment, prolonging burst firing (Longstaff, 2000; Bernard et al, 2001). Hyper-phosphorylation of NR2B receptors may lead to hypersensitivity to the endogenous transmitter, inducing neuronal hyperexcitability and epilepsy (Arias et al, 2002). Tyrosine phosphorylation of NR2A and NR2B receptors is altered by kainic acid-induced status epilepticus (Moussa et al, 2001).

There is much evidence which demonstrates the role of polyamines in the enhancement of NMDA receptor activity (Matsumoto et al, 1993). The possibility that polyamines may be involved in epilepsy stems from the observation that the administration of polyamines, particularly spermine results in convulsions. S-adenosylmethionine decarboxylase, a regulatory enzyme in polyamine synthesis, has been demonstrated to be increased in regions of active epileptogenic cortical discharge in samples from patients with intractable epilepsy, indicating polyamines may be involved with maintenance of hypersynchronous discharges mediated through the NMDA receptor (Morrison et al, 1994). Furthermore 1-napthylacetyl

spermine an analogue of the polyamine toxin, Joro spider toxin, (JSTX) suppressed hippocampal epileptic discharges mediated through non-NMDA receptors (Kanai et al, 1992). Finally there have been reports of increases in brain polyamine concentrations following kindling, suggesting that polyamines are involved in neuronal excitability in the epileptic brain (Hayashi et al, 1993).

1.6. Competitive polyamine antagonists:

Various compounds have been suggested to be polyamine antagonists. In theory, a polyamine-like molecule (polyamine analogue) could be a suitable competitive antagonist. However, polyamine analogues often show some polyamine-like effects. Three such analogues, Arcaine, 1,10-diaminodecane and diethylenetriamine were shown to be partial agonists and not pure antagonists (Doyle et al, 1998). More recently, the novel polyamine analogue, N^1-dansylspermine (Seiler et al, 1998), has been shown to stimulate the negative polyamine site (Chao et al, 1997). Kirby et al (2004), investigated the effects of N^1-dansylspermine on spermine induced CNS excitation. N^1-dansylspermine was very effective in antagonizing the effects of spermine, an effect thought to be mediated through the antagonism on the positive polyamine modulatory site on the NMDA receptor macrocomplex. Thus, in vivo, at appropriate concentrations N^1-dansylspermine may be a highly potent polyamine antagonist (Kirby et al, 2004).

1.6.1 Novel compounds:

Polyamine analogues are derivatives of the naturally found polyamines, whereby they are similar in structure. The structural similarity may mean that such analogues may interact with polyamine receptors, transport mechanisms, and synthesis pathways. Over the past few years it has become very evident there are two distinct categories of polyamine analogues: the polyamine antimetabolite and the polyamine mimetic (Wallace et al, 2003). The antimetabolite analogues result in polyamine

depletion and a decrease in cell growth. The mimetics decrease growth without necessarily producing significant polyamine depletion (Wallace et al, 2003).

Previous studies have examined the N^1-dansyl derivatives of spermine and spermidine. It was found that these derivatives are potent NMDA channel blockers. Some are several hundred-thousand-fold more potent than the natural polyamines (Chao et al, 1997). The N-benzyl polyamines, (di and tri-benzyl polyamines) have been shown to be potent NMDA receptor antagonists (Igarashi et al, 1997). The tri-benzyl-polyamines (such as TB3-4), which are derived from spermidine and contain a benzyl substitution on each of the three amino groups, showed high selectivity for NMDA channels over AMPA channels, and are potent channel blockers (Igarashi et al, 1997). The benzyl-polyamines represent a new class of NMDA receptor antagonists, which could be useful products to study as NMDA channel blockers and may have novel therapeutic potential as neuroprotective or anticonvulsant agents (Igarashi et al, 1997).

It is widely known that plants can conjugate polyamines with various phenolic acids giving mono-, di- and tri-amides, some of which contain hydroxycinnamic acid (Fixon-Owoo et al, 2003). These are similar in structure and composition to the other acylated polyamine conjugates found in venoms of predaceous spiders and wasps, which inhibit neurotransmission and presumably have evolved to immobilize prey for capture (Fixon-Owoo et al, 2003). Further studies have shown that icv administration of Joro spider toxin (JSTX-3) produced several different behaviours (Himi et al, 1990). These behavioural features consisted of an increase in motor activity with staggering gait, (ataxia) - lasting for days, abnormal behaviour such as stretching of the hind legs (stretched sideways and forward with buttocks up) or standing in the hind legs, grooming, tremor and muscle relaxation followed by death from suppression of breathing (Himi et al, 1990). The similarity of plant derived hydroxycinnamic acid (coumaric) amides and invertebrate polyamine toxins has been investigated by Blagbrough and Usherwood (1990; 1992). They tested N^1-coumaroyl spermine and analogues in a locust breve-muscle preparation. The

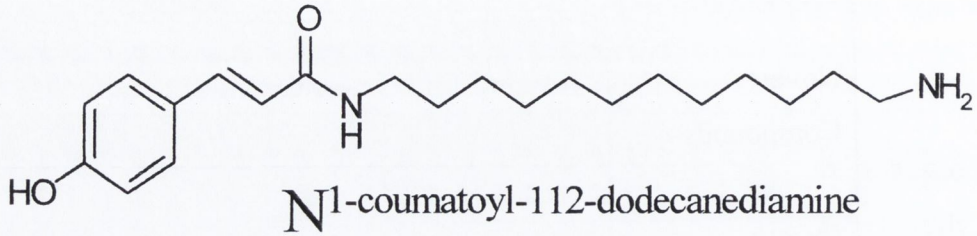
findings from this study suggested that plant hydroxycinnamoyl conjugates of polyamines (N^1, N^8-coumaroyl spermidine and N^1-courmaroyl spermine), may mimic the arthropod toxins and are active at glutamate-dependant neuromuscular junctions of crustaceans (Fixon-Owoo et al, 2003).

A method of extracting hydroxycinnamic acid amides from plant tissue has been developed (Panagabko et al, 2000). In Fixon-Owoo et al's (2003) study, initially aryl substituted cinnamic acid amides were isolated, these were then expanded by preparing coumaric acid monoamides with a variety of di-, tri- and tetra-amines, which are presented below Figure 1.11. In this study the prelimary investigation was to see if these compounds were active inhibitors of glutamatergic receptors in crustaceans. The acylpolyamines were shown to work similarly to spider and wasp toxins and showed strong activities at mammalian glutamate receptors, especially the NR1/NR2B NMDA receptors. The structure-activity relationship showed strong parallels with that of philanthotoxin, argiotoxin and non-acylated polyamine analogues at the various receptor preparations (Fixon-Owoo et al, 2003). The results are displayed on Table 1.10:

Novel Compounds	AMPA GluR2	AMPA GluR1/GluR2	NMDA NR1/NR2B
Bu 31b	13%	11%	98%
Bu 37b	8%	9%	12%
Bu 33b	2%	1%	3%
Bu 40b	15%	13%	28%
Bu 36b	18%	16%	26%
Bu 43b	52%	50%	95%

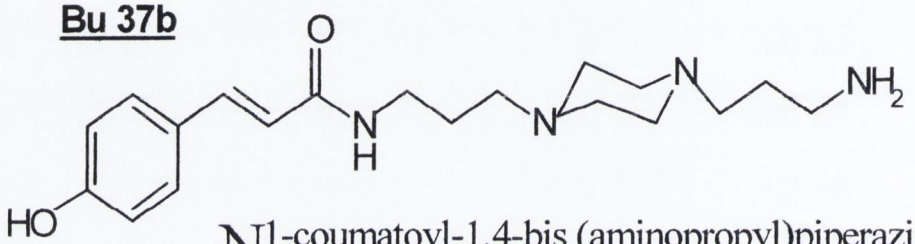
Table 1.10: The percentage inhibition of Novel compounds on NMDA and AMPA receptors. Results taken from findings by Fixon-Owoo et al 2003.

Bu 31b



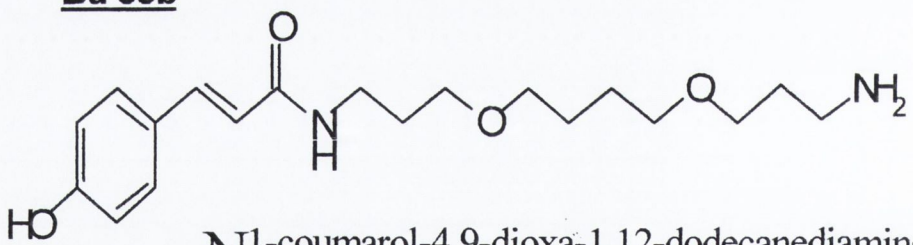
N1-coumatoyl-112-dodecanediamine

Bu 37b



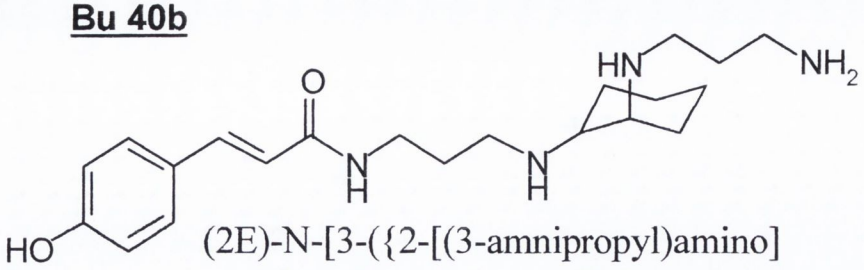
N1-coumatoyl-1,4-bis (aminopropyl)piperazine

Bu 33b



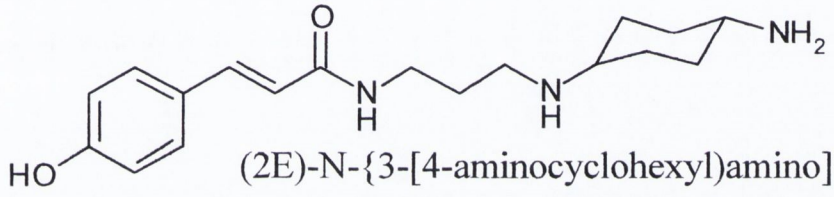
N1-coumarol-4,9-dioxa-1,12-dodecanediamine

Bu 40b



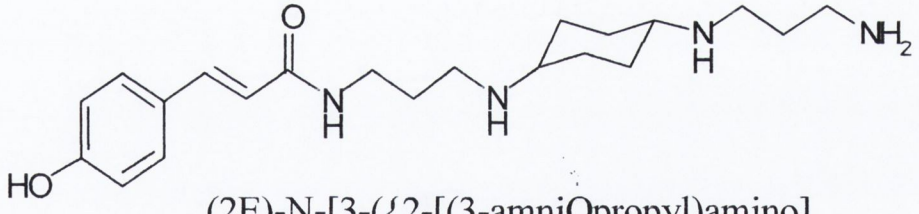
(2E)-N-[3-({2-[(3-amnipropyl)amino]
cyclohexyl}amino)propyl]-3-(4-hydroxyphenyl)
prop-2-enamide

Bu 36b



(2E)-N-{3-[4-aminocyclohexyl)amino]
propyl}-3-(4-hydroxyphenyl)prop-2-enamide

Bu 43b



(2E)-N-[3-({2-[(3-amniOpropyl)amino]
cyclohexyl}amino)propyl]-3-(4-hydroxyphenyl)
prop-2-enamide

Figure 1.11: Molecular structures of Novel Polyamines from Brock University Canada.

1.7 Aims:

The overall aim of this study was to establish a better understanding of the effects and mechanism of action of the polyamines putrescine, spermidine and spermine. In the first strand of this study, the effect of novel polyamine analogues was investigated *in vivo*, in both the spermine induced CNS excitation model and also in the $ACTH_{1-24}$ behavioural induced model. To facilitate a better understanding of the mechanism of action of the polyamines, the effect of a range of non-competitive polyamine antagonists NMDA antagonists and calcium antagonists was assessed.

These compounds were administered on their own in the whole mammal to see whether or not they developed the polyamine behavioural profile as seen with putrescine, spermidine or spermine alone.

In the third strand of this study, the concentrations of putrescine, spermidine and spermine in the mouse brain during the different behavioural stages in each model was assessed by HPLC analysis of polyamine levels.

Chapter 2.

2.0 Materials & Methods.

Laca mice, both male and female were used throughout the entire investigation. Previous publications from the same laboratory used this strain of mouse. For consistency reasons it was logical to use *Laca* mice as previously used and also maintaining consistency throughout the various investigations within this study.

2.1 Spermine Behavioural Model:

2.1.1 Direct Administration of Spermine and Antagonists:

Female *Laca* mice weighing between 20 and 25g were obtained from the Bioresources Unit Trinity College Dublin. The mice were housed in groups of 4-6 under a 12 hour light/dark cycle (light 7am - 7pm) and given food and water *ad libitum*. 100μg Spermine (Sigma, Ireland) dissolved in 0.9% sterile saline was administered through the intracerebroventricular (i.c.v) route (by freehand) as described by Brittain (1966). The injection site was 1mm to the left of the midline and 2mm rostral to a line joining the anterior base of the ears. The injection was achieved using a Hamilton microlite syringe calibrated to administer exactly 20μl and using a 27-gauge needle to a depth of 3mm. The depth was achieved by using a length of PVC tubing attached to the needle to allow it to penetrate the skull to this depth. The weight range is essential, as in the weight range 20-25g, the skull has not fully calcified and a "soft" spot is present. The microlite syringe was held in a vertical position via a clamp attached to a retort stand. The mouse was restrained to enable it to be held in position on the needle while the drug/s were being administered slowly. The mice were then replaced in the cage and allowed to recover.

2.1.2 Assessment Profile:

The assessment methodology is based on that established by Doyle & Shaw, (1998). The spermine-induced CNS excitation observation began 2 hours after spermine injection and subsequently at 30 minute intervals over a 7.5 hour period. Previous work has shown that there are 2 phases of spermine induced effects (Doyle & Shaw, 1998). The first is antagonised by a wide range of drugs, while the second responds more specifically and is of most interest in this study. A scoring system was established to monitor the degree of body tremor over this period. The body tremor worsens over time, and terminates in a fatal tonic convulsion. The scoring system used was 1: slight tremor, 2: moderate tremor, 3: severe tremor, 4: tonic convulsion - survived, 5: fatal tonic convulsion. Assessment of the degree of tremor was achieved subjectively by lifting the mouse by the tail and feeling the degree of tremor. Due to the subjective nature of the assessments, the scoring system utilises only 3 levels of tremor to reduce scope for error in severity scoring. All experiments were carried out with the experimenter blind as to which treatment had been administered.

2.1.3 Polyamine Antagonists:

The novel polyamine analogue Bu 31b, N1-coumaroyl-1, 12-dodecanediamine, was administered through either the intracerebroventricular route or the intraperitoneal route. Bu 31b was dissolved in 0.9% sterile saline and placed in an ultra-sonic bath (Decon FS4006) at room temperature for 10 minutes. Solubilisation was produced by addition of a maximum of 2 drops of Tween 80. Administration of Bu 31b through the intracerebroventricular route used the same methodology as described for spermine in section 2.1.1. Bu 31b was administered alone or co-administrated with 100μg Spermine. Intraperitoneal (i.p) administration of Bu 31b occurred 30 minutes prior to Spermine injection (i.c.v) using a 1ml syringe (Omnifix -F) and a Microlance 3 sterile needle. The volume administered was 0.1ml/10g body weight.

Similarly, Bu 37b, (B), N1-coumaroyl-1,4bis(aminopropyl) piperazine was also administered through either the i.c.v and i.p routes using the same procedure

described for Bu 31b. Bu 37b was dissolved in 0.9% saline, placed in an ultra-sonic bath for 10 minutes at room temperature containing 2 drops of Tween 80 to aid solubilisation.

Bu 33b, N1-coumaroyl-4,9-dioxa-1,12-dodecanediamine, Bu 40b, 2E)-N-[3-({2-[(3-amnipropyl) amino]cyclohexyl}amino) propyl]-3-(4 hydroxyphenyl)prop-2-enamide, Bu 36b, 2E)-N-{3-[4-aminocyclohexyl)amino]propyl}-3-hydroxyphenyl)prop-2- enamide and Bu 43b, 2E)-N-[3-{2-(3-aminopropyl)amino]cyclohexyl} amino)propyl]-3-(4-hydroxyphenyl)prop-2-enamide were each administered at varying concentrations through the intracerebroventricular route and the intraperitoneal route. Each of the drugs were dissolved in 0.9% sterile saline and either administered alone or co-administered with Spermine (i.c.v) or given (i.p) 30 minutes prior to Spermine administration.

The novel polyamine analogue N^1-Dansylspermine was dissolved in 0.9% sterile saline and either administered alone or co-administered with 100μg Spermine i.c.v. N^1-Dansylspermine was obtained through a synthesis process in the laboratory by Rhona Prendergast, Department of Pharmachemistry, Trinity College Dublin, following a methodology by Seiler (1998).

2.1.4 Calcium Antagonists and NMDA Antagonists:

The calcium antagonist Nitrendipine (Research Biochemical Incorporated N-144) was dissolved in 0.2ml 100% ethanol and then placed in an ultra-sonic bath for 15 minutes and 0.9% sterile saline added to produce a 5% ethanol solution. 2 drops of Tween 80 was added for solubilisation. Nitrendipine was administered through the i.p route 30 minutes prior to Spermine injection i.c.v. The volume administered was 0.1ml/10g body weight.

Another calcium antagonist investigated was Nisoldipine (Bayer). Nisoldipine was dissolved in a solution of alcohol/saline (35:65). This was then diluted with 0.9% saline (i.e. 1 in 10 dilution) to yield a 3.5% alcohol solution. Solubilisation was

aided by adding 2 drops of Tween 80. Administration was via the i.p route (0.1ml/10g) 30 minutes prior to Spermine injection.

Memantine, a noncompetitive polyamine antagonist was dissolved in 0.9% sterile saline and administered through the intraperitenial route (0.1ml/10g) 30 minutes prior to Spermine injection.

Ifenprodil (Tartrate salt, from the Research Biochemicals International was administered via the intraperitoneal route (0.1ml/10g). Ifenprodil was dissolved in 0.9% sterile saline. Eliprodil (Synthelabo Recherché, Bagneux, France) was also administered through the intraperitoneal route (0.1ml/10g). Eliprodil was dissolved in 0.9% sterile saline with 1 drop of Tween 80 for solubilisation.

2.1.5 Data Analysis:

The Mann-Whitney U-test was used to assess the statistical significance of the differences between treatment and controls. Results are expressed as the median score of the CNS excitation of each group and interquartile ranges (IQR). The Median CNS excitation scores were plotted against time (hours).

2.2 ACTH Behavioural Model:

2.2.1 Direct Administration of $ACTH_{1-24}$ (AdrenoCorticoTropic Hormone) and Antagonists:

Male *Laca* mice weighing between 20 and 25g were obtained from the Bioresources Unit Trinity College Dublin. The mice were housed groups of 4-6 under a 12 hour light/dark cycle, (light 7am - 7pm) and given food and water *ad libitum*. Direct administration of $ACTH_{1-24}$ ($ACTH_{1-24}$ Human, Synthetic fragment, Sigma Irl, into the cerebral ventricles was via the intracerebroventricular route. $ACTH_{1-24}$ was dissolved in 0.9% sterile saline and was administered by freehand using the method described by Brittain (1966). The injection procedure for intracerebroventricular administration followed the same procedure as for Spermine administration (Section 2.1.1). Before the administration of $ACTH_{1-24}$, the mouse body temperature was

recorded using a KJT-Thermocouple Thermometer (*Hanna* Instruments) by inserting the probe in the rectum. Once the mouse was injected with $ACTH_{1-24}$ they were placed in a rectangular transparent Perspex box (31.5cm length x 21.5cm width x 20.5cm depth), which was further divided into 4 equilateral chambers (16cm length x 11cm width x 20.5 depth).

2.2.2 Assessment Profile:

Each mouse was observed for a 1 hour duration and quantitative scores of behaviours were noted including, yawning, stretching, tail grooming, penile erections and frequency of grooming. Behaviours were recorded on videotape and the frequency of grooming later calculated per minute of grooming time. Once the 1hour duration was complete the animal's temperature was recorded again.

2.2.3 Antagonists Assessed:

N^1 Dansylspermine and the novel compounds Bu 31b, Bu 37b, Bu 33b, Bu 40b, Bu 36b and Bu 43b were co-administered with $ACTH_{1-24}$ through the intracerebroventricular route. Each of these compounds was dissolved using the same technique as the spermine experimental procedure, (section 2.1.3).

Nisoldipine, Nitrendipine and Memantine were given through the intraperitoneal route 30 minutes prior to $ACTH_{1-24}$ i.c.v (0.1ml/10g dose volume). Temperature was recorded before initial injection and after the 1-hour observation. Nisoldipine, Nitrendipine and Memantine were dissolved using the same techniques used for the spermine model, (section 2.1.4)

Ifenprodil was administered via the intraperitoneal route 30 minutes prior to $ACTH_{1-24}$ i.c.v. Eliprodil was also administered through the intraperitoneal route 30 minutes prior to $ACTH_{1-24}$ i.c.v as previously described, (section 2.1.4).

Arcaine (Sulfate salt, from Sigma Irl) and α–Difluoromethylornithine DFMO, (Hydrochloride, Sigma Irl) were dissolved in 0.9% sterile saline and were co-

administered with $ACTH_{1-24}$ through the intracerebroventricular route. Putrescine (Dihydrochloride, Sigma) was dissolved in 0.9% sterile saline and administered alone through the intracerebroventricular route.

2.2.4 Data Analysis:

The average for each behaviour was counted and the standard error of mean (S.E.M) was evaluated. Statistical analysis was performed by analysis of variance using One-Way ANOVA. Post-Hoc analysis was performed using Tukey HSD.

2.3 HPLC Experimental Procedure, the Analysis of Polyamines, $ACTH_{1-24}$ and CRF:

2.3.1 Tissue Preparation:

Spermine or ACTH administration was achieved as described in sections 2.1.1 and 2.2.1. Once behavioural observations were complete, the animals were culled by cervical dislocation followed by decapitation. The brain was extracted, by initially removing the fur around the head, then with a small sharp scissors, the scull was cut along the midline. Then making two small cuts at the side of the head with a very small forceps the scull was peeled back to reveal the brain. A small spatula was placed under the brain and gently agitated to sever all connective tissue connections thereby allowing the release of the brain without any lesions or damage. Each brain once removed was weighed and then manually homogenised in 0.5ml, 0.4M perchloric acid solution in an Eppendorf tube. The samples were then placed in an ultra-sonicator for 15minutes and then vortexed for 1-2 minutes to give a homogeneous solution. Samples were then centrifuged for 15 minutes at 4000rpm to remove the cell debris. At this stage the solution had separated into the natant and the upper layer, the supernatant. 100µl of the supernatant, which contained protein, was transferred to a new eppendorf tube, and a 1 in 10 dilution with HPLC water carried out. The samples were frozen at -20°C, until ready for use in the dansylation reaction. The remainder of the supernatant was kept in a separate eppendorf tube

and placed in the freezer at -20°C for future use. The maximum time samples were frozen for was approximately 1 month.

2.3.2 Preparation of Standard Curve:

Initially stock standards of polyamines were made (solution 1), by adding the following to a scintillation vial:

Solution 1:

1. Putrescine 161.1mg
2. Spermidine 254.6mg
3. Spermine 348.2mg
4. N^1Acetylspermidine 260.2mg
5. N^8Acetylspermidine 260.2mg
6. 1,7-diamino heptane (internal standard) 130.2mg

Weighed polyamines were dissolved in 100ml of 0.01M HCL. The final concentration of each polyamine in solution 1 was 100μmol/ml in 0.01M HCL.

Polyamine standards solutions were made consisting of 1.25, 2.5, 5, 7.5, 10, 12.5, 25, 37.5 and 50μM of all the polyamines, Putrescine, Spermidine, Spermine. The standard solutions employed for N^1 and N^8Acetylspermidine, were as follows; 0.0625, 0.125, 0.25, 0.375, 0.5, 0.625 and 1.25μM. The blank consisted of HPLC water, instead of the polyamine standards.

2.3.3 Dansylation Reaction:

100μl of the brain sample was placed into an eppendorf tube along with 150μl of Borax buffer pH 10.2 (boric acid dissolved in HPLC grade distilled water and spiked with 0.04M NaOH), at pH 10.2 and 50μl internal standard (1,7 diamino heptane). The internal standard is needed as an internal control. Finally 25μl of dansyl chloride concentration was added and vortexed. Once vortexed the sample was heated for 30 minutes at 60°C in complete darkness. 10μl praline (100mg ml^{-1})

was added, this stops the reaction by binding to any excess dansyl chloride remaining converting it to dansyl proline, which is separated early in the HPLC run. This was heated for another 30 minutes at 60°C in the dark and then left to stand for another 10 minutes to allow to cool. 200µl of toluene was added, this allows the polyamines to disperse into the solvent layer (top layer). The sample was vortexed very well. 100µl from the solvent layer was pipetted into a new eppendorf and place into a vacuum centrifuge at 50 °C for 5-10 minutes until dry. A yellow layer/film was present at the bottom of the eppendorf. 100µl of the top layer was removed and placed into new vials. A vacuum centrifuge was used to remove the toluene at 50°C for 20 minutes. 0.5ml of acetnitrile was added to the dry sample immediately. 6µl of this was injected in the HPLC system or were stored at -18°C. Below the reaction of polyamines with dansyl chloride is illustrated (Figure 2.1).

Putrescine:

$$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_3\text{-}NH_2 + 2Dans\text{-}Cl \rightarrow$$
$$Dans\text{-}HN\text{-}(CH_2)_4\text{-}NH\text{-}Dans + 2HCL$$

Spermidine:

$$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_3\text{-}NH_2 + 3Dans\text{-}Cl \rightarrow$$
$$Dans\text{-}NH\text{-}(CH_2)_3\text{-}N(Dans)\text{-}(CH_2)_3\text{-}NH\text{-}Dans + 3HCL$$

Spermine:

$$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH\text{-}(CH_2)_3\text{-}NH_2 + 4Dans\text{-}Cl \rightarrow$$
$$Dans\text{-}NH\text{-}(CH_2)_3\text{-}N(Dans)\text{-}(CH_2)_4\text{-}N(Dans)\text{-}(CH_2)_3\text{-}NH\text{-}Dans + 4HCL$$

Figure 2.1 Dansylation reaction with polyamines.

2.3.4 Analytic Technique:

For quantitative determination of polyamines a reverse-phase HPLC system was used, as it gives a convenient and satisfactory final separation. The stationary phase used to pack the column was ultrasphere octadecylsulphate with a column length of

33mm and width 4.6mm coupled with a Phenomenex C18 ODS guard column. The phenomenex C18 ODS column was attached to a Shimadzu HPLC apparatus. This consisted of (i) a Shimadzu system controller; model SCL-10A VP, (ii) 2 Shimadzu Liquid Chromatographs; model LC-10AT UP, (iii) a Shimadzu Fluorescence Detector; model RF-10A XL, (iv) a Shimadzu Auto Injector; model SIL-10 AD UP.

2.3.5 Gradient Elution System used:

This technique involved the altering of the composition of the mobile phase during the chromatographic run, using a weak eluting solvent over the course of the separation. The system consisted of using water and acetonitrile.

58% Acetonitrile for 2 minutes
58% Acetonitrile to 100% Acetonitrile over 3.33 minutes
100% Acetonitrile for 2 minutes
100% Acetonitrile to 58% Acetonitrile over 10 seconds
58% Acetonitrile for 4 minutes

Dansylated polyamine dried pellets (section 2.3.3), were dissolved in 1ml of acetonitrile, vortexed for 30 seconds and left to stand for 30 minutes. This was repeated 4 times. The mixture was transferred to individual injection vials and placed in the autosampler. 10µl of the sample was drawn into the HPLC column. The sample was then separated by partitioning between a polar mobile phase and a non-polar Phenomenex C18 stationary phase, allowing hydrophobic compounds to bind more readily. Eluant compounds were detected using the Shimadzu Fluorescence detector with a Xenon lamp. All polyamines are fluorescent after dansylation and therefore act as Fluorophores with excitation at 340nm and emission at 510nm.

2.3.6 Fluorescence Detection:

Fluorescence detection is typically three orders of magnitude more sensitive than UV detection. HPLC procedures are used routinely for assays in the low ng/ml

range and p/mol range. Fluorescence detection is a highly effective technique as it can discriminate between interference and background peaks, called "Ghost Peaks". Few analytes posess natural fluorescence, hence a reagent such as dansyl chloride is useful for this type of detection. Light passes from the lamp through an excitation filter which provides monochromatic light at 340nm. This excited light passes through the column effluent in the flow cell causing sample molecules to emit light at a higher wavelength, 510nm. A second filter is positioned to collect light at an angle of 90^{o} to the original direction of excitation, resulting in light from the sample to fluoresce onto the photomultiplier tube for quantification of the emission signal. Data was collected using the software package JCL 6000 windows chromatography data system, where it was transformed onto a graph and polyamine retention time and concentration were assessed.

2.3.7 Data analysis for HPLC Fluorescence Detection:

A response factor giving the concentration of component which produces a unit detector response was calculated using the following equation for polyamines, putrescine, spermidine and spermine:

$$\text{Response Factor (R)} = \frac{C/A}{C_i/A_i}$$

Whereby, C = concentration of polyamine standard (μM, nmol ml^{-1})

A = peak area of polyamine standard

Ci = concentration of internal standard (μM, nmol ml^{-1})

Ai = peak area (height) of internal standard

The concentration of the polyamine in the 100μl supernatant (for tissue polyamines) was as follows:

$$Cm = Am \times R \times (Ci/Ai)$$

Cm = concentration of polyamines in 100μl supernatant (μM, nmol ml^{-1})

Am = peak area for internal standard

Ci = concentration of internal standard (μM, nmol ml^{-1})

Ai = peak area (height) of internal standard

Finally, the tissue polyamine concentration (Ct, nmol g^{-1}) was calculated

$$Ct = \frac{Cm \times V}{W.V}$$

Whereby, W = weight of tissue (g)

V = 0.5ml (volume of tissue homogenate)

The detector response for N^1-acetylspermidine and N^8-acetylspermidine was calculated using the following equation for polyamines, putrescine, spermidine and spermine:

$$Cm = (C/A) \times Am.$$

Whereby, Cm = concentration of N^1 and N^8-acetylspermidine in 100µl supernatant (µM, nmol ml^{-1})

A = peak area of N^1 and N^8-acetylspermidine standard

Am = peak area supernatant

The tissue concentration was calculated using the same equation as for the polyamines.

2.3.8 Statistical analysis:

Statistical analysis was performed by analysis of variance using One-Way ANOVA. The average for each polyamine level at different stages both in the Spermine behavioural model and the $ACTH_{1-23}$ behavioural model was counted and the standard error of mean (S.E.M) was evaluated. A probability value of less than 0.05 was considered statistically significant. Post-Hoc analyses was performed using Tukey HSD.

2.4 Behavioural Drug Screening Model:

2.4.1 Preparation and Assessment of Animals:

The novel polyamine analogues, Bu 31b, Bu 37b, Bu 33b, Bu 40b, Bu 36b and Bu 43b are very novel compounds and very little information regarding their biological

effects is known. The novel polyamines had not previously been screened to assess their behavioural effects when given directly to an animal. A behavioural assessment profile was developed using locomotor activity, (LMA count), rotarod performance and an Irwin profile assessment investigating both autonomic and CNS effects of these novel drugs. Male *Laca* mice were injected by either i.c.v (20µl volume) or i.p (0.1ml/10g dose volume) administration with one of the 6 novel polyamine compounds, Bu 31b, Bu 37b, Bu 33b, Bu 40b, Bu 36b and Bu 43b, or vehicle. The doses used were the highest concentrations used in the spermine model, (0.1ml/10g dose volume), administered i.c.v or i.p. For control groups each animals was given saline containing Tween 80 using either i.c.v or i.p administration. The temperature of each animal was taken before administration of drugs and at the end of the first batch of behavioural tests. Directly after i.c.v or i.p administration the animals were put into a locomotor activity Perspex box (see section 2.4.2). After locomotor activity assessment for 1 hour, they were then individually placed on a rotating rod for 2 minutes (see section 2.4.3). After rotarod assessment, each of the animals were assessed using an adapted Irwin profile assessment tool (section 2.4.4) Each animal was kept for 5 days and weight and temperature was measured on each of the 5 days. On day 3 and day 5 the LMA, rotarod and Irwin profile assessments were carried out again.

2.4.2 Spontaneous Locomotor Activity (LMA):

The mice were placed individually in an activity monitor, (AM1051 Activity Monitor, Benwick Electronics, Essex, UK), which consisted of 5 clear Perspex boxes, 42cm length x 21cm width x 20cm depth, with a caged top. Each of the boxes was positioned within a frame, equipped with infrared beams along the length and width of the frame. LMA was recorded automatically electronically on a computer by counting the number of breaks in the beam every 5 minutes over a 1 hour duration.

2.4.3 Rotarod Assessment:

The rotarod assessment consisted of examining balance and co-ordination when placed on a rotating rod (Palmer Recording Drum, UK) at two different speeds. At the first speed the rod rotated at 2 revolutions per minute, and at the second speed, the rod rotated at 4 revolutions per minute. Once the animal was balanced on the rod the drum was switched on at the lower speed for one minute. If the animal maintained its balance at this speed, the speed was increased for a second minute. At any stage during these two minutes if the animal fell off the rod the experiment was terminated and the time at which it fell was recorded. The time totals were added together as the final result for each mouse on that day (maximum of 120 seconds).

2.4.4 Irwin Profile Assessment:

The Irwin profile assessed behaviours attributed to reflect CNS and autonomic nervous system functioning. These behaviours are listed below:

Behavioural Profile CNS:	Normal Score:
Alertness: 1: Very Sedated 2: Moderately sedated 3: Slightly sedated 4: Normal 5: More alert and active movement 6: Very alert, excited, moving rapidly, jumping	4
Stereotypy: 0: Normal 1: Head shake 2: Circulating	0
Vocalisation: 0: None 1: Provoked during handling 2: Spontaneous on plate	0
Pelvic Elevation: 0: Markedly flattened 1: Barely touches plate	2

2: Normal (3mm elevation)
3: Elevated

Grooming: 0
0: None, resting
1: Casual scratching, groom
2: Vigorous scratch, groom

Tail Elevation: 0
0: Dragging, normal
1: Horizontally Extended
2: Straub tail

Tremor: 0
0: None
1: Slight tremor
2: Moderate Tremor
3: Severe Tremor

Convulsion: 0
0: None
1: Clonic
2: Tonic - survived
3: Tonic – fatality

Behavioral Profile Autonomic:	Normal Score:

Salivation: 0
0: None
1: Slight margin around mouth
2: Very wet around mouth

Piloerection: 0
0: None
1: Coat stood on end

Palpebral Closure: 0
0: Eyes wide open
1: Eyes half shut
2: Eyes closed

Body Posture: 3
0: Completely flattened
1: Lying on side
2: Lying prone
3: Sitting or standing
4: Rearing on hind legs
5: Repeated vertical leaping

Limb Tone: 4

0: No resistance
1: Slight resistance
2: Moderate resistance
3: Marked resistance
4: Extreme resistance

Abnormal Gait: 0

0: Normal
1: Fluid but abnormal (staggers)
2: Limited movement only (staggers slowly)
3: Incapacitated

Writhing: 0

0: Normal
1: Slight twisting of body
2: Moderate twisting
3: Severe twisting

In addition the animals were observed for behaviours which are associated with the Spermine model (section 2.1.1), and also the behaviours of the ACTH model (section 2.2.1).

2.4.5 Data Analysis:

Data for the LMA test, Rotarod, weight and temperature was expressed as mean and ± S.E.M (Standard Error of Mean). One-Way ANOVA was used to assess the significance of treated animals, the difference between the control and drug treated animals. Post-Hoc analyses was performed using Tukey HSD. Data analysis for the Irwin profile used a scoring system for each of the behaviours (section 2.4.4). Data was expressed as Median score values and interquartile range. For statistical purposes the Mann-Whitney U-test was used to asses the difference between test group and control.

Chapter 3.

3.1 Introduction.

As previously discussed excitatory Amino Acids (EAA) have been reported to play and important role in the events leading to a seizure. The most widely and frequently associated EAA receptor with epilepsy is the NMDA (N-Methyl-D-Aspartate) receptor. This receptor has two modulatory polyamine sites, one excitatory and one inhibitory. The excitatory polyamine site positively modulates the NMDA receptor activation, and may contribute to seizure pathophysiology. Another possible mechanism of action for polyamines, is their influence on voltage-activated Ca^{2+} channels. The possible role of spermine in seizures is the focus of this study.

The aim of the work presented in this chapter was to evaluate the antagonist potential of known polyamine antagonists and 6 novel polyamine analogues on spermine induced CNS excitation. The effectiveness of these putative polyamine antagonists in comparison to known NMDA antagonists and Ca^{2+} channel antagonists was assessed. The investigation concentrated on examining the role of NMDA and L-type calcium channel mechanisms involved in spermine induced effects.

In this investigation a number of classes of drugs were used, including the polyamine antagonists N^1-Dansylspermine, ifenprodil, eliprodil and arcaine. The non-competitive open channel blocker memantine and the L-type calcium antagonists nisoldipine and nitrendipine were also used.

Assessment of CNS excitation in the spermine treated mice used a scoring system (score from 1 to 5) to quantify the degree of tremor produced (Doyle et al, 1998), every 30mins from 2 to 7.5 hours after spermine administration. The median (+/- interquartile range) CNS excitation score for each treatment was calculated. Statistical significance of the difference between test and control was calculated using the Mann-Whitney U-test. Spermine treatment in the absence of antagonist produced a median score of 5 at 7.5hr after spermine administration.

3.2 Results.

3.2.1 Ifenprodil

At 3mg/kg Ifenprodil caused a notable but not statistically significant reduction in the development of CNS excitation (Figure 3.1 and Table 3.1). 10mg/kg of ifenprodil caused a significant inhibition of CNS excitation. The final dose of 20mg/kg also significantly reduced the development of the CNS excitation caused by spermine administration. Figure 3.1 and Table 3.1.

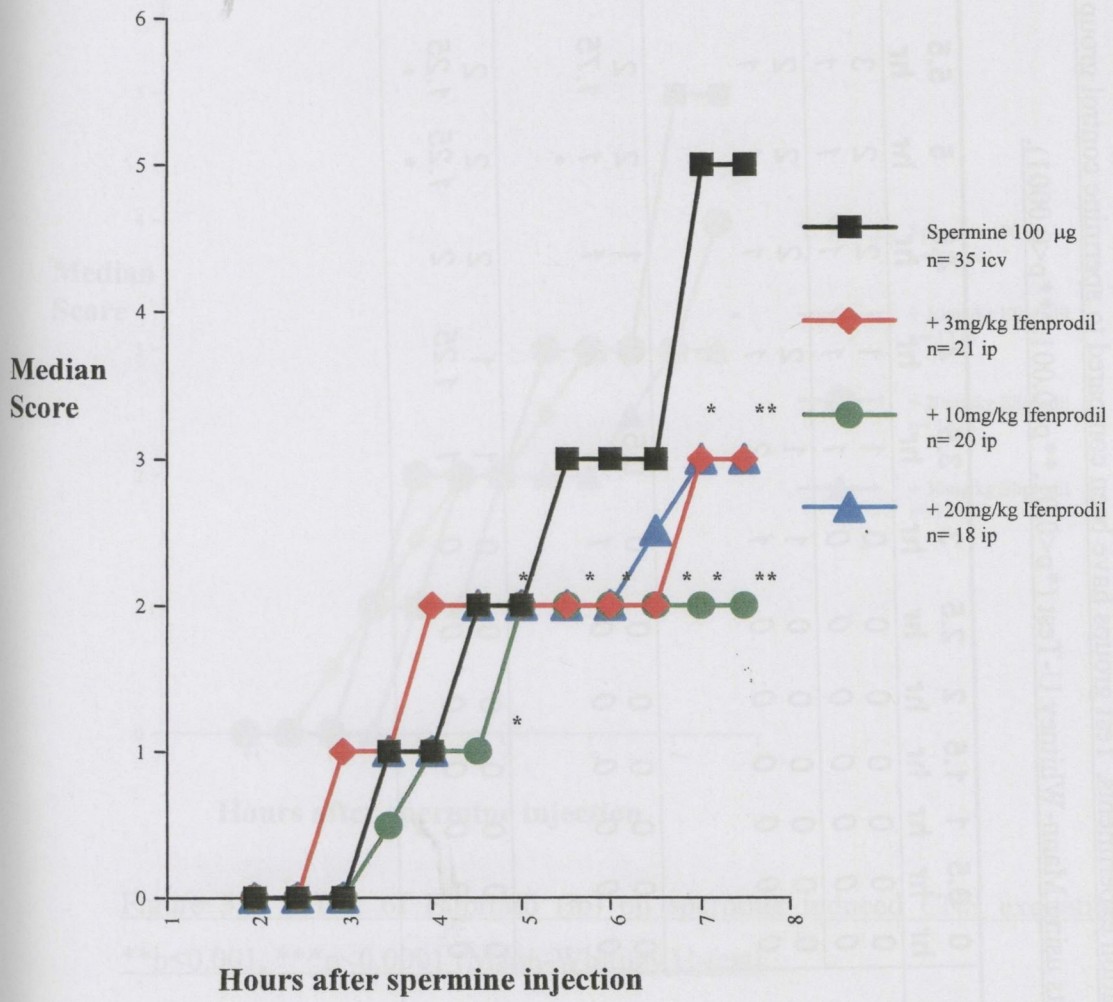


Figure 3.1: Effect of Ifenprodil (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.1: The effect of Ifenprodil on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 3mg Ifenprodil	0	0	0	0	0	0	1	1	2	2	2	2	2	2	3	3
ip n= 21	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*2*	*1*	*1*	*1*	*1*	*1.5*	*3*	*3*	*3*
+ 10mg Ifenprodil	0	0	0	0	0	0	0	0.5	1	1	2	2	2	2	2	2
ip n= 20	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*2*	*1*	*1* *	*1.75*	*1.75*	*2.5* *	*3* *	*3* **
+ 20mg Ifenprodil	0	0	0	0	0	0	0	1	1	2	2	2	2	2.5	3	3
ip n= 18	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1.25*	*2*	*1.25* *	*1.25* *	*2.25* *	*4.25*	*4.25* *	*4.25* **

3.2.2 Eliprodil.

10mg/kg or 20mg/kg of Eliprodil did not produce any statistically significant reduction in the median score value of CNS excitation. 30mg/kg produced a stastical significant decrease in CNS excitation at 7.5 hours $p<0.01$, (Figure 3.2 and Table 3.2). Overall Eliprodil was not very effective in reducing the development of CNS excitation induced by spermine.

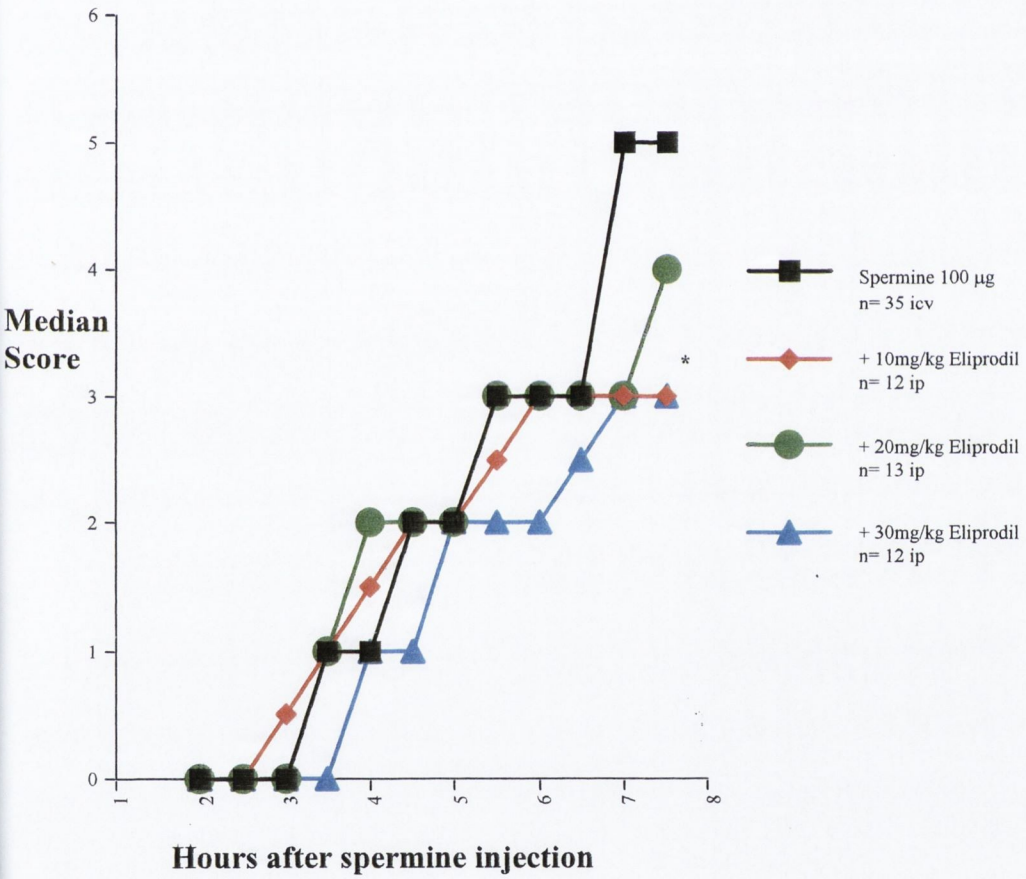


Figure 3.2: Effect of Eliprodil (ip) on spermine induced CNS excitation. *$p<0.01$, **$p<0.001$, ***$p<0.0001$ (Mann-Whitney U-test).

Table 3.2: The effect of Eliprodil on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10mg Eliprodil	0	0	0	0	0	0	0.5	1	1.5	2	2	2.5	3	3	3	3
ip n= 12	*0*	*0*	*0*	*0*	*1*	*1*	*1.75*	*1.75*	*1*	*0.75*	*1*	*1*	*1*	*0.75*	*0*	*1* *
+ 20mg Eliprodil	0	0	0	0	0	0	0	1	2	2	2	3	3	3	3	4
ip n= 13	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1.5*	*1*	*1.5*	*1.5*	*1*	*1.25*	*1*	*2*	*2*
+ 30mg Eliprodil	0	0	0	0	0	0	0	0	1	1	2	2	2	2.5	3	3
ip n= 12	*0*	*0*	*0*	*0*	*0*	*0*	*0.75*	*0.75*	*1.75*	*1.75*	*1.75*	*1.75*	*2*	*3.25*	*3.75*	*3* *

3.2.3 Arcaine

Arcaine was co-administered with spermine (see section 2.1.4). Arcaine produced a dose-dependant reduction in spermine induced CNS excitation. What is interesting from figure 3.3 is the late development of the CNS excitation with administration of Arcaine. This delay was apparent with the lowest dose of arcaine at 5 and 5.5 hours with $p<0.001$ and $p<0.01$ respectively, (Table 3.3). 20μg of Arcaine also produced a significant delay in CNS excitation, (Table 3.3). 30μg provided the most significant reduction in the development of tonic convulsions (Figure 3.3 and Table 3.3).

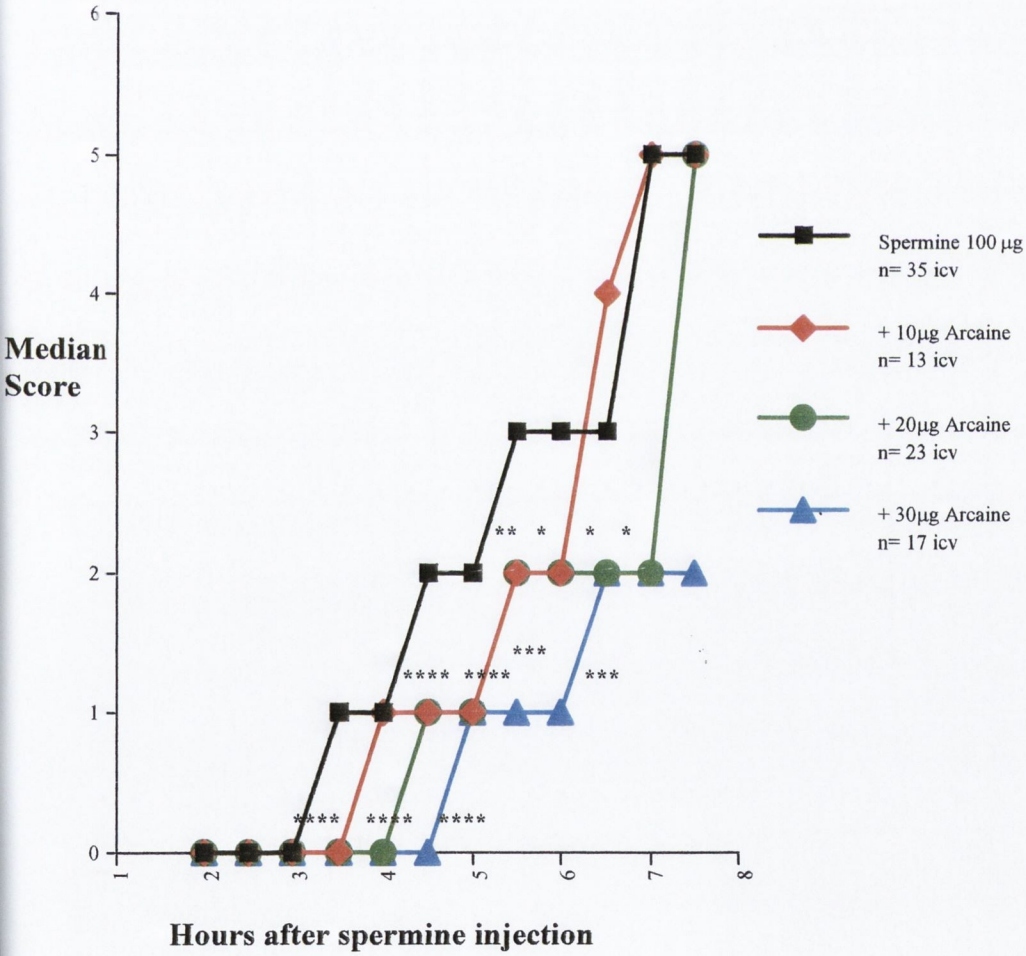


Figure 3.3: Effect of Arcaine (icv) on spermine induced CNS excitation. *$p<0.01$, **$p<0.001$, ***$p<0.0001$, ****$p<0.00001$ (Mann-Whitney U-test).

Table 3.3: The effect of Arcaine on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistical significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10μg Arcaine	0	0	0	0	0	0	0	0	1	1	1	2	2	4	5	5
icv n=13	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*
											**	*				
+ 20μg Arcaine	0	0	0	0	0	0	0	0	0	1	1	2	2	2	2	5
icv n= 23	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0*	*1*	*1*	*1*	*3*	*3*
								***	***	***	***	**	*	*	*	
+ 30μg Arcaine	0	0	0	0	0	0	0	0	0	0	1	1	1	2	2	2
icv n= 17	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*4*	*3*
								*	**	***	***	***	***	**	*	

3.2.4 Memantine

A low dose of 5mg/kg was given 30 minutes prior to spermine injection. From Figure 3.4 it may be seen that the 5mg/kg dose of Memantine significantly reduced the development of the CNS excitation caused by spermine administration, at 5.5, 6, 6.5, 7 and 7.5 hours. At 10mg/kg Memantine only reduced CNS excitation development at 3.5, 4 and 5 hours hours. 20mg/kg of Memantine also successfully reduced the development of the CNS excitation from 3.5 hours to 7.5 hours,(Figure 3.4 and Table 3.4).

