Perinatal Opiates and the Sexual Differentiation of the Rodent Central Nervous System.

By

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EXPERIMENTS

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Chapter 1

Introduction

The aim of this investigation was to study the effects that opiate administration, during the perinatal period, might have on various aspects of sexual differentiation of the central nervous system in the rodent. The processes of particular interest were,

1. Differentiation of masculine and feminine sexual behaviour in the adult golden hamster.
2. The levels of hypothalamic biogenic amines in the brain of the golden hamster on days 4 and 12 after birth.
3. The volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in the adult rat.
4. The number of motoneurones in the spinal nucleus of the bulbocavernosus (SNB) in the adult rat.
5. Plasma testosterone levels in 2 day old male hamsters.

To give a better understanding of the work carried out for this thesis I will give a brief outline of the various processes of sexual differentiation taking place in the mammal from the time of conception leading up to the perinatal period.

Mammalian Sexual differentiation

Sexual differentiation occurs as a sequential array of events. The process of mammalian sexual differentiation begins with the establishment of the chromosome complement of the individual at fertilization with the pairing of the

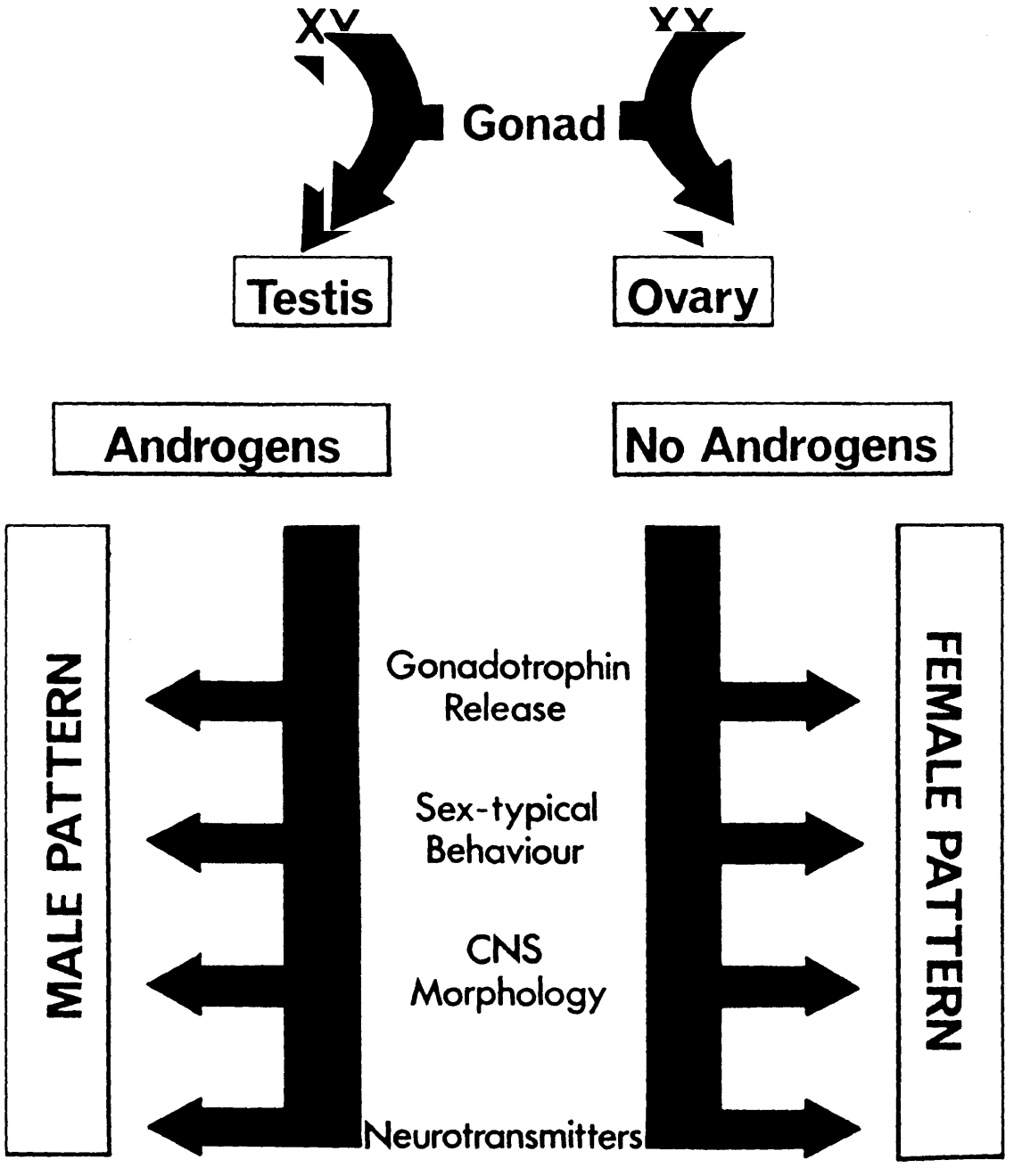
XY
XX
Gonad
Testis
Ovary
Androgens
No Androgens
MALE PATTERN
FEMALE PATTERN
Gonadotrophin Release
Sex-typical Behaviour
CNS Morphology
Neurotransmitters

Fig 1:1

gametes. The genetic information carried in the egg and sperm will, under normal circumstances, determine the phenotypic sex, that is , XX results in a female phenotype and XY results in a male phenotype.

Thus under the direction of the genotypic sex, the indifferent gonads develop as either testes or ovaries. In males, once the testes have differentiated, the interstitial Leydig cells begin to secrete testosterone, which then acts to direct development of the internal sex ducts, the external genitalia, somatic tissue and finally the nervous system along male lines. If the gonads become ovaries, all subsequent differentiation will be along female lines. (see Fig. 1:1)

Chromosomal Sex

In most mammals, the chromosomes consist of xn pairs of autosomes plus 2X chromosomes in females and 1X + 1Y in males. Thus, the possession of a Y chromosome results in maleness, and is responsible for triggering gonadal differentiation to form testes. The development of karyotyping techniques (Moorhead et al, 1960; Guthrie, 1962) revealed that some individuals with supernumerary X chromosomes ie, XXY or XXXY have a male phenotype although they may be sterile (Davis, 1981). When the X and Y chromosomes pair to form the XY body during spermatogenesis, the X chromosome is inactivated, thus the supernumerary X may be coding for some factor which

inhibits spermatogenesis (McLaren & Monk, 1981). While it is not uncommon for XO females (Turner's Syndrome) to be born and reach adulthood, no male individual has been recorded as YO, which implies that a minimum of 1 X is required for survival.

Females inherit 1 X chromosome from the mother and the other from the father, while males always inherit a maternal X and a paternal Y. During normal development, one of the X chromosomes in every somatic cell is randomly inactivated (heteropycnosis); thus all daughter cells in a clone will have the same X chromosome inactivated (Lyon, 1962). This inactive X can be visualised as a Barr body in cells taken from a female (Barr & Bertram, 1949). The female body is therefore a mosaic of cells, some have the maternal X inactive while others have the paternal X inactive. Where there are more than two X's there will be a Barr body for each supernumerary X chromosome. Heteropycnosis does not occur in the germ cells and, during oogenesis, both X chromosomes are active.

It has become apparent that the genes which determine testicular differentiation are situated on the short arm of the Y chromosome, since loss of the long arm has no effect on testicular development; neither does translocation of the long arm onto an autosome (see review, Davis, 1981). Duplication of the long arm and thus loss of the short arm results in ovarian development (Gordon & Ruddle, 1981).

Where on the short arm of the Y chromosome are these genes located? Eichwald and Silmser, (1955) described

transplantation experiments which they had carried out on highly inbred strains of mice. Here skin grafts from male mice were rejected by females of the same strain, yet, the reciprocal grafts were not. They deduced that this was caused by a chromosome histocompatability gene, which was subsequently named the H-Y antigen. Using serological assays to measure the male antigen, Wachtel et al (1975) found that where the male antigen was detected, testes were usually present. Thus they proposed that the H-Y antigen was the testis- determining gene.

Cattanach et al, (1971) described an autosomally-inherited condition in mice which causes genetic females to develop as phenotypic males (Sxr) which have small testes and are sterile as adults. They found no evidence that the Sxr gene could have come from the Y chromosome and proposed that it was an autosomal gene mutation. However, in 1982, Singh and Jones reported that DNA hybridization techniques indicate that the Sxr gene consists of DNA which is related to that found on the Y chromosome. They suggested that the Sxr gene is transferred to the distal end of the X chromosome from the distal end of the Y chromosome in the carrier male XY_{Sxr} during meiosis. These findings were confirmed by Evans et al (1982) who reported that XY_{Sxr} males have an abnormal Y chromosome possessing an extra dark-staining distal body. Evans and his colleagues, (1982) were able to show that this dark-stained body was transferred via crossing over from one chromatid of the Y

to one chromatid of the X during meiosis and the Sxr gene was contained within this body. Since X inactivation is a random procedure, offspring from XY_{Sxr} males could be expected to be a mixture of XX_{Sxr} females, XX_{Sxr} males and XX_{Sxr} intersexes. This indeed was shown to be the case by Cattenach et al (1982), although there appeared to be an imbalance towards males. Further research by McLaren and Monk, (1982) indicated that when the X chromosome carrying the Sxr gene is preferentially inactivated, fertile XX_{Sxr} females can be produced as a result of the Sxr gene being inactivated with its carrier X chromosome. Thus, although testicular development takes place in the absence of a Y chromosome in the Sxr mouse, these males are always sterile as adults, probably due to the XX chromosome composition. Shapiro et al, (1982) demonstrated increased serological H-Y in XY_{Sxr} carrier males. XX_{Sxr} males are H-Y positive, whereas XX_{Sxr}, males have testes but are H-Y negative (McLaren et al, 1984). This indicates that the H-Y antigen is not responsible for the testes-determining factor in mice or in man (Simpson et al, 1987), but is located very close to the testes-determining gene (Tdy) and may be necessary for normal spermatogenesis (Burgoyne et al, 1986).

A few other regions of the Y chromosome have been isolated and sequenced genetically, but so far the exact switch which triggers testicular differentiation has not been identified; in fact, it has been suggested that the Y chromosome acts merely as a switch on a cascade of events

leading to testicular differentiation which involves autosomal genes as well as the sex chromosomes.

Gonadal differentiation.

The gonadal primordium develops on the coelomic surface of the mesonephros and is composed of:-

1) coelomic epithelial cells, which give rise to Sertoli cells in males and granulosa cells in females,

2) stromal cells derived from gonadal mesenchyme which form the interstitial cells

3) primordial germ cells (PGC's) which migrate into the gonad from their original location in the wall of the yolk sac endoderm.

The PGCs migrate from the yolk sac via the dorsal mesentery of the hind gut to the genital ridge. Proliferation of the coelomic epithelial cells occurs around the same time as the PGC's are accumulating in the gonad. These cells aggregate to form cord-like structures which surround the PGC's.

Testicular Differentiation.

The first morphological sign of sexual dimorphism in the gonad is the differentiation of the pre-Sertoli cells, identified by their abundant clear cytoplasm and their aggregation to form testicular cords in the fetal testis (Jost & Magre, 1984). Myoid cells form around the testicular cords and Leydig cells differentiate within the

interstitial tissue. Testicular differentiation occurs early in development, but varies according to species and length of gestation, i.e. day 13 post-coitum in the rat (Jost, 1972), day 12 in the mouse (Taketo et al, 1985), day 10 in the golden hamster (MacKay et al, 1989) and between the 6th and 7th week in humans. After differentiation, the pre-Sertoli cells secrete a protein of molecular weight 125Kd which acts locally to induce the regression of the Mullerian ducts, (primitive female sex ducts) (Josso et al, 1977). It has also been suggested that the pre-Sertoli cells may induce subsequent development of both the peritubular cells and the Leydig cells, but this has yet to be demonstrated (McLaren, 1987). Leydig cells secrete testosterone, the major androgen responsible for the masculinization of the genital tract.

Experimental evidence has indicated that gonadal differentiation is not dependent on the presence of germ cells as their surgical removal (Zuckerman, 1960), destruction (Merchant, 1975), absence or gross reduction of numbers due to mutation (Mintz, 1957; McCoshen, 1983), does not prevent differentiation taking place. Nor is testicular cord formation essential for the development of a testis. In in vitro culture conditions, which appear to prevent testicular cord formation, Sertoli cells produced Mullerian inhibitory substance at the normal time (Magre & Jost, 1984); Leydig cells differentiated and were capable of steroidogenesis (Patsavoudi et al, 1985).

On arrival in the gonad, primordial germ cells rapidly

increase in number by mitotic division. In males, once the optimal number has been reached i.e. 5000 in the mouse, further proliferation ceases until puberty. Thereupon the germ cells enter meiosis and continue to proliferate by meiotic division throughout life. In contrast to this, female germ cells proliferate by mitosis, reaching a peak of 7,000,000 by the fifth month of development in humans; they then enter the first meiotic division and become surrounded by a single layer of granulosa cells to form the primordial follicles. The majority are then lost by cell degeneration and by the age of 7 years numbers have been reduced to around 40,000 (Pinkerton et al, 1961).

Ovarian Differentiation.

Ovarian differentiation occurs later than that of the testis, and is characterised by two successive stages. Proliferating germ cells first enter meiosis; this is followed by the development of follicles around some of the germ cells. Byskov, (1978) has suggested that germ cells are induced to enter meiosis by a hormone (meiosis-inducing substance) produced by the rete ovarii, since they do not enter meiosis if cultured in the absence of the rete. However, this finding has still not been confirmed. MacLaren, (1984) and Francavilla & Zambini, (1985) have reported that a factor produced by the testicular cords prevents the germ cells from entering meiosis. In contrast to testicular development, where testis cord formation

occurs in the medulla of the gonad, ovarian follicles accumulate within the cortex. The cells in the ovary develop the capacity to synthesize oestrogen very early in development. Although elegant studies by Jost, (1953) showed that the ovary, and therefore its hormonal output, is not essential for the differentiation of the female genital tract, the diameter of the Mullerian duct in castrated male or female rabbit fetuses is less than that of normal female fetuses. Thus oestrogens may play a role in stabilizing the female sex ducts. It is known that the fetal ovary develops the ability to aromatize testosterone to oestradiol at a very early age (George & Wilson, 1978).

