# THE EFFECT OF STEROIDS ON SEXUAL DIFFERENTIATION OF THE RAT CENTRAL NERVOUS SYSTEM

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بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيْمِ

*IN THE NAME OF ALLAH*

*THE MOST BENEFICENT AND MERCIFUL*

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## ABBREVIATIONS

**ADR** - Adrenaline

**AFP** - Alpha fetoprotein

**CNS** - Central nervous system

**DA** - Dopamine

**DHBA** - Dihydroxy benzylamine

**DHTB** -5α Dihydroxy testosterone benzoate

**DLN** - Dorsolateral nucleus

**HCl** - Hydrochloric acid

**5HIAA** - 5 Hydroxy indoleacetic acid

**HPLC-ECD** - High performance liquid chromatography with Electrochemical detection.

**HRP** - Horse radish peroxidase

**5HT** - 5 Hydroxytryptamine

**mCPP** - m-Chlorophenylpiperazine

**MHPG** - 3 methyl 4 hydroxy phenyl glycol

**NA** - Noradrenaline

**p-CPA** - P-chlorophenyl alanine

**RDLN** - Retrodorsolateral nucleus

**SNB** - Spinal nucleus of bulbocavernosus

**TP** - Testosterone propionate

**VM** - Ventromedial nucleus

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   11th Joint Meeting of British Endocrine Societies, Harrogate (March, 1992).

2. Taher, A., Duncan, A., Payne, A.P. & Gilmore, D.P.
   Do androgens and serotonin combine to control motor neuron numbers in the male lumbosacral spinal cord?
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## SUMMARY

Gonadal steroids have an organisational effect on the sexual differentiation of the mammalian central nervous system. In rodents, sex differences appear to be dependent upon the levels of circulating androgens during a limited perinatal period.

Some motor neuron groups in the lumbar and sacral spinal cord exhibit marked sex differences and the number of neurons present are regulated by perinatal androgen levels. These groups include the cremasteric nucleus, the spinal nucleus of bulbocavernosus (SNB) and the dorsolateral nucleus (DLN) which innervate perineal muscles responsible for penile erection and genital reflexes. Muscles are absent and neuron numbers are much reduced in adult females, although they develop similarly in both sexes until birth.There is a considerable body of evidence to show that the normal regression in neuron numbers in females can be prevented by androgen administration during the perinatal period. However, it is less clear whether supplementary androgen treatment given to males at this time can lead to supranormal neuron numbers and the present study uses an unworked strain of rats.

Serotoninergic neurons establish contact with these motor neuron groups early in development and the evidence suggests that serotonin suppression also increases the number of surviving neurons.Evidence has come from male or female rodents,while the present study uses males only. The use of 5HT receptor subtype agonists/antagonists in this field is entirely novel.

To examine the role of androgens in sexual differentiation of the spinal cord, pregnant female Albino-Swiss rats received 500 Ìg per day (administered subcutaneously) of either testosterone propionate (TP) or dihydrotestosterone benzoate (DHTB) during the last four days (17-21) of pregnancy. Alternatively, pups received these treatments for the first four days after birth (200Ìg per day subcutaneously). As adults, treated and untreated control males were killed by an overdose of ether and perfused with mammalian Ringer plus lignocaine followed by buffered 10% formalin. The spinal cord was removed by the blow out method and processed. The lumbosacral cord was sectioned at 100 Ìm on a vibratome and the number of neurons in the SNB, DLN, retrodorsolateral nucleus (RDLN) and ventromedial nucleus (VM) counted after staining with thionine.

Untreated control males possessed just under 200 SNB neurons. Motor neuron numbers in males which were treated prenatally with TP and postnatally with DHTB did not differ from controls. However, males which had been treated prenatally with DHTB, or postnatally with TP had significantly raised numbers of motor neurons in the SNB (+15-20%). Males treated postnatally with TP also had significantly raised numbers of motor neurons in the DLN. Perinatal androgens may thus increase motor neuron numbers in male rodents above normal levels.

To examine the role of biogenic amines in sexual differentiation of the spinal cord, male Albino-Swiss rats were given TP (200 µg/day subcutaneously) on Days 1-3, 4-6

and 7-9 postnatally. Controls were left untreated. Half of each group was also injected with the serotonin inhibitor p-Chlorophenylalanine (pCPA) 200 mg/kg intraperitoneally on Days 1-14 after birth. As adults, motor neuron numbers were counted in the SNB and DLN nuclei. Postnatal androgen and pCPA treatments both significantly raised SNB and DLN neuron numbers in males by some 15-20%. The two treatments did not have an additive effect. Preliminary data suggests that $5HT_1$ and $5HT_2$ receptor antagonists also increase motor neuron numbers when they are administered during the postnatal period.

For the determination of serotonin and 5 hydroxy indolacetic acid (5HIAA) in the lumbar and thoracic region of the spinal cord of developing Albino-Swiss rats, groups of animals were decapitated on postnatal Days 4, 12, 14 or 22. The spinal cord was removed and separated into lumbar and thoracic parts. The sections were homogenized and centrifuged at 3000 r.p.m. for 10 minutes. Amine concentrations in the resulting supernatant were measured by high performance liquid chromatography with electrochemical detection (HPLC ECD).

There were no sex differences in serotonin and 5HIAA concentrations at Day 4 after birth. Sex differences were however present on Day 12. The amount of serotonin in the lumbosacral region of female rats was 0.37ng/mg and in males 0.07ng/mg. The amount of 5HIAA in the lumbosacral region of the female rat was 0.08ng/mg and in the male 0.02ng/mg. Sex differences were also observed on postnatal Day 14. The amount of serotonin in female rats at that

time was 0.30ng/mg and in the males 0.07ng/mg. There were no sex differences in 5HIAA content on Day 14. On Day 22 after birth, the serotonin concentration in the lumbar region of female rats was 4.71ng/mg and in the males 4.36ng/mg. The 5HIAA concentration in female rats at the age of 22 days after birth was 1.47ng/mg and in the males 1.40ng/mg.

These studies have revealed that perinatal androgen treatment may increase motor neuron numbers. Evidence also suggests that serotonin suppression also increases the number of surviving motor neurons.

# GENERAL INTRODUCTION

## 1. INTRODUCTION

The brain, like the reproductive system, is inherently female, and remains so if not exposed to gonadal hormones at a critical period of its development. Androgens, secreted by the developing testes, act in an inductive capacity on the undifferentiated brain to bring about its masculinization during a limited period restricted to fetal and early post-natal life (Breedlove, 1984).

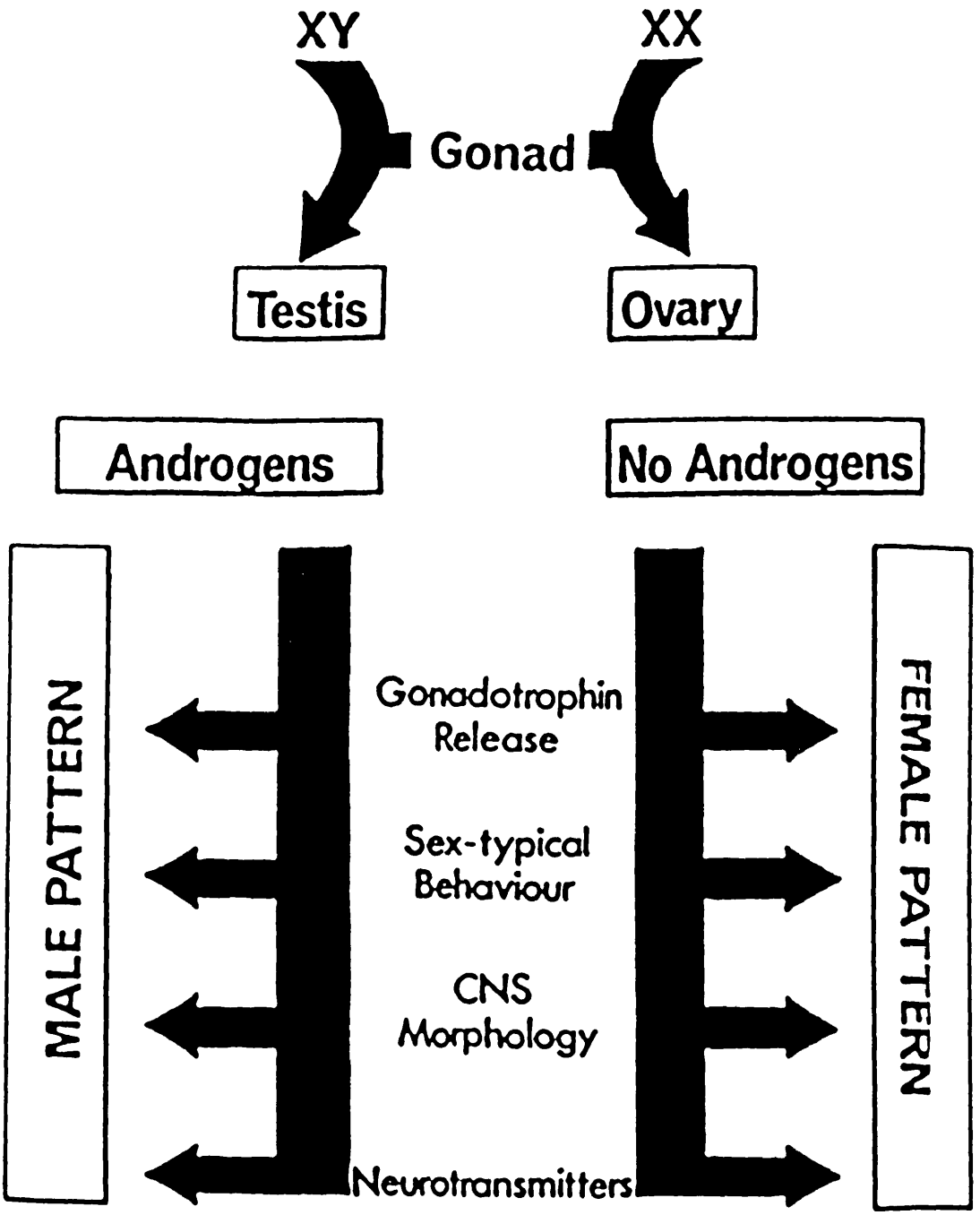
Dorner (1981) published evidence of the involvement of both steroid hormones and neurotransmitters in the sexual differentiation of the brain. Sex differences have been found in the levels of neurotransmitters in the hypothalamus, amygdala and mesencephalon (Siddiqui and Gilmore, 1988; Siddiqui, Gilmore and Clark, 1989).

The influence of exogenous steroids in altering brain development is clearly dose and time-dependent; both factors may vary for each functional system undergoing differentiation (Gorski, 1968; 1971).

Sexual differentiation of the brain is dependent on oestradiol (McEwen et al., 1977) whereas the masculinization of the external genitalia is dependent on 5 $\alpha$-dihydrotestosterone (DHT) (Breedlove, 1984). Both hormones are derived from testosterone, the former by its aromatization and the latter by the action of the enzyme 5$\alpha$-reductase.

**Figure 1**

Mammalian sexual differentiation.


XY
XX
Gonad
Testis
Ovary
Androgens
No Androgens
MALE PATTERN
FEMALE PATTERN
Gonadotrophin Release
Sex-typical Behaviour
CNS Morphology
Neurotransmitters

It is clear that the action of steroid hormones on the developing brain may result in sexually dimorphic patterns of behaviour such as sexual behaviour (see Baum et al., 1990).

The androgens elicit permanent structural changes in the brain, particularly in the medial preoptic region of the anterior hypothalamus, to bring this differentiation. Masculinization of the rodent brain is controlled by testicular hormones acting during the early perinatal period (Barraclough and Gorski, 1961). Female rats treated with androgens during the first few days of life will, at puberty, show a few ovulatory cycles prior to becoming permanently sterile. This process is termed the delayed anovulatory syndrome.

For complete masculinization to occur, testosterone needs to be converted to oestradiol-17$\beta$ (McDonald & Doughty, 1974). Oestrogen treatment before Day 5 postnatally has been shown to be just as effective as testosterone in causing anovulatory sterility in female rats (Christensen and Gorski, 1978).

It has been suggested that differences between male and female in both behaviour and physiology may be the result of differences in the functional organization of the brain (Gorski,1973; McEwen et al.,1974). Sexual differentiation of the neural control of reproductive function in mammals is thought to result from exposure of the brain to testicular androgens during a limited

critical period of neural differentiation (Gorski, 1971).

In the rat there is evidence however to suggest that the initial mode of androgen action may involve its intraneuronal aromatization to oestrogen (Naftoline et al.,1971) in such neural sites as the preoptic area, hypothalamus and amygdala. The mediation of the action of testosterone by oestradiol has also been proven in the golden hamster(Carter et al.,1972) and in the mouse ( Toran - Allerand, 1976).

In the present study the Albino Swiss rat was chosen because behavioural dimorphism is apparent in response to testosterone treatment. In addition there is relatively little sexual dimorphism in brain size, simplifying the interpretation of possible neuronal differences.

## 2. SEXUAL DIFFERENTIATION IN THE CENTRAL NERVOUS SYSTEM (CNS)

The CNS shows sex differences in the control of gonadotrophin release and in the regulation of many types of behaviour. There is strong evidence to indicate that neurotransmitters exert a vital role in the regulation of hypothalamic stimulatory and inhibitory hormone production and in their secretion both in fetal and adult life (Dorner, Hecht and Hinz, 1976; Reznikov, 1978). These hypothalamic neurotransmitters in turn control, either directly or indirectly, the synthesis and release of anterior pituitary hormones (Barraclough and Wise, 1982). It would seem that androgens acting neonatally promote the

development of aminergic axonal processes and interneuronal connections (Siddiqui et al., 1989). Evidence has now accumulated to indicate that the brain neurotransmitter systems become sexually diferentiated through the action of testicular androgens during a critical period restricted to fetal and/or early postnatal life (Dohler et al., 1982; Vaccari, 1980; De Vries et al., 1984; see De Vries, 1990).

It has been suggested that there are specific sex centres within the brain and other regions of the CNS which are responsive to gonadal hormones and indispensable for the cyclic release of the ovulating hormones in the female and for the expression of behaviour in both sexes.

As early as 1936 Pfeiffer demonstrated that sexual differentiation ofthe brain in the rat is determined by the hormonal state at a specific developmental period. He also concluded that the anterior pituitary gland of the male rat became differentiated during development by exposure to testicular hormones and could thereafter not support cyclic gonadotrophin secretion. Harris and Jacobson (1952) transplanted the pituitary of a male rat to a new position under the hypothalamus of a hypophysectomised female, where it could be revascularised by the hypophyseal portal vessels. Later it became clear it was the CNS and not the anterior pituitary that became sexually differentiated. In many developing mammals plasma concentrations of androgens are higher in the male than in the female only during a limited critical period (Weisz and Ward, 1980). Phoenix

et al., (1959) proposed that during development steroid hormones have the capacity to organize or establish, at least in functional terms, the neural circuits that will regulate neuroendocrine function in the adult.

## 3. POSSIBLE SITES OF SEXUAL DIFFERENTIATION IN THE CENTRAL NERVOUS SYSTEM

The actions of sex steroids on brain tissue have been examined from a variety of perspectives. The regional localization of steroid-concentrating areas in the brain have been explored by autoradiography (Pfaff and Keiner, 1973). It has been found that such areas include:-

a) Preoptic hypothalamic structures such as the medial preoptic area, the medial anterior hypothalamus, ventromedial nucleus, the arcuate nucleus and the ventral premammillary nucleus.

b) Limbic structures such as the medial and cortical nuclei of the amygdala, the lateral septum, the bed nucleus of the stria terminalis, the diagonal band of Broce, the olfactory tubercle, the ventral hippocampus and the prepiriform and entorhinal cortex.

Labelled cells were also found in the lateral and ventrolateral portions of the mesencephalic central grey matter (Pfaff and Keiner, 1973). Compared to the above mentioned regions most other regions of the nervous system, including the spinal cord, have a very small number of labelled cells.

The sum of the available evidence from many investigators (Dohler et al., 1982; Jacobson and Gorski, 1981; Arnold and Gorski, 1984), indicates that the sexually dimorphic nucleus of the preoptic area (SDN-POA) appears to be a morphological marker of the process of sexual differentiation of the rat brain. Jacobson et al., (1985) demonstrated that in an autoradiographic study following administration of [$^3$H] thymidine to rats on Day 18 of gestation, the size, number and location of labelled cells within the medial preoptic area was determined. It appeared that the labelled cells grew in size during the early postnatal period and also migrate from the more ventral aspects of the medial preoptic area (MPOA) to aggregate and form the (SDN-POA). Furthermore, there is a significant decrease in the number of labelled cells by day 32 post-fertilization (day 10 of postnatal life).

Swaab and Fliers (1985) described a sexually dimorphic cell group in the preoptic area of the human hypothalamus. The medial preoptic area is a relatively large and complex region in the hypothalamus and is implicated in the control of gonadotrophin release and sexual behaviour in many species (Ayoub et al., 1983; Gorski, 1971).

Autoradiographic studies on rat diencephalon and limbic structures have shown that the final cell divisions of the neuroblasts which give rise to preoptic, arcuate or amygdaloid neurons occur 13-18 days after conception. At the neonatal stage the neurophil matrix of these areas

is still characterized by the presence of extracellular space, the presence of growth cones and paucity of synapses (Arai and Matsumoto,1978); thus the environment of these areas is easily accessible to sex steroids.

Arai (1981) described that sex steroids played a significant role in modulating post-natal neuronal maturation and neural circuit formation. Sexual dimorphism of morphological parameters has also been demonstrated in certain brain areas where receptors for sex steroids are abundant.

## 4. SEXUAL DIMORPHISM IN THE CNS

There are morphological sex differences in various regions of the mammalian CNS. Testicular androgens, acting during the perinatal period, may alter functional neuronal development in the following ways:

1. By changing the number of receptors for the various neurotransmitters (Arimatsu et al., 1981), or by affecting the enzymes controlling synaptic transmission (McEwen, 1983).
2. By interference with maturational or metabolic aspects of the receptor system (Vertes and King, 1971).
3. By altering membrane properties (Toran-Allerand, 1984).

The morphological consequences of perinatal exposure of the CNS to endogenous and exogenous hormones are numerous. Some important sex differences in the CNS brought about by androgens acting during the perinatal period are summarized in Table 1.

Table 1

STEROID-DEPENDENT STRUCTURAL DIMORPHISM IN THE VERTEBRATE CNS (MODIFIED FROM TORAN-ALLERAND, 1984).

| CYTOLOGICAL DIFFERENCE | REGION | ANIMAL |
|---|---|---|
| Neuronal numbers (Gorski et al., 1978; Breedlove and Arnold, 1980; 1983b) | POA, amygdala, spinal cord, vocal centres | Rat, mouse, song-bird, frog |
| Neuronal size (Breedlove and Arnold, 1980; 1983b) | POA, ventromedial area, amygdala, habenula, hippocampus, cerebral cortex, vocal centres. | Rat, mouse, monkey, song-bird, frog |
| Dendritic length/branching (Greenough et al., 1977; Ayoub et al., 1982) | POA, suprachiasmatic, vocal centres | Rat, monkey, song-bird, hamster, frog |
| Dendritic spines (Ayoub et al., 1982) | POA, hippocampus, vocal centres | Rat, monkey, song-bird |
| Number of synapses (Ayoub et al., 1982) | Arcuate and suprachiasmatic nuclei | Rat |
| Type of synapses | Suprachiasmatic nucleus | Rat |
| Synaptic organization (Raisman and Field, 1973; Nishizuaka and Arai, 1981) | POA, arcuate nucleus, amygdala | Rat |
| Synaptic organelles (Le blond, 1982) | Arcuate and supra chiasmatic nuclei, amygdala | Rat |
| Axonal density (De vries et al., 1981) | Hippocampus (sympathetic); septum and habenula (vasse pressinergic) | Rat |
| Regional nuclear volume (Gorski et al., 1978; Breedlove and Arnold, 1981) | POA, spinal cord, amygdala, vocal centres | Rat, mouse, frog, song-bird |
| Volume of neural structures (Fitch et al., 1990; Pappas et al., 1978) | Cerebral cortex<br>Corpus callosum | Rat<br>Human |
| Besides these, some other structural dimorphisms have also been noted. These are:- | | |
| Neuronal number (Wright et al., 1983) | Superior cervical ganglion | Rat |
| Size (Meites and Kragt, 1973) | Pituitary gland | Rat |
| Number of granuale cells (Segovia et al., 1986) | Accessory olfactory bulb | Rat |

## 5. DEVELOPMENT OF THE SPINAL CORD

When the neural tube is closing its wall consists of a single layer of columnar neural epithelial cells, the extremities of which abut on internal and external limiting membranes. The columnar cells increase in length and develop numerous longitudinally disposed microtubules. The borders of their luminal ends are firmly attached to adjacent cells by junctional complexes, the cytoplasmic aspect of the complexes being associated with a dense paraluminal web of microfilaments (Watterson, 1965). It is proposed that this disposition of organelles imparts a slight wedge of conformation on the cells, resulting in neural groove and eventually neural tube formation (Grays, 1989).

As the cells elongate their nuclei become clustered at varying depths in the deeper parts of the wall (near the internal limited membrane) and, for a period, the epithelium is pseudostratified with an inner nucleated zone. The outer zone is composed of cytoplasmic processes of the cells. Soon, however, some peripheral cytoplasmic processes become detached from the(basal) external limiting membrane, the cells appear close to the inner membrane. There is mitotic division of the cells, which migrate outwards to take up an intermediate position in the wall of the tube (Grays, 1989).

## 6. STRUCTURE AND SEX DIFFERENCES IN THE RAT SPINAL CORD

**MACROSCOPIC STRUCTURE:-**

This cylindrical mass of nervous tissue represents the original neural tube, much modified in its development. Being firmly anchored rostrally to the bulging brain it is pulled up during development and in the adult reaches only to the level of the 1st or 2nd lumbar vertebrae. The roots of the lumbar, sacral and coccygeal nerves are greatly elongated to form a thick bundle called the cauda equina. The spinal cord is flattened ventrodorsally and has two swellings; the rostral forms the brachial plexus, the caudal the lumbar and sacral plexuses.

**MICROSCOPIC STRUCTURE:-**

The spinal cord consists of nerve cell bodies and their processes. The cell bodies of the neurons and the complete glial cells are massed together in columns.