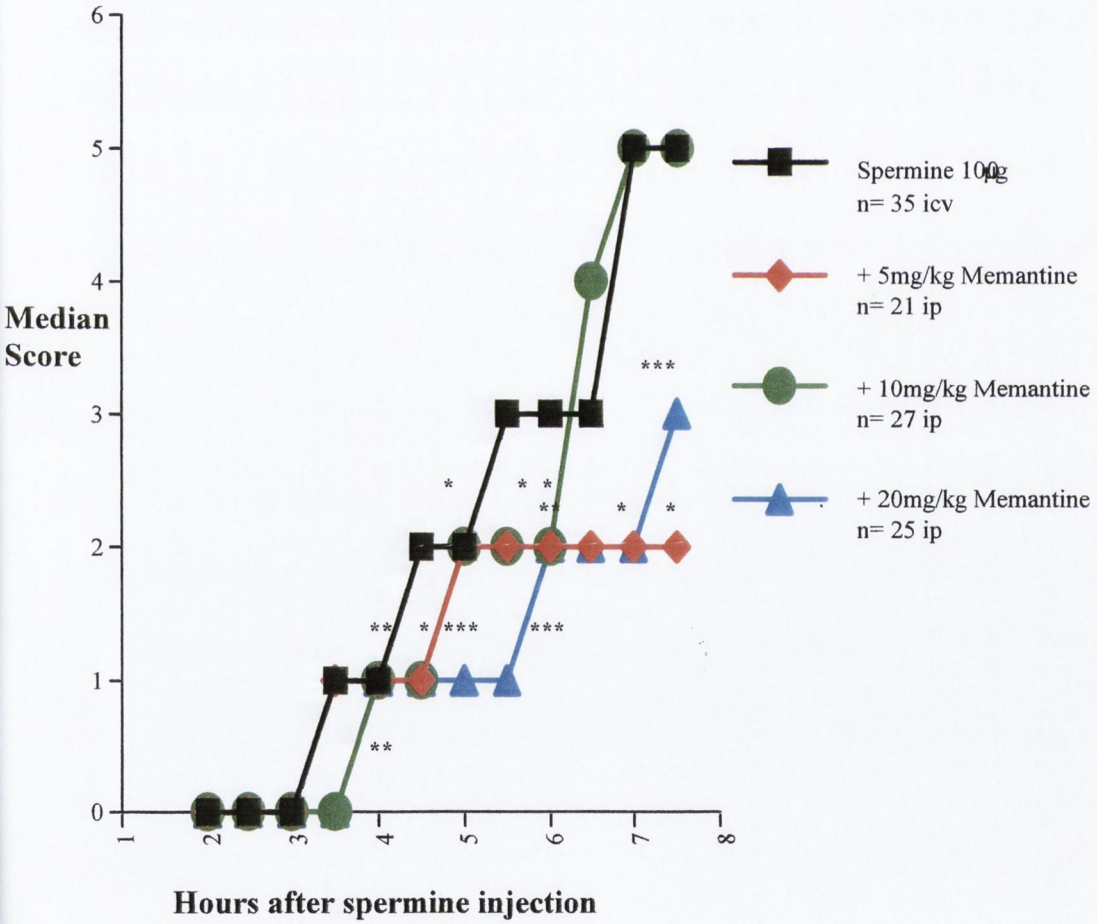


Figure 3.4: Effect of Memantine (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.4: The effect of Memantine on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100µg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 5mg Memantine	0	0	0	0	0	0	0	1	1	1	2	2	2	2	2	2
ip n= 21	*0*	*0*	*0*	*0*	*0*	*0*	*0.5*	*1*	*2*	*1*	*1.5*	*1.5*	*1.5*	*1.5*	*3.5*	*3.5*
												*	*	**	*	*
+ 10mg Memantine	0	0	0	0	0	0	0	0	1	1	2	2	2	4	5	5
ip n= 27	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*
								**	**		*					
+ 20mg Memantine	0	0	0	0	0	0	0	0	1	1	1	1	2	2	2	3
ip n= 25	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.5*	*1*	*1*	*1*	*1*	*1.5*	*2*	*2*	*1.5*
								*	*	*	***	**	**	***		***

3.2.5 Nisoldipine

Figure 3.5 exhibits the results obtained for Nisoldipine. 2mg/kg provided a marked reduction in the development of the CNS excitation. Statistically significant differences were seen from 3 hours (Table 3.5). 5mg/kg of Nisoldipine also significantly inhibited the onset of the spermine induced CNS excitation, with statistically significant differences from 3.5 hours. Table 3.5 illustrates these results. The final dose of 10mg/kg also showed some inhibition of CNS excitation. However the inhibition was not as pronounced at this dose. Statistically significant differences were observed at 3.5 hours and 7.5 hours Figure 3.5 and Table 3.5.

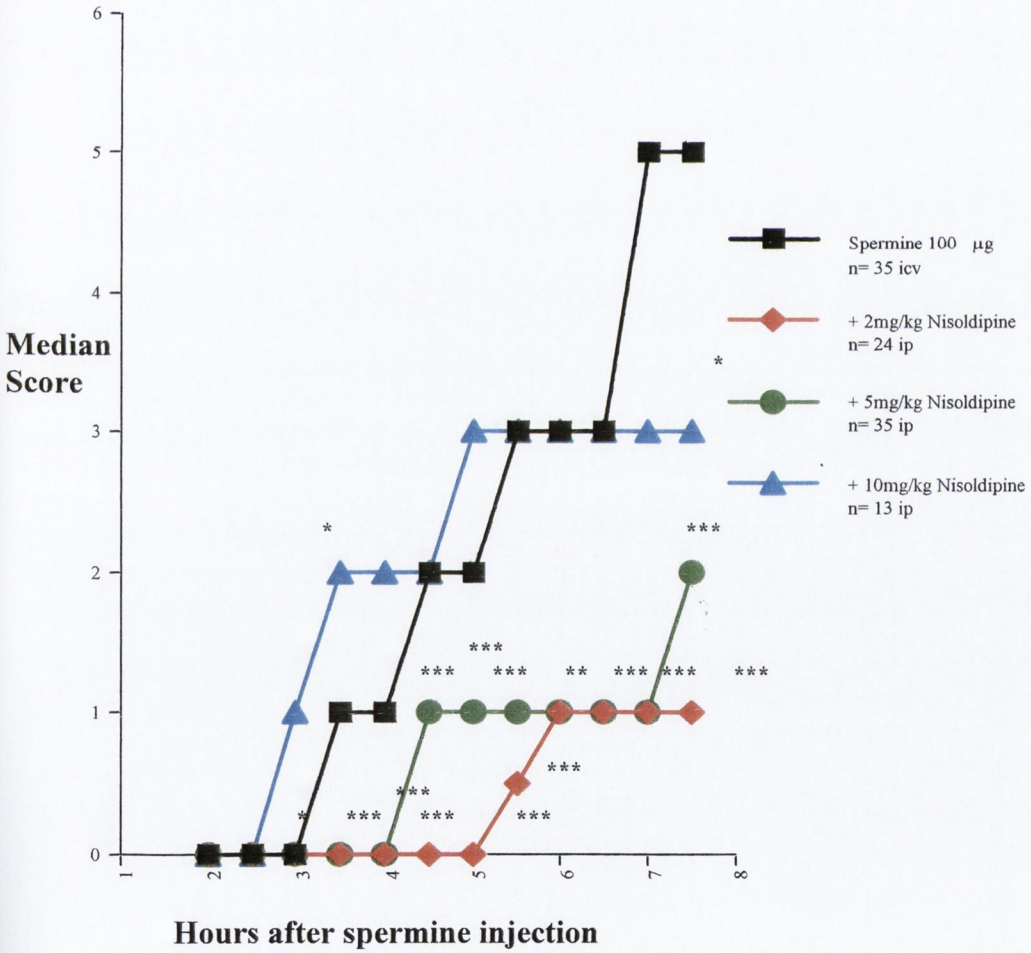


Figure 3.5: Effect of Nisoldipine (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.5: The effect of Nisoldipine on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 2mg Nisoldipine	0	0	0	0	0	0	0	0	0	0	0	0.5	1	1	1	1
ip n= 24	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*2*	*2*	*3.5*	*4*
							*	***	***	***	***	***	**	***	***	***
+ 5mg Nisoldipine	0	0	0	0	0	0		0	0	1	1	1	1	1	1	2
ip n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*2*
								***	***	***	***	***	***	***	***	***
+ 10mg Nisoldipine	0	0	0	0	0	0	1	2	2	2	3	3	3	3	3	3
ip n= 13	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*2*	*1*	*2*	*2*	*2*	*3*	*3*
								*								*

3.2.6 Nitrendipine

From Figure 3.6 it may be seen that at the dose of 5 mg/kg Nitrendipine reduced the effects of spermine. A significant effect of Nitrendipine is apparent from 4.5 hours (Figure 3.6 and Table 3.6). 10mg/kg provided a better inhibition of spermine induced CNS excitation than 5mg/kg. A statistically significant effect is observed from 3.5 hours, Figure 3.6 and Table 3.6 illustrate these values. 20mg/kg Nitrendipine also significantly reduced the CNS excitation which was apparent from 4.5 hours (Figure 3.6 Table 3.6).

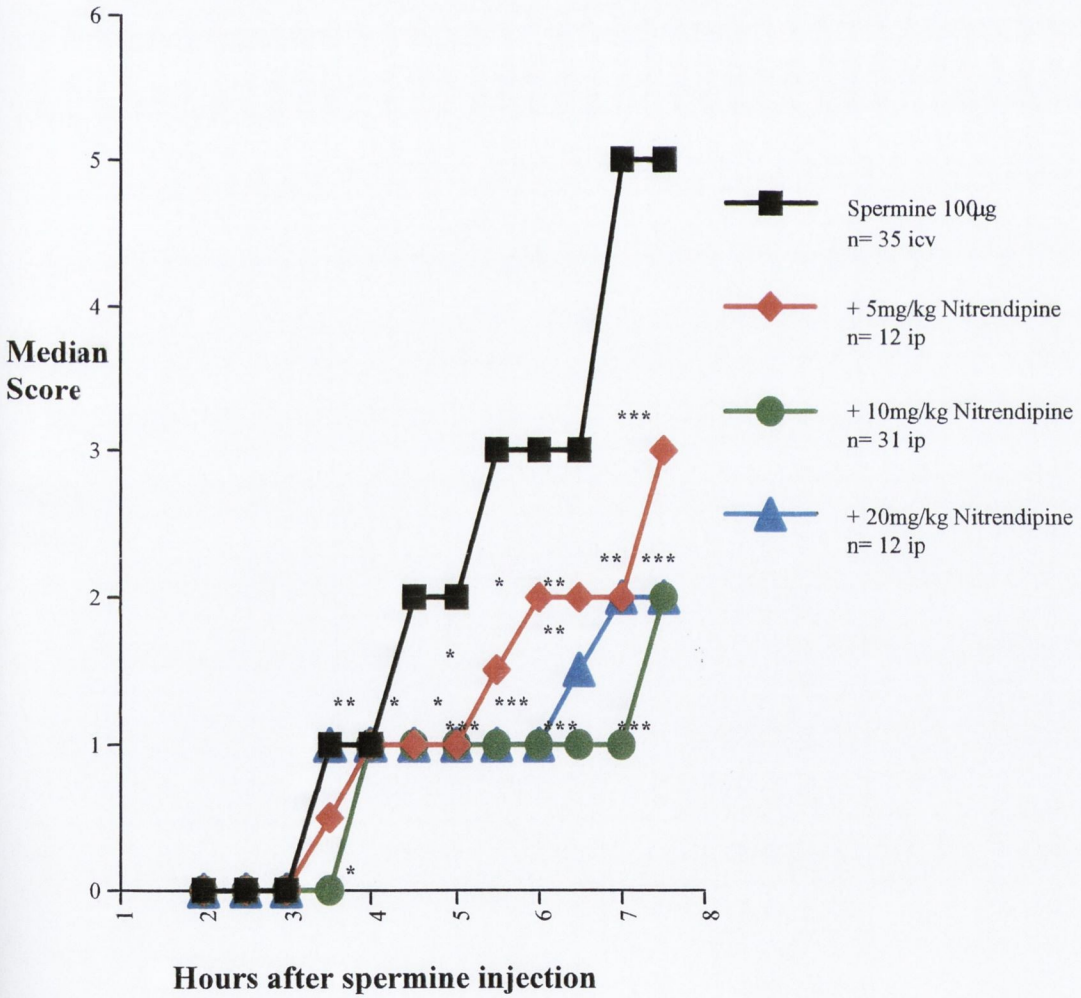


Figure 3.6: Effect of Nitrendipine (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.6: The effect of Nitrendipine on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 5mg Nitrendipine	0	0	0	0	0	0	0	0.5	1	1	1	1.5	2	2	2	3
ip n= 12	*0*	*0*	*0*	*0*	*0*	*0*	*0.75*	*1*	*0*	*0*	*1*	*1*	*1.75*	*1.75*	*1*	*1*
										*	*	*	*	**	**	***
+ 10mg Nitrendipine	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	2
ip n= 31	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0*	*1*	*1*	*1*	*1*	*1*
								*	**	***	***	***	***	***	***	***
+ 20mg Nitrendipine	0	0	0	0	0	0	0	1	1	1	1	1	1	1.5	2	2
ip n= 12	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0.75*	*0.75*	*1.75*	*1.75*	*1.75*	*1.75*	*4*	*4.25*
										*	**	**	**	**	**	**

3.2.7 N^1-Dansylspermine

At 5µg/20µl, N^1-Dansylspermine had no effect on the development of the CNS excitation. There was no significant difference between test and control at any time point (Figure 3.7 and Table 3.7). A dose of 10µg showed a significant reduction in CNS excitation score from 5 to 7 hours following spermine administration, see table 3.7 for individual values.

The highest dose of N^1-Dansylspermine used was 20µg/20µl. The maximum median score value obtained here was 2 at the final time point of 7.5 hours indicating that this dose was reducing the development of the CNS excitation. Statistically significant differences were observed from 3.5 hours see Table 3.7 and Table 3.7.

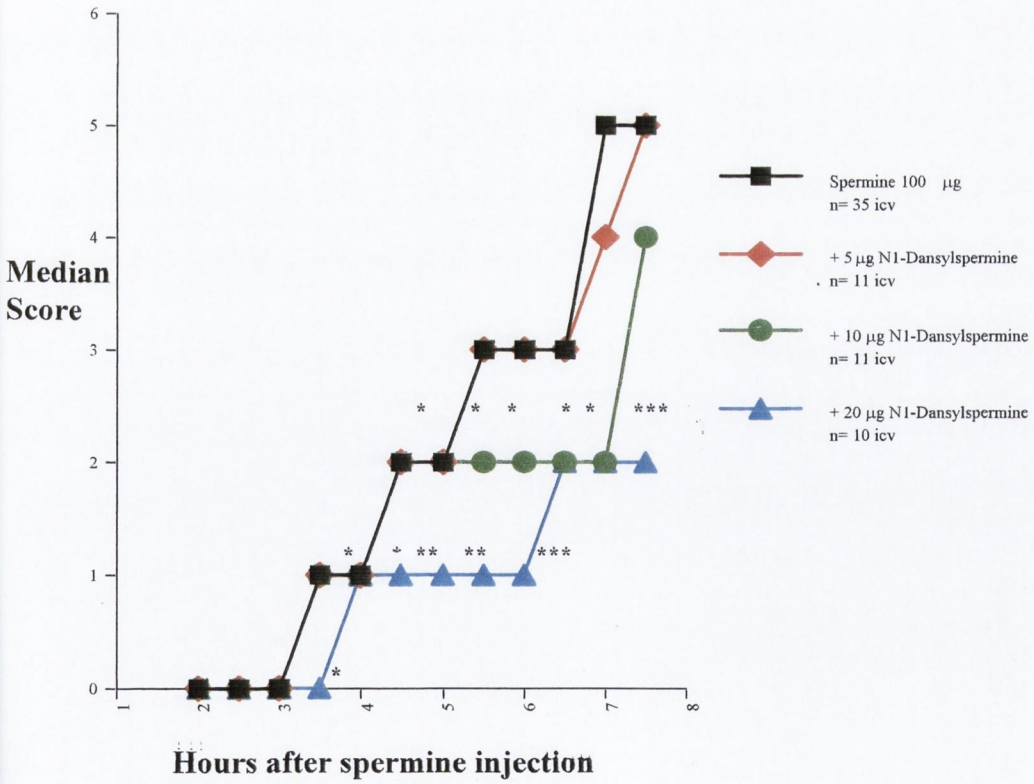


Figure 3.7: Effect of N^1-Dansylspermine (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.7: The effect of N^1- Dansylspermine on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 5μg N^1-Dansylspermine	0	0	0	0	0	0	0	1	1	2	2	3	3	3	4	5
icv n=11	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0*	*1*	*1*	*3*	*2*	*2*
+10μg N^1-Dansylspermine	0	0	0	0	0	0	0	1	1	2	2	2	2	2	2	4
icv n= 11	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*2*	*2*	*2* *	*2* *	*2* *	*2* *	*2* *	*3*
+20μg N^1-Dansylspermine	0	0	0	0	0	0	0	0	1	1	1	1	1	2	2	2
icv n= 10	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0* *	*1* *	*1* *	*1.25* **	*1* **	*1* ***	*1* ***	*1* ***	*0.25* ***

3.3 Effect of Novel Compounds (icv administration).

The novel compounds were co-administered with spermine through the i.c.v route as described in section 2.1.1. The initial dose used was 10μg/20μl which was co-administered with 100μg of spermine to establish a safe dose range which would show an effect. This was used as the standard starting dose for each of the novel compounds. Depending on the result from using 10μg the succeeding doses were either lower or higher.

3.3.1 Novel Compound Bu 31b

From Figure 3.8 it may be seen that at 10μg there was no effect on spermine-induced CNS excitation by Bu 31b. However the dose of 20μg showed a remarkable inhibition of the spermine induced CNS excitation. The inhibition was statistically significant from the 4 hour time point (Figure 3.8 and Table 3.8). Bu 31b at 30μg also caused a significant decrease in spermine induced CNS excitation. Statistical significance of difference was seen from the 5 hour time point (Figure 3.8 and Table 3.8).

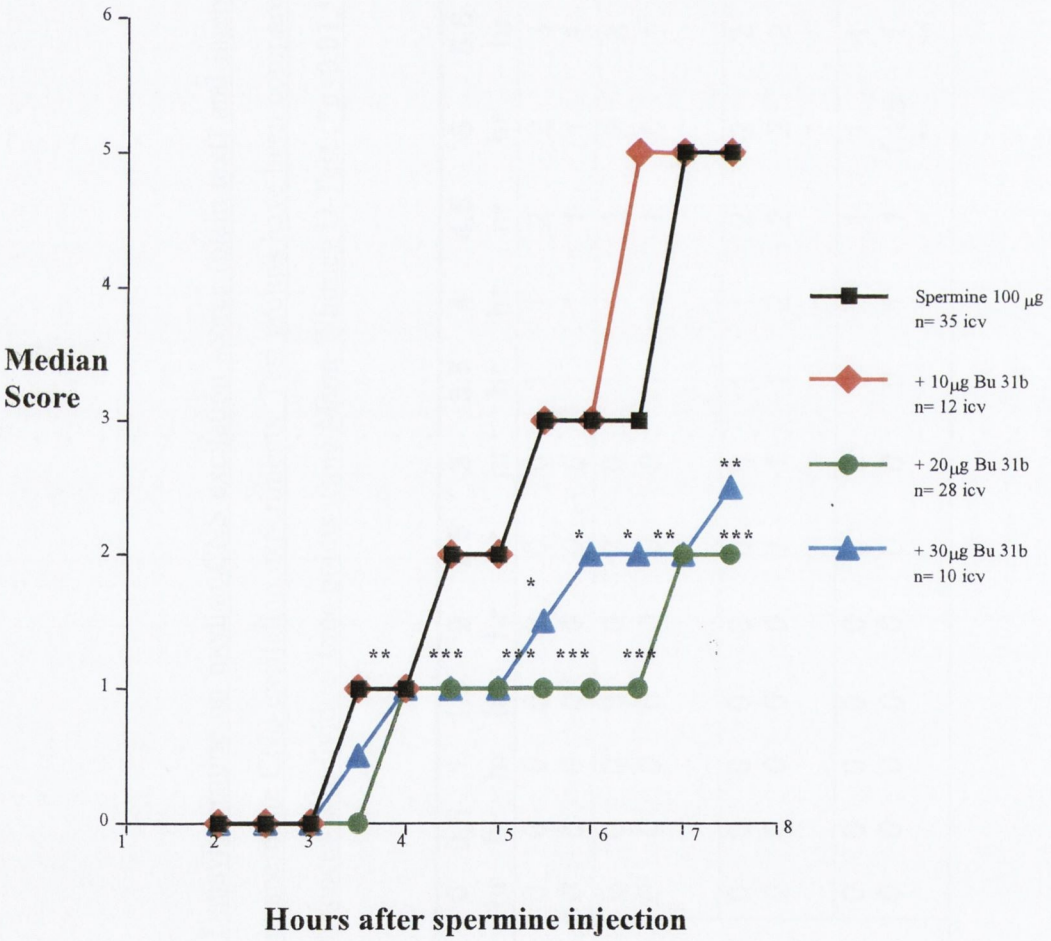


Figure 3.8: Effect of Bu 31b (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.8: The effect of Bu 31b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100µg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10µg Bu 31b	0	0	0	0	0	0	0	1	1	2	2	3	3	5	5	5
icv n=12	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.75*	*0*	*1*	*0.75*	*2.5*	*2.5*	*2*	*2*	*2*
+ 20µg Bu 31b	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	2
icv n= 28	*0*	*0*	*0*	*0*	*0*	*0*	*0.75*	*1*	*1*	*1*	*1*	*1*	*1*	*1.75*	*1.75*	*3.5*
									**	***	***	***	***	***	***	***
+ 30µg Bu 31b	0	0	0	0	0	0	0	0.5	1	1	1	1.5	2	2	2	2.5
icv n= 10	*0*	*0*	*0*	*0*	*0*	*0.25*	*1*	*1*	*1.25*	*1.25*	*1*	*1.25*	*2*	*1.25*	*1.25*	*1.25*
											*	*	*	*	**	**

3.3.2 Novel Compound Bu 37b

Figure 3.9 represents the results for the investigation of Bu 37b (icv) on spermine induced CNS excitation. Again 10μg/20μl was the initial dose used. From Figure 3.9 it can be seen that at 10μg there is a difference between treatment and control. Although both terminate with a median score value of 5, the progression in animals administered Bu 37b is much quicker than that of spermine alone, (Figure 3.9 and Table 3.9). At 20μg/20μl there is no statistically significant difference between spermine alone and Bu 37b treated animals. Finally at 30μg/20μl, Bu 37b showed a trend towards some improvement in inhibiting the spermine induced CNS excitation. However, the effect was not statistically significant (Figure 3.9 and Table 3.9).

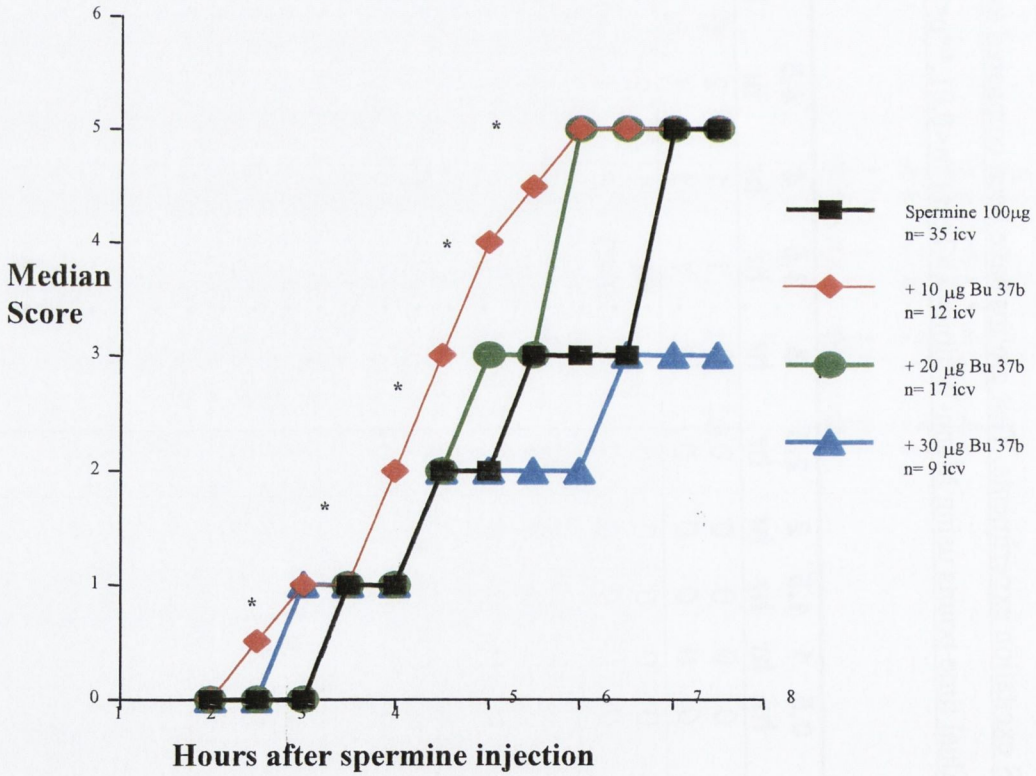


Figure 3.9: Effect of Bu 37b (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.9: The effect of Bu 37b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10μg Bu 37b	0	0	0	0	0	0.5	1	1	2	3	4	4.5	5	5	5	5
icv n=12	*0*	*0*	*0*	*0*	*0*	*1* *	*0* *	*0.75*	*0.75* *	*2.75* *	*2* *	*2*	*2*	*0*	*0*	*0*
+ 20μg Bu 37b	0	0	0	0	0	0	0	1	1	2	3	3	5	5	5	5
icv n= 17	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*2.5*	*3*	*3*	*3*	*2*	*1*	*0*
+ 30μg Bu 37b	0	0	0	0	0	0	1	1	1	2	2	2	2	3	3	3
icv n= 9	*0*	*0*	*0*	*0*	*0*	*0.5*	*1*	*1*	*1*	*1*	*1.5*	*1*	*1*	*1*	*1*	*2*

3.3.3 Novel Compound Bu 33b

Figure 3.10 shows the results for the effect of Bu 33b on the CNS excitation of spermine. The initial dose of 10μg/20μl had no effect on spermine induced CNS excitation (Figure 3.10). Interestingly the higher dose of 20μg/20μl proved to be more effective at reducing the CNS excitation from 3.5 hours, (Figure 3.10 and Table 3.10). Finally the highest dose of 30μg/20μl showed no statistically significant effect on the development of the CNS excitation behaviour.

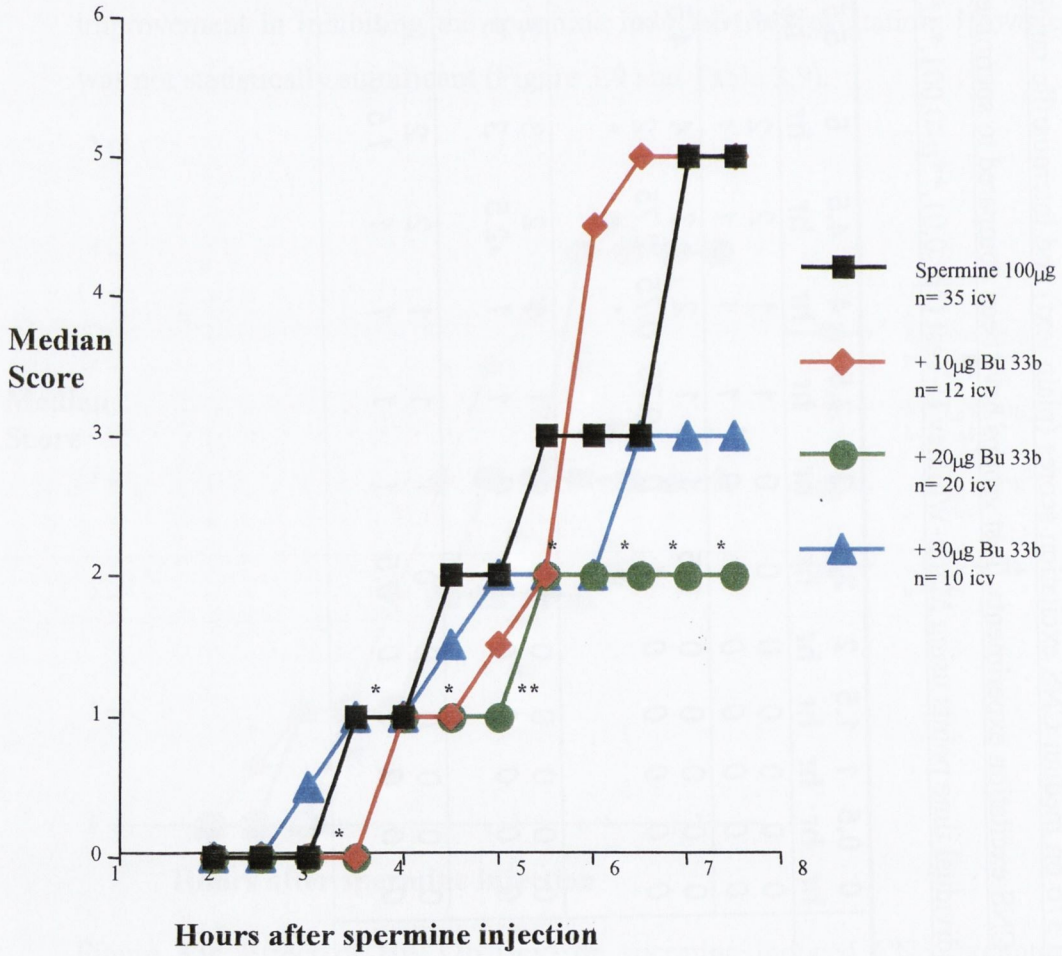


Figure 3.10: Effect of Bu 33b (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.10: The effect of Bu 33b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10μg Bu 33b	0	0	0	0	0	0	0	0	1	1	1.5	2	4.5	5	5	5
icv n=12	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1.5*	*1*	*3*	*3*	*3*	*3*	*1.5*
+ 20μg Bu 33b	0	0	0	0	0	0	0	0	1	1	1	2	2	2	2	2
icv n= 20	*0*	*0*	*0*	*0*	*0*	*0*	*0.75*	*1*	*1*	*0.75*	*1*	*1*	*2.75*	*3*	*4*	*3.75*
								*	*	*	**	*		*	*	*
+ 30μg Bu 33b	0	0	0	0	0	0	0.5	1	1	1.5	2	2	2	3	3	3
icv n= 10	*0*	*0*	*0*	*0*	*0.25*	*1*	*1*	*2*	*1*	*1*	2	*1.25*	*1.25*	*3*	*3*	*3*

3.3.4 Novel Compound Bu 40b

Figure 3.11 displays the results found for the effect of Bu 40b on spermine induced CNS excitation. Bu 40b was co-administered with 100μg/20μl of spermine through the intracerebroventricular route. At the dose of 10μg it may be seen that there was no inhibition of CNS excitation behaviour. In fact a significant potentiation was observered at 5.5 hours as there was a more rapid development of the CNS excitation in animals given Bu 40b in comparison to the administration of spermine alone (see Figure 3.11). The middle dose of 20μg/20μl of Bu 40b showed one statistically significant difference at 3.5 hours. However a maximum median score of 5 was observered from 6 hours, (Figure 3.11 and Table 3.11). The final dose of 30μg/20μl proved to be very interesting. In fact a significant potentiation was observered from 2 hours to 4.5 hours and there was a more rapid development of the CNS excitation, cumulating with a maximum median score value of 5 from 6.5 hours (Figure 3.11 and Table 3.11).

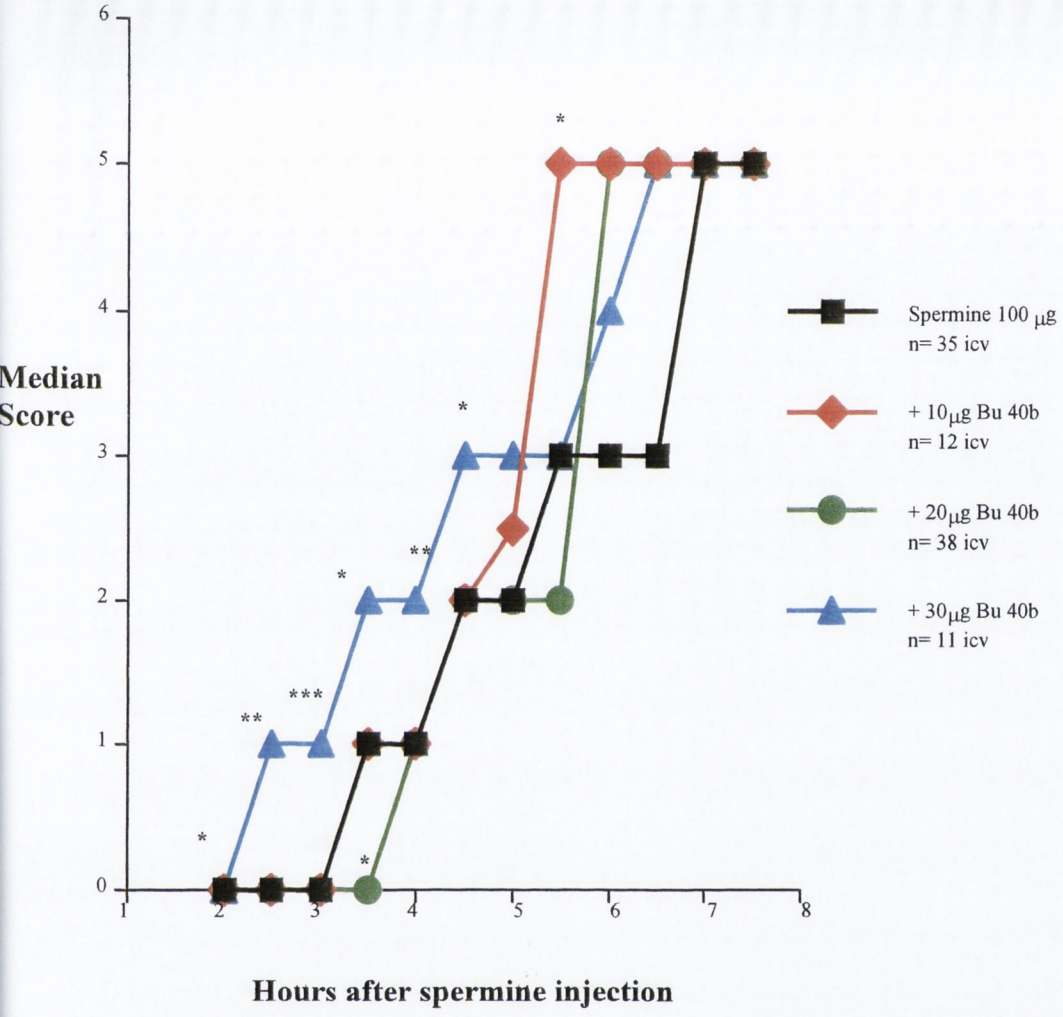


Figure 3.11: Effect of Bu 40b (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.11: The effect of Bu 40b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100µg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10µg Bu 40b	0	0	0	0	0	0	0	1	1	2	2.5	5	5	5	5	5
icv n=12	*0*	*0*	*0*	*0*	*0*	*0*	*0.75*	*1*	*2*	*3*	*3*	*2* *	*2*	*2*	*2*	*0.75*
+ 20µg Bu 40b	0	0	0	0	0	0	0	0	1	2	2	2	5	5	5	5
icv n= 38	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1* *	*2*	*1*	*2*	*3*	*3*	*3*	*2*	*0.5*
+ 30µg Bu 40b	0	0	0	0	0	1	1	2	2	3	3	3	4	5	5	5
icv n= 11	*0*	*0*	*0*	*0*	*1* *	*1* **	*1* ***	*1* **	*0* **	*1* *	*1*	*2*	*2*	*2*	*0*	*0*

3.3.5 Novel Compound Bu 36b

Bu 36b was co-administered through the icv route with 100μg spermine in 20μl at a starting dose of 10μg. From Figure 3.12 it may be seen that this dose was very significantly effective by reducing the maximum median score value to 1.5 at 7.5 hours, hence a lower dose of 5μg was also used in this study. The low dose of 5μg showed no significant effect. A 20μg dose of Bu 36b also significantly inhibited the response to spermine in comparison to control, (Figure 3.12 and Table 3.12).

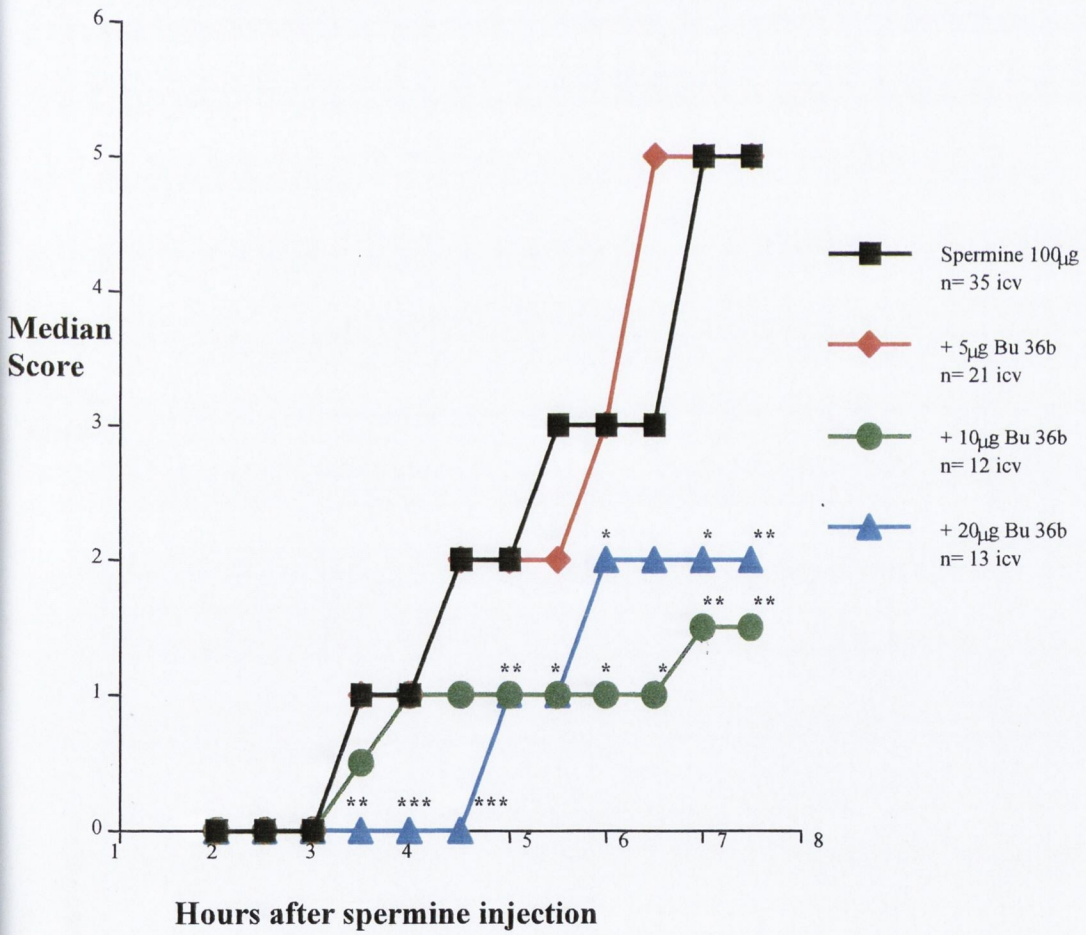


Figure 3.12: Effect of Bu 36b (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.12: The effect of Bu 36b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 5μg Bu 36b	0	0	0	0	0	0	0	1	1	2	2	2	3	5	5	5
icv n=21	*0*	*0*	*0*	*0*	*0*	*0*	*0.5*	*1*	*1.5*	*1*	*2*	*2.5*	*3*	*3*	*2.5*	*2*
+ 10μg Bu 36b	0	0	0	0	0	0	0.5	1	1	1	1	1	1	1	1.5	1.5
icv n= 12	*0*	*0*	*0*	*0*	*0*	*0*	*0.75*	*1*	*1.75*	*1.75*	*1*	*1.75*	*1.75*	*2*	*3.5*	*3.5*
											**	*	*	*	**	**
+ 20μg Bu 36b	0	0	0	0	0	0	0	0	0	0	1	1	2	2	2	2
icv n= 13	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.5*	*1*	*2*	*2*	*2*	*4.5*	*4*	*4*
								**	***	***	*	*	*		*	**

3.3.6 Novel Compound Bu 43b

The initial dose used as before was 10μg/20μl. From Figure 3.13 it can be seen that this proved to have a significant effect, hence a lower dose of 2μg was used to see if this had an effect. 2μg showed no effect reducing the spermine induced CNS excitation. Bu 43b at 10μg significantly inhibited the spermine induced behavioural profile development, by a maximum median score value of 1, Figure 3.13 and Table 3.13. 20μg of Bu 43b totally inhibited CNS excitation over the 7.5 hour period of the study, a maximum score value of 0, Figure 3.13 and Table 3.13 illustrates this.

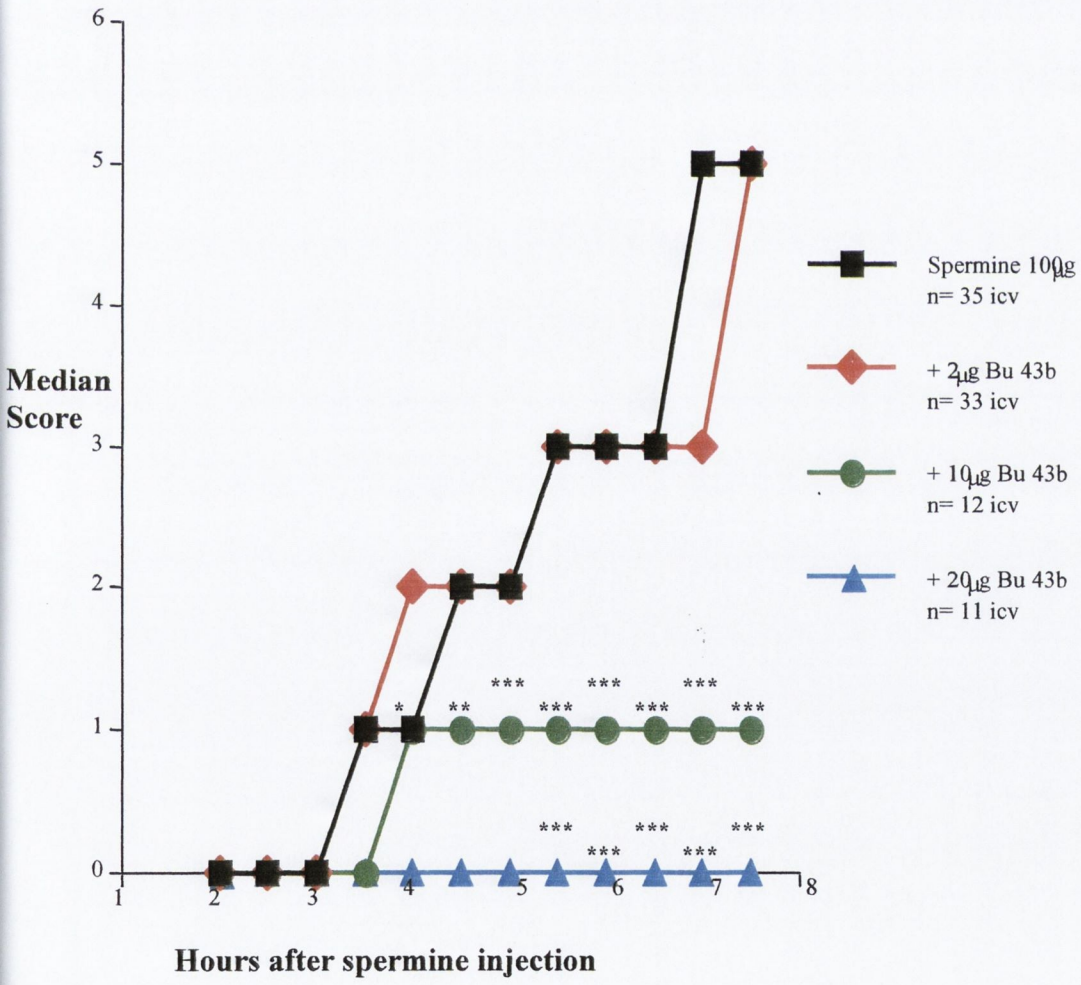


Figure 3.13: Effect of Bu 43b (icv) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.13: The effect of Bu 43b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 2μg Bu 43b	0	0	0	0	0	0	0	1	2	2	2	3	3	3	3	5
icv n=33	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1.5*	*1*	*0*	*1*	*2.5*	*3*	*3*	*3*	*2*
+ 10μg Bu 43b	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
icv n= 12	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0*	*0*	*0*	*0*	*1*	*1*	*1*
									*	**	***	***	***	***	***	***
+ 20μg Bu 43b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
icv n= 11	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*
												***	***	***	***	***

3.4 Effect of Novel Compounds (ip administration).

The next step taken with the novel compounds was to investigate their potential as possible polyamine antagonists through peripheral administration. This would give an indication of whether or not these novel compounds had the ability to penetrate and cross the blood-brain -barrier.

The standard dose of 10mg/kg was the initial dose used to investigate activity through a peripheral route. As previously described each of the compounds were administered via the intraperitoneal route 30 minutes prior to spermine administration. The assessment methodology used was by assessing the degree of tremor through the tail (Section 2.1.2). The Mann-Whitney U-test was used for analysis of significant differences between test and control.

3.4.1 Novel Compound Bu 31b

Figure 3.14 displays the results for Bu 31b when administered through the intraperitoneal route. At 10mg/kg it can be seen that Bu 31b has had an inhibitory effect in the development of the CNS excitation behaviour, apparent from 3.5 hours, with a maximum median score of 3 obtained throughout (Figure 3.14 and Table 3.14). 20mg/kg was also effective in reducing the CNS excitation induced by spermine. The maximum median score value was 2 from the 5 hour time point, clearly indicating a statistically significant inhibitory response. Similarly, with 30mg/kg, the maximum median score value obtained was 2 from 6 hours onwards (Figure and Table 3.14).

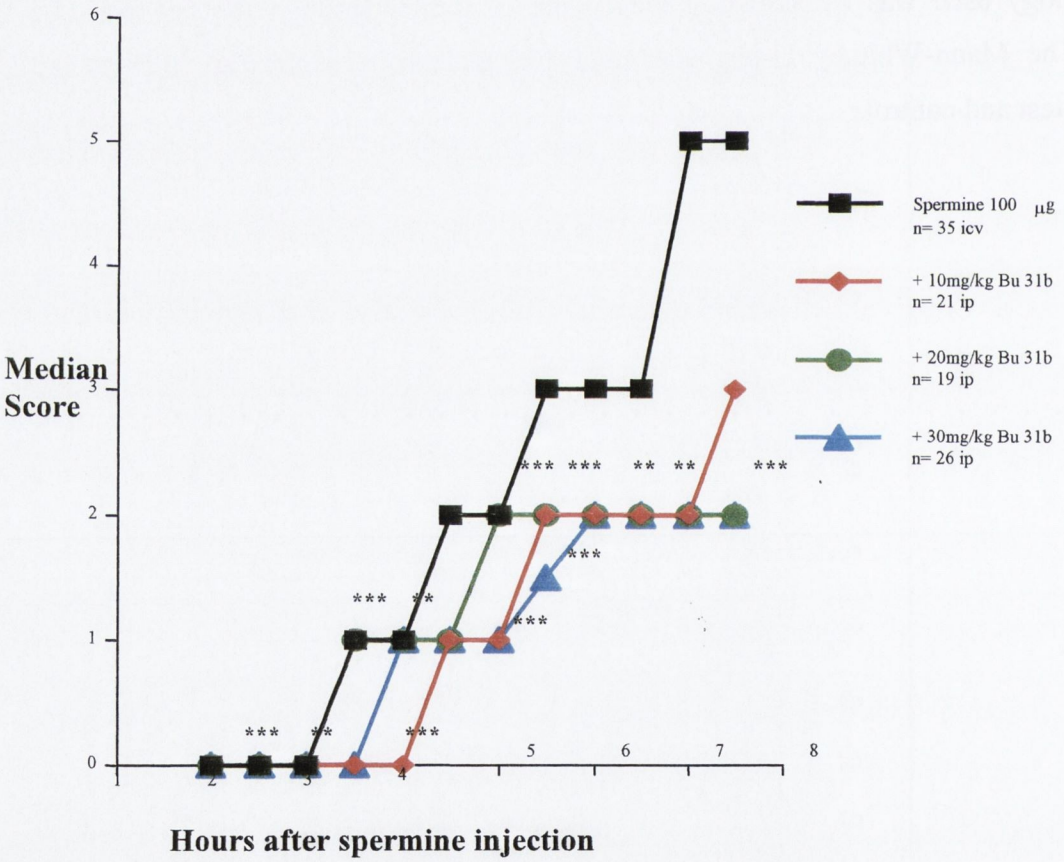


Figure 3.14: Effect of Bu 31b (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001(Mann-Whitney U-test).

Table 3.14: The effect of Bu 31b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001, ****p<0.00001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100µg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10mg Bu 31b	0	0	0	0	0	0	0	0	0	1	1	2	2	2	2	3
ip n= 21	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*1*	*1.5*	*1.5*	*1*	*1*	*2*	*2*	*3*
								**	***	**	***	***	***	**	**	
+ 20mg Bu 31b	0	0	0	0	0	0	0	1	1	1	2	2	2	2	2	2
ip n= 19	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*3*	*2*
											**	**	***	**	**	**
+ 30mg Bu 31b	0	0	0	0	0	0	0	0	1	1	1	1.5	2	2	2	2.5
ip n= 26	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0*	*1*	*1*	*1*	*2.5*	*4*	*3.25*
							***	***	***	**	***	***	***	**	*	**

3.4.2 Novel Compound Bu 37b

A 10mg/kg dose had no effect on reducing the CNS excitation caused by spermine, Figure 3.15. Similarly the middle dose of 20mg/kg also proved to have little effect in reducing the CNS excitation. Once again the maximum median score value of 5 was obtained from time point 6.5 hours onwards. There was no significant differences between test and control. The final concentration of 30mg/kg also had no effect in reducing the development of the CNS excitation behaviour (Figure 3.15 and Table 3.15).

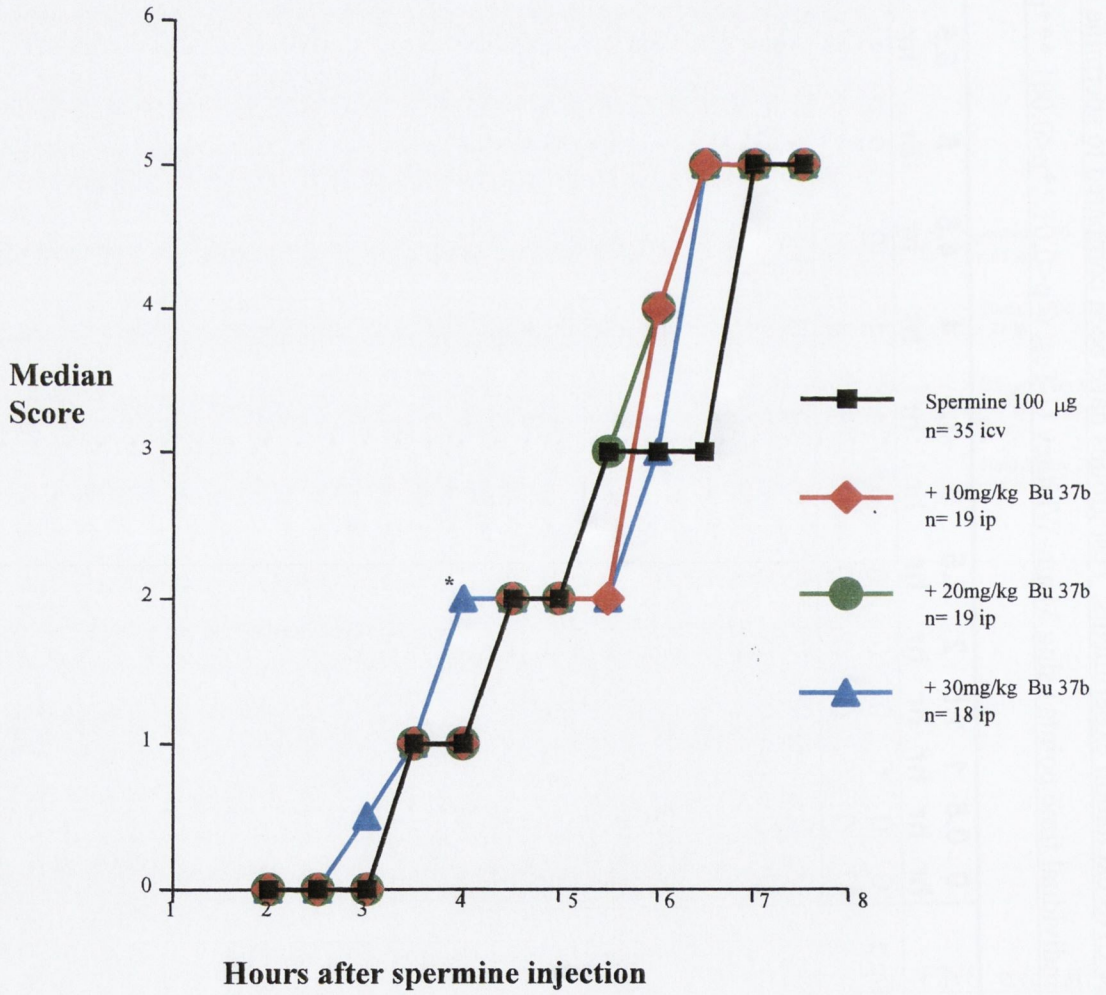


Figure 3.15: Effect of Bu 37b (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.15: The effect of Bu 37b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10mg Bu 37b	0	0	0	0	0	0	0	1	1	2	2	2	4	5	5	5
ip n= 19	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*0*	*3*	*3*	*3*	*2*	*0*
+ 20mg Bu 37b	0	0	0	0	0	0	0	1	1	2	2	3	4	5	5	5
ip n= 19	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*2*	*3*	*2*	*2*	*2*	*2*
+ 30mg Bu 37b	0	0	0	0	0	0	0.5	1	2	2	2	2	3	5	5	5
ip n= 18	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1* *	*0*	*1*	*1.25*	*3*	*3*	*3*	*3*

3.4.3 Novel Compound Bu 33b

At 10mg/kg Bu 33b significantly reduced spermine induced CNS excitation with a maximum median score of 2, from 3.5 hours (Figure 3.16 and Table 3.16). 20mg/kg and 30mg/kg also caused significant inhibition of the development of the CNS excitation behaviour.