Endocrine function of gonad

The gonads function as endocrine organs both in adulthood and during prenatal development. In the male, the capacity to produce androgens develops with the differentiation of the Leydig cells (Wilson & Siiteri, 1973). In humans, proliferation of these cells and the concommitant increase in testosterone production coincides with the establishment of the male sex ducts, external genitalia and the CNS (Bloch, 1979). Leydig cell numbers then decrease, and by week 21 of gestation have reach a low level which is maintained postnatally (Bloch, 1964). Other mammals show a similar pattern of development (Wilson & Siiteri,1973; Noumura et al, 1966). However, in rodents, where sex differentiation of the CNS occurs very late in gestation or early in the postnatal period, there is a peak

of testosterone production around the time of birth (Pang et al,1979; Pang & Tang, 1984). A similar postnatal peak in testosterone levels has been reported in human male offspring by some investigators (Forest et al, 1975; Forest & Cathiard, 1975; Dawood & Saxena, 1977), but not by others (Abramovich & Rowe, 1973; Mizuno et al, 1968).

The importance of gonadal secretions during development was first recognized by Lillie, (1917) who proposed that secretions (hormones) produced by the bovine testes were being carried in the blood to their female twin and causing virilisation of the genital tract; these masculinized females were dubbed freemartins. Castration and testosterone replacement experiments by Jost (1953) substantiated the proposals of Lillie that testicular compounds were responsible for the phenomenon of the freemartin. Jost however, also determined that two testicular products were required for complete masculinization of the female. These are:

1) Mullerian inhibiting substance, required for regression of the female sex ducts

2) Testosterone, required for maintenance and development of the Wolffian ducts.

By transplantation experiments in rabbits, Jost showed that a testis transplanted onto the mesosalpinx resulted in ipsilateral regression of the Mullerian duct.

Internal Reproductive Tract.

Working with the Flanders giant rabbit, Jost (1947) castrated fetuses *in utero* prior to gonadal differentiation and noted that, regardless of genetic sex, the offspring were born as phenotypic females, with normal oviducts, uteri and vaginae. However, he found that if a piece of testis was transplanted on to one of the ovaries of a fetal rabbit, the reproductive tract ipsilateral to the graft would be masculinized.

Prior to differentiation of the internal genitalia, both the Wolffian duct (male) and the Mullerian duct (female) presumptive tract anlagen are present. Thus the reproductive tract is capable of becoming either male or female depending on the hormone milieu at the time of differentiation. Jost's castration and transplantation experiments, coupled with the proposals of Lillie on the cause of the freemartin condition, indicate that the testes play a crucial role in determining the phenotypic sex of an animal. Injections of testosterone into castrated animals stabilize the Wolffian duct, but are unable to trigger regression of the Mullerian duct. Therefore two testicular products are necessary for complete masculinization. Nathalie Josso, (1972) isolated the second substance, Jost's Mullerian inhibiting substance (MIS), from fetal calf testes and identified it as a non-steroidal 125K Dalton dimer which caused regression of the Mullerian duct in organ culture. MIS is secreted by the pre-Sertoli cells

immediately on their differentiation, and regression of the duct begins almost at the same time as testicular cord formation occurs (Blanchard & Josso 1974).

Testosterone is the androgen which is necessary for the differentiation of Wolffian ducts to form seminal vesicles, vas deferens and epididymis and also for the maturation of spermatic tubules (Wilson & Lasnitzki, 1971; Wilson, 1973). In the rabbit there is continued high testosterone production to day 25 post coitum. This correlates well with the stabilization of the Wolffian duct which is completed by day 24. Similar results have been reported in the rat (Noumura et al, 1966). This is further borne out by enzyme studies. Thus, 17β hydroxysteroid dehydrogenase Δ5,isomerase activity in Leydig cells is indicative of testosterone production, and the amount of testosterone produced tends to correlate with the temporal sequence of Leydig cell proliferation and regression. The increase in testosterone production also correlates with the sequence of reproductive tract differentiation (Bloch, 1979). Initially testicular production of androgens is under autonomous control (Wilson et al, 1983). However in vivo studies have indicated that within a few days, production is under the control of LH (Wilson et al, 1983) . This can be demonstrated experimentally; pituitary destruction during gestation results in decreased testis weight and activity (Eguchi et al, 1975: Tseng, 1975)

As has been mentioned previously, the ovaries apparently have little function in establishing the female

reproductive tract, as is indicated by its differentiation even in their absence. However the ovaries exhibit aromatase activity (which converts testosterone to oestrogen) very early in development. In fact, rabbit ovaries are reported to acquire this capability at the same time as the testis becomes capable of producing testosterone (Milewich et al, 1977). Again, timing appears to be important in female reproductive tract differentiation; if the Mullerian duct has not been induced to regress by MIS at the appropriate time, it stabilizes and goes on to form the Fallopian tubes, uterus and upper part of the vagina. In contrast, in the absence of testosterone, the Wolffian duct will regress.

External genitalia.

Only at a late stage in Wolffian duct stabilization do the cells within the duct develop the capacity to convert testosterone to 5α-dihydrotestosterone (DHT) (Wilson & Lasnitzki, 1971). However, levels of the enzyme responsible for this conversion, 5α-reductase, are particularly high in the tissues of the urogenital sinus in males prior to differentiation (Manson 1989). These tissues differentiate into the prostate, scrotum and penis. A human genetic deficiency of the enzyme results in male pseudohermaphroditism, whereupon the prostate and external genitalia are poorly developed and the offspring may not be recognised as males. However, at puberty these tissues

become responsive to testosterone and the individuals develop masculine characteristics, ie. penile growth, deepening of the voice and increased muscle mass in response to the increase in testosterone at this time (Imperato-McGinley 1979). This condition can be induced experimentally by giving rats 5α-reductase inhibitors over the critical period when masculinization of the external genitalia takes place (Imperato-McGinley et al, 1985). In contrast, in the testicular feminization syndrome, the tissues of the urogenital system have greatly reduced numbers of androgen receptors, or have receptors that are in some way defective, and therefore cannot respond to the high levels of testosterone produced either during development or puberty (Griffin & Durrant, 1982; Mowszowicz et al, 1989). Thus these individuals have testes, but no male reproductive tract or external genitalia. Such individuals are therefore regarded as females and quite often it is not until in adulthood, and married, that their true gender becomes apparent, e.g. when attending a clinic for infertility (Short, 1972). A similar condition has been identified in the mouse (Lyon & Hawkes, 1970) and is caused by an X-linked disorder. DNA hybridization analyses have shown that the gene specifying the androgen receptor is localized on the X chromosome in man, rat and mouse (Migeon et al, 1981; Chang, Kokontis & Liao, 1988). Thus while Wolffian duct stabilization is dependent upon testosterone, masculinization of the urogenital sinus requires conversion of the androgen to dihydrotestosterone.

In the female the genital tubercle (which forms the phallus in males) becomes the clitoris, the urethral folds become the labia minora while the genital swelling differentiates into the labia majora.

The adrenal gland normally produces corticosteroids and very small amounts of androgens. However if corticosteroid production is abnormally low, under the influence of ACTH stimulation from the pituitary the gland increases in size to compensate (congenital adrenal hyperplasia). This condition, termed the adrenogenital syndrome, results in over production of androgens which can result in varying degrees of masculinization of the female external genitalia e.g. enlargement of the clitoris and fusion of the labia (New et al, 1983). In male offspring this increase in androgen output can cause precocious puberty (Short, 1972). Similar abnormalities have been brought about by treatment of pregnant women with synthetic progestins (e.g. diethylstilboestrol) to fend off a threatened miscarriage (Hayles & Nolan, 1958; Johnson & Everitt, 1988).

Sexual differentiation of the CNS.

Many aspects of CNS function show sex differences. These include physiological controls such as gonadotropin release, sensory capacities, and many types of social and non-social behaviour. This suggests that male and female brains differ functionally and/or structurally. Like the reproductive tract and genitalia, the immature brain

appears to be indifferent and to differentiate during development according to the presence or absence of testosterone or its metabolites. Here androgens are acting to organise the CNS and its capacities.

Differentiation of the hypothalamus-hypophyseal-gonadal axis and sex-typical behaviour.

This section deals with the perinatal establishment of adult patterns of hormone release. However, it is necessary first to discuss the differences in these patterns in the adult mammal. In adulthood, female sex hormones are produced both tonically and in a cyclic pattern, while male hormones are released tonically (Neill,1972). In the adult mammal, the hypothalamus controls the pattern of hormone secretion from the anterior pituitary. The anterior pituitary releases many hormones of which three are important in reproduction; these are luteinising hormone (LH), follicle stimulating hormone (FSH), and prolactin (PRL). Prolactin output is controlled by prolactin inhibitory factor (PIF),which is now believed to be dopamine (MacLeod, 1976). LH and FSH secretion is controlled by gonadotrophin releasing hormone (GnRH) produced in the hypothalamus. Pulses of GnRH are carried via the hypophyseal portal system to the adenohypophysis where they stimulate the output of the gonadotrophins. These in turn are released into the general circulation and carried to their target organs, the gonads. The pulsatile secretion of GnRH is controlled by a pulse generator

located within the medial basal hypothalamus (Knobil, 1974): frequency and amplitude of pulses are modified by feedback mechanisms (Karsch, 1984). There are three of these feedback mechanisms, (see fig.1:2)

1) A long loop:- from the gonad to the hypothalamus and anterior pituitary; high levels of circulating gonadal hormones act to suppress further gonadotrophin and GnRH release.

2) A short loop:- from the anterior pituitary to the hypothalamus; LH and FSH suppresses GnRH secretion.

3) An ultra short loop:- in which the hypothalamus acts upon itself; high concentrations of GnRH suppress its own production.

All three are negative feed-back systems in males, but in females the long-loop system can at times be positive. Also involved in gonadotrophin release at the hypothalamic level are a number of neurotransmitters. Of particular interest are the monoamines, noradrenaline (NA), dopamine (DA) and serotonin (5HT), the amino acid -aminobutyric acid (GABA), and the endogenous opioids, the enkephalins and endorphins. Dopamine, as already mentioned, acts as an inhibitor of prolactin release. Noradrenaline fibres synapse with GABA neurones in the POA; GABA neurones synapse with LHRH neurons and are probably inhibitory. It is thought that NA may stimulate LHRH release during the preovulatory surge by reducing this inhibition (see reviews Wilson, 1979; De Vries, 1990). Serotonin appears to play a

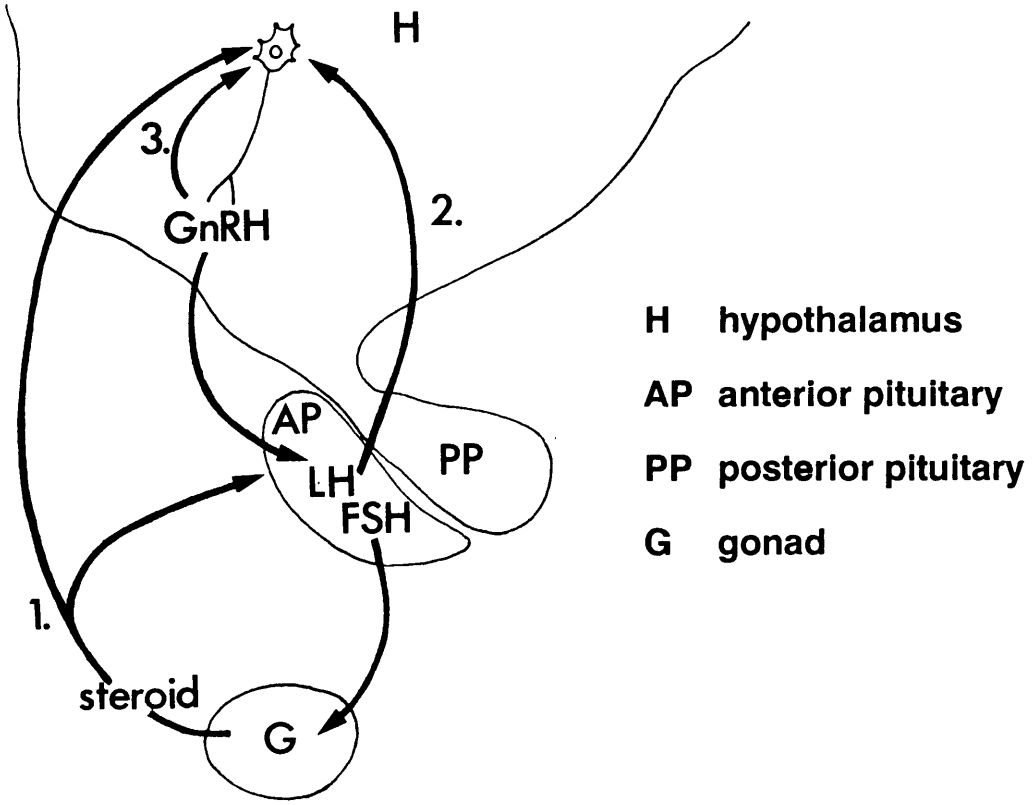

Fig.1:2 Feedback mechanisms of the hypothalamo-pituitary-gonadal axis.

1. **Long loop feedback.**

2. **Short loop feedback.**

3. **Ultra-short loop feedback.**

dual role in gonadotrophin regulation, since it has been reported to act in an inhibitory manner and to facilitate the release of the preovulatory LH surge (see review, Wilson 1979). The endogenous opioids may act either on the NA/GABA/GnRH pathway by inhibiting NA release, or directly on GnRH cells (Miller et al, 1985), as they are reported to synapse directly onto these neurones (Leranth et al, 1988).

In the male, LH stimulates the testicular Leydig cells to produce testosterone which acts synergistically with FSH to stimulate the Sertoli cells and induce spermatogenesis (de Kretser,1984). The Leydig cells also produce small amounts of oestrogen and high levels of circulating testosterone plus oestrogen act via the long-loop system to suppress gonadotrophin production. The testosterone acts in the medial basal hypothalamus to reduce the frequency of GnRH pulses, while oestrogen acts directly on the pituitary to lower the amplitude of LH pulses (Karsch, 1984).

In the female, the pattern of reproductive hormone secretion is more complicated and is linked to the maturation and release of ripe oocytes, the preparation of the uterus for implantation, and the induction of appropriate behaviour patterns which maximise the chances of copulation and fertilisation. Tonic release of LH stimulates the ovarian follicular cells to produce androgens and oestrogen. FSH induces the granulosa cells to aromatize the testosterone produced in the follicular cells to oestradiol (Flowerdew, 1987). Oestradiol in the general circulation stimulates further release of LH and

FSH which in turn causes further production of oestrogens until the levels reach the threshold at which surges of LH FSH and PRL are induced (Neill & Smith, 1974). It is believed that oestrogen acts on the anterior hypothalamus-suprachiasmatic nucleus-preoptic area to induce these surges (Brown-Grant 1977; Goodman, 1978; Peterson & Barraclough, 1989). During the period of oestrogen production the ovarian follicles are ripening and preparing for the release of the oocytes. The surge of LH at this time causes massive swelling of the follicles destined to be ruptured (Schwartz, 1969): and leads to atresia of follicles not destined to ovulate (Greep, 1961). Furthermore it promotes the switching from oestrogen production to progesterone production (Hori et al, 1970). This is followed by the rupture of the follicles; the released oocytes are swept into the infundibulum of the oviduct prior to fertilization. The cells of the ruptured follicle form the corpus luteum and produce progesterone which acts on the endometrium to prepare for implantation. The high levels of circulating oestrogen and progesterone at this time act to induce estrous behaviour in the female (Feder et al,1971). In the absence of pregnancy, the corpus luteum regresses as another cycle begins.