## 7. SEXUALLY DIMORPHIC NUCLEI IN THE SPINAL CORD

There are morphological sex differences in various areas of the mammalian CNS. One of the important sites is the lumbosacral region of the spinal cord containing sexually dimorphic motor neuron groups, the spinal nucleus of bulbocavernosus (SNB), the dorsolateral nucleus (DLN) (Breedlove, 1984; Currie et al., 1990; Davidson et al., 1990) and cremasteric nucleus (Kojima & Sano, 1984). In this study the SNB and DLN were examined in detail. .pa

**SPINAL NUCLEUS OF BULBO CAVERNOSUS (SNB)**

**Situation:-**

In the rat the SNB neurons are located dorsomedially in the ventral funiculus and at the medial border of the ventral horn close to the central canal between regions $L_5$ to $S_1$ of the spinal cord. The SNB is situated 50 to 250 µm from the midline and 200 to 400 µm below the ventral margin of the central canal (Breedlove and Arnold, 1981; Wee and Clemens, 1987).

**Characteristics:-**

The neurons are large (30-50µm in diameter) multipolar, and stain densely for Nissl substances. The cells are comparable to Onuf's nucleus in the dog and human (Schroder, 1980). These motor neurons innervate striated penile muscles involved in copulatory reflexes in the male rat (Sachs, 1982; Hart and Melese-D'Hospital, 1983) and mouse (Elmore and Sachs, 1988). They project to the bulbocavernosus (BC) or levator ani (LA) muscles (Breedlove and Arnold, 1980; Schroder, 1980; Veyama et al., 1987) as well as to the anal sphincter (McKenna and Nadelhaft, 1986).

In the male rat the SNB is composed of around 200 cells and in the female about 40 (Currie et al., 1990; Davidson et al., 1990).

**DORSOLATERAL NUCLEUS (DLN)**

This is the other sexually dimorphic nucleus present in the spinal cord. It forms a cluster of cells within the dorsolateral aspect of the ventral horn of the spinal cord.

The neurons are also large, multipolar and dense staining with clear nuclei that have a prominent nucleolus. The DLN neurons project to the ischiocavernosus (IC) muscles, as well as to the urethral sphincter (McKenna and Nadelhaft, 1986; Micevych et al., 1985; Schroder, 1980; Veyama et al., 1987).

In the male rat the number of DLN neurons is about 26 per 100Ìm section of spinal cord between $L_5$ to $S_1$ and in the female it is about 14 (Cowburn and Payne, 1992).

These motor neurons and their target muscles are known to accumulate androgens and are dependent on testosterone or DHT for their maintenance (Breedlove and Arnold, 1983b). Castration of adult male rats results in a decreased size of some of the SNB motor neurons and a reduction of muscle mass, but their number is unchanged (Breedlove and Arnold, 1981). However, in some strains of mice the number of SNB neurons is also reduced by castration (Wee and Clemens, 1987). Androgen administration prevents this reduction.

Autoradiographic studies have revealed that all motor neurons in the lumbar region of the rat spinal cord are capable of accumulating testosterone and DHT, but not oestrogen (Breedlove and Arnold, 1983b). Nevertheless, those motor neurons involved in specially masculine functions take up significantly more label than those not involved in such functions, such as the retrodorsolateral nucleus (RDLN), which innervates leg muscles (Breedlove and

Arnold, 1980).

Both the SNB-innervated muscles, (the levator ani, and the bulbocavernosus) and the SNB motor neurons are dependent upon the hormone milieu during the perinatal period for their survival. During the prenatal period the perineal muscles develop in both sexes as do the motor neurons which innervate them (Cihak et al., 1970; Nordeen et al., 1985). After birth there is substantial neuronal cell death, but this is less in normal males and in females given testosterone and DHTB on the 2nd day postnatally than in untreated females (Nordeen et al., 1985). In untreated females and in androgen-insensitive mutant (Tfm) males the motor neuron numbers fall to less than a third of those in control males. Soma size is reduced in the remaining cells and the muscle completely disappears (Breedlove and Arnold, 1981). Prenatal exposure of males to the drug flutamide, plus neonatal castration, completely sex-reverses the SNB numbers (Breedlove and Arnold, 1983a).

Treatment of Sprague-Dawley rat pups postnatally with oestradiol does not prevent SNB cell death in females or in males castrated at birth (Breedlove et al., 1982). However, this finding may be due to strain differences as oestradiol benzoate has been reported to enhance SNB neuron survival in female Albino-Swiss rats (Currie et al., 1990). The effect of androgens on the survival of motor neurons and their target muscles is not yet fully understood. It has been shown that testosterone will support BC neuron survival in the absence of a descending supraspinal neural

input (Fishman and Breedlove, 1988). Neuromuscular junctions are already established before androgen exposure is necessary for their survival. Therefore, androgens are not necessary for the initial formation of the junction (Rand and Breedlove, 1987). From both of these findings, it has been suggested that androgens could act in the following way:

1) Directly upon -
   a) muscles and
   b) neurons
2) Androgens act on muscle and this in turn promotes motor neuron survival via trophic substance released by the muscle.

## 8. STEROIDOGENESIS

The steroids comprise a large group of compounds all derived from a common sterol precursor cholesterol. Cholesterol is a lipid and it is synthesised from acetate in many tissues of the body (Johnson and Everitt, 1988). It is a molecule of major importance in maintaining structural integrity of the plasma membrane of the cell. Steroids can exert profound effects on the morphogenesis and survival of specific neurons resulting in marked sex differences in CNS structure (see Arnold and Gorski, 1984).

## 9. MECHANISM OF ACTION OF STEROID HORMONES

When a steroid encounters a compatible receptor within a target tissue and combines with it, the receptor undergoes a conformational change. After being activated in this way, the steroid receptor complex is able to bind to a specific DNA sequence in the chromatin, the so-called acceptor site. The interaction between the complex and the chromatin acceptor site is associated with an increase in production of mRNA. Following this early rise in mRNA production and in the continuing presence and binding of the complex to the chromatin, a more general stimulation of nucleolar and transfer RNA synthesis occurs and the synthetic machinery of the cell increases (see Johnson and Everitt, 1988).

## 10. MODE OF ACTION OF STEROID HORMONE IN CNS

In the brain, cells binding oestrogen are found predominantly in the medial preoptic area, the tuberal hypothalamus and in specific regions such as the amygdala and in the region of the midbrain deep to the tectum (Pfaff and Keiner, 1973; Barley et al., 1977). The distribution of neurons concentrating tritiated testosterone is similar to those that concentrate oestrogen, though not identical. However, interpretation is complicated by the fact that testosterone is extensively converted to oestradiol in the brain (Lieberburg et al., 1977). The aromatizing enzyme system that converts testosterone to oestradiol is

concentrated in those areas that have oestrogen receptor sites.

Some possible mechanisms by which steroids might act to bring about sexual differentiation of the CNS are as follows:

1. They may stimulate neuronal proliferation (Arnold and Gorski, 1984).
2. They may prevent neuronal death (Oppenheim, 1981).
3. There is a possibility that steroids might alter neuronal specification, migration or morphological development (Gorski, 1984).
4. Steroids in the vicinity of the SDN-POA may promote the aggregation of these neurons (Jacobson et al., 1985).

The neurons of the medial preoptic area have been reported to become post-mitotic by about day 16 of gestation in rats (Anderson, 1978). However, Jacobson and Gorski (1981) have revealed that mitotic activity in those cells which eventually form the neurons of the SDN-POA is still occurring on day 18, almost two days after these neurons stop dividing.

In the rat most SNB neuronal mitotic divisions occur on day 12 of gestation with a few taking place as late as day 14 (Breedlove, 1983). Since testosterone propionate injection of the female will augment adult SNB neuron numbers, when the injections are begun on Day 16 of gestation or even postnatally, it is unlikely that the administration alters SNB proliferation. This conclusion is strengthened considerably by the observation from

thymidine autoradiographic studies that females exposed to tritiated thymidine on Day 12 of gestation and then injected with TP postnatally, possess a masculinized SNB whose neurons are labelled with thymidine. This indicates that the neurons present in the SNB have already proliferated by Day 12, before the androgen injection (Breedlove and Arnold, 1983b).

## 11. BRAIN STEROID RECEPTORS AND THEIR ROLE

The brain's response to steroid hormones has been reported to alter during its development (Donovan and Van der Werff ten Bosch, 1959). Interaction of steroid hormones with specific brain receptors may be the basis of the central action of the hormone (Kato and Onouchi, 1977).

It has been postulated that oestrogen receptors in the neonatal brain play an exclusive role in inducing sexual differentiation of the hypothalamus of the rat (Barley et al., 1977; Naftolin and Ryan, 1975). However, hypothalamic oestrogen receptors appear only in very small amounts at seven days of age (Plapinger and McEwen, 1973; Kato et al., 1974).

## 12. ROLE OF AROMATIZATION IN THE SEXUAL DIFFERENTIATION OF THE BRAIN

McDonald and Doughty (1974) were the first to propose that sex behaviour was dependent on the conversion of androgens to oestrogens within the brain.

Aromatization requires specific enzymes which are only present in certain brain areas including the anterior and medial basal hypothalamus and amygdala. Neurons in these regions contain high affinity oestrogen-binding macromolecules and "receptors" which bind the locally produced oestradiol. However, there is a great deal of species variability. Even within a species differences exist between regions and between the types of sexually differentiated functions with respect to the extent to which oestrogen is required for sexual differentiation of the CNS.

In rats and mice, for which brain sexual differentiation depends on aromatization, intrahypothalamic implants of testosterone or oestradiol-17β are equally effective in eliciting masculinization of reproductive function and sexual behaviour (Christensen and Gorski, 1978). Anti-oestrogens have been shown to block both testosterone- and oestradiol-induced masculinization and aromatase inhibitors attenuate the masculinizing effect of both endogenous and exogenous testosterone (Doughty et al., 1975; Brown-Grant, 1974; McEwen et al., 1977). Non-aromatizable androgens such as DHTB appear to be largely ineffective in bringing about sexual differentiation (McDonald and Doughty, 1974). Testosterone-induced sexual differentiation is inhibited by androgen antagonists, as well as by oestrogen antagonists (Gladue and Clemens, 1978).

## 13. OESTRADIOL BINDING PROTEIN (α FETOPROTEIN) IN THE BRAIN AND ITS ROLE

In placental mammals, the fetus is continually exposed to endogenous oestrogen carried in the blood from the placenta. If oestrogen formation within the brain plays a vital role in sexual differentiation, then it follows that the female fetus must somehow be protected from the effects of circulating oestrogen.

This protection in rodents is brought about by the binding of these oestrogens to α-fetoprotein (McEwan et al., 1975; Vannier and Raynaud, 1975). Because α-fetoprotein does not bind testosterone, this hormone is free to enter the brain where it can be converted to oestrogen and interact with cellular oestrogen receptors (Plapinger and McEwen, 1973; Ali et al., 1981). The administration of antibodies to α-fetoprotein in newborn female rats produces effects on sexual development that resemble those of oestradiol injections (Mizejewski et al., 1980).

## 14. SEROTONIN PATHWAYS IN THE BRAIN

Serotonin (5-hydroxytryptamine: 5HT) has been proposed as a regulator of neuronal development (Lauder, 1983). There are many reports that neurotransmitters and neuropeptides can act as trophic agents during development of the nervous system (for review, see Mattson, 1988). Serotonin is a neurotransmitter found in certain neurons of the brain stem, which have extensive axonal projections in

the central nervous system (Wilson and Molliver, 1991).

The two main sources of ascending serotoninergic projections are the dorsal raphe (DR) and the median raphe (MR) (Consolazione and Cuello, 1982). The DR is the larger and contains the highest density of serotoninergic cell bodies in the brain (Pfister and Danner, 1980). The ascending projections of the raphe nuclei travel via two main routes which converge at the level of the hypothalamus in the medial forebrain bundle (Takagi et al., 1980).

The two raphe nuclei project to a variety of forebrain areas, the main targets being the olfactory bulb, the hypothalamus, the septal area, the thalamus, the caudate-putamen, the hippocampal region, the amygdala and the cerebral cortex (Molliver, 1987).

Anterograde and retrograde tracer studies have implicated the striatum as the major target for ascending DR fibres, whereas MR fibres primarily project to targets in the ventral forebrain, including the nucleus accumbens and the hippocampus (Vertes and Martin, 1988).

In simplified terms the most prominent groups are the nucleus raphe pallidus, nucleus raphe obscurus and nucleus raphe magnus which give rise to descending projections to the spinal cord.

Serotonin (5HT) neurons caudally directed from raphe nuclei invade the spinal cord at embryonic Day 14 and reach the caudal most levels by embryonic Days 16-17 (Rajaofetra et al, 1989). Axons are seen by embryonic Day 15 at a

cervical level and at upper thoracic levels, to invade the presumptive gray matter from the anterior and lateral funiculi. By embryonic Day 16, at thoracic levels, the anterior horn and the intermedio-lateral columns are profusedly innervated by very thin varicose fibres (Rajaofetra, 1989).

After birth serotonin innervation forms a diffuse network at the thoracic level of the intermediolateral column and at the cervical and lumbar levels of the anterior horn. The adult pattern is reached by postnatal Day 21. The growth of axons toward the dorsal horn becomes noticeable by embryonic Day 19 at all spinal levels, when fibres invade the neck of the horn from the lateral funiculus and innervation proceeds diffusely until postnatal Day 5. By postnatal Day 7 thin fibres course dorsally and laterally along the border of the gray matter and ramify profusely.

## 15. SEROTONINERGIC INFLUENCE ON SEXUAL DIFFERENTIATION OF THE RAT BRAIN AND ON SEXUAL BEHAVIOUR

The possibility has been raised that neurohumoral agents which normally act as neurotransmitters in the adult brain might play a role during embryogenesis in the regulation of brain development (Lauder et al., 1980; McMahon, 1974). The result of a combined fluorescence histochemical and [$^3$H] thymidine autoradiographic study on the development of monoamine cells of the nucleus locus coeruleus, raphe nuclei and substantia nigra in the rat

(Stensas, 1967) led to the speculation that such monoaminergic neurons might regulate the onset of differentiation of cells to which they will ultimately project in the adult (Lauder and Bloom, 1974).

Neurotransmitters and neuropeptides have been reported to act as trophic agents during development of nervous system (Mattson, 1988). Serotonin has been implicated in the development of the nervous system in both invertebrates and vertebrates and may influence cell division, migration, morphogenesis and synaptogenesis (Forda and Kelly, 1985; Lauder et al., 1981; Gromova et al., 1983).

Earlier studies have sugested that the serotonin may act as an intermediary in the events leading to the sexually dimorphic organization of the brain (Dorner, 1981). Sex differences in the levels of serotonin have been demonstrated and these may be altered by neonatal exposure to testosterone (Giulian et al., 1973; Walker and Timiras, 1980; Siddiqui et al., 1989).

It is thought likely that the steroids influence neuronal organization since there are steroid- dependent sexually dimorphic neuronal structures in specific hypothalamic and amygdaloid areas (Arai, 1981; Matsumoto & Arai, 1980; Raisman and Field, 1973). It follows from this that neurotransmitter activity might also be different in the sexes and may well be involved in sexual differentiation (Wilson et al., 1986). This

hypothesis is supported by the fact that administration of pharmacological agents, known to alter neurotransmitter activity, can effect sexually differentiated behaviour in adulthood (Hull et al., 1984).

Since 5HT systems have an influence on proliferative differentiation of neuronal and glial precursor cells in the developing nervous system (Lauder et al., 1982) and assuming there is sexually differentiated 5 HT activity induced by neonatal steroids, perhaps 5HT may be involved in the sexual differentiation of neuronal structures (Wilson et al., 1986).

The organizational effects of serotonin upon the CNS may be provided by comparing those areas which exhibit sexual dimorphism with other regions that do not. A cluster of neurons in the medial preoptic area of the rat brain, called the sexually dimorphic nucleus, is larger in males than females (Bleir et al., 1982; Swaab and Hoffman, 1988). This nucleus, which is proportionally more dense in the female, is surrounded by a relatively dense distribution of serotonin immunoreactive fibres (Simerly et al., 1984). Furthermore, manipulation of serotonin levels, before birth has been reported to increase the size of the sexually dimorphic nucleus of the medial preoptic area (Handa et al., 1986; Jarzab et al., 1990).

Perinatal treatments which alter serotonin biosynthesis also affect sexually dimorphic behaviours in adulthood (Hyppa et al., 1972; Jarzab & Dohler, 1984). Additionally in adulthood, serotonin administration has

been shown to inhibit masculine reproductive behaviour (Gessa et al., 1970). In homotypical sexual activity, neonatal 5HT does not appear to have any influence on the development of feminine behaviour and seems to be inhibitory for masculine behaviour. Possibly the fall in 5HT activity on day 14 in males specifically removes an antagonistic influence on the androgenizing and defeminizing activity of neonatal testosterone, while the rise in 5HT activity in the females may have a protective effect against any circulating testosterone (Wilson et al., 1986). Thus the indolaminergic system would appear to play a role in the expression of sex specific patterns of brain growth and development and in particular affect these structures of the brain associated with the control of masculine sexual behaviour in adulthood.

Serotonin has been shown to affect the development of serotoninergic neurons themselves and also their targets within the CNS (Forda and Kelly, 1985; Gromova et al., 1983). It is well known that motor neurons are surrounded by the terminals of a descending pathway of serotoninergic fibres utilising a wide variety of transmitters, including indoleamines, enkephalines and substance P (Micevych et al., 1985; Tashiro et al., 1989).

The motor neuron group in the first lumbar segment is the cremasteric nucleus which shows a sexually dimorphic pattern of serotonin innervation (Kojima & Sano, 1984). The sexually dimorphic nucleus of the medial preoptic area also

shows sexually dimorphic patterns of serotonin innervation (Simerly et al., 1984); this pattern can be altered by perinatal hormone manipulation (Simerly et al., 1985). Moreover serotonin manipulation during development can alter the size of the sexually dimorphic medial preoptic nucleus (Handa et al., 1986).

In relation to the above we have attempted to determine whether serotonin manipulation during early development could alter the number of certain groups of sexually dimorphic motor neurons, the SNB and DLN present in the lumbar and sacral part of the spinal cord, which have a serotoninergic input (Newton & Hamill, 1989; Tashiro et al., 1989).

## 16. SEX DIFFERENCES OF NEUROTRANSMITTERS IN THE CENTRAL NERVOUS SYSTEM

All the major monoamine systems act as developmental regulators in the immature brain. They include serotonin (Shemer et al., 1991), dopamine (Kalsbeck et al., 1987) and noradrenaline (Parnaveals and Blue, 1982). Sex differences have been found in the neurotransmitter content and enzyme activity of the noradrenergic, dopaminergic and serotoninergic systems (Vaccari, 1980).

Elevated serotonin levels in the female rat brain have been reported to be present as early as the 12th postnatal day (Ladosky and Gaziri, 1970; Giulian et al., 1973; Siddiqui et al., 1989) and in the hamster (Johnston et al., 1990).

The fact that the critical period for steroid-induced masculinization of the brain is limited to the first 10 days of life, whereas sex differences in brain serotonin levels are observed only after this period, favours the assumption that the reduced serotonin levels seen in the male rodent are a consequence of masculinization rather than a cause, or could be a secondary stage of differentiation.

## AIMS OF THE STUDY

The purpose of this investigation was to examine the effects of androgen and serotonin manipulation upon sexual differentiation of the CNS. In particular it was hoped to learn more about:-

1. The involvement of steroids in the development of sexual dimorphism in the mammalian CNS.
2. The inter-relationship between steroids and brain serotonin concentrations in bringing about sexual differentiation of the CNS.
3. It was also planned to investigate in more detail differences in the sexually dimorphic nuclei present in the lumbosacral region of the spinal cord, and to determine whether sex differences might exist in concentrations of biogenic amines in the lumbar and thoracic portions of the developing rat spinal cord.

# MATERIALS AND METHODS

**ANIMALS**:-

The Albino-Swiss rats used in the study were obtained from laboratory stock established in the Anatomy Department of the University of Glasgow. The animals were maintained in a room at a temperature of 20 $\pm$ 2$^{o}$C. They were housed in North Kent Plastic cages measuring 45-28-22cm and allowed food and water ad libitum.

**BREEDING**:-

Female rats of this strain have regular four day oestrous cycles. They were time-mated and checked for sperm in the vagina the following morning. The day following the positive sperm test was denoted as Day 1 of pregnancy. The pregnant rats were then transferred to a fresh cage and, when approaching term, were checked every morning and evening for the presence of litters. Any new litters detected in the morning were presumed to have been born during the previous night. Newborn rats were collected and sexed by measuring their anogenital distance. The distance in the male is about two-three times longer than that in the female. At 21-23 days postpartum the animals were weaned and rehoused according to sex.

**EXPERIMENT 1**:- **Sex differences in motor neuron numbers in the lumbosacral spinal cord.**

The purpose of this experiment was to determine whether any differences exist in certain motor neuron groups between male and female.

Fifteen untreated adult male and 15 untreated adult female Albino Swiss rats were killed and the lubosacral spinal

cord removed for counting the motor neurons.

**EXPERIMENT 2:- Perinatal androgen treatment and its effect on sexually dimorphic spinal motor neurons in male rats.**

The purpose was to observe any changes in the number of SNB and DLN motor neurons in the lumbosacral spinal cord of male rats.

Pregnant rats were given 500 µg/day of testosterone propionate (TP) or 500 µg/day of dihydrotestosterone benzoate (DHTB) subcutaneously during the last four days (17-21) of pregnancy. Five Albino-Swiss pups were injected subcutaneously with DHTB (200 µg/day) on Days 1-4 postnatally.

The hormones were injected in arachis oil (0.1ml) which was supplied by Sigma Chemical Co. Ltd. The male pups born to these TP-, DHTB-treated mothers were used.

Five pups were taken from prenatal TP-treated mothers and 11 pups were taken from prenatal DHTB-treated mothers. Controls (n = 15) received buffer only. Controls and treated rats were maintained as previously described.

**EXPERIMENT 3:- Effects of a non-steroidal anti-androgen (flutamide) on sexually dimorphic spinal motor neurons in male rats.**

The purpose of this experiment was to determine the number of SNB and DLN motor neurons in lumbosacral spinal cord after anti-androgen treatment perinatally.