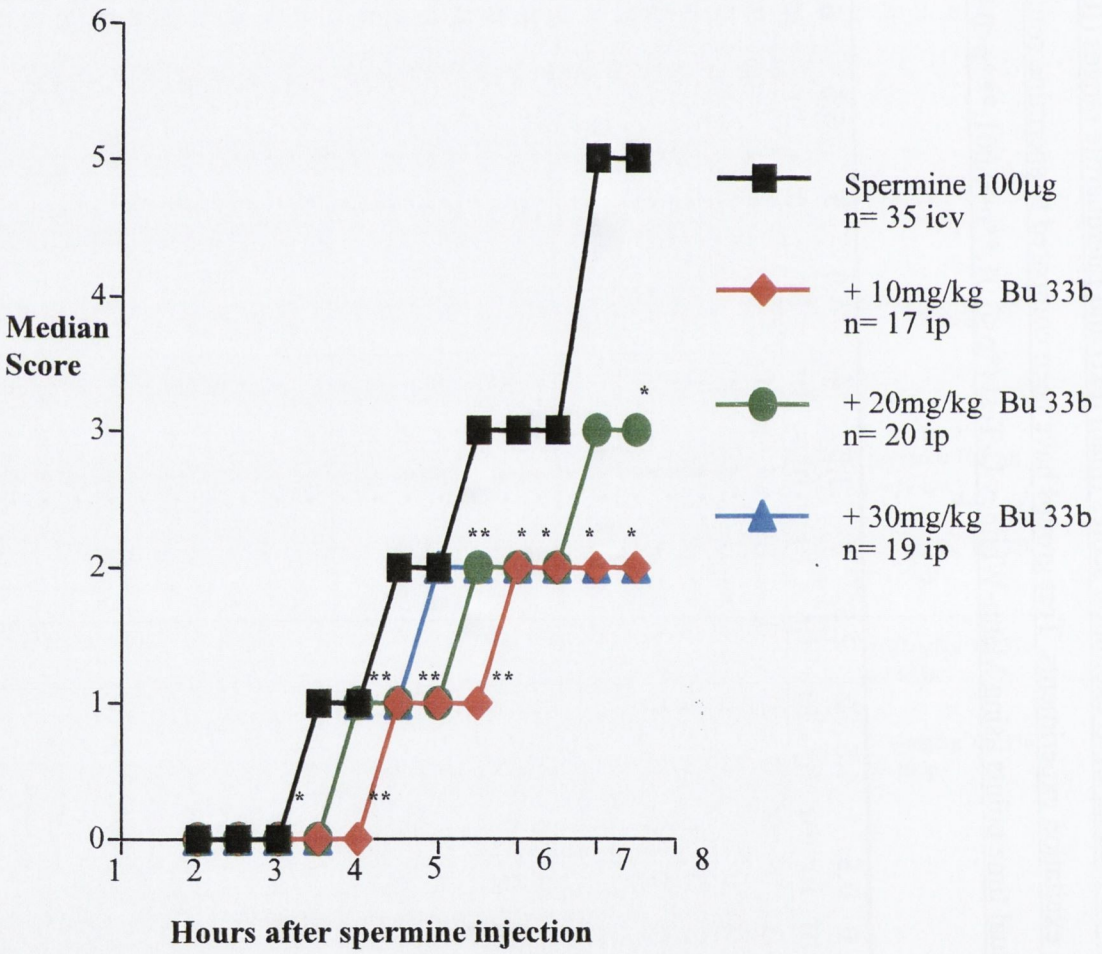


Figure 3.16: Effect of Bu 33b (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.16: The effect of Bu 33b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100µg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10mg Bu 33b	0	0	0	0	0	0	0	0	0	1	1	1	2	2	2	2
ip n= 17	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.5*	*1*	*1*	*1*	*1*	*2*	*3.5*	*3*	*3*
								*	**	**	**	**	*		*	
+ 20mg Bu 33b	0	0	0	0	0	0	0	0	1	1	1	2	2	2	3	3
ip n= 20	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*0.75*	*0.75*	*1*	*1*	*1*	*2*	*3*	*3*
											**	**	**	*		*
+ 30mg Bu 33b	0	0	0	0	0	0	0	0	1	1	2	2	2	2	2	2
ip n= 19	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0*	*1*	0	*3*	*3*	*3*	*3*
									*		*					*

3.4.4 Novel Compound Bu 40b

10mg/kg Bu 40b had no effect on the spermine induced CNS excitation. However at 20mg/kg Bu 40b had a mild effect on the development of the CNS excitation induced by spermine. Only one time point showed a statistically significant difference, (7.5 hours), Figure 3.17 and Table 3.17. At the higher dose of 30mg/kg, Bu 40b had no effect on inhibiting spermine induced CNS excitation.

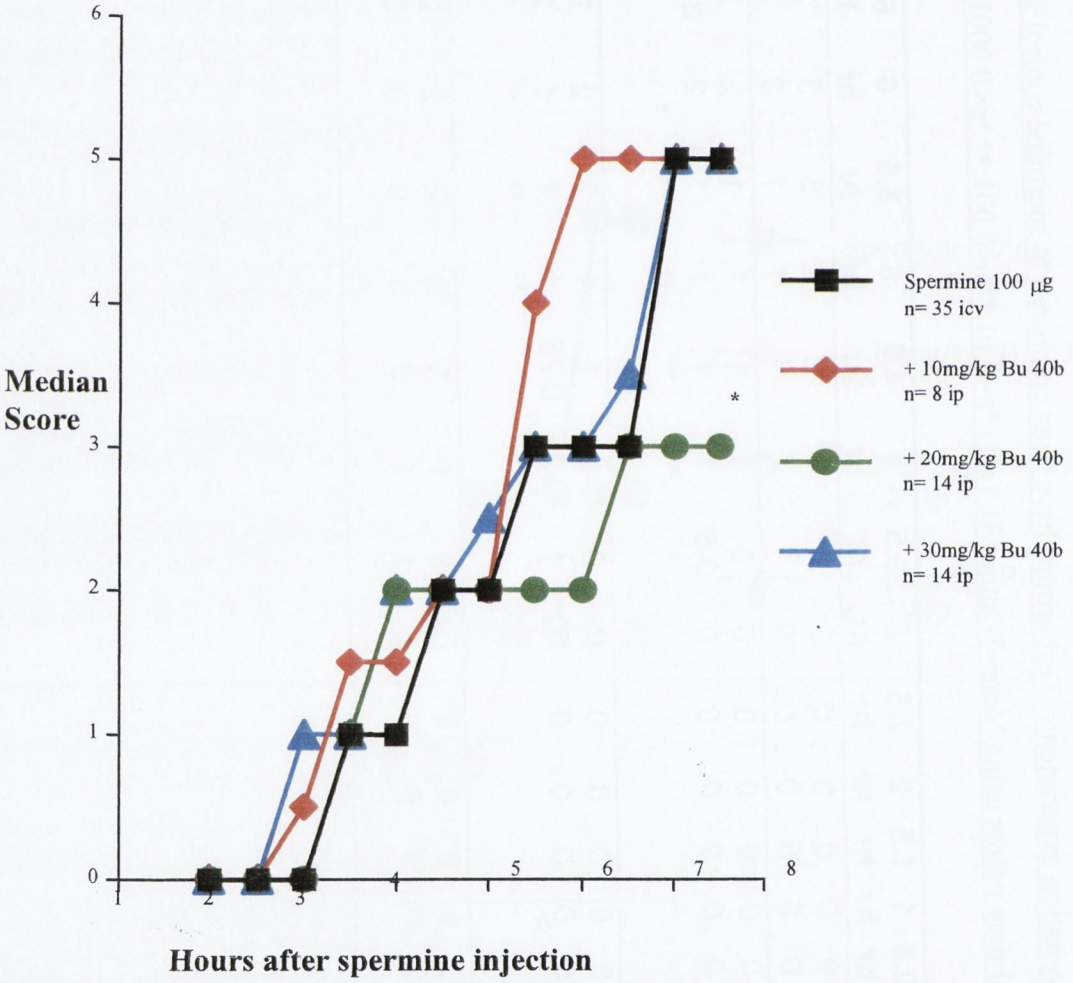


Figure 3.17: Effect of Bu 40b (i.p) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.17: The effect of Bu 40b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10mg Bu 40b	0	0	0	0	0	0	0.5	1.5	1.5	2	2	4	5	5	5	5
ip n= 8	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1.75*	*1*	*0.75*	*1.75*	*3*	*2.75*	*0.75*	*0*	*0*
+ 20mg Bu 40b	0	0	0	0	0	0	0	1	2	2	2	2	2	3	3	3
ip n= 14	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0*	*0.25*	*1*	*3*	*3*	*3*	*3* *
+ 30mg Bu 40b	0	0	0	0	0	0	1	1	2	2	2.5	3	3	3.5	5	5
ip n= 14	*0*	*0*	*0*	*0*	*0*	*0.25*	*1*	*1.25*	*1*	*2.25*	*1.25*	*3*	*3*	*3*	*3*	*2.25*

3.4.5 Novel Compound Bu 36b

Figure 3.18 displays the results obtained from Bu 36b. A10mg/kg dose had no significant effect on CNS excitation. 20mg/kg caused a significant reduction in CNS excitation from 3.5 hours, (Figure 3.18 and Table 3.18). 30mg/kg also caused a significant reduction in spermine induced CNS excitation (see Table 3.18).

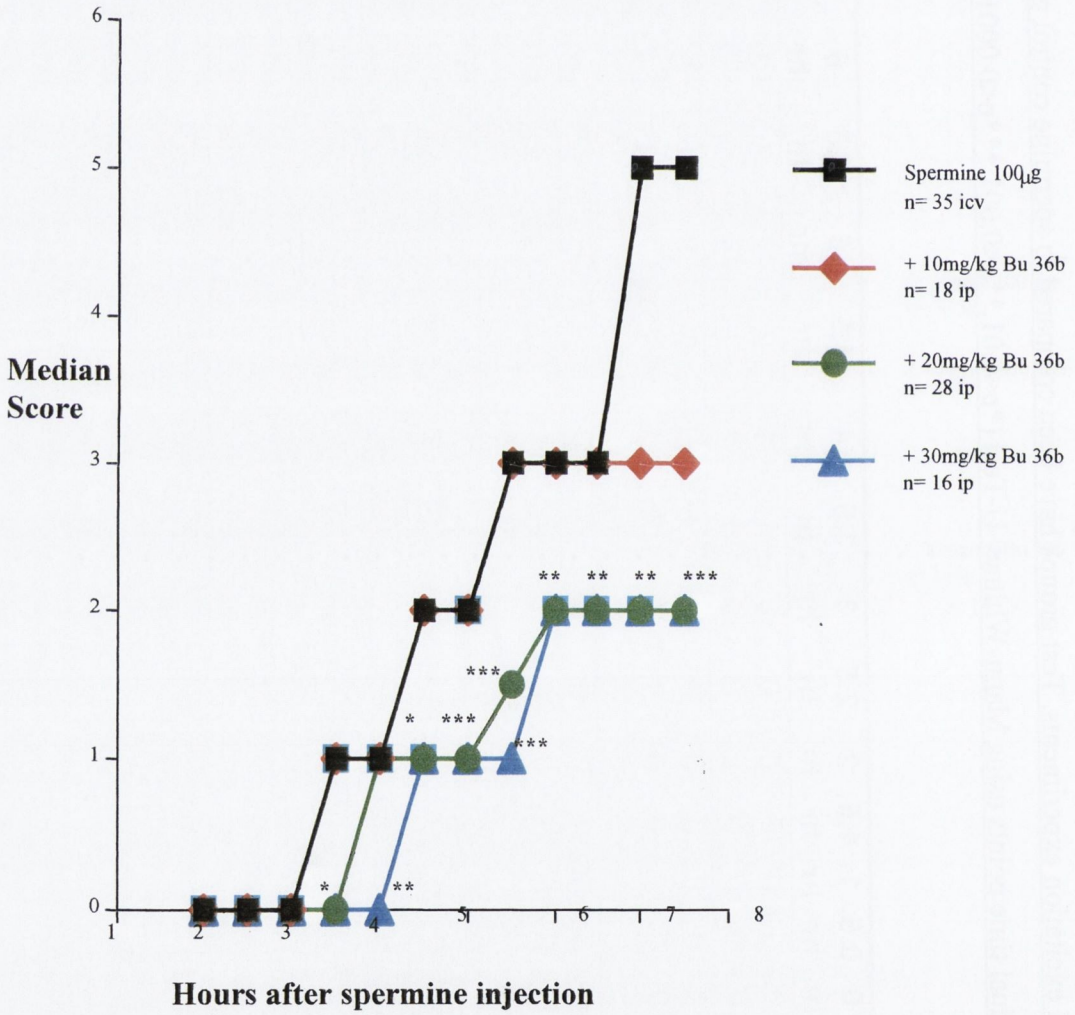


Figure 3.18: Effect of Bu 36b (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.18: The effect of Bu 36b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100μg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 10mg Bu 36b	0	0	0	0	0	0	0	1	1	2	2	3	3	3	3	3
ip n= 18	*0*	*0*	*0*	*0*	*0*	*0.25*	*1*	*1*	*1*	*1*	*1*	*1.25*	*3*	*2*	*2*	*2*
+ 20mg Bu 36b	0	0	0	0	0	0	0	0	1	1	1	1.5	2	2	2	2
ip n= 28	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*0.75*	*1*	*1*	*1.75*	*2*	*1*	*1*
								*		*	***	***	**	**	***	***
+ 30mg Bu 36b	0	0	0	0	0	0	0	0	0	1	1	1	2	2	2	2
ip n= 16	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*0.75*	*1.75*	*1*	*1*	*1*	*1*
								*	**	**	***	***	**	***	***	***

3.4.6 Novel Compound Bu 43b

Figure 3.19 presents the results for the effect of Bu 43b on the CNS excitation induced by spermine in mice. Three dose levels were examined as before. The initial dose used was 10mg/kg. This significantly reduced the CNS excitation induced by spermine, so a lower dose of 5 mg/kg was also used. 5mg/kg of Bu 43b was also an effective concentration, as it reduced the spermine CNS excitation, with a maximum median score 3 at 7.5 hours, (Figure 3.19 and Table 3.19). At the highest dose of 20mg/kg a maximum median score of 2 was observed at 7.5 hours, indicating significant inhibition of CNS excitation (Figure 3.19 and Table 3.19).

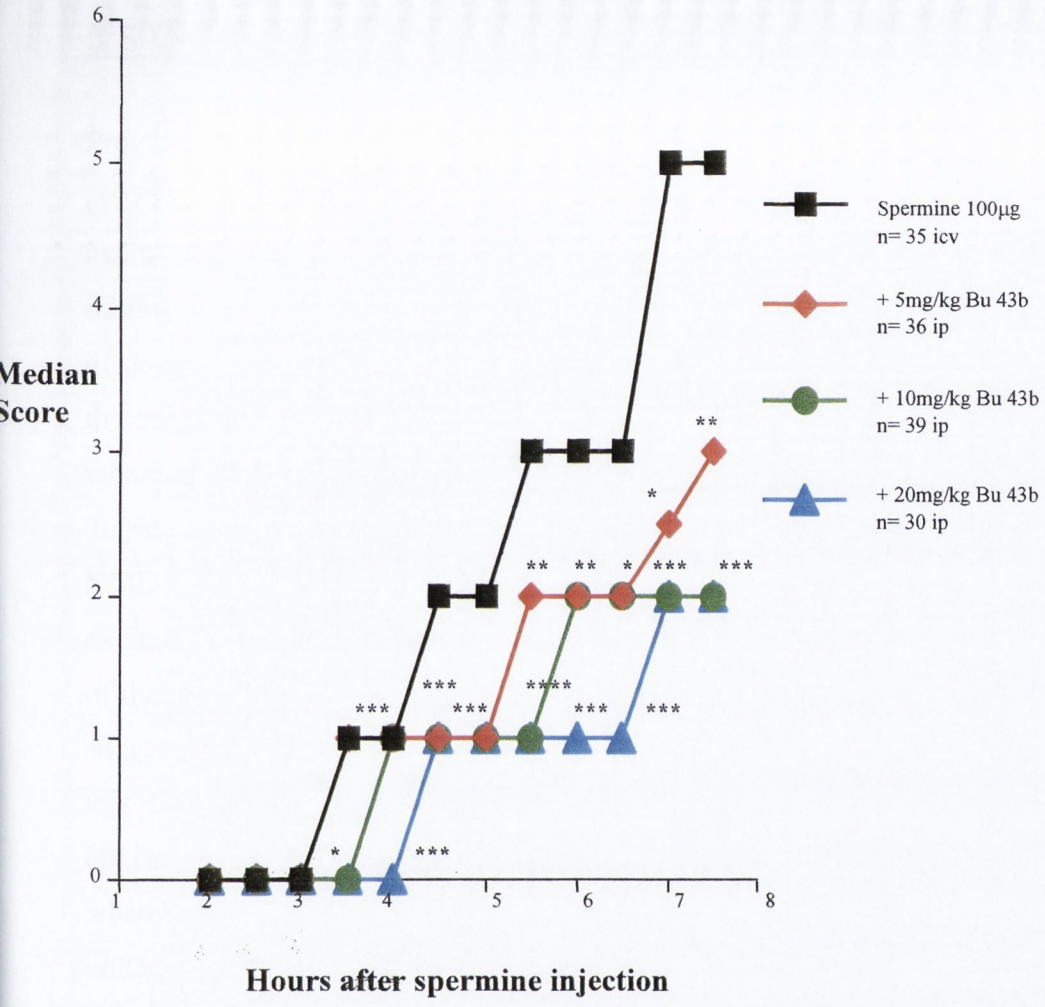


Figure 3.19: Effect of Bu 43b (ip) on spermine induced CNS excitation. *p<0.01, **p<0.001, ***p<0.0001 (Mann-Whitney U-test).

Table 3.19: The effect of Bu 43b on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) recorded over the time range in spermine CNS excitation experiments. Test groups have been compared to spermine control group for statistically significant differences at individual time points using Mann-Whitney U-Test (*p<0.01,**p<0.001,***p<0.0001).

Drugs	**0 hr**	**0.5 hr**	**1 hr**	**1.5 hr**	**2 hr**	**2.5 hr**	**3 hr**	**3.5 hr**	**4 hr**	**4.5 hr**	**5 hr**	**5.5 hr**	**6 hr**	**6.5 hr**	**7 hr**	**7.5 hr**
Control Spermine 100µg	0	0	0	0	0	0	0	1	1	2	2	3	3	3	5	5
icv n= 35	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*3*	*3*	*2*	*2*
+ 5mg Bu 43b	0	0	0	0	0	0	0	1	1	1	1	2	2	2	2.5	3
ip n= 36	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*3*	*4*	*3.5*
											***	**	**	*	*	**
+ 10mg Bu 43b	0	0	0	0	0	0	0	0	1	1	1	1	2	2	2	2
ip n= 39	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*2*
								*	***	***	***	***	***	***	***	***
+ 20mg Bu 43b	0	0	0	0	0	0	0	0	0	1	1	1	1	1	2	2
ip n= 30	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0.25*	*1*	*1*	*1*	*2*	*1.25*	*1*	*1*	*1.25*
								**	***	***	***	***	***	***	***	***

3.5 Discussion

Previous studies carried out in this laboratory have supported findings by Anderson et al (1975), which demonstrated the existence of two phases of spermine-induced CNS excitation. The initial phase was not examined here, as a wide variety of drugs seem to inhibit this phase. The second phase was more interesting to examine as only a minority of drugs inhibit the second phase, demonstrating a selectivity of response. In addition, the majority of the animals injected with spermine developed the second phase effects whereas not all animals were consistent in developing the first phase of effects (Kirby, 2000). Many of the previous studies carried out in this laboratory have used known NMDA antagonists, which seem to be very effective in reducing spermine induced CNS excitation (Doyle & Shaw, 1996; Doyle & Shaw, 1998; Kirby et al, 2004). The ability of these NMDA antagonists to inhibit the development of CNS excitation has led to the suggestion that the NMDA receptor is involved, at least in part, in the development of this CNS excitation. Spermine has both stimulatory and inhibitory effects at the NR1A/NR2A and NR1A/NR2B receptors (Williams, 1994; Williams et al, 1994), whereby it has four mechanisms of action: 1:glycine-independant stimulation, 2: voltage-dependent block, 3: increase in the affinity for glycine and 4: decrease in the affinity for NMDA and glutamate (Williams, 1994). Therefore the effect of spermine on NMDA depends on concentrations of glutamate, glycine and spermine, and the membrane potential, as well as the NMDA subunits present in the receptor complex (Williams, 1994; Williams et al, 1994).

Ifenprodil was originally developed as a vasodilating agent based on its activity as an α_1-adrenergic antagonist (Carter et al, 1990). Ifenprodil is known to block several other types of receptors and channels, such as voltage-dependent Ca^{2+} channels (Carter et al, 1997) and σ sites (Hashimoto et al, 1993; Carter et al, 1997). The phenylethanolamine ifenprodil has been known to be a class of NMDA receptor antagonist since the 1980's. Through the identification of the NMDA receptor subunits, ifenprodil was shown to be a selective antagonist for the NR1/NR2B subunit of NMDA receptors (Chenard et al, 1999; Yamakura et al, 1999; Grimwood et al, 2000).

Previous studies have suggested that the effects of ifenprodil are through polyamine binding sites. Ifenprodil is not a pure antagonist at the polyamine site on the NMDA (Carter et al, 1990; Williams et al, 1991; Beart et al, 1992; Carter et al, 1992; Beart et al, 1995; Carter et al, 1997). It has been suggested that the interaction of ifenprodil with polyamines is through an allosteric interaction between the two (Williams, 2001). Ifenprodil inhibits NMDA receptors in the absence of extracellular polyamines, indicating that ifenprodil is not a pure competitive antagonist at the polyamine site (Williams, 2001). Spermine has the ability to reduce the affinity of NMDA receptors for ifenprodil and ifenprodil can reduce the affinity for spermine (Williams, 2001). Studies have shown ifenprodil to be neuroprotective and additionally lack the motor and behavioural side effects associated with other NMDA antagonists (Carter et al, 1990). Furthermore, Chenard et al (1999) have investigated ifenprodil and found that at relevant concentrations there is a significant overlap and interaction involved in glycine-insensitive polyamine stimulation. Indicating, there is a significant overlap and interaction involved in glycine-insensitive polyamine stimulation (Chenard et al, 1999). The evidence above indicates that there is some correlation between the binding of polyamines and ifenprodil to the NMDA receptor possibly on the NR2B subunit.

Previous studies have demonstrated that high doses of ifenprodil reduce the spermine induced CNS excitation when administered in combination therapy with D-CPP or 7-chlorokynurenate (Doyle & Shaw, 1996). Ifenprodil administered as monotherapy did not cause a significant inhibition of the CNS excitation at each of the dose levels used. However, a significant inhibition was observered when a sub-effective dose of D-CPP or 7-chlorokynurenate was administered (Doyle & Shaw, 1996).

Gallagher et al (1996) reported that the first two thirds of the amino-terminal domain of the NR2B subunit are essential for both polyamine stimulation and ifenprodil inhibition. Interestingly arginine-337 is critical for ifenprodil binding at this region, but is not essential for polyamine binding. It has been suggested that a positively charged argenine-337 is critically required for high affinity ifenprodil inhibition in this region (Gallagher et al, 1997; Gallagher et al, 1998). Furthermore, glutamate-201 on the NR2B

subunit is critical for polyamine stimulation but not for ifenprodil inhibition (Gallagher et al, 1997; Gallagher et al, 1998). This evidence suggests that there are two distinct sites on the NR2B subunit for the interaction of ifenprodil and polyamines independently from each other. Kew et al (1996), found that spermine is able to allosterically change affinity for ifenprodil and not through a direct competitive interaction (Kew et al, 1996).

Eliprodil is a structural analogue of ifenprodil and is also reported to be strongly selective for the NR1/NR2B subunits (Avenet et al, 1997), however mutation studies suggest that eliprodil does not necessarily have a common structural determinant to ifenprodil (Gallagher et al, 1998). Eliprodil is shown to have neuroprotective activity in experimental brain trauma (Toulmond et al, 1993) and also exhibits antidepressant-like effects (Layer et al, 1995). Previous studies using Eliprodil have shown that Eliprodil is an antagonist, which acts at the polyamine regulatory site on the NMDA receptor, the same site as ifenprodil (Toulmond et al, 1993). Eliprodil antagonizes the effects of NMDA on cyclic GMP production as well as NMDA induced effects on acetylcholine and dopamine release (Carter et al, 1988). Eliprodil also inhibits NMDA-evoked depolarization (Balster et al, 1994). In addition to the NMDA receptor action, eliprodil can also block voltage-activated L- and N-type Ca^{2+} channels, with an IC_{50} of 1.48μM (I_{max} = 87%) (Biton et al, 1994). Walz et al (1999) demonstrated that a combination of Eliprodil with a glycine site antagonist (L-701, 324), showed significant anticonvulsant activity in amygdala kindled rats, although this combination showed less anticonvulsant activity than ifenprodil. This correlates with recent findings that eliprodil was less effective in inhibiting NMDA-induced currents in vitro then ifenprodil (Avenet et al, 1997).

In the present study eliprodil, was not very effective against the development of CNS excitation. This is in agreement with previously published work from this laboratory (Doyle & Shaw, 1996). However, eliprodil did cause some reduction in convulsions in comparison to control.

Arcaine is a naturally occurring structural analogue of the polyamines (Doyle & Shaw, 1998). Arcaine was initially proposed to be a competitive antagonist at the polyamine site in the NMDA receptor. However other evidence suggests that arcaine is not a pure competitive antagonist at polyamine sites. Reynolds (1990) initially suggested that arcaine was effective as a competitive antagonist of spermidine. However, Donevan et al (1992), demonstrated that arcaine inhibits NMDA activity by blocking the open NMDA receptor channel in the absence of exogenous polyamines. Similarly arcaine produced a voltage-dependent reduction in NMDA receptor whole-cell currents and reductions in single-channel conductance and open time in the absence of polyamines. It may be suggested that arcaine is enhancing the inhibitory actions of polyamine agonists, to shift the biphasic concentration-response for enhancement of receptor binding (Rock et al, 1992). Arcaine has an opposing effect on responses of NMDA receptors to polyamines and acts in a competitive manner (Sacann et al, 1990; Maciver et al, 1991). In more recent studies Arcaine has been shown to be an inverse agonist at the polyamine binding site and can act as an open channel blocker of the NMDA receptor (Pritchard et al, 1994). Arcaine, given *in vivo* alone produced polyamine-like behaviours, suggesting that arcaine has some agonist activity. It also potentates the development of the first phase of the spermine-induced effect, but did show some antagonist action for the development of the second phase (Doyle & Shaw, 1998). In the present study the results obtained showed that arcainè produced a dose-dependant decrease in spermine induced CNS excitation. Arcaine, inhibited the development of tonic convulsions, indicating its antagonist activity, which is consistent with the results found by Doyle and Shaw (1998). These findings are consistent with the suggestion that arcaine may act as an inverse agonist or possibly a partial agonist at the stimulatory site (Pritchard et al, 1994).

Memantine has been shown to block NMDA-evoked currents and displace MK-801 from its binding sites (Bormann et al, 1989; Kornhuber et al, 1989). Memantine has also been shown to be neuroprotective in animal models of focal or global ischemia, and is well tolerated in humans, (Nankai et al, 1995). Previous studies have indicated that the mode of action of memantine on the NMDA receptor is predominately through an

antagonist response via open-channel blockade in a non-competitive manner (Chen et al, 1992; Chen et al, 1997). In the present study the results for memantine as monotherapy indicates that memantine inhibits the development of spermine-induced CNS excitation. These results suggest involvement of NMDA receptor action in spermine effects. In house studies have found that memantine administered twice, 30 minutes before spermine injection and 30 minutes after spermine injection, also inhibited the development of CNS excitation (Kirby, 2000).

It was also of great interest to investigate the involvement of voltage gated calcium channel activity in spermine induced effects. The initial investigation of calcium channels was done by Doyle (1993), whereby nisoldipine was shown to have a mild inhibitory effect against spermine induced CNS excitation. Two dihydropyridine L-type calcium channel blockers were used, namely, Nisoldipine and Nitrendipine. These interact at binding sites on the $\alpha 1$ subunit of the L-type voltage gated channel. It has been suggested there is an intimate relationship between polyamines and calcium channels, as polyamines have been shown to allosterically modulate dihydropyridine binding to L-type calcium channels (Schoemaker, 1992). Polyamines have been documented to be permissive in the role of membrane calcium fluxes. The highly positively charged polyamines promote calcium channel gating by displacing calcium from the inner membrane surface (Schoemaker, 1992). Nifedipine was shown to antagonize seizures induced by γ-HCH a $GABA_A$ antagonist and BayK-8644 (L-type Ca^{2+} channel agonist, but only partially protects against convulsions induced by NMDA and had no effect on convulsions by Kainic acid (Sola et al, 2001). Dolin et al (1988) reported that nitrendipine and nimodipine are antagonists for certain types of convulsions, such as pentylenetetrazol and ethanol withdrawal seizures. Nitrendipine has also been shown to decrease benzodiazepine withdrawal seizures (Dolin et al, 1990). Other studies using patch-clamp recordings on postnatal rat retinal ganglion cells, have found that low doses of calcium channel antagonists, nifedipine and nimodipine, can protect against NMDA and non-NMDA receptor-mediated neurotoxicity (Sucher et al, 1991).

In previous studies nisoldipine has been shown to be only slightly less potent than nitrendipine at antagonizing pentylenetetazole-induced seizures (Moran et al, 1990). It has been demonstrated that nisoldipine can antagonize NMDA-induced seizures more potently than MK-801 or nitrendipine (Palmer et al, 1993). Other studies have examined nifedipine and nimodipine, and found them to have no effect on the direct inhibition of MK-801 binding in the mouse brain (Skeen et al, 1993; Filloux et al, 1994). Previous in house studies have shown the effect of nisoldipine on spermine-induced CNS excitation. A significant decrease in the CNS excitation score was observed, indicating that nisoldipine can reduce the effects of spermine (Doyle, 1993; Kirby, 2000; Doyle et al, 2004). It is possible that L-type calcium channels may play a role in the development of spermine induced CNS excitation, as L-type channel antagonists (nisoldipine, nitrendipine and verapamil) reduced the CNS excitation effects of spermine action (Doyle et al, 2004). The evidence suggests that there may be a modulation of spermine induced effects by calcium channel antagonists (Doyle et al, 2004).

Nitrendipine was also examined in the present study. Extensive research has reported the high potency of nitrendipine as a calcium channel antagonist (Dolin et al, 1990). Nitrendipine decreases ethanol withdrawal seizures when given acutely (Dolin et al, 1990). Nitrendipine has also been shown to suppress NMDA/glycine-mediated calcium influx by a direct interaction with the NMDA receptor and has also been shown to reduce NMDA evoked currents (Skeen et al, 1993; Skeen et al, 1994). Furthermore nitrendipine inhibits the binding of MK-801 to mouse brain indicating a direct interaction of nitrendipine and the NMDA-activated ion channel (Filloux et al, 1994). These findings illustrate an unusual status whereby nitrendipine has relatively equal affinity of L-type voltage sensitive calcium channels and NMDA receptors (Filloux et al, 1994). Nitrendipine may be acting through these mechanisms to antagonise the effects of spermine.

Nitrendipine dose-dependently reduced the effect of spermine CNS excitation in the present study. These results correlate to the findings of Doyle (1993) and Kirby (2000),

whereby it was reported that nitrendipine had an inhibitory effect on the development of the spermine-induced CNS excitation. Further to this it may also be concluded that L-type calcium channels are involved in the development of spermine induced CNS excitation and tonic convulsions, but as of yet the mechanism still remains to be elucidated (Doyle et al, 2004). Nitrendipine was more effective than nisoldipine, which may suggest that its effect may have been mediated partially through L-type Ca^{2+} channel activity, and partially through NMDA receptor inhibition.

N^1-dansylspermine is a structural analogue of spermine consisting of a spermine backbone with a large alkyl group attached to one of the terminal amino groups to distinguish it from the polyamines. It is very stable and is relatively easy to synthesise (Chao et al, 1997). Initial studies have found that N^1-dansylspermine was able to block the NMDA receptor through binding to the inhibitory polyamine site (Chao et al, 1997). N^1-dansylspermine was found to be a potent voltage-dependant blocker that could differentially block or penetrate recombinant NMDA receptors expressed in *Xenopus laevis* oocytes (Chao et al, 1997). A deeper investigation of the binding ability of N^1-dansylspermine has shown that N^1-dansylspermine greatly blocked the NMDA receptor in the M2 region of the NR2B subunit receptor (Kashiwagi et al, 1997). N^1-dansylspermine has also been shown to be an effective calmodulin antagonist and cytotoxic agent, which are properties intrinsic to spermine at high concentrations (Seiler et al, 1998). Kirby et al (2004) showed that N^1-dansylspermine was a potent antagonist of the CNS effects of spermine and its mode of action is through the positive polyamine site on the NMDA receptor. N^1-dansylspermine induces a voltage-dependent channel block in recombinant NMDA receptors at EC_{50} in the range of 0.3-16μM, dependant on receptor subunit composition (Chao et al, 1997). However, N^1-dansylspermine, administered through a peripherial route had no effect on spermine induced CNS excitation (Kirby, 2000). This may be attributed to the lack of penetration of this bulky molecule through the blood brain barrier (Kirby, 2000).

In the present study N^1-dansylspermine was only administered through the icv route to ensure CNS delivery. The results obtained here show once again that N^1-

dansylspermine in a dose dependant manner reduced spermine induced CNS excitation. The results suggest that N^1-dansylspermine may be blocking the effects of spermine binding to the stimulatory site on the NMDA receptor and therefore reducing the CNS excitation. This concurs with previous findings (Kirby et al, 2004).

The most important findings of this study were the results obtained for the novel compounds from Brock University Canada. These compounds are novel, hence only one other study has been carried out with these compounds. Fixon-Owoo et al (2003) studied these compounds (initially in insects), where it was found that these compounds showed no notable toxicity. The insects used were the European corn borer (*Ostrinia nubilalis*), the tobacco budworm (*Heliothis verescens*), the oblique banded leaf roller (*Choristoneura rosaceana*) and rice weevil (*Sitophilus oryzae*) (Fixon-Owoo et al, 2003). The investigation examined excitatory post-synaptic potentials (EPSP), of glutamate receptors NMDA, δ2 and AMPA taken from abdominal exterior muscles of crayfish and mammals. Fixon-Owoo et al (2003), screened 13 compounds at a concentration of 10μM for activity at NMDA, AMPA and δ2 receptors. The NMDA receptors contained NR1A and NR2B subunits; homomeric AMPA receptors expressed the GluR2(Q) subunit, which contains Q in the M2 loop region (Hume et al 1991); heteromeric GluR1/GluR2(Q) receptors; and δ2 receptors expressed from the constitutively active δ2(A654T) mutant (Fixon-Owoo et al, 2003). The results obtained showed clearly that some of these compounds are inhibitors of glutamate receptors (Fixon-Owoo et al, 2003).

Bu 31b showed 98% inhibition at the NR1/NR2B NMDA receptor subunits, 22% at δ2, 13% at AMPA GluR2 and 11% at AMPA GluR1/GluR2 (Fixon-Owoo et al, 2003). This indicates a high level of specificity for the NMDA receptors. In the present study it was interesting to examine the effect of this compound in vivo by two routes of and administration, (icv or ip administration). The results obtained for icv administration indicate that, at the higher doses used, Bu 31b inhibited the development of the spermine-induced convulsions. The results suggest that Bu 31b is antagonizing the

effects of spermine perhaps at the polyamine stimulatory site of the NMDA receptor, at the higher dose level.

The results obtained for ip administration showed that Bu 31b had caused a remarkable inhibition of the spermine-induced CNS excitation at each dose level. This illustrates, firstly that this compound given through a peripheral route is penetrating the blood brain barrier and having an effect. Secondly, Bu 31b is an antagonist of spermine-induced convulsions, an effect possibly mediated at the stimulatory polyamine site on the NR1/NR2B NMDA receptor. This finding is consistent with the results of Fixon-Owoo et al (2003).

Bu 37b in the study by Fixon-Owoo et al 2003, showed 12% inhibition at the NR1/NR2B NMDA receptor subunits, 4% at δ2, 8% at AMPA GluR2 and 9% at AMPA GluR1/GluR2. It was concluded that this compound was not an effective inhibitor of glutamate receptors.

In the present study, the findings obtained would indicate that Bu 37b had very little effect on the spermine-induced convulsions when given icv. At the low dose Bu 37b produced a worsened profile than that of the controls. Although the higher doses showed some inhibition in a dose-dependant manner, this would indicate that Bu 37b at a high dose has antagonist potential by binding to the stimulatory polyamine site. However, it was not very effective, which correlates to the previous studies (Fixon-Owoo et al, 2003).

Bu 37b administered ip, had no effect on inhibiting the development of the spermine-induced convulsions. There is a question as to whether it crossed the blood brain barrier. The evidence presented here correlates with the results of Fixon-Owoo et al (2003), whereby Bu 37b showed very little inhibition on the NR1/NR2B NMDA receptor subunits.

In the Fixon-Owoo study (2003), Bu 33b showed 3% inhibition at the NR1/NR2B NMDA receptor subunits, 0% at δ2, 2% at AMPA GluR2 and 1% at AMPA GluR1/GluR2. This indicates that this compound is ineffective at inhibiting of glutamate receptors (Fixon-Owoo et al, 2003).

In the present study the results obtained for icv administration indicate that Bu 33b is, at some dose levels effective at reducing the spermine-induced CNS excitation, (in particular the effect seen for 20μg). This would indicate that this compound is antagonizing the spermine-induced NMDA convulsions. This finding is in contrast to the results obtained by Fixon-Owoo et al (2003), where this compound had no inhibition on the glutamate receptors, although the effects in vivo shown here were obtained at high doses.

Following i.p. administration, Bu 33b also reduced the effects of spermine. Hence this compound, or a metabolite of this compound has the ability to cross the blood brain barrier. The fact that Bu 33b was not shown to interact effectively with the NMDA receptor in vitro, may suggest that Bu 33b could be antagonizing the spermine-induced effects through a mechanism not related to NMDA, perhaps via a voltage sensitive Ca^{2+} channel. Ca^{2+} channel antagonists have been shown in this study and other studies to antagonize the spermine-induced CNS excitation (Doyle, 1993; Kirby, 2000; Doyle et al, 2005). Further exploration of the mechanism of inhibition by B3 33b is needed.

In the in vitro study by Fixon-Owoo (2003), Bu 40b showed 28% inhibition at the NR1/NR2B NMDA receptor subunits, 0% at δ2, 15% at AMPA GluR2 and 13% at AMPA GluR1/GluR2 (Fixon-Owoo et al, 2003).

In this study, Bu 40b did not have a very pronounced inhibitory effect on the spermine-induced convulsions through either the icv or ip route of administration. The results from icv administration showed, Bu 40b potentiates the effects of spermine induced CNS excitation, illustrating agonist activity. There is some evidence to suggest that Bu 40b may have penetrated the blood brain barrier as at 20mg/kg i.p. there was some

inhibition of the spermine-induced CNS excitation. The results found here show a relationship with the results found with Fixon-Owoo et al (2003), in that, Bu 40b has some interaction with glutamate receptors but not a high affinity for them.

In the Fixon-Owoo study (2003), Bu 36b showed 26% inhibition at the NR1/NR2B NMDA receptor subunits, 4% at δ2, 18% at AMPA GluR2 and 16% at AMPA GluR1/GluR2 (Fixon-Owoo et al, 2003).

Bu 36b produced interesting results in the present study. Through the icv route, Bu 36b produced significant inhibition at the higher doses of 10 and 20μg. Thus, it could be possible that Bu 36b is acting as an antagonist at the polyamine stimulatory site of the NMDA receptor, hence the reduction in the development of the CNS excitation. This does not preclude the notion that it could also be acting at another site as the findings from Fixon-Owoo et al (2003), showed Bu 36b had relatively low inhibition of the glutamate receptors in vitro. As discussed for Bu 33b, it is possible that Bu 33b may interact with Ca^{2+} channel activity, although further work is needed to investigate this. Similarly, the results obtained for Bu 36b through ip administration indicated inhibition of the spermine-induced convulsions. Therefore, Bu 36b has the ability to penetrate the blood brain barrier.

The final novel compound to be investigated was Bu 43b. Fixon-Owoo et al (2003) showed 95% inhibition at the NR1/NR2B NMDA receptor subunits, 43% at δ2, 52% at AMPA GluR2 and 50% at AMPA GluR1/GluR2.

In the present study it may be seen that Bu 43b caused a remarkable reduction in the onset of the spermine-induced CNS excitation. Upon administration through the icv route, in a dose-dependant manner, it greatly inhibited the spermine-induced convulsions. 20μg had the most marked reduction. No CNS signs of excitation was observed within the 7.5 hour period. This illustrates the potency of BU 43b in this behavioural model. The results for i.p. administration also showed remarkable

inhibition, indicating that Bu 43b, or an active metabolite crosses the blood brain barrier.

Bu 43b may act as a competitive antagonist at the polyamine stimulatory site of the NMDA receptor. There is supportive evidence for this in the previous in vitro studies whereby Bu 43b showed high, 95%, inhibition at the NR1/NR2B NMDA receptor subunits.

3.6 Conclusion

This study investigated the effect of a variety of NMDA antagonists, voltage sensitive Ca^{2+} channel antagonists (L-type) and polyamine analogues on spermine induced CNS excitation.

Ifenprodil antagonised spermine-induced convulsions. Eliprodil too inhibited the spermine-induced convulsions but was not as potent as ifenprodil. These findings are consistant with the findings of Doyle (1993). Arcaine decreased the spermine-induced CNS excitation in a dose-dependant manner showing arcaine may have some antagonist actions at the stimulatory polyamine site or act as an inverse agonist at the inhibitory site, consistent with previous findings. Memantine also partially protected against the development of CNS excitation. This results suggest that spermine causes CNS excitation, at least in part, by a stimulatory action at the NMDA receptor.

Both nisoldipine and nitrendipine, L-type calcium channel antagonists, demonstrated their ability to inhibit the development of the spermine convulsions, supporting a role for voltage sensitive calcium channels in spermine induced effects. However in addition, these drugs have been shown to exert influence on the NMDA receptor, as described previously. N^1-dansylspermine was shown to be a potent polyamine antagonist in vivo in this study, consistent with previous studies (Kirby et al, 2004). This effect could be mediated through an antagonist action on the polyamine stimulatory site of the NMDA receptor.

The findings of greatest significance in this study are the findings for the 6 novel compounds from Brock University, Canada. The most impressive results were the results obtained for Bu 31B and Bu 43b, which showed a high level of inhibition of spermine CNS excitation. This evidence suggests that both of these compounds may be competitive polyamine antagonists, previously reported (Murphy et al, 2003). Their mechanism of action is probably through the polyamine stimulatory site of the NR1/NR2B subunit of the NMDA receptor. This would also correlate to the findings of Fixon-Owoo et al (2003) whereby Bu 31b and Bu 43B were shown to have high inhibitory potential at these receptor sub-units. Bu 37b and Bu 40b had no effect on spermine CNS excitation, showing these are not potent polyamine receptor antagonists, which is consistent the in vitro findings whereby both these compounds were very poor inhibitors of the NR1/NR2B NMDA receptor subunits (Fixon-Owoo et al, 2003).

Finally, interestingly, the results obtained for Bu 33b and Bu 36b, show that both these compounds had an inhibition effect on the development of spermine-induced convulsions (Murphy et al, 2003). This suggests that they may be polyamine antagonists. However, Fixon-Owoo et al (2003), showed that both these compounds had low or relatively low (Bu 36b), inhibitory effects with the NR1/NR2B NMDA receptors subunits in vitro. This has given rise to the suggestion that the antagonistic action of both Bu 33b and Bu 36b may be mediated through a further mechanism, as yet not elucidated. It is possible, for example, that their effect may be mediated by L-type Ca^{2+} voltage-activated channels as these have been implicated in the spermine-induced convulsions. Unfortunately the animals were not kept for 24 hours to investigate if there was a delayed effect in the development of the convulsions versus the initial antagonist effect. Further work is necessary to test this hypothesis.

Chapter 4.

4.1 Introduction.

As mentioned in section 1.3.5, direct administration of $ACTH_{1-24}$ into the cerebral ventricles of male Laca mice leads to the development of behavioural excitation characterised by recurrent episodes of stretching and yawning, known as the stretching yawning syndrome (SYS), penile erections (PE) and excessive grooming (G) (Ferrari et al, 1963). These effects begin to show 15-30 minutes after injection and last for several hours (Ferrari et al. 1963; Bertolini et al, 1969; Gispen et al, 1975; Bertolini et al, 1981).

Previous studies have indicated that $ACTH_{1-24}$ stimulates brain Ornithine decarboxylase (ODC) activity. DFMO (α– diflurомethylornithine), an irreversible inhibitor of mammalian ODC, antagonises some of the $ACTH_{1-24}$ behavioural effects, which suggests a link between $ACTH_{1-24}$ and the ODC-polyamine system. DFMO inhibited the induced SYS and PE (Genedani et al, 1984). This evidence supports the idea that ODC and polyamines may have a role in the onset of $ACTH_{1-24}$-induced behavioural syndrome (Bertolini et al. 1968; Poggioli et al, 1984). The effects of Ifenprodil on ACTH-induced behavioural syndrome have been previously studied and it was found that Ifenprodil significantly reduced both SYS and PE behaviours in rats (Genedani et al, 1994).

The effect of a range of compounds on $ACTH_{1-24}$ induced behaviours was assessed in the present study. Initially to establish a control, 3μg of $ACTH_{1-24}$ in 20μl was given through the i.c.v route (Section 2.2.1). The mean and the standard error of mean (S.E.M) was calculated for each behaviour. Statistical analysis was performed by analysis of variance using One-Way ANOVA. Post-hoc analysis Tukey's test was used.

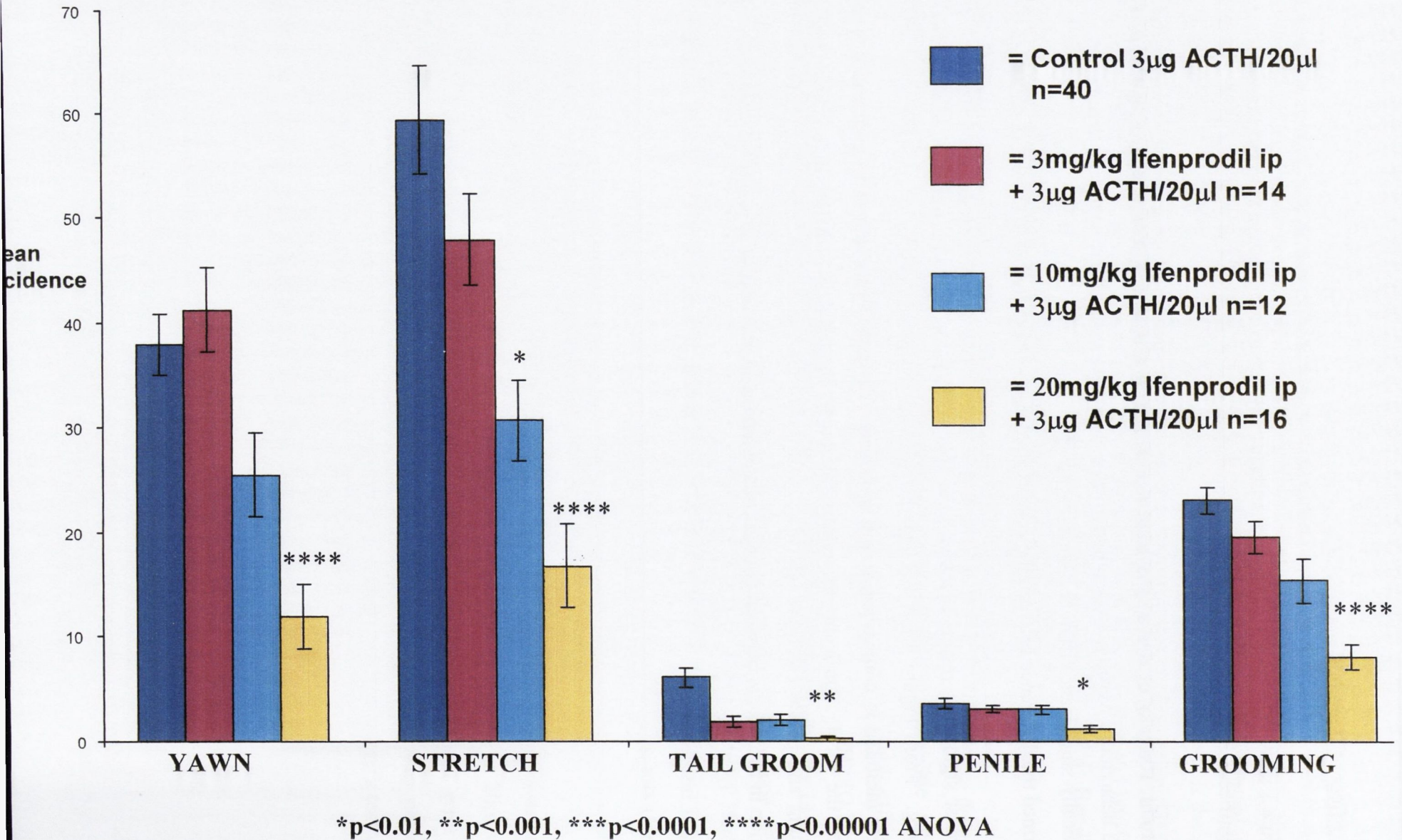
4.2 Results.

4.2.1 Effect of Ifenprodil in the $ACTH_{1-24}$ model:

Figure 4.1 shows the results obtained for ifenprodil. The lowest dose of 3mg/kg caused no significant effect on any of the behaviours (Fig 4.1).

Ifenprodil, 10mg/kg caused a significant reduction in the incidence of behaviours. Each of the behaviours were reduced, although stretching was the only behaviour to show a statistically significant difference between test and control (Fig 4.1). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The most significant results obtained with ifenprodil were with the highest dose of 20mg/kg. From Figure 4.1 it is clear that each of the behaviours were much more strongly inhibited in comparison to the two doses. Yawning, stretching and grooming showed the most significant differences, although, the effect of ifenprodil on tail grooming and penile erections was also significant. Post-hoc analysis revealed the reduction in behaviours between the control and treatment group ($p<0.001$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

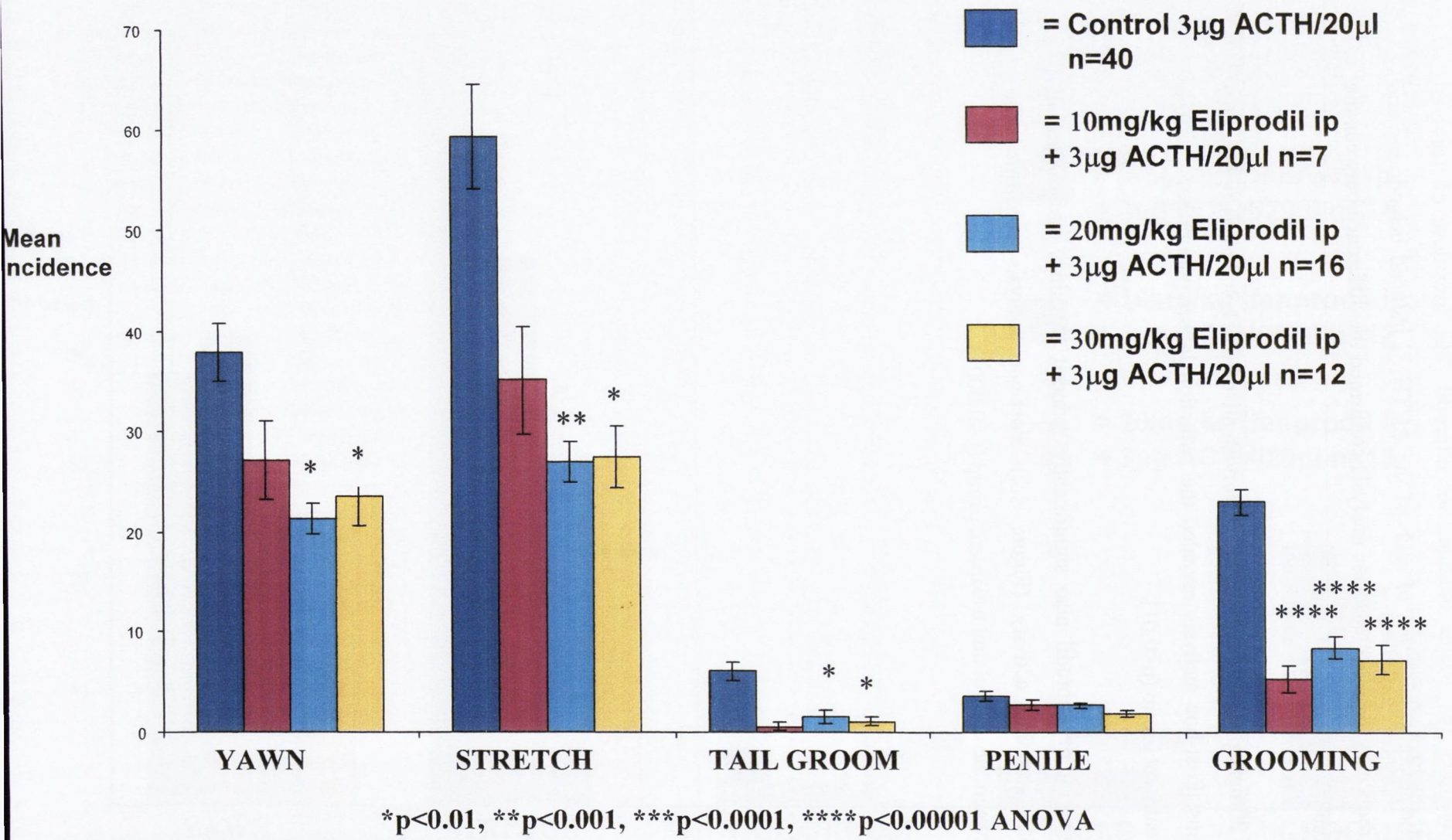
Figure 4.1: Effect of 3, 10 and 20mg of Ifenprodil ip on $ACTH_{1-24}$ (3µg /20µl icv) Induced Animal Behaviours Over 1 Hour in *laca* mice. Results

4.2.2 Effect of Eliprodil in the $ACTH_{1-24}$ model:

Figure 4.2 illustrates the results obtained for eliprodil. The low dose of 10mg/kg showed a trend towards reduction of each of the $ACTH_{1-24}$ induced behaviours, and significantly reduced grooming. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.05$).