Environmental factors also play a role in controlling gonadatrophin secretion: thus visual, olfactory and tactile stimulation can induce increases in GnRH output. Experiments with mice revealed that initial exposure to a

female rapidly increases LH pulses in the male; these decreased with time of exposure, but rapidly increased again when a new female was placed with the male (Coquelin & Bronson 1979). Sheep and deer are seasonal breeders with a relatively long gestation and therefore mate in the autumn. These species go through periods of anestrus and testicular quiescence outwith the breeding season. In such animals the pineal gland responds to daylength and suppresses GnRH release during the periods of long daylength. As daylength shortens, the inhibitory actions of the pineal are removed and gonadotrophin and steriod production increase (Lincoln, 1984). In some species the females are induced ovulators, that is ovulation only occurs if copulation has taken place. Rabbits, hares and cats are examples of induced ovulators. In these females the stimulation of the cervix during copulation triggers the release of a surge of gonadotrophin which in turn induces ovulation (Sadleir, 1969; Conaway, 1971).

Perinatal sexual differentiation of gonadotrophin releasing patterns.

In long gestation species, brain sexual differentiation takes place prenatally, while in short gestation mammals, it occurs in the early postnatal period (see review MacLusky & Naftolin, 1981). In rats the critical period runs from late gestation to the early postnatal period, while in the hamster sexual differentiation of the CNS occurs in the postnatal period only.

In 1936 Pfeiffer reported a series of experiments in rats which indicated that the presence of testes during the perinatal period is necessary for the masculinization of the pattern of gonadotrophin release from the pituitary. Subsequently many experiments have confirmed that masculinization of the rodent brain is controlled by testicular hormones acting during the early perinatal period (Barraclough & Gorski 1961; Harris 1970). There is a time limit after which the androgen will be ineffective in causing masculinization; a single dose of TP on days 2 or 5 resulted in 100% anovulatory sterility; on day 10, TP caused sterility in some females but not all, while the same dose on day 20 had no effect (Barraclough, 1961). Male rats castrated on or before day 3 were capable of sustaining cyclic release of gonadotrophins in adulthood, whereas male rats castrated on day 5 postnatally could not (Harris, 1970). However, low doses of testosterone over several days postnatally will cause permanent anovulatory sterility. Female rats treated in this manner will, at puberty, show a few ovulatory cycles and then become permanently sterile. This process is termed the delayed anovulatory syndrome (Swanson & van der Werff ten Bosch, 1964).

Administration of various androgenic metabolites led to the conclusion that testosterone itself is not responsible for male differentiation in those regions of the rat brain controlling gonadotrophin release. For complete

masculinization to occur, testosterone needs to be converted to oestradiol-17β (McDonald & Doughty 1974). Oestrogen treatment before day 5 postnatally was shown to be as effective as testosterone in causing anovulatory sterility in female rats (Gorski, 1963; Dörner et al, 1971; Christensen & Gorski, 1978). Similarly antioestrogen administration prevents the masculine pattern of gonadotrophin release in the androgenized female rat (McDonald & Doughty, 1972). Immunocytochemical studies reveal large numbers of oestrogenic receptors located within the hypothalamus and other sexually dimorphic areas in both male and female rats. (Naftolin et al 1975; Toran-Allerand et al 1980). Nonetheless, not all CNS sexual differentiation is oestrogen-activated; a few areas have a high concentration of testosterone receptors and a deficit of oestrogen receptors. These regions, e.g. spinal nucleus of Bulobcavernosus (SNB) appear to require 5α-reductase activity to become committed to their masculine role (Breedlove & Arnold, 1983c).

A circulating oestrogen-binding substance α-fetoprotein (AFP) is present in high levels in the fetus, neonate and pregnant female, but is not found in non-pregnant females or adult males (Raynaud et al, 1971). AFP was initially thought to prevent plasma oestrogens from reacting with the intracellular receptors within susceptible areas of the brain (MacLusky & Naftolin, 1981). However, contradictory evidence has since come to light regarding the function of AFP. Steroid-binding proteins, including AFP, transport

steroids in an inactive form. AFP is not synthesized within neurones, but as it has been detected within brain cells (Benno & Williams, 1978; Schachter and Toran-Allerand, 1982) it must have been transported there via endocytosis. Therefore, AFP does not, as was originally believed, prevent oestrogen from reacting with brain cells, but may act to control the level of interaction. This would suggest that oestrogens may also be required for feminization of the brain, since Tamoxifen (an oestrogen antagonist) given postnatally to female rats causes permanent anovulatory sterility (PAS), and reduces feminine receptivity. However it is unable to masculinize sexual behaviour (Döhler et al, 1976). For masculinization to be completed a combination of oestrogen and testosterone may be required. On the other hand , perhaps higher levels of oestrogen, (enhanced by aromatization of testosterone), may be necessary before masculinization can take place. Such a phenomenon would in part explain some of the reported changes in adult sexual behaviour. Females exposed to higher levels of oestrogen than normal during the perinatal period, show less adult feminine sexual behaviour or more masculine sexual behaviour than expected, depending on the dosage. Similarly, males deprived of threshold levels of oestrogen display more feminine and less masculine behaviour than expected. Oestradiol has been reported to enhance neuronal survival (Toran-Allerand, 1976), induce dendritic arborization in hypothalamic explants (Toran-Allerand 1984)

and enhance hypothalamic synaptic formation (Matsumoto & Aria 1976). It is thus possible that by these processes oestrogen, depending on the dose, imposes a feminine or masculine pattern on the immature developing brain.

Can other androgen metabolites masculinize the brain during the critical postnatal period? This seems unlikely as DHT administered during the critical postnatal period has little or no effect on hormone secretion patterns in females or males castrated at birth, neither do other 5α-reduced androgens (McDonald & Doughty 1972; Aria, 1972; Whalen & Luttge 1971; Tonjes et al 1987).

Sexual differentiation of behaviour.

Many aspects of behaviour are sexually dimorphic; these include aggression, courtship and copulation, maternal and play behaviour. All of these forms of behaviour require perinatal exposure to testicular hormones and /or their metabolites to be masculinized. This is demonstrated by exposing female neonates to androgens or by castrating males on day 1 postnatally. Perinatally androgenized female rats display less maternal behaviour in adulthood than normal, while neonatally castrated males show more maternal behaviour (Quadagno & Rockwell, 1972; Bridges et al, 1973). Similarly, female rats exposed to androgens during the critical period of differentiation display more rough and tumble play (male-typical behaviour) as pups (Olioff & Stewart, 1978; Tonjes et al, 1987) and more aggression as adults (Van de Poll, 1978) than untreated

females.

Adult sexual behaviour is activated by gonadal hormones after puberty. Masculine sexual behaviour patterns (which, in the rodent, include sniffing the female, licking the vulval region, mounting, intromission and ejaculation) require exposure to testicular androgens within the first five days of life (Whalen & Edwards, 1967; Paup, Coniglio & Clemens, 1972). Absence of androgens during this period results in a failure to display these behaviours even when androgens are administered in adulthood. Moreover, a genetic male deprived of masculinizing hormones during the critical period will respond behaviourally to oestrogen and progesterone priming in adulthood by displaying feminine sexual behaviour (i.e.lordosis) to a normal male. Defeminization, that is loss of the ability to display feminine copulatory behaviour in adulthood after the appropriate priming, also occurs after normal or experimental exposure to androgens postnatally in both the rat (Whalen & Nadler, 1963) and the golden hamster, (Debold & Whalen, 1975). Female hamster pups treated with androgens within 5 days of birth are, as adults, capable of displaying all parameters of masculine sexual behaviour. However, they have a reduced capacity to display feminine behaviour (Payne, 1976 & 1979; Debold & Whalen, 1975). Castration of male pups within 2 days of birth has the opposite affect, i.e. it feminizes and demasculinizes adult behaviour. Whereas replacement of testosterone at the same

time prevents this (Whalen & Edwards, 1967; Swanson, 1971). Thus, testicular hormones are required for organization of the appropriate sexual behaviour patterns. It was originally believed that testosterone was responsible for masculinization of sexual behaviour, but subsequent studies indicate that, in rodents, androgens must be aromatized to oestrogens to be effective (Luttge & Whalen, 1970; Paup, Coniglio & Clemens, 1972). Futhermore, antioestrogens such as MER25 and Tamoxifen, given concurrently with testosterone or oestrogen prevents the masculinisation of behaviour (McDonald & Doughty, 1972; Döhler et al, 1984c). Additional studies have shown that masculinization and defeminization are two separate processes, and that it is possible to masculinize female golden hamsters with exogenous androgens without defeminizing them (Payne, 1979). Defeminization without masculinization can result in an animal which responds to neither androgens nor oestrogens in adulthood (Dohler et al, 1984c).

The exact location of the areas within the hypothalamus which control sexual behaviour have not yet been totally elucidated. Pfaff & Sakuma, (1979) and DeJonge et al, (1989) reported that masculine sexual behaviour is markedly inhibited by lesion in the medial preoptic area of the adult rat, and adult gerbil (Commins & Yahr, 1984). On the other hand, Hennessey et al,(1986) reported that POA lesions increased the display of lordosis in adult male rats. Feminine sexual behaviour is inhibited by lesion of the ventromedial hypothalamus (Malsbury et al, 1977; Pfaff

& Sakuma, 1979).

The establishment of behavioural patterns during the postnatal period is a complex process which probably involves neurotransmitters and/or peptides as well as gonadal hormones. Of particular interest is the role of serotonin (5HT) in this process. In 1970 Ladosky & Gaziri reported a temporary sex difference in the levels of 5HT in the hypothalamus of the rat on day 12 postnatally. Serotonin levels rise in both male and females from birth (Schmidt & Saunders-Bush, 1971) but at day 12 females have significantly more hypothalamic 5HT than males. Giulian et al, (1973) has suggested that the increased levels of 5HT observed in females results from a greater synthesis, while Gaziri & Ladosky, (1973) argued that the difference is due to increased monoamine oxidase activity in males. The fact that this sex difference is androgen-dependent (Ladosky & Gaziri, 1972) suggests that 5HT may be involved in the process of sexual differentiation. Serotonin has been implicated in the control of adult sexual behaviour (Dewsbury et al, 1972); thus increased 5HT levels reduce lordosis in female rabbits, hamsters and mice (Meyerson et al, 1973), whereas decreased 5HT levels increase lordosis (Zelman et al, 1973, 1977). Perinatal manipulation of serotonin has been equally effective in altering patterns of behaviour in adulthood. Hyyppa et al, (1972) and Wilson et al, (1986) reported that reduction of 5HT postnatally by p-chlorophenylalanine (pCPA) subsequently enhances

masculine sexual behaviour in adult male rats. In contrast, Farabollini et al, (1988) found no difference in male rat sexual behaviour after perinatal administration of pCPA, nor did Johnston et al, (1990) in hamsters. However the latter authors did find that pCPA given during the second week of life increased feminine sexual behaviour in male hamsters, while decreasing it in females. This would suggest that 5HT may be involved in the defeminization process. In contrast, increasing 5HT levels during the perinatal period appears to have little effect on feminine sexual behaviour in either male or female rats (Gladue et al, 1977). Although NA and DA do not show any apparent differences in levels between the sexes during development, they have been reported to be involved in the control of sexual behaviour in the adult (Agmo & Fernandez, 1989; Vathy & Etgen, 1989).

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[illegible] ventricular diameter nucle
[illegible] is greater in male than in fe
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[illegible] differences (Sennwis et al, 1986).

Differences in CNS morphology.

Male and female brains, while differing slightly in size, were always thought to be morphologically similar. However, over the past 20 years it has become apparent that there are sex differences in the morphology of certain areas of the mammalian CNS. Listed below are some of these.

1. Differences in nuclear and nucleoli size in the medial preoptic area, (Dörner & Staudt, 1968) and the ventromedial nucleus (Dörner & Staudt, 1969) of the rat. The nuclei and nucleoli volumes of the female are greater than those of the male.
2. Numbers of dendritic spine synapses of non-strial origin in the medial preoptic area of the rat; females have more spine synapses than do males (Raisman & Field, 1973).
3. Organization of synapses in the amygdala of the rat; there are greater numbers of shaft synapses in the male than in the female (Nishizuka & Arai, 1981).
4. Dendritic branching patterns in the hamster preoptic area are sexually dimorphic (Greenough et al,1977).
5. The volume of the sexually dimorphic nucleus of the preoptic area is greater in male than in female rats (Gorski et al, 1978).
6. Male rats have a greater number of both light and dark accessory olfactory bulb granule cells than do females, neonatal castration of males or female androgenization reverses these differences (Segovia et al, 1986).

7. There is a greater number of motor neurones in the spinal nucleus of bulbocavernosus in the male than in the female rat, this is determined by postnatal androgen levels (Breedlove & Arnold, 1980)

8.The volume of the parastrial nucleus of the rat preoptic area is greater in females than in males (del Abril et al, 1990)

9. The corpus callosum of the male rat is larger than that of the female (Fitch et al, 1990).

Of these morphologically different areas, those which have been most extensively studied are the sexually dimorphic nucleus of the preoptic area (SDN-POA) and the spinal nucleus of bulbocavernosus (SNB). These will be dealt with in detail since they are examined in this study.

SDN-POA

The preoptic area of the hypothalamus has been shown to be important in the organization of adult sexual behaviour patterns and in the control of the preovulatory LH surge in females (see above). The findings of Dörner & Staudt, (1968,1969) that cell body size within the area is sexually dimorphic, and the elegant studies by Raisman & Field (1973), into the sex differences in the synaptic organization of the POA lie at the foundation of a considerable accumulation of knowledge over the past 20 years. Gorski et al's (1978) finding that the volume of the medial preoptic area is greater in male than in female

adult rats led to this region being named the SDN-POA. Further research indicated that this difference in volume is under the influence of testicular hormones and that the difference becomes manifest during the perinatal period (Jacobson et al, 1980). Castration of male rat pups on the day of birth and up to 24 hours postnatally reduced the volume of the adult SDN-POA to approximately 50% of that of controls (Handa et al, 1985), but did not wholly sex reverse it. Administration of TP or EB postnatally to females increased the volume, but not to that of control males (Gorski et al, 1978). However, if the treatment was extended such that it covered both the pre-and postnatal period, the volume of the female SDN-POA was increased to a size equal to that of normal males (Döhler et al, 1982). Since the discovery of the SDN-POA in rats, the same structure has been identified in gerbils (Yahr & Commins, 1983), guinea-pig (Hines et al, 1982; Byne & Bleier, 1987), hamster and mouse (Bleier et al, 1982), ferret (Tobet et al, 1986) and man (Swaab & Hofman, 1988).