Pregnant rats were given flutamide 10mg/kg body weight/day)

subcutaneously during the last four days (17-21) of pregnancy. In another series rat pups (n = 10) were injected with flutamide (20mg/kg of body weight) subcutaneously on Days 1-14 postnatally. Ten pups were taken from prenatal flutamide treated mothers. Controls (n = 15) received buffer only. Controls and treated rats were maintained as previously described.

**EXPERIMENT 4:- Postnatal effects of TP and p-chlorophenylalanine on sexually dimorphic spinal motor neurone groups.**

The purpose of this experiment was to observe the effect of either TP or pCPA, or TP and pCPA administered together, on the number of neurons in the SNB and DLN of male rats.

Albino Swiss pups were injected subcutaneously with TP (200µg daily) on Days 1-3 (n = 11), or Days 4-6 (n = 15), or Days 7-9 (n = 8) postnatally. Half of these rats also received pCPA (200mg/kg body weight i.p.) on Days 1-3 (n = 7), or Days 4-6 (n = 11) or Days 7-9 (n = 2) postnatally. Controls (n = 15) and treated rats were maintained as before.

**EXPERIMENT 5:- The effects of a 5HT agonist and antagonist administered postnatally on the sexually dimorphic spinal motor neuron groups.**

Male rat pups were injected intraperitoneally with either ritanserin (2mg/kg body weight) (n = 21) or m-chlorophenylpiperazine (1.5mg/kg body weight) (n = 14) or methysergide (2.5mg/kg body weight) (n = 7) on Days 1-14 after birth. Controls were treated with the vehicle

propylene glycol (n = 15). The rats were maintained as the previous group.

**EXPERIMENT 6:- Sex differences in the concentrations of serotonin and 5HIAA in the lumbosacral and thoracic regions of the spinal cord in rats.**

This experiment was designed to examine sex differences and age-related changes in biogenic amine levels in the rat lumbosacral and thoracic spinal cord.

Twentyeight male and twentyeight female rats aged 4, 12, 14 or 22 days, were killed and the lumbosacral spinal cord removed to measure the concentrations of the biogenic amines present.

Removal of the Spinal Cord:- Adult rats were killed by an overdose of ether and the animals decapitated. The skin of the back was slit on both sides of the midline, so that the vertebral column was exposed. The vertebral canal was opened at the fourth lumbar vertebra and lower sacral vertebra (fifth). The whole block was taken out.

An 18-gauge hypodermic needle was fixed to a 50 c.c. syringe filled with 0.9% normal saline and inserted into the rostral end of the vertebral canal. Pressure was exerted on the syringe plunger and the spinal cord was extruded at the end of the vertebral canal into a petri dish containing alcohol, formaldehyde and acetic acid (AFA) fixative.

**SAMPLE PREPARATION FOR MOTOR NEURON COUNT**:-

After its removal, the spinal cord was immersed in fixative for 24 hours. The fixative contained 5% alcohol, 90% formaldehyde and 5% acetic acid. It was then immersed in Millonig's buffer and placed in a microwave oven for 20 minutes and maintained at 37$^{o}$C.

The lumbosacral enlargements of the spinal cords were chosen for sectioning. Melted agar (0.7%) was placed in a small plastic tray. The spinal cord was carefully placed in this and the agar allowed to cool for 15-20 minutes. The block containing the segment of spinal cord was fixed on a Bio-rad microcut H-1200 vibratome plate, and sectioned serially at a thickness of 100µm. The sections were immersed in Millonig's buffer until further processing.

For staining, the sections were placed in 0.3% thionine for two minutes, then dehydrated as follows (three minutes in each).

70% alcohol

90% alcohol

Absolute alcohol (x 3)

The sections were placed in two changes of xylene, five minutes in each. The sections were then mounted in DPX and examined under the light microscope.

Sample preparation for measurement of biogenic amines using high performance liquid chromatography with electrochemical detection (HPLC-ECD):-

After removal, the samples of spinal cord were frozen in liquid nitrogen and stored at -80$^{o}$C.

Homogenization:-

Prior to homogenization the samples were weighed and placed in a glass homogenizer, kept in ice which contained 600 l of 0.1M HCl, to which 100 µg/1 of an internal standard, dihydroxylbenzylamine (DHBA), had been added. The tissue was homogenized gently before being centrifuged at 3000 r.p.m. for 10 minutes at 4°C. The resulting supernatant was either injected directly into the HPLC or snap frozen in liquid nitrogen and stored at -80°C until assayed. All samples were analysed within one month of their preparation. 20 µl of supernatant was injected into the HPLC column and the concentration of the amines was measured using an electrochemical detector. At least two injections per sample were analysed.

High performance liquid chromatography with electrochemical detection:-

**Definition:-** HPLC is a method of separating different components of a mixture by a successive series of stages. In this experiment the apparatus used for the analysis of biogenic amines consisted of -

a) A microparticulate high resolution chromatographic column
b) A pump to force the fluid through the column
c) A detector, able to measure separated compounds quantitatively (the minium detectable quantity is 0.05 to 0.10ng/20 microlitres of supernatant).

The system included:

a) An injection port

b) A pressure gauge

c) A guard column

d) A solvent reservoir

e) A data processor/recorder

Instrumentation:-

Standards and samples were loaded onto the column through a Rheodyne 7125 injection valve, fitted with a 20ul injection loop, using a Hamilton syringe. The main analytical column was protected from deterioration by the samples first passing through a guard column (Anachem). The analytical column itself consists of a 15cm x 0.46cm stainless steel tube (Rainin Instruments). This was purchased pre-packed with microsorb (5µm) silica particles coated with a hydrocarbon.

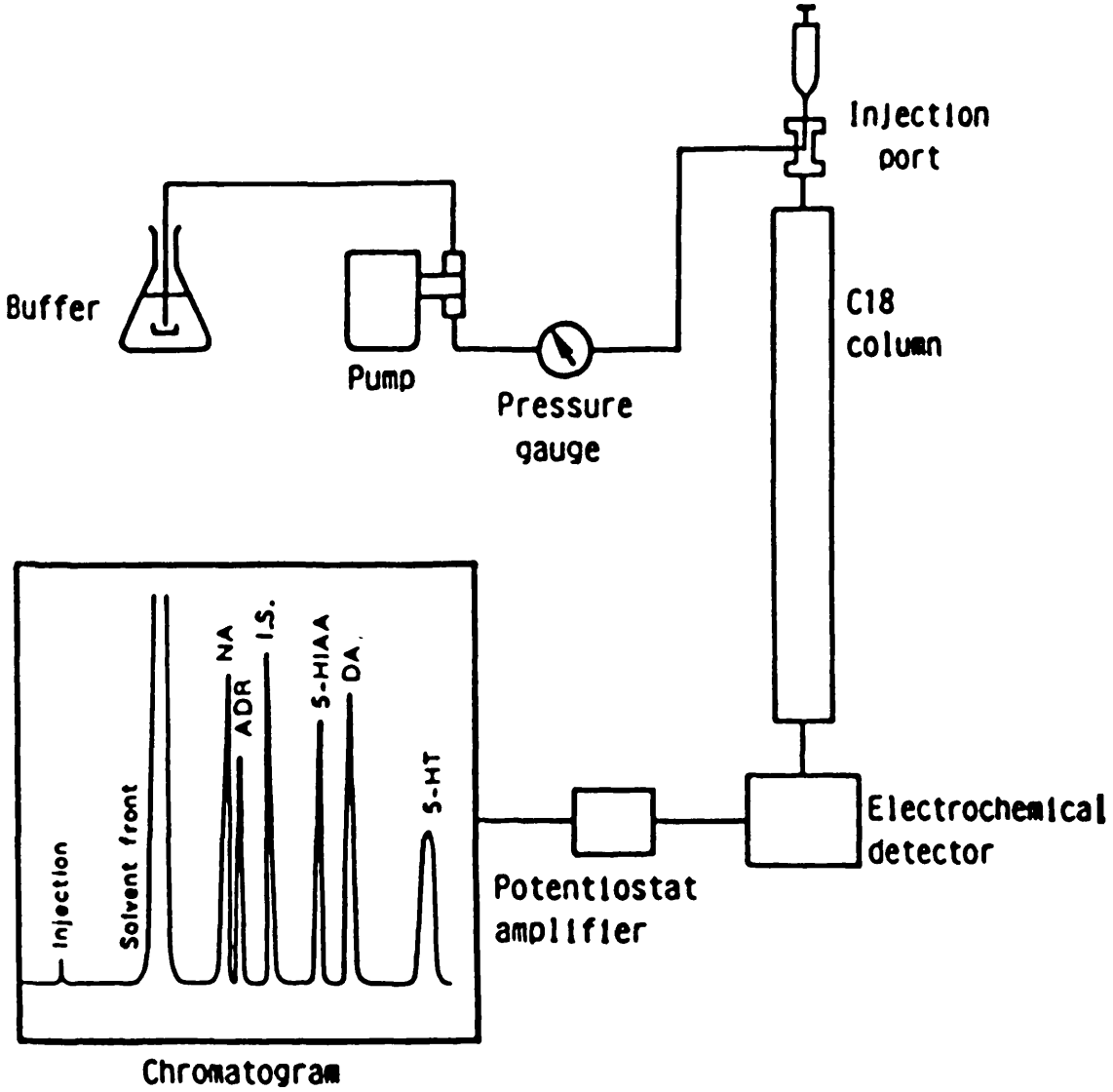
The guard column was frequently repacked, with Shandon ODS Hypersil (5µm particle size). Compounds were detected by a Bioanalytical system flow cell TL-5 and an LC-3A electrochemical detector. The potential was set at 0.70v with reference to an Ag/AgCl reference electrode (sensitivty 1nA, time constant 5 seconds). The output signal was recorded and analysed by a Trivector Trio integrator.

The mobile phase was pumped through the system at a rate of 0.6ml/minute (modified from the method of Siddiqui and Gilmore, 1986).

pa

**Figure 2**

Schematic diagram of HPLC apparatus.


Injection port
Buffer
Pump
Pressure gauge
C18 column
Injection
Solvent front
NA
ADR
I.S.
5-HIAA
DA
5-HT
Potentiostat amplifier
Electrochemical detector
Chromatogram

Mobile phase:-

This is the liquid, pumped through the system along with the sample. It consists of -

- 0.1M citric acid - 6.74g/1
- 0.1M sodium acetate - 4.81g/1
- 19mM glacial acetic acid 1.15ml/1
- 126 mM Na EDTA - 47mg/1
- 5% (v/v) methanol - 50ml/1
- 2% (v/v) tetrahydrofuran (THF)
- 431mM sodium actyl sulphate
- Heptane sulphonic acid - 100mg/1
- Deionized water to make a total volume 1 litre

This was adjusted to a pH of 4.9 - 5.1 with sodium hydroxide to ensure complete ionisation of the biogenic amine molecules. Deionized water, and Analar grade reagents were always used.

Solvent pretreatment:-

The solvent was filtered under pressure through a Millipore solvent clarification kit with a 0.45 aqueous filter before use, and then degassed with helium for 15 minutes. Degassing the mobile phase reduced the possibility of air bubbles forming in the pump and detector under the high pressure conditions. Air bubbles can disrupt solvent flow through the column and cause severe baseline noise. THF was then added, and the buffer and THF were very gently mixed taking care not to introduce any air.

Solvent pumping system:-

This provides a constant supply of mobile phase to the column. The small particles used to pack modern liquid chromatography columns offer substantial resistance to flow. A high pressure pump is therefore required to deliver solvent to the column. This pumping system can deliver solvent at precise flow rates with a relatively pulse-free output at pressures up to 5000 psi.

Sample loading:-

The samples were introduced to the top of the column via the injection valve. The rheodyne valve was then turned from the 'load' to the 'inject' position thereby diverting mobile phase through the loop to carry the sample into the column.

Electrochemical detection:-

This operates on the principle that compounds capable of oxidation and reduction in an electrical field result in the passage of current, and the magnitude of this current is a measure of the quantity of compound oxidised or reduced.

The potentiostat system consists of a carbon working electrode, a platinum wire auxillary electrode, and a reference electrode (Ag/AgCl), all contained in the detector. Each chemical reaction has a threshold voltage related to its redox potential and the potentiostat, a form of feedback voltage control, allows this voltage to be set for the particular compound to be measured. This

introduces some measure of selectivity into the system. For catecholamines and indoleamines a positive potential of +0.70v is used.

The potentiostat amplifier has two functions in the system -

a) It maintains the constant preset potential across the electrochemical detector.

b) It amplifies the nanoampere oxidation current and provides a proportional output voltage which is displayed on the recording integrator.

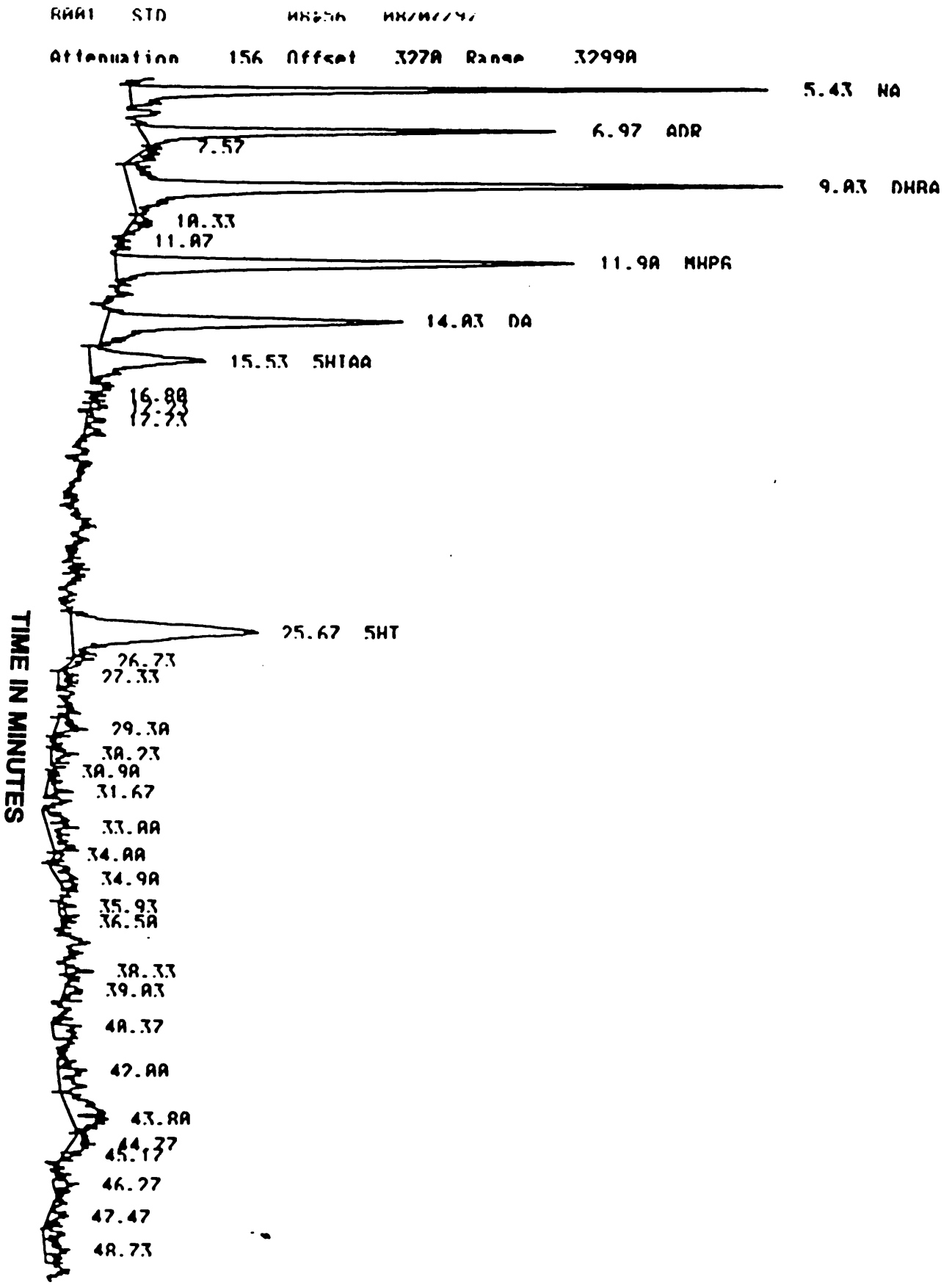
As oxidisable bands of each compound pass the detector, the current (and resultant voltage) rises and falls as a function of time, to yield a liquid-chromatography electrochemical chromatogram. In this system the first peak on the chromatogram, known as the solvent front, represents the oxidation of hydrochloric acid, which has the shortest retention time on the column and is the first band to pass through the detector. This peak is followed by noradrenaline (NA), then by adrenaline (ADR), dopamine (DA), 5HIAA and finally by serotonin (see Fig. 3).

Calculation of the result:-

The biogenic amine content of the samples was derived from the peak heights using calibration curves constructed at least twice daily from chromatograms of standard amine solution. Since the same quantity of internal standard (1ng/20µl) had been added to the standard mixture and to all the samples, it was possible to calculate the

**Figure 3**

Trace produced when 20 µl of a mixture of standards is injected into the HPLC system. Each peak represents a concentration of 1ng.The minium detectable quantity of the biogenic amines was 0.05-0.10 ng/20 microlitre of supernatant dependent on the noise level of the system.


Attenuation 156 Offset 3278 Range 32998
5.43 NA
6.97 ADR
7.57
9.83 DHRA
10.33
11.87
11.98 MHPG
14.83 DA
15.53 5HIAA
16.88
25.67 5HT
26.73
27.33
29.38
30.23
30.98
31.67
33.88
34.88
34.98
35.93
36.58
38.33
39.83
40.37
42.88
43.88
44.27
45.17
46.27
47.47
48.73
TIME IN MINUTES

## TABLE 2: Calculation of the concentration of amines in a standard mixture

RAA1 STD AA:56 AA/A7/97
Method RASI C3AA Re-analysed
Method Text RASI C3AA

Sample
Weight 1.AA Volume 1.AA

| Report Retention Time | Intall Relative Time | Peak Area | Response Factor | Peak Conc | Peak Name | Peak Code |
|---|---|---|---|---|---|---|
| 5.43 | A.6A | 167229 | 1.195 | A.75 | NA | |
| 6.97 | A.77 | 129457 | 1.263 | A.62 | ADR | |
| 7.57 | A.84 | 11551 | 1.AAA | A.A4 | | |
| 9.A3 | 1.AA | 264972 | 1.AAA | 1.AA | DHBA | I.S. |
| 1A.33 | 1.14 | 9611 | 1.AAA | A.A4 | | |
| 11.A7 | 1.23 | 2151 | 1.AAA | A.A1 | | |
| 11.9A | 1.32 | 2A2394 | 1.565 | 1.2A | MHPG | |
| 14.A3 | 1.55 | 156187 | A.74A | A.44 | DA | |
| 15.53 | 1.72 | 76241 | A.811 | A.23 | 5HIAA | |
| 16.8A | 1.86 | 2126 | 1.AAA | A.A1 | | |
| 17.23 | 1.91 | 3535 | 1.AAA | A.A1 | | |
| 17.73 | 1.96 | 18339 | 1.AAA | A.A4 | | |
| 25.67 | 2.84 | 161138 | 1.31A | A.8A | 5HT | |
| 26.73 | 2.96 | 7122 | 1.AAA | A.A3 | | |
| 27.33 | 3.A3 | 8265 | 1.AAA | A.A3 | | |
| 29.3A | 3.24 | 12542 | 1.AAA | A.A5 | | |
| 3A.23 | 3.35 | 7377 | 1.AAA | A.A3 | | |
| 3A.9A | 3.42 | 821 | 1.AAA | A.AA | | |
| 31.67 | 3.51 | 11896 | 1.AAA | A.A4 | | |
| 33.AA | 3.65 | 266A3 | 1.AAA | A.1A | | |
| 34.AA | 3.76 | 2814 | 1.AAA | A.A1 | | |
| 34.9A | 3.86 | 14117 | 1.AAA | A.A5 | | |
| 35.93 | 3.98 | 4787 | 1.AAA | A.A2 | | |
| 36.5A | 4.A4 | 2632 | 1.AAA | A.A1 | | |
| 38.33 | 4.24 | 5231 | 1.AAA | A.A2 | | |
| 39.A3 | 4.32 | 7425 | 1.AAA | A.A3 | | |
| 4A.37 | 4.47 | 12995 | 1.AAA | A.A5 | | |
| 42.AA | 4.65 | 22653 | 1.AAA | A.A9 | | |
| 43.8A | 4.85 | 42286 | 1.AAA | A.16 | | |
| 44.77 | 4.96 | 13A45 | 1.AAA | A.A5 | | |
| 45.17 | 5.AA | 3616 | 1.AAA | A.A1 | | |
| 46.27 | 5.12 | 8686 | 1.AAA | A.A3 | | |
| 47.47 | 5.25 | 11968 | 1.AAA | A.A5 | | |
| 48.73 | 5.39 | 21235 | 1.AAA | A.AA | | |
| | | 1444879 | | 6.11 | | |

concentration of each amine relative to the internal standard peak area.

Standard solution:-

**Stock solution** - Noradrenaline bitartrate
- Adrenaline bitartrate
- Dopamine hydrochloride
- 5-hydroxyindole acetic acid
- 5-hydroxytryptamine (serotonin)
- 3, 4 dihydroxy benzylamine as the internal standards.

They were dissolved in 100ml of 0.1M HCl. This stock solution was stable for up to six weeks when stored in the dark at $4^{o}C$. The concentration was 2mg/100ml of 0.1M HCl.

Every week a working standard solution was made by diluting 125Ìl of the stock solution in 100ml of 0.1M HCl. This was used to calibrate the chromotography system for amine anlysis. An internal standard of 1ng/20µl of DHBA was included in both the sample homogenizing medium and in the standard solutions.

Analysis of spinal cord samples:-

The ratio of the amine peak areas to internal standard peak area is known as the response factor and is a measure of the recovery of the amine per unit of internal standard. A trivector integrating computer was used to calculate the actual amount of amine present in each sample.

Statistical Analyses:-

In Experiment 1 the significance of differences between the motor neuron numbers of control males and females was determined using the Students t-test. In other experiments, the motor neuron counts were compared between groups using one-way analyses of variance (F) followed by confidence interval analysis (Statistical Graphics System: Statistical Graphics Corporation) to reveal inter-group differences.

# RESULTS

**Experiment 1: Sex differences in motor neuron numbers in the lumbosacral spinal cord of control male and female rats** (The results are sumarised in Figures 4 - 10).