The dose of 20mg/kg significantly reduced almost all of the behaviours, except for penile erections. Post-hoc analysis revealed the reduction in behaviours between the control and treatment group ($p<0.01$).

Similarly, 30mg/kg of eliprodil also significantly reduced yawning, stretching, tail grooming and grooming activity (Figure 4.2). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.0001$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

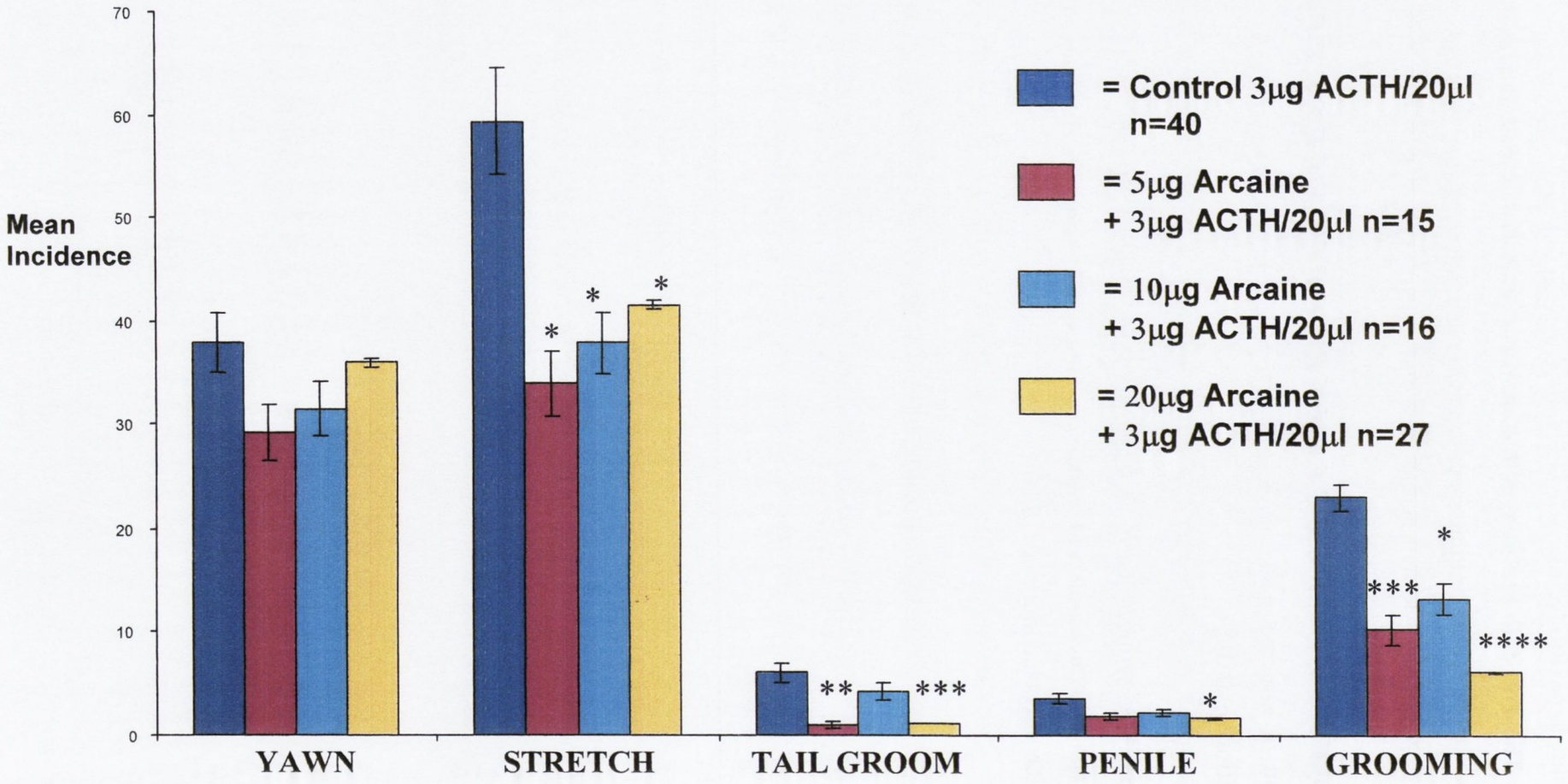
Figure 4.2: Effect of 10, 20 and 30mg of Eliprodil ip on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.2.3 Effect of Arcaine in the $ACTH_{1-24}$ model:

Arcaine was co-administered through the icv route with $ACTH_{1-24}$. Figure 4.3 shows the results for arcaine. The low dose of 5μg significantly reduced stretch, tail grooming and grooming indicating a strong inhibitory effect with arcaine. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.001$).

10μg of arcaine did not have as marked a reduction in behaviours in comparison to 5μg. Significant inhibition of stretching and grooming was observed. Post-hoc analysis confirmed this inhibition ($p<0.01$).

The higher dose of 20μg reduced each of the $ACTH_{1-24}$ induced behaviours in comparison to the control values, except yawning (Figure 4.3). Post-hoc analysis revealed this reduction in behaviours also between the control and treatment group ($p<0.01$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

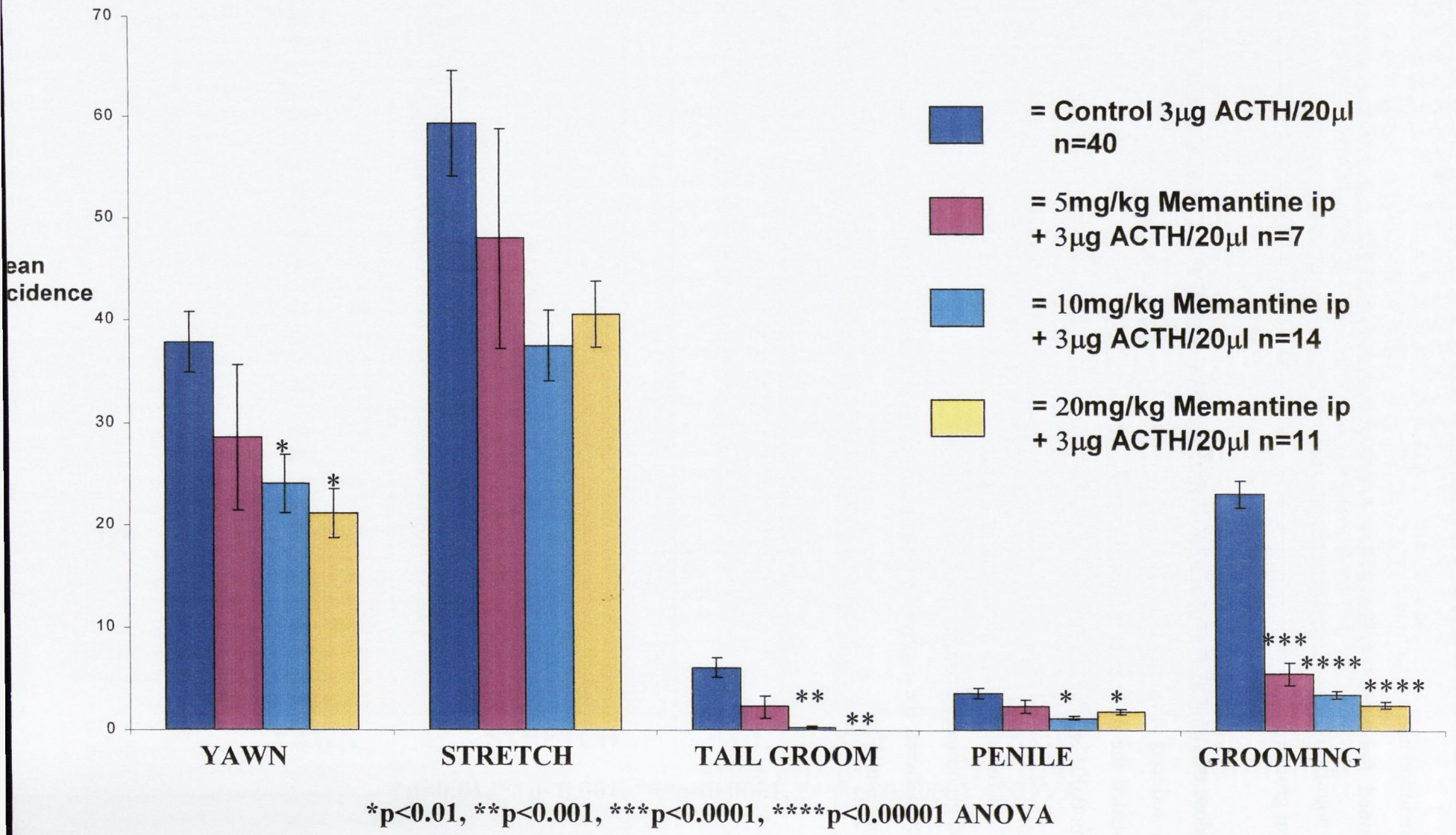
Figure 4.3: Effect of 5, 10 and 20μg of Arcaine icv on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ±s.e.m.

4.2.4 Effect of Memantine in the $ACTH_{1-24}$ model:

5mg/kg memantine had a slight inhibitory effect on each behaviour in comparison to the results obtained for $ACTH_{1-24}$ alone. However, only the effect on grooming was statistically significant. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The higher dose of 10mg/kg was more effective at reducing each of the behaviours. It significantly reduced yawning, tail grooming, penile erections and grooming. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.0001$, $p<0.01$ and $p<0.001$ respectively).

20mg/kg of memantine also significantly reduced yawning, tail grooming, penile erections and grooming in comparison to the control values. Post-hoc analysis showed the difference between the control and treatment group ($p<0.01$, $p<0.001$, $p<0.02$ and $p<0.001$ respectively).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

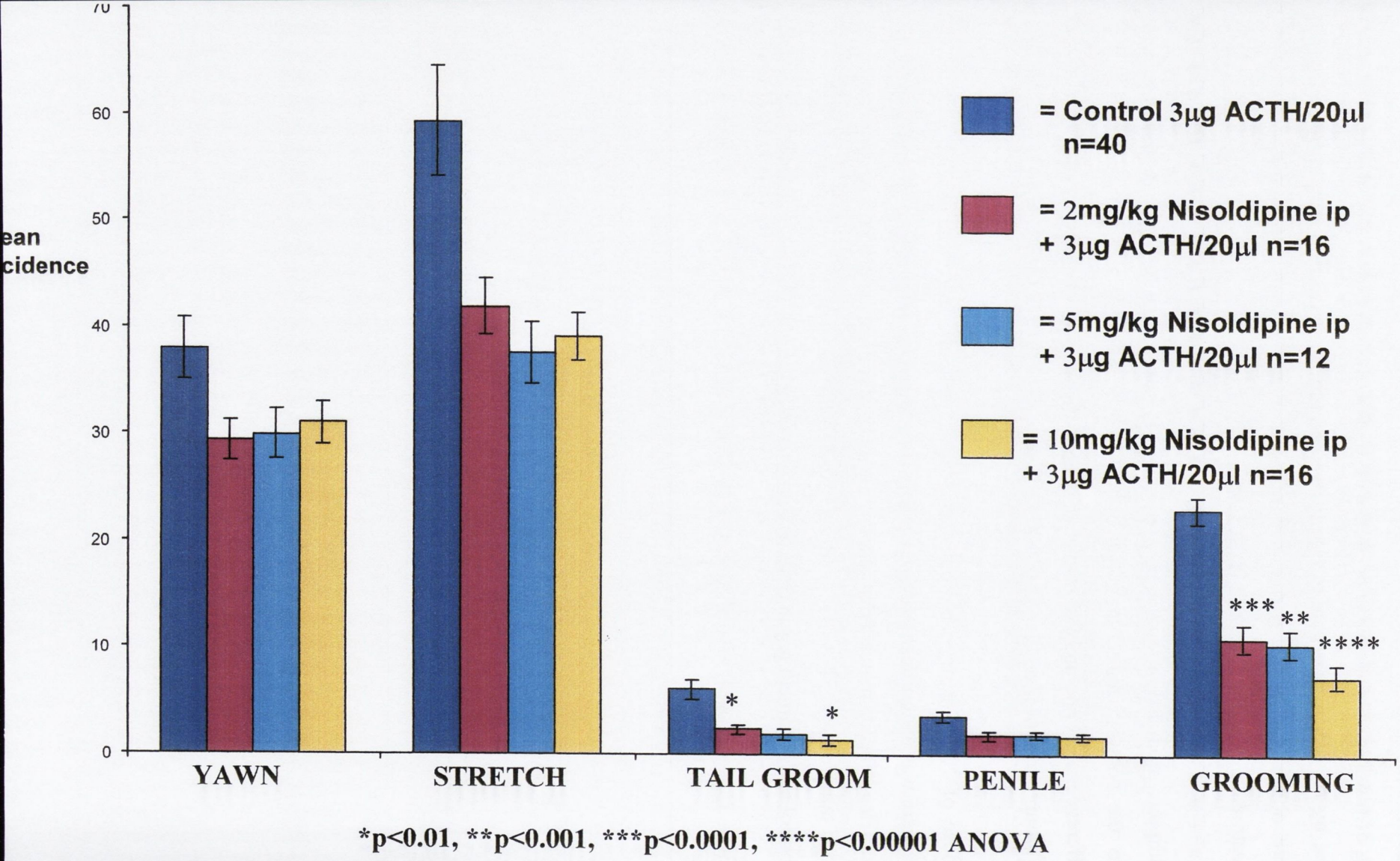
Figure 4.4: Effect of 5, 10 and 20mg of Memantine ip on $ACTH_{1-24}$ (3µg/ 20µl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ±s.e.m.

4.2.5 Effect of Nisoldipine in the $ACTH_{1-24}$ model:

Figure 4.5 illustrates the results obtained for nisoldipine. From the graph it may be seen that at 2mg/kg of nisoldipine caused a considerable reduction in two of the behavioural effects induced by $ACTH_{1-24}$. Statistically significant differences in tail grooming and grooming were observed. Post-hoc analysis confirmed these findings between the control and treatment group ($p<0.01$ and $p<0.001$).

5mg/kg nisoldipine produced a noticeable reduction in all of the behaviours in comparison to the control values, although only grooming showed a statistically significant difference. Post hoc analysis confirmed the difference between the control and treatment group ($p<0.001$).

The highest dose of 10mg/kg of nisoldipine also caused a slight reduction in each behaviour, but there was a significant difference between test and control in two of the five behaviours. These results indicate that nisoldipine had a significant inhibitory effect on grooming and tail grooming. Post-hoc analysis revealed this reduction in behaviours between the control and treatment group ($p<0.01$ and $p<0.001$).



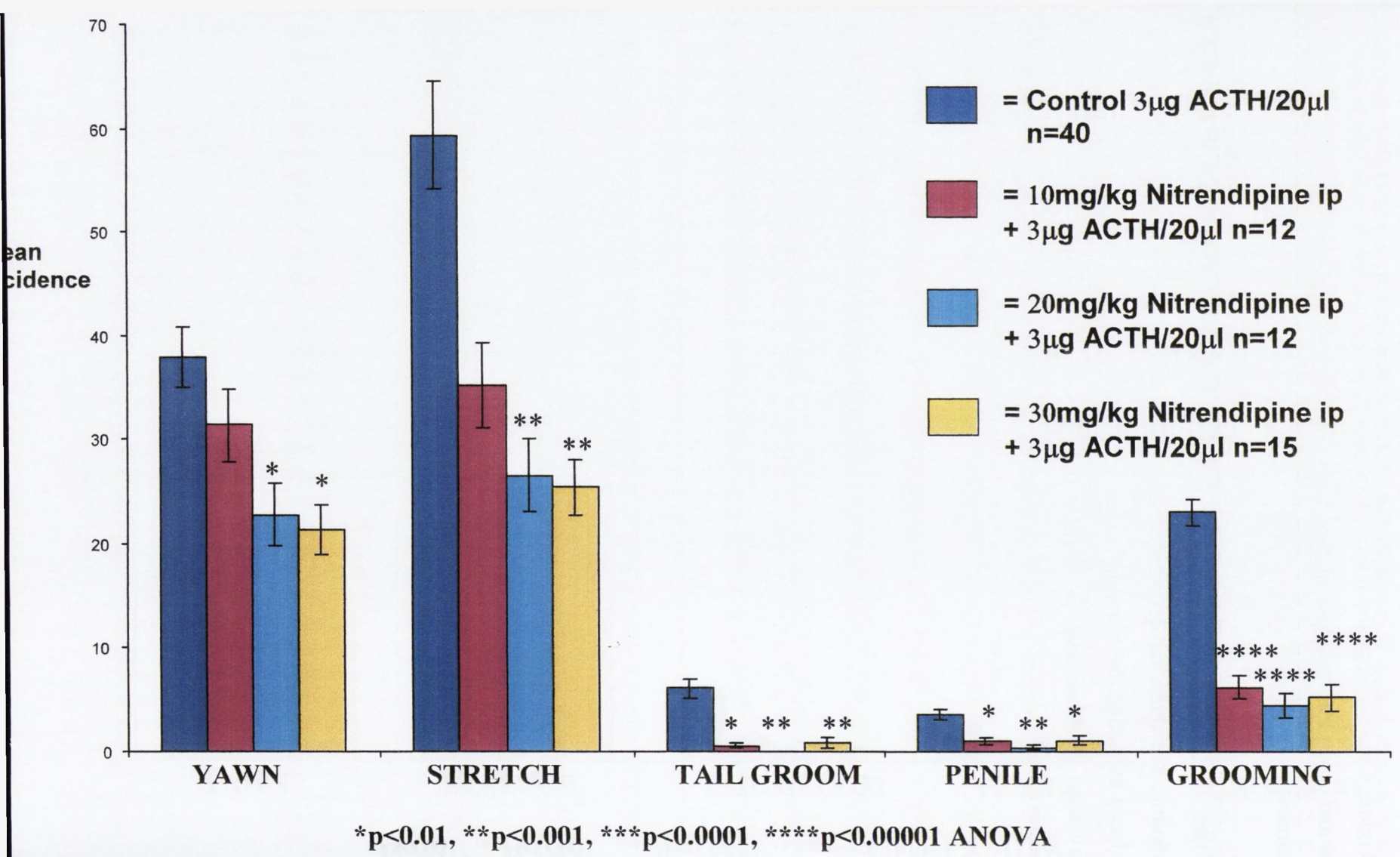
*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

Figure 4.5: Effect of 2, 5 and 10mg of Nisoldipine ip on $ACTH_{1-24}$ (3µg/ 20µl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.2.6 Effect of Nitrendipine in the $ACTH_{1-24}$ model:

Figure 4.6 illustrates the results obtained for Nitrendipine. Nitrendipine 10mg/kg, (as indicated on Figure 4.6), significantly inhibited the development of tail grooming, penile erections and grooming. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$ and $p<0.001$).

The higher doses of 20mg/kg and 30mg/kg significantly reduced each of the behaviours. Yawning, stretching, tail grooming, penile erection and grooming were all significantly reduced indicating that nitrendipine has a very potent effect on the $ACTH_{1-24}$ induced behaviours. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.01$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

Figure 4.6: Effect of 10, 20 and 30mg of Nitrendipine ip on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ±s.e.m.

4.2.7 Effect of Putrescine in the $ACTH_{1-24}$ model:

From Figure 4.7 it may be seen that putrescine co-administered with $ACTH_{1-24}$ via the icv route at each dose level had a marked effect on the $ACTH_{1-24}$ behaviours. The low dose of 120μg had a dramatic and very significant effect on the behaviours yawning, stretching and grooming. Tail grooming and penile erections were also significantly reduced. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The middle dose of 240μg also had a dramatic inhibitory effect on the $ACTH_{1-24}$ behaviours as this also reduced the mean incidence for each behaviour. Significant differences were observed for most of the behaviours, except for tail grooming. Post-hoc analysis revealed the reduction in behaviours in the treated group compared to control ($p<0.001$).

The highest dose of 480μg also had a large impact on the mean incidence for each behaviour. However, this dose was toxic to the animals, and half of those treated ($n=4$) suffered from a convulsion resulting in fatality at this dose level. The results presented are the findings from the four surviving animals. Yawning, stretching and grooming were significantly inhibited at this dose level. Post-hoc analysis revealed this finding between groups ($p<0.001$).

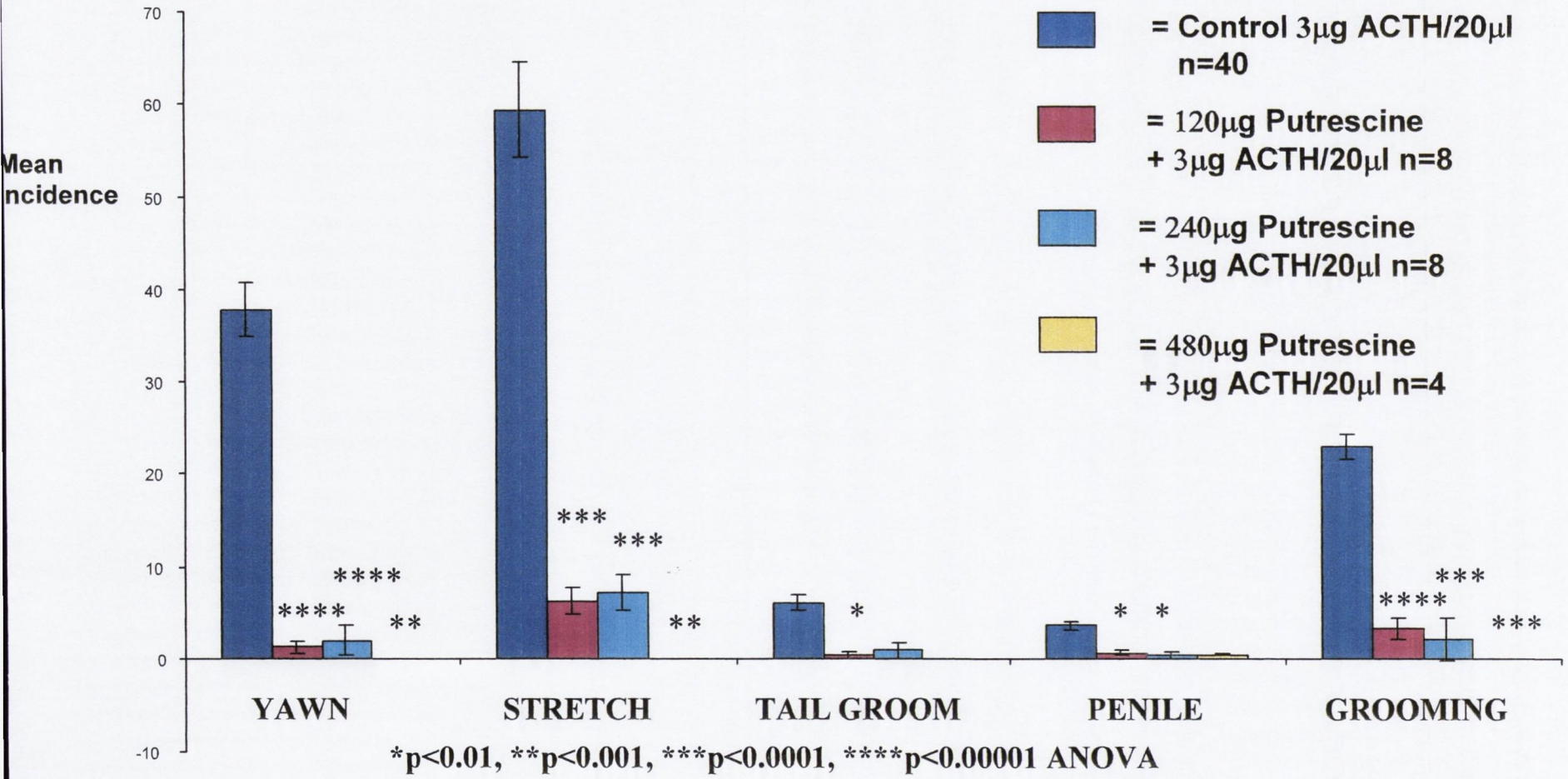
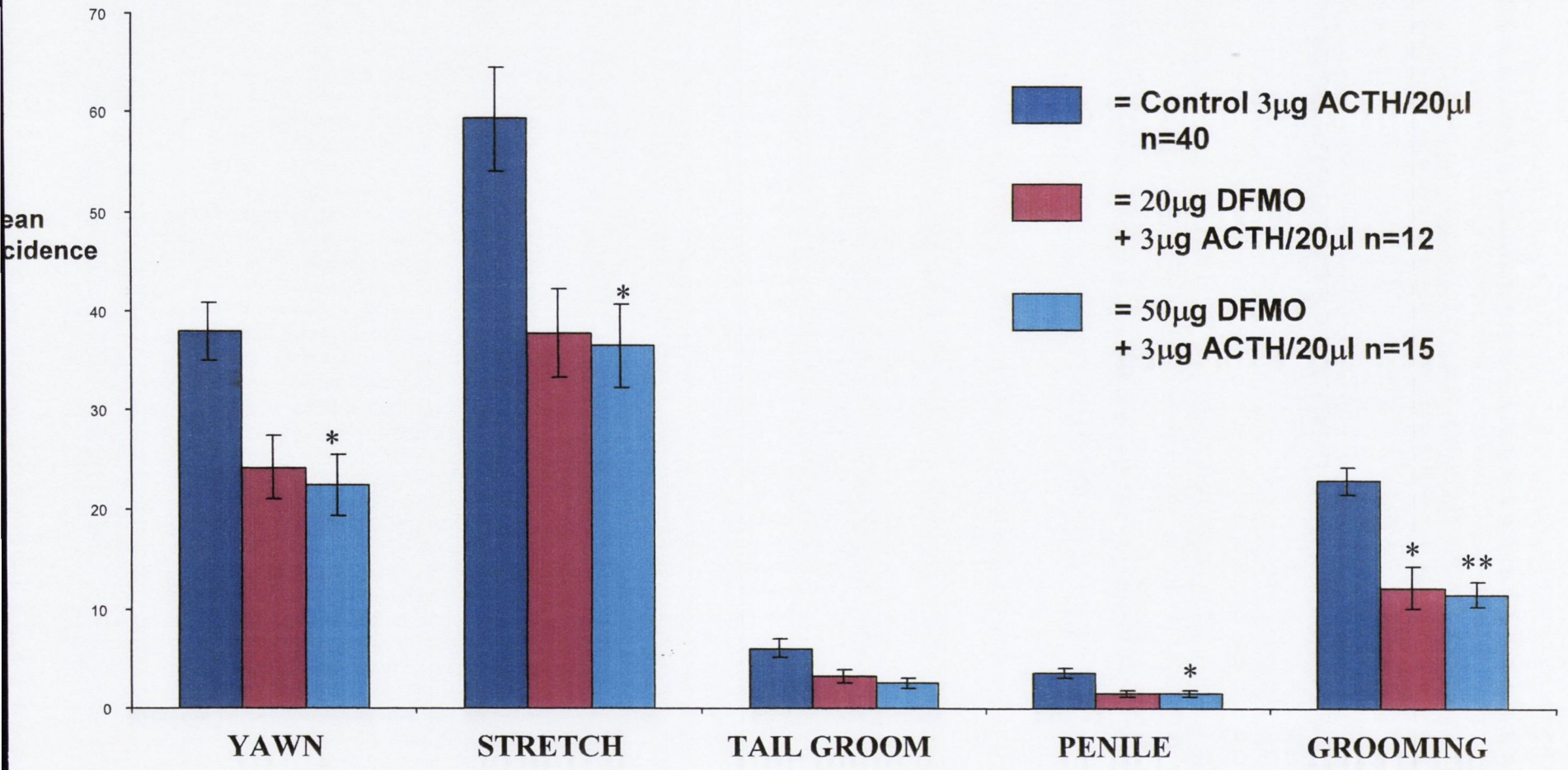


Figure 4.7: Effect of 120, 240 and 480μg of Putrescine icv on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.2.8 Effect of DFMO in the $ACTH_{1-24}$ model:

Figure 4.8 indicates the results obtained for the effect of DFMO on the $ACTH_{1-24}$ induced behaviours. Three doses, 20, 50 and 100μg were administered, but the highest dose of 100μg resulted in each animal having a fatal tonic convulsion and was excluded from this study. From the Figure 4.8 it may bee seen that at the low dose of 20μg DFMO had a significant inhibitory effect on the grooming behaviour induced by $ACTH_{1-24}$. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

50μg provided a further reduction in the mean incidence for each behaviour. A significant reduction was observed in four of the five parameters. These including yawning, stretching, penile reactions and grooming (Figure 4.8). Post-hoc analysis revealed this difference also ($p<0.01$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

Figure 4.8: Effect of 20, and 50μg of α -Difluromethylornithine (DFMO) icv on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ±s.e.m.

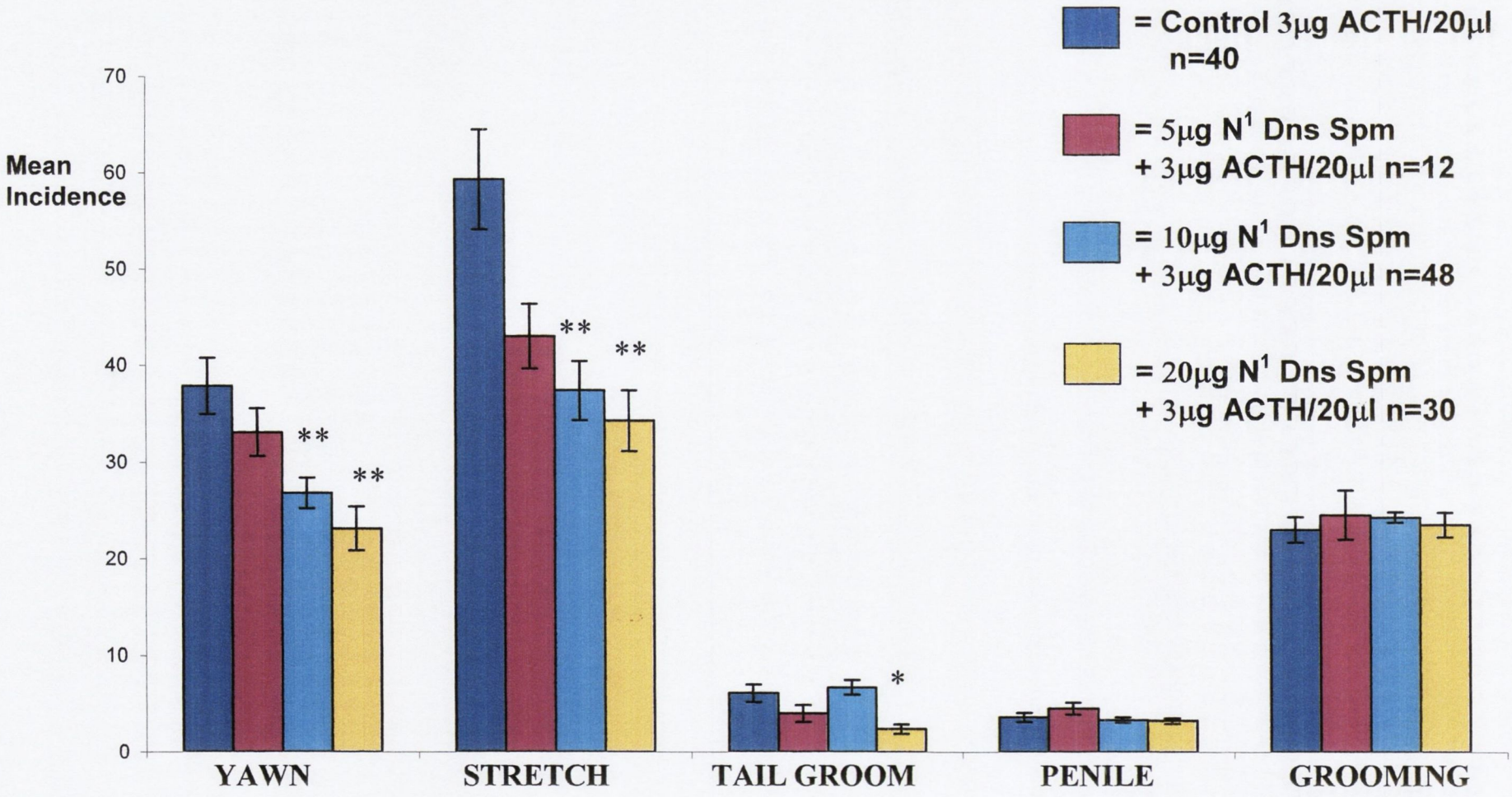
4.3 Novel Compounds

4.3.1 Effect of N^1-Dansylspermine in the $ACTH_{1-24}$ model:

Figure 4.9 displays the results obtained for N^1-dansylspermine. N^1-dansylspermine was co-administered with $ACTH_{1-24}$ through the icv route. Three dose levels were given to see this drug's potential at antagonizing the behavioural effects of $ACTH_{1-24}$. From the graph it may be seen that at the low dose of 5μg of N^1-dansylspermine has no significant effect, but caused a slight reduction in three of the five behaviours, these included yawning, stretching and tail grooming. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The middle dose of 10μg significantly reduced yawning and stretching. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.001$). N^1-dansylspermine at this dose had no effect on tail grooming, penile erection or grooming.

The highest dose of 20μg of N^1-dansylspermine significantly reduced the behaviours of yawning, stretching and tail grooming. Post-hoc analysis confirmed this difference ($p<0.0001$). .No effect on the behaviour of grooming or penile erections was observed.



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

Figure 4.9: Effect of 5, 10 and 20μg of N^1-Dansylspermine icv on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.3.2 Effect of Bu 31b in the $ACTH_{1-24}$ model:

Figure 4.10 displays the results for Bu 31b. Bu 31b was co-administered with $ACTH_{1-24}$ through the icv route. From the graph it may be seen that the low dose of 10μg significantly reduced the behaviours of stretching, tail grooming and grooming. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$, $p<0.01$ and $p<0.0001$).

The middle dose of 20μg inhibited the development of each of the $ACTH_{1-24}$ behaviours. At this dose level it can be seen that this compound produced a marked effect in this model. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.001$).

The highest dose of 30μg produced similar findings to those seen with the 20μg dose. It also caused a marked reduction in each of the $ACTH_{1-24}$ induced behaviours. The 30μg dose had the most pronounced effect in inhibiting the $ACTH_{1-24}$ behaviours. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.001$). Overall these findings, show that this compound is very effective in this model, (Figure 4.10).

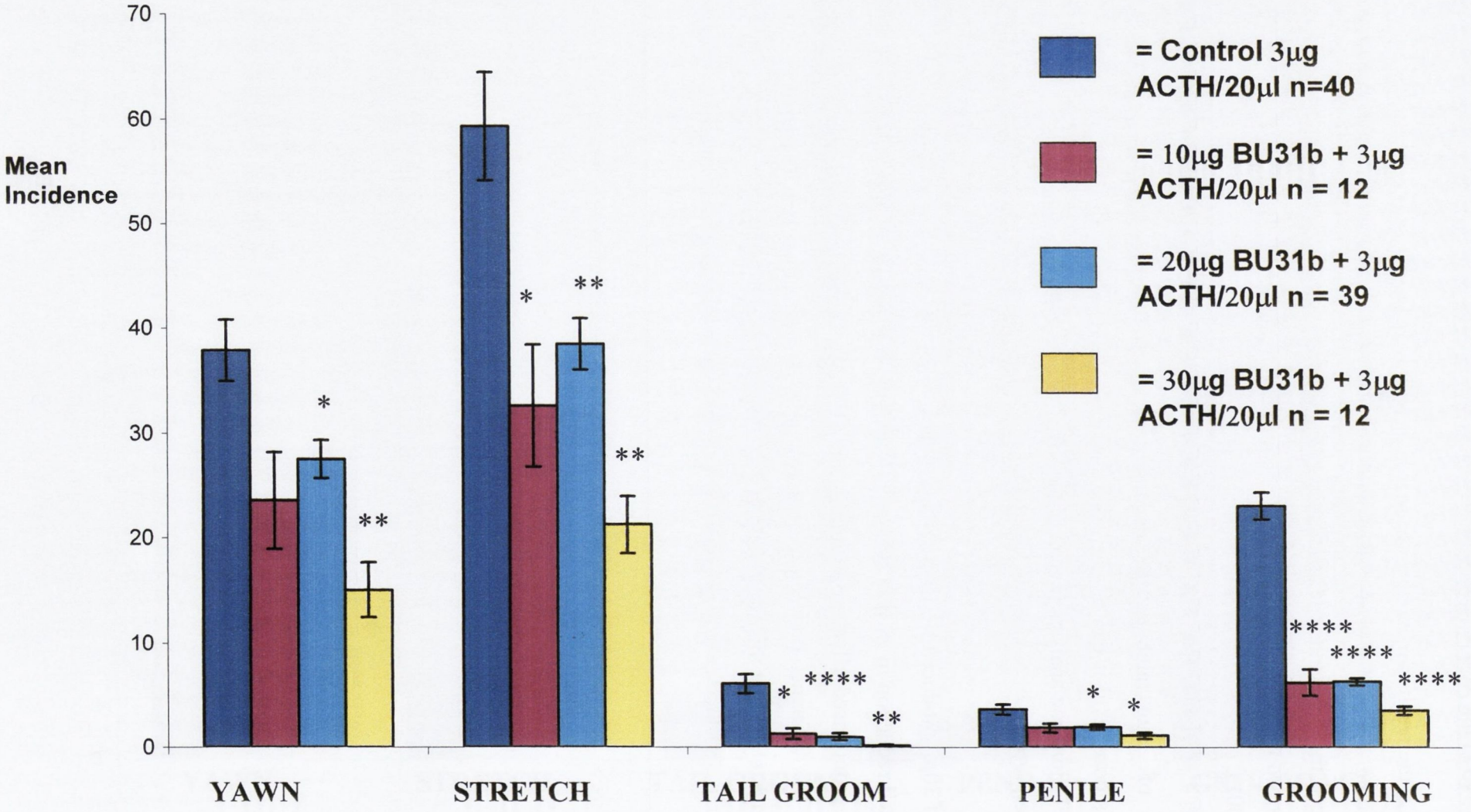


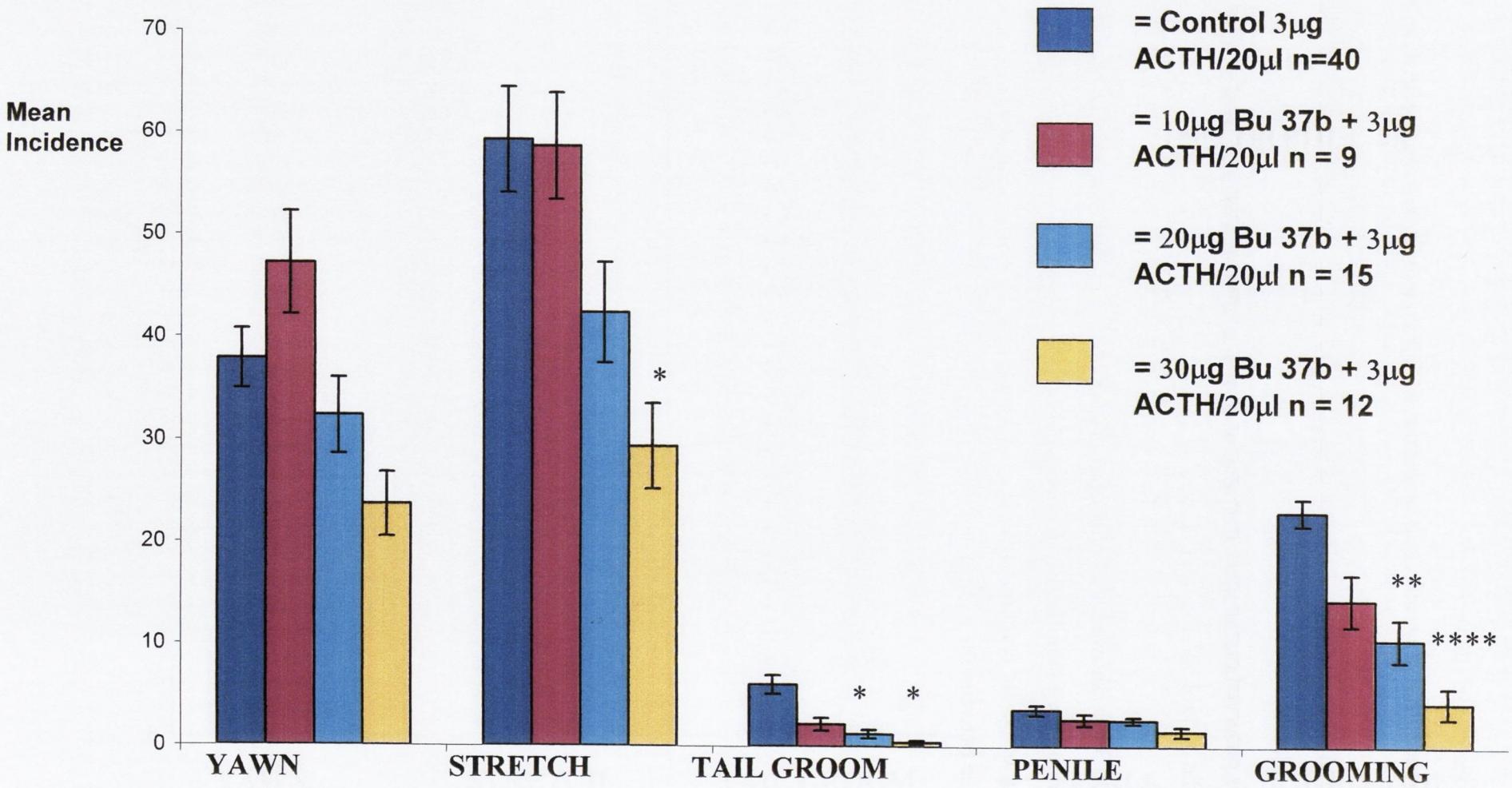
Figure 4.10: Effect of 10, 20 and 30µg of Bu 31b icv on $ACTH_{1-24}$ (3µg/ 20µl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.3.3 Effect of Bu 37b in the $ACTH_{1-24}$ model:

Figure 4.11exhibits the results obtained for Bu 37b. From the graph it may be seen that the low dose of 10μg had little or no effect on any of the $ACTH_{1-24}$ induced behaviours.

The middle dose of 20μg provided more significant findings. At this dose level there was a significant inhibition in the incidences of tail grooming and grooming. No significant effect was observed on the other parameters. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The highest dose of 30μg showed the most pronounced response. Stretching, tail grooming and grooming were significantly reduced, although no significant effect was observed on yawning or penile erections with Bu 37b (Figure 4.11). Post-hoc analysis revealed the difference between the control and treatment group ($p<0.001$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

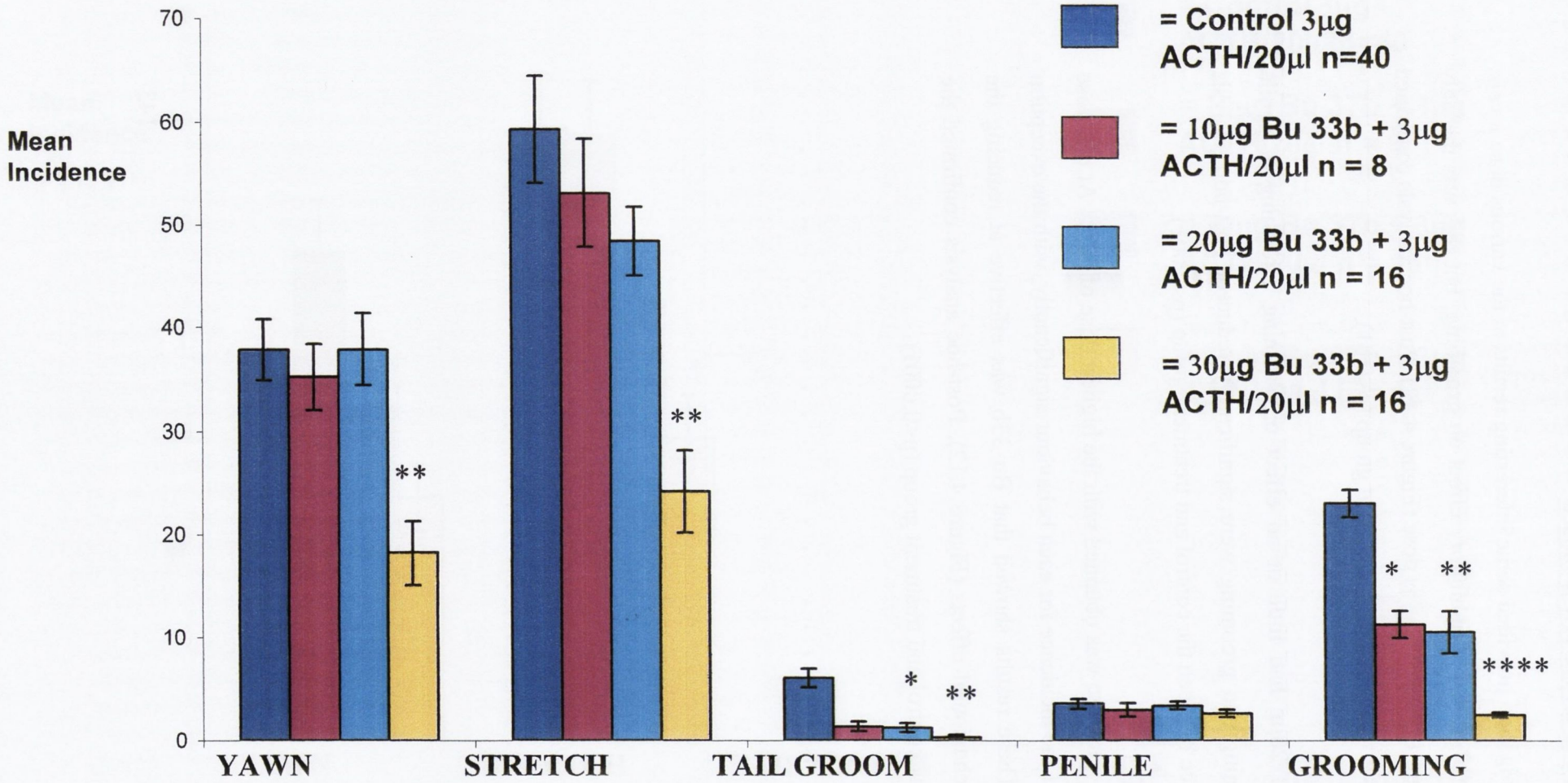
Figure 4.11: Effect of 10, 20 and 30µg of Bu 37b icv on $ACTH_{1-24}$ (3µg/ 20µl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.3.4 Effect of Bu 33b in the $ACTH_{1-24}$ model:

Bu 33b in this investigation provided some interesting results at the various dose levels. The lowest dose of 10μg had an inhibitory effect on grooming, but this was the only statistically significant finding with this dose (Figure 4.12). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The middle dose of 20μg had little or no effect on yawning, stretching or penile erection. Tail grooming and grooming were significantly reduced. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.001$).

The most significant response was obtained with the highest dose of 30μg. At this dose level Bu 33b reduced the incidence for each behaviour significantly, with the exception of penile erection. These results showed that Bu 33b was effective at reducing the $ACTH_{1-24}$ induced behavioural effects (Figure 4.12). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.0001$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

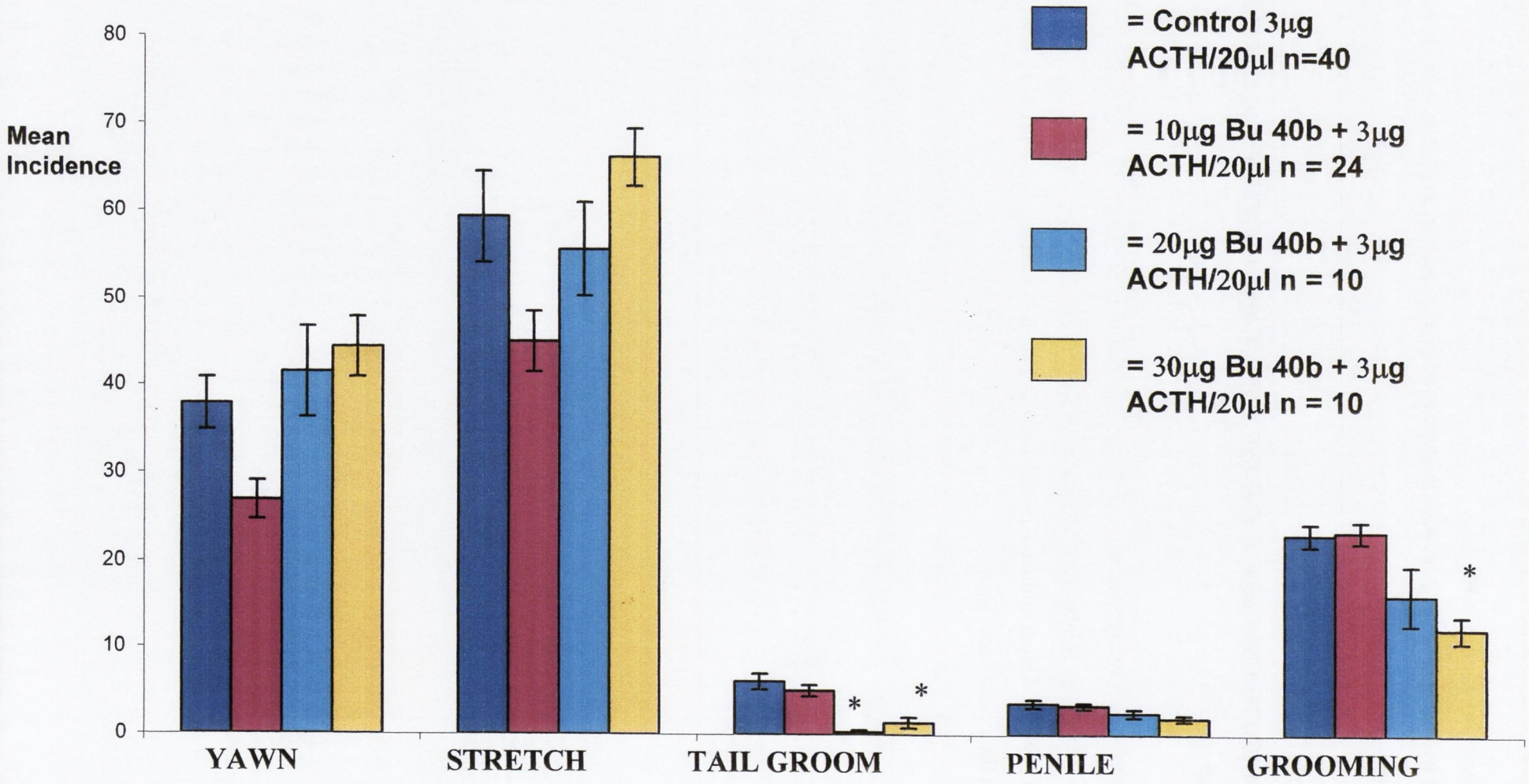
Figure 4.12: Effect of 10, 20 and 30µg of Bu 33b icv on $ACTH_{1-24}$ (3µg/ 20µl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.3.5 Effect of Bu 40b in the $ACTH_{1-24}$ model:

The results displayed on Figure 4.13 show the effect of Bu 40b on the behavioural syndrome induced by $ACTH_{1-24}$. The lowest dose had no statistically significant effect on any of the parameters.

The middle dose of 20μg had a statistically significant effect on tail grooming, which it inhibited (Figure 4.13). Post-hoc analysis revealed the difference between the control and treatment group ($p<0.01$).