The neurones which make up the SDN-POA migrate from the periventricular area to form the nucleus during gestation. Tritiated thymidine can be used to label cells undergoing mitosis. By administering this to pregnant rats on day 18 post-coitum Jacobson et al, (1980) were able to locate mitotic neurones in the ependyma of the 3rd ventricle and follow their migration to the SDN-POA. The number of labelled neurones decreased over this period indicating cell death, a common feature of CNS development. From

these observations, and the reports that oestrogens can stimulate neuronal survival (Toran-Allerand, 1981) and enhance dendritic arborization (Toran-Allerand, 1980), Gorski, (1984) suggested that the surge of testicular hormones present in male rats on day E18 may promote neuronal survival during a period when cell death is taking place. It is also of interest to note that 5HT is involved in the determining size of the SDN. Handa et al (1986) investigated the effects of serotonin depletion on the volume of the nucleus and found that pCPA administered from day 8 of gestation significantly reduced nuclear size at birth. However this change in size is not maintained into adulthood (Hines & Gorski, 1986) More recently, Bloch & Gorski, (1988) have described the SDN-POA in terms of the contribution from each of three specific cytoarchitectonic divisions of the medial preoptic nucleus (MPN). The SDN-POA is located within the medial area of the MPN (MPNm) with contributions from the anteroventral division of the MPN (MPNav), the lateral division of the MPN (MPNl) and the central division of the MPN (MPNc). In males the contributions from the MPNc and the MPNav are greater while in the female the contribution from the other regions of the MPN than these two was greater. These findings reveal that the SDN-POA has a different cytoarchitectonic composition in the two sexes, and that, in the male at least since the relative contribution from the MPN, MPNl and MPNm is altered by neonatal castration, the input is

controlled by testicular hormones during development.

SNB

One of the more striking examples of a sexually dimorphic area within the CNS is that of the (SNB), which in the rat consists of some 200 neurones in males but only 40 in females. The cells of the SNB form a discrete nucleus within the dorsomedial aspect of the ventral horn between lumbar regions L5 and S1 in the spinal cord. The SNB cells are large multipolar motor neurones with extensive dendritic arborisation which innervate the perineal muscles bulbocavernosus (BC) and levator ani (LA). These two striated muscles, together with the ischiocavernosus (IC), mediate penile reflexes in the male rat (Sachs 1982; Hart & Melese d'Hospital 1983), and mouse (Elmore & Sachs 1988). The BC and LA are attached exclusively to the penis, while the IC is attached to the penis and ischium. The IC is innervated by a subpopulation of motor neurones in the dorsolateral nucleus (DLN), the remainder of which innervate the hind limb via the sciatic nerve. The SNB is comparable to Onuf's nucleus in the dog and in humans.

These motor neurones and their target muscles are known to accumulate androgens and are dependent on testosterone or DHT for their maintenance (Breedlove & Arnold, 1983). Castration of adult male rats results in decreased size of the soma of motor neurones and a reduction of muscle mass, but does not reduce the number of SNB neurones (Breedlove &

Arnold, 1981). However, in some strains of mice the SNB neurone numbers are also reduced (Wee & Clemens, 1987); androgen administration prevents this. Autoradiographic studies have revealed that all motor neurones in the lumbar regions of the rat spinal cord are capable of accumulating testosterone and dihydrotestosterone, but not oestrogen (Breedlove & Arnold, 1983). Nevertheless, those motor neurones involved in specifically masculine functions (e.g. SNB) were seen to take up significantly more label than those not involved in sexual functions e.g. the retrodorsal nucleus (RDLN) which innervates the leg (Breedlove & Arnold, 1980). Breedlove and Arnold (1983) repeated these autoradiographic experiments in females and reported that there is a significant sex difference in androgen uptake, male motor neurones accumulating more testosterone than those in the female.

Both the muscles and SNB nucleus are dependent upon the hormone milieu during the perinatal period for their survival. During the prenatal period both sexes develop these perineal muscles and the motor neurones which innervate them (Cihak et al, 1970; Nordeen et al, 1985). At birth there is substantial neuronal cell death, but this is less in normal males or in females given testosterone or DHT on day 2 postnatally than in control females (Nordeen et al, 1985). In untreated females and in androgen-insensitive mutant (Tfm) males, the motor neurone numbers fall to less than one third of those in control males; soma

size is reduced in the remaining cells and the muscles completely disappear (Breedlove & Arnold, 1981). Prenatal exposure of males to the anti-androgen flutamide, plus neonatal castration, completely sex-reverses the SNB (Breedlove & Arnold, 1983).

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 a strain difference as oestrdiol benzoate has been reported to enhance SNB neurone survival in female albino Swiss rats (Currie et al, 1990). Thus it appears that in some strains this particular nucleus is exclusively dependent upon testosterone and/ or its 5α-reduced metabolites for its survival, while in others it is not.

Exactly how androgens promote the survival of the motor neurones and target muscles is not yet fully understood. However, it has been shown that testosterone will support BC survival in the absence of a neural input (Fishman & 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 & Breedlove, 1987). From these two finding it has been suggested that androgens act by stabilizing the muscle and thus promote SNB survival via trophic substance released by it.

With the exception of gonadal differentiation itself,

all of the processes of sexual differentiation described here have required exposure to testicular androgens for masculinization. That animals and humans have been reported with ambiguous sexuality demonstrates the plasticity and sensitivity of the developing systems. Anything, exogenous or endogenous, which interferes with androgen synthesis, release or uptake may therefore cause incomplete masculinization. In addition, interference with the development of those parameters involved in sexual differentiation, such as retardation or precocious development of neural structures may also result in altered sexuality.

Of particular interest, is the effect that exposure to opiates during this period of plasticity, might have on the sexual differentiation of the CNS.

Opiates.

Opiate-type drug abuse has been widespread throughout the world for many years. Unfortunately for the addicts, one of the more common side-affects of chronic drug abuse is loss of reproductive function. Male addicts report loss of libido, failure to sustain erection and failure to ejaculate. Ironically, opiates initially heighten sexual arousal and were once claimed to be aphrodisiacs, but as tolerance builds up, ever higher doses are needed, resulting in a progressive decline in sexual function (Cushman, 1972; Hanbury, Cohen & Stimmel, 1977; Smith *et*

al, 1982). Female addicts are reported to suffer loss of libido, anorgasmia, amenorrhoea and anovulation (see review by Pfaus & Gorzalka, 1986). Nonetheless, many women do become pregnant, and continue to abuse drugs throughout pregnancy. Babies born to opiate abusers suffer the classic withdrawal symptoms, " Cold Turkey" at birth and often have to be weaned gradually off the drug (Harper et al, 1977). Follow up assessment of babies exposed to opiates during the perinatal period have revealed that these children still have poorer motor control and learning abilities when compared to infants of similar socio-economic status 18 months after birth (Rosen & Johnson, 1982).

Investigations into how opiates might act to suppress reproductive function have shown that heroin administration results in a reduction of serum LH levels and a concommitant lowering of serum testosterone (Akabori & Barraclough, 1986; Purohit et al, 1979; Genazzani & Petraglia, 1989). The action of opiates on LH levels is believed to be mediated via inhibition of GnRH release.

The discovery of specific opiate receptors in the mammalian CNS (Pert & Snyder, 1973; Terenius, 1973; Simon et al, 1973) suggested that there must be endogenous opiate-like substances produced which would recognise these receptors. The enkephalins were the first of these endogenous substances to be reported, (Hughes, (1975) and Hughes et al, (1975). Subsequently, β-endorphin, a 31-amino-acid peptide was isolated by Li & Chung (1976), and

was shown to have potent morphine-like activity both *in-vivo* and *in-vitro*. Since then many more opiate-like peptides have been isolated and characterised. To avoid confusion between the exogenous opiates and the endogenous opiate-like peptides, the endogenous peptides are referred to as the opioids. The roles that these substances play within the body are many and varied. They are reported to be involved in regulation of gonadotropin release (Diez-Guerra *et al*, 1986; Gilbeau *et al*, 1984; Dyer *et al*, 1988), sexual behaviour (Pfaus & Gorzalka, 1986), respiration (Hindmarsh & Sankaran, 1985), gastrointestinal activity (Kreek *et al*, 1983) and modulation of pain sensation (Bausbaum & Fields, 1984). In addition, opioids have been shown to be active during development in the regulation of cell proliferation (Zagon & McLaughlin,1987) and synaptic organization (Hauser *et al*, 1987). Since exogenous opiates are known to alter the ontogeny of opiate receptors during development (Hammer *et al*, 1989) it would be very surprising if in doing so, they did not also interfere with the role of the endogenous opioids in neural development.

The identification of opiate receptors and opioid neural systems within the hypothalamus suggested a role for these in the control of reproduction (Kornblum *et al*, 1987; Martini *et al*, 1989). That opiates inhibited LH release and sexual behaviour lent support to this theory. Furthermore the administration of the opiate antagonist naloxone or the more potent naltrexone reversed the

inhibitory effects of morphine on sexual behaviour and gonadotrophin release. In addition, naloxone enhanced sexual behaviour in the absence of opiate drugs (Pfaus & Gorzalka, 1987; Murphy, 1981).

Opiate receptors have been separated into the sub-types, μ, δ and k, according to the types of opiate/opioid which bind to them. Morphine binds to both μ and δ receptors but is 50 times more potent at the μ-receptor, the enkephalins also bind preferably to the μ receptor. β-endorphin binds with equal affinity to both μ and δ receptors while dynorphin binds to k receptors. Investigations into the ontogeny of the opioid receptors have revealed that they are first detectable within the rat CNS on embryonic day 14 (Kent et al, 1982) and in the human brain by week 20 of gestation (Magnan et al, 1988; Magnan & Tiberi, 1989). The early development of these receptors and the fact that their patterns of distribution change with time (Tavani et al, 1985; Petrillo et al, 1987) suggests that they may play a vital role in development. Equally interesting is the finding that receptor concentration is altered by manipulation of gonadal hormones in the adult (Hahn & Fishman, 1979) and neonatal rat (Hammer, 1985). Further reports have indicated that exposure to morphine during the developmental period alters receptor distribution and density (Tsang & Ng, 1980; Tempel et al, 1988), and inhibits both DNA synthesis in vivo (Kornblum et al, 1987) and neuronal outgrowth in vitro (Davila-Garcia & Azmitia, 1989).

Opioids act as neurotransmitters to inhibit or activate other neurones, and are known to block LH secretion by inhibiting noradrenergic transmission in the preoptic area (Dyer et al, 1988; Gopalan et al, 1989). The potent opiate antagonist naloxone, has the opposite effect in that it amplifies LH release (Van Vugt et al, 1982). Because opiates apparently play a marked role in development, and reduce androgen levels in both adults (see above) and neonates (Ward et al, 1983) it is likely that exposure to such drugs during the critical period of sexual differentiation, would be detrimental to the masculinization of the rodent CNS. This project therefore set out firstly to examine the changes (if any) in the adult sexual behaviour of golden hamsters exposed to a long-acting form of morphine (Duromorph) over the perinatal period. If changes were observed to occur, a second aim was to examine other an over the perinatal period.

[illegible]logy). The results from the tests demonstrate that while both forms o[illegible]d analgesia, the effects of morphin[illegible]rm and the response latency to the

Appendix 1

All of the experiments discussed in this thesis involve the administration of morphine to pregnant females and/or their pups. Handling of animals is stressful to them. This, therefore had to be minimized to avoid the effects of stress interfering with the action of the drug. Therefore a long-acting form of morphine, Duromorph, was chosen for these experiments. Treatment was reduced to a maximum of two injections per day using Duromorph. Duromorph is a long-acting aqueous suspension of morphine of which 95% is in crystaline form. Before the experiments were begun, it was important to establish that

a) the drug was effective and

b) that it was indeed long-acting.

Three experiments were carried out to establish that Duromorph was suitable for use in this investigation. These experiments were not carried out by the author, but by other members of staff as part of a pilot study, prior to the main investigation. Two studies compared Duromorph with morphine sulphate to determine how long the drug was effective.

i) The first experiment compared the analgesic effects of the two drugs using a standard hot-plate test. This test was carried out in the Anatomy Dept by Mrs. J Odber (Psychology). The results from the tests (shown in appendix 1:1) demonstrate that while both forms of morphine rapidly caused analgesia, the effects of morphine sulphate were short-term and the response latency to the heat was

almost back to control levels within two hours of administration. In contrast, Duromorph treated animals continued to display analgesic effects 12 hours after treatment. ii) This experiment and the following one, were carried out in the Department of Materia Medica, Western Infirmary, Glasgow by Mr.G Forrest.

Pregnant female hamsters were treated with Duromorph and culled at various times after treatment. Blood was removed and the serum analysed chromatographically using High Performance Liquid Chromatography coupled to a U.V. detector. Concentrations of morphine and its metabolites Morphine-6-glucuronate (M-6-G) and morphine-3-glucuronate (M-3-G) were measured (see appendix 1:2).

The concentrations of both morphine and its inactive metabolite M-3-G decline fairly quickly from the blood while the active metabolite M-6-G [known to be x 20 more potent than morphine itself (Pasternak et al, 1987) was still present in the blood 24 hours after administration. iii) Further chromatographic analyses of the concentrations of the drugs in brain tissue of mothers and of fetuses were carried out (see appendix 1:3). These investigations indicated that morphine does cross the placental barrier and accumulates in the fetal brain to a greater extent than in the adult.

Appendix 1:1

Hot Plate test Temp. 45.2 °C

Time from treatment	response latency	n
control	11.4 ± 1.3	30
morphine sulphate 15mins	19.4 ±3.5	8
30 mins	35.1 ± 0.8 **	6
1 hour	48.6 ± 5.0 **	6
2 hours	19.5 ± 3.3	5
duromorph 30 mins	26.1 ± 5.5 **	10
1 hour	30.6 ± 3.5 **	11
4 hours	32.8 ± 4.6 **	12
6 hours	32.7 ± 4.6 **	11
8 hours	20.9 ± 2.8 *	8
12 hours	24.8 ± 4.5 **	6
24 hours	17.4 ± 3.1	6

Latency of response to the hot plate in hamsters treated with morphine sulphate or Duromorph.
Mean seconds ± SEM

* differs from controls $p<0.05$
** differs from controls $p<0.01$

Appendix 1:2

Plasma Concentrations of morphine and its metabolites.