The SNB:-

Control males had a total 197.0 ± 8.3 motor neurons, while control females averaged 40.0 ± 1.5. This difference was highly significant ($t = 11.08$, $df = 28$, $p < 0.001$).

The DLN:-

In this region male rats had 24.6 ± 1.0 neurons per 100 Ìm section, while females averaged 10.8 ± 1.1. This difference was also highly significant ($t = 19.46$, $df = 28$, $p < 0.001$).

The RDLN:-

With regard to the RDLN, control males had a total of 26.3 ± 1.2 motor neurons per 100 Ìm section, while females averaged a total of 25.9 ± 2.2. This difference was not significant.

VM numbers:-

With regard to the VM motor neurons male rats had 5.8 ± 0.4 per 100 Ìm section, and females averaged 6.7 ± 0.7. The difference was not significant.

Overall, male rats have significantly more SNB and DLN motor neurons than do females, confirming that sexual dimorphism is present in the two motor neuron groups in this region of the spinal cord.

**Figure 4**

Photograph of a section of spinal cord from an intact male Albino-Swiss rat at level L5 showing the spinal nucleus of bulbocavernosus (SNB), dorsolateral nucleus (DLN), retrodorsolateral nucleus (RDLN) and ventromedial nucleus (VM). Stained with thionine. Magnification x 54

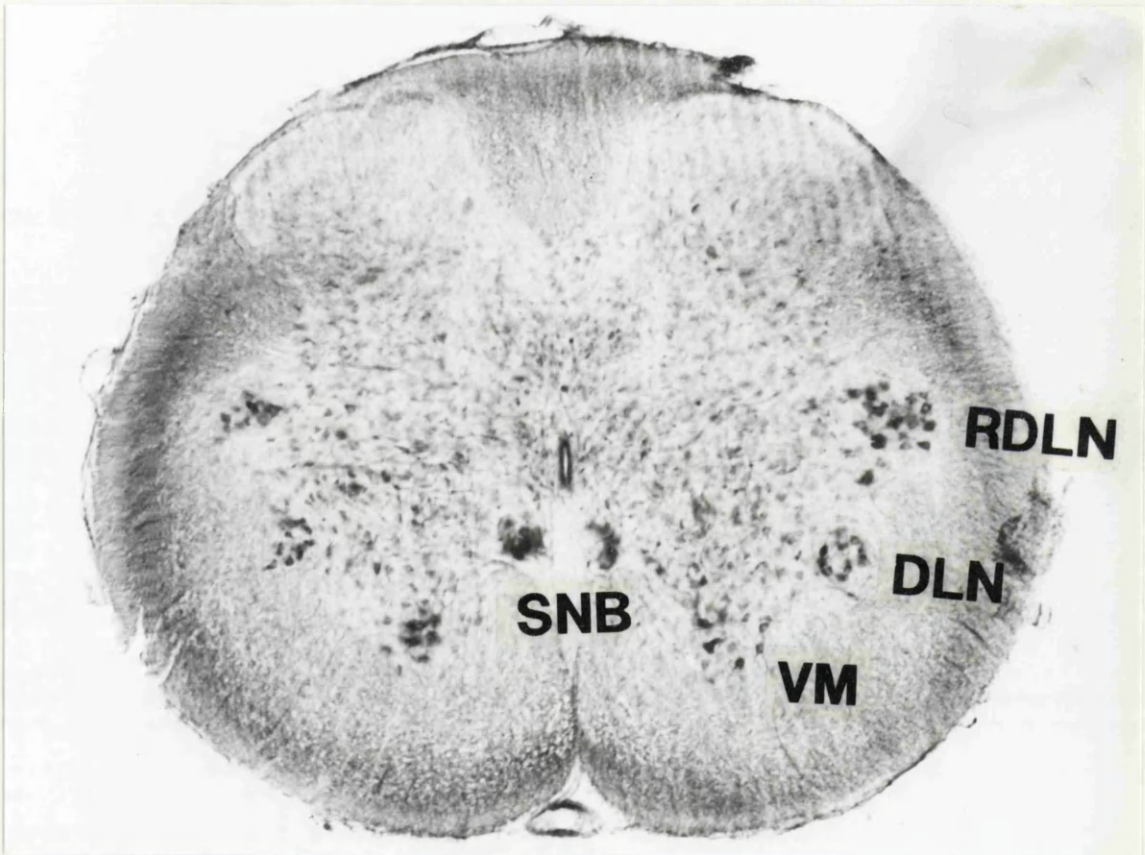

RDLN
DLN
SNB
VM

**Figure 5**

Photograph of a section of spinal cord from an intact female Albino-Swiss rat at level L5 showing the spinal nucleus of bulbocavernosus (SNB), dorsolateral nucleus (DLN), retrodorsolateral nucleus (RDLN) and ventromedial nucleus (VM). Stained with thionine. Magnification x 66.9

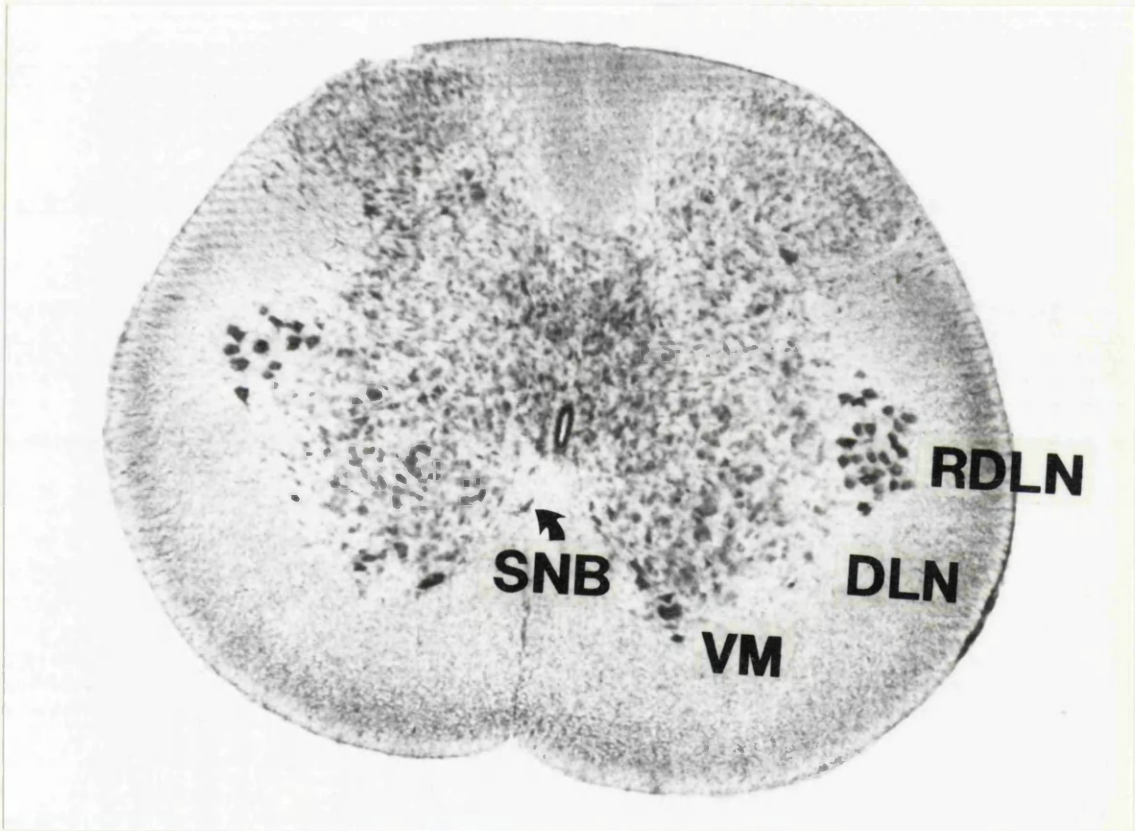

RDLN
SNB
DLN
VM

**Figure 6**

Photograph of a section of spinal cord from an intact male Albino-Swiss rat at level lumbar six (L6) showing the spinal nucleus of bulbocavernosus (SNB), dorsolateral nucleus (DLN), retrodorsolateral nucleus (RDLN) and ventromedial nucleus (VM). Stained with thionine.
Magnification x 47.5

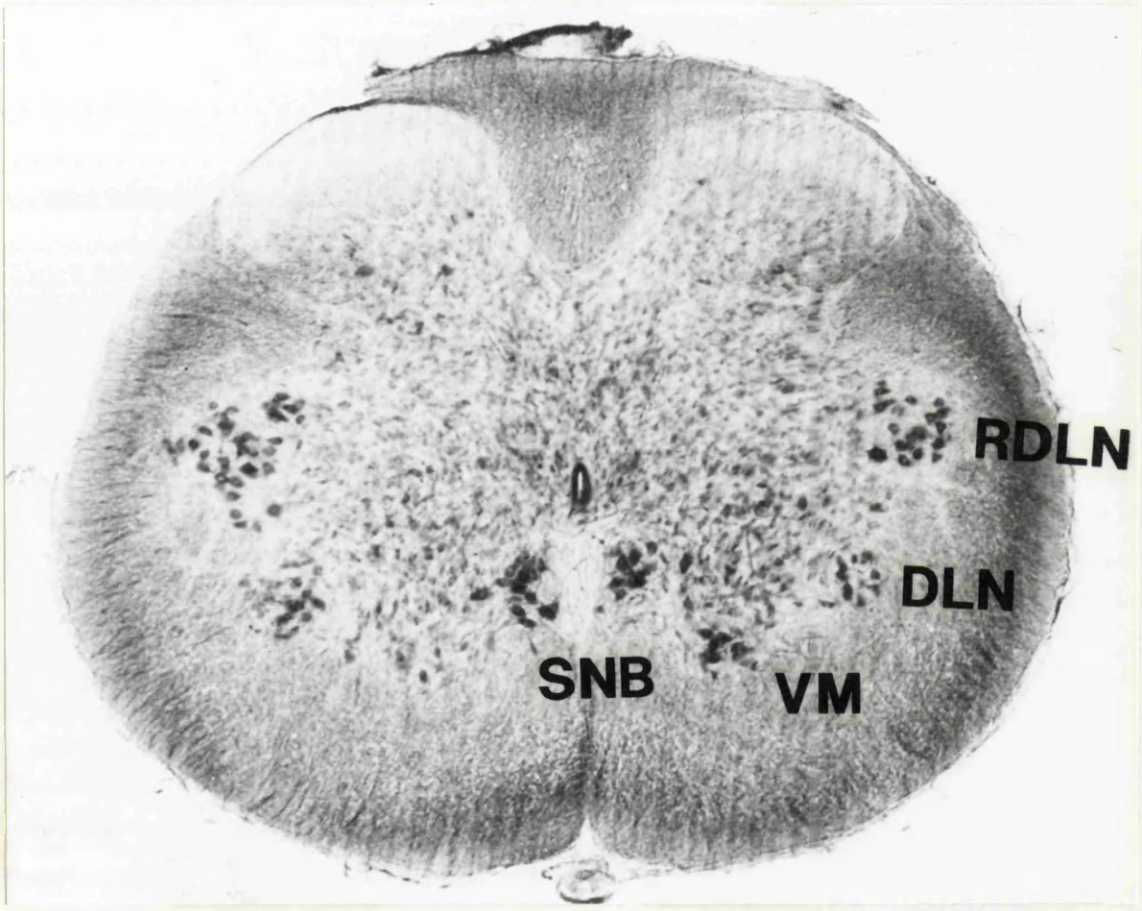

RDLN
DLN
SNB
VM

**Figure 7**

Photograph of a section of spinal cord from an intact female Albino-Swiss rat at level L6, showing the spinal nucleus of bulbocavernosus (SNB), dorsolateral nucleus (DLN), and retrodorsolateral nucleus (RDLN). Stained with thionine.
Magnification x 51.4

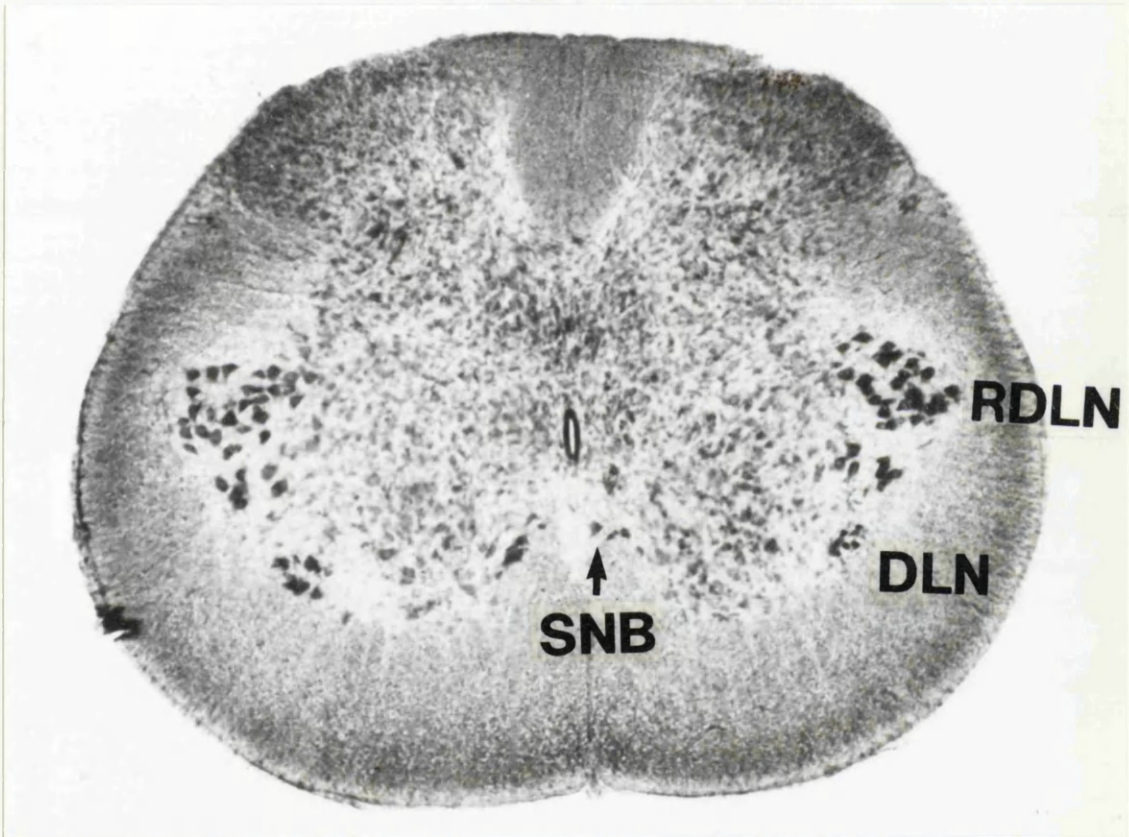

RDLN
DLN
SNB

**Figure 8**

Photograph of a section of spinal cord from an intact male Albino-Swiss rat at level S1, showing the spinal nucleus bulbocavernosus (SNB) and ventromedial nucleus (VM). Stained with thionine.
Magnification x 67.9

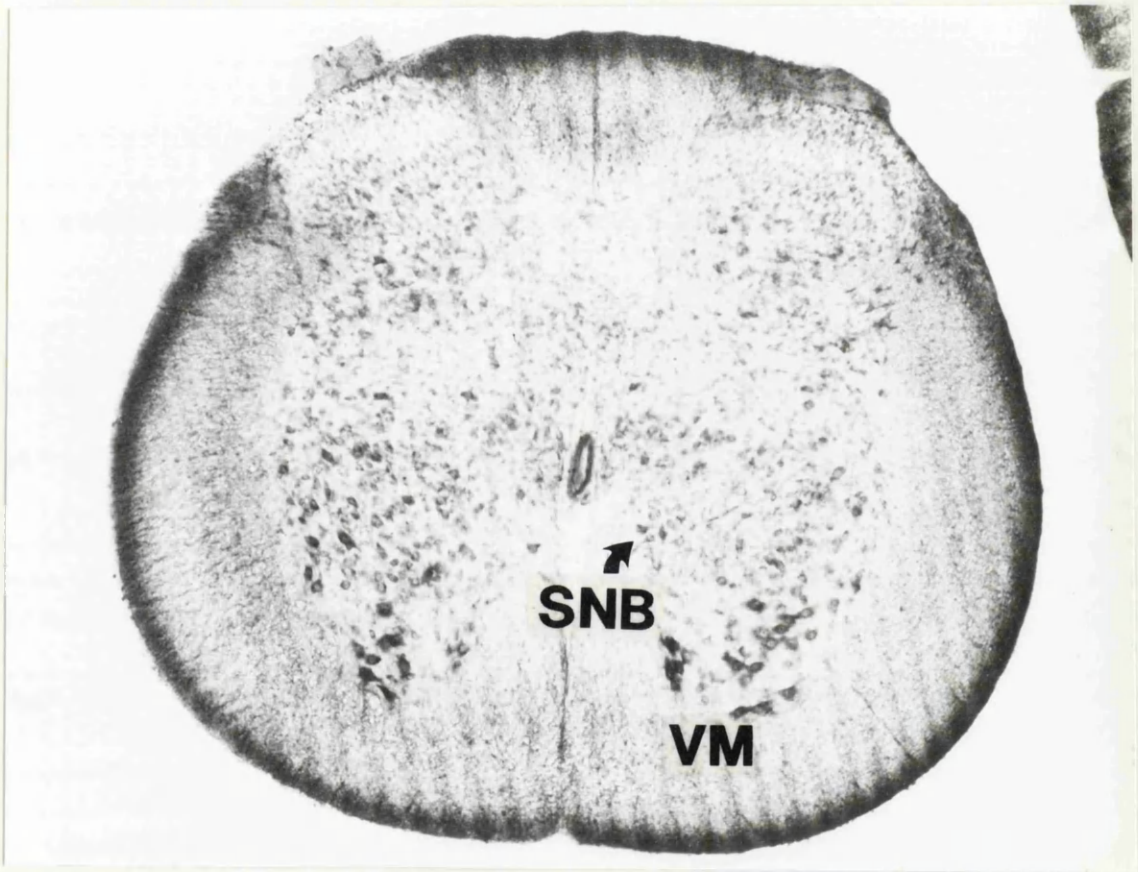

SNB
VM

**Figure 9**

Photograph of a section of spinal cord from an intact female Albino-Swiss rat at level S1, showing the ventromedial nucleus (VM). Stained with thionine. Magnification x 75

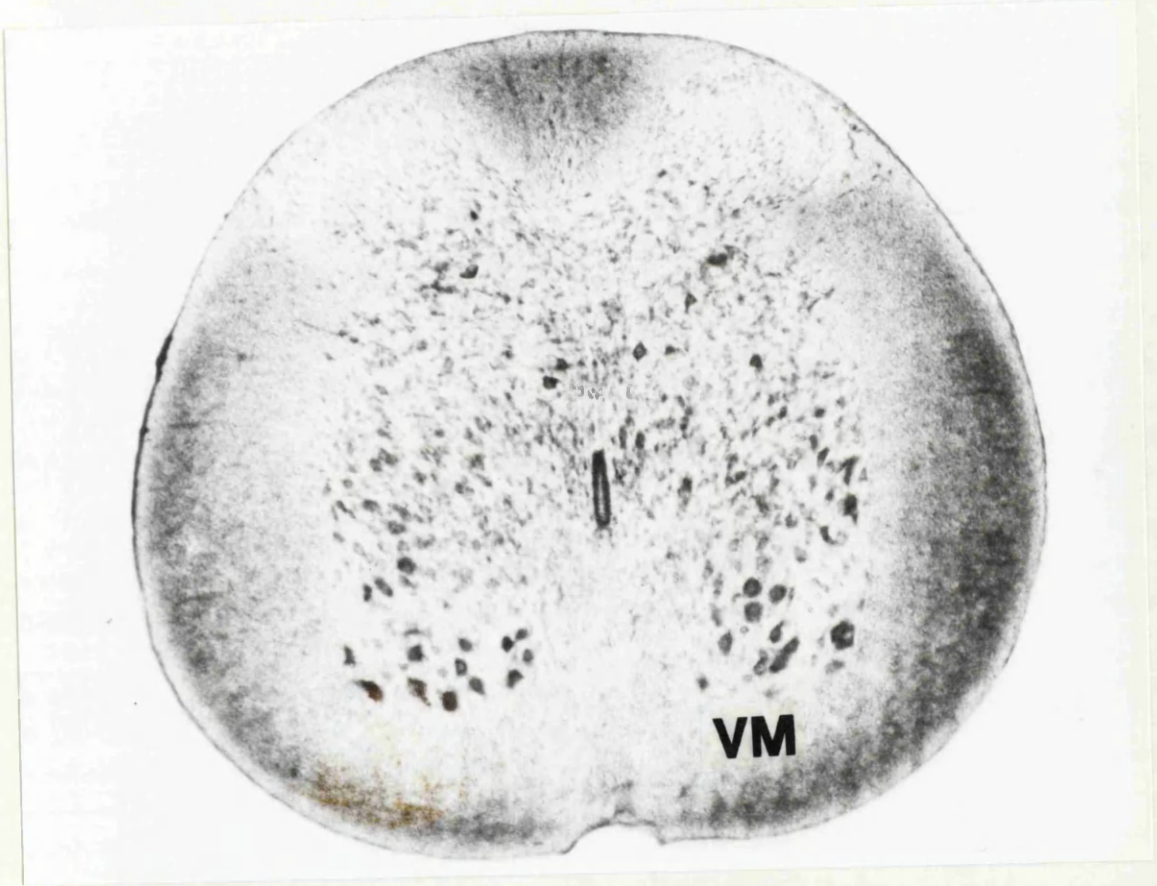
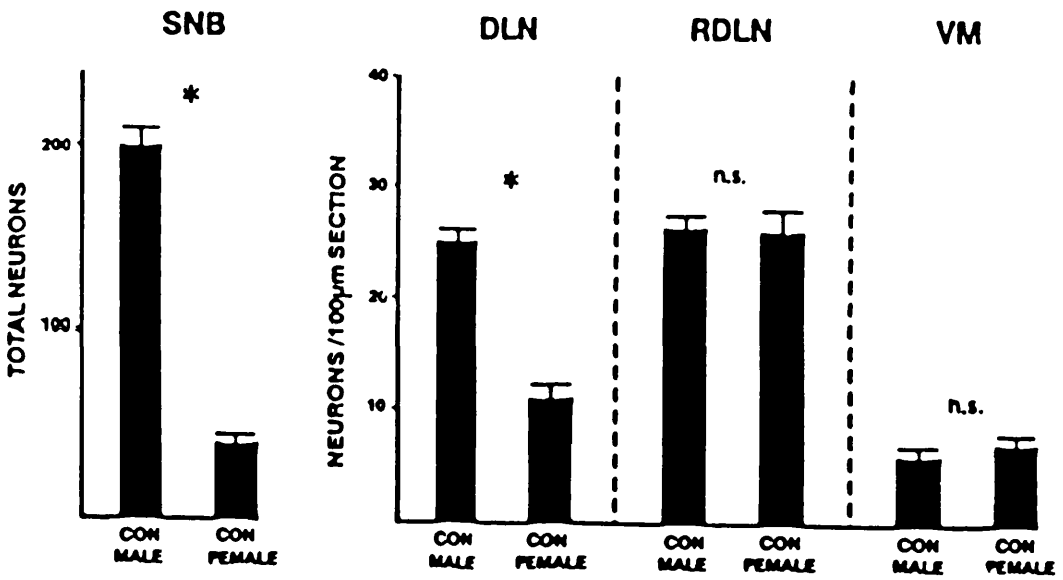

VM

**Figure 10**

Histograms showing the sex differences in motor neuron groups, i) spinal nucleus bulbocavernosus (SNB), ii) dorsolateral nucleus (DLN), iii) retrodorsolateral nucleus (RDLN) and v) ventromedial nucleus (VM) present in lumbosacral region of spinal cord of Albino-Swiss rats.
Values are mean ± SEM.

terms

CON MALE = Untreated control male
CON FEMALE = Untreated control female


SNB
DLN
RDLN
VM
TOTAL NEURONS
NEURONS / 100µm SECTION
200
100
40
30
20
10
*
*
n.s.
n.s.
CON MALE
CON FEMALE
CON MALE
CON FEMALE
CON MALE
CON FEMALE
CON MALE
CON FEMALE
* Sexes differ p < 0.001

**Experiment 2: Perinatal androgen treatment and its effect on sexually dimorphic spinal motor neurons in male rats. (The results are summarised in Fig. 11).**

In this experiment the SNB and DLN neuron numbers were counted in control males and in males which had been pre or postnatally exposed to TP or DHTB.