The largest dose used of 30μg significantly inhibited two of the behaviours, these were tail grooming and grooming. These results indicate that Bu 40b had some inhibitory effect on some of the $ACTH_{1-24}$ induced behaviours. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.05$ and $p<0.01$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

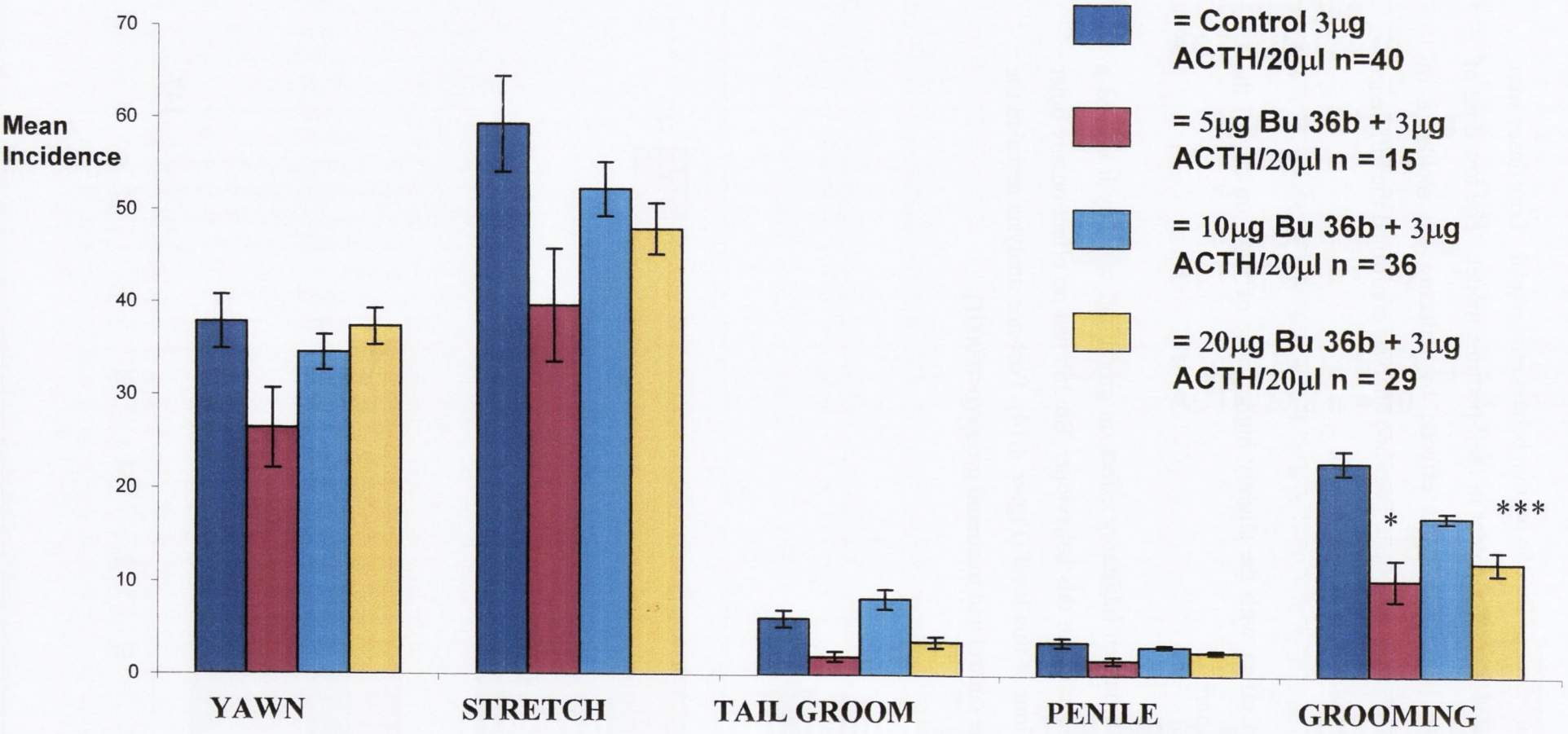
Figure 4.13: Effect of 10, 20 and 30µg of Bu 40b icv on $ACTH_{1-24}$ (3µg/ 20µl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.3.6 Effect of Bu 36b in the $ACTH_{1-24}$ model:

Figure 4.14 illustrates the effect of Bu 36b on this behavioural model. Low doses were used here as this compound was very potent in the Spermine model. The low dose of 5μg did inhibit the $ACTH_{1-24}$ behavioural effects, by reducing the incidence of grooming to a statistically significant extent. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.001$).

There was no significant effect with the slightly higher dose of 10μg on any of the $ACTH_{1-24}$ induced behaviours.

The highest dose of 20μg had an inhibitory effect on grooming, whereby it caused a statistically significant reduction in this behaviour. Bu 36b had no effect on any other $ACTH_{1-24}$ induced behaviour at this level (Figure 4.14). Post-hoc analysis revealed the same findings between the control and treatment group ($p<0.0001$).



*p<0.01, **p<0.001, ***p<0.0001, ****p<0.00001 ANOVA

Figure 4.14: Effect of 5, 10 and 20μg of Bu 36b icv on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

4.3.7 Effect of Bu 43b in the $ACTH_{1-24}$ model:

Figure 4.15 displays the results obtained for Bu 43b. Three low dose levels were used as this compound proved to be very potent in the Spermine model. The lowest dose of 5μg produced a significant inhibitory response on grooming (Figure 4.15). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

The middle dose of 10μg also produced an inhibitory response in the development grooming, but no effect on any the other parameters was observed. Post-hoc analysis revealed the difference between the control and treatment group ($p<0.001$).

The highest dose of 20μg had the most marked ability to inhibit the development of the $ACTH_{1-24}$ behaviours. At this dose level Bu 43b reduced stretching, tail grooming and grooming to a statistically significant extent (Figure 4.15). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

4.4 Discussion:

Previous studies have looked at the effects of Ifenprodil on $ACTH_{1-24}$-induced behavioural syndrome (Genedani et al, 1994). Ifenprodil is a non-competitive polyamine antagonist at the stimulatory site of the NMDA receptor complex, which is sensitive to polyamines (Schoemaker et al, 1990). Genedani et al, (1994) showed that Ifenprodil significantly reduced SYS and PE induced by i.c.v. injection of $ACTH_{1-24}$. In the present study, at the high dose of 30mg/kg ifenprodil reduced all the ACTH induced effects measured.

Ifenprodil is a known NMDA receptor antagonist as previously described (Section 3.5). In addition, Ifenprodil inhibits ornithine decarboxylase activity to the same extent as DFMO (Badolo et al, 1998). Ifenprodil may be inhibiting $ACTH_{1-24}$ effects through binding to the NMDA receptor. It has been hypothesised that excitatory amino acids, in particular those acting at the NMDA receptor, are involved in the central actions of $ACTH_{1-24}$ (Genedani et al, 1994). It has been suggested that, $ACTH_{1-24}$ could exert its effects by modulation of NMDA receptor activation (Spruijt et al, 1994). More recent evidence has shown that ifenprodil is a more selective antagonist at a subset of NMDA receptors containing the NR2B subunit (Chenard et al, 1999). Hence it is plausible that here too ifenprodil could be mediating it's antagonistic effects through the NMDA receptor, in particular the NR2B subunit. Alternatively, ifenprodil could be having its effect through the inhibition of ODC activity. $ACTH_{1-24}$ stimulates adrenal ornithine decarboxylase (ODC) activity (Levine et al, 1975). Genedani et al, (1984) looked into the effect of α-difluoromethylornithine (DFMO) on $ACTH_{1-24}$ induced behavioural syndrome. DFMO reduced the $ACTH_{1-24}$ induced behaviours. As, ifenprodil inhibits ornithine decarboxylase activity to the same extent as DFMO (Badolo et al, 1998), hence ifenprodil could be mediating inhibition of behaviours through this mechanism. Other mechanisms for ifenprodil's activity are through σ receptors and voltage sensitive calcium channels, which may also play a role in the inhibition of the $ACTH_{1-24}$ behavioural profile (Hashimoto et al, 1993; Carter et al, 1997).

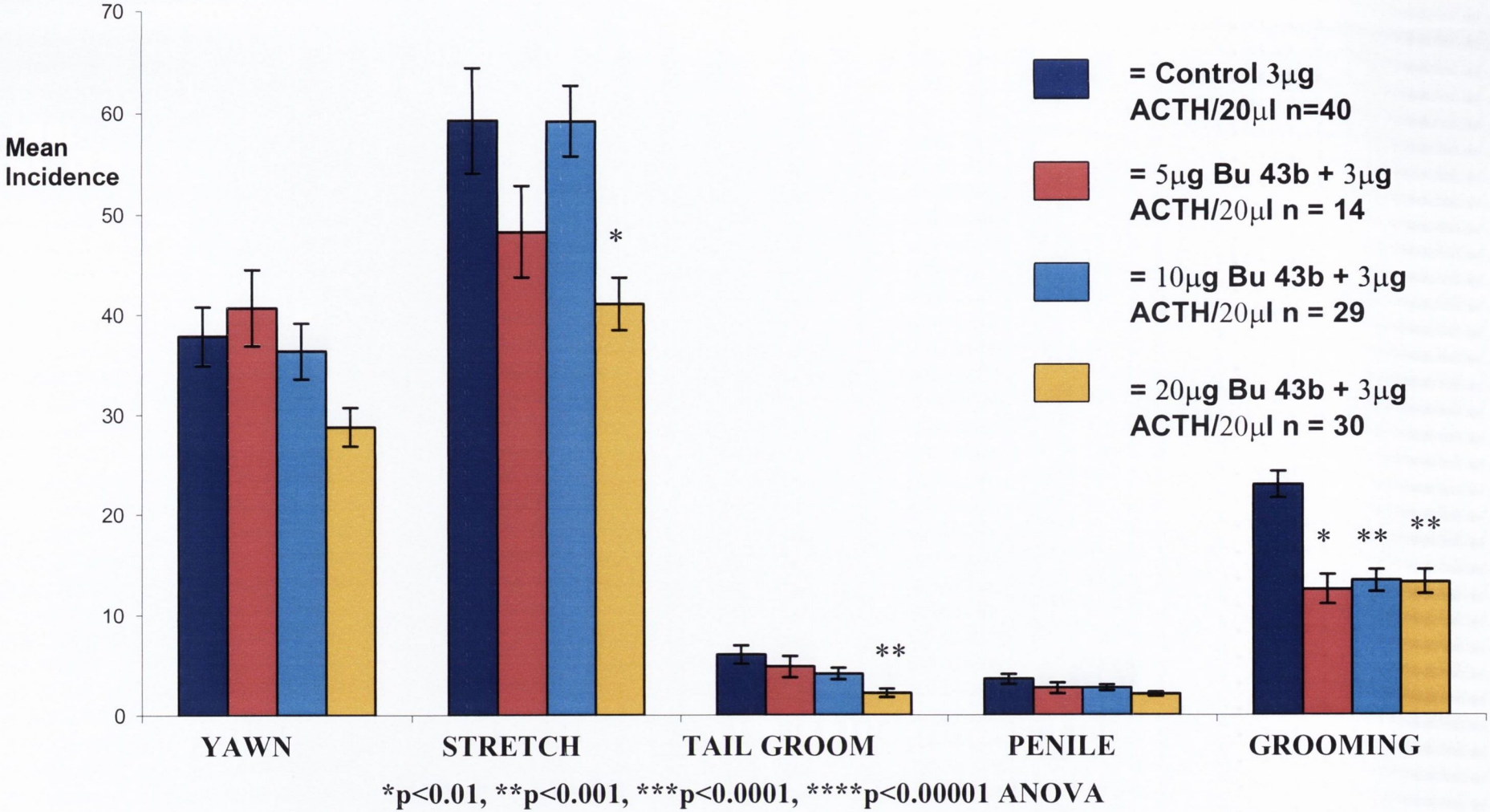


Figure 4.15: Effect of 5, 10 and 20μg of Bu 43b icv on $ACTH_{1-24}$ (3μg/ 20μl icv) Induced Animal Behaviours Over 1 Hour in *Laca* Mice. Results expressed as x ± s.e.m.

Another involvement could be with nitric oxide, as it is known that NO synthase is involved in the development of the $ACTH_{1-24}$ behaviours (Poggioli et al, 1995). NO has been shown to induce yawning and penile erections, through brain nitrergic pathways, while stretching and grooming involves peripheral nitrergic pathways (Poggioli et al, 1995). Even more interestingly, NO has also been implicated in the induction of penile erections and yawning produced by NMDA (Melis et al, 1997). Finally it is likely that $ACTH_{1-24}$ binds to the MC4 melanocortin receptors which mediates grooming, yawning and stretching but not penile erections (Argioloas et al, 2000). These melanocortin receptors have been found to be coupled to adenylate cyclase or to phosphatidyl-inositol/ Ca^{2+} mediated signaling systems in the CNS (Argioloas et al, 2000). This evidence emphesises an involvement of calcium in the development of $ACTH_{1-24}$ induced behaviours. Intracellular calcium levels could be influenced by ifenprodil through an NMDA or voltage sensitive calcium channel mechanism. The present study provides evidence there is some interaction between ifenprodil and $ACTH_{1-24}$ but the mechanism is not fully elucidated.

The results found for eliprodil showed that eliprodil reduced $ACTH_{1-24}$ induced behaviours. Grooming in particular was significantly reduced, while yawning, stretching and tail grooming were significantly reduced at the high doses. This illustrates that eliprodil is inhibiting at least some of the behavioural effects of $ACTH_{1-24}$.

Eliprodil is a structural analogue of ifenprodil, and also strongly selective for the NR1/NR2B subunits of the NMDA receptor (Avenet et al, 1997). Eliprodil, (as previously described see Section 3.5), has been shown to be more potent in its ability to block voltage-activated L- and N- type calcium channels than ifenprodil (Biton et al, 1994). In this study, it is reasonable to suggest that eliprodil's effects may be mediated at least in part, through the blocking of calcium channels as previous studies have indicated that calcium is of key importance in the occurrence of the $ACTH_{1-24}$ behaviours. However, eliprodil could also be mediating its effects directly through its interaction with the NMDA receptor, in particular the NR1/NR2B subunits, in a similar

manner to ifenprodil. The findings from this investigation have supported the idea that the different components of the $ACTH_{1-24}$-induced behavioural syndrome in particular SYS and the PE, may involve different neural pathways (Genedani et al, 1984). From the previous text it is possible that other mechanisms are also involved also, e.g MC4 melanocortin receptor activity.

Arcaine has been described as an antagonist (Donevan et al, 1992) and later a partial agonist (Rock et al, 1992), at the polyamine sites of the NMDA receptor (see section 3.5). In the present study, arcaine inhibited the $ACTH_{1-24}$ behavioural effects, except for yawning, illustrating that this polyamine antagonists is effective in reducing $ACTH_{1-24}$ behaviours. Arcaine didn't have as marked reduction as the previous two compounds. It is not unreasonable to suggest that the possible mechanism of action is mediated through the blocking of the NMDA receptor or voltage sensitive Ca^{2+} channels, but further work is necessary to ascertain the mechanism.

Memantine also had an inhibitory response on the $ACTH_{1-24}$ behaviours, whereby it reduced yawning, tail grooming, penile erections and grooming behaviours at 10 and 20mg/kg. These results indicate that memantine can cross the blood brain barrier and have an inhibitory effect on the development of these behaviours. The mechanism of action is uncertain, but as memantine is known to be a NMDA antagonist, acting via the open-channel, it is likely that this underpins its action. In doing this it would be inhibiting Ca^{2+} influx, which is essential to the $ACTH_{1-24}$ behaviours. The effectiveness of memantine in this model suggests that the effect of ifenprodil, eliprodil and arcaine, may be attributed, at least in part, to NMDA receptor inhibition via polyamine antagonism on the NR2B subunit.

Previous studies have indicated that Ca^{2+} channel blockers have an inhibitory effect on the $ACTH_{1-24}$ induced behaviours (Poggioli et al, 1993; Poggioli et al, 1995; Vergoni et al, 1995). In the present study two calcium channel blockers were used to investigate their effects. Nisoldipine, (previously mentioned see section 3.5), significantly inhibited

grooming and tail grooming. These results highlight that grooming and tail grooming may be the behaviours most readily influenced by blocking L-type Ca^{2+} channels.

Nitrendipine induced a more significant inhibition of the $ACTH_{1-24}$ induced behaviours than nisoldipine. At each dose level there was significant inhibition of tail grooming, penile erections and grooming. This highlights that forms of grooming in particular, may be influenced by L-type Ca^{2+} channels. At the higher dose levels of 20 & 30mg/kg yawning and stretching were also inhibited. Probably the most likely mechanism for the effects of nitrendipine, is that nitrendipine is mediating its antagonist effect's through binding sites on the $\alpha 1$ subunit of the L-type voltage gated channel (Schoemaker, 1992). It is also not unreasosnable to suggest that nitrendipine may be mediating some of its inhibitory effects through the NMDA receptor, as nitrendipine has been shown to directly reduce NMDA evoked currents by suppression of NMDA/glycine-mediated calcium influx (Skeen et al, 1993; Skeen et al, 1994). The results presented here illustrate nitrendipine was a more effective drug than nisoldipine in reducing the $ACTH_{1-24}$ induced behaviours. Although, a higher dose of nitrendipine was used in comparison to nisoldipine. Nisoldipine has previously been demonstrated to antagonize NMDA-induced seizures more potently than MK-801 or nitrendipine (Palmer et al, 1993).

The polyamine putrescine, a direct product of ODC synthesis, blocked the $ACTH_{1-24}$ induced behaviours extremely effectively. If polyamines are involved in the development of the $ACTH_{1-24}$ effects, one might expect putrescine to cause an increase in the $ACTH_{1-24}$ behavioural effects. However, the high levels of putrescine may prevent further ODC activation. And it is possible that putrescine physiologically antagonizes spermine and spermidine effects. The results presented here suggest that putrescine is not directly responsible for the profile of $ACTH_{1-24}$ behaviours. Putrescine is also known to interact with DNA/RNA, and it is possible that this may stimulate another cascade within cell that negates $ACTH_{1-24}$ effects.

The present results obtained for DFMO are largely concurrent with previous findings using DFMO (Genedani et al, 1984). The results here show that DFMO significantly inhibits the effects of $ACTH_{1-24}$. The results, in conjunction with previous findings indicate there is an interaction between ODC activity and $ACTH_{1-24}$ induced effects. This implicates ODC and polyamine synthesis in the behaviours.

N^1-dansylspermine (as previously described Section 3.5), is an analogue of the polyamine spermine. It has been shown, through binding studies and behavioural studies to be an antagonist at the NMDA receptor. It has also been shown to be an effective calmodulin antagonist and cytotoxic agent (Seiler et al, 1998). In this study N^1-dansylspermine had an inhibitory effect on the some of the $ACTH_{1-24}$ induced behaviours. It had no effect on grooming and penile erections, which interstingly were inhibited by arcaine in this study. N^1-dansylspermine may have mediated its inhibition through the NMDA receptor, although it had a different profile of effects to any of the NMDA antagonists (memantine, ifenprodil and eliprodil), investigated in this study.

It was of interest to investigate the novel compounds from Brock University in this behavioural model also. The results for Bu 31b indicate that this novel compound had an inhibitory effect on the entire $ACTH_{1-24}$ behavioural profile. Already mentioned is the fact that little is known about the mechanism of action of this compound. Only one publication is available on this compound and this describes the synthesis of the compound and the interaction with the NMDA receptor in vitro (Fixon-Owoo et al, 2003). Bu 31b was shown to have a very high affinity for the NR1/NR2B subunit of the NMDA receptor, with an inhibition of 98% (Fixon-Owoo et al, 2003). With the results presented here, it could be hypothesized that this compound is acting at a polyamine site on the NMDA receptor to inhibit the $ACTH_{1-24}$ induced behaviours.

The present results obtained for compound Bu 37b indicate there is some inhibition of the $ACTH_{1-24}$ behavioural effects, with forms of grooming being the most affected. This profile of effects is strikingly similar to the effects of nisoldipine making a possible link to L-type Ca^{2+} channel activity. This finding also strongly emphasizes the notion of

different neuronal pathways involved in the different behaviours, mentioned previously. In the study by Fixon-Owoo et al (2003), Bu 37b was shown to be a poor glutamate receptor inhibitor.

Bu 33b also had an inhibitory effect on the $ACTH_{1-24}$ induced behaviours. At the highest dose it reduced yawning, stretching, tail grooming and grooming significantly. No effect on penile erection was observed which could be attributed to the different mechanisms involved in the generation of the behaviours. Previous studies have shown that Bu 33b was a very poor inhibitor of the glutamate receptors (Fixon-Owoo et al, 2003). It is possible Bu 33b may be inducing its effects through voltage sensitive calcium channel (VSCC) Ca^{2+} channel as previous studies have shown that Ca^{2+} antagonists block the development of the $ACTH_{1-24}$ behaviours.

The results obtained for the novel compound Bu 40b show that Bu 40b had little effect on the $ACTH_{1-24}$ induced behaviours. Previous studies with the compound have found that this compound is a poor inhibitor of the glutamate receptors (Fixon-Owoo et al, 2003). It is possible that Bu 40b could be mediating its mild inhibitory effects through Ca^{2+} voltage sensitive calcium channel (VSCC) channel blockage as previously described.

Bu 36b was not very effective at reducing the development of $ACTH_{1-24}$ induced effects, with the exception of a significant inhibition of grooming. Bu 40b is a poor inhibitor of the $ACTH_{1-24}$ induced behaviours. Previous studies have shown that Bu 40b has poor inhibitor of glutamate receptors, especially NR1/NR2B subunit types (Fixon-Owoo et al, 2003), NMDA receptors have been indicated in the development of behaviours, such as yawning and stretching. As there is some inhibition of grooming it might be suggested that Bu 36b is having an effect mediated through interaction with Ca^{2+} VSCC as this too is implicated with the development of the $ACTH_{1-24}$ behaviours.

The present results indicate Bu 43b reduces some of the $ACTH_{1-24}$ induced behavioural effects, in moderate degree. Bu 43b has been shown to be a very potent antagonist at

the NR1/NR2B subunit of the NMDA receptor with 95% inhibition reported (Fixon-Owoo et al, 2003). It would seem plausible to suggest that the inhibition here is attributed to the impact on the NMDA receptor. Another possible mechanism of inhibition of Bu 43b on $ACTH_{1-24}$ induced behaviours could be attributed to voltage sensitive calcium channel (VSCC) inhibition.

4.5 Conclusion

In conclusion it appears there may be a link between the polyamines and the $ACTH_{1-24}$ induced behavioural profile of effects. The NMDA receptor in particular the NR1/NR2B subunit could be involved. L-type Ca^{2+} channels may be involved in the development of these behaviours. There are different neuronal pathways stimulated for the different behaviours as only certain behaviours were inhibited with different drugs used. Novel polyamine analogues showed a profile of effects which more closely mirrored the effect of arcaine than N^1-dansylspermine. Grooming and tail grooming were most affected by the novel polyamine analogues.

Chapter 5.

5.1 Introduction.

To further understand the synthesis and metabolism of polyamines it was of interest to undertake a study to determine the concentration of polyamines in the mouse brain from the two behavioural models previously used. The concentration of polyamines varies from species to species and from region to region within the brain. In this study mouse brains were used. Due to the blood-brain barrier the exchange of polyamines between blood and brain are limited, hence all brain polyamines are synthesised in the brain (Shaw, 1979). In this study the whole brain was used, rather than sections of the brain.

Polyamine analysis has been performed using a wide variety of techniques, these include thin-layer and paper chromatography, electrophoresis, liquid chromatography in amino acid analyzers, immunochemical methods, enzymatic methods, gas chromatography and high-performance liquid chromatography (HPLC) (Muskiet et al, 1995). Polyamines and their metabolites do not contain functional groups that allow sensitive and selective on-line detection without derivatisation, therefore pre or post-column derivatisation followed by spectrophotometric flurometric detection are applied. The most commonly employed method uses pre-column derivatisation with dansyl chloride in aqueous solution, followed by derivative purification and reverse-phase HPLC/fluorimetry (Muskiet et al, 1995). In this study this was the method applied to samples from the spermine behavioural model and the $ACTH_{1\text{-}24}$ behavioural model. Levels of putrescine, spermidine, spermine, N^1-acetylspermidine and N^8-acetylspermidine were assessed at various behavioural stages in both models.

5.1.1 Materials and Methods. (For a full details see Chapter 2).

As previously described (see Chapter 2), spermine (100μg) was administered through the icv route to each animal. Assessment of CNS excitation in the spermine treated mice used a scoring system (score from 1 to 5) to quantify the degree of tremor produced (Doyle et al, 1998), from 2 hours after injection, every 30mins for 7.5 hours. 1= slight tremor, 2= moderate tremor, 3= severe tremor, 4= tonic convulsion but survived and 5= fatal tonic convulsion (Doyle et al 1998). As the spermine (100μg) induced CNS excitation developed, Stage 1 to 5, animals (n=73), were culled at the various score values at various times over the 7.5 hours after spermine administration. Brains were extracted immediately in each of the groups and placed in 1 ml of 0.4M perchloric acid. For the control group animals were given saline and culled at various time points to eliminate bias. In relation to the $ACTH_{1-24}$ induced behavioural model, $ACTH_{1-24}$ was administered as previously described (see Chapter 2), animals were culled at different time points over 1 hour duration, every 15 minutes (n=50). For control, saline was administered and the animals culled after 1 hour. To investigate the mechanism of action of $ACTH_{1-24}$, the effects of administration of putrescine and DFMO in changes in polyamines levels induced by $ACTH_{1-24}$, was investigated.

Each brain, once removed was weighed and then manually homogenised in 1ml of 0.4M perchloric acid solution, sonicated for 15 minutes and then vortexed for 1-2 minutes to give a homogeneous solution. Samples were then centrifuged for 15 minutes at 4000rpm. 100μl of the supernatant was diluted 1 in 10 with HPLC water. The samples were stored in a freezer for subsequent HPLC analysis (for full details on HPLC analysis and procedure on polyamine derivatisation see Chapter 2).

5.1.2 Detection of Polyamines.

Polyamines were derivatised with dansyl chloride and levels measured via HPLC described in chapter 2. The following high sensitivity standards were used to calculate the levels of putrescine, spermidine and spermine measured in the samples 1.25, 2.5, 5, 7.5, 10, 12.5, 25, 37.5 and 50μM. Typical standard curves are seen in Figure 2. The following very high sensitivity standards were used for the estimation of N^1-acetylspermidine and N^8-acetylspermidine at 0.0625, 0.125, 0.25, 0.375, 0.5, 0.625 and 1.25μM. Typical standard curves are seen in Figure 5.2. Retention times were in close proximity, but peaks were sharp and separable. The retention times were reproducible and no broadening of peaks was experienced. Figure 5.3 illustrates the peaks for the polyamines, putrescine, spermidine, spermine and the internal standard 1,7 diaminoheptane. Figure 5.4 displays the chromatogram peaks for N^1-acetylspermidine and N^8-acetylspermidine. Retention times were as follows; putrescine ~2.9 minutes, spermidine ~ 4.2 minutes and spermine ~4.9 minutes. N1-acetylspermidine ~ 9.7 minutes and N8-acetylspermidine ~ 9.0 minutes. A response factor giving the concentration of component which produces a unit detector response was calculated (See Chapter 2, section 2.3.7):

Data Analysis:

Analysis of significant differences was performed by analysis of variance using One-Way ANOVA. A probability values of less than 0.05 was considered statistically significant. The mean polyamine level (g wet weight tissue) in each experimental condition (± S.E.M) was calculated. Post-Hoc analyses was performed using Tukey.

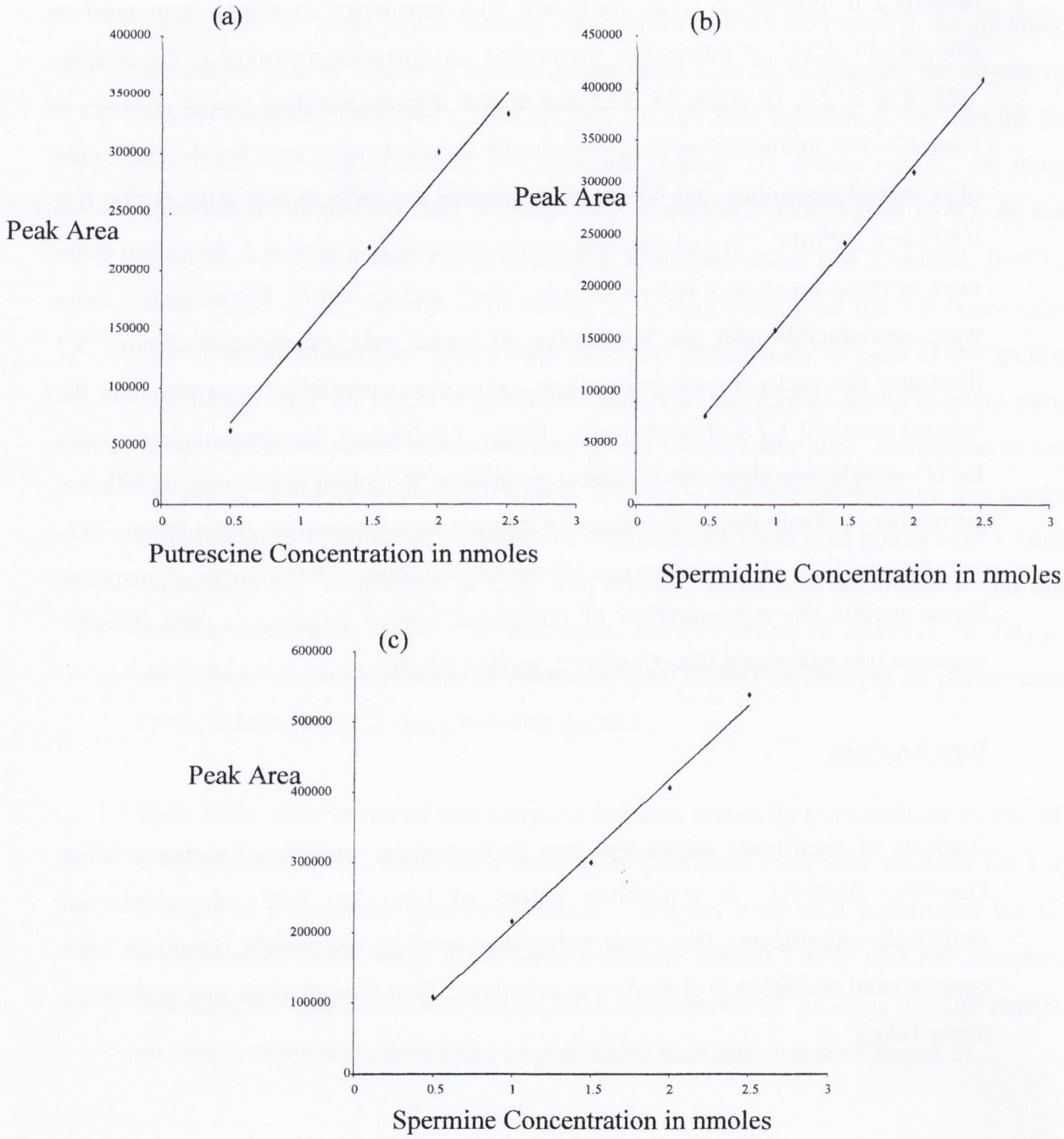
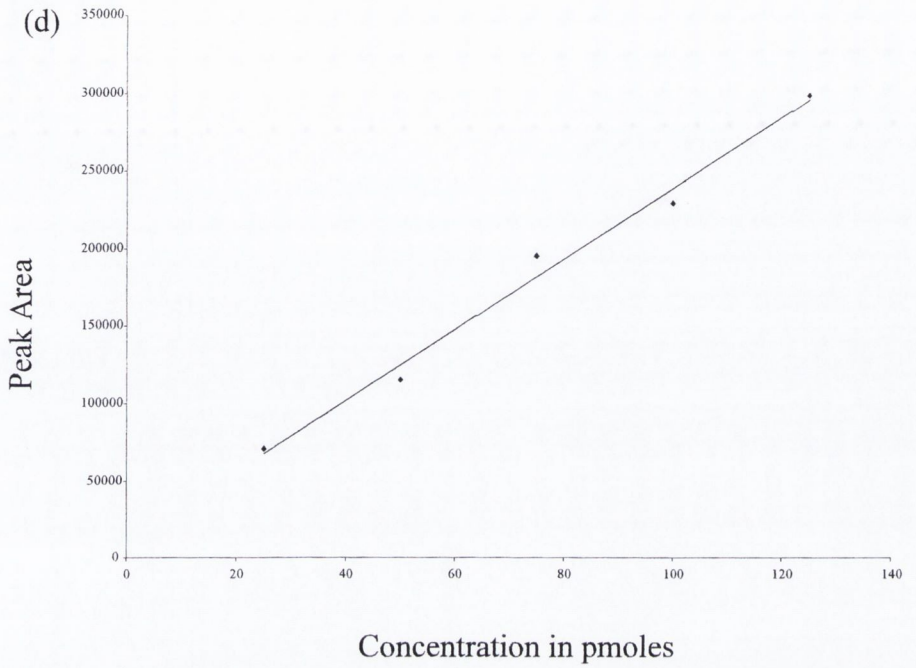


Figure 5.1: Standard curves for HPLC analysis of polyamines putrescine (a), spermidine (b) and spermine (c) measured in nmoles



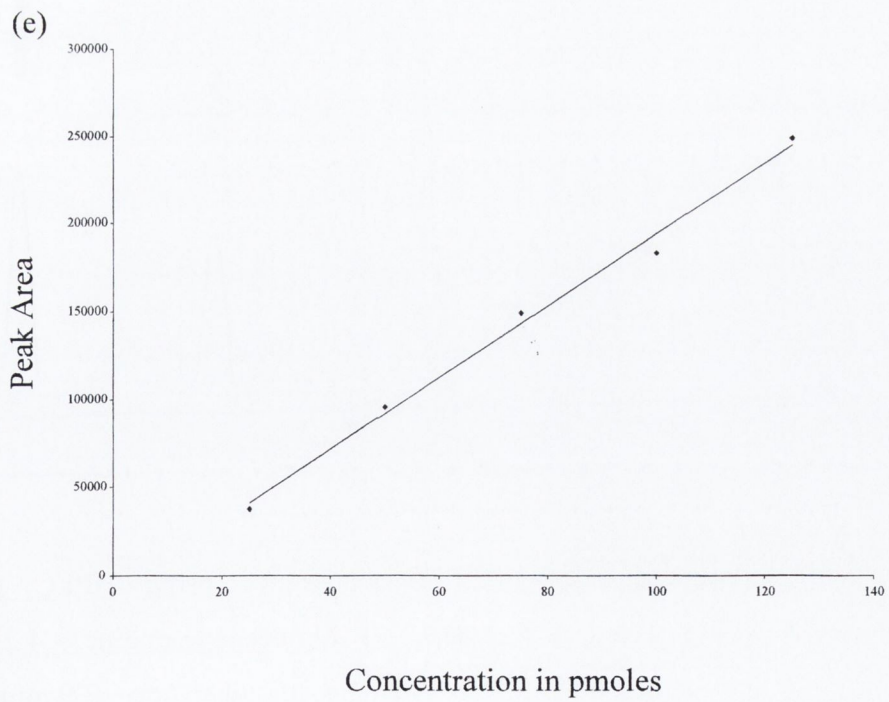


Figure 5.2: Standard curves for HPLC analysis of polyamines N1-acetylsperrmidine (d) and N8-Acetylspermidine (e) measured in pmoles.

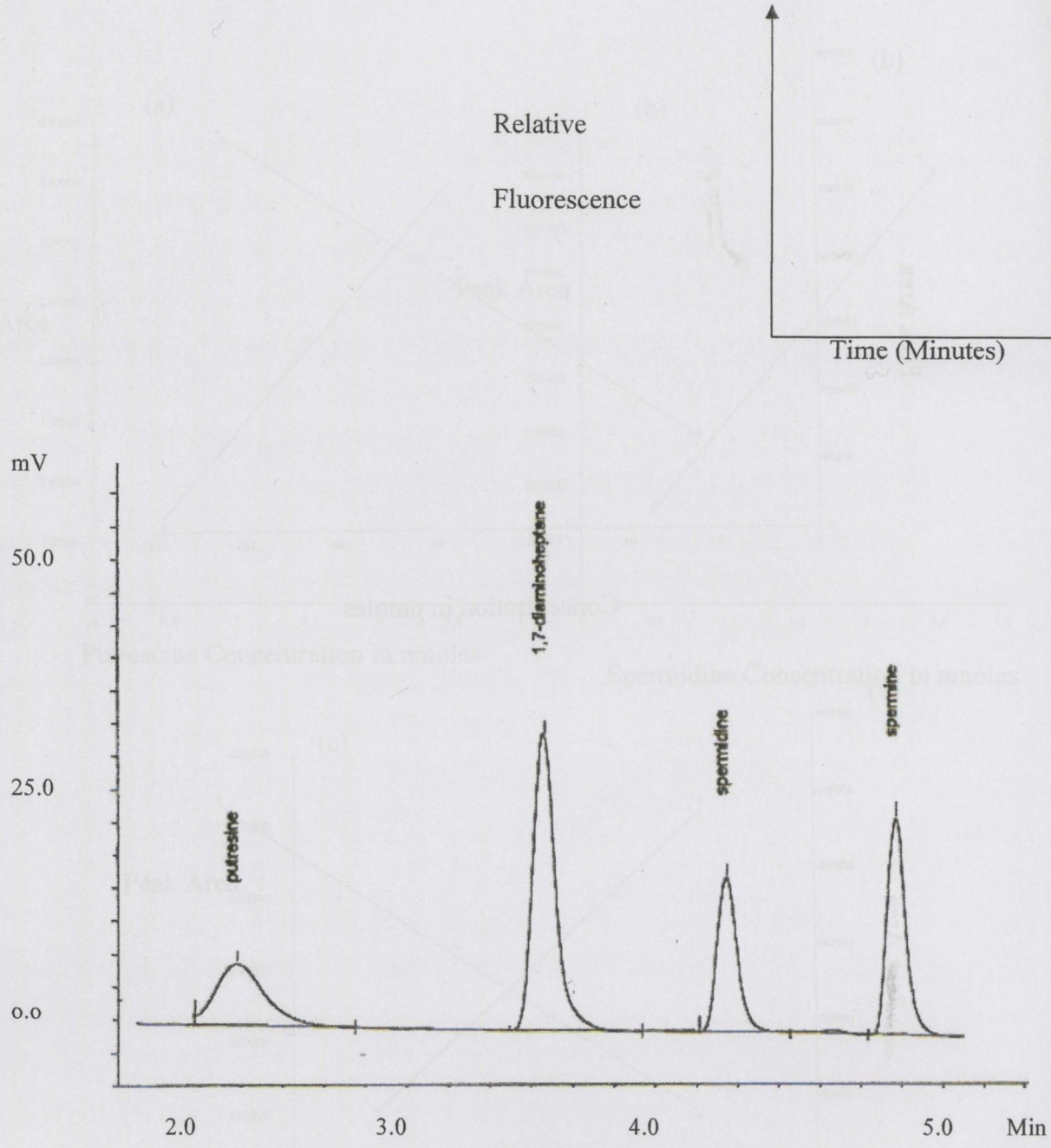


Figure 5.3: Fluorimetric detection of polyamines using HPLC following dansylation. Retention times: putrescine ~2.93 minutes, spermidine ~ 4.2 minutes, spermine ~ 4.9 minutes and internal standard 1,7 diaminoheptane ~ 3.9 minutes

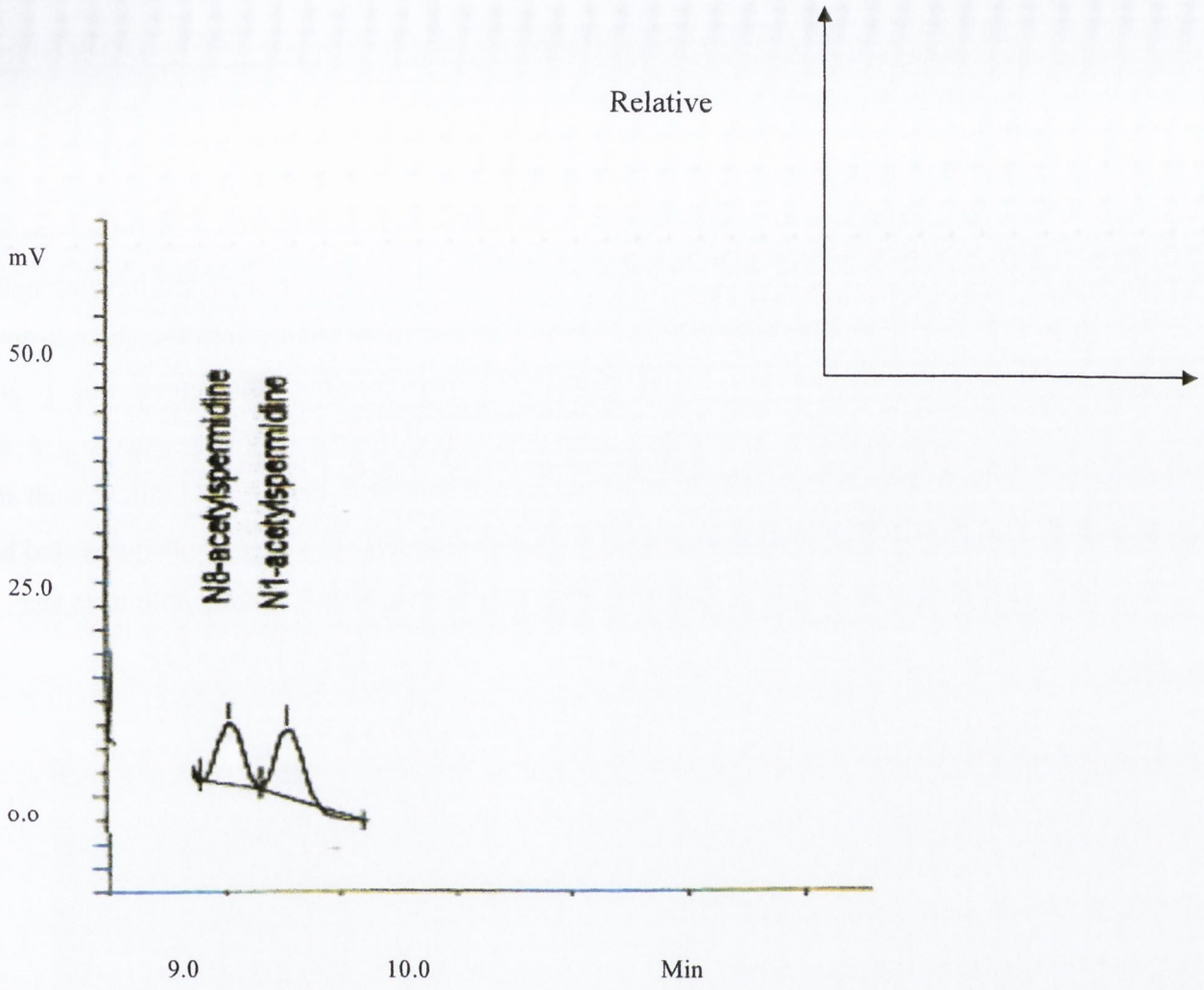


Figure 5.4: Fluorimetric detection of polyamines using HPLC following dansylation. Retention times: N^1-acetylspermidine ~ 9.7 minutes and N^8-acetylspermidine ~ 9.0 minutes.

5.2 Effect of 100μg/20μl Spermine on polyamine concentration levels.

As previously described (section 2.1.1) following administration of 100μg spermine icv to Laca mice, the behavioural effects observed consist of the development of CNS excitation in the form of tremor, the tremor worsens over time and culminates in tonic convulsions (Doyle et al, 1998). The scoring system used was 1: slight tremor, 2: moderate tremor, 3: severe tremor, 4: tonic convulsion - survived, 5: fatal tonic convulsion. The level of polyamine concentrations in the brain at each stage of the CNS excitation has never been studied, until now. The results presented here, show the polyamine levels in mouse brain at each of the CNS excitation stages.

5.2.1 Summary of changes in total polyamine levels in the spermine model.

Figure 5.5. From the graph it may be seen that there is a statistically significant increase in the concentration of total polyamines following spermine administration in comparison to control. Most prominent is the highest polyamine concentrations at score 3 and score 4 in comparison to the saline treated group. There is a steady increase in the level of total polyamines from score 0 to score 4. Further to this there is a dramatic drop in the level of polyamines at score 5, whereby the level of total polyamines has retuned to almost the saline levels. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.05$, $p<0.05$, $p<0.03$, $p<0.01$, $p<0.01$ and $p<0.05$).

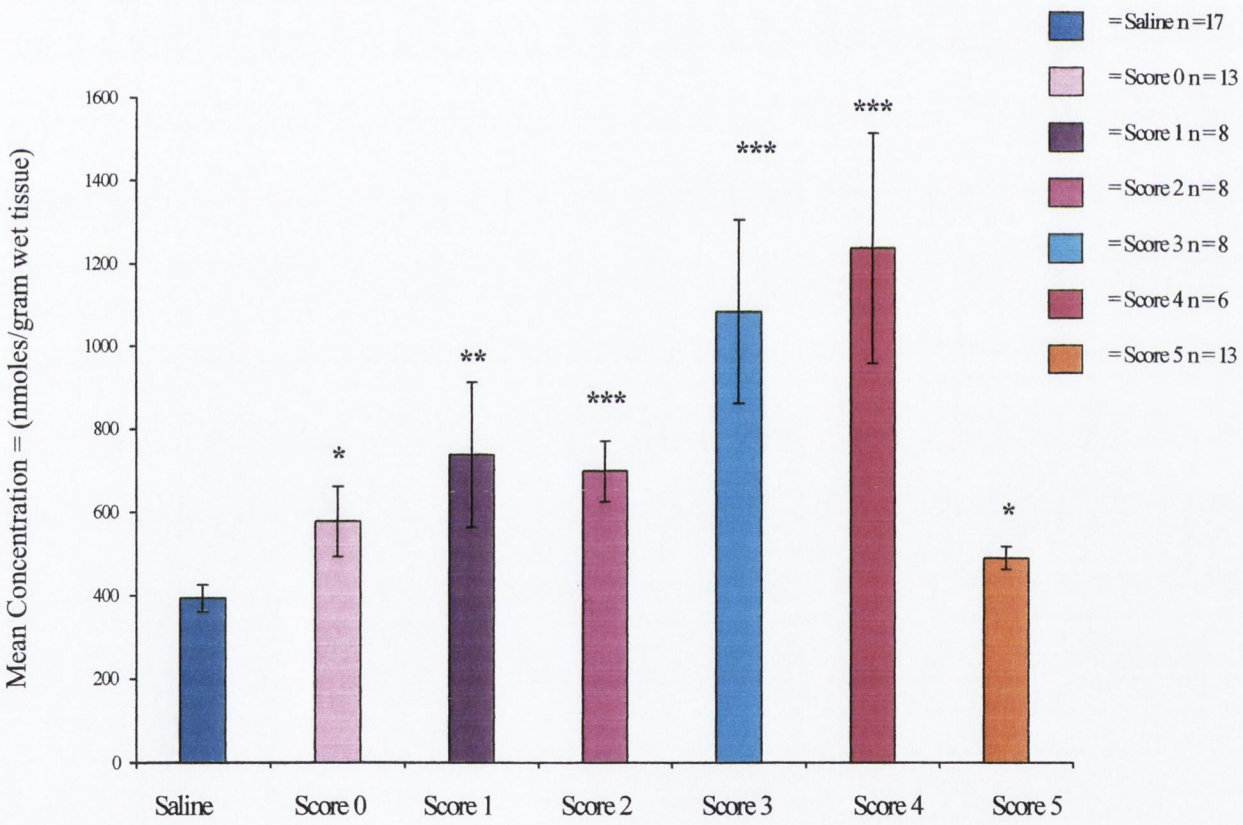


Figure 5.5: Effect of spermine (100μg icv) on total polyamine concentration in mouse brain in samples taken at each of the CNS excitation stages (1-5). Results are expressed as x± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.2.2 Effect of the administration of 100μg spermine on individual polyamine concentrations at median score 0.

From the graph it may be seen that there are two axes presented, the first axis is for the higher levels of putrescine, spermidine and spermine, while the secondary axis, is for the levels of N^1-acetylspermidine and N^8-acetylspermidine. Both are expressed in nmoles/gram of wet tissue.

In figure 5.6 (a), the high levels of spermine present prior to any behavioural change (score 0) in brain tissue is evident. This was to be expected, as spermine was the drug administered to the animals. Both the levels of putrescine and spermidine in the spermine treated group are similar to that of the saline control group. From figure 5.6 (b), in the saline treated group there is very little N^1-acetylspermidine and N^8-acetylspermidine present. However the spermine treated group shows a dramatic increase in the level of N^1-acetylspermidine in comparison to the control group. There is no change in the level of N^8-acetylspermidine following spermine treatment. Post-hoc analysis revealed this difference for spermine and N^1-acetylspermidine ($p<0.05$).

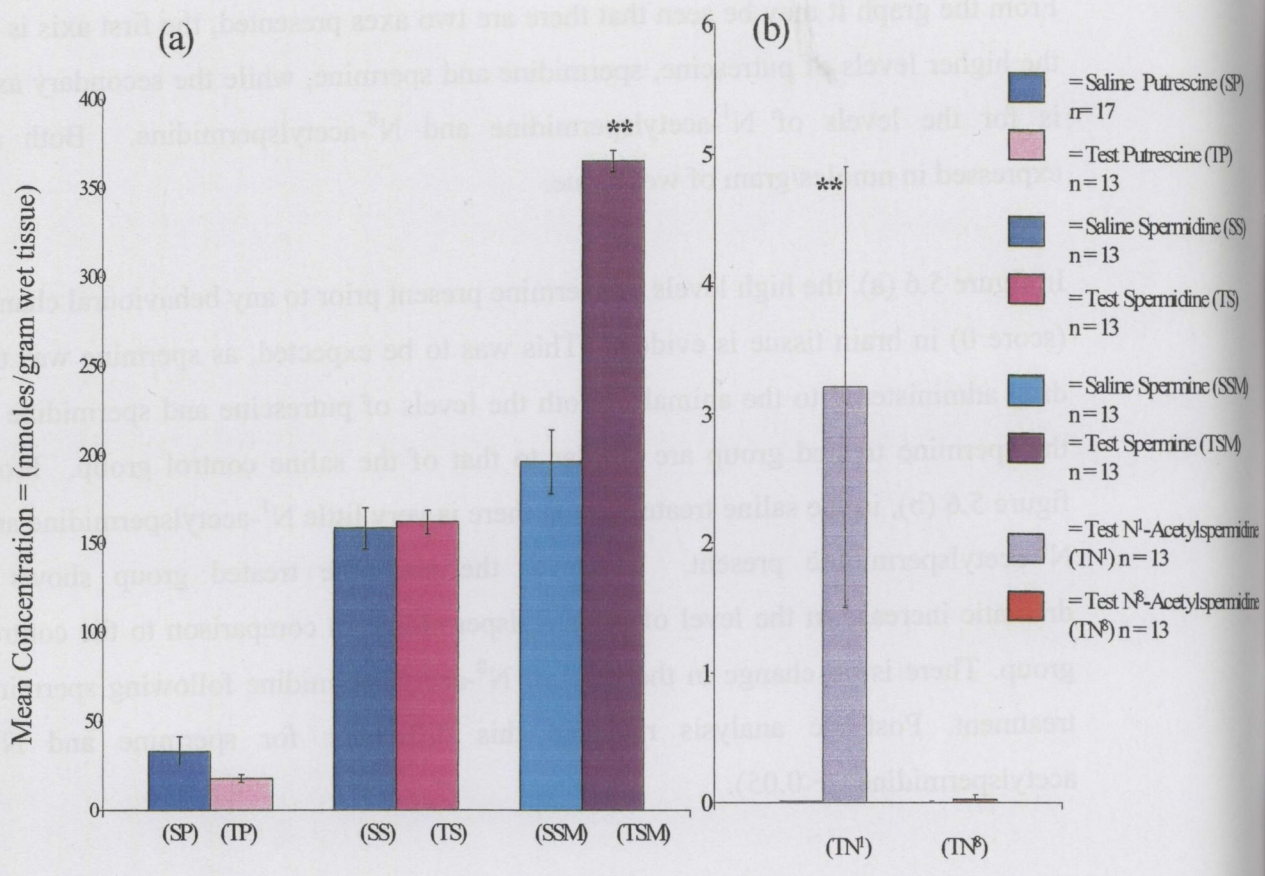


Figure 5.6: Effect of spermine (100μg icv) on polyamine levels in mouse brain in samples taken prior to the development of behavioural changes representing CNS excitation stages (0). Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.2.3 Effect of the administration of 100μg spermine on individual polyamine concentrations at median score 1.

From graph 5.7 (a) there is a significant increase in the level of putrescine present in comparison to the control level. There is also an increase in the level of spermine present, in comparison to the saline group. Spermidine levels remain unchanged. From figure 5.7 (b) there is an increase in the level of N^1-acetylspermidine in comparison to the control. There was a slight but statistically insignificant, increase in the level of N^8-acetylspermidine in comparison to the control level. Post-hoc analysis revealed this difference ($p<0.01$, $p<0.05$ and $p<0.01$).

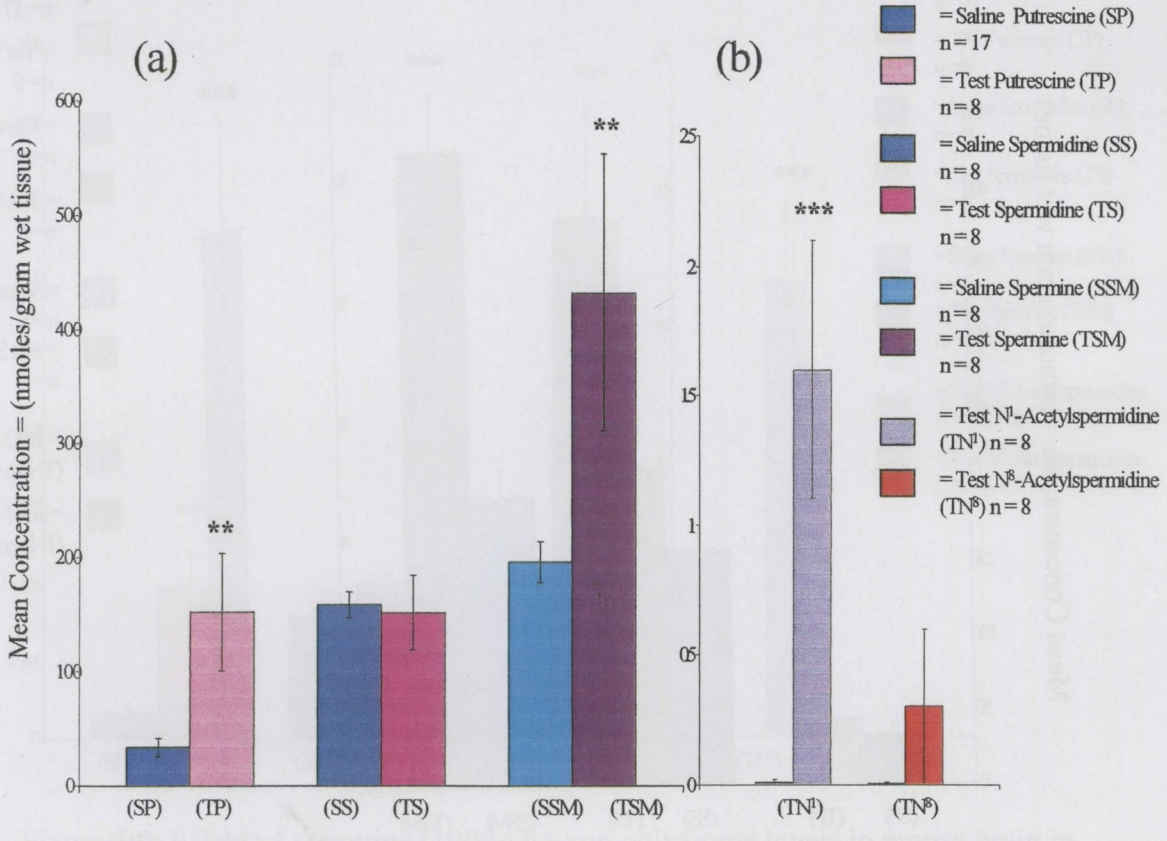


Figure 5.7: Effect of spermine (100μg icv) on polyamine levels in mouse brain in samples taken when the animals were displaying a slight tremor (stage 1). Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.2.4 Effect of the administration of 100μg spermine on individual polyamine concentrations at median score 2.