Time after injection	morphine	M-3-G	M-6-G
30 mins	4893	21076	436
1 hour	451	8078	261
3 hours	233	4082	364
5 hours	78	1549	928
8 hours	31	605	68
24 hours	...	30	87

1:2 Plasma levels of morphine and its metabolites, morphine-3-glucuronate (M-3-G) and morphine-6-glucuronate (M-6-G) (ng/ml) after duromorph (10mg/kg) injection.

Appendix 1:3

Concentrations of morphine and its metabolites

	n	morphine	M-3-G	M-6-G
adult plasma	5	0.18 ± 0.2	6.47 ± 4.9	0
adult brain	5	1.03 ± 3.8	0.31 ±0.35	0.84 ±0.5
fetal brain	5	3.7 ± 2.2	3.62 ±3.2	0

1:3 Levels of morphine and its metabolites in adult female plasma and brain and in fetal brain (μg/ml)

Chapter 2

The effects of perinatal opiate administration on the differentiation of sexual behaviour patterns.

Whalen & Edwards (1967) showed that if male rodents are castrated at birth, they are unable to display the normal patterns of male sexual behaviour after appropriate androgen priming in adulthood. However, if the same or similarly-treated males are primed with oestrogen and progesterone they will display the feminine lordotic posture when placed with a stud male. In contrast, female rats or hamsters, given a single injection of testosterone or TP before day 5 postnatally, display reduced amounts of feminine behaviour as adults and exhibit all aspects of masculine copulatory behaviour when placed with an oestrous female (Paup et al, 1972; Payne, 1979). These results demonstrate that masculinization and defeminization of adult sexual behaviour require exposure to testicular hormones during the early postnatal period.

Analysis of androgen levels during development have revealed that there is a surge of testosterone which corresponds to the period of masculinization of reproductive tract and genitalia in mammals (Warren et al,1973). In addition, in the rat, there is a significant rise in testosterone during embryonic days 18 and 19 (Weisz & Ward, 1980) and on the day of birth (Slob et al, 1980). It appears that these two androgen peaks are related to masculinization of the CNS and that both are necessary for

complete masculinization. The E18-19 peak seems to prime the CNS and enable it to react to the later postnatal surge of androgen (Perakis & Stylianopoulou, 1986). Altering the timing of the late gestational surge of testosterone results in males which show a decrease in masculine, and an increase in feminine, sexual behaviour. Maternal stress during the last week of pregnancy in rats has been shown to cause a premature surge of testosterone, such that it appears on day E17 and levels of the androgen decline over days 18-19 (Ward & Weisz, 1980). It has been suggested that such stress causes an increase in β-endorphin (an endogenous opioid) in the mother and/or the pups, and that this suppresses LH release in the pups leading to the feminization of behaviour (Ward, Orth & Weisz, 1983; Ward & Ward, 1985). Evidence for this is that,

1 If naltrexone (an opiate antagonist) is given prior to episodes of stress, no feminization of adult behaviour is reported in male pups born to these mothers (Ward et al, 1986).

2 If exogenous opiates are given to pregnant females over the same period, their male offspring show similar feminization of adult behaviour to those prenatally stressed (Ward, et al, 1983).

Prenatal stress has also been reported to have adverse effects on reproductive function in female rats and mice. Although it did not appear to affect sexual behaviour per se, prenatal stress resulted in changes to the oestrous

cycle, (Herrenkohl & Politch, 1978; Harvey & Chevins, 1987) and reduced fertility and fecundity in female offspring (Herrenkohl, 1979). In other studies prenatal exposure to morphine sulphate from day 5 to day 12 of gestation did significantly reduce both the quality and quantity of adult proceptive and receptive behaviour in female rats (Vathy et al, 1983). On the other hand, postnatal exposure to the opiate antagonist naltrexone significantly enhanced lordosis response in adult female rats, but did not affect mounting behaviour in males (Meyerson et al, 1988).

The results discussed above suggest that the endogenous opioids may be involved in the establishment of copulutory behaviour patterns, both pre- and postnatally. Therefore, administration of exogenous opiates during this critical period may well interfere with the sexual differentiation of behaviour.

Studies of Adult Sexual Behaviour

The golden hamster Mesocricetus auratus Waterhouse, was used in these studies because adult sexual behaviour in this species is almost sexually exclusive. Adult males show very little feminine sexual behaviour while normal adult females display long bouts of lordosis, the receptive posture, and seldom if ever display any masculine sexual behaviour (Tiefer, 1970).

Initially experiments were undertaken to determine whether morphine given over different times during the critical period would have any effect on sexual behaviour,

and then, would chronic administration of the opiate enhance or suppress the effect.

Five treatment groups were established. These were:-

1 Duromorph given pre + postnatally (n=18 females, 26 males)

2 Duromorph given prenatally only (n=19 females, 18 males)

3 Duromorph given postnatally only (n=28 females, 25 males)

4 Duromorph given prior to mating, throughout pregnancy and in decreasing amounts during lactation (i.e. chronic administration) (n=13 females, 10 males)

5 Untreated controls, virgin males and females housed and treated in a similar manner to the experimental animals from birth (n=15 females, 19 males)

Materials and methods

All animals were kept in an air-conditioned light reversal room, 9 hours dull red light and 15 hours bright light. Female hamsters were placed with vigorous stud males for 2-3 hours on the afternoon of oestrus. The day of mating was taken as day 0. Gestation in hamsters lasts 16 days. After mating the females were housed individually in PVC cages measuring 26 x 24 x 10 cm and allowed access to food and water ad libitum.

Treatments

Pre + Postnatal

1. Duromorph (10mg/kg) was administered i.p. twice daily to

females from day 13 of pregnancy until parturition. Pups were treated similarly from the day of birth for four days.

Prenatal only

2. Duromorph (10mg/kg) i.p. twice daily to females from day 13 of pregnancy until parturition.

Postnatal only

3. Duromorph (10mg/kg) i.p. twice daily to pups from day of birth for four days.

Chronic

4. Females in oestrus were treated once daily with Duromorph (10mg/kg) i.p. four days prior to mating and then throughout pregnancy. They were gradually weaned off the opiate during lactation with the dose being reduced by approximately 10% per day.

The pups remained with their natural mothers until weaned at day 21. They were then sexed and housed with same sex litter-mates, 3-4 per cage under reversed lighting conditions. At 3 months of age, they were gonadectomised under sodium pentabarbitone anaesthesia (Sagatal). The testes, epididymides and seminal vesicles were removed from the males and the ovaries from the females. After a recovery period of three weeks, each animal received a subcutaneous injection of oestradiol benzoate (OB)(5μg) dissolved in peanut oil. Twenty-four hours later 500μg progesterone (Prog) was administered sc. in peanut oil. Four to six hours after the second injection, each animal

was tested for its ability to display feminine sexual behaviour when placed with a vigorous stud male.

Sexual behaviour of a receptive female hamster consists of a depression of the back, with the head and tail raised (lordosis), see fig 2:1. A normal female will remain in this position between successive mounts and dismounts by the males, and may remain so throughout the test period. Each test period lasted for 10 minutes.

The tests were carried out in a plexiglass cage measuring 38 x 24 x 16 cm in a room with normal lighting. Previous studies have shown that, although the test is carried out during the dark period, the animals are undisturbed by the bright conditions. Each test session was recorded on video using an overhead camera, and the information stored for later analysis.

This first test was merely to acclimatise the animals to the test conditions. Although data were recorded, they were not used in the subsequent analyses. A second test was carried out one week later after a second priming with O.B. and Prog. The data collected during this test are reported below. Those aspects of behaviour used in the analysis were as follows:-

1. The latency to lordosis (seconds)
2. The total time spent during the test session in lordosis (seconds)
3. The longest single episode of lordosis (seconds)

Following the second test for feminine behaviour, each animal was given s.c. injections of testosterone

propionate (TP) (1.5mg thrice weekly) for four weeks. They were then tested on three consecutive days for their ability to display masculine sexual behaviour towards a receptive female.

In the hamster, masculine sexual behaviour consists of:-

1. Licking and sniffing the female's vulval region.
2. Mounting the female and palpating her flanks with his forepaws.
3. Intromission, the penetration of the vagina with the penis.
4. Self grooming of his own genitalia after each mount.
5. Ejaculation, which occurs after several mounts with intromission.

These behaviours are shown in figs 2:1-2:4

The test period lasted for ten minutes on each day. As with female behaviour, tests were recorded on video and the specific aspects of behaviour used for analysis were as follows:-

1. Latency to mount (secs, average for three tests)
2. Total number of mounts (regardless of orientation) over the three test periods.
3. Total number of genital self - grooms from the three tests.
4. Total number of intromissions from three tests.

Statistics

Data were analysed by one-way ANOVA followed by inter-

group comparisons using Least Significant Difference computations. In Expt 1. the chronically-treated groups were each compared with controls using Student's t-test.

Results:

Feminine Sexual Behaviour in Perinatally - treated Hamsters

Females: (See Table 2:1 Fig.2:5)

Control females adopt the lordotic stance within 60 seconds of being placed with a stud male, and remain in that position for most of the test period. Exposure to morphine had little effect on the females' ability to display typical receptive behaviour. However, exposure to morphine prenatally significantly increased the latency to lordosis; and while it reduced both the total and maximum length of lordosis; these did not reach significance.

Males: (See Table 2:2 Fig 2:6)

In comparison to females, normal male hamsters show little feminine sexual behaviour; only 53% of controls displayed any lordosis and even then the duration was relatively low. However, exposure to morphine significantly increased the amount of feminine behaviour displayed by males. Of those treated postnatally, 88% showed feminine copulatory behaviour and 92% of those treated pre + postnatally did so. Pre + postnatal morphine exposure significantly decreased latency and increased the total length of lordosis displayed by males. Postnatal

treatment alone also decreased lordosis latency, but did not increase the total amount of lordosis, while prenatal morphine exposure was ineffective.

Masculine Sexual Behaviour in Perinatally-treated hamsters.

Females: (See Table 2:3, Fig. 2:7)
There were no significant differences between morphine-treated animals and controls in their ability to display masculine sexual behaviour.

Males: (See Table 2:4, Fig.2:8)
All of the control males (100%) showed mounting behaviour; morphine exposure had little effect on masculine sexual behaviour in males. Although there was a slight decrease in the numbers of males mounting in the pre + postnatally-treated and in the postnatally-treated groups, this was not significant. Nor were there any significant differences in any other aspect of masculine behaviour in the morphine-treated groups.

Sexual behaviour as displayed by hamsters chronically exposed to morphine during development.

Feminine behaviour

Females (See Table 2:5, Fig. 2:9)
As with those acutely exposed to morphine during the critical perinatal period (see above), chronically-treated females showed no significant change in their ability to

display feminine sexual behaviour.

Males (See Table 2:6, Fig. 2:10)
Males chronically exposed to morphine showed a significantly reduced latency to lordosis. Although total and maximum periods of lordosis were increased, these did not reach significance.

Masculine Sexual Behaviour

Females (See Table 2:7, Fig 2:11)
Chronic morphine treatment had no effect on the females' ability to display masculine sexual behaviour.

Males (See Table 2:8, Fig. 2:12)
In contrast to males treated acutely with morphine, those exposed throughout development showed a surprising and marked increase in their masculine sexual behaviour. All aspects of male copulatory behaviour were significantly enhanced by chronic exposure to morphine during gestation.

Discussion

Females seem to be least affected by exposure to opiates during the period of sexual differentiation. Prenatal exposure to morphine did significantly increase latency to lordosis and slightly decrease the total time spent in lordosis. This may suggest that opiate exposure during this period has interfered with the mechanisms involved with response. That the total time spent in

lordosis was decreased is probably due to the delay in the females response to the advances of the male. In contrast, prenatal opiate exposure has little effect on the ability of the male hamster to display feminine sexual behaviour, although it appears to have enhanced some aspects of masculine sexual behaviour such as intromission scores. This is contrary to the findings in rats, where prenatal morphine exposure has a marked effect on both feminization and demasculinization of males (Ward, Orth & Weisz, 1983). Since prenatal manipulation of androgens has little effect in the golden hamster (Nucci & Beach, 1971) the present findings would suggest that the opiates are acting on some other factor (eg the amines), or perhaps general neural development to produce these effects.

Pre + postnatal and postnatal morphine administration exerted no apparent changes in the males ability to display mounting when placed with a receptive female. However both treatments have interfered with the defemininzation process, such that these males show significantly reduced latency to lordosis and more time in lordosis compared to controls. In addition, the number of males in each treatment group displaying lordosis was increased from 53% in controls to 92% and 88% in the pre + post and postnatal groups respectively. With regard to the changes in behaviour in males the present findings suggest that, in the golden hamster, the defeminization process occurs, [or is at least most sensitive to manipulation] postnatally. However, extending manipulation to cover the late prenatal

and early postnatal period results in a less defeminized male.

Morphine given to females prior to and throughout pregnancy had a different effect on their offspring. Chronic exposure to morphine during development appears to have had no significant effect on either masculine or feminine sexual behaviour in females. In contrast, in males, the changes are more marked and more surprising! Feminine sexual behaviour was increased but only lordosis latency was significantly reduced. The number of males displaying lordosis and the total amount of lordosis were increased but not significantly. On the other hand, masculine sexual behaviour in males chronically exposed to morphine was most significantly enhanced when compared with that of controls. Why this should be so is unknown, but it does suggest that long-term exposure to opiates, ie throughout gestation and lactation, is less detrimental to the developing male than is acute exposure. On the other hand it is possible that the findings recorded after acute exposure are caused by withdrawal of that drug rather than by the drug itself. Lichtblau & Sparber (1984) published a review on the effects of opiates during development, and are very critical of conclusions drawn from work involving short-term exposure, suggesting that opiate withdrawal is as harmful, if not more so, than the drug itself.