There was a significant variance between the five groups for the numbers of SNB ($F = 10.59$, d.f. 4x49, $P < 0.001$) and DLN ($F = 8.58$, d.f. 4x49, $p < 0.001$) neurons.

Intergroup comparisons using confidence interval analyses showed that males treated postnatally with TP have significantly more SNB and DLN neurons in adulthood than controls ($p < 0.01$) while prenatal treatment with DHTB also signficantly increased SNB neuron numbers ($p < 0.01$). No other differences were statistically significant.

**Experiment 3: Effects of a non-steroidal anti-androgen (flutamide) on sexually dimorphic spinal motor neurons in male rats (see Fig. 12)**

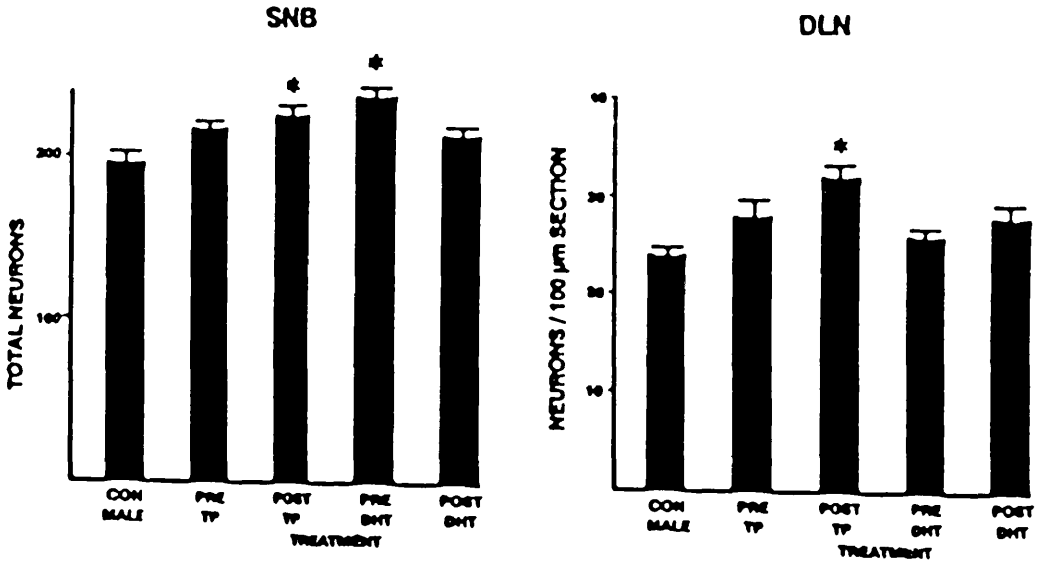
In this experiment the number of SNB and DLN motor neurons was examined, in control male rats and in rats treated pre- or postnatally with flutamide. There was a significant variance over the three groups for SNB numbers ($F = 46.94$, df 2x26, $p < 0.001$) but not for DLN numbers ($F = 1.37$, df 2x26, $p < 0.27$). Intergroup comparisons using confidence interval analyses showed that SNB numbers in males treated prenatally with flutamide were significantly lower than in control males ($p < 0.01$).

**Figure 11**

Histograms showing the effect of perinatal hormone treatment on two sexually dimorphic motor neuron groups i) spinal nucleus bulbocavernosus (SNB) and ii) dorsolateral nucleus (DLN) present in lumbosacral region of spinal cord of male Albino-Swiss rats. Values are mean ± SEM.

terms

CON MALE = untreated control male
PRE TP = prenatal testosterone propionate treated
POST TP = postnatal testosterone propionate treated
PRE DHT = Prenatal dihydrotestosterone benzoate treated
POST DHT = Postnatal dihydrotestosterone benzoate treated



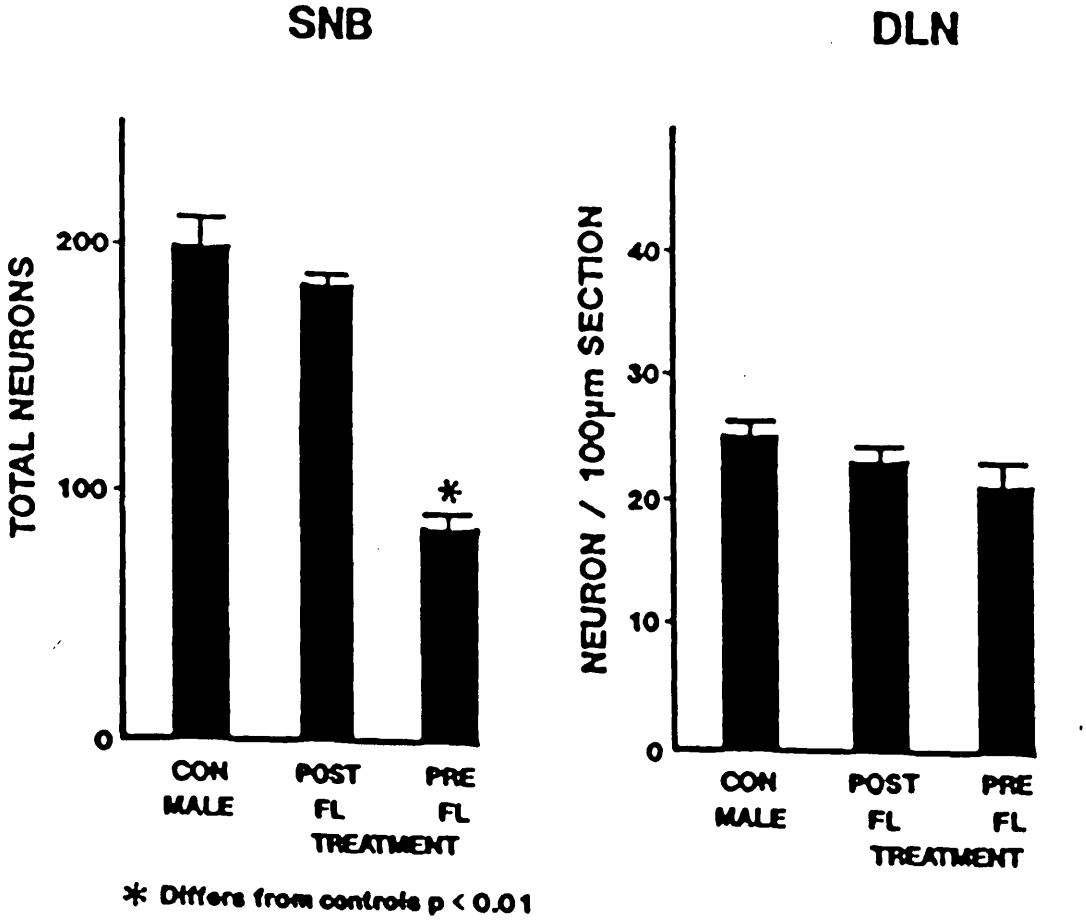

* Differs from controls $p < 0.01$

**Figure 12**

Histograms showing the effect of perinatal flutamide (antiandrogen) treatment on two sexually dimorphic motor neuron groups, the spinal nucleus of bulbocavernosus (SNB) and the dorsolateral nucleus (DLN) present in the lumbosacral region of the spinal cord of male Albio-Swiss rats.
Values are mean ± SEM.

terms

CON MALE = untreated control male
POST FL = postnatal flutamide treated
PRE FL = prenatal flutamide treated


SNB
DLN
TOTAL NEURONS
200
100
0
*
CON MALE
POST FL
PRE FL
TREATMENT
NEURON / 100µm SECTION
40
30
20
10
0
CON MALE
POST FL
PRE FL
TREATMENT
* Differs from controls p < 0.01

**Experiment 4: Postnatal effects of TP and p-Chlorophenylalanine on sexually dimorphic spinal motor neuron numbers.**

For SNB neurons, two-way analysis of variance shows a significant effect for day of androgen treatment ($F = 7.75$, $df = 3$, $p < 0.001$), for pCPA treatment versus buffer ($F = 9.69$, $df = 1$, $p < 0.01$) and for interactions between the two factors ($F = 9.37$, $df = 3$, $p < 0.001$). Briefly, ignoring any effects of pCPA, males treated with androgens on days 1-3 or 4-6 have more SNB neurons than controls. If treatment is delayed until days 7-9 after birth, there is no effect. Secondly, ignoring any effect of androgens, pCPA-treated males have more SNB neurons than buffer-treated males. However, consideration of individual groups (see Figures 13, 14) shows that this is primarily due to the group receiving pCPA alone. In no case do males treated with androgens and pCPA possess more SNB neurons than comparable males treated with androgens alone.

For DLN neurons, two-way analysis of variance shows a significant effect for day of androgen treatment ($F = 9.15$, $df = 3$, $p < 0.001$) but not for pCPA treatment versus buffer ($F = 3.44$, $df = 1$, $p < 0.07$). Briefly ignoring any effects of pCPA, males treated with androgen on Days 1-3 or 4-6 have more DLN neurons than controls. If treatment is delayed until Days 7-9 after birth there is little effect. Secondly, ignoring any effect of androgens, pCPA treated males have more DLN neuron than buffer treated males but this was not significant and in no case do males treated

**Figure 13**

Histograms showing the effect of postnatal testosterone propionate (TP) treatment on two sexually dimorphic motor neuron groups, the spinal nucleus of bulbocavernosus (SNB) and the dorsolateral nucleus (DLN) present in the lumbosacral region of the spinal cord of male Albino-Swiss rats.
Values are mean $\pm$ SEM.

terms

CON MALE = untreated control male

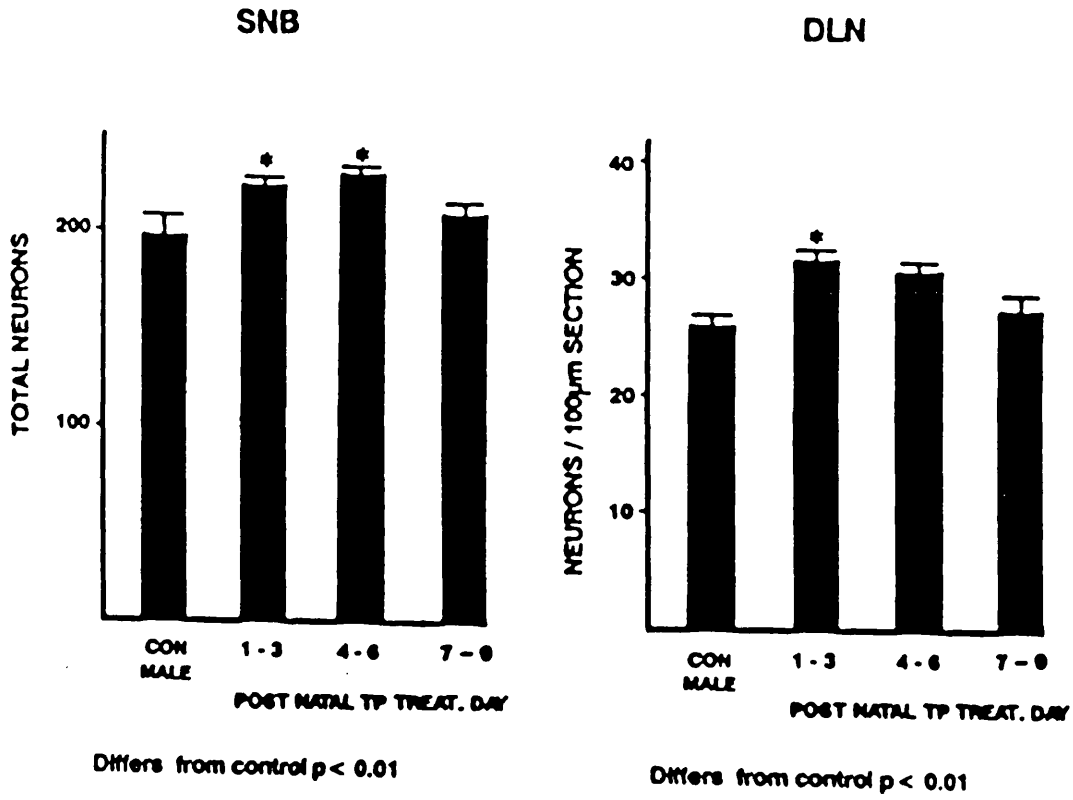

SNB
TOTAL NEURONS
200
100
*
*
CON MALE
1 - 3
4 - 6
7 - 9
POST NATAL TP TREAT. DAY
Differs from control p < 0.01
DLN
NEURONS / 100µm SECTION
40
30
20
10
*
CON MALE
1 - 3
4 - 6
7 - 9
POST NATAL TP TREAT. DAY
Differs from control p < 0.01

**Figure 14**

Histograms showing the effect of postnatal testosterone propionate (TP) and p-Chlorophenyl alanine (a 5HT synthesis inhibitor) treatment on sexually dimorphic motor neuron groups, the spinal nucleus of bulbocavernosus (SNB) and the dorsolateral nucleus (DLN) present in the lumbosacral region of the spinal cord of male Albino-Swiss rats.
Values are mean ± SEM.

terms

CON MALE = untreated control male
TP + pCPA = testosterone propionate and
p-Chlorophenylalanine treated

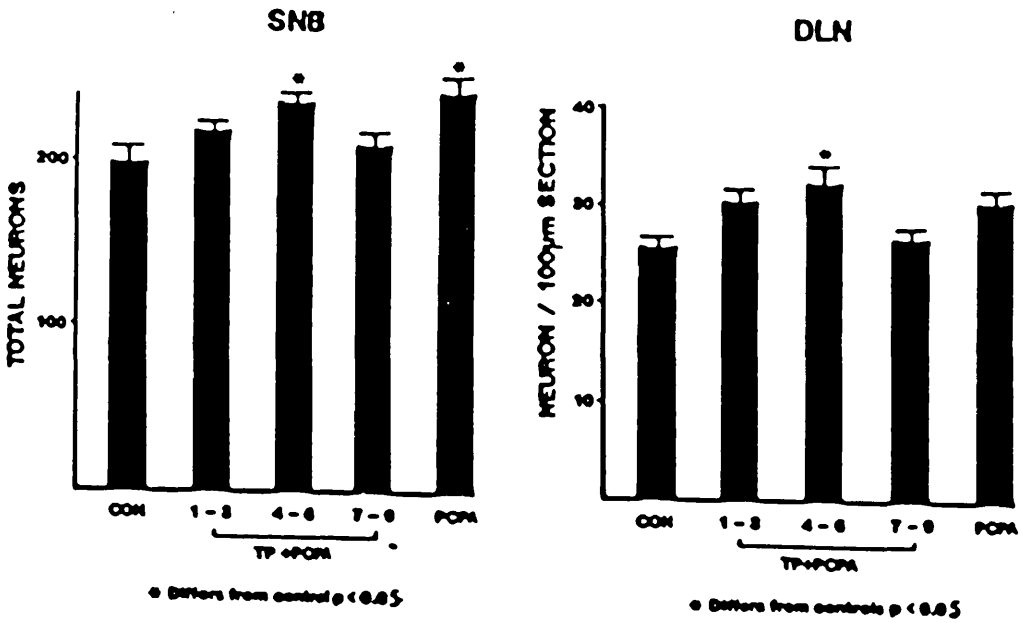
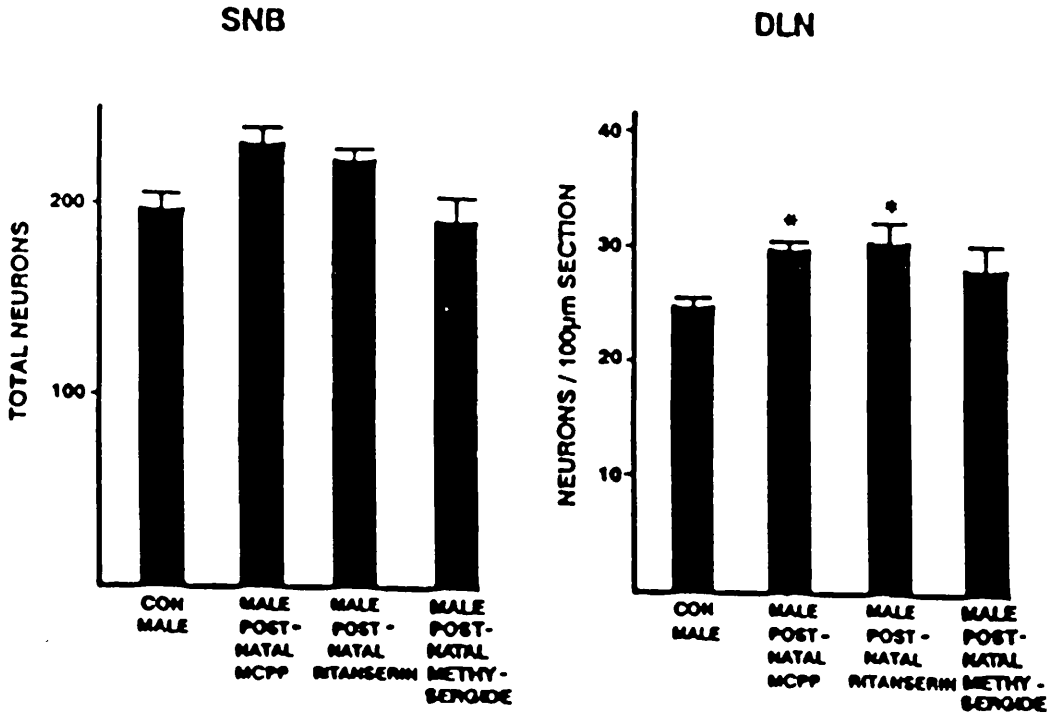

SNB
TOTAL NEURONS
200
100
CON
1 - 3
4 - 6
7 - 9
POPA
TP +POPA
* Differs from control p < 0.05
DLN
NEURON / 100μm SECTION
40
30
20
10
CON
1 - 3
4 - 6
7 - 9
POPA
TP+POPA
* Differs from controls p < 0.05

**Figure 15**

Histograms showing the effect of postnatal m-Chlorophenyl piperazine (a 5HTIC agonist), ritanserin (a 5HT2 antagonist) and methysergide (a general 5HT antagonist) treatment on sexually dimorphic motor neuron groups, the spinal nucleus of bulbocavernosus (SNB) and the dorsolateral nucleus (DLN) present in the lumbosacral region of the spinal cord of male Albino-Swiss rats.
Values are mean ± SEM.

terms

CON MALE = untreated control male
mCPP = m-Chlorophenyl piperazine


SNB
DLN
TOTAL NEURONS
200
100
NEURONS / 100µm SECTION
40
30
20
10
CON MALE
MALE POST-NATAL MCPP
MALE POST-NATAL RITANSERIN
MALE POST-NATAL METHY-BEROIDE
* Differs from control p < 0.05

with androgen and pCPA possess more DLN neurons than comparable males treated with androgen alone.

**Experiment 5: Effects of a serotonin agonist and antagonist administered postnatally on the sexually dimorphic spinal motorneurons (see Fig. 15).**

SNB and DLN neuron numbers were counted in control males and in males which received on Days 1-14 after birth either the 5HT1B, 5HT1C agonist and 5HT2, 5HT3 antagonist m-cholorophenylpiperazine or the general 5HT antagonist methysergide or the 5HT1C and $5HT_2$ antagonist ritanserin. The drug m-CPP was chosen as it is usually described as a 5HT1B agonist (Gardner, 1988; Kinstein & Spear, 1988; Samanin, 1991). However some behavioural effects can be blocked by antagonists of 5HT1C/5HT2 receptors (mianserin, metergoline) but not by pure 5HT2 antagonist (ritanserin), nor by 5HT1A/ 5HT1B antagonists (cyanopindolol, propranolol). This suggests that m-CPP may also act as a 5HT1C agonist.

There was a significant variance over the four groups for SNB ($F = 3.28$, d.f. 3x30, $P < 0.05$) and DLN ($F = 7.35$, df 3x30, $p < 0.001$) neurons. Intergroup comparison using confidence interval analyses showed that DLN neuron numbers were significantly higher in ritanserin and mCPP treated males than in controls ($p < 0.05$).