From figure 5.8 (a) there are no significant changes observed in putrescine or spermidine levels. However spermine levels are increased 2 fold in comparison to saline control levels. From figure 5.8 (b), N^1-acetylspermidine levels are significantly increased in spermine treated animals. Post-hoc analysis confirmed the difference between control and treated ($p<0.0001$ and $p<0.0001$ respectively).

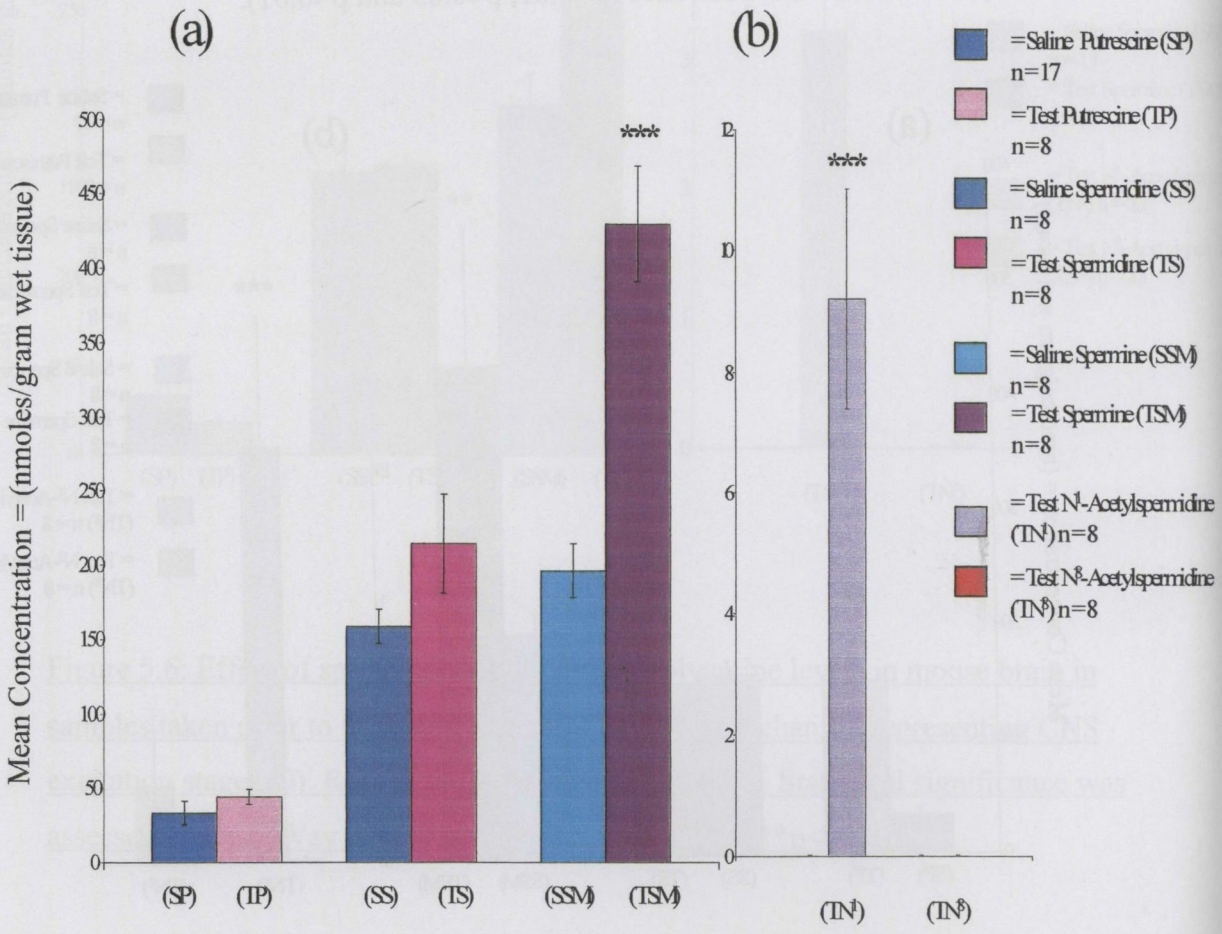


Figure 5.8: Effect of spermine (100μg icv) on polyamine levels in mouse brain in samples taken when the animals were displaying a moderate tremor (stage 2). Results are expressed as x ± s.e.m. Statistical significance was assessed by One - Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.2.5 Effect of the administration of 100μg spermine on individual polyamine concentrations at median score 3.

From figure 5.9 (a), there is an increase in putrescine levels of almost 6 fold in comparison to untreated saline group. There is also a 3 fold increase in spermine in the treated group. In figure 5.9 (b), the level of N^1-acetylspermidine is high, consistent with previous score stages. While there is very little trace of N^8-acetylspermidine in the samples. Post-hoc analysis revealed this difference for putrescine, spermine and N^1-acetylspermidine ($p<0.0001$).

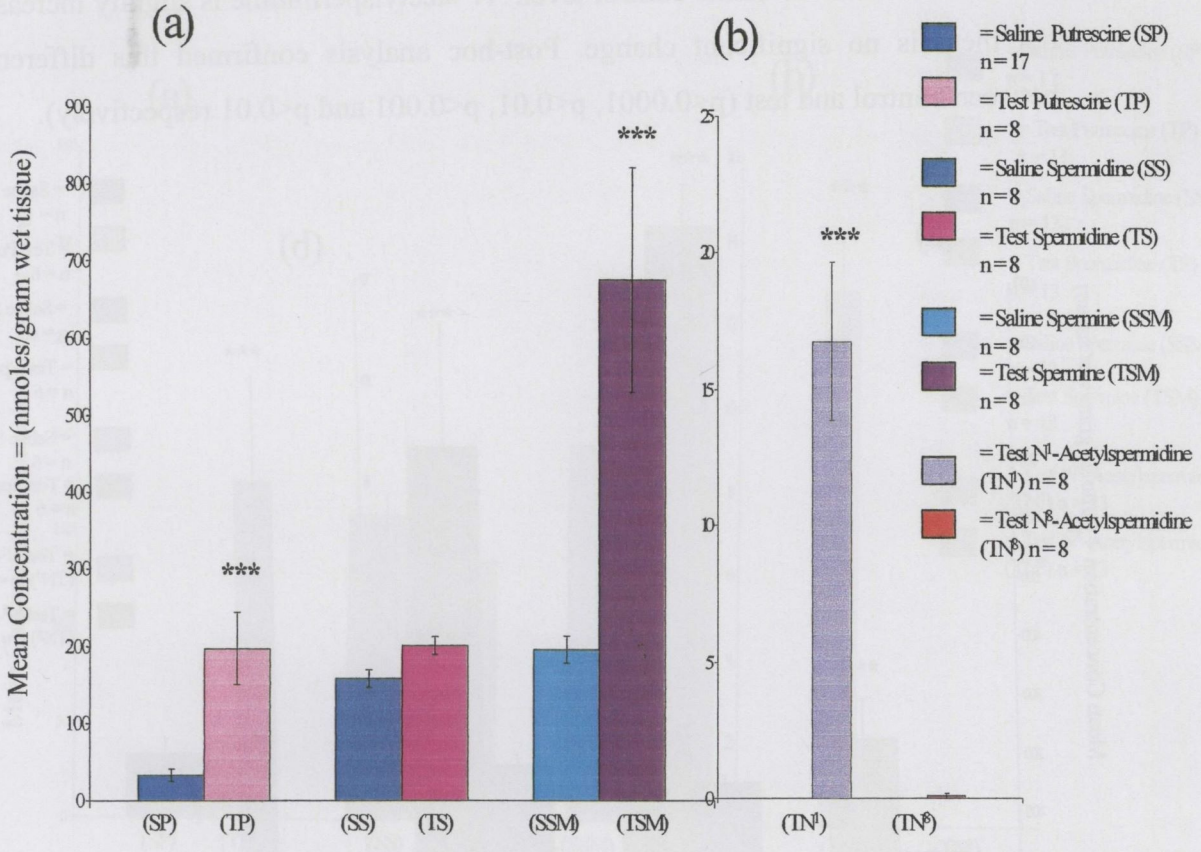


Figure 5.9: Effect of spermine (100μg icv) on polyamine levels in mouse brain in samples taken when the animals were displaying a severe tremor (stage 3). Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.2.6 Effect of the administration of 100μg spermine on individual polyamine concentrations at median score 4.

From figure 5.10 (a) and (b), it is evident that, putrescine, spermidine, spermine and N^1-acetylspermidine are increased in spermine treated animals in comparison to saline treated controls. A median score of 4 is characterised by a tonic convulsion, but the animals survive. Putrescine levels are 7 times higher, the increase in spermidine is double the amount found in the saline samples, while spermine concentration is increased by 4 times the control amount. N^1-acetylspermidine levels are 8 times the saline control level. N^8-acetylspermidine is slightly increased but there is no significant change. Post-hoc analysis confirmed this difference between control and test ($p<0.0001$, $p<0.01$, $p<0.001$ and $p<0.01$ respectively).

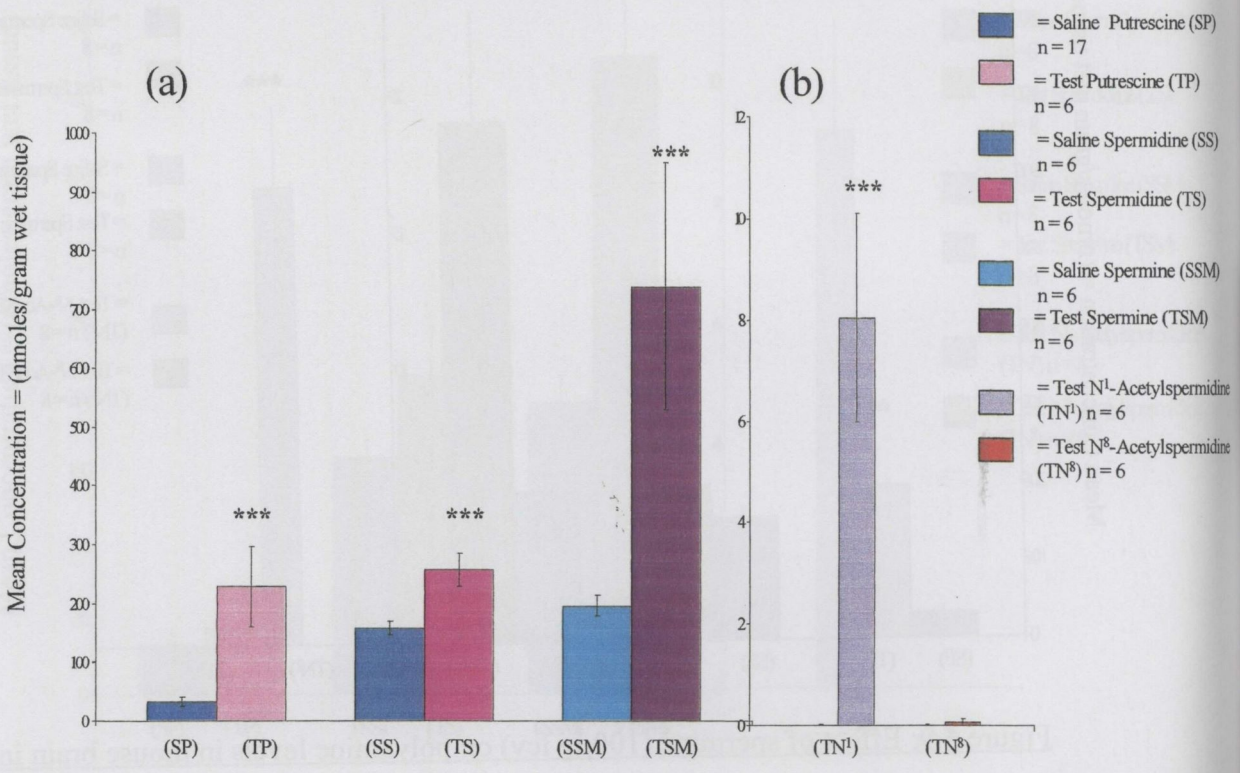


Figure 5.10: Effect of spermine (100μg icv) on polyamine levels in mouse brain in samples taken following a tonic convulsion which was survived (stage 4). Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.2.7 Effect of administration of 100μg spermine on individual polyamine concentrations at median score 5.

From figure 5.11 (a), it may be seen that spermidine levels are slightly lower in the spermine treated group following a fatal tonic convulsion, although not statistically significant. However the level of spermine is significantly higher in the treated group in comparison to the non-treated group. From figure 5.11 (b), N^1-acetylspermidine is also considerable higher in comparison to the saline treated levels. Post-hoc analysis confirmed the difference for spermine and N^1-acetylspermidine ($p<0.01$ and $p<0.0001$).

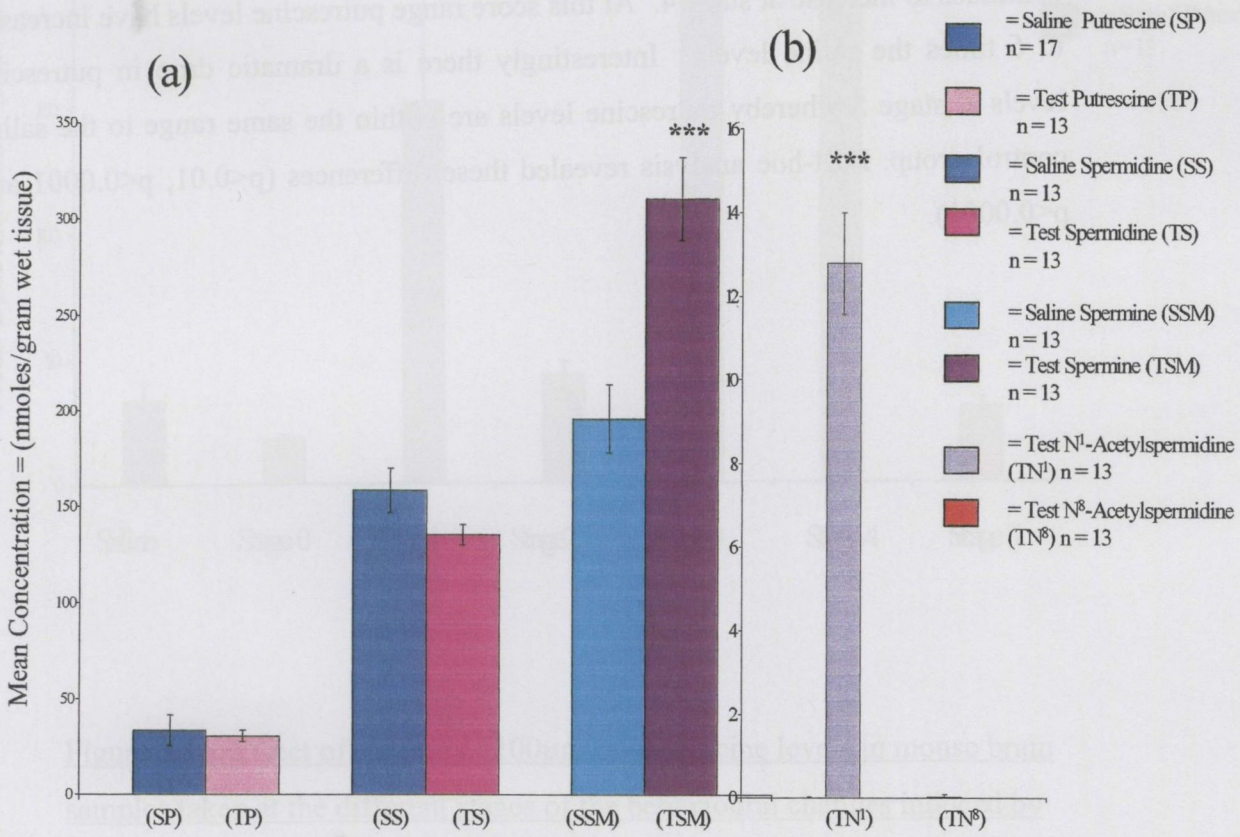


Figure 5.11: Effect of spermine (100μg icv) on polyamine levels in mouse brain in samples taken following a fatal tonic convulsion (stage 5). Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.2.8 Effect of spermine (100μg icv) on putrescine concentrations at each median score.

The following graphs show the same data presented in Figures 5.5 to 5.11. However in these figures, the change in the individual polyamines through the 5 stages of behavioural changes induced by spermine is demonstrated. Figure 5.12 illustrates the level of putrescine at each behavioural stage, and is compared to the level of putrescine in the saline control. From the graph it may be seen there is an increase in putrescine levels at stage 1 in comparison to the control value. This then drops at stage 2, but at stage three there is a bigger increase in the level of putrescine which continues to increase at stage 4. At this score range putrescine levels have increased to 6 times the saline levels. Interestingly there is a dramatic drop in putrescine levels at stage 5 whereby putrescine levels are within the same range to the saline control group. Post-hoc analysis revealed these differences ($p<0.01$, $p<0.0001$ and $p<0.0001$).

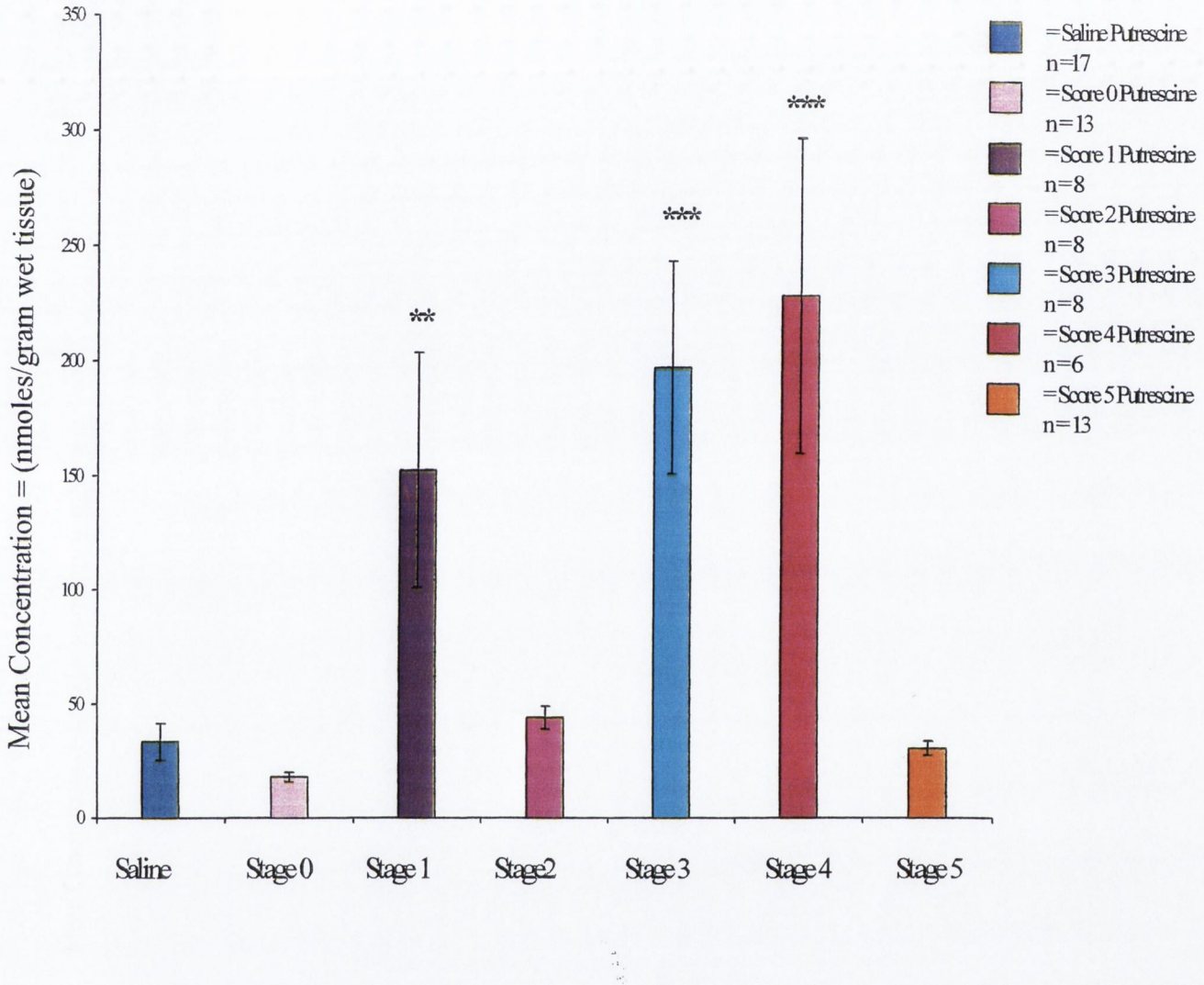


Figure 5.12: Effect of spermine (100μg) on putrescine levels in mouse brain samples taken at the different stages of the behavioural changes induced by spermine. Stage 0: no CNS excitation, stage 1: slight tremor, stage 2 moderate tremor, stage 3: severe tremor, stage 4: tonic convulsion which survived and stage 5: a fatal tonic convulsion. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.2.9 Effect of spermine (100μg icv) on spermidine concentrations at each median score.

The following graph displays the results obtained for the levels of spermidine within each median score range. From figure 5.13, there is a little increase in spermidine at stage 2 and 3 but no statistical difference was observed. However, at stage 4 spermidine levels were significantly raised in comparison to the control saline group. Similarly to putrescine, spermidine levels decreased dramatically at score 5 to within the saline level range. Post-hoc analysis revealed an increase in spermidine levels at stage 4 ($p<0.01$).

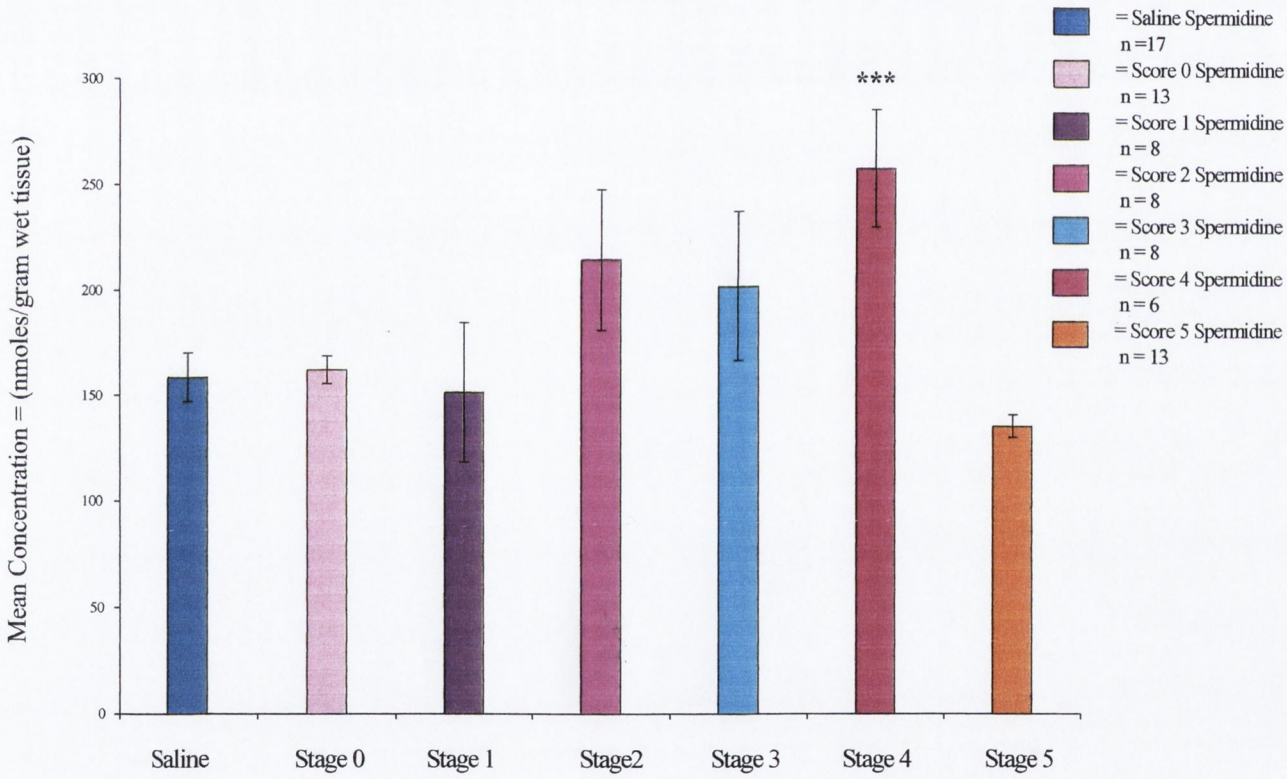


Figure 5.13: Effect of spermine (100μg) on spermidine levels in mouse brain samples taken at the different stages of the behavioural changes induced by spermine. Stage 0: no CNS excitation, stage 1: slight tremor, stage 2 moderate tremor, stage 3: severe tremor, stage 4: tonic convulsion which survived and stage 5: a fatal tonic convulsion. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.2.10 Effect of spermine (100μg icv) on spermine concentrations at each median score.

From the graph it may be seen there are significant differences in spermine levels at each stage of the spermine induced effects. This is not surprising as 100μg of spermine was administered, hence the high spermine levels obtained. However what is interesting is the increase in spermine concentrations as the behavioural development occurs, from score 0 to score 5. From the graph what is very apparent is, as the behaviour develops there is an increase in spermine levels, particularly at stage 3 and 4. Interestingly here once again there is a dramatic decrease in the level of spermine at stage 5. Post-hoc analysis revealed this difference for spermine at each stage ($p<0.05$, $p<0.03$, $p<0.0001$, $p<0.0001$, $p<0.0001$ and $p<0.01$).

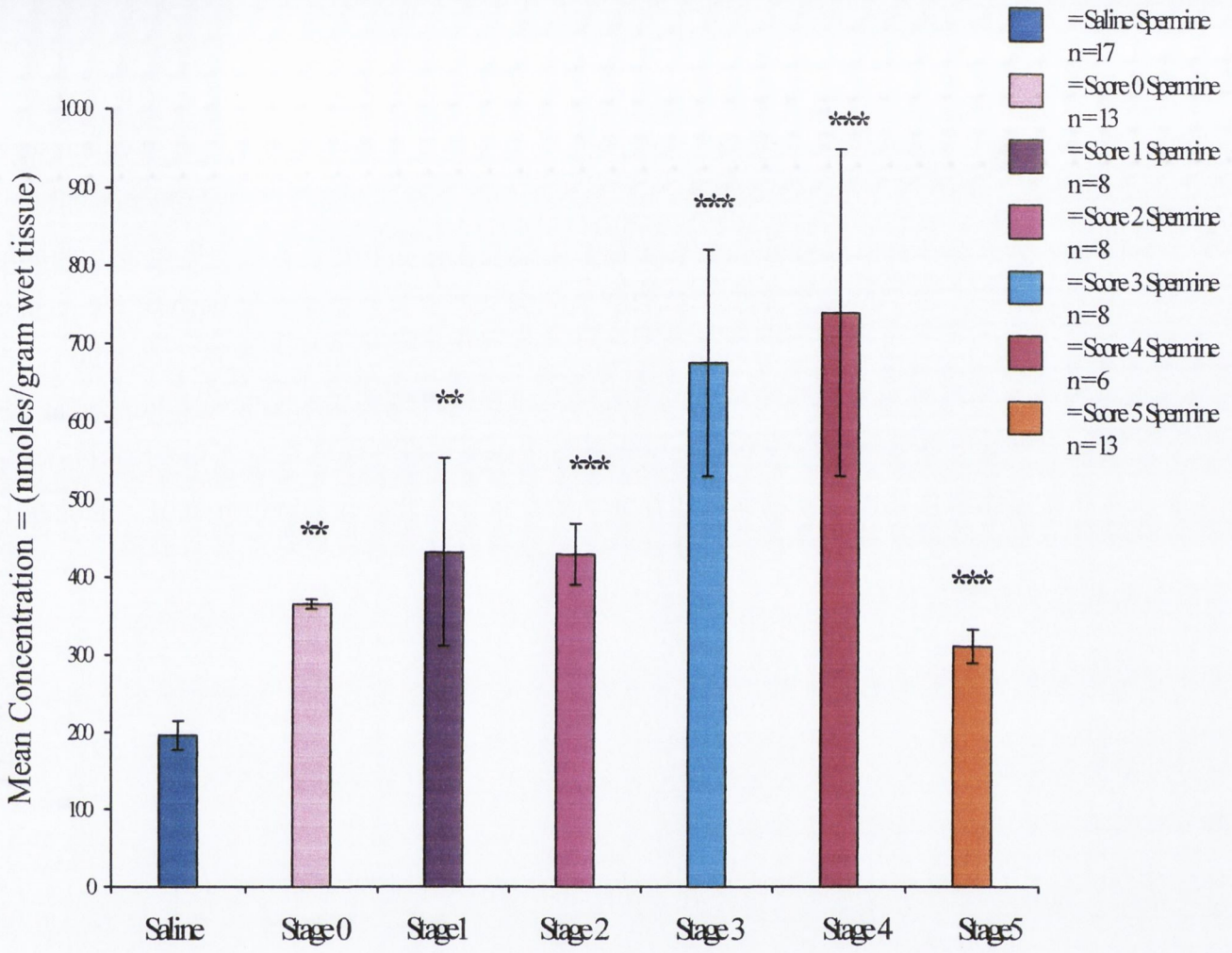


Figure 5.14: Effect of spermine (100μg) on spermine levels in mouse brain samples taken at the different stages of the behavioural changes induced by spermine. Stage 0: no CNS excitation, stage 1: slight tremor, stage 2 moderate tremor, stage 3: severe tremor, stage 4: tonic convulsion which survived and stage 5: a fatal tonic convulsion. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.2.11 Effect of spermine (100μg icv) on N^1-acetylspermidine polyamine concentrations at each median score.

Figure 5.15 displays the level of N^1-acetylspermidine across all median score values. From the graph it may be seen that N^1-acetylspermidine, was present only in tiny amounts (0.01nmoles/gram) in the control animals. After 100μg of spermine administration, there was an increase in N^1-acetylspermidine at each of the median score values. The largest increase in N^1-acetylspermidine concentration was observed at stage3. The high level at stage 5 is interesting, as levels dropped at this stage for each of the other polyamines examined (Figures 5.12, 13 & 14). Post-hoc analysis revealed this difference for N^1-acetylspermidine ($p<0.01$, $p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.01$ and $p<0.0001$).

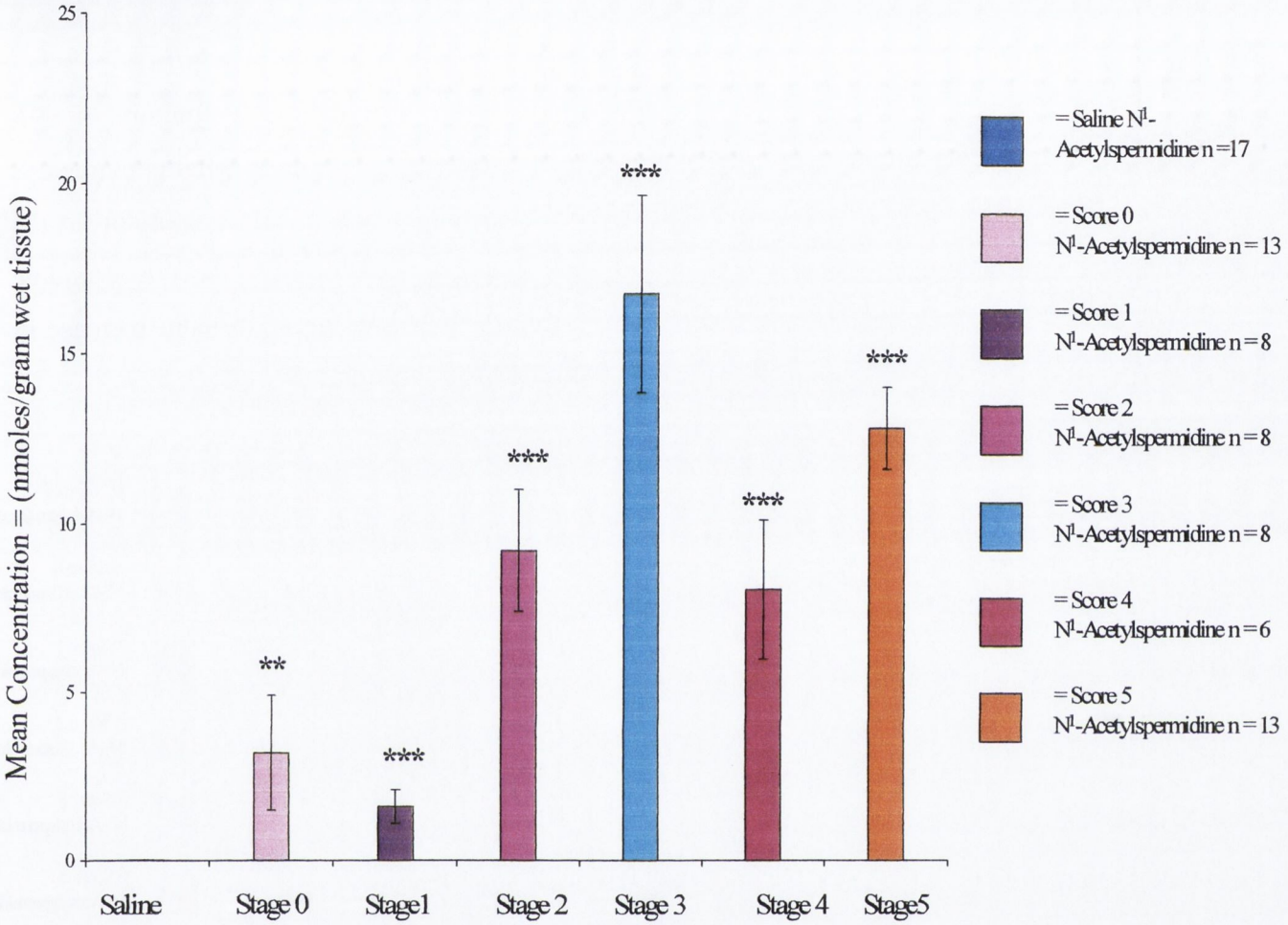


Figure 5.15: Effect of spermine (100μg) on N^1-acetylspermidine levels in mouse brain samples taken at the different stages of the behavioural changes induced by spermine. Stage 0: no CNS excitation, stage 1: slight tremor, stage 2 moderate tremor, stage 3: severe tremor, stage 4: tonic convulsion which survived and stage 5: a fatal tonic convulsion. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.2.12 Effect of spermine (100μg icv) on N^8-acetylspermidine polyamine concentrations at each median score.

Figure 5.16 illustrates the findings obtained for the investigation of N^8-acetylspermidine over each of the median score values. Initially what is evident, is the very low concentrations of N^8-acetylspermidine present in comparison to the levels obtained for N^1-acetylspermidine. The concentration of N^8-acetylspermidine values here did not rise above 1nmole/gram. There was no significant increase in N^8-acetylspermidine following administration of spermine.

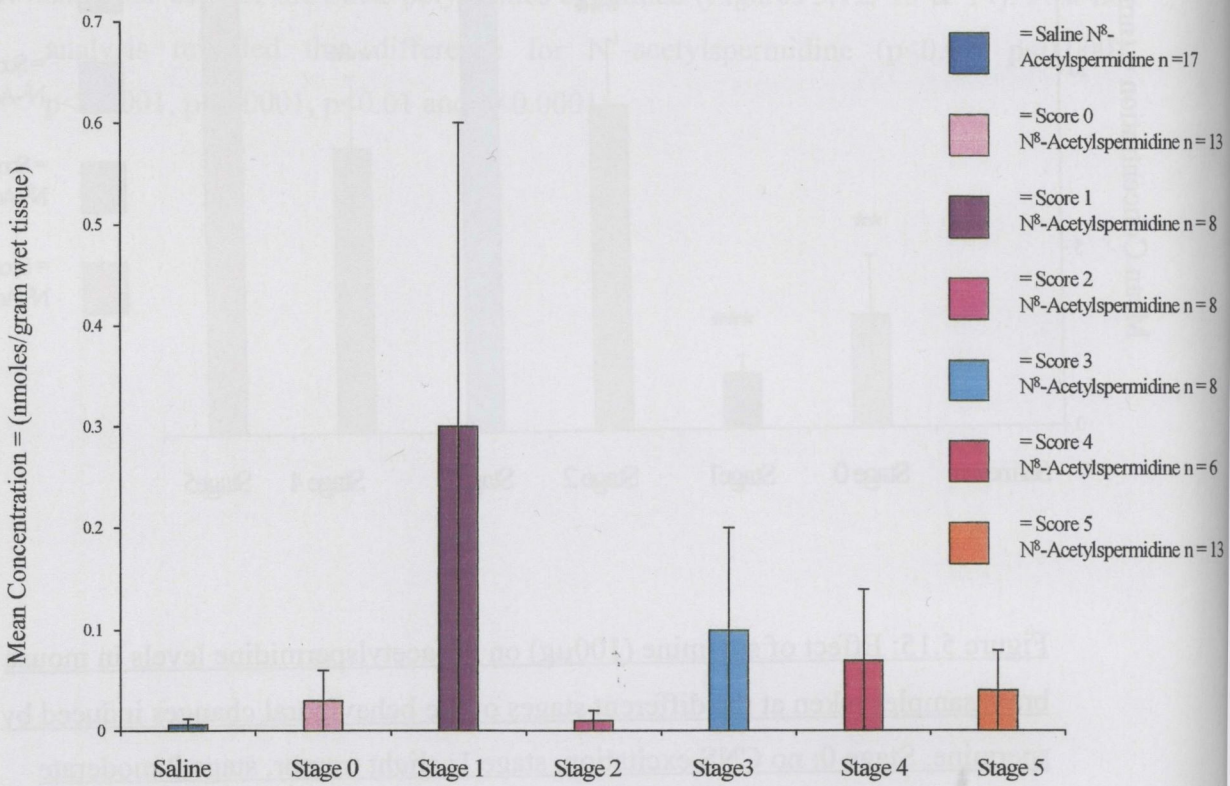


Figure 5.16: Effect of spermine (100μg) on N^8-acetylspermidine levels in mouse brain samples taken at the different stages of the behavioural changes induced by spermine. Stage 0: no CNS excitation, stage 1: slight tremor, stage 2 moderate tremor, stage 3: severe tremor, stage 4: tonic convulsion which survived and stage 5: a fatal tonic convulsion. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.3 Effect of 3μg/20μl Adrenocorticotrophic hormone ($ACTH_{1-24}$) on polyamine concentration levels.

As with the previous study, the purpose of this investigation was to examine the effects of administration of 3μg icv of $ACTH_{1-24}$ (Adrenocorticotrophic hormone) on polyamine levels and to correlate any changes with the behavioural effects (Chapter 4). The behavioural effects consist of the development of a syndrome inducing yawning, stretching, tail grooming, penile erections and increased level of grooming (Ferrari et al, 1963). The results presented here, show the brain polyamine concentrations at 15 minute time intervals over a 1 hour duration, following administration of 3μg $ACTH_{1-24}$. The effects of DFMO or putrescine on the level of polyamines after 1 hour in animals treated with 3μg $ACTH_{1-24}$was also assessed. The mean concentration is expressed in nmoles/gram wet tissue.

5.3.1 Effect of 3μg $ACTH_{1-24}$ on total polyamine concentrations at 15 minute time intervals over 1 hour.

Figure 5.17 illustrates the total level of all polyamines measured at 15 minute time intervals over a 1 hour duration following $ACTH_{1-24}$ administration. It may be seen from the graph that there is no significant difference between the saline treated group and the 3μg $ACTH_{1-24}$ treated group at any time point over the 1 hour duration. The level of polyamines in the saline group was slightly higher than that of the saline group used for the investigation of spermine induced polyamine levels (Figure 5.17).

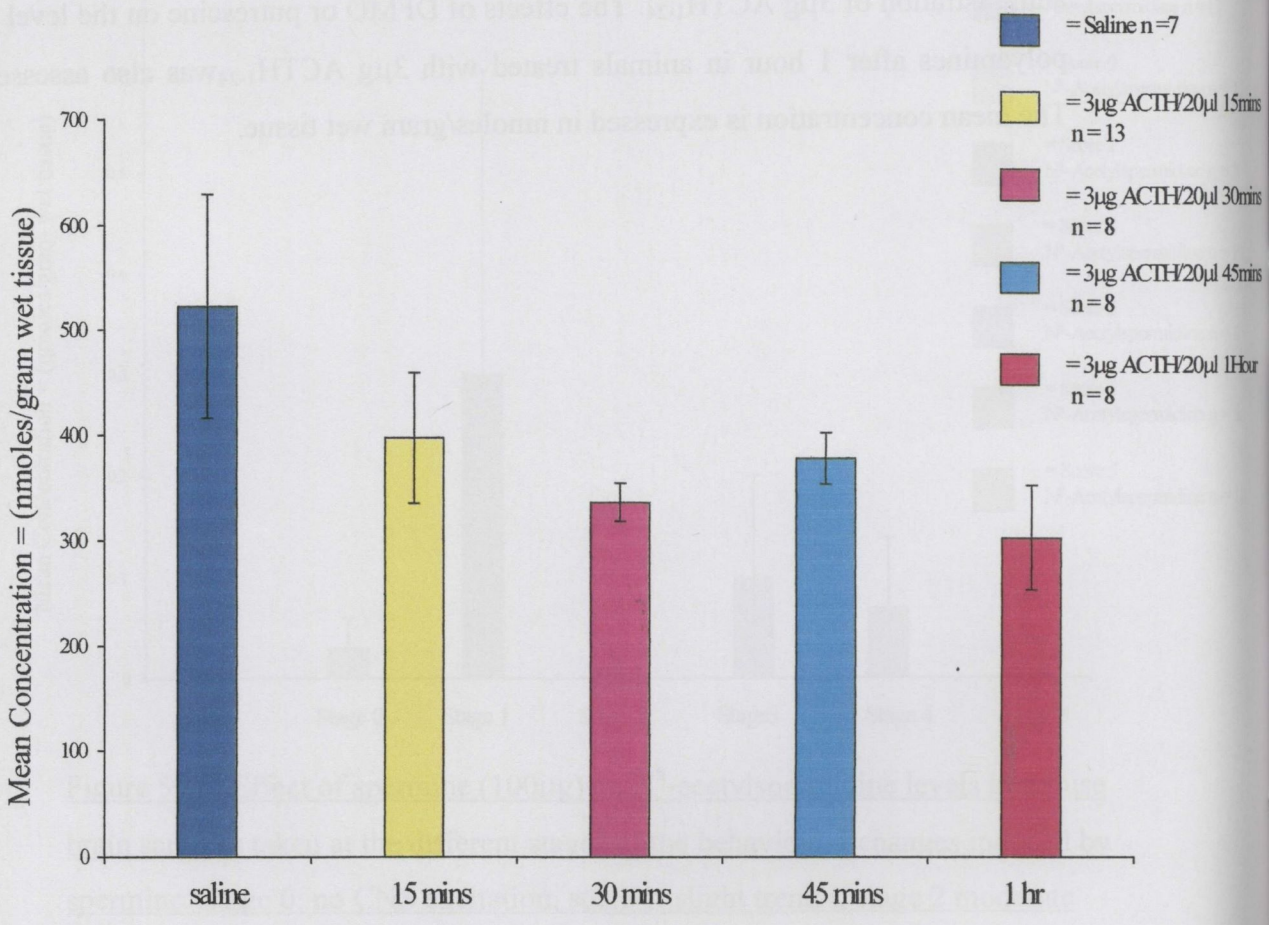


Figure 5.17: Effect of 3μg $ACTH_{1-24}$ on total polyamine concentrations over the first hour following administration. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.3.2 Effect of 3μg $ACTH_{1-24}$ on putrescine concentrations at 15 minute time intervals over 1 hour.

To understand the involvement of polyamines in the $ACTH_{1-24}$ induced behavioural syndrome, it was important to examine the effects of $ACTH_{1-24}$ on levels of individual polyamines. Figure 5.18 displays the results obtained for putrescine levels at the 15 minute intervals. It may be seen that at 15 and 30 minutes following $ACTH_{1-24}$ administration there was no trace of putrescine, while at 45 minutes there was a large increase in putrescine concentrations. At 1 hour there is still an increased level of putrescine present in comparison to the saline putrescine level, however this is not statistically significant. Post-hoc analysis confirmed this difference for at 45 minutes ($p<0.0001$).

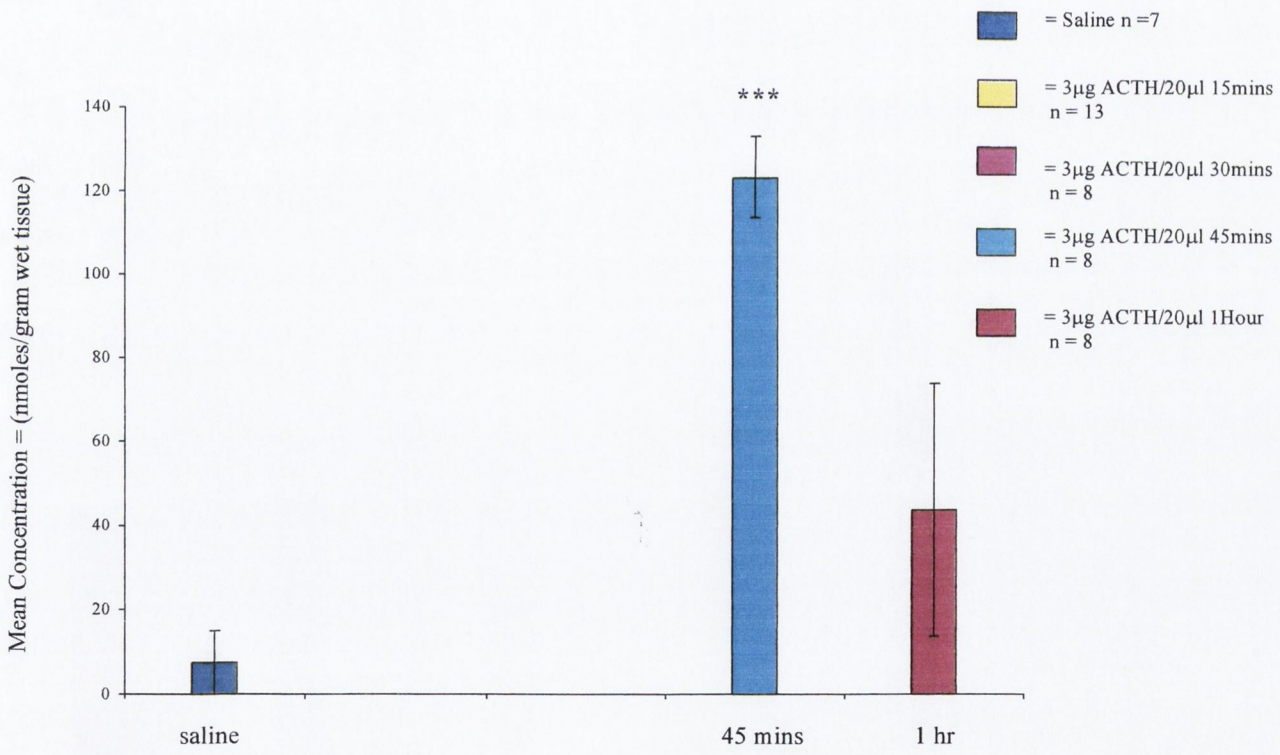


Figure 5.18: Effect of 3μg $ACTH_{1-24}$ on Putrescine concentrations at 15 minute time points over 1 hour. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.3.3 Effect of 3μg $ACTH_{1-24}$ on spermidine concentrations at 15 minute time intervals over 1 hour.

Figure 5.19 illustrates the findings for spermidine after 3μg $ACTH_{1-24}$ treatment. From the graph it may be seen that spermidine levels are high in the saline group, (300nmol/g) in comparison to control spermidine levels in the CNS excitation model (Figure 5.9). There are two statistically significant differences, one at 45 minutes and the second after 1 hour. There was a decrease in spermidine at these two time points in comparison to the control saline treated group. There was also a non-significant decrease at 15 and 30 minutes. Post-hoc analysis confirmed the difference between control and test at 45 and 60 minutes ($p<0.04$ and $p<0.02$).

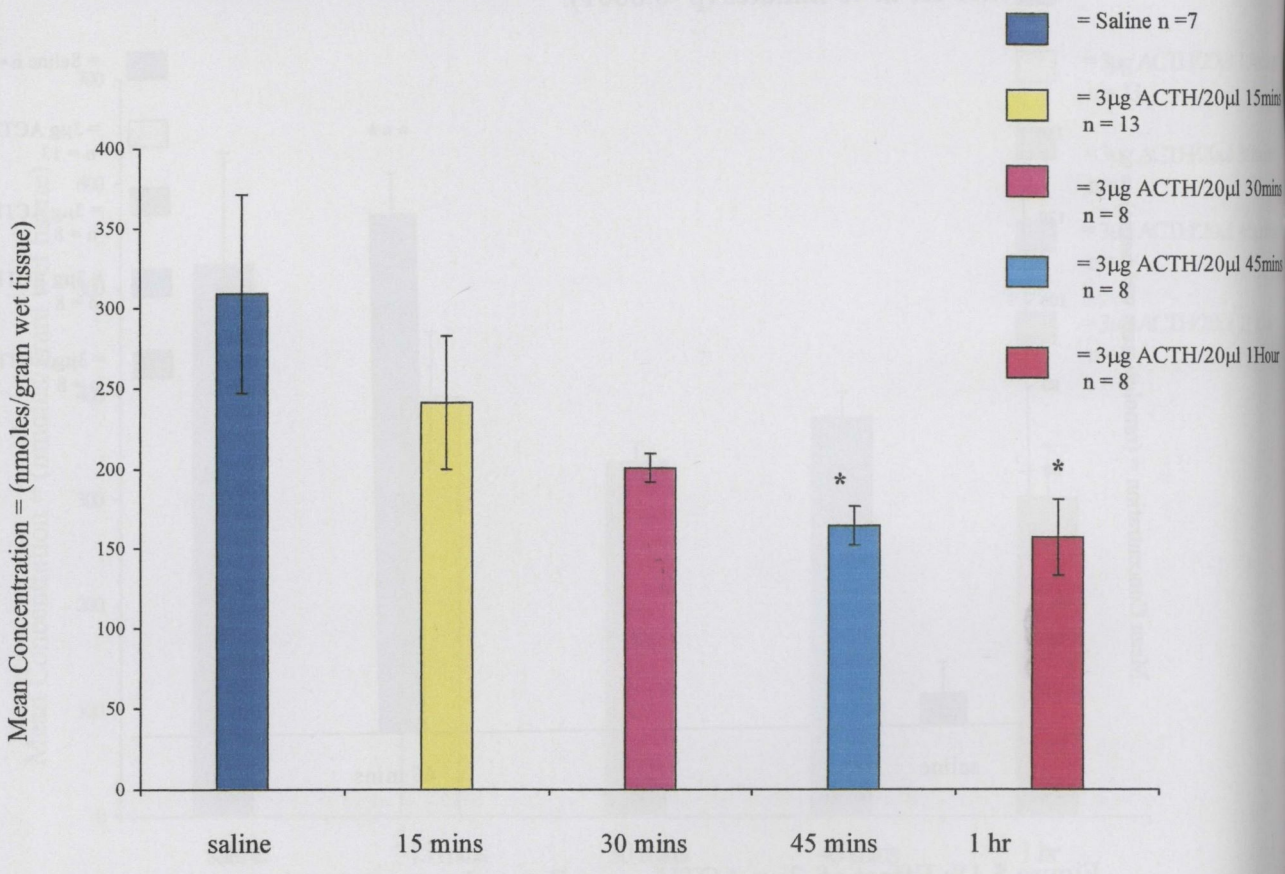


Figure 5.19: Effect of 3μg $ACTH_{1-24}$ on Spermidine concentrations at 15 minute time points over 1 hour. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.3.4 Effect of 3μg $ACTH_{1-24}$ on spermine concentrations at 15 minute time intervals over 1 hour.

Figure 5.20 shows spermine levels after the administration of 3μg $ACTH_{1-24}$. From this figure it may be seen there is a significant decrease in spermine levels at 45 minutes and 1 hour in comparison to the saline control levels. Post-hoc analysis revealed the decrease at 45 and 60 minutes in comparison to control ($p<0.01$ and $p<0.03$).There is a decreasing trend seen from 15 minutes to 1 hour. However there are no statistically significant difference at 15 or 30 minutes.