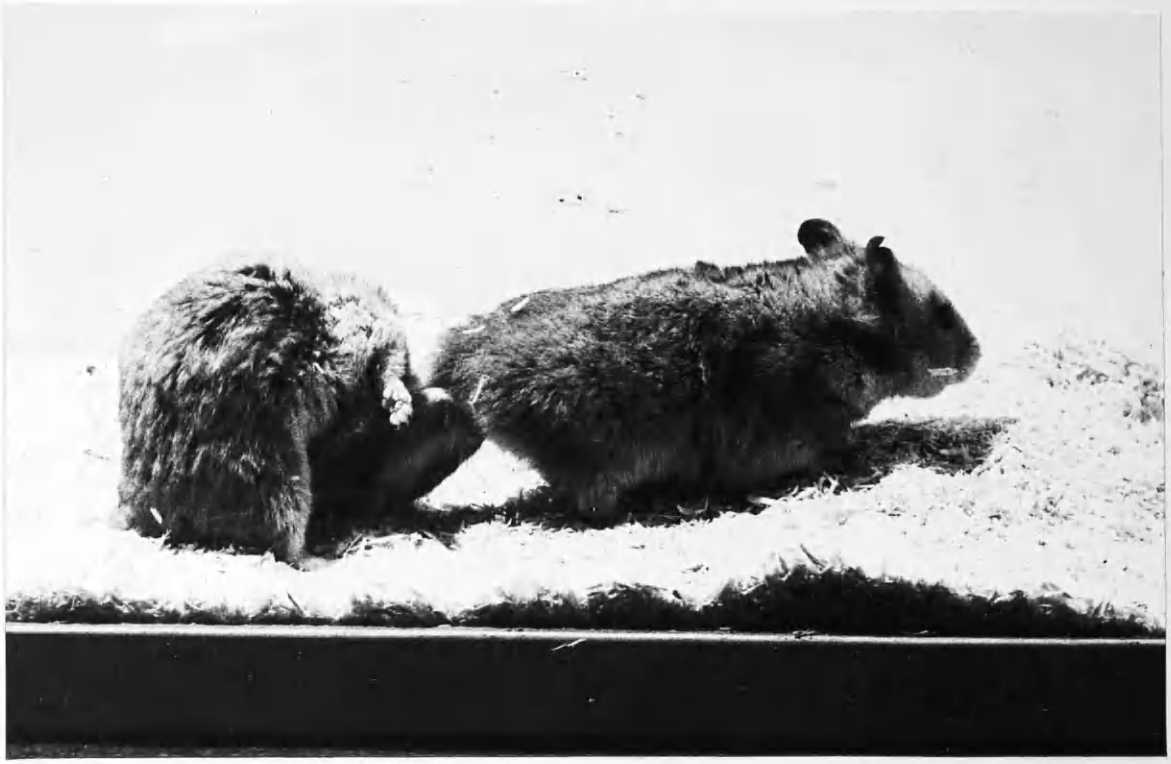


Fig.2:1 Male licking the perineal area of a receptive female. Female in lordosis.

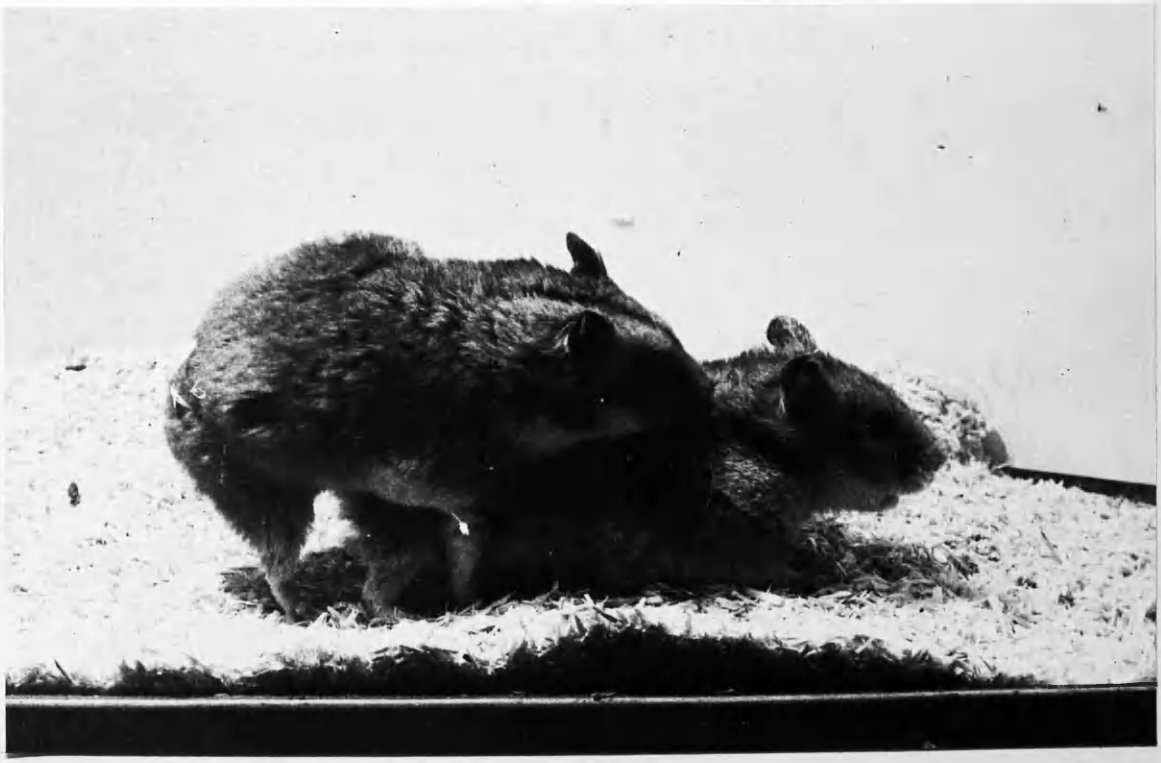


Fig.2:2 Male mounting a receptive female.

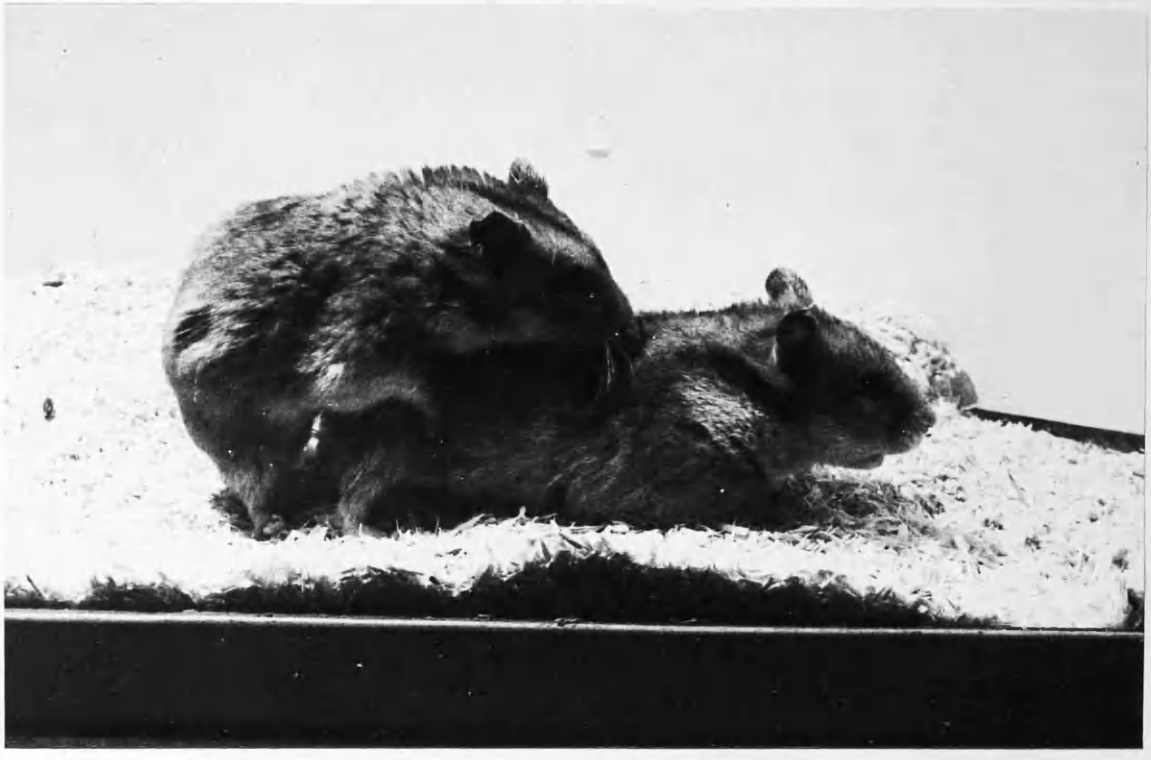


Fig.2:3 Intromission

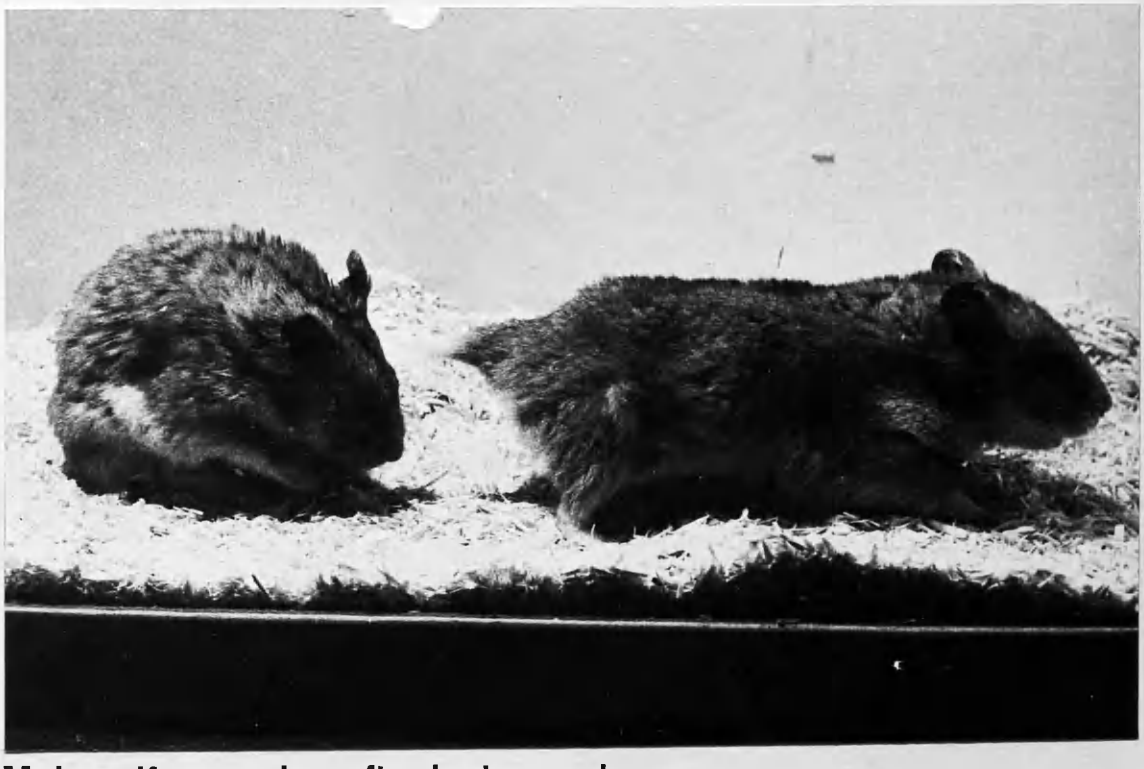


Fig.2:4 Male self grooming after last mount.
Female remaining in lordosis between mounts.

Experiment 1:

Feminine Sexual Behaviour in the Adult Golden Hamster.

Female

treatment	prenatal morphine	pre+post morphine	postnatal morphine	control	F value
n	19	18	28	15	
lordosis latency	145±48 **	13±3	73±26	25±4	3.79 p<0.01
total lordosis	365±46	498±15	445±35	431±44	1.62 n.s.
maximum lordosis	264±44	256±27	298±38	322±60	0.45 n.s.
% showing behaviour	89%	100%	96%	100%	

Table 2:1. Female hamsters treated perinatally with morphine.

Males

treatment	prenatal morphine	pre & post morphine	postnatal morphine	control	F value
n	18	26	25	19	
lordosis latency	360±61	123±33 **	187±43 **	360±60	6.45 p<0.01
total lordosis	76±27	165±25 **	126±22	76±23	3.36 p<0.02
maximum lordosis	26±9	47±8	42±7	28±8	1.64 n.s.
% showing behaviour	50%	92%	88%	53%	

Table 2:2 Male hamsters treated perinatally with morphine.

Mean scores in seconds ±SEM.

** differs from controls p<0.01

Experiment 1:

Feminine Sexual Behaviour in the Golden Hamster exposed to morphine over the perinatal period.

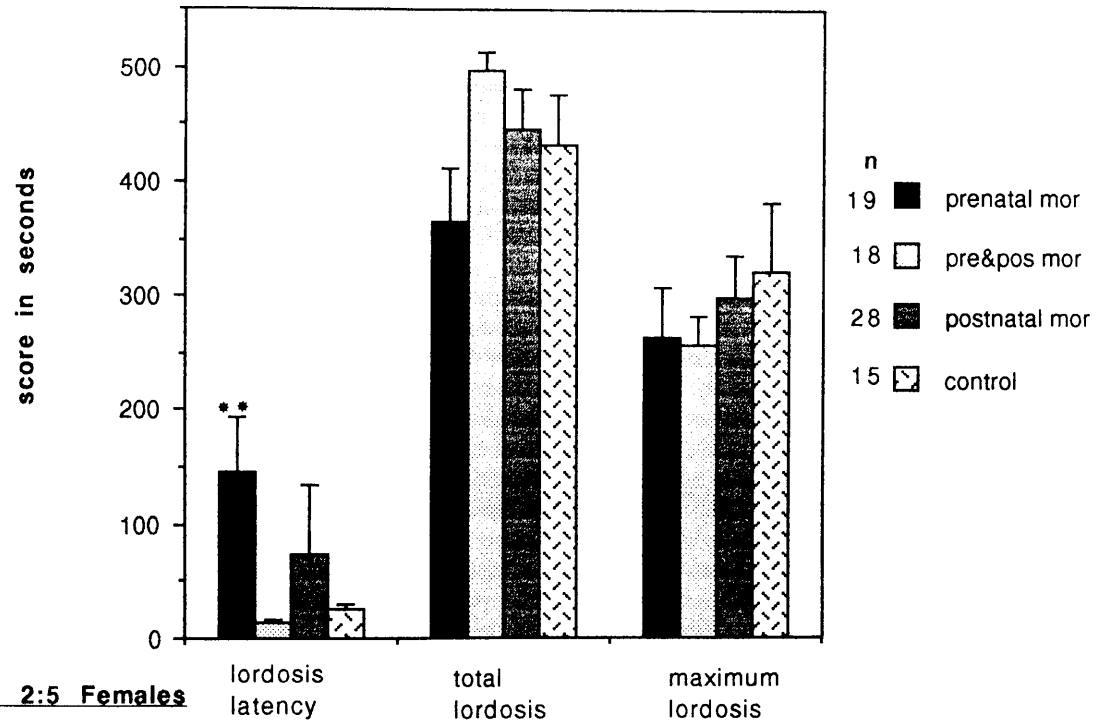


Fig 2:5 Females

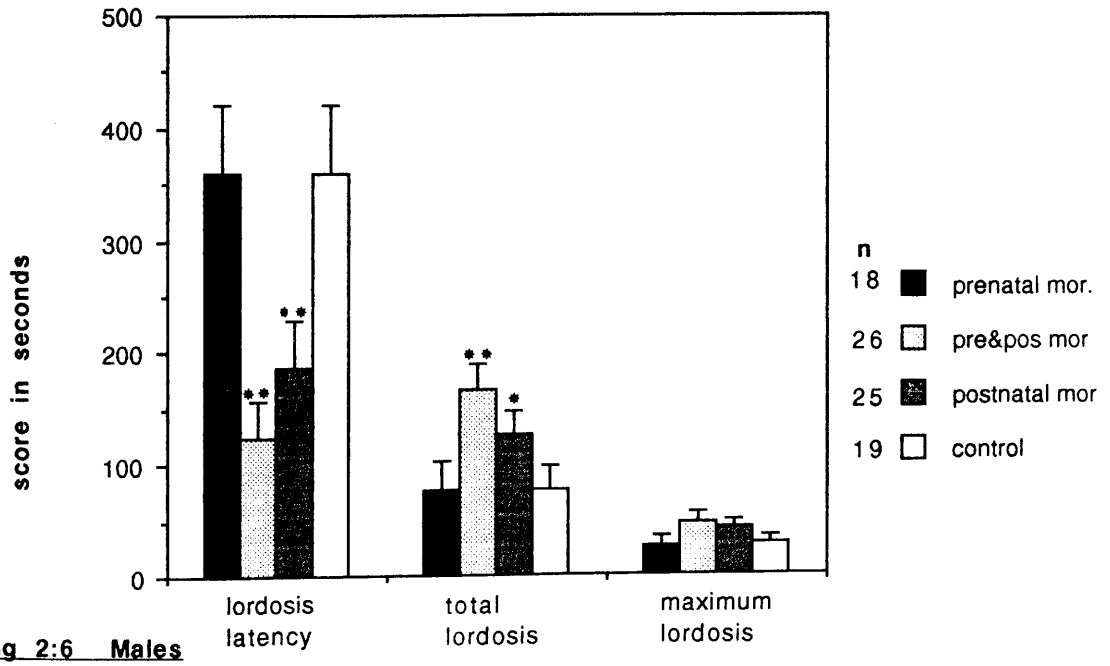


Fig 2:6 Males

** differs from controls p<0.01

Experiment 1:

Masculine Sexual Behaviour in the Adult Golden Hamster.