**Experiment 6: A) Sex differences in the concentrations of serotonin and 5HIAA in the lumbosacral region of the spinal cord in rats (Table 4).**

1. **Postnatal Day 4**

At this age there were no significant sex differences seen in the concentrations of serotonin and 5HIAA in the lumbosacral region of the spinal cord.

2. **Postnatal Day 12**

Although concentrations of both serotonin and 5HIAA were higher in the lumbosacral regions of the spinal cords of female rats at this age the differences did not reach significance.

3. **Postnatal Day 14**

Again while the lumbosacral region of the spinal cords of female rats had higher concentrations of serotonin than did those of males, the difference was not statistically significant; nor were there any significant sex differences in 5HIAA levels.

4. **Postnatal Day 22**

Although female rats once again had higher concentrations of both serotonin and 5HIAA than did the males, the difference did not reach statistical significance.

**B) Sex differences in the concentrations of indoleamines in the thoracic region of the spinal cord of rats (Table 5).**

1. **Postnatal Day 4**

The spinal cords of male rats contained more serotonin and

5HIAA than did those of females; but the differences were not statistically significant.

2. **Postnatal Day 12**

At this age the spinal cords of female rats contained higher concentrations of serotonin than did those of the males, but 5HIAA concentrations were higher in the latter. These differences were not statistically significant.

3. **Postnatal Day 14**

There were higher concentrations of both serotonin and 5HIAA in the spinal cords taken from the thoracic regions of female rats than that in those of males. However, the differences were not statistically significant.

4. **Postnatal Day 22**

Although spinal cords collected from the thoracic region of female rats possesed higher concentrations of both serotonin and 5HIAA than did those taken from males the difference was again not significant.

**Table 3**

**Average weight of the sample of the spinal cord of rats (in mg).**

| Age in days | No.of animals | Male | | Female | |
|---|---|---|---|---|---|
| | | Lumbar | Thoracic | Lumbar | Thoracic |
| 4 | 5 | 12.60±1.98 | 8.20±2.13 | 17.00±4.67 | 11.80±2.66 |
| 12 | 5 | 34.80±12.73 | 23.40±7.44 | 30.40±10.85 | 22.60±7.38 |
| 14 | 5 | 34.80±12.40 | 27.80±9.89 | 31.50±8.99 | 28.80±8.17 |
| 22 | 6 | 40.16±12.32 | 47.17±7.92 | 44.83±14.12 | 35.50±10.35 |

Values are mean ± SEM

20 microlitre of supernatant was injected in to the HPLC (Column).

**Table 4**

**Comparisons of serotonin and 5HIAA concentrations in the lumbosacral regions of the spinal cord of male and female rats (Concentrations in ng/mg).**

| AGE IN DAYS | No. of Animals | Serotonin | | 5HIAA | |
|---|---|---|---|---|---|
| | | Male | Female | Male | Female |
| 4 | 5 | 0.15±0.04 | 0.15±0.03 | 0.04±0.00 | 0.04±0.00 |
| 12 | 5 | 0.07±0.02 | 0.37±0.14 | 0.02±0.00 | 0.08±0.03 |
| 14 | 5 | 0.07±0.08 | 0.30±0.04 | 0.04±0.01 | 0.04±0.02 |
| 22 | 6 | 4.36±0.18 | 4.71±0.15 | 1.40±0.05 | 1.47±0.06 |

Values are mean ± SEM.

**Table 5**

**Comparison of the serotonin and 5HIAA concentrations in the thoracic region of the spinal cords of male and female rats (Concentration in ng/mg).**

| AGE IN DAYS | No. of Animals | Serotonin | | 5HIAA | |
|---|---|---|---|---|---|
| | | Male | Female | Male | Female |
| 4 | 5 | 0.60±0.08 | 0.18±0.06 | 0.36±0.05 | 0.05±0.02 |
| 12 | 5 | 0.08±0.03 | 0.25±0.03 | 0.03±0.00 | 0.01±0.00 |
| 14 | 5 | 0.08±0.03 | 0.19±0.04 | 0.03±0.00 | 0.04±0.01 |
| 22 | 6 | 2.77±0.36 | 3.10±0.69 | 1.02±0.13 | 0.67±0.10 |

Values are mean ± SEM

**TABLE 6**

**Ratio of 5HIAA to serotonin levels in the lumbosacral and thoracic region of the spinal cords of male and female rats ( concentration in ng/mg ).**

| Age in days | No.of animals | Lumbar male | Female | t | Thoracic male | Female | t |
|---|---|---|---|---|---|---|---|
| 4 | 5 | 0.24±0.12 | 0.30±0.13 | 0.27 | 0.67±0.16 | 0.27±0.08 | 1.00 |
| 12 | 5 | 0.54±0.29 | 0.37±0.21 | 0.45 | 0.75±0.31 | 0.06±0.03 | 1.60 |
| 14 | 5 | 0.81±0.41 | 0.21±0.16 | 1.22 | 0.59±0.19 | 0.52±0.41 | 0.15 |
| 22 | 6 | 0.32±0.02 | 0.31±0.03 | 0.06 | 0.38±0.04 | 0.27±0.07 | 0.48 |

Values are mean ± SEM

All t values are n.s

Terms:

Ratio= 5HIAA/5HT.

## DISCUSSION

### SEX SPECIFIC NEURONAL NUMBERS IN THE LUMBOSACRAL SPINAL CORD SECTIONS OF THE MALE AND FEMALE ALBINO-SWISS RAT

The mammalian CNS exhibits morphological sex differences in neuronal size and number, and in the synaptic connections between neurons and cell types (Segovia et al., 1986). All these differences appear to be established during a limited period in perinatal life, and are dependent on the hormonal environment during that time (Arnold and Gorski, 1984; Sengelaub et al., 1989).

Neonatal manipulation of the androgen environment in the rat will bring about sexually dimorphic anatomical and functional changes in the brain, especially in terms of the neural control of sexual physiology and behaviour, by causing permanent changes in neuronal organisation (Gorski, 1971; Naumenko et al., 1985).

The present study has confirmed previous work which has shown that structural sex differences also occur in the lumbosacral region of the rat spinal cord (Breedlove and Arnold, 1980; Arnold, 1984; Breedlove, 1984). The results of the present study are also in agreement with those of Davidson et al., (1990) and Currie et al., (1990 who observed that male Albino Swiss rats possess significantly more SNB neurons than do the females. Furthermore the present work also confirms that by Jordan et al., (1982) who found that Sprague-Dawley male rats have more DLN motor neurons than do females. The present

research also indicates that there are no sex differences in the neurons making up the RDLN. This finding conflicts with that of Jordan et al., (1982), who suggested that there may be a minor sex difference favouring the female in neuron numbers in the RDLN. They also found a small sex difference in RDLN neuronal numbers in one experiment, but not in a second one (Jordan et al., 1982). The present work also confirms previous studies that there is no sex difference in the neurons making up the VM.

Sexual dimorphism in the number of SNB neurons is dependent upon androgens acting during an early critical period of development. If genetically female rats are exposed to androgens just prior to and after birth, they will have more and larger SNB neurons than will unexposed females. Conversely, if genetic males are deprived of androgens perinatally, by the prenatal administration of the antiandrogen flutamide followed by castration at birth, there will be a reduction in the number of SNB neurons and SNB target muscles (Arnold, 1984).

The decrease in the number of DLN neurons in female rats is probably due to the absence of the ischiocavernosus muscles, which are innervated by these neurons in the male (Schroder, 1980).

**PERINATAL ANDROGEN TREATMENT AND ITS EFFECTS ON THE SEXUALLY DIMORPHIC MOTOR NEURON SYSTEM**

Morphological studies of the developing CNS have led to the hypothesis that maximal sensitivity to gonadal

hormones may be associated with the appearance of receptors for androgens and oestrogens which serve as signals for the onset of the 'critical period' of sexual differentiation (Vito and Fox, 1982).

In the present study male rats treated prenatally with TP show some increase in the numbers of SNB neurons compared to control males. This finding is similar to that of Arnold (1984), who reported that TP acting prenatally is most effective in increasing SNB neuron numbers.

The SNB motor neurons and their target musculature accumulate androgens (Breedlove and Arnold, 1980, 1983c) and therefore at least two potential sites for androgen action are available. For example, it is possible that androgens might aid the survival of the SNB motor neurons by acting directly on them. Moreover, the fact that SNB motor neurons of both sexes have their axons at their target musculature well before the onset of cell death, suggests that androgens could act here to influence motor neuron survival and the development of sex differences (Sengelaub and Arnold, 1986). Similarly androgens can maintain DLN neurons and their target musculature (Breedlove and Arnold, 1980). Male rats given TP prenatally also possess a higher number of DLN neurons than do their controls. An increased number of DLN neurons has also been shown by Jordan et al., (1982), who suggested that an exogenous source of TP in the male may exert a super-masculinising influence on DLN neuron numbers.

In this present study male rats treated prenatally with DHTB also possessed significantly more SNB neurons than did the control males. This finding differs from that of Breedlove and Arnold (1983b) and Arnold (1984), who suggested that prenatal DHTB administration cannot increase SNB neuron numbers in Sprague Dawley rats. There may therefore be a strain difference as regards the development of the SNB complex. This possibility is supported by the finding of strain differences in SNB neuron numbers in mice (Wee and Clemen, 1986).

The research findings reported here are similar to those of Davidson et al., (1990), who found that prenatal DHTB administration to male Albino Swiss rats maintained higher numbers of SNB neurons. The present research is also in agreement with that of Breedlove (1986) who suggested that treating female rats with DHTB prenatally could raise SNB neuron numbers without affecting the perineal musculature. Conversely male rats treated with DHTB prenatally do not possess more DLN neurones than do control males.

The present study also indicates that SNB neuron numbers are significantly reduced in male rats whose mothers were treated with the non-steroidal anti-androgen flutamide during the last four days of pregnancy. In contrast DLN neuron numbers were not significantly altered from those of the controls. Furthermore there was no significant change in the SNB and DLN neuron numbers in male rats treated with flutamide postnatally from Days 1-

14.

The decrease in number of SNB neurons after treatment with flutamide, the increase in their number after treatment with androgens in the present and previous studies (Breedlove and Arnold, 1983b; Breedlove et al., 1982), and the feminine appearance of the SNB in testicular feminization mutation (tfm) rats (Breedlove and Arnold, 1981), leaves little doubt that androgens are normally responsible for the sexually dimorphic development of the SNB system.

From the results of the present study it is apparent that the SNB system is hormonally sensitive during both the immediate prenatal and postnatal period. Similarly, perinatal androgen treatment of female rats also demonstrates the hormone sensitivity (Breedlove and Arnold, 1983b). The target muscles of the SNB neurons have androgen receptors in adulthood (Dube et al., 1976). The SNB motor neurons accumulate androgens (Breedlove and Arnold, 1980) and both the SNB and target muscles respond morphologically to TP in adulthood (Breedlove and Arnold, 1981).

McEwen et al., (1977) reported that oestradiol is primarily responsible for masculinisation of the brain. However, there is now evidence to indicate that androgens guide the masculine development of the SNB system. This is because:

1) The non-aromatizable androgen DHTB, when given

immediately after birth, brings about a greater number and an increase in size of the SNB motor neurons (Breedlove and Arnold, 1983b).

2) A single injection of TP (1mg), but not OB (100ug) to female pups on Day 2 of life masculinises the number of SNB neuronal cells present in adulthood (Breedlove et al., 1982).

Finally, the present study reveals that prenatal administration of flutamide demasculinises the SNB. This is probably due to interference with androgen, but not oestrogen action. It would suggest that oestrogen receptors do not play a role in the development of the SNB nucleus since flutamide is ineffective in blocking tritiated oestradiol uptake in rat uterine extracts (De Bold et al., 1981). On the other hand, flutamide effectively blocks DHTB uptake by receptors in the rat kidney and brain (De Bold et al., 1981).

Injection of TP neonatally results in a significant increase in the number of SNB neurons in the adult female rat. This masculinization of the female spinal cord confirms the hypothesis that perinatal androgens direct the sexually dimorphic development of the SNB and suggests that the critical period for androgen action extends at least partially into the postnatal period. Breedlove et al., (1982) showed that OB had no effect postnatally, but it was only given on one day and so this may not be good enough evidence for the lack of activity of OB in this system. It is possible that

oestrogen as well as androgens are responsible for the sexual differentiation of the SNB postnatally.

**POSTNATAL DHTB AND ITS EFFECT ON SEXUALLY DIMORPHIC MOTOR NEURONS IN THE SNB AND DLN**

The results from this experiment reveal that postnatal administration of DHTB to male rats does not significantly alter either SNB or DLN neuronal numbers. Earlier work by Currie et al.,(1990) has demonstrated that DHTB is effective in maintaining SNB motor neurons in female Albino Swiss rats.

The present finding differs from that of Davidson et al., (1990) who found that postnatal administration of DHTB to female rats brought about a two to threefold increase in neuronal numbers in the SNB compared with those in the controls. In this study the increased number of DLN neurons does not reach significance.

Overall, TP and DHTB have a different pattern of effect. TP is most effective in masculinising the SNB and DLN postnatally, whereas the effect of DHTB is to masculinise the SNB prenatally.

Some studies using Sprague-Dawley rats suggest that perinatal DHTB administration can maintain SNB neuron numbers (Breedlove and Arnold, 1983b; Arnold, 1984), while the work of others indicates that this is not so (Sengelaub et al., 1989). In the present study (involving Albino-Swiss rats), DHTB was the most effective of the treatments given prenatally, and this may suggest strain differences

in development of the SNB complex. Discrepancies may be due to timing and dosage; when DHTB has acted, it has not been as effective as testosterone (Arnold & Gorski, 1984).

The disparate effects of TP and DHTB may imply that these androgens are metabolized at different rates or do not have equal access to relevant receptors. The effects of DHTB bear on the issue of which metabolites of testosterone might be involved in the masculinisation process. The hypotheses of Nordeen et al., (1985) and Sengelaub and Arnold (1986) are that the prenatal increase in SNB motor neurons is due to their migration from the lateral motor columns. The timing of DHTB treatment in this study coincides with that period of migration.

Davidson et al., (1990) suggested that female rats treated with DHTB either prenatally or postnatally possessed readily identifiable perineal muscles. Breedlove (1986) suggested that hormones act on the SNB system primarily by maintaining perineal muscles and that neuron survival is an indirect consequence.

## POSTNATAL EFFECTS OF ANDROGENS AND OF THE SEROTONIN SYNTHESIS INHIBITOR p-CHLOROPHENYLALANINE ON THE SEXUALLY DIMORPHIC MOTOR NEURONS OF THE SPINAL CORD OF RATS

Male rats treated with TP on Days 1-3 and 4-6 postnatally possess significantly more SNB neurons than do control males. However the number of SNB neurons do not increase in male rats treated on Days 7-9 postnatally.

This finding confirms that of Nordeen et al., (1985) who suggested that androgens normally prevent cell death occurring in sexually dimorphic spinal motor neurons, and permanently increase the number of motor neurons which are retained into adulthood. DLN neurons were increased significantly in male rats treated with TP on Days 1-3 postnatally, but not in those treated later, agreeing with the findings of Jordan et al., (1982).

Androgens could act on the SNB motor neurons, preventing them from dying, stimulating axonal outgrowth and enhancing their ability to maintain synapses on the target muscle (Sengelaub & Arnold, 1986). Since muscle development requires proper innervation by motor neurons (Jacobson, 1978), it is possible that the muscles develop normally only if androgens first act on the SNB motor neurons.

The site of accumulation of androgens and their action is in the SNB motor neurons and upon androgen receptors in the target muscles of adult rats. However, androgens also have morphological effects on SNB neurons and their targets in adulthood. Castration reduces the size and strength of the levator ani muscle fibres and androgens prevent this decline (Buresova and Gutmann, 1971).

Sengelaub and Arnold (1986) suggested that during the period in which the increase in SNB motor neurons occurs, horse radish peroxidase-labelled cells can be seen outside

the SNB, midway between it and the lateral motor neuron column. These cells are located in areas where motor neurons are not found in more mature animals, and are not seen after the period of increase in SNB motor neuron numbers. As the increase in SNB neuronal numbers is sexually dimorphic and influenced by androgens, it is possible that the lateral to medial migration of motor neurons into the SNB region may itself be subject to regulation by androgens (Sengelaub and Arnold, 1986, 1989). The DLN and SNB both have a similar pattern of steroid accumulation, since they accumulate label after injection of both tritiated TP or DHTB but not of OB (Breedlove and Arnold, 1980).

In adult rats, DLN neurons accumulate androgens (Breedlove and Arnold, 1980) and the ischiocavernosus target muscle is also androgen sensitive (Hays, 1965; Wainman & Shipounoff, 1941). While it is clear that early TP administration exerts a masculinising influence in some sections of the female DLN, the hormone's effect does not appear to depend on sex. We have shown that an exogenous source of TP in the intact male may exert a super masculinising influence on DLN neuron numbers.

A major finding of the present experiment was that the SNB and DLN neuronal numbers increase significantly in male rats treated with TP and pCPA postnatally on Days 4-6. The results of the present experiments also indicate that the number of SNB neurons increased significantly in male rats treated with pCPA postnatally on Days 1-14, although

DLN neuronal numbers were unaltered. This finding is in agreement with that of Cowburn and Payne (1992) and suggests that 5HT and androgens may interact to control neuron numbers.

Although there is clear evidence for the organizational role of androgens in determining sex differences, the role of serotonin and its interaction with hormones is still to be clarified. The observed neonatal sex differences of 5HT and 5HIAA (higher in female) in the hypothalamus preoptic area indicates that the release of 5HT is reduced in neonatal males. It has been suggested that reduced 5HT concentration in the male has functional significance in that it removes an inhibitory influence of the serotoninergic system on testosterone action during the neonatal period (Wilson et al., 1986).

It thus appears that neonatal 5HT activity may specifically antagonise the effects of neonatal testosterone, since reducing 5HT activity by pCPA over either the first or second week of life enhances the masculinizing effect of endogenous testosterone in males and the defeminizing effect of exogenous testosterone in females. On the other hand raising 5HT antagonises the masculinizing and defeminizing effect of exogenous testosterone (Wilson et al., 1986). The fact that PCPA acts similar to androgen suggests the possibility that 5HT may act to antagonise the organizational effect of androgen (Wilson et al., 1991 ).

Serotonin itself has been shown to affect the development of serotoninergic neurons, and their targets within the CNS (Gromova et al., 1983; Forda & Kelly, 1985). There are several sexually dimorphic regions within the CNS with serotoninergic input, including groups of motor neurons in the lumbar and sacral regions of the spinal cord (Kcjima et al., 1984; Newton & Hamill, 1989). In the rat serotonin-containing terminals reach most caudal parts of the spinal cord by embryonic Days 16-17, then regress to the more restricted adult pattern of distribution (Rajaofetra et al., 1989; Wallace & Lauder, 1983).

There are functional serotoninergic receptors in those parts of the fetal forebrain and brainstem containing serotoninergic cell bodies (Whitaker-Azmitia & Azmitia, 1987). In the mature brain, a functional serotoninergic receptor responds to decreased serotonin (such as might occur through the administration of PCPA) by upregulation of receptor numbers. Although the neonatal PCPA treatment caused a reduction in brain 5HT and 5HIAA levels over the period of administration (Wilson et al., 1986 ), by adulthood the levels did not differ from the controls (Wilson et al., 1986; Farabollini et al., 1988). It is possible, however that there was also a permanent change in sensitivity in some class of 5HT receptor as has been shown for receptors in other neuronal systems after their neonatal modification (Meyerson, 1985; Breese et al., 1985). A sex difference has been seen in plasma testosterone levels in control neonates (George and Ojeda,

1982) and persists in PCPA-treated neonates (Handa et al., 1986). In the present study, the increased number of SNB neurons following post natal PCPA treatment, extends and parallels the observations of Lauder et al., (1976, 1978). They reported a decrease in brain serotonin levels in rat pups following PCPA treatment to pregnant dams. This decrease may be an important factor directing both the proliferation and/or migration of neurons which will receive serotoninergic innervation in adulthood (Lauder et al., 1981).

Studies on the sexually dimorphic nucleus in the pre-optic area of the rat have shown that some of the neurons which eventually form this area proliferate at a significantly later age than neurons in the surrounding regions. These neurons migrate from Days 16-21 of gestation (Jacobson & Gorski, 1981) at a time when serotonin fibres are present in the hypothalamus (Wallace & Lauder, 1983). The depletion of serotonin during fetal life will cause an increase in the sexually dimorphic nuclear volume of the preoptic area of female neonates. Similarly, in the present study, serotonin depletion may have increased the number of motor neurons by altering steroid hormone production or the proliferation of motor neurons.

Moreover, it has been shown that serotonin is inhibitory to male reproductive behaviour in the adult (Gessa et al., 1970). An inhibition of serotonin

biosynthesis during development will result in a more male-like pattern of brain development (Handa et al., 1986). It is clear that the masculinisation of the brain during the critical period of sexual differentiation is androgen-dependent; androgens increase the size of the sexually dimorphic SNB and DLN when administered during the perinatal period. It is possible that serotonin depletion during the perinatal period increases neuronal numbers by either influencing cell division (Forda & Kelly, 1985) or interacting with androgens to control neuron numbers.

**POSTNATAL EFFECTS OF SEROTONIN AGONISTS AND ANTAGONISTS ON SEXUALLY DIMORPHIC MOTOR NEURONS IN THE MALE LUMBOSACRAL SPINAL CORD.**

Data from the experiments described here indicate that the $5HT_2$ receptor antagonist ritanserin when administered from Days 1-14 did not significantly alter SNB neuron numbers. However DLN neuron numbers were increased significantly by the same treatment.

Vertebrate motor neurons posses distinct serotoninergic receptors which mediate the various facilitatory and inhibitory effects of this transmitter (Kiehn, 1991). Ritanserin is an extremely potent and very long-acting serotonin $S_2$ receptor blocker (Leysen et al., 1985). An interesting characteristic of 5HT2 receptors is that their blocade facilitates the expression of other 5HT receptor activity.This has been shown behaviourally (Backus et al., 1990 ), and electrophysiologically in the prefrontal

cortex and lateral geniculate (but not in dorsal raphe) where blocade of 5HT2 receptors facilitated the neuronal inhibitory effect of activating 5HT1 receptors (Lakoski and Aghajanian, 1985). Probably ritanserin has the ability to form an energetically stable complex with the serotonin $S_2$ receptor site (Leysen et al., 1985) and thereby blocking the action of serotonin.