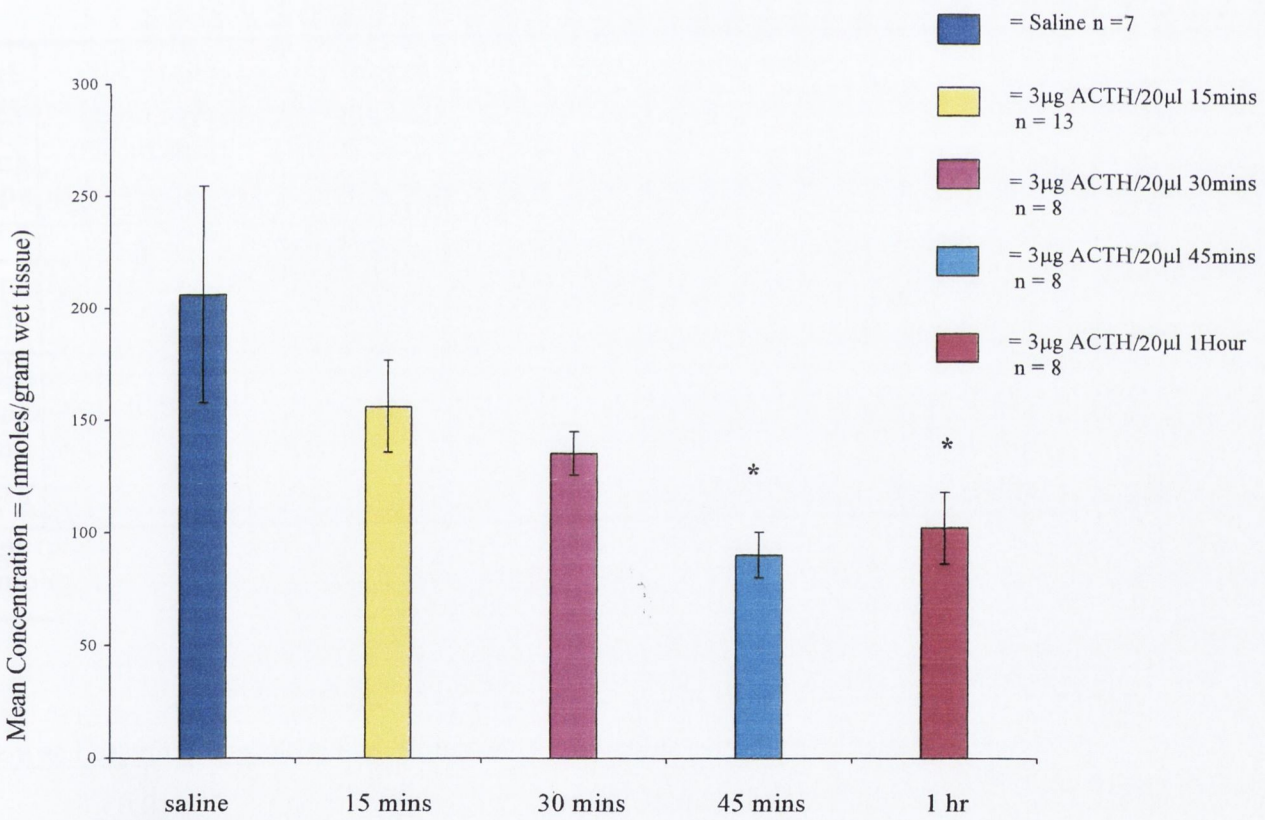


Figure 5.20: Effect of 3μg $ACTH_{1-24}$ on Spermine concentrations at 15 minute time points over 1 hour. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *p<0.05, **p<0.01, ***p<0.001.

5.3.5 Effect of 3μg $ACTH_{1-24}$ on N^1-acetylspermidine and N^8-acetylspermidine concentrations at 15 minute time intervals over 1 hour.

Table 5.1 illustrates the results obtained for N^1-acetylspermidine and N^8-acetylspermidine. From the table it may be seen that there was very little of both N^1-acetylspermidine and N^8-acetylspermidine present in the saline treated group and also in the $ACTH_{1-24}$ treated group. No significant changes were observed following treatment.

	Saline 20μl icv (n = 7)	**3μgACTH /20μl icv 15 mins (n = 13)**	**3μgACTH /20μl icv 30 mins (n = 8)**	**3μgACTH /20μl icv 45 mins (n = 8)**	**3μgACTH /20μl icv 1 hr (n = 8)**
N^1Acetyl Spermidine nmoles/gram	**0**	0	0.35 ± 0.25	0	0
N^8Acetyl Spermidine nmoles/gram	0.08 ± 0.08	0	0	0	0

Table 5.1: Effect of 3μg $ACTH_{1-24}$ on N^1- acetylspermidine and N^8-acetylspermidine concentrations over a 1 hour duration. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.3.6 Effect of 3μg $ACTH_{1-24}$ and 3μg $ACTH_{1-24}$+ 20, 50 and 100μg α-difluoromethylornithine (DFMO) on all polyamine concentrations over 1 hour.

Table 5.2 illustrates the results obtained for administration of various concentrations of α-difluoromethylornithine (DFMO). From the table it may be seen that DFMO had no significant effect on any of the polyamine level following treatment.

	3μg ACTH /20μl icv (n = 8)	**3μg ACTH + 20μg DFMO /20μl icv (n = 12)**	**3μg ACTH + 50μg DFMO /20μl icv (n = 15)**	**3μg ACTH + 100μg DFMO /20μl icv (n = 4)**
Putrescine nmoles/gram	**43.71 ± 30.02**	0	16.48 ± 9.13	0
Spermidine nmoles/gram	**157.12 ± 23.8**	131.92 ± 15.51	232.37 ± 115.96	126.2 ± 44.67
Spermine nmoles/gram	**102.15 ± 16.03**	77.58 ± 10.22	125.19 ± 59.18	73.9 ± 26.07
N^1Acetyl Spermidine nmoles/gram	**0**	0.36 ± 0.2	0.06 ± 0.04	0
N^8Acetyl Spermidine nmoles/gram	**0**	0	0.03 ± 0.03	0

Table 5.2: Effect of 3μg $ACTH_{1-24}$ versus 3μg $ACTH_{1-24}$ + 20, 50 and 100μg/20μl of DFMO on all polyamine concentrations over a 1 hour duration. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.3.7 Effect of 3μg $ACTH_{1-24}$ and 3μg $ACTH_{1-24}$+ 120, 240 and 480μg putrescine on all polyamine concentrations over 1 hour.

Table 5.3 illustrates the results obtained for putrescine over 1 hour. From the table it may be seen that there was significant changes in the level of putrescine at the higher concentrations administered of 240 and 480μg/20μl. While none of the other polyamines were affected, no significant changes were observed following treatment. Post-hoc analysis revealed this difference for putrescine at the higher doses of 240 and 480μg ($p<0.05$ and $p<0.001$ respectively).

	3μg ACTH/20μl icv (n = 8)	**3μg ACTH + 120μg Putrescine /20μl icv (n = 8)**	**3μg ACTH + 240μg Putrescine /20μl icv (n = 4)**	**3μg ACTH + 480μg Putrescine /20μl icv (n = 4)**
Putrescine nmoles/gram	**43.71 ± 30.02**	1027± 656.83	753.4 ± 414.69 *	3265.6± 30.02 ***
Spermidine nmoles/gram	**157.12 ± 23.8**	139.78 ± 13.6	158.02 ± 3.21	147.2 ± 12.19
Spermine nmoles/gram	**102.15 ± 16.03**	84.36 ± 8.41	93.97 ± 3.05	90.26 ± 9.89
N^1Acetyl Spermidine nmoles/gram	**0**	0	0	0
N^8Acetyl Spermidine nmoles/gram	**0**	0	0.09 ± 0.09	0

Table 5.3: Effect of 3μg $ACTH_{1-24}$ versus 3μg $ACTH_{1-24}$ +120, 240 and 480μg/20μl of Putrescine on all polyamine concentrations over a 1 hour duration. Results are expressed as x ± s.e.m. Statistical significance was assessed by One -Way ANOVA, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

5.4 Discussion:

Polyamines are widely, but heterogeneously distributed in the brain (Shaw and Pateman, 1973). The levels of polyamines vary in concentration from location to location. In general the overall concentrations for each of the polyamines are as follows; Putrescine levels range from 3 to 23nmol/g in the brain. Spermidine levels range from 235nmol/g to 1016nmol/g. Spermine levels range from 157nmol/g to 506nmol/g (Seiler & Schmidt-Glenewinkel, 1975; Al-Deen, 1982).

Spermine induced behaviours:

In the spermine CNS excitation model, there is a progressive increase in the levels of polyamines from score 0 to score 4, with a rapid decline at score 5. A possible hypothesis, could be from the synthesis and metabolism of polyamines, previously described.

At score 0 (Figure 5.2), putrescine and spermidine levels are comparative to the control value, while spermine and N^1-acetylspermidine are elevated. This is not surprising as spermine was administered. In relation to the metabolism of polyamines the increased presence of N^1-acetylspermidine indicates the retro-conversion pathway is stimulated. As there is an increase in N^1-acetylspermidine it may be hypothesised that spermidine is being converted to putrescine. In conjunction with this, spermine levels are slightly raised, which could indicate that the conversion of spermidine to spermine is also occuring simultaneously (Shaskan & Snyder, 1973).

At stage 2 spermine levels stay high, which is not surprising as spermine was administered. There is another increase in N^1-acetylpermidine at this stage, which would indicate a stimulation of the retro-conversion pathway. In the next stage of progression, score 3 (Figure 5.5), there is a significant increase in putrescine levels, perhaps reflecting the previous stimulation of the retro-conversion pathway. There

is also a surge in N^1-acetylspermidine, larger than before, signifying the increased stimulation of the retro-conversion pathway. Spermine levels are also high at this stage, perhaps reflecting some synthesis of spermine. Most interesting is the results obtained for score 4 (Figure 5.6). Each of the polyamines, putrescine, spermidine and spermine are at high concentrations. Similarly N^1-acetylspermidine levels are high. At this stage behaviourally the animals have a tonic convulsion but survive. The high levels of spermine are particularly interesting here as spermine has a high affinity for the positive modulatory site on the NMDA receptor, which could result in the behavioural response. This does not preclude that putrescine and spermidine could be producing an effect, but the likelihood is the effects are mediated by the very high levels of spermine. In general at this stage each of the polyamines are higher than previous stages, this could indicate that there is an increase in synthesis of polyamines, but it also indicates the retro-conversion pathway is active, due to the high levels of N^1-acetylspermidine present here also.

The polyamine concentrations for the final behavioural stage score 5 (Figure 5.7) are interesting. Levels of putrescine, spermidine and spermine have dramatically decreased in comparison to the previous levels. Spermine continues to remain high which would indicate spermine is causing the final behavioural effect, a fatal tonic convulsion. It could be suggested there is severe neuronal damage at this stage from the previous levels at score 4. It is known that extracellular spermine is cytotoxic (Sharmin et al, 2001). N^1-acetylspermidine levels remain elevated indicating that the retro-conversion pathway is activated and spermidine is being broken into its derivative.

The above findings may be further dissected by examining the findings for each of the individual polyamines at the different score levels over 7.5 hours. Interestingly there is the increase in concentration of the main polyamines, putrescine, spermidine and spermine as progression of the CNS excitation behaviour develops, in particular at scores 3 and 4 (Figure 5.8, 5.9 & 5.10 respectively). N^1-acetylspermidine (Figure 5.11), was elevated at different score stages throughout the progression of the CNS

excitation, indicating that the retro-conversion pathway is active at the different stages. N^8-acetylspermidine showed no significant change. N^8-acetylspermidine, an acetylated polyamine, is synthesised from spermidine by the action of nuclear N^8-acetyltransferase (Moinard et al, 2005). The unchanged levels of N^8-acetylspermidine correlate with spermidine levels which were relatively unchanged throughout the 5 stages. This data suggests that polyamine metabolism through the action of SSAT and PAO is dominant over the action of N^8-acetyltransferase under these conditions. High polyamine levels may cause oxidative stress from the interconversion pathway, which results in the formation of hydrogen peroxide (Hoet et al, 2000). It is possible that the production of H_2O_2 could be contributing to spermine toxicity culminating in a fatal tonic convulsion.

The results presented here indicate there is strong evidence that a cascade of events occurs during the behavioural profile, in particular the metabolic transformation of spermine into its derivatives, via the retro-conversion pathway. Similarly there is strong evidence that there is increased synthesis of polyamines. Previous studies have examined the effects of MDL 72527, an inactivator of polyamine oxidase, and found that MDL 72527 significantly reduced the neurotoxicity of spermidine (Doyle & Shaw, 1994).

$ACTH_{1-24}$ induced behaviours:

The link between $ACTH_{1-24}$ and polyamines is uncertain, but previous studies have shown that there is some connection between the two. Many ideas have been postulated to the mechanisms involved but few have shown a clear insight. The purpose of this part of this study was to elucidate in some way the underlying mechanisms.

There was no change in the overall brain polyamine concentrations when measured every 15 minutes over a 1 hour duration. This finding conflicts with the suggestion

that $ACTH_{1-24}$ is stimulating ODC, hence having a knock-on effect on polyamine synthesis and increasing the overall levels of polyamines within the brain.

It was previously shown that putrescine levels are particularly elevated (Tinter et al, 1979). Figure 5.14 illustrates the individual findings for putrescine alone. The results presented here are consistent with Tinter's where it was reported that putrescine levels were elevated in all brain regions (Tinter et al, 1979). The present results indicate a time effect of this, which would be concurrent with previous findings. It is known that ODC has a short half-life of 10-20 minutes, hence it would be expected that, after ODC stimulation there would be an upsurge of polyamine synthesis, beginning with putrescine synthesis. This may account for the significant elevation of putrescine at 45 minutes which then declined at 1 hour. This evidence supports the hypothesis that ODC and polyamines may have a role in the onset of $ACTH_{1-24}$-induced behavioural syndrome (Bertolini et al, 1968; Poggioli et al, 1984). Levine et al (1975), investigated the action of $ACTH_{1-24}$ of adrenal ODC activity and discovered that $ACTH_{1-24}$ stimulates transcription of new mRNA, which is involved in regulation of adrenal ODC synthesis. They also examined the influence of cyclic AMP on ornithine decarboxylase and found that stimulation of ornithine decarboxylase was not dependant on the early peak of cAMP but may be influenced following sustained levels of $ACTH_{1-24}$.

Figure 5.15 illustrates the results found for Spermidine over the 1 hour duration. There was a decline in the concentration of spermidine. This could be attributed to the rate of polyamine turnover being high, but the cerebral concentration may not reflect the synthesis rate. Catabolism may also be increased so the several-fold increase in synthesis by ODC may result in an increase in turnover, with only a modest increase in content of individual polyamines (Tinter et al, 1979). However, no increase in N^1-acetylspermidine or N^8-acetylspermidine was observed. It may be hypothesised that spermidine is being converted to putrescine this process is the reverse process of the first step in the retro-conversion pathway, whereby the enzyme N^1-Acetylspermidine deacetylase, releases acetate to form putrescine from

spermidine. The polyamines produced by this process can be re-utilised for polyamine synthesis, indicating that the polyamine synthesis is a cyclic process (Seiler, 1991). At 45 minutes the rise in putrescine levels corresponded with the drop in spermidine levels, illustrating the cyclic process, whereby there was a reduction in spermidine at 45 minutes and at 1 hour, while putrescine concentration levels were elevated.

The results for spermine Figure 5.16, showed a similar profile to spermidine, whereby there was a significant decrease in spermine concentration found at 45 minutes and 1 hour. The depletion of spermine could also be attributed to the stimulation of the reverse process of the retro-conversion pathway.

Table 5.1 illustrates results for the level of polyamine derivatives N^1-Acetylspermidine and N^8-Acetylspermidine. There was no change observed for both N^1-Acetylspermidine and N^8-Acetylspermidine levels, these levels were very low throughout, perhaps indicating low retro-conversion activity.

Table 5.2 displays the results for DFMO. From the table it may be seen DFMO did not cause a statistical significant depletion in the level of all polyamines in the whole brain. However it is widely know that DFMO is an inhibitor of the rate-limiting enzyme ODC in polyamine synthesis (Genedani et al, 1984). It is possible that DFMO is inhibiting the effects of $ACTH_{1-24}$ by reducing the effect of $ACTH_{1-24}$ on ODC hence decreasing the level of polyamine synthesis. It is already known that DFMO ($\bar{\alpha}$ difluromethylornithine) an irreversible inhibitor of mammalian ODC antagonises the $ACTH_{1-24}$ behavioural effects (Genedani et al, 1984). It has also been shown that DFMO selectively inhibits both $ACTH_{1-24}$-induced polyamine increase and CKII nuclear accumulation suggesting a link between intracellular polyamines and nuclear CKII, which may be secondary messengers for regulation, distribution and activity (Filhol et al, 1991).

Table 5.3 provides the results for each of the polyamines after putrescine and $ACTH_{1-24}$ administration. From the table it may be seen that there was a significant statistical difference between control and test at 240μg and 480μg in the level of putrescine while each of the other polyamines were not affected. The high level of putrescine could be due to the high level of putrescine administered. It is not unreasonable to suggest that $ACTH_{1-24}$ is still having an effect on ODC, by the stimulation of ODC production. This in turn would stimulate the retro-conversion pathway leading to an over production of polyamines, resulting in an increase in putrescine levels, as putrescine is the first polyamine synthesised in the retro-conversion pathway (Seiler, 1991). This would be in keeping with previous findings (Tinter et al, 1979; Genedani et al, 1984).

From the results of this study, there is not strong evidence that raised levels of polyamines may contribute to the development of $ACTH_{1-24}$ induced behaviours. $ACTH_{1-24}$ significantly raised putrescine levels 45 minutes following administration, and reduced spermine and spermidine levels at 45 and 60 minutes following administration. Therefore there is not strong evidence of stimulation of polyamine synthesis, nor, due to a lack of significant change in N^1-acetylspermidine, is there strong evidence of substantial increase in the breakdown of polyamines through the retroconversion pathway. This data questions the role of the polyamines in the onset of $ACTH_{1-24}$ induced behaviours.

Chapter 6.

6.1 Introduction.

It is important for the purpose of future investigations to have an understanding of the biological effects of the novel compounds from Brock University Canada. There are substantial side effect limitations associated with NMDA receptor antagonists, and poor therapeutic ratios, hence this has led to an active search for NMDA receptor antagonists with a greater window of opportunity between therapeutic and adverse effects (Dawson et al, 2001).

Many studies have been performed to evaluate the behaviour of glutamate receptor antagonists, in particular extensive work has been carried out NMDA receptor antagonists. These agents have psychoactive properties in humans and morphologically damage neurons in cerebral cortex of rats. NMDA antagonists, (MK-801, tiletamine, ketamine and $_{D}$-2-amino-5-phosophonopentanoate (D-AP5) can cause neurotoxic side effects consisting of pathomorphological changes in neurons of the cingulated and retrosplenial cerebral cortices, which are reversible at low doses (Olney et al, 1991). Other studies have shown that the NMDA antagonists, MK-801, NPC 12626 [2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid], CPP [3-(2-carboxypiperizin-4-yl)propyl-1-phosphonic acid] and PCP, impair performance on sensorimotor, learning and memory intensive tasks (Genovese et al, 1991; Clissold et al, 1991). While there are similarities in some of the behavioural effects between competitive and PCP-like NMDA antagonists, there are some differences, suggesting it may be possible to develop NMDA-antagonists with less side-effects (Willetts et al, 1990).

The method of assessment used in the present study was a behavioural assessment profile based on locomotor activity (LMA) count, rotarod performance and an Irwin profile investigating the CNS and autonomic effects of these novel drugs. Assessment of locomotor dysfunction and motor coordination using a rotarod, can be used as an index of CNS dysfunction and can highlight adverse effects produced by CNS active drugs (Dawson et al, 2001). The SHIRPA or Irwin protocol is a primary screening

process developed by Irwin in 1968, which is widely used for drug screening. This screening method provides a behavioural observational profile and functional profile. All parameters are scored to provide a quantitative assessment which enables comparison of results over time and between research groups (www.mgu.har.mrc.ac.uk).

Male *Laca* mice were injected using either i.c.v or i.p. administration with one of the 6 novel compounds. The highest tolerated doses from the spermine induced CNS excitation study were used (spermine behavioural model) (Section 2.1). For a full description see Section 2.4.

Data from the LMA, Rotarod, weight and temperature assessments was expressed as mean and $\pm$ S.E.M (Standard Error of Mean) and statistical significance of results was assessed using a One-Way ANOVA. Post hoc test was done using Tukey ($p<0.05$).

Data analysis of the Irwin profile data, which used a scoring system, produced median score values $\pm$ interquartile range. For statistical purposes the Mann-Whitney U-test was used to assess the difference between test group and control.

6.2 Behavioural effects of novel compounds

6.2.1 Behavioural effects of Bu 31b administered alone.

Bu 31b was administered either through the i.c.v route or i.p route at the highest concentration used for the spermine model (see Chapter 3). The effect of Bu 31b on locomotor and rotarod activity, weight and temperature of mice over 5 days can be seen on Table 6.1. From Table 6.1 it may be seen that Bu 31b caused a significant reduction in LMA when administered through the icv route, on day 1. No significant difference was observed on day 3 or 5, although there was a trend towards increased LMA activity. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$). Bu 31b given through the ip route had no effect on locomotor activity.

Bu 31b had no significant effect on rotarod performance when administered ether by the icv route or the ip route. Bu 31b administered i.c.v caused a significant reduction in weight on days 2, 3, 4 and 5 (Table 6.1). Post-hoc analysis confirmed a significant drop in weight this on these days ($p<0.01$ and 0.05, 0.05). Temperature was not significantly affected by Bu 31b administered i.c.v or i.p.

Bu 31b had no effect on locomotor activity, rotarod, weight or temperature on any of the days when administered i.p, (results not displayed).

Given	LMA	RR	Weight	Temp
Saline 20µl icv n=6 Day 1	1234.33 ± 284.4	92.17 ± 17.71	22.87 ± 0.31	36.52 ± 0.35
Bu 31b 30µg icv n=6 Day 1	**144.17** ** ± 135.05	39.17 ± 17.99	24.18 ± 0.92	37.85 ± 0.13
Saline 20µl icv n=6 Day 2	-	-	23.73 ± 0.3	36.27 ± 0.22
Bu 31b 30µg icv n=6 Day 2	-	-	**20.58** ** ± 0.98	37.12 ± 0.26
Saline 20µl icv n=6 Day 3	1204.5 ± 232.53	108.17 ± 8.88	22.93 ± 0.41	36.87 ± 0.25
Bu 31b 30µg icv n=6 Day 3	1814 ± 328.07	111.5 ± 8.5	**19.10** ** ± 0.98	36.65 ± 0.35
Saline 20µl icv n=6 Day 4	-	-	24.93 ± 0.35	35.92 ± 0.29
Bu 31b 30µg icv n=6 Day 4	-	-	**20.72**** ± 0.9	37.17 ± 0.46
Saline 20µl icv n=6 Day 5	949.33 ± 203.22	113 ± 7	24.07 ± 0.64	36.98 ± 0.24
Bu 31b 30µg icv n=6 Day 5	1680.17 ± 281.35	108.67 ± 11.53	**19.97** ** ± 0.94	37.0 ± 0.55

Table 6.1: The effect of Bu 31b i.c.v on Locomotor activity (LMA), weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *p<0.05, **p<0.01, ***p<0.001.

6.2.2 Behavioural effects of Bu 37b administered alone.

Table 6.2 displays the results obtained for the effect of Bu 37b icv on locomotor activity. It may be seen there was a decrease on day 1, while on each of the other two days there was no significant effect. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.02$).

Bu 37b had no significant effect on rotarod performance, weight and temperature when administered ether by the icv route (Table 6.2). Bu 37b had no effect on locomotor activity, rotarod, weight or temperature on any of the days when administered i.p, (results not displayed).

Given	LMA	RR	Weight	Temp
Saline 20µl icv n=6 Day 1	1234.33 ± 284.4	92.17 ± 17.71	22.87 ± 0.31	36.52 ± 0.35
Bu 37b 30µg icv n=6 Day 1	**228.17**** ± 106.43	87.17 ± 16.66	24.13 ± 0.53	38.07 ± 0.37
Saline 20µl icv n=6 Day 2	-	-	23.73 ± 0.3	36.27 ± 0.22
Bu 37b 30µg icv n=6 Day 2	-	-	24.83 ± 0.51	38.07 ± 0.33
Saline 20µl icv n=6 Day 3	1204.5 ± 232.53	108.17 ± 8.88	22.93 ± 0.41	36.87 ± 0.25
Bu 37b 30µg icv n=6 Day 3	1392.33 ± 260.95	120	25.5 ± 0.45	38.08 ± 0.36
Saline 20µl icv n=6 Day 4	-	-	24.93 ± 0.35	35.92 ± 0.29
Bu 37b 30µg icv n=6 Day 4	-	-	26.13 ± 0.39	37.48 ± 0.65
Saline 20µl icv n=6 Day 5	949.33 ± 203.22	113 ± 7	24.07 ± 0.64	36.98 ± 0.24
Bu 37b 30µg icv n=6 Day 5	1157.17 ± 158.93	120	27.15 ± 0.39	37.35 ± 0.29

Table 6.2: The effect of Bu 37b icv on Locomotor activity (LMA), rotoarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *p<0.05, **p<0.01, ***p<0.001.

6.2.3 Effect of Bu 33b administered alone.

Table 6.3 illustrates the results describing the effect of icv administration of Bu 33b on locomotor activity. There was a significant decrease in LMA obtained compared to control on day 1 while on day 3 and day 5 there was no significant effect (Table 6.3). Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.04$). Bu 33b had no significant effect on rotarod performance, temperature or weight on any day tested. There was no significant effect found for Bu 33b when administered through the ip route on locomotor activity, rotarod, weight or temperature, (results not displayed).

Given	LMA	RR	Weight	Temp
Saline 20µl icv n=6 Day 1	1234.33 ± 284.4	92.17 ± 17.71	22.87 ± 0.31	36.52 ± 0.35
Bu 33b 30µg icv n=6 Day 1	**291.17*** ± 82.98	81.67 ± 18.72	24.01 ± 0.44	37.78 ± 0.34
Saline 20µl icv n=6 Day 2	-	-	23.73 ± 0.3	36.27 ± 0.22
Bu 33b 30µg icv n=6 Day 2	-	-	24.42 ± 0.41	37.67 ± 0.2
Saline 20µl icv n=6 Day 3	1204.5 ± 232.53	108.17 ± 8.88	22.93 ± 0.41	36.87 ± 0.25
Bu 33b 30µg icv n=6 Day 3	1690.67 ± 161.1	112.83 ± 7.17	24.5 ± 0.47	37.67 ± 0.41
Saline 20µl icv n=6 Day 4	-	-	24.93 ± 0.35	35.92 ± 0.29
Bu 33b 30µg icv n=6 Day 4	-	-	25.11 ± 0.55	36.87 ± 0.44
Saline 20µl icv n=6 Day 5	949.33 ± 203.22	113 ± 7	24.07 ± 0.64	36.98 ± 0.24
Bu 33b 30µg icv n=6 Day 5	1265.67 ± 263.1	120	25.36 ± 0.54	38.02 ± 0.25

Table 6.3: The effect of Bu 33b icv on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *p<0.05, **p<0.01, ***p<0.001.

6.2.4 Behavioural effects of Bu 40b icv administered alone.

Table 6.4 (a) displays the results found for the effect of Bu 40b on locomotor activity. From the table it may be seen there was a considerable reduction in the amount of locomotor activity on day 1 when Bu 40b was administered through the icv route. Post-hoc analysis confirmed the reduction in LMA on day 1 icv ($p<0.01$).

Bu 40b when administered through the icv route had no effect on rotarod performance, weight or temperature.

Given	LMA	RR	Weight	Temp
Saline 20µl icv n=6 Day 1	1234.33 ± 284.4	92.17 ± 17.71	22.87 ± 0.31	36.52 ± 0.35
Bu 40b 30µg icv n=6 Day 1	**165.67**** ± 79.82	120	21.9 ± 0.73	37.28 ± 0.45
Saline 20µl icv n=6 Day 2	-	-	23.73 ± 0.3	36.27 ± 0.22
Bu 40b 30µg icv n=6 Day 2	-	-	22.91 ± 0.78	36.87 ± 0.36
Saline 20µl icv n=6 Day 3	1204.5 ± 232.53	108.17 ± 8.88	22.93 ± 0.41	36.87 ± 0.25
Bu 40b 30µg icv n=6 Day 3	921.67 ± 244.73	120	22.88 ± 0.76	37.4 ± 0.34
Saline 20µl icv n=6 Day 4	-	-	24.93 ± 0.35	35.92 ± 0.29
Bu 40b 30µg icv n=6 Day 4	-	-	23.17 ± 0.66	36.47 ± 0.43
Saline 20µl icv n=6 Day 5	949.33 ± 203.22	113 ± 7	24.07 ± 0.64	36.98 ± 0.24
Bu 40b 30µg icv n=6 Day 5	903.33 ± 202.74	119.83 ± 0.17	23.05 ± 0.73	36.0 ± 0.41

Table 6.4 (a): The effect of Bu 40b icv on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *p<0.05, **p<0.01, ***p<0.001.

6.2.5 Behavioural effects of Bu 40b ip administered alone.

Table 6.4 (b) displays the results found for the effect of Bu 40b ip on locomotor activity. From the table it may be seen there was a considerable reduction in the amount of locomotor activity on day 1 when Bu 40b was administered by the peripheral route. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.01$).

Bu 40b when administered through the ip route had no effect on rotarod performance, weight or temperature given ip.

Given	LMA	RR	Weight	Temp
Saline ip n=6 Day 1	523.33 ± 133.65	120	22.1 ± 0.38	37.5 ± 0.15
Bu 40b 30mg ip n=6 Day 1	**139.17*** ± 22.13	120	25.11 ± 0.75	36.48 ± 0.25
Saline ip n=6 Day 2	-	-	22.6 ± 0.41	35.97 ± 0.45
Bu 40b 30mg ip n=6 Day 2	-	-	26.22 ± 0.78	36.92 ± 0.23
Saline ip n=6 Day 3	1047.33 ± 221.69	120	23.93 ± 0.51	36.87 ± 0.35
Bu 40b 30mg ip n=6 Day 3	1614.67 ± 305.87	120	27.01 ± 0.92	37.27 ± 0.49
Saline ip n=6 Day 4	-	-	23.71 ± 0.65	36.27 ± 0.29
Bu 40b 30mg ip n=6 Day 4	-	-	26.67 ± 1	37.43 ± 0.24
Saline ip n=6 Day 5	927.5 ± 233.7	120	24.75 ± 0.73	35.42 ± 0.48
Bu 40b 30mg ip n=6 Day 5	904.83 ± 238.47	120	27.59 ± 1.12	37.03 ± 0.44

Table 6.4 (b): The effect of Bu 40b ip on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *p<0.05, **p<0.01, ***p<0.001.

6.2.6 Behavioural effects of Bu 36b icv administered alone

Bu 36b decreased locomotor activity in comparison to control when administered through the icv route on day 1 (Table 6.5 (a)). Post-hoc analysis established the difference between control and treatment group ($p<0.01$). No change on day 3 and day 5 was observed.

Bu 36b had a significant effect on weight reduction on day 4 when given i.c.v. Post-hoc analysis confirmed the difference between the control and treatment group ($p<0.05$). Bu 36b had no effect on rotarod performance or temperature when given icv, (Table 6.5 (a)).

Given	LMA	RR	Weight	Temp
Saline 20µl icv n=6 Day 1	1234.33 ± 284.4	92.17 ± 17.71	22.87 ± 0.31	36.52 ± 0.35
Bu 36b 30µg icv n=6 Day 1	**226.33**** ± 93.63	120	23.51 ± 0.62	37.3 ± 0.3
Saline 20µl icv n=6 Day 2	-	-	23.73 ± 0.3	36.27 ± 0.22
Bu 36b 30µg icv n=6 Day 2	-	-	23.23 ± 0.84	37.38 ± 0.34
Saline 20µl icv n=6 Day 3	1204.5 ± 232.53	108.17 ± 8.88	22.93 ± 0.41	36.87 ± 0.25
Bu 36b 30µg icv n=6 Day 3	965.67 ± 170.51	120	22.54 ± 0.76	37.35 ± 0.13
Saline 20µl icv n=6 Day 4	-	-	24.93 ± 0.35	35.92 ± 0.29
Bu 36b 30µg icv n=6 Day 4	-	-	**22.83*** ± 0.68	37.62 ± 0.13
Saline 20µl icv n=6 Day 5	949.33 ± 203.22	113 ± 7	24.07 ± 0.64	36.98 ± 0.24
Bu 36b 30µg icv n=6 Day 5	1242.33 ± 176.98	120	23.07 ± 0.65	37.53 ± 0.35

Table 6.5 (a): The effect of Bu 36b icv on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *$p<0.05$, **$p<0.01$, ***$p<0.001$.

6.2.7 Effect of Bu 36b ip administered alone.

Bu 36b caused a significant drop in temperature on day 1 when given i.p (Table 6.5(b)). Bu 36b given through the peripheral route reduced LMA on day 1, but not day 3 or 5 (Table 6.5 (b)). Post-hoc analysis revealed LMA and temperature was reduced in the treatment group in comparison to control on day 1 ($p<0.01$ and $p<0.0001$ respectively).

Coordination was also examined as before. It was found that Bu 36b had no effect on rotarod performance or weight on any of the observation days, when administered through the ip route (Table 6.5 (b)).

Given	LMA	RR	Weight	Temp
Saline ip n=6 Day 1	523.33 ± 133.65	120	22.1 ± 0.38	37.5 ± 0.15
Bu 36b 30mg ip n=6 Day 1	**26.33**** ± 22.36	120	24.06 ± 0.79	**36.62**** ± 0.21
Saline ip n=6 Day 2	-	-	22.6 ± 0.41	35.97 ± 0.45
Bu 36b 30mg ip n=6 Day 2	-	-	23.56 ± 0.41	35.98 ± 0.55
Saline ip n=6 Day 3	1047.33 ± 221.69	120	23.93 ± 0.51	36.87 ± 0.35
Bu 36b 30mg ip n=6 Day 3	1437.17 ± 234.76	120	22.97 ± 0.51	36.25 ± 0.7
Saline ip n=6 Day 4	-	-	23.71 ± 0.65	36.27 ± 0.29
Bu 36b 30mg ip n=6 Day 4	-	-	25.66 ± 0.65	36.45 ± 0.12
Saline ip n=6 Day 5	927.5 ± 233.7	120	24.75 ± 0.73	35.42 ± 0.48
Bu 36b 30mg ip n=6 Day 5	949.33 ± 193.94	120	25.39 ± 0.73	35.9 ± 0.26

Table 6.5 (b): The effect of Bu 36b ip on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *$p<0.05$, **$p<0.01$, ***$p<0.001$.

6.2.8 Effect of Bu 43b icv administered alone.

Bu 43b when administered through the icv route reduced locomotor activity on day 1 in comparison to control, while LMA on the other two days was unaffected, Table 6.6(a). Post-hoc analysis revealed LMA was reduced on day 1 ($p<0.05$).

From table 6.6(a) it may be seen that Bu 43b had no effect on rotarod performance or weight when administered through the icv route.

Given	LMA	RR	Weight	Temp
Saline 20µl icv n=6 Day 1	1234.33 ± 284.4	92.17 ± 17.71	22.87 ± 0.31	36.52 ± 0.35
Bu 43b 30µg icv n=6 Day 1	**468.5*** ± 146.64	120	22.61 ± 0.92	37.47 ± 0.47
Saline 20µl icv n=6 Day 2	-	-	23.73 ± 0.3	36.27 ± 0.22
Bu 43b 30µg icv n=6 Day 2	-	-	23.11 ± 0.12	37.55 ± 0.51
Saline 20µl icv n=6 Day 3	1204.5 ± 232.53	108.17 ± 8.88	22.93 ± 0.41	36.87 ± 0.25
Bu 43b 30µg icv n=6 Day 3	846.0 ± 193.43	120	22.59 ± 1.14	36.87 ± 0.47
Saline 20µl icv n=6 Day 4	-	-	24.93 ± 0.35	35.92 ± 0.29
Bu 43b 30µg icv n=6 Day 4	-	-	24.29 ± 1.16	36.7 ± 0.42
Saline 20µl icv n=6 Day 5	949.33 ± 203.22	113 ± 7	24.07 ± 0.64	36.98 ± 0.24
Bu 43b 30µg icv n=6 Day 5	785.33 ± 130.38	120	23.51 ± 1.22	37.50 ± 0.29

Table 6.6(a): The effect of Bu 43b icv on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *$p<0.05$, **$p<0.01$, ***$p<0.001$.

6.2.9 Effect of Bu 43b ip administered alone.

Bu 43b also reduced LMA activity when given through the peripheral route on day 1 (Table 6.6(b)). Post-hoc analysis revealed LMA was reduced in the treatment group on day 1 ($p<0.01$).

Bu 43b had a significant effect on lowering temperature on day 1 when given i.p (Table 6.6(b)). Post-hoc analysis confirmed a reduction in temperature on day 1 ($p<0.001$).

Bu 43b when administered through the ip route had no effect on rotarod performance or weight. Table 6.6 (b) illustrates the results of these findings.

Given	LMA	RR	Weight	Temp
Saline ip n=6 Day 1	523.33 ± 133.65	120	22.1 ± 0.38	37.5 ± 0.15
Bu 43b 30mg ip n=6 Day 1	**9.83**** ± 3.65	120	21.99 ± 1.37	**30.87** *** ± 0.38
Saline ip n=6 Day 2	-	-	22.6 ± 0.41	35.97 ± 0.45
Bu 43b 30mg ip n=6 Day 2	-	-	21.35 ± 1.10	35.9 ± 0.23
Saline ip n=6 Day 3	1047.33 ± 221.69	120	23.93 ± 0.51	36.87 ± 0.35
Bu 43b 30mg ip n=6 Day 3	1340.33 ± 316.4	120	21.15 ± 1.17	36.5 ± 0.33
Saline ip n=6 Day 4	-	-	23.71 ± 0.65	36.27 ± 0.29
Bu 43b 30mg ip n=6 Day 4	-	-	22.54 ± 0.87	35.53 ± 0.3
Saline ip n=6 Day 5	927.5 ± 233.7	120	24.75 ± 0.73	35.42 ± 0.48
Bu 43b 30mg ip n=6 Day 5	1008.83 ± 235.71	120	22.19 ± 1.18	34.48 ± 0.24

Table 6.6(b): The effect of Bu 43b ip on Locomotor activity (LMA), rotarod, weight and temperature over 5 days. Results are presented as mean ± SEM. One-Way ANOVA *$p<0.05$, **$p<0.01$, ***$p<0.001$.

6.3 Irwin profile behavioural effects of novel compounds

In the present study, none of the novel compounds tested had any effect on two of the behaviours examined. None of the compounds tested caused stereotypy or convulsions.

6.3.1 Effect of Bu 31b on CNS behaviours.

Tables 6.7 (a) & (b) display the results for CNS behaviours. Bu 31b given through the ip route (Table 6.7 (b)) increased vocalisation on day 1 and day 5. Pelvic elevation was also altered with Bu 31b. On day 1 (icv) (Table 6.7 (a)) there was a decrease found in the elevation of the pelvis, while on day 3 (ip) (Table 6.7 (b)) there was an increase in pelvic elevation in comparison to the saline controls. Bu 31b produced a tremor on day 1, 3 and 5 when administered through the icv route. On day 3 and day 5 a tremor was detected following ip administratio

Table 6.7 (a): The effect of Bu 31b i.c.v on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline 20µl icv n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 31b 30µg icv n=6 Day 1	3 *0*	1 *1*	**0.5** ** *1*	0 *0*	0 *0*	**1**** *1*
Saline 20µl icv n=6 Day 3	4 *0*	0 *0.25*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 31b 30µg icv n=6 Day 3	4 *0.5*	0 *0.5*	2 *0.5*	0 *0*	0 *0*	**1** ** *0.25*
Saline 20µl icv n=6 Day 5	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 31b 30µg icv n=6 Day 5	4 *0.25*	0 *0*	2 *0.5*	0 *0*	1 *2*	**1**** *0.25*

Table 6.7 (b): The effect of Bu 31b i.p on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline ip n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 31b 30mg ip n=6 Day 1	4 *0*	**1** ** *0*	3 *0.25*	0 *0*	0 *0*	0 *1*
Saline ip n=6 Day 3	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 31b 30mg ip n=6 Day 3	4 *0*	0 *0*	**3** ** *1*	0 *0.75*	0 *0*	**1** * *1*
Saline ip n=6 Day 5	4 0	0 0	2 0	0 0	0 0	0 0
Bu 31b 30mg ip n=6 Day 5	4 *0*	2 *0*	3 *1.25*	0 *0*	0 *0*	**1** ** *0.25*

6.3.2 Effect of Bu 31b on Autonomic behaviours.

Bu 31b had several effects on autonomic behaviours. Bu 31b administered through the icv route had an effect on limb tone and gait on day 1, whereby there was a decrease in limb tone and an abnormal gait on that day, Table 6.8 (a).

Bu 31b had an effect on limb tone and gait when administered through the ip route in comparison to the control on each of the three observation days. In relation to limb tone there was a decrease in resistance over the 5 days which got progressively worse culminating in a median score of 2 on day 5. The results for abnormal gait showed that the animals moved in the form of a stagger over the 5 days. Score of 1 Bu 31b also had an effect on palpebral closure on day 3 after ip administration, whereby the animals eyes were found to be half shut, (Table 6.8 (b)).

Table 6.8 (a): The effect of Bu 31b i.c.v on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline 20µl icv n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 31b 30µg icv n=6 Day 1	0 *0*	0 *0*	2.5 *1*	**3 *** *3*	**2 *** *2*	0 *0*
Saline 20µl icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 31b 30µg icv n=6 Day 3	0 *0*	0 *0.25*	3 *0*	4 *0*	0 *2*	0 *0*
Saline 20µl icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 31b 30µg icv n=6 Day 5	0 *0.25*	2 *0*	3 *0.5*	4 *0.75*	0 *0.75*	0 *0*

Table 6.8 (b): The effect of Bu 31b i.p on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline ip n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 31b 30mg ip n=6 Day 1	0 *0*	0 *0.25*	3 *0.25*	**3** ** *0.5*	**1** ** *1*	0 *0*
Saline ip n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 31b 30mg ip n=6 Day 3	0 *0*	**1** * *1*	3 *0.25*	**3** ** *1.25*	**1** ** *1.5*	0 *0*
Saline ip n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 31b 30mg ip n=6 Day 5	0 *0*	0 *0*	3 *0.25*	**2** * *2*	**1** * *0*	0 *0*

6.3.3 Effect of Bu 37b on CNS behaviours.

Bu 37b had several effects on the CNS behaviours examined. Bu 37b administered through the icv route caused an increase in activity of vocalisation only on day 1 of investigations. Tremor was also affected on day 3 and day 5 in comparison to the saline control. On day 5 there was a median score value of 1 for this behaviour (Table 6.9 (a)).

Bu 37b administered through the peripheral route had an effect on pelvic elevation, whereby on day 3 there was an increase in elevation in the pelvis in comparison to the control, while on day 5 there was a decrease in pelvic elevation (Table 6.9 (a)). Tremor was increased on each of the days after Bu 37b was administered through the ip route. On day 1 and 3 animals displayed a slight tremor (median score was 1), while on day 5 there was an increase in tremor to a median score value of 1.5 which is between the slight tremor and moderate tremor (Table 6.9 (a)).

Table 6.9 (a): The effect of Bu 37b i.c.v on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline 20µl icv n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 37b 30µg icv n=6 Day 1	4 *0.25*	**1** * *1.25*	2 *1*	0 *0*	0 *0.5*	0 *0.25*
Saline 20µl icv n=6 Day 3	4 *0*	0 *0.25*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 37b 30µg icv n=6 Day 3	4 *0.25*	0 *0.25*	2 *0*	0 *0*	0 *0.5*	**0** * *2*
Saline 20µl icv n=6 Day 5	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 37b 30µg icv n=6 Day 5	4 *0*	1 *2*	2 *0.25*	0 *0*	0 *0*	**1** ** *0.25*

Table 6.9 (b): The effect of Bu 37b i.p on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline ip n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 37b 30mg ip n=6 Day 1	4 *0*	0 *0*	2 *0.5*	0 *0*	0 *0.5*	**1** ** *0.25*
Saline ip n=6 Day 3	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 37b 30mg ip n=6 Day 3	4 *0*	0 *0*	**3** ** *0.25*	0 *0*	0 *0*	**1** *** *0.25*
Saline ip n=6 Day 5	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 37b 30mg ip n=6 Day 5	4 *0*	0 *0*	**1** *** *0*	0 *0*	0 *0*	**1.5** ** *1*

6.3.4 Effect of Bu 37b on Autonomic behaviours.

Table 6.10 (a) illustrates the results obtained for the autonomic behavioural effects of Bu 37b. Bu 37b given through the icv route only had an effect on body posture on day 5. Bu 37b on day 1 had an adverse effect on gait when given icv (Table 6.10 (a)).

Bu 37b administered through the peripheral route had several, autonomic effects. There was a progressive abnormality in gait on day 1, 3 and 5 (Table 6.10 (b)). On day 5 body posture, limb tone and gait were greatly affected. The animals were lying between prone and on their side, had hardly any limb tone and were incapacitated.

Table 6.10 (a): The effect of Bu 37b i.c.v on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline 20µl icv n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 37b 30µg icv n=6 Day 1	0 0.25	0 *0*	3 *0*	4 *0.75*	**1**** *2*	0 *0*
Saline 20µl icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 37b 30µg icv n=6 Day 3	0 0	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Saline 20µl icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 37b 30µg icv n=6 Day 5	0 0	0 *0*	**2 *** *0*	4 *0*	0 *2*	0 *0*

Table 6.10 (b): The effect of Bu 37b i.p on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline ip n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 37b 30mg ip n=6 Day 1	0 0	0 *0.25*	**3 *** *0.5*	**4 *** *1*	**0 *** *1.5*	0 *0*
Saline ip n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 37b 30mg ip n=6 Day 3	0 0	0 *0*	3 *0*	3.5 *3*	**1 **** *1.25*	0 *0*
Saline ip n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 37b 30mg ip n=6 Day 5	0 0	0 *0*	**1.5**** *1*	**0.5**** *1*	**3 ***** *0*	0 *0*

6.3.5 Effect of Bu 33b on CNS behaviours.

Bu 33b had several effects on the CNS behaviours, which are shown on Table 6.11 (a). Bu 33b administered through the icv route had an effect on vocalisation on day 5 only, where there was an increase in this behaviour and the animals were spontaneously vocal. Tail elevation was also seen on day 1 after icv administration. Tremor was also affected on each of the days after administration of Bu 33b. Each of the days showed a median score value of 1 indicating a slight tremor in the animals, (Table 6.11 (a)).

The peripheral administration of Bu 33b also produced interesting results, whereby pelvic elevation and tremor were affected. On day 1 there was a decrease in pelvic elevation, which seemed to recover on day 3 and was showing signs of an increase in elevation, which is later seen on day 5. The results obtained for day 5 show a maximum median score of 3 indicating that the pelvis was raised considerably.

Tremor was also caused when Bu 33b was administered through the peripheral route. Table 6.11 (a) displays these results. It can be seen that on day 3 and day 5 the test group showed a slight tremor in comparison to the controls.

Table 6.11 (a): The effect of Bu 33b i.c.v on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline 20µl icv	4	0	2	0	0	0
n=6 Day 1	*0*	*0*	*0*	*0*	*0*	*0*
Bu 33b 30µg icv	4	0	2	0	**2***	**1 ***
n=6 Day 1	*0*	*0.5*	*0*	*0*	*2*	*0.5*
Saline 20µl icv	4	0	2	0	0	0
n=6 Day 3	*0*	*0.25*	*0*	*0*	*0*	0
Bu 33b 30µg icv	4	0	2	0	0	**1 ***
n=6 Day 3	*0*	*2*	*0*	*0*	*0*	*0.25*
Saline 20µl icv	4	0	2	0	0	0
n=6 Day 5	*0*	*0*	*0*	*0*	*0*	*0*
Bu 33b 30µg icv	4	**2 ***	2	0	0	**1 **
n=6 Day 5	*0*	*2*	*0*	*0.25*	*2*	*0.25*

Table 6.11 (b): The effect of Bu 33b i.p on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline ip n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 33b 30mg ip n=6 Day 1	4 *0*	0 *2*	**1** * *1*	0 *0*	0 *0*	0.5 *1*
Saline ip n=6 Day 3	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 33b 30mg ip n=6 Day 3	4 *0*	0 *0*	2.5 *1*	0 *0*	0 *0*	**1** ** *0.5*
Saline ip n=6 Day 5	4 0	0 *0*	2 *0*	0 0	0 *0*	0 *0*
Bu 33b 30mg ip n=6 Day 5	4 *0*	0 *0.5*	**3** *** *0*	0 *0*	0 *0*	**1** *** *0*

6.3.6 Effect of Bu 33b on Autonomic behaviours.

Bu 33b showed no effects on the autonomic behaviours, Table 6.12 (a).

Limb tone was affected on day 1 after ip administration of Bu 33b, whereby there was a decrease in tone, median score 2, indicating a moderate resistance in comparison to the saline control and normal score.

Bu 33b administered ip produced an abnormality in gait, whereby on day 1 the animals were incapacitated. There seemed to be a recovery from this and on day 3 and 5 the animals showed fluid movement but staggered slightly, median score values of 1 (Table 6.12 (b)).

Table 6.12 (a): The effect of Bu 33b i.c.v on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline 20µl icv n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 33b 30µg icv n=6 Day 1	0 *1*	0 *0*	3 *0*	4 *0*	0 *0.5*	0 *0*
Saline 20µl icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 33b 30µg icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Saline 20µl icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 33b 30µg icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*

Table 6.12 (b): The effect of Bu 33b i.p on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline ip	0	0	3	4	0	0
n=6 Day 1	*0*	*0*	*0*	*0*	*0*	*0*
Bu 33b 30mg ip	0	0	2	**2** *	**3** *	0
n=6 Day 1	*0*	*0*	*1*	*2.25*	*3*	*0*
Saline ip	0	0	3	4	0	0
n=6 Day 3	*0*	*0*	*0*	*0*	*0*	*0*
Bu 33b 30mg ip	0	0	3	4	**1** **	0
n=6 Day 3	*0*	*0*	*1*	*3*	*1*	*0*
Saline ip	0	0	3	4	0	0
n=6 Day 5	*0*	*0*	*0*	*0*	*0*	*0*
Bu 33b 30mg ip	0	0	2.5	3	**1** ***	0
n=6 Day 5	*0*	*0*	*1*	*2*	*0.25*	*0*

6.3.7 Effect of Bu 40b on CNS behaviours.

Bu 40b had several effects on the CNS behaviours investigated. Bu 40b when administered through the icv route had an effect on vocalisation on day 3 and day 5 whereby the animals were vocal spontaneously. Tremor was also present, on each of the 3 days examined. It is worth noting that on day one the median score value was 2 indicating a moderate tremor, while on day 3 and 5 the median score value was 1 indicating a slight tremor, Table 6.13 (a) illustrates each of these findings.

When Bu 40b was administered through the peripheral route it also produced interesting results, namely an effect on pelvic elevation and tremor. On day 1 and 3 there was a significant variability in pelvic elevation amoung the test group.

The results obtained for Bu 40b in relation to tremor showed that Bu 40b had an effect on this behaviour over each of the days investigated. On day 1 there was a slight tremor recorded which got progressively worse over day 3 and day 5 where the median score was 2, indicating a moderate tremor. Table 6.13 (b) displays each of these results.

Table 6.13 (a): The effect of Bu 40b i.c.v on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline 20µl icv n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 40b 30µg icv n=6 Day 1	4 *0.25*	1 *2*	2 *0*	0 *2*	0 *2*	**2** ** *1.25*
Saline 20µl icv n=6 Day 3	4 *0*	0 *0.25*	2 *0*	0 *0*	0 *0*	0 0
Bu 40b 30µg icv n=6 Day 3	4 *0*	**2** ** *0.5*	2 *0*	0 *0*	0 *0*	**1** *** *0.25*
Saline 20µl icv n=6 Day 5	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 40b 30µg icv n=6 Day 5	4 *0*	**2** ** *0.5*	2 *0*	0 *0*	0 *0*	**1** ** *0.25*

Table 6.13 (b): The effect of Bu 40b i.p on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline ip n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 40b 30mg ip n=6 Day 1	4 *0*	0 *2*	**2** *** *2*	0 *0*	0 *0*	**1** *** *0.25*
Saline ip n=6 Day 3	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 40b 30mg ip n=6 Day 3	4 *0*	0 *2*	**2** *** *2*	0 *0*	0 *0*	**2** *** *0.25*
Saline ip n=6 Day 5	4 0	0 *0*	2 *0*	0 0	0 *0*	0 *0*
Bu 40b 30mg ip n=6 Day 5	4 *0*	0 *2*	1 *0.75*	0 *0*	0 *0*	**2** ** *1*

6.3.8 Effect of Bu 40b on Autonomic behaviours.

Bu 40b had several effects on the autonomic behaviours when administered through the peripheral route. It had only one effect when administered through the icv route on body posture on day 5, Table 6.13 (a).

Gait was also affected, on each of the days investigated. Day 1 showed the animals having fluid movement with a staggered motion. Day 3 showed the animals getting progressively worse. The animals had limited movement i.e staggered slowly, which gave the median score of 2. Bu 40b ip had an effect on limb tone whereupon there was a moderate resistance, median score 2.5, obtained on day 1 of investigation, Table 6.14 (b). On day 5 the animals seem to improve and the movement was similar to day 1 (Table 6.14 (b)).