Females

treatment	prenatal morphine	pre & post morphine	postnatal morphine	control	F value
n	18	18	27	12	
mount latency	592±30	597±6	586±7	574±12	1.66 n.s.
total mounts	0	0	0	1	2.33 n.s.
total grooms	0	0	0	1	1.16 n.s.
total intromit	0	0	0	0	0 n.s.
% showing mounting	11%	11%	15%	40%	

Table 2:3. Females treated perinatally with morphine.
Mount latency :- average latency over three tests, group mean in seconds ± SEM:
All other parameters, total count over three tests. Group means ± SEM

Males

treatment	prenatal morphine	pre & post morphine	postnatal morphine	control	F value
n	18	25	25	17	
mount latency	352±32	434±18	381±29	369±26	1.79: n.s.
total mounts	27±5	14±3	22±4	15±2	2.83: p<0.04
total grooms	23±5 *	10±2	17±3	10±2	4.04 p<0.01
total intromit	16±4 **	3±1	9±3	3±1	6.62: p<0.01
% showing behaviour	100%	92%	92%	100%	

Table 2:4. Male hamsters treated perinatally with morphine.
Scores: As in Table 2:3

* Differs from control $p<0.05$
** Differs from controls $p<0.01$

Experiment 1:

Masculine sexual behaviour in the Golden Hamster exposed to morphine over the perinatal period.

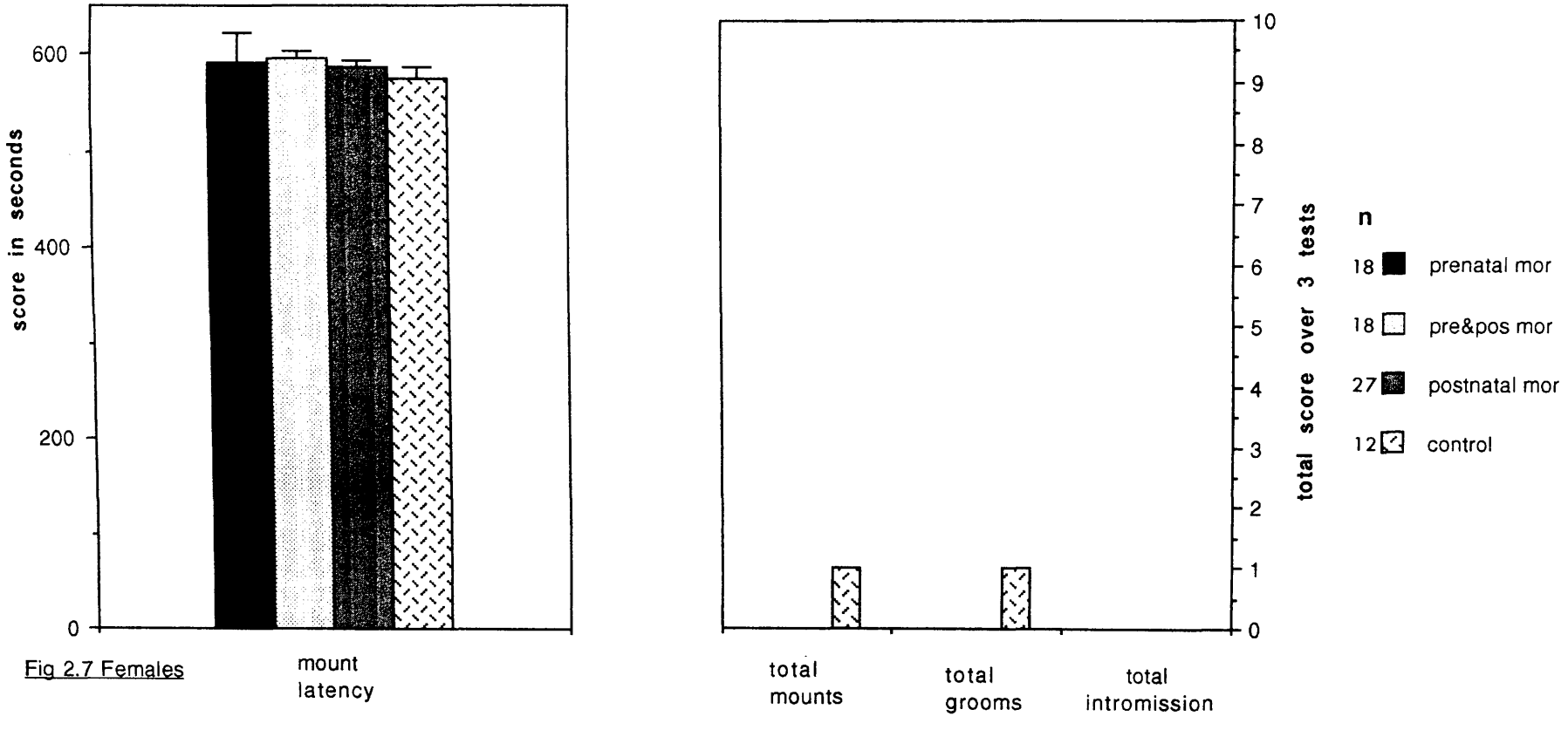


Fig 2.7 Females

Experiment 1:

Masculine sexual behaviour in the Golden Hamster exposed to morphine over the perinatal period.

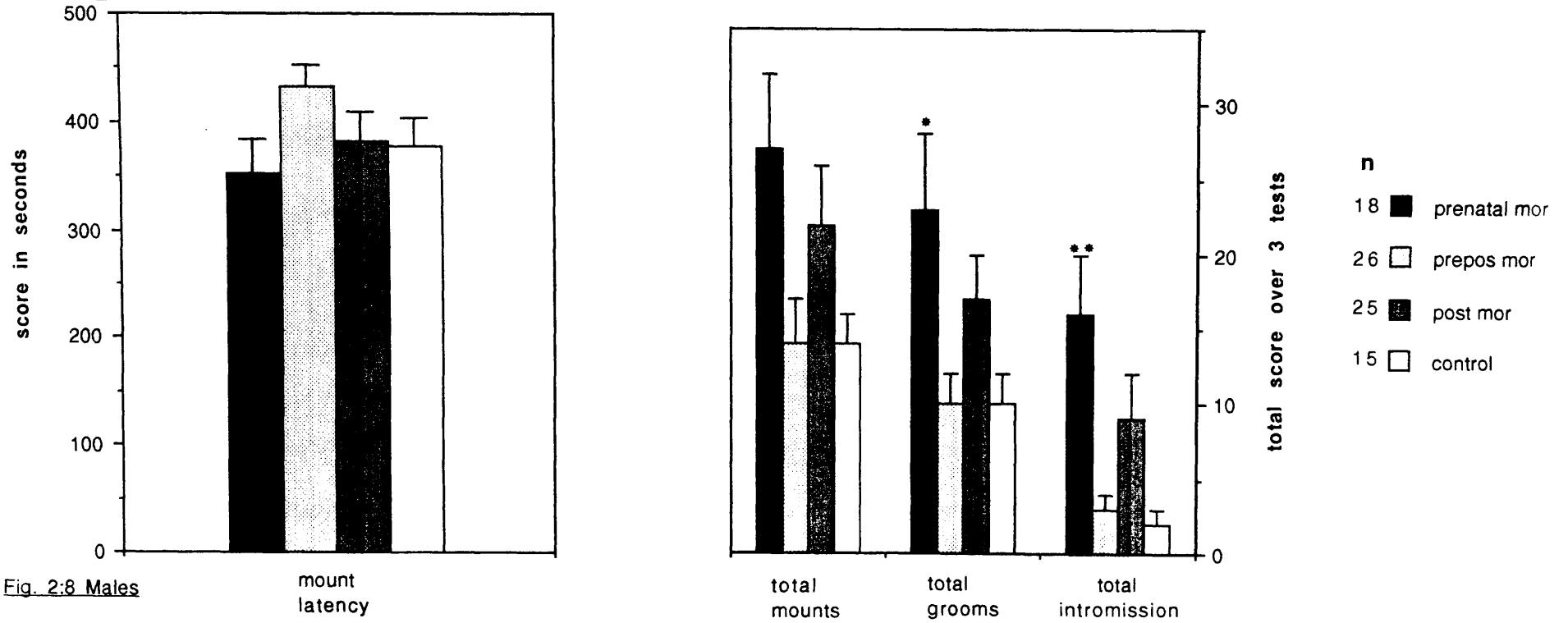


Fig. 2:8 Males

* differs from controls $p<0.05$

Experiment 1:

Feminine sexual behaviour in the adult Golden Hamster born to mothers exposed to morphine prior to and throughout pregnancy and into lactation.

Females

Treatment	n	lordosis latency	total lordosis	maximum lordosis	% showing lordosis
control	15	25±4	431±43	322±60	100%
chronic	13	117±60	380±63	263±58	92%
t value		1.65 n.s.	0.68 n.s.	0.70 n.s.	

Table 2:5 Females exposed throughout development to morphine. Scores in seconds Group mean ± SEM

Males

Treatment	n	lordosis latency	total lordosis	maximum lordosis	% showing lordosis
control	19	360±60	76±23	28±8	53%
chronic	10	118±55	163±46	43±14	90%
t value		2.62 $p<0.01$	1.88 n.s.	0.98 n.s.	

Table 2:6 Feminine sexual behaviour as displayed by adult male hamsters exposed throughout development to morphine.
Scores in seconds. Group means ± SEM.

Experiment 1:
Feminine sexual behaviour in the Golden Hamster chronically exposed to morphine during development.

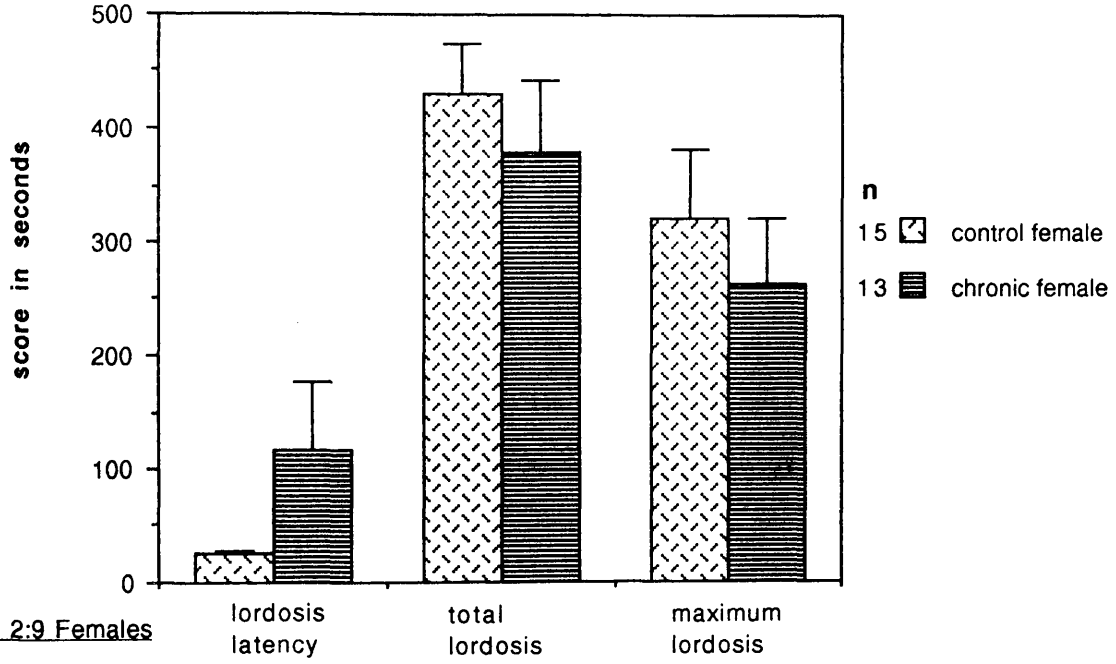


Fig. 2:9 Females

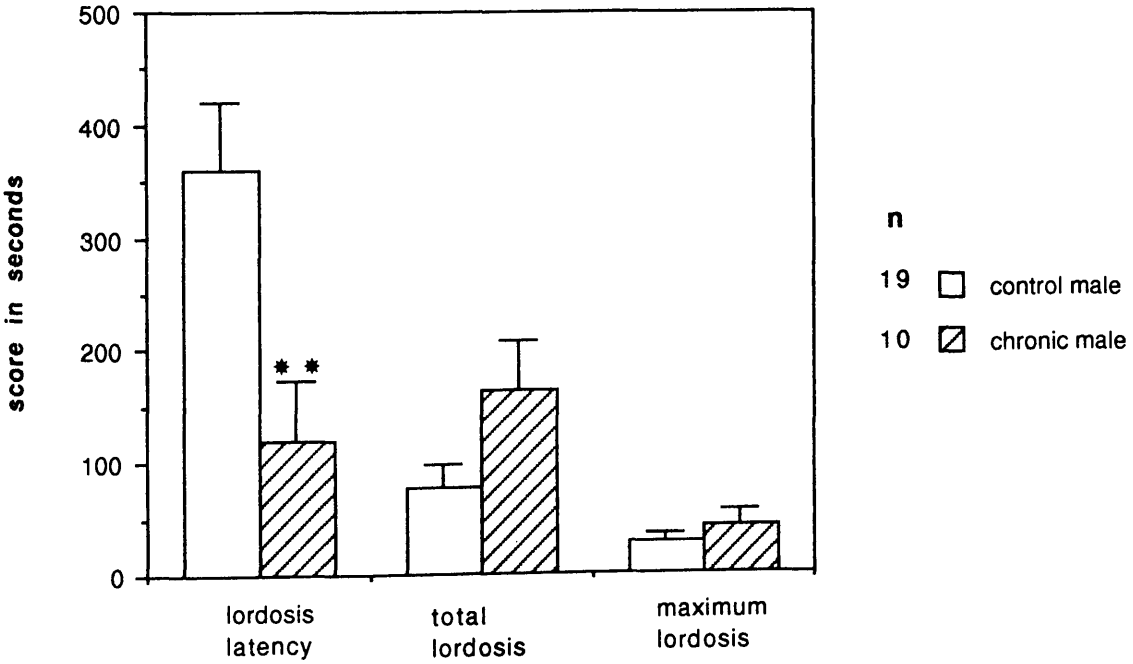


Fig. 2: 10 Males

** differs from controls $p<0.01$

Experiment 1

Masculine sexual behaviour in adult Golden Hamsters born to mothers exposed to morphine prior to, and throughout pregnancy and into lactation.

Females

treatment	n	mount latency	total mounts	total grooms	total intros	% showing behaviour
control	12	574+12	1	1	0	50%
chronic	13	568+13	1	1	0	54%
t value		0.4 n.s.	0.55 n.s.	1.03 n.s.	0	

Table 2:7 Masculine sexual behaviour as displayed by adult female hamsters exposed to morphine throughout development.

Mount latency = average over 3 tests in seconds. Other scores total scores over three test periods, group mean ± SEM

Males

treatment	n	mount latency	total mounts	total grooms	total intros	% showing behaviour
control	17	377±27	14±2	10±2	2±1	100%
chronic	10	266±47	45±10	43±10	27±7	100%
t value		2.32 $p<0.03$	3.94 $p<0.01$	4.35 $p<0.01$	4.55 $p<0.01$	

Table 2:8 Masculine sexual behaviour as displayed by adult male hamsters exposed to morphine throughout development.

Mount latency = average over 3 tests in seconds.
Other scores are totals over three test periods.

Experiment 1:

Masculine sexual behaviour in the Golden Hamster chronically exposed to morphine during development.

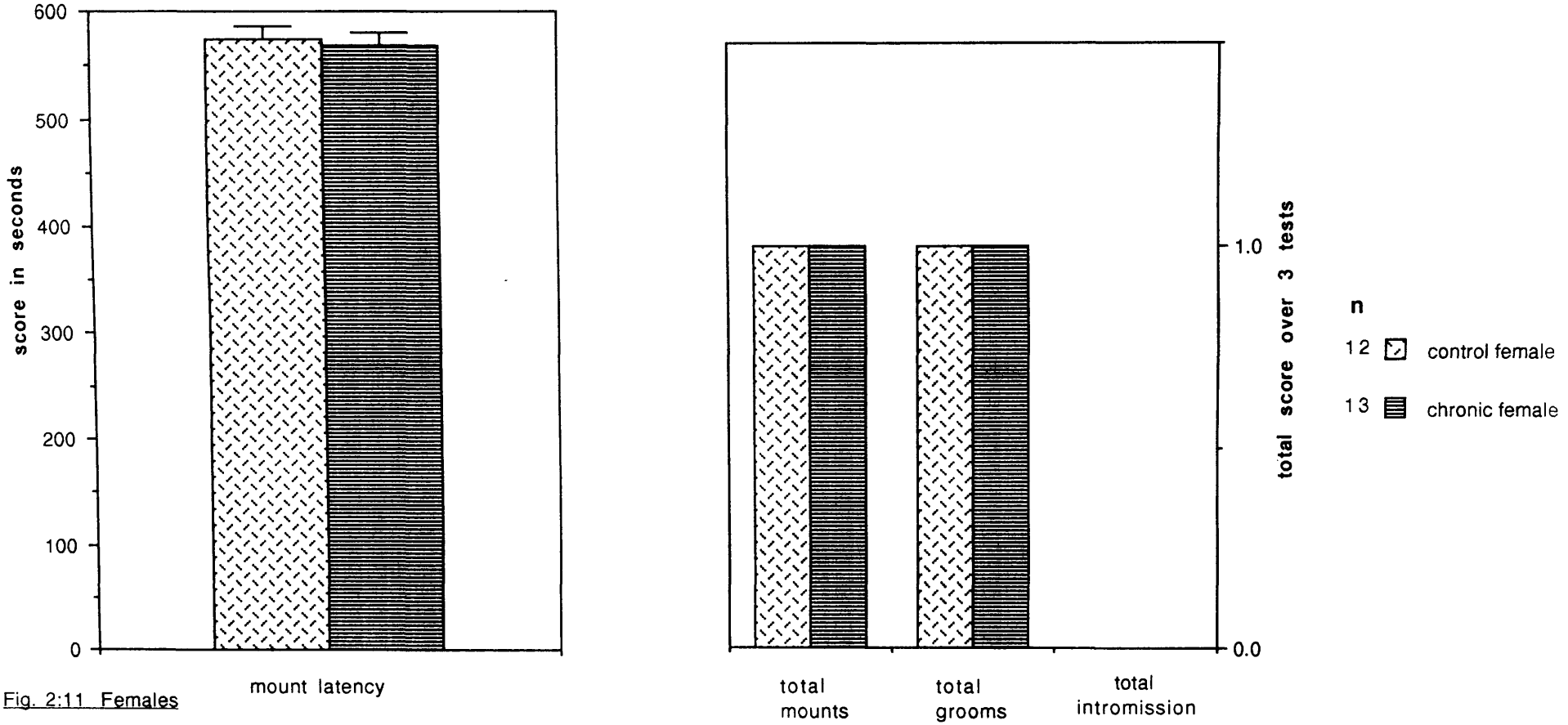


Fig. 2:11 Females

Experiment 1:

Masculine sexual behaviour in the Golden Hamster chronically exposed to morphine during development.

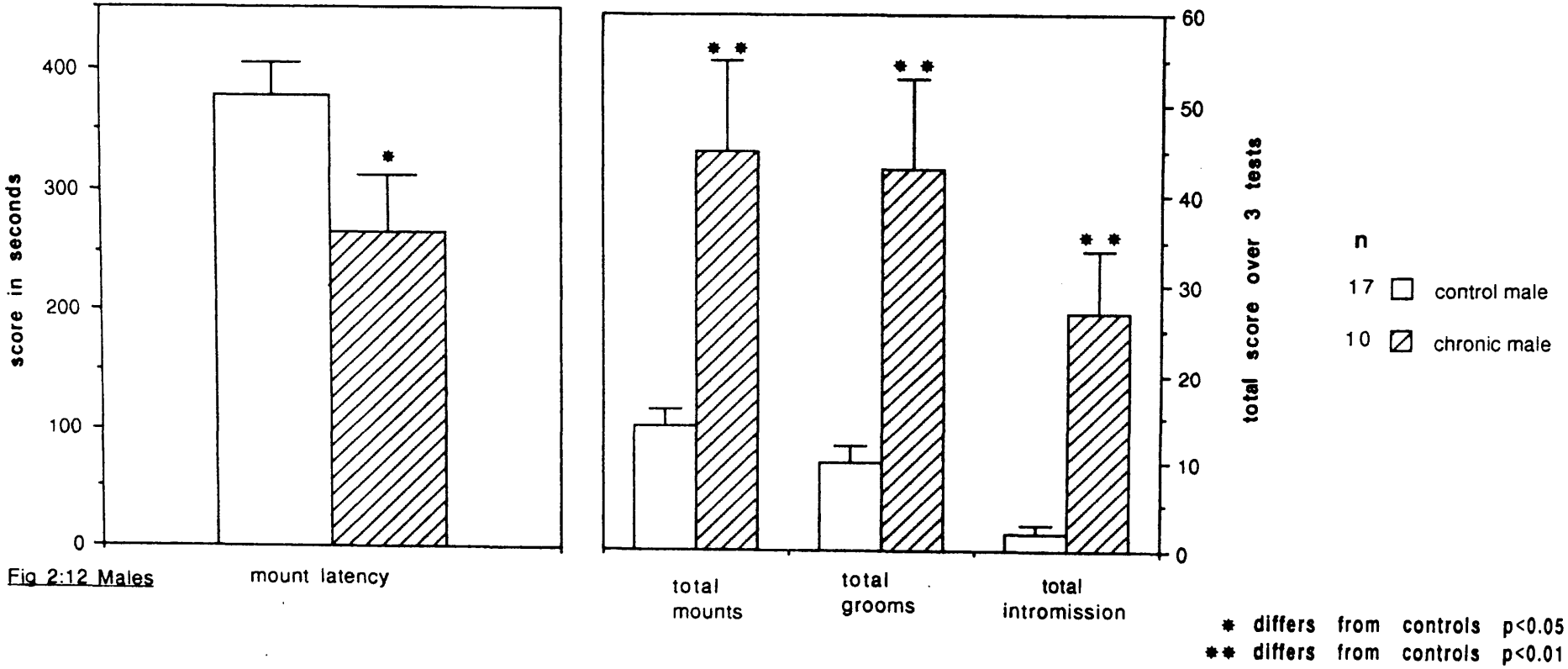


Fig 2:12 Males

Experiment 2

Does testosterone counteract the effect of perinatal opiate exposure?

Having found that perinatal morphine exposure does affect the adult sexual behaviour of hamsters, further investigations were set up to determine whether alterations caused by morphine exposure could be counteracted by concurrent administration of testosterone. The reasoning behind this was that it has been reported that morphine reduces testosterone levels, and that if that is the cause of the behavioural changes then testosterone administration should prevent these changes occuring.

The following experimental groups were set up:-

Prenatal

1. Duromorph (10mg/kg) ip twice + 500μg testosterone sc once daily were given to females from day 13 of pregnancy to day of parturition. (n=6 females, 14 males)

Pre + postnatal

2. Duromorph (10mg/kg) ip twice + 500μg testosterone sc once daily were given to females from day 13 of pregnancy to day of parturition. In addition, 10mg/kg Duromorph ip was given twice daily to pups from the day of birth for 4 days. (n=16 females, 15 males)

Postnatal

3. Duromorph (10mg/kg) ip twice + 100μg testosterone sc once daily were given to pups from the day of birth for 4 days.(n=10 females, 17 males)

All other conditions, treatments and behavioural tests were as in Expt. 1. In particular, all animals were tested for feminine and masculine sexual behaviour as adults.

Results:

Feminine Sexual Behaviour

Females (See Table 2:9 and Fig.2:13)

In Expt 1 it was shown that perinatal morphine administration increased lordosis latency in adulthood. This effect was reversed by concurrent perinatal testosterone administration. The maximum single episode of lordosis was significantly reduced in both the prenatal morphine + T and pre + postnatally morphine + T groups, but neither lordosis latency nor total lordosis in these two groups differed significantly from controls. In contrast, concurrent administration of T with morphine postnatally significantly reduced feminine sexual behaviour, resulting in increased lordosis latency and decreased total and maximum lordosis.

Males: (See Table 2:10, Fig. 2:14)

Concurrent administration of testosterone with morphine had a more marked affect on feminine behaviour in males. In the prenatally-treated group, males injected with both morphine and testosterone did not show less feminine sexual behaviour than did males treated with morphine alone. In contrast, testosterone given with morphine either pre + postnatally or postnatally only, significantly reduced the

amount of feminine sexual behaviour in males. In addition, fewer males in the latter 2 groups displayed feminine behaviour, 35% in each compared with 92% treated with morphine pre + postnatally and 88% treated with morphine postnatally.

Masculine Sexual Behaviour

Females: (See Table 2:11, Fig.2:15)

Masculine sexual behaviour was increased in all treatment groups given testosterone, and the number of females in ech group displaying mounting behaviour was also significantly increased. Postnatal morphine + T had the most marked effect in that all females in this group mounted. Mount latency was reduced and the total number of mounts was significantly increased.

Males: (See Table 2:12, Fig.16)

Males treated prenatally and pre + postnatally with morphine and testosterone showed significantly more masculine sexual behaviour than did males treated with morphine only over the same periods. Postnatal morphine + testosterone did not alter sexual behaviour when compared with morphine only. However, testosterone treatment over all three periods enhanced intromission scores when compared with controls, although in the case of postnatal testosterone it was not significant.

Discussion

Morphine + testosterone treatment during the perinatal period does affect feminine sexual behaviour in females when compared with controls, but the timing of treatment appears to be an important factor. Although latency to lordosis is increased in all groups, only postnatal testosterone and morphine caused a significant increase in latency when compared to the administration of morphine only. Similarly, females treated with morphine + T showed less lordosis in total, and the maximum single episode of lordotic behaviour was also reduced. These behavioural changes which were recorded in the postnatally treated groups are most likely to have been caused by the androgen rather than the opiate, and are in agreement with many other reports that postnatal androgen administration reduces feminine sexual behaviour in the rodent (Debold & Whalen,1975; Payne, 1976; Goy & McEwen, 1980).

Testosterone given concurrently with morphine pre + postnatally and postnatally reversed the feminizing effects of morphine on the males' ability to display lordosis and reduced the number of males displaying feminine sexual behaviour. Surprisingly, testosterone given prenatally actually enhanced feminine **and** masculine behaviour in males suggesting a role for prenatal androgens in masculinization but not defeminization of the CNS. Antiandrogens given prenatally have been reported to impair male sexual behaviour in adult hamsters (Swanson, 1971).

Perinatal exposure to testosterone in conjunction with morphine resulted in all three groups of females displaying masculine sexual behaviour. There was also an increase in the numbers of females mounting in each group. This is contrary to previous reports on masculinization of the female hamster, where prenatal androgens were unable to cause masculinization (Nucci & Beach, 1971). The role of the opiate must also be considered when discussing the present findings, and it is possible that alterations to neural systems as a result of opiate exposure have allowed them to become more sensitive to androgens or to remain sensitive over a longer time period. Exposure to exogenous opiates during the neonatal period has been shown to down-regulate opioid receptors in the CNS (