The administration of the general $5HT_1$ and $5HT_2$ antagonist methysergide to the male rat on Days 1-14 postnatally did not significantly alter either SNB or DLN neuronal numbers. Methysergide is a general serotonin antagonist. It acts mainly at $5HT_2$ receptor sites, but at $5HT_1$ sites also (White and Newman, 1983). There is a pharmacological similarity between $5HT_2$ and 5HT1C receptors. Methysergide can act noncompetitively at 5HT1C receptors. It alone can diminish the synaptic responses in some of the motor neurons of the spinal cord (Wu Sy et al., 1991) As methysergide blocks both the $5HT_2$ and 5HT1C receptor sites, serotonin activity is decreased.

In the present study, methysergide did not alter the motor neuron numbers present in the sexually dimorphic lumbosacral region of the spinal cord. The reason why it did not act to do so is unclear, but perhaps the dose of methysergide used here was insufficient. But other possibilities are that its various actions may affect the system in opposite ways.For example the receptor binding profiles of various 5HT antagonists show unequivocally that these compounds differ considerably in their primary and

secondary action toward different receptors (Leysen et al., 1980). The analysis of the receptor binding profiles offers an interesting opportunity to help classify the relationship between receptor occupation and observed pharmacological and therapeutic effects. Methysergide produces antagonistic effects at the 5HT2 and 5HT1 receptors and may also exert agonistic effects (Colpaert et al., 1979); it is also a DA agonist via its metabolites (Leysen et al., 1980). It is thus possible that due to its agonistic and antagonistic activities the net result is not to have any significant effect.

The present experiments also indicate that DLN neuron numbers increase significantly in male rats treated with the 5HT1B and C agonist, m-chlorophenyl piperazine (mCPP) postnatally on Days 1-14, whereas SNB neuron numbers are unaltered. mCPP produced an effect on the central serotoninergic system in animals compatible with direct agonist activity on postsynaptic serotonin receptors (Mueller et al., 1985 ). The neuroendocrine response to serotoninergic change may be mediated as much by post synaptic receptors as by presynaptic serotoninergic availability ( Siever et al., 1991). It is possible that 5HT1B and 5HT1C receptors may be presynaptic and therefore act to auto-regulate endogenous 5HT release. Thus the 5HT1 agonist may inhibit endogenous 5HT release and together with its 5HT2 antagonist activity may act in the same way

as ritanserin.

Ritanserin brings about behavioural and biochemical effects such as hormonal and temperature changes in rodents and nonhuman primates compatible with a direct stimulatory action on central 5HT receptors (Aloi et al., 1984). It is also known that mCPP is not only a 5HT1C agonist (Kennet and Curzon, 1988) but also a $5HT_2$ (Simansky and Schechter, 1987) and $5HT_3$ antagonist (Kilpatrick et al., 1987). It is, therefore, possible that the agonistic activity of mCPP is not marked in comparison to its antagonistic activity.

The increase in DLN neuron numbers following administration of mCPP confirms the superiority of its antagonistic action. As we know, decreased 5HT levels increase the sexually dimorphic motor neuron numbers. On the other hand, Melzacka et al., (1979) have shown that mCPP is produced as a metabolite of trazodone in rats. Originally trazodone was described as having serotonin antagonist properties (Silvestrini et al., 1968). Maj et al., (1979) reported that trazodone produces the pharmacological effects of serotonin antagonists in small doses in rats, but the effect of the agonist (after some delay) in large doses.

**BIOGENIC AMINE LEVELS IN THE LUMBOSACRAL AND THORACIC REGIONS OF THE SPINAL CORD IN ALBINO SWISS RATS**

The results from the present experiments indicating that there are no significant sex differences in serotonin and 5HIAA levels in the lumbar region on Day 4 postnatally are in agreement with earlier findings by Hardin (1973) and Gladue et al., (1977). Giulian et al., (1973) reported that in rats hypothalamic serotonin levels are approximately similar in both sexes until Day 8 of life, after which they become higher in the female, the difference reaching significance by Day 12.

Ladosky and Gaziri (1970) and Giulian et al., (1973) reported that whole brain serotonin levels were lower in the male than the female at 12 days of age. Similarly Gladue et al., (1977) and Johnston et al., (1990) and Wilson et et al., (1986) found that at this age, serotonin levels were significantly lower in the male hypothalamus.

In an earlier study, Hardin (1973) reported that sex differences occurred in the brain serotonin content of the neonatal rat, and that these were due to differences in the activity of the enzyme system synthesizing this neurotransmitter, and not to the monoamine oxidase system responsible for its breakdown. Work by Bourgoin et al., (1977) indicated that high free tryptophan levels are present in the newborn female rat brain, but a dramatic decrease occurs during the later part of postnatal life.

Although the findings of the present study with regard to sex differences in indoleamine levels in the spinal cord are not significant, this may be revealed by further work using a larger number of animals.

Neurochemical studies have revealed that in certain areas of the CNS neurotransmitter sythesis, content and metabolism show sex differences, and that these also may be under the influence of sex steroids in adulthood (Vaccari, 1980). Sex differences in brain levels of 5HT have been reported, particularly in the second week of life and this is under the control of the previous androgen environment (Wilson et al., 1991). Thus levels of 5HT and 5HIAA are higher in the whole hypothalamus and in the preoptic area of the female compared to the male during the first 12 to 14 days after birth. Furthermore perinatal androgenization of the female results in masculinization of the 5HT system, with a reduction in indolamine concentrations (Wilson et al., 1986 ).

Gaziri and Gladue (1973) suggested that such sex differences are due to higher monoamine oxidase activity in male rats on Day 12, and that activity of this enzyme can be altered by the presence of androgens during a critical perinatal period. Castration of male rats at birth leads to serotonin levels similar to those seen in females (Ladosky and Gaziri, 1970), whereas treatment of newborn females with androgens results in a reduced brain serotonin content by Day 12 (Giulian et al., 1973; Wilson et al., 1986).

It is therefore possible that the lack of circulating testosterone in the 12 day old female brings about an increased synthesis of serotonin. Watts and Stanley (1984) reported that increased serotonin concentrations seen in the female rat are due to the serotoninergic neurons in this sex possessing a greater indoleamine storage capacity and also greater enzymatic activity, with a higher rate of serotonin synthesis, than in the male.

## MECHANISM OF CONTROL OF CNS DEVELOPMENT BY HUMOURAL FACTORS SUCH AS ANDROGENS AND AMINES

Steroid hormone exposure during early development has been proven responsible for certain male neural structures differing from those in the female. The precise role of neurotransmitters in bringing this about within the CNS is difficult to characterise. However, considerable evidence has accumulated to suggest that the neuroendocrine system is vulnerable to internal and external environmental influences during the limited critical period of sexual differentiation. In the experiments presented here the steroidal milieu of male rats was manipulated during the period when the rat brain monoaminergic neurons were acquiring a particular pattern of distribution for adult life.

The action of androgens and amines on the developing CNS have not yet been fully elucidated. However, they may affect the CNS development via

- Cell division and proliferation
- Prevention of neuronal death
- Cellular specification and migration
- Neuron and target muscle interaction
- Synaptogenesis
- Neurite formation

In this study particular attention has been paid to the effects of androgens and the neurotransmitter serotonin upon cellular and morphological parameters such as neuron numbers, size and extent of the dendritic tree.

a) Steroids may stimulate neuronal proliferation:- This is indicated by the injection of tritiated thymidine on the 12th day of gestation resulting in the concentration of radioactivity in the nuclei of more than 90% of SNB cells in adulthood. However, no SNB cell nuclei were ever heavily labelled with thymidine if it was injected on the 15th day of gestation or later (Breedlove, 1984). This indicates that the completion of neurogenesis occurs by Day 14. All lumbar motor neurons in either sex were postmitotic by Day 15 of gestation (Breedlove and Arnold, 1983b). In male rats TP production does not commence until Day 16 (Warren et al., 1973; Feldman and Bloch, 1978) or perhaps Day 15 (Picon, 1976), and measureable sex differences in plasma TP levels do not occur until even later on Day 18 of gestation (Weisz and Ward, 1980).

Therefore, androgen-dependent sex differences in the adult number of SNB cells (Breedlove and Arnold, 1983a,b) are not due to proliferation of these cells, because androgens are not present in males before completion of neurogenesis.

b) Androgens probably prevent the programmed cell death of motor neurons:-

There is evidence to support the view that androgens affect the SNB by sparing developing motor neurons from programmed cell death. Nurcombe et al., (1981) reported that 45% of rat brachial motor neurons die between birth and Day 6 of life. This period correlates well with the time during which neonatal androgenization of females can cause the retention of many neurons making up the SNB. If females are given androgen on Days 1, 3 and 5 of life, more SNB cells are seen in adulthood than in control animals. However, if the androgen is given on Days 7, 9 and 11, no significant increase in adult SNB numbers is observed (Breedlove and Arnold, 1983b).

It may be that androgen treatment after Day 7 fails to increase SNB numbers, because motor neuronal death is already completed, and there are no further locally available cells to be rescued. The time course of death of the lumbar motor neurons may be slightly different. Correlation between the end of cell death and the completion of the critical period for altering SNB numbers suggests that androgens may act by preventing the death of motor neurons.

c) Steroids influence motor neuronal specification and migration:-

The sexually differentiating process might involve the specification or rearrangement of neurons (Arnold, 1984). Androgens secreted by the testes might stimulate motor neurons to acquire the role of SNB neurons which would involve innervation of the penile muscles and migration of neurons to the correct position. In females, where no androgens are present, these SNB homologues might acquire another functional role and assume another position in the spinal cord. Thus androgens might alter the number of neurons in the SNB without affecting total neuron numbers in the spinal cord. However, a study by Breedlove (1983) suggests that androgens may regulate neuronal specificity by influencing motor neurons which innervate specific muscles.

d) Androgens act on muscles which in turn help to maintain the motor neuron numbers.

Breedlove and Arnold (1983b) found that if females are injected prenatally with TP, they possess a masculinised SNB and retain the perineal muscles. Similarly, injections of DHTB cause retention of the muscles, but no masculinisation in SNB neuron numbers. Breedlove (1983) mentioned that the perineal muscles in DHTP-injected females may be innervated by motor neurons outside of the SNB, perhaps by neurons which do not normally innervate the perineal muscle.

By injecting horse radish peroxidase (HRP) into the

bulbocavernosus muscle, Breedlove (1983) found that many neurons outside the SNB innervate this muscle in the DHTB-injected females compared to their TP-injected counterparts. The above finding suggests that androgens alter the pattern of connections between motor neurons and muscles. However, it is unclear at present whether androgens normally masculinise the SNB system by influencing neuronal specification or the positioning of neurons in the spinal cord.

To understand the mechanism by which androgens masculinize the SNB system, we also need to know the site of action of androgen. If androgens prevent motor neuronal death, conceivably they could do this by acting only on the target muscles, preventing their involution and thus providing the SNB neurons with a stable target. Because motor neurons deprived of targets will die (Buresova et al., 1971), androgen regulation of SNB target muscle is one possible mechanism of regulation of SNB neurons. Alternatively androgens could act exclusively on SNB motor neurons, preventing them from dying, stimulating axonal outgrowth and enhancing their ability to maintain synapse on the target muscles (Sengelaub and Arnold, 1986).

The monoamines which act as neurotransmitters in the brain during later life might play a role during embryogenesis in the regulation of brain development (Lauder and Bloom, 1974; Laurence and Burden, 1973; McMahon, 1974). They are demonstrable by biochemical assay

one week prior to birth in the rat brain (see Baker & Quay, 1969). The results of a combined fluorescence histochemical and [$3_H$] thymidine autoradiographic study on the development of the monoamine cells of the nucleus locus coeruleus, raphe nuclei and substantia nigra in the rat (Lauder and Bloom, 1974) led to the speculation that the monoaminergic neurons might regulate the onset of differentiation of cells to which they will ultimately project in the adult. This hypothesis was drawn from the findings that these monoamine cells begin their differentiation very early in gestation (locus coeruleus: Days 10-13, raphe nuclei: and substantia nigra, Days 11-15), several days prior to the appearance of their prospective target cells and appeared to be capable of synthesising transmitters and elaborating processes soon thereafter - long before they themselves are innervated (Lauder and Bloom, 1975).

The early-forming neurons influence the subsequent differentiation of their presumptive target cells (Lauder and Krebs, 1976). In the present study, the sexually dimorphic motor neurons increase in number due to perinatal androgen treatment and by the depletion of serotonin levels. The increase in number of sexually dimorphic motor neurons by the manipulation of both androgens and amines during the critical period for sexual differentiation indicates that these substances interact regardless of their underlying mechanism of action.

## CONCLUSION

1. Sex differences are present in the numbers of SNB and DLN neurons, but not in those in the RDLN and VM.
2. Postnatal TP or pCPA administration can increase the number of surviving neurons in both the SNB and DLN.
3. Prenatal administration of DHTB will increase the number of neurons present in the SNB.
4. The period of maximum effectiveness of TP, and of TP and pCPA administered concurrently, is the immediate postnatal period Days 1-3 and Days 4-6 after birth respectively. But in case of DHTB treatment, the period of maximum effectiveness is during prenatal life from Days 17-21.
5. There is a close relationship between androgen levels and brain serotonin concentrations for maintaining motor neuron numbers. This confirms that androgens and serotonin play an important role in the sexual differentiation of CNS.

Further studies will be needed to examine how manipulation of serotonin levels react with androgens around the time of sexual differentiation to alter motor neuron numbers in the rat spinal cord.

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# APPENDIX

**STATISTICAL ANALYSIS OF EXPERIMENT 4:**

ANALYSIS OF VARIANCE FOR SNB

| Source of variation | Sum of square | d.f | Mean square | F-ratio | Sig.level |
|---|---|---|---|---|---|
| Main effects | 9788 | 4 | 2447.1 | 9.83 | <0.001 |
| TP treat | 5790 | 3 | 1930.2 | 7.75 | <0.001 |
| p-CPA | 2414 | 1 | 2414.0 | 9.69 | <0.002 |
| 2-Factor interactions | 7000 | 3 | 2333.4 | 9.37 | <0.001 |
| TP treat + p-CPA | 7000 | 3 | 2333.4 | 9.37 | <0.001 |
| Residual | 16183 | 65 | 248.9 | | |
| Total (corr) | 32972 | 72 | | | |

TABLE OF MEANS FOR SNB

| GROUP | N | MEAN | SEM | 95% CONFIDENCE FOR MEAN | |
|---|---|---|---|---|---|
| TP treat | | | | | |
| nil | 19 | 206.15 | 6.17 | 198.92 | 213.38 |
| TP 1-3 days | 18 | 222.66 | 3.87 | 215.23 | 230.09 |
| TP 4-6 days | 26 | 228.96 | 3.08 | 222.78 | 235.14 |
| TP 7-9 days | 10 | 207.00 | 4.44 | 197.03 | 216.96 |
| p-CPA | | | | | |
| nil | 48 | 213.12 | 2.94 | 208.57 | 217.67 |
| p-CPA | 25 | 228.72 | 3.95 | 222.41 | 235.02 |
| Tp treat + p-CPA | | | | | |
| nil nil | 14 | 193.85 | 4.48 | 185.43 | 202.28 |
| nil p-CPA | 5 | 240.60 | 8.20 | 226.50 | 254.69 |
| TP1-3 days nil | 11 | 226.45 | 4.82 | 216.95 | 235.95 |
| TP&p-CPA1-3 days | 7 | 216.71 | 6.23 | 204.80 | 228.62 |
| TP 4-6 days nil | 15 | 223.93 | 4.09 | 215.79 | 232.07 |
| TP&p-CPA 4-6days | 11 | 235.81 | 3.97 | 226.31 | 245.32 |
| TP 7-9 days nil | 8 | 208.25 | 3.64 | 197.10 | 219.39 |
| TP&P-CPA 7-9 days | 2 | 202.00 | 22.00 | 179.71 | 224.28 |
| TOTAL | 73 | 218.46 | 1.84 | 214.77 | 222.15 |

MULTIPLE RANGE ANALYSIS FOR SNB BY TP TREAT

Method: 95 percent confidence intervals

| GROUP | N | MEAN | HOMOGENOUS GROUPS |
|---|---|---|---|
| NIL | 19 | 206.16 | * |
| TP 1-3 days | 18 | 222.66 | ** |
| TP 4-6 days | 26 | 228.96 | * |
| TP 7-9 days | 10 | 207.00 | ** |

MULTIPLE RANGE ANALYSIS FOR SNB BY PCPA

METHOD 95 percent confidence intervals

| GROUP | N | MEAN | HOMOGENOUS GROUPS |
|---|---|---|---|
| NIL | 48 | 213.12 | * |
| p-CPA | 25 | 228.72 | * |

FIGURE 16

The effects of postnatal testosterone propionate (TP) treatment on the sexually dimorphic spinal nucleus of bulbcavernosus (SNB) present in the lumbosacral region of the spinal cord of the male Albino Swiss rats.

TERMS

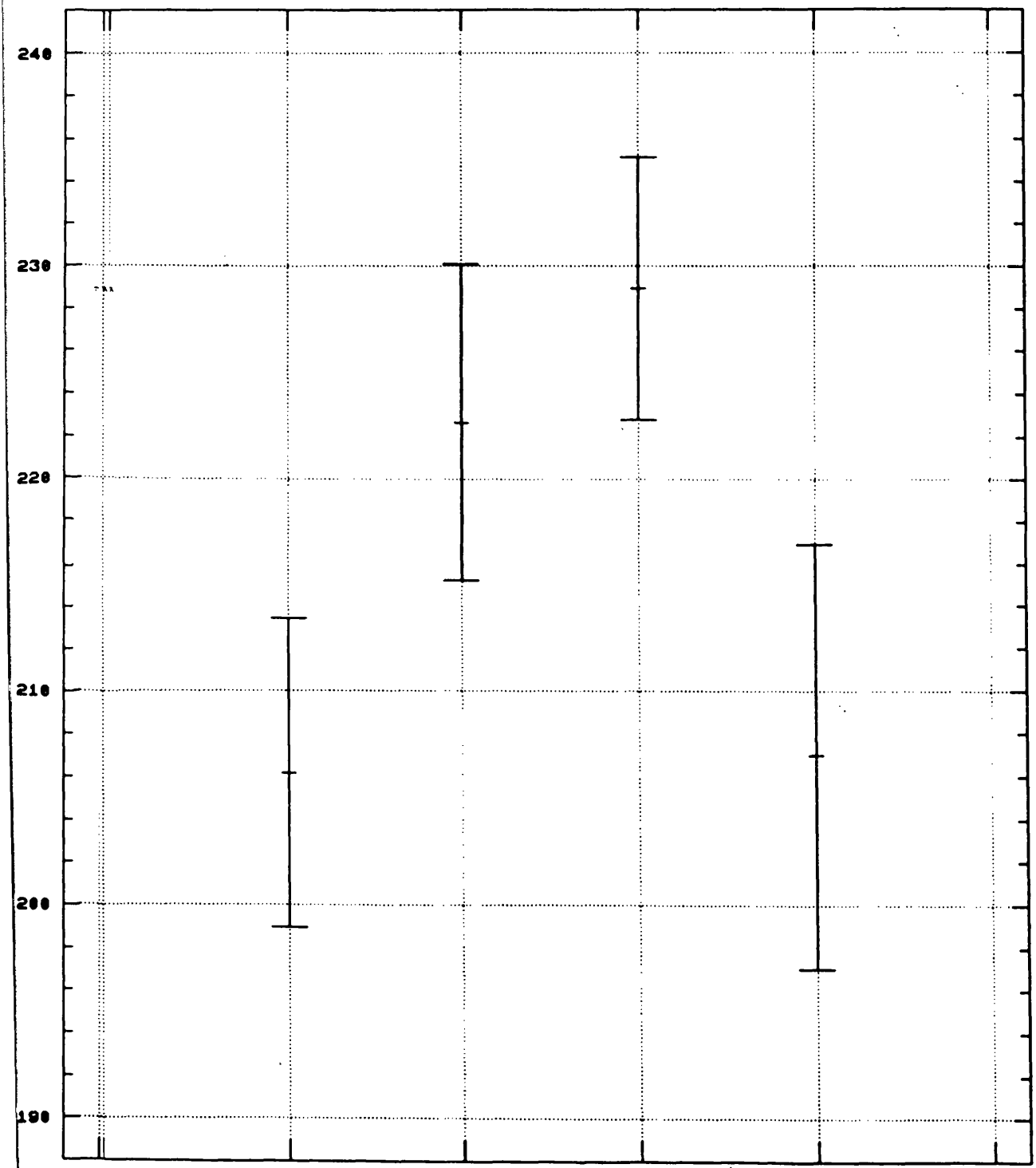
TP 1-3 =Testosterone propionate treatment from days 1-3.

TP 4-6 = Testosterone propionate treatment from days 4-6.

Tp 7-9 = Testosterone propionate treatment from days 7-9.

95 Percent Confidence

Intervals for Factor Means




Level of TP treatment

FIGURE 17

The effects of p-chlorophenylalanine (pcpa) treatment on the sexually dimorphic spinal nucleus of bulbocavernosus(SNB) present in the lumbosacral region of the spinal - cord of male Albino Swiss rats and comparison with control animals.

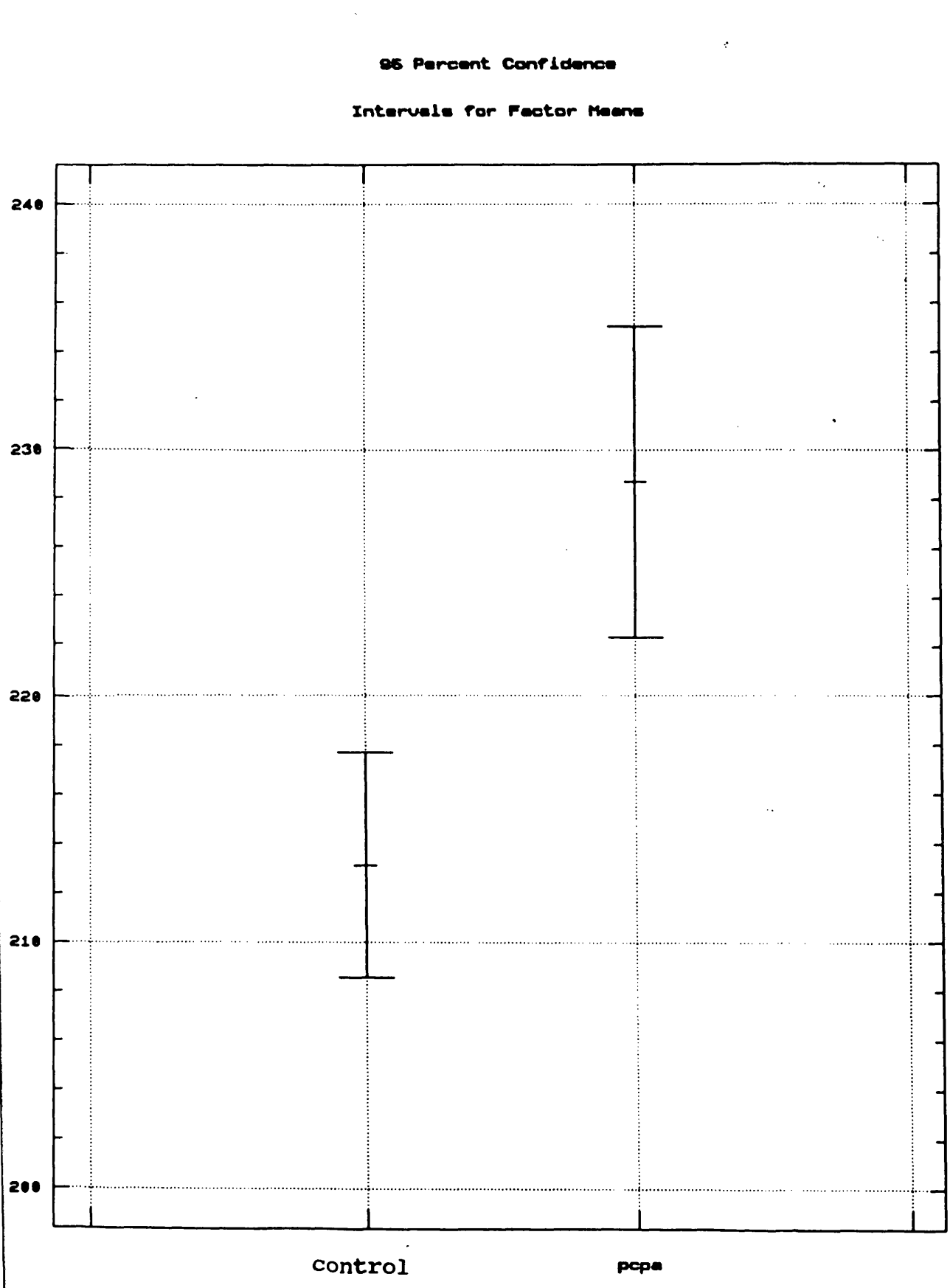
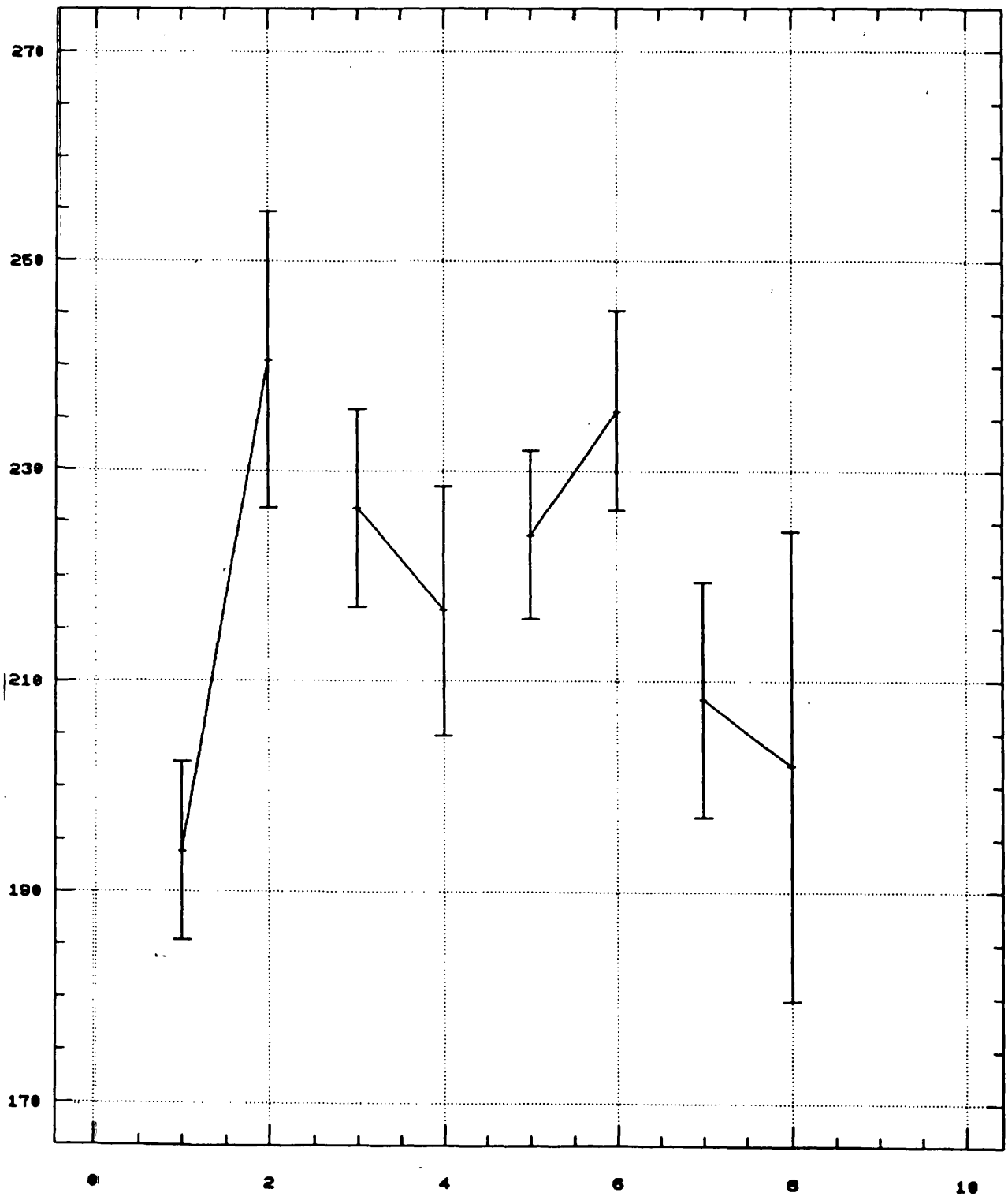

95 Percent Confidence
Intervals for Factor Means
240
230
220
210
200
control
pcpa
level of pcpa treatment

FIGURE 18

The effects of testosterone propionate (TP) and p-cholor-phenylalanine (pcpa) treatment on the sexually dimorphic spinal nucleus of bulbocavernosus(SNB) present in the lumbosacral region of the spinal cord of male Albino Swiss rats and comparison between the two treatment.




Level of TP treatment by pcpa

ANALYSIS OF VARIANCE FOR DLN

| Source of variation | Sum of square | d.f | Mean square | F-ratio | Sig.level |
|---|---|---|---|---|---|
| Main effects | 440 | 4 | 110.1 | 8.68 | <0.001 |
| Tp treat | 348 | 3 | 116.1 | 9.15 | <0.001 |
| p-CPA | 43 | 1 | 43.6 | 3.44 | n.s |
| 2- Factor interactions | | | | | |
| TP treat& p-CPA | 163 | 3 | 54.5 | 4.29 | <0.007 |
| Residual | 824 | 65 | 12.7 | | |
| Total (corr) | 1424 | 72 | | | |

TABLE OF MEANS FOR DLN

| GROUP | N | MEAN | SEM | 95% CONFIDENCE FOR MEAN | |
|---|---|---|---|---|---|
| TP treat | | | | | |
| Nil | 19 | 25.92 | 0.91 | 24.29 | 27.56 |
| TP 1-3 days | 18 | 31.59 | 1.01 | 29.92 | 33.27 |
| TP 4-6 days | 26 | 30.76 | 0.71 | 29.36 | 32.15 |
| TP 7-9 days | 10 | 27.55 | 1.04 | 25.30 | 29.80 |
| P-cpa | | | | | |
| Nil | 48 | 28.46 | 0.65 | 27.43 | 29.48 |
| p-CPA | 25 | 30.82 | 0.78 | 29.40 | 32.24 |
| TP treat & p-CPA | | | | | |
| Nil Nil | 14 | 24.41 | 0.84 | 22.50 | 26.31 |
| Nil p-CPA | 5 | 30.18 | 1.33 | 27.00 | 33.36 |
| TP 1-3days Nil | 11 | 32.54 | 1.37 | 30.40 | 34.69 |
| TP&p-CPA1-3days | 7 | 30.10 | 1.41 | 27.41 | 32.79 |
| TP 4-6 days Nil | 15 | 29.53 | 0.82 | 27.69 | 31.37 |
| TP&p-CPA4-6 days | 11 | 32.43 | 1.09 | 30.28 | 34.58 |
| TP 7-9 days Nil | 8 | 27.91 | 0.95 | 25.40 | 30.43 |
| TP&p-CPA 7-9days | 2 | 26.10 | 4.55 | 21.07 | 31.13 |
| TOTAL | 73 | 29.27 | 0.42 | 28.43 | 30.10 |

MULTIPLE RANGE ANALYSIS FOR DLN BY TP TREAT

Method: 95 percent confidence intervals

| GROUP | N | MEAN | HOMOGENOUS GROUP |
|---|---|---|---|
| Nil | 19 | 25.93 | * |
| TP 1-3 days | 18 | 31.59 | * |
| TP 4-6 days | 26 | 30.76 | ** |
| TP 7-9 days | 10 | 27.55 | ** |

MULTIPLE RANGE ANALYSIS FOR DLN BY PCPA

Method: 95 percent confidence interval

| GROUP | N | MEAN | HOMOGENOUS GROUPS |
|---|---|---|---|
| NIL | 48 | 28.42 | * |
| p-CPA | 25 | 30.82 | * |

FIGURE 19

The effects of testosterone propionate (TP) treatment on the sexually dimorphic dorsolateral nucleus(DLN) present in the lumbosacral region of the spinal cord of male Albino Swiss rats.

TERMS

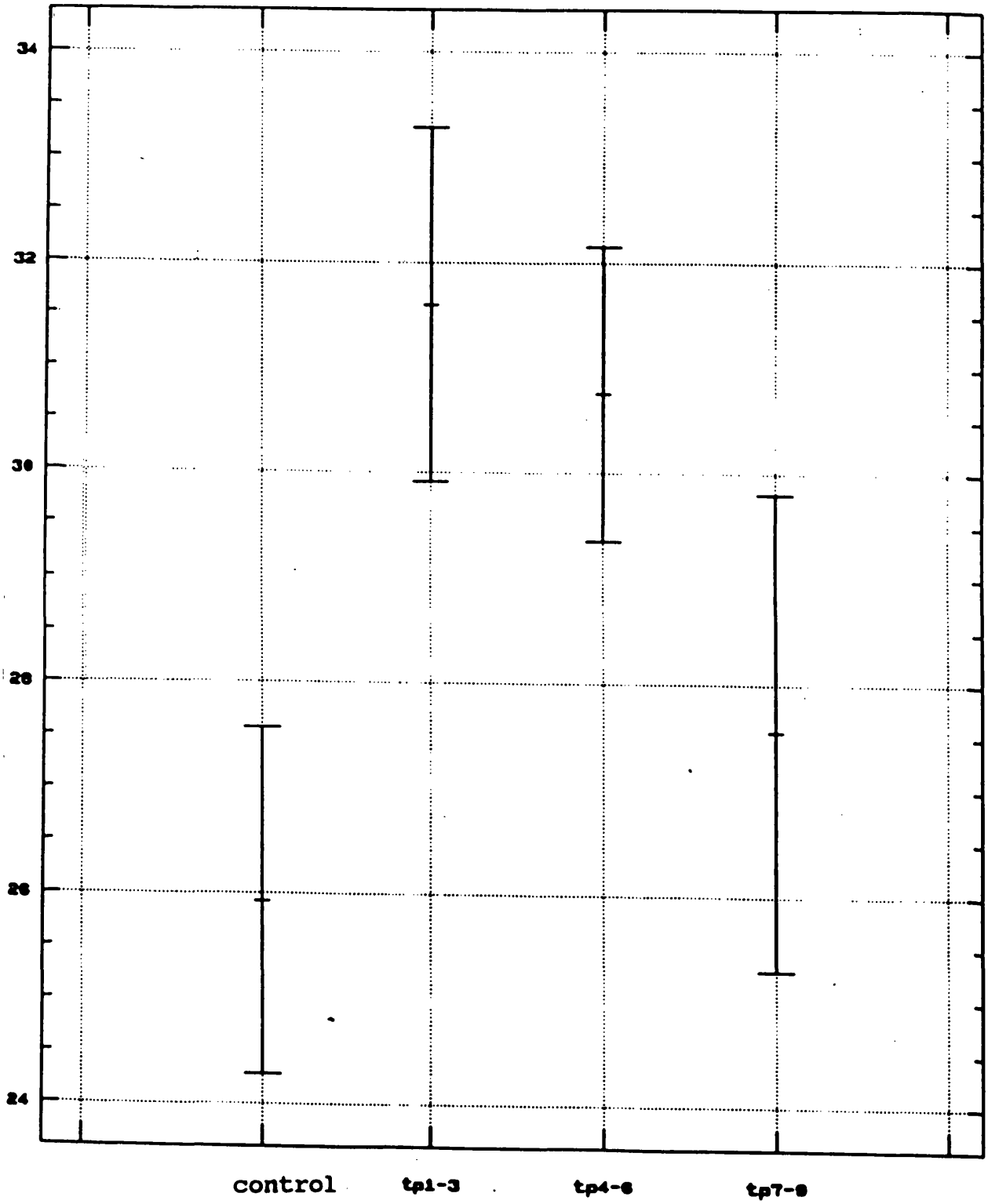
tp 1-3 = Testosterone propionate treatment from 1-3 days.

tp 4-6 = Testosterone propionate treatment from 4-6 days.

tp 7-9 = Testosterone propionate treatment from 7-9 days.

95 Percent Confidence

Intervals for Factor Means



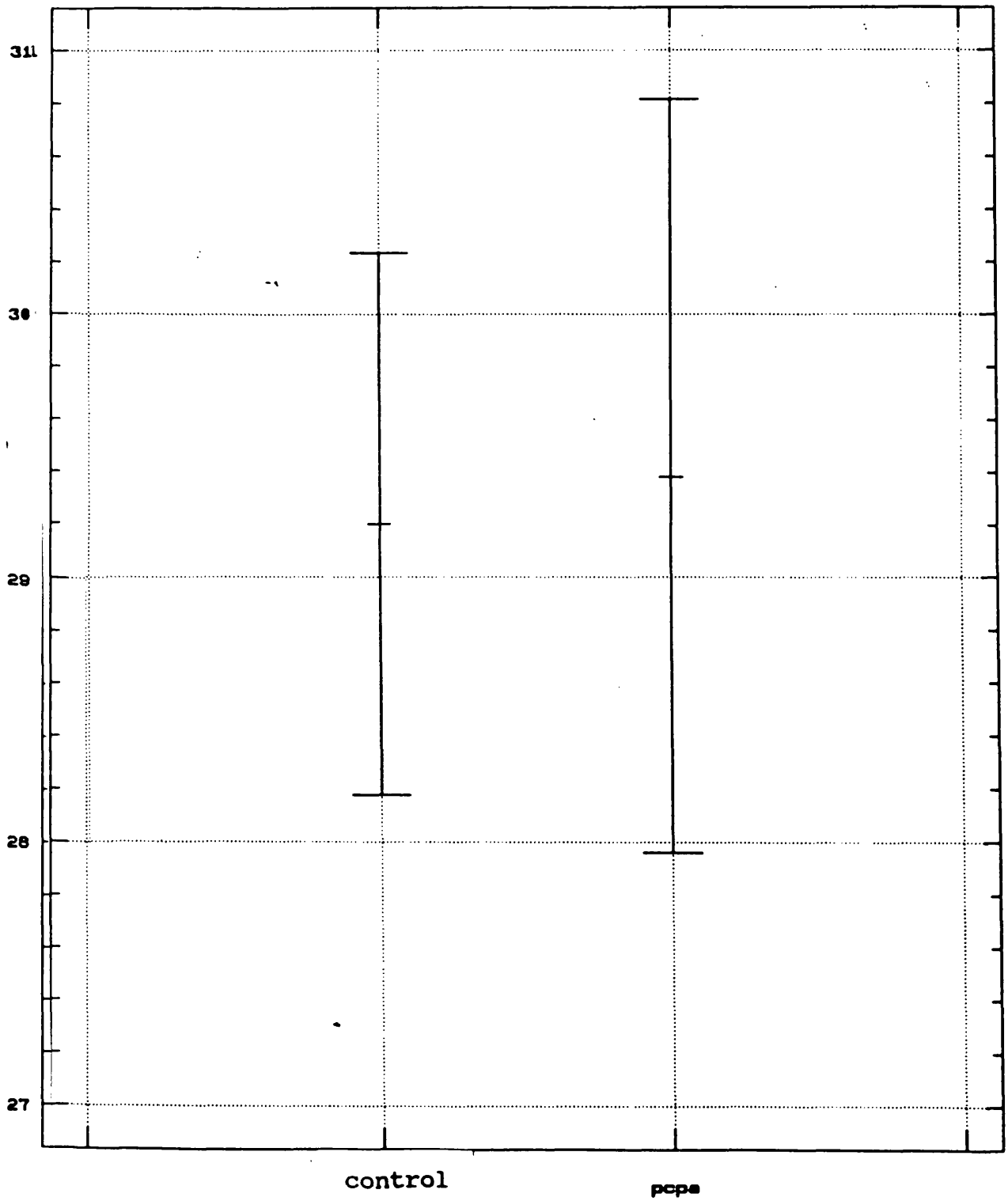

Level of TP treatment

FIGURE 20

The effects of p-cholorophenylalanine (pcpa) treatment on the sexually dimorphic dorsolateral nucleus (DLN) present in the lumbosacral region of the spinal cord of male Albino Swiss rats and comparison with control animals.

95 Percent Confidence

Intervals for Factor Means



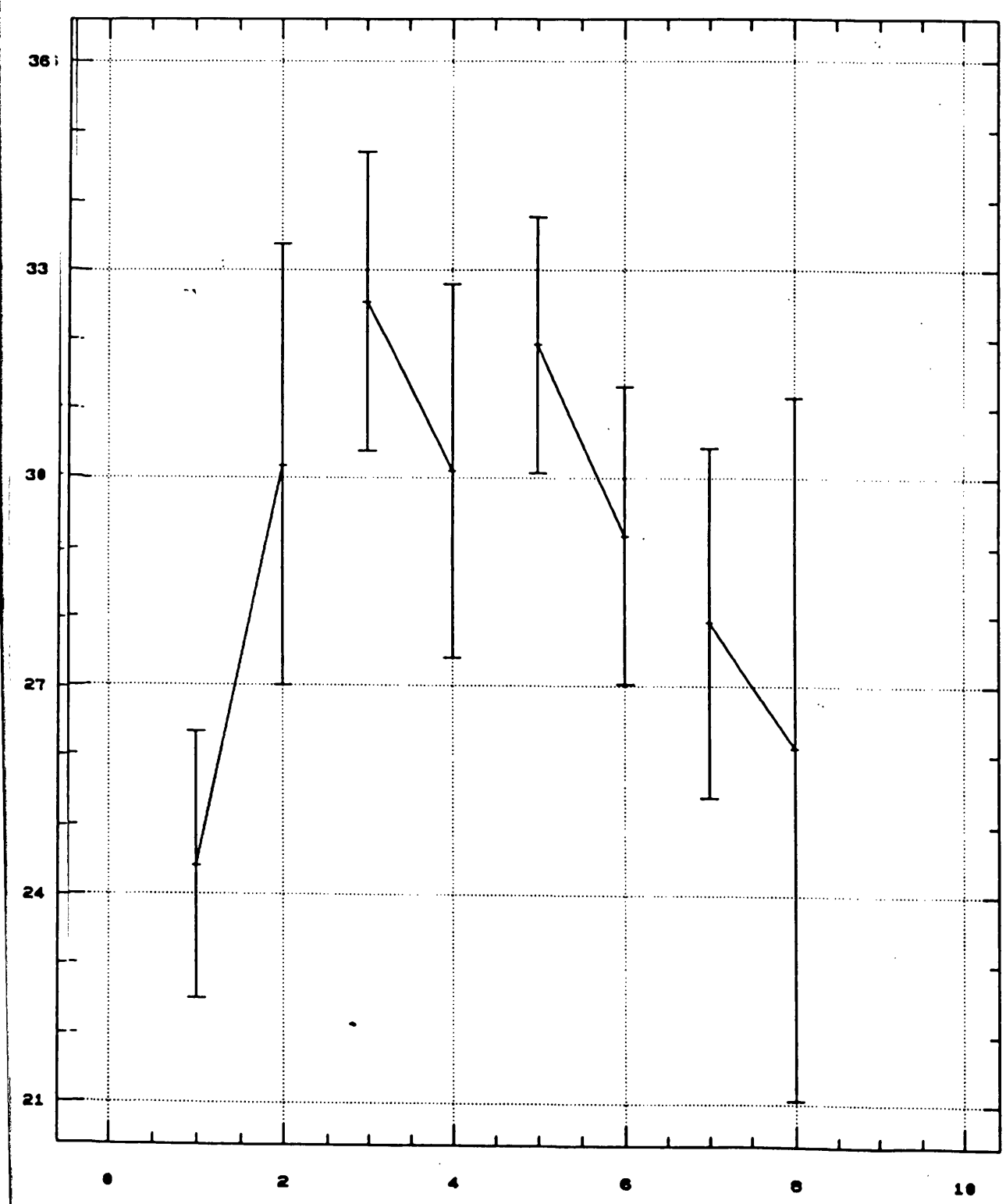

level of pcpa treatment

FIGURE 21

The comparison of the effects of testosterone propionate (TP) and p-cholorophenylalanine (pcpa) treatment on the sexually dimorphic dorsolateral(DLN) present in the lumbosacral region of the spinal cord of male Albino Swiss rats.

95 Percent Confidence

Intervals for Factor Means




Level of TP treatment by pcpa