Table 6.14 (a): The effect of Bu 40b i.c.v on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline 20µl icv n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 40b 30µg icv n=6 Day 1	0 *1*	0 *0*	3 0	4 *0*	0 *0*	0 *0*
Saline 20µl icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 40b 30µg icv n=6 Day 3	0 *0*	0 *0*	3 0	4 *0*	0 *0*	0 *0*
Saline 20µl icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 40b 30µg icv n=6 Day 5	0 *0*	0 *0*	**2*** 0	4 *0*	0 *0*	0 *1.25*

Table 6.14 (b): The effect of Bu 40b i.p on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline ip n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 40b 30mg ip n=6 Day 1	0.5 *1*	0 *0*	3 *1.75*	**2.5** ** *1.25*	**1.5** ** *1.25*	0.5 *1*
Saline ip n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 40b 30mg ip n=6 Day 3	0 *0*	0 *0*	2.5 *1.25*	3.5 *1*	**2** ** *1.25*	0 *0*
Saline ip n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 40b 30mg ip n=6 Day 5	0 *0*	0 *0*	2.5 *1*	3.5 *1*	**1** *** *0*	0 *0*

6.3.9 Effect of Bu 36b on CNS behaviours.

Table 6.15 displays the results for Bu 36b whereby it had several effects on CNS behaviours. When Bu 36b was administered through the icv route it had an effect on tremor, whereupon there was a slight tremor detected on each of the days investigated (Table 6.15 (a)).

Bu 36b given through the peripheral route had an effect on alertness. The results obtained for this behaviour showed that on day 1 the animals were slightly sedated in comparison to the saline control group.

Pelvic elevation was also affected, on day 1, 3 and 5. The results obtained showed that the pelvis was elevated in comparison to the control group.

Table 6.15 (b) also shows the effects of Bu 36b on tremor on each of the 3 days under investigation. On all of the days tested, a median score value of 1 was obtained signifying a slight tremor detected.

Table 6.15 (a): The effect of Bu 36b i.c.v on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline 20µl icv n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 36b 30µg icv n=6 Day 1	5 *2.25*	0 *0.5*	2 *0.25*	0 *0*	0 *0*	**1** ** *1.25*
Saline 20µl icv n=6 Day 3	4 *0*	0 *0.25*	2 *0*	0 *0*	0 *0*	0 0
Bu 36b 30µg icv n=6 Day 3	4 *1*	0 *0.5*	2 *0*	0 *0*	0 *0*	**1** *** *0*
Saline 20µl icv n=6 Day 5	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 36b 30µg icv n=6 Day 5	4 *1*	0 *0.5*	2 *0*	0 *0*	0 *0*	**1** ** *0*

Table 6.15 (b): The effect of Bu 36b i.p on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline ip n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 36b 30mg ip n=6 Day 1	**3** *** *0*	0 *0*	**3** *** *0*	0 *0*	0 *0*	**1** *** *0*
Saline ip n=6 Day 3	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 36b 30mg ip n=6 Day 3	4 *0*	0 *2*	**3** * *1*	0 *0*	0 *0*	**1** ** *0.25*
Saline ip n=6 Day 5	4 0	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 36b 30mg ip n=6 Day 5	4 *0*	0 *0.5*	**3** ** *0.25*	0 *0*	0 *0*	**1** * *1*

6.3.10 Effect of Bu 36b on Autonomic behaviours.

Table 6.16(a) illustrates the autonomic behavioural effects of Bu 36b. From the table it may be seen that Bu 36b did not produced many significant difference on autonomic behaviours, when administered icv. The only significant finding was an increased variability in body posture on day 5.

Bu 36b administered through the peripheral route had an effect on piloerection, whereby by the animal's coats were shown to be standing on end, only on day 1.

Bu 36b also affected body posture on day 5 after ip administration. The animals were shown to be lying prone in comparison to the normal posture of sitting or standing.

Bu 36b also had an effect on limb tone on day 5. There was a decrease observed.

Abnormal gait was also caused by the novel compound Bu 36b on day 3 and day 5. Day 1 had a median score of 1 signifying fluid movement in the animals but with a stagger and on day 5 the animals had limited movement and staggered slowly when they did move, median score 2 (Table 6.16 (b)).

Table 6.16 (a): The effect of Bu 36b i.c.v on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline 20µl icv n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 36b 30µg icv n=6 Day 1	0 *0*	0 *0*	3 *2.25*	4 *1*	0 *0.75*	0 *0*
Saline 20µl icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 36b 30µg icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Saline 20µl icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 36b 30µg icv n=6 Day 5	0 *0*	0 *0*	**3 *** *2*	4 *2*	1 *1.25*	0 *0*

Table 6.16 (b): The effect of Bu 36b i.p on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline ip n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 36b 30mg ip n=6 Day 1	**1***** *0*	0 *0*	3 *0.25*	4 *0.25*	0 *0.25*	0 *0*
Saline ip n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 36b 30mg ip n=6 Day 3	0 *0*	0 *0*	2 *1*	3.5 *1*	**1 * *1*	0 *0*
Saline ip n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 36b 30mg ip n=6 Day 5	0 *0*	0 *0*	**2 *** *1*	**3 *** *1*	**2 **** *1*	0 *0*

6.3.11 Effect of Bu 43b on CNS behaviours.

Bu 43b had several effects on the CNS behaviours, when administered through the icv route and the ip route. Bu 43b icv caused a significant variability in pelvic elevation in comparison to control on day 3. On day 5 there was an elevation in the pelvis of the Bu 43b treated group whereby the median score value was 3, (Table 6.17 (a)).

Tremor was also caused when Bu 43b was administered directly in the brain, on day 1, 3 and 5. the results obtained showed there was a slight tremor found in each of these days in comparisons to the control group, Table 6.17 (a).

From the table 6.17 (b), it may be seen that Bu 43b ip had an effect on alertness on day 1. Moderate sedation was observed, by the median score of 2 in comparison to the control group.

Pelvic elevation was also affected as there was initially a reduction in the elevation of the pelvis on day 1, median score 1, while on the other two days, 3 and 5 there was a marked increase in the elevation of the pelvis of the animals in the Bu 43b treated group, median scores 3 on both days, (Table 6.17 (b)).

Table 6.17 (a): The effect of Bu 43b i.c.v on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline 20µl icv	4	0	2	0	0	0
n=6 Day 1	*0*	*0*	*0*	*0*	*0*	*0*
Bu 43b 30µg icv	4	0	2	0	0	**1** *
n=6 Day 1	*1*	*0*	*1*	*0*	*0*	*1*
Saline 20µl icv	4	0	2	0	0	0
n=6 Day 3	*0*	*0.25*	*0*	*0*	*0*	0
Bu 43b 30µg icv	3.5	0	**2** ***	0	0	**1** *
n=6 Day 3	*1*	*0*	*0.5*	*0*	*0*	*1*
Saline 20µl icv	4	0	2	0	0	0
n=6 Day 5	*0*	*0*	*0*	*0*	*0*	*0*
Bu 43b 30µg icv	4	0	**3** **	0	0	**1** **
n=6 Day 5	*0*	*0.5*	*0.25*	*0*	*0*	*0.25*

Table 6.17 (b): The effect of Bu 43b i.p on median CNS excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Alertness** (normal: 4)	**Vocalisation** (normal: 0)	**Pelvic Elevation** (normal:2)	**Grooming** (normal:0)	**Tail Elevation** (normal:0)	**Tremor** (normal:0)
Saline ip n=6 Day 1	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 43b 30mg ip n=6 Day 1	**2** *** *0.25*	0 *0*	**1** *** *0*	0 *0*	0 *0*	0 *0*
Saline ip n=6 Day 3	4 *0*	0 *0*	2 *0*	0 *0*	0 *0*	0 *0*
Bu 43b 30mg ip n=6 Day 3	4 *0*	0 *0.5*	**3** ** *0.25*	0 *0*	0 *0*	0.5 *1*
Saline ip n=6 Day 5	4 0	0 *0*	2 *0*	0 0	0 *0*	0 *0*
Bu 43b 30mg ip n=6 Day 5	4 *0*	0 *0.5*	**3** ** *0.25*	0 *0*	0 *0*	0 *0.25*

6.3.12 Effect of Bu 43b on Autonomic behaviours.

Bu 43b showed reduced limb tone on day 5 when administered i.c.v (Table 6.18 (a)).

Table 6.18 (b) displays the results for Bu 43b administered through the peripheral route. On day 1, palpebral closure, body posture, limb tone and gait were all affected. The animals were shown to have eyes half closed, were lying prone, had reduced resistance in limb tone and limited slow staggering movement. These descriptions are represented by the relevant median scores for the behaviours, Table 6.18 (b).

Bu 43b on day 3 and day 5 had an affect on limb tone and gait. Limb tone was reduced in comparison to controls, but there was an improvement in comparison to day 1, although the animals displayed fluid movement but with a stagger, Table 6.18 (b).

Table 6.18 (a): The effect of Bu 43b i.c.v on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline 20µl icv n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 43b 30µg icv n=6 Day 1	0 *0*	0 *0*	3 *1*	4 *0*	0 *0*	0 *0*
Saline 20µl icv n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 43b 30µg icv n=6 Day 3	0 *0*	0 *1*	2.5 *1*	3.5 *1.25*	0 *2*	0 *0*
Saline 20µl icv n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 43b 30µg icv n=6 Day 5	0 *0*	0 *0*	3 *0*	***2*** ** *0.75*	0 *0.25*	0 *0*

Table 6.18 (b): The effect of Bu 43b i.p on median Autonomic excitation scores (plain text) and interquartile ranges (IQR-*italics*) over 5 days. Test groups have been compared to saline group using Mann-Whitney U-Test (*p<0.05,**p<0.01,***p<0.001).

Given	**Piloerection** (normal:0)	**Palpebral Closure** (normal:0)	**Body Posture** (normal:3)	**Limb Tone** (normal:4)	**Abnormal Gait** (normal:0)	**Writhing** (normal:0)
Saline ip n=6 Day 1	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 43b 30mg ip n=6 Day 1	0 *0*	**1** ** *0.25*	**2** ** *0.5*	**2** ** *0.75*	**2** ** *1*	0 *0*
Saline ip n=6 Day 3	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 43b 30mg ip n=6 Day 3	0 *0*	0 *0*	3 *0*	**3** ** *0.25*	**1** * *1*	0 *0*
Saline ip n=6 Day 5	0 *0*	0 *0*	3 *0*	4 *0*	0 *0*	0 *0*
Bu 43b 30mg ip n=6 Day 5	0 *0*	0 *0*	3 *0*	**3** * *1*	**1** ** *1*	0 *0*

6.4 Discussion.

In order to fully understand the mechanisms of action of compounds and their respective side effects, it is essential that various behavioural techniques are established as a measurement of activity. Many studies have evaluated the behavioural effects of glutamate receptor antagonists, it has been found that these agents have psychotomimetic properties in humans. Extensive work has been carried out on NMDA receptor antagonists. Studies have shown that NMDA antagonists can cause neurotoxic side effects consisting of pathomorphological changes in neurons of the cingulated and retrosplenial cerebral cortices, which are reversible at low doses (Olney et al, 1991). Other studies have shown NMDA antagonist agents impair performance on sensorimotor, learning and memory-intensive tasks (Genovese et al, 1991; Clissold et al 1991), unfortunately these were not assessed in this study.

Both competitive and non-competitive NMDA receptor antagonists have similar actions, e.g cerbroprotective, anticonvulsant properties and the ability to counteract catalepsy (Bubser et al, 1991). Non-competitive NMDA receptor antagonists induce rotarod performance locomotion (Kew et al, 2000), stereotyped sniffing, stereotypy and rearing (Bubser et al, 1991). NMDA antagonists have shown to reduce response rates and disrupt learning and memory (Willetts et al, 1990; Porsolt et al, 2002). NMDA antagonists have shown to alter body posture, gait, sensory response, arched back, piloerection, tremors and autonomic activity (Kew et al, 2000).

In this study, assessment of locomotor activity was employed. Locomotor dysfunction can be used as an index of CNS dysfunction and can highlight adverse effects produced by CNS active drugs (Dawson et al, 2001). MK-801 has been show to increase locomotor activity following peripheral administration (Liljequist et al, 1991; Babcock et al, 2002). MK-801 has also been shown to produce a typical motor syndrome characterized by head weaving, body rolling, ataxia and salivation (Liljequist et al, 1991).

The rotarod test is also a good index of toxicity (Porsolt et al, 2002). A normal animal is able to walk up and down the rotarod without falling off at varying speeds. When suffering with ataxia, commonly animals adopt a fixed position with the tail wrapped around the rod and adjust to the movement of the rod until the momentum of the rod and the animals body weight cause the animal to fall off the rod.

The Irwin profile provides a behavioural and functional profile based on observation. In this study many parameters were investigated as this would provide a very good profile of activity for each of the novel compounds investigated. In particular, behaviours were selected to highlight any polyamine-like effects, such as pelvic elevation, tremor and convulsions. The presence or absence of behaviours may highlight the involvement of certain neurotransmitter systems in the mechanism of action of novel compounds. For example dopaminergic, GABA, acetylcholine, opioids and finally excitatory amino acids. It is important to summaries expectations: If animals were displaying any stereotypy this would signify possible dopaminergic, serotoninergic or opiate action (Millan, 1991). Reduced alertness, vocalization and increased grooming may reflect central dopamine stimulation e.g. amphetamine (Hinz et al, 1996). Tail elevation would signify possible opiate activity. Salivation changes in body posture, limb tone, gait and palpebral closure may all illustrate changes in muscarinic activity (Rogers et al, 1997). Piloerection is an indicator of a decrease body temperature, which is a polyamine-like activity (Anderson et al, 1975). Finally writhing is used as an index of the abnormal pH (Rogers et al, 1997).

The most pronounced and consistent changes caused by the novel compounds in this study were changes in locomotion and gait (body posture and limb tone) and tremor. These changes could be caused by interaction with any number of neurotransmitter systems, inducing dopaminergic, muscarinic and glutamatergic. However, it is striking that the behaviours produced closely mirror effects, following spermine or spermidine administration. The nature of this study makes it impossible to draw any conclusions regarding possible interactions with many

classical neurotransmitter systems, so the discussion is centred mainly around the presence of polyamine-like effects.

Previously mentioned were the findings obtained for the novel compounds by Fixon-Owoo et al (2003). On investigation of these compounds it was discovered that they showed no notable toxicity towards the European corn borer (*Ostrinia nubilalis*), the tobacco budworm (*Heliothis verescens*), the oblique banded leaf roller (*Choristoneura rosaceana*) and rice weevil (*Sitophilus oryzae*) (Fixon-Owoo et al, 2003). It was also discovered that these compounds are inhibitors of crayfish glutamate receptors and mammalian NMDA, δ2 and AMPA receptors (Fixon-Owoo et al, 2003).

Generally, it is expected that male mice gain weight on a daily basis of 0.5 to 1g per day. The results obtained for the saline group in this study showed that the animals did put on weight over 5 days of observation following saline through the icv route or the ip route.

As previously mentioned in Section 3.5, Bu 31B showed 98% inhibition at the NR1/NR2B NMDA receptor subunits, 22% at δ2, 13% at AMPA GluR2 and 11% at AMPA GluR1/GluR2 (Fixon-Owoo et al, 2003). This indicates that this compound is an effective antagonist at NMDA receptors in particular. In this part of the study it was interesting to see the effect of Bu 31b on locomotor activity. There was a decrease in locomotor activity following administration, which is similar to the findings when spermine or spermidine are administered (Gimenez-Llort et al, 1996) and opposite to the effect of MK-801 (Liljequist et al, 1991; Babcock et al, 2002) and JSTX-3 (Himi et al, 1990).

Bu 31b hindered weight gain on day 2, 3, 4 and 5 when administered through the icv route. The result here is in keeping with the effects of the polyamines on weight as previous studies have shown that polyamines can cause anorexia (Shaw, 1972;

Anderson & Shaw, 1974; Anderson et al, 1975; Kohno et al, 1981a, Kohno et al, 1981b).

Bu 31b also was shown to have an effect on temperature when administered icv (Table 6.1). Once again this may be signifying that Bu 31b is producing behavioral effects similar to that of the polyamines, which also produce hypothermia.

Bu 31b produced some effects on CNS behaviours. These findings are consistent with previous findings found for polyamines and spider toxin derived polyamine antagonists (JSTX-3) (Himi et al, 1990). Pelvic elevation was seen in animals after ip administration. This spermidine-like effect in the animals indicated acute toxicity. Effects on vocalistation and the onset of a tremor were also observed. Spermine consistently causes tremor. Therefore, these effects could illustrate polyamine-like effects.

Bu 31b also produced significant autonomic effects. On day 1 (i.c.v) Bu 31b had an effect on limb tone and caused an abnormal gait, which progressively worsened over the 5 days. The change in pelvic elevation and loss of limb tone would lead to an abnormal gait. Administration through the i.p route produced more toxicity than i.c.v administration which could be attributed to the compound being broken down through metabolism, to produce a more active metabolite. Alternatively, as the ip dose was double the icv dose, in relative terms, the greater level of side effects could reflect the higher level of Bu 31b reaching peripherial and central receptors. This would suggest that Bu 31b readily crosses the blood brain barrier.

Bu 37b showed 12% inhibition at the NR1/NR2B NMDA receptor subunits, 4% at δ2, 8% at AMPA GluR2 and 9% at AMPA GluR1/GluR2 in the study by Fixon-Owoo et al (2003). It was concluded that this compound was a poor antagonist of glutamate receptors (Fixon-Owoo et al, 2003). Bu 37b on day 1 caused a significant decrease in locomotor activity, which was not seen on any of the other two days.

As previously mentioned, spermine and spermidine also produce this hypomotility (Shaw, 1972).

Bu 37b had very little effect on CNS induced behaviors when given icv. On day 1 there was slight vocalization, only when the animals were handled. Bu 37b also caused the development of a tremor on day 3 and 5, perhaps indicating spermine like symptoms developing. Bu 37b given ip caused an increase in pelvic elevation, which could be a spermidine-like effect (Doyle et al, 1994). Tremor was also more severe when Bu 37b was administered i.p. As discussed for Bu 31b this could be the result of active metabolites from the original compound which had the ability to cross the blood-brain barrier. Alternatively, it is not unreasonable to suggest that the high i.p dose in comparison to the icv dose produced the greater level of side effects. This would imply that Bu 37b also penetrates the blood brain barrier effectively.

There were two autonomic effects produced by Bu 37b when administered icv over the 5 days. This was on day 1 and 5 on gait and body posture, where the animals were lying prone, indicating toxicity. The limited adverse effects of this compound which reflect the fact that Bu 37b been shown to be a poor glutamate receptor antagonist (Fixon-Owoo et al, 2003). It is known that spermidine can cause quadriplegia, and the effects observed may suggest Bu 37b is generating spermidine-like effects. When Bu 37b was administered through the ip route there were multiple effects produced. Bu 37b affected body posture, limb tone and caused abnormal gait. The animals were lying between prone and on their side, had hardly any resistance in terms of limb tone and were incapacitated. This signifies considerable toxicity, plus illustrating further spermidine-like effects.

Bu 33b showed 3% inhibition at the NR1/NR2B NMDA receptor subunits, 0% at δ2, 2% at AMPA GluR2 and 1% at AMPA GluR1/GluR2 (Fixon-Owoo et al 2003). From the present study it was found that Bu 33b produced hypomotility on day 1 of investigations when given icv, which is in keeping with findings for spermine and

spermidine (Shaw, 1972; Anderson & Shaw, 1974; Anderson et al, 1975; Kohno et al, 1981; Kohno et al, 1981b).

Bu 33b produced mild tremor on each of the experimental days. It did not get progressively worse with time. This polyamine-like effect is similar to a mild spermine-like action. As there was evidence of a tail elevation, it is possible that Bu 33b is affecting opiate homeostasis, as a tail elevation or straub tail is a clear indication of opiate activity. There is some evidence that there are interactions between NMDA receptor antagonists and morphine (Bespalov et al, 1998), which could be why Bu 33b is producing a straub tail. Given peripherally Bu 33b had an effect on pelvic elevation, which increased over time and by day 5 the animals were shown to have a raised pelvis indicating similar effects to spermidine (Doyle et al, 1994). The effects were more pronounced following i.p administration. Similarly, to the previously discussed novel compounds these behaviours could be caused by a metabolite of the compound rather than the compound itself. Alternatively, as a higher dose was given peripherally than directly icv, more compound could have reached the brain, illustrating the ability of Bu 33b to cross the blood brain barrier.

Interestingly, Bu 33b had no effect on the autonomic behaviors given icv, but administered i.p it had an effect on limb tone and caused abnormal gait. The animals displayed a high carriage or abnormal gait with splayed digits, leading to a "tip-toe walking" behavior, which involves an arched back and severe splaying of digits when walking (Doyle et al, 1994). In conjunction with CNS effects it is evident that this compound administered i.p causes similar behavioural characteristics to spermidine.

Bu 40b has been previously shown to cause 28% inhibition at the NR1/NR2B NMDA receptor subunits, 0% at δ2, 15% at AMPA GluR2 and 13% at AMPA GluR1/GluR2 (Fixon-Owoo et al, 2003). From this study on the whole animals Bu 40b had an effect on locomotor activity when given i.c.v or i.p. This compound

produced hypomotility, similarly to polyamine agonists and appears to cross the blood brain barrier well.

Bu 40b given i.c.v caused a moderate tremor on day 1, which decreased to a slight tremor on day 3 and 5. This could be due to Bu 40b having an agonist action on the polyamine binding sites, possibly on glutamate receptors, as it has been shown to have some activity at these receptors (Fixon-Owoo et al, 2003). Through the i.p route of administration tremor was also caused, this worsened with time. This could be due to a number of reasons, firstly, higher doses were given through the ip route of administration, secondly, it could be due to increased levels of an active metabolite of Bu 40b. Furthermore, it is possible that metabolic alteration of this and the other compounds produces a metabolite that is a potent agonist at glutamate receptors.

Bu 40b had a significant effect on body posture when administered through the i.c.v route. Bu 40b administered peripherally did have an adverse effect on limb tone and gait, producing similar behavioural effects to those observed when spermidine is administered alone (Doyle & Shaw, 1994).

In the Fixon-Owoo study (2003), Bu 36b showed 26% inhibition at the NR1/NR2B NMDA receptor subunits, 4% at δ2, 18% at AMPA GluR2 and 16% at AMPA GluR1/GluR2 (Fixon-Owoo et al, 2003). Bu 36b had an inhibitory effect on locomotor activity on day 1 when administered i.c.v and i.p. This finding is similar to that of spermine and spermidine, whereby these polyamines impair motion. This finding also implies that Bu 36b crosses the blood brain barrier. Another effect which is seen for polyamines is anorexia and significant weight loss was seen on day 4 when Bu 36b was given i.c.v. Bu 36b i.p also on day 1 caused hypothermia, another polyamine-like effect.

Bu 36b caused a slight tremor on each of the days examined, which is a spermine-like effect. This is possibly mediated through a polyamine binding site on

glutamate receptors, as previously described (Section 1.5.1). Bu 36b had no significant effect on any of the autonomic behaviours through icv administration showing limited toxic effects of this compound.

Administration of Bu 36b through the peripheral route did cause alterations in the physiology of the animals. There was a reduction in alertness on day 1, similar to that experienced with spermine and spermidine (Shaw, 1972; Anderson & Shaw, 1974; Anderson et al, 1975; Kohno et al, 1981a; Kohno et al, 1981b). There was also an increase in pelvic elevation over the 5 days possibly due to the development of tip-toe walking, a spermidine-like effect (Doyle & Shaw, 1994). Tremor was also present on all experimental days, which is a spermine-like effect. Again these alterations could be due to the higher dose given through the ip route of administration or due to the metabolites of the compound, which result in active agonists crossing the blood-brain barrier. Piloerection was also observed on day 1, which is a sign of a decrease in body temperature, which is consistent with the findings for temperature on that day. Body posture, limb tone and gait were affected over time. This could signify a development of spermidine-like effects.

Bu 43b had an inhibitory effect on locomotor activity on day 1 when administered i.c.v and i.p. This finding is similar to spermine and spermidine, whereby these polyamines impair motion. Bu 43b i.p on day 1 caused hypothermia, another polyamine-like effect.

CNS and autonomic observations showed that Bu 43b i.c.v caused tremor, pelvic elevation and reduced limb tone. As previously discussed the development of tremor is a spermine-like activity. The development of pelvic elevation and a decrease in limb tone are typical spermidine-like characteristics.

Bu 43b administered through the ip route produced several different effects on CNS and autonomic behaviours. In particular on day 1, there was sedation, decrease in pelvic elevation, the eyes were seen half closed, the animals were lying prone,

reduced limb tone and there was a staggered movement. These effects are very similar to what is seen on initial administration of spermine and spermidine and the spider toxin JSTX-3 (Himi et al, 1990). Pelvic elevation increased with time and limb tone increased to a marked resistance along with an improvement in abnormal gait. It is possible that these behaviours are still characteristic polyamine-like actions.

6.5 Conclusion.

In conclusion, the findings presented here indicate that these compounds have some toxicity. No convulsions were observed, neither was salivation, stereotypy, circling and very little writhing was present. Most importantly no animals were lost in this investigation.

It is very uncertain, what is causing several of the different behavioural effects. What can be hypothesized is that there seems to be several neurological systems triggered on administration of these compounds. This could be through direct interaction or it could be due to the metabolism of the compounds to active metabolites, which are producing these behaviours. The compounds were more active on administration through the peripheral route. This is possibly reflecting good capability to cross the bbb, and may reflect a comparative higher i.p dose compared to i.c.v dose.

Further to this, what is evident is that many of these compounds have behavioural effects similar to that of the polyamines, spermine and spermidine, perhaps indicating partial agonist activity. What is interesting is that some of the compounds produce the same effects as JSTK-3, a polyamine antagonist.

The compounds which produced the least toxicity were Bu 37b, Bu 33b and Bu 40b. This is interesting as these compounds were shown to have low inhibitory activity on glutamate receptors. Bu 31b, Bu 43b and Bu 36b showed more behavioural

effects generally. Bu 31b and Bu 36b have previously been shown to be highly potent inhibitors of NMDA receptors (Fixon-Owoo et al, 2003).

Further assessment of these compounds would need to be done, to ascertain their potential as therapeutic agents, as currently there is limited information available on each of these novel polyamine analogues.

Chapter 7.

7.1 Further Discussion.

Spermine CNS excitation model:

Chapter 3, investigated the effects of compounds on spermine-induced CNS excitation. Previous studies have used known NMDA antagonists, which seem to be very effective in reducing spermine induced CNS excitation (Doyle & Shaw, 1996; Doyle & Shaw, 1998; Kirby et al, 2004). The ability of these NMDA antagonists to inhibit the development of CNS excitation has led to the conclusion that the NMDA receptor is involved at least in part, in the development of this excitation.

In this study, various antagonists were investigated to develop a better understanding of the connection between the NMDA receptor and spermine induced CNS excitation. Well known NMDA antagonists and calcium channel antagonists were used to inhibit spermine-induced convulsions, ifenprodil, eliprodil, arcaine, memantine and others, along with the novel compounds. Table 7.1 illustrates the percentage of inhibition of both the old compounds and the novel compounds at the most effective doses for each. Appendix 1 shows the percentage (%) of inhibition for each compound and each dose.

Spermine Model

Old compounds		
Ifenprodil	10mg = 42%	
Eliprodil	30mg = 34%	
Arcaine	20μg = 64%	
Memantine	20mg = 48%	
Nisoldipine	2mg = 82%	
Nitrendipine	10mg = 64%	
N^1Dansylspermine	20μg = 56%	
Novel Compounds	**icv**	**ip**
Bu 31b	20μg = 60%	30mg = 48%
Bu 37b	30μg = 20%	0%
Bu 33b	20μg = 48%	10mg = 56%
Bu 40b	0%	20mg = 20%
Bu 36b	20μg = 60%	30mg = 56%
Bu 43b	20μg = 100%	20mg = 64%

Table 7.1. The percentage of inhibition of old and novel compounds on spermine induced CNS excitation.

Table 7.1 showed the highest percentage inhibition (82%) with nisoldipine at a dose of 2mg and the second highest with nitrendipine at 10mg (64%). Both Nitrendipine have shown to suppress NMDA/glycine-mediated calcium influx by a direct interaction with the NMDA receptor and has also been shown to reduce NMDA

evoked currents (Skeen et al, 1993; Skeen et al, 1994). Nisoldipine can inhibit the effects of spermine which in part could be mediated through L-type calcium channels (Doyle, 1993; Kirby, 2000 & Doyle et al, 2004). What is known is that both nisoldipine and nitrendipine interact with binding sites on the α1 subunit of the L-type voltage gated Ca^{2+} channel, and there is a known relationship between polyamines and calcium channels, whereby polyamines allosterically modulate dihydropyridine binding to L-type calcium channels (Schoemaker, 1992). The results presented here suggest that nisoldipine and nitrendipine may be mediating their effects through the NMDA receptor or L-type calcium channels, or more than likely through both.

Arcaine produced a dose-dependant decrease in spermine induced CNS excitation with the highest inhibition of 64 % at 20μg. These findings are consistent with Doyle and Shaw's (1998), where it has been suggesting arcaine is a competitive antagonist, partial agonist or an inverse agonist at the stimulatory polyamine site (Pritchard et al, 1994). Memantine also partially protected against the development of CNS excitation probably through an NMDA antagonist response via open-channel blockade (Chen et al, 1992; Chen et al, 1997). This is further signified by the % of inhibition at 20mg by 48%.

Ifenprodil at a dose of 10mg/kg showed 42% inhibition, this inhibition is thought to be mediated through a direct selective antagonist effect on the NMDA receptors containing the NR2B subunit (Chenard et al, 1999). Eliprodil had 34% inhibition at a higher dose of 30mg/kg. Eliprodil's action may be mediated through NR1/NR2B subunits (Avenet et al, 1997), or by blocking several other types of receptors or ion channels, such as voltage-dependent Ca^{2+} channels (Biton et al, 1994; Carter et al, 1997). These findings are consistent with previous studies (Doyle & Shaw, 1996).

N^1-dansylspermine inhibited the effects of spermine by 56% at 20μg. It is reasonable to suggest that N^1-dansylspermine is blocking the effects of spermine by binding to the stimulatory polyamine site on the NMDA receptor as previously

suggested by Kirby et al (2004). Also, Chao et al (1997) showed N^1-dansylspermine was able to block the NMDA receptor through binding to the inhibitory polyamine site, showing subunit specificity for NMDA receptor by greatly blocking the receptor at a mutated trytophan residue on the M2 region of the NR2B subunit and also permeating the channels containing this mutation (Kashiwagi et al, 1997). It is uncertain as to where N^1-dansylspermine is mediating its effects as N^1-dansylspermine has affinity for both inhibitory and stimulatory sites on the NMDA receptor.

Probably the most interesting findings were with the novel compounds. In a previous study by Fixon-Owoo et al, (2003), they examined NMDA and AMPA receptors and the affinity that Bu 31b, Bu 37b, Bu 33b, Bu 40b, Bu 36b and Bu 43b had on these glutamate receptors.

From Table 7.1 it may be seen that Bu 43b had the highest percentage of inhibition at 20μg icv and 20mg ip. Most interesting are the icv results which demonstrated an astonishing 100% inhibition. These results reflect that Bu 43b is the most effective novel compound, showing a high level of competitive antagonist activity in reducing the spermine CNS excitation convulsions. This is possibly mediated through the polyamine stimulatory site of the NR1/NR2B subunit of the NMDA receptor, as Bu 43B was shown to have high inhibitory affinity for these receptor units (Fixon-Owoo et al, 2003). Similarly the results for Bu 31b showed high inhibition (Table 7.1), which could be attributed to the above suggestion as Bu 31b also has high affinity for this sub-class of NMDA receptors.

Bu 36b showed a high level of inhibition at the individual doses, 60% icv and 64% ip. However, Bu 36b had an inhibitory effect on the development of spermine-induced convulsions. Fixon-Owoo et al, (2003), showed that this compound had a low level of inhibition on the NR1/NR2B NMDA receptors subunits in vitro (Fixon-Owoo et al, 2003). This evidence suggests that Bu 36b may be mediating its effects through an alternative means, possibly L-type calcium channels, as it shows a high

percentage of inhibition. Similarly the results obtained for Bu 33b showed reasonable inhibition, but as with Bu 36b, it was a poor inhibitor of NR1/NR2B (Fixon-Owoo et al, 2003), which further questions its mechanism of action and other mediators for the development of spermine-induced CNS excitation behaviours.

Bu 37b and Bu 40b had very little effect on reducing the spermine CNS excitation, showing that these are not potent polyamine antagonists. These compounds were also shown to be poor inhibitors of the NR1/NR2B NMDA receptor subunits (Fixon-Owoo et al, 2003). Although both these compounds did demonstrate a fraction of inhibition, Bu 37b icv of 20% at 30μg, and Bu 40b of 20% at 20mg, it is uncertain their mechanism of action.

The possible antagonistic action of Bu 33b, Bu 40b, Bu 37b and Bu36b may be mediated through another mechanism. L-type voltage-activated Ca^{2+} channels have been implicated in the spermine-induced convulsions due to the effectiveness of L-type channel blockers in this model. It is possible that the novel polyamine analogues may mediate their effects, at least in part, through action involving blockade of L-type calcium channels. Additional work is needed to explore this further.

In conclusion, the evidence presented here suggests that L-type calcium channels are involved in the development of spermine induced CNS excitation, due to the known compounds nisoldipine and nitrendipine, which were the most effective in inhibiting the development of spermine effects. Unfortunately, the mechanism still remains to be elucidated (Doyle et al, 2004). In addition, the evidence presented in this study further confirms that the modulation of spermine induced effects is through the NMDA receptor, in particular the NR1/NR2B NMDA receptors subunits, at least in part, is due to the effective novel compound Bu 43b, which showed the most inhibition in this study and the highest affinity for this NMDA subunit (Fixon-Owoo et al, 2003).

The results from Chapter 5 illustrate that in the spermine CNS excitation model (Chapter 3), an increase in polyamine levels is associated with CNS excitation. There is a progressive incline in the levels of polyamines from score 0 to score 4, with a rapid decline at score 5. The results indicate there is strong evidence that a cascade of events occurs during the behavioural profile, in particular the metabolic transformation of spermine into its derivatives, through the retro-conversion pathway. N^1-acetyspermidine was elevated at different score stages throughout the progression of the CNS excitation, indicting the retro-conversion pathway is active at the different stages. Similarly, spermine levels increased from stage 0-4, indicating that the injection of spermine stimulates the synthesis of polyamines. As previously described, activation of the NMDA receptor stimulates ODC activity, and this could be the mechanism by which spermine levels increase through the stages. It is likely that the raised spermine levels directly contribute to the increasing CNS excitation score, and ultimately the fatal tonic convulsion.

Adrenocorticotrophic hormone ($ACTH_{1-24}$):

The second behavioural model employed to investigate the polyamines used Adrenocorticotrophic hormone ($ACTH_{1-24}$). The behaviours produced are characterised by recurrent episodes of stretching yawning syndrome (SYS), penile erection (PE) and by excessive grooming (G), the effects beginning 15-30 minutes after treatment with $ACTH_{1-24}$, and lasting for several hours (Ferrari et al, 1963; Bertolini et al, 1969, Gispen et al, 1975; Bertolini et al, 1981). $ACTH_{1-24}$ stimulates adrenal Ornithine Decarboxylase (ODC) activity, and therefore ODC and polyamines may have a prominent role in the onset of some of the $ACTH_{1-24}$-induced behaviours (Bertolini et al, 1968; Tinter et al, 1979; Poggioli et al, 1984).

$ACTH_{1-24}$ Model

Old compounds		Novel Compounds	
Ifenprodil	20mg/kg = 73%	Bu 31b	30μg = 76%
Eliprodil	30mg = 58%	Bu 37b	30μg = 64%
Arcaine	5μg = 50%	Bu 33b	30μg = 64%
Memantine	10mg = 64%	Bu 40b	30μg = 30%
Nisoldipine	10mg = 49%	Bu 36b	5μg = 47%
Nitrendipine	20mg = 72%	Bu 43b	20μg = 39%
N^1Dansylspermine	20μg = 28%		
Putrescine	480μg = 99.9%		
DFMO	50μg = 48%		

Table 7.2. The percentage of inhibition of old and novel compounds on Adrenocorticotrophic hormone ($ACTH_{1-24}$) behaviours.

Table 7.2 showed ifenprodil showed the highest percentage of inhibition of 73% at 20mg. It is thought that ifenprodil mediates its effects by modulation of NMDA receptor activation (Genedani et al, 1994; Spruijt et al, 1994), in particular the NR2B subunit (Chenard et al, 1999), or through the inhibition of ODC activity as Ifenprodil inhibits ornithine decarboxylase activity to the same extent as DFMO (Badolo et al, 1998). Finally, as $ACTH_{1-24}$ activity is known to be Ca^{2+} dependent. It is possible the effect maybe through interference with Ca^{2+} influx channels as ifenprodil blocks voltage-dependent Ca^{2+} channels (Carter et al, 1997).

Nitrendipine showed the second highest percentage of inhibition, 72% at 20mg. It is possible that this inhibition may be mediated directly through L-type calcium

channels as Ca^{2+} channel blockers which have previously been shown inhibitory effects on the $ACTH_{1-24}$ induced behaviours (Poggioli et al, 1993; Poggioli et al, 1995; Vergoni et al, 1995). Nitrendipine could also mediate its effects through the NMDA receptor, as nitrendipine in particular has been shown to directly interact with the NMDA receptor (Palmer et al, 1993; Skeen et al, 1993; Skeen et al, 1994). Nisoldipine, the other calcium channel antagonist, also showed almost half the percetage of inhibition, again illustrating that a Ca^{2+} influx is involved in $ACTH_{1-24}$ behaviours (Poggioli et al, 1993; Poggioli et al, 1995; Vergoni et al, 1995).

Memantine reduced $ACTH_{1-24}$ behaviours showed no effect on stretching. Memantine showed an inhibition of 64% at 10mg. These results further emphasise the link between NMDA receptor activity and $ACTH_{1-24}$ effects, as memantine is an NMDA antagonist. Eliprodil showed a 58% inhibition at the higher dose of 30mg. This inhibition is probably mediated through the same mechanisms as ifenprodil. Eliprodil is strongly selective for the NR1/NR2B subunits of the NMDA receptor (Avenet et al, 1997). Ca^{2+} influx is essential to the $ACTH_{1-24}$ behaviours (Poggioli et al, 1993; Poggioli et al, 1995; Vergoni et al, 1995), while eliprodil is potent at blocking voltage-activated L- and N- type calcium channels (Biton et al, 1994), therefore, eliprodil could be mediating its effects through both these mechanisms.

Arcaine inhibited $ACTH_{1-24}$ behaviours by half (50%) at 5μg, although arcaine had no effect on yawning. This result, as with memantine, illustrates a link between NMDA receptor activity and $ACTH_{1-24}$ effects. In this study N^1-dansylspermine had an inhibitory effect (28%), on only some of the $ACTH_{1-24}$ induced behaviours. The results for N^1-dansylspermine indicate that there may be different mechanisms involved in the different behaviours, which is consistent with previous findings (Poggioli et al, 1995). N^1-dansylspermine may have mediated its inhibition through the NMDA receptor.

Putrescine had a dramatic inhibitory effect on the development of $ACTH_{1-24}$ effects, 99.9%. These findings for putrescine suggest that $ACTH_{1-24}$ may generally stimulate

the brain ODC and enhance cerebral content of polyamines. However, in the presence of high levels of putrescine, a negative feedback mechanism was possibly initiated, thereby stopping the cycle. The results, in conjunction with previous findings, indicate there is a correlation between the ODC enzyme activity and $ACTH_{1-24}$ activity, but also suggests that another cascade, perhaps under the control of ODC, but not polyamine related, may be involved in the development of the behaviours.

From Table 7.2 it may be seen that from the novel compounds, Bu 31b showed the highest percentage of inhibition at 76%. Bu 31b is known to interact at the NR1/NR2B subunit of the NMDA receptor (Fixon-Owoo et al, 2003). The result presented illustrates the interaction between NMDA and $ACTH_{1-24}$ for the development of the behaviours. The second most effective novel polyamine analogues in this model were Bu 33b and Bu 37b, both at 64%. These compounds were not very effective in the spermine CNS excitation model, and have poor affinity for the NR2B subunit of the NMDA receptor indicating there are other mechanisms involved in the development of $ACTH_{1-24}$ induced behaviours, and some mediated through NMDA in particular NR1/NR2B and another via calcium channels. There seems Ca^{2+} may have an influence on the development of $ACTH_{1-24}$ behaviours, it may be hypothesized that the novel analogues may be inducing their effects through inhibiting Ca^{2+} channel activity as previous studies have shown that Ca^{2+} antagonists block the development of the $ACTH_{1-24}$ behaviours (Poggioli et al, 1993; Poggioli et al, 1995; Vergoni et al, 1995). However, further work is necessary to elucidate the mechanism of action of the novel analogues.

Interestingly, Bu 43b, which was very effective in the spermine CNS excitation model and known to have high affinity for the NR2B subunit, was not as effective in the $ACTH_{1-24}$ model with only 39% inhibition. Similarly, Bu 36b also showed limited inhibition at 47%.

In conclusion, the highest percentage of inhibition was seen from the novel compounds with Bu 31b, followed by ifenprodil at 73%. These results show that there is some link between the polyamines and the $ACTH_{1-24}$ induced behavioural profile of effects. The NMDA receptor, in particular, the NR1/NR2B subunit could be involved, and there is evidence that L-type Ca^{2+} channels may also be involved. There are different mechanisms involved in different behaviours as only certain behaviours were inhibited by the different drugs used.

Results from Chapter 5, are consistent with previous findings following $ACTH_{1-24}$ administration, whereby putrescine levels were elevated, indicating an upsurge in polyamine synthesis with putrescine being the first polyamine synthesised. However, levels of spermidine and spermine were decreased. Collectively the results from Chapters 4 and 5 suggest that polyamines may not be crucial mediators of the $ACTH_{1-24}$ behaviours, although they (and their inhibitors) can influence the development of $ACTH_{1-24}$ effects. The link between polyamine activity and $ACTH_{1-24}$ activity is not fully clarified and further work is needed in this area.

Behavioural effects of the novel polyamine analogues:

Chapter 6 investigated the novel compounds administered alone in the whole animal. Various methods were used as a medium of assessment (Section 2.4). The findings indicate that these compounds have some toxicity and polyamine-like agonist effects.

All of the novel polyamine analogues showed some polyamine-like effects. Bu 31b and Bu 36b caused anorexia. While Bu 31b, Bu 36b and Bu 43b caused hypothermia. Bu 33b also caused a related piloerection. All of the compounds showed significant autonomic effects, showing an effect on limb tone and abnormal gait. Some of the abnormalities presented as a high carriage or abnormal gait with splayed digits, leading to a spermidine-like "tip-toe walking" behaviour, involved an arched back and severe splaying of digits when walking (Doyle et al, 1994). Bu 37b

had very little effect on CNS induced behaviors given i.c.v. Bu 31b, Bu 43b, Bu 37b, Bu 33b and Bu 33b caused an increase in pelvic elevation, which could be the result of a spermidine-like effect (Doyle et al, 1994). Bu 36b and Bu 43b were shown to cause sedation and decrease alertness.

In addition, each of the compounds caused a mild or moderate tremor. This polyamine-like effect is similar to a mild spermine-like action. Bu 33b may also affect opiate homeostasis, as a tail elevation or straub tail was observed. There is strong evidence that there are interactions between NMDA receptor antagonists and the opiate system (Bespalov et al, 1998)

Bu 37b, Bu 33b and Bu 40b were the least active. This is interesting as these compounds were shown to have low inhibitory activity on glutamate receptors in the Fixon-Owoo study (2003). Bu 31b, Bu 43b and Bu 36b showed more behavioural activity generally. Bu 31b and Bu 36b were shown to be highly potent inhibitors of the NMDA receptors (Fixon-Owoo et al, 2003). It is interesting to note that Bu 31b, Bu 36b and Bu 43b were the most effective of the analogues tested in the spermine CNS excitation model.

7.2 Conclusion.

Two behavioural models were used to investigate the antagonist potential of various compounds. In relation to the most effective model to use it would seem prudent to suggest that the spermine induced CNS excitation model is the better of the two for the investigation of polyamine antagonists. In the $ACTH_{1-24}$ model there are too many unknown factors or mechanisms whereby $ACTH_{1-24}$ is mediating its effects. Ifenprodil, eliprodil, arcaine, memantine and N^1-dansylspermine were effective in both models. This evidence combined with previous findings would suggest that these compounds are elucidating their effects through NMDA receptors in particular the NR1/NR2B subunits in both models. It is not unreasonable given the evidence that these compounds could also be mediating their effects through calcium

channels in particular the L-type Ca^{2+} channels. The influence of L-type Ca^{2+} channels is highlighted in both models as there was inhibition in the development of both sets of behaviours when nisoldopine and nitrendipine were administered.

As there is limited information available on the novel compounds, it was interesting to investigate their antagonist potential *in vivo*. Bu 31b illustrated produced significant inhibition in both behavioural models. In the spermine model, Bu 36b and Bu 43b had the most potent effects. Bu 36b and Bu 43b had very little effect on $ACTH_{1-24}$ induced behaviours, perhaps illustrating $ACTH_{1-24}$ is elucidating it's effect through alternative mechanisms. This is further emphasised by the pronounced inhibition of Bu 33b in this model. Bu 33b had little effect in the spermine model. Bu 40b was only mildly effective in either model, and showed the least overall biological effects. The mechanism of action of these polyamine analogues may involve NMDA receptor inhibition, or possibly, an action via L-type Ca^{2+} channel inhibition, but more work is needed to clarify this issue. Bu 36b, Bu 43b, Bu 33b and Bu 31b in particular are worthy of further investigation.

The findings from chapter 5 have further developed an understanding in the mechanisms involved in the development of the behaviours studied. In the spermine induced excitation model, previous findings using polyamine antagonists hypothesised that the mechanism of action was mediated through an increase in polyamine, particularly spermine level. The results in this study have illustrated that there is indeed an increase in polyamine synthesis and turnover and this likely underpins the development of the fatal tonic convulsions. The $ACTH_{1-24}$ findings showed that although polyamine analogues can inhibit some ACTH effects, it is uncertain that ODC stimulation of polyamine synthesis underpins development of the behavioural effects to any great extent. The evidence presented from the behavioural models further extends the understanding of the underlying mechanisms involved. Known NMDA antagonists inhibited the development of behaviours in both models. The influence of these underlines NMDA involvement in both behavioural models. Furthermore, calcium channel antagonists, nisoldopine and

nitrendipine, also inhibited the development of behaviours in both models, highlighting that perhaps both models are elucidating their effects in-part through L-type Ca^{2+} channels.

When administered alone, the simple most obvious finding following administration of the novel analogues was the development of spermidine or spermine-like effects, suggesting polyamine-like effects at high doses of these compounds.

An optimum dose was needed to find an inhibition in behaviours. The initial dose used was 10mg/kg or 10μg/20μl. Depending on the reaction, doses was either increased or decreased. Whilst investigating the initial dose many animals were used. Future studies would require a much smaller amount of animals to be used.

7.3 Future studies.

In the spermine model more recent potential polyamine antagonists should be investigated. Recent developments, have led to the discovery of NR2B subtype selective receptor antagonists (McCauley, 2005). These compounds include phenols, divided further into piperidines and aminocyclohexanes. Other compounds include quinolines, amidines, heterocycles and peptides (McCauley, 2005). Various pharmaceutical companies have isolated compounds within each of these categories and they are currently under investigation. As part of future investigations, it would be interesting to procure these products and examine their antagonist potential in inhibiting spermine induced CNS excitation behaviours.

Recent studies have shown the influence of polyamines, spermine and spermidine, on inward rectifier potassium channels. It is thought that polyamines lead to neuronal damage by increasing rectification of Kir channels and increasing sensitivity to NMDA receptors, by increasing excitability. Due to the influence on these Kir channels, it would seem prudent to investigate the potential of potassium antagonists in the spermine induced CNS excitation model.

Within the $ACTH_{1-24}$ behavioural model, it would be interesting to examine e.g bicuclline, a selective competitive $GABA_A$ antagonist. Through the synthesis of polyamines it is know that Putrescine can be rapidly transformed into 4-aminobutyric acid (GABA) and its lactin and GABA may be further metabolised (Seiler, 1991). It is also known that polyamines can influence the $GABA_A$ receptor system (Brackley et al, 1990; Seiler, 1991).

Two further compounds which would be of interest to investigate in the future would be Ionomycin a Ca^{2+} ionophore and Oubain a Na^{2+} ionophore. Dot et al, (2000) investigated these two compounds in relation to their ability to inhibit the uptake of polyamines at the membrane surface. The effect of these compounds on $ACTH_{1-24}$-induced behavioural effects would be interesting to study.

Another compound is pinacidil, a potassium channel opener. Previous studies in rats have shown that pinacidil inhibited the behaviour effects of $ACTH_{1-24}$. This evidence indicates that the complex mechanism of $ACTH_{1-24}$ induced behavioural syndrome involves in part the closure of potassium channels in target neurones (Vergoni et al, 1995). A possible further study in this behavioural model would be to examine CRF, as CRF may act as a neurotransmitter within the CNS in response to stress which could implicate a further involvement of the adrenal hormonal system.

The primary investigations in examining the effects of $ACTH_{1-24}$ have looked at the behaviours in male mice or rats, for future investigation it would be of tremendous interest to investigate the effect of $ACTH_{1-24}$ in female mice. It would be anticipated that the same effects would be seen as of that which occurs in male mice in that these compounds would inhibit the behaviour effects of $ACTH_{1-24}$ administration. However, it is important to understand the influence of polyamines on female hormones. It has been already reported that ODC activity rises sharply in the late proestrous stage just before ovulation. Also reported was the increase of putrescine

and spermidine in the ovary. Uterine ODC can fluctuate during the estrous cycle, with highest activity during estrous (Raina & Janne, 1975). Another part of this investigation could involve administering $ACTH_{1-24}$ at the various stages of the estrous cycle and assessing behaviour.

Finally, it would be essential to investigate the therapeutic potential of the novel polyamine analogues from this study in a variety of other disease models. These could include a model of cerebral ischaemia, Alzheimer's disease, schizophrenia, and depression.

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Appendix 1.

Percentage of inhibition of compounds on spermine induced CNS excitation

Spermine Model

Old compounds			
Ifenprodil	3mg/kg = 20%	10mg/kg = 42%	20mg/kg = 26%
Eliprodil	10mg/kg = 14%	20mg/kg = 8%	30mg = 34%
Arcaine	10μg = 16%	20μg = 40%	30μg = 64%
Memantine	5mg/kg = 40%	10mg = 12%	48mg/kg = 48%
Nisoldipine	2mg/kg = 82%	5mg/kg = 68%	10mg = -5%
Nitrendipine	5mg/kg = 44%	10mg = 64%	20mg/kg = 54%
N^1Dansylspermine	5μg = 4%	10μg = 28%	20μg = 56%
Novel Compounds			
Bu 31b icv	10μg = -8%	20μg = 60%	30μg = 46%
ip	10mg/kg = 48%	20mg/kg = 40%	30mg/kg = 48%
Bu 37b icv	10μg = -44%	20μg = -20%	30μg = 20%
ip	10mg/kg = -8%	20mg/kg = -12%	30mg/kg = -10%
Bu 33b icv	10μg = 0%	20μg = 48%	30μg = 24%
ip	10mg/kg = 56%	20mg/kg = 40%	30mg/kg = 44%
Bu 40b icv	10μg = -26%	20μg = -8%	30μg = -36%
ip	10mg/kg = -26%	20mg/kg = 20%	30mg/kg = -12%
Bu 36b icv	10μg = -4%	20μg = 58%	30μg = 60%
ip	10mg/kg = 16%	20mg/kg = 50%	30mg/kg = 56%
Bu 43b icv	5μg = 4%	10μg = 68%	20μg = 100%
ip	5mg/kg = 38%	10mg/kg = 52%	20mg/kg = 64%

Appendix 2.

Percentage of inhibition of compounds on ($ACTH_{1-24}$) behaviours.

$ACTH_{1-24}$ Model

Old compounds			
Ifenprodil	3mg/kg = 20%	10mg/kg = 38%	20mg/kg = 73%
Eliprodil	10mg/kg = 52%	20mg/kg = 52%	30mg = 58%
Arcaine	5μg = 50%	10μg = 32%	20μg = 48%
Memantine	5mg/kg = 43%	10mg = 64%	20mg/kg = 63%
Nisoldipine	2mg/kg = 42%	5mg/kg = 45%	10mg = 49%
Nitrendipine	10mg/kg = 58%	20mg = 72%	30mg/kg = 66%
N^1Dansylspermine	5μg = 7%	10μg = 10%	20μg = 28%
Putrescine	120μg = 99.8%	240μg = 99.8%	480μg = 99.9%
DFMO	20μg = 44%	50μg = 48%	
Novel Compounds			
Bu 31b	10μg = 57%	20μg = 54%	30μg = 76%
Bu 37b	10μg = 20%	20μg = 40%	30μg = 64%
Bu 33b	10μg = 32%	20μg = 31%	30μg = 64%
Bu 40b	10μg = 14%	20μg = 28%	30μg = 30%
Bu 36b	5μg = 47%	10μg = 4%	20μg = 26%
Bu 43b	5μg = 19%	10μg = 19%	20μg = 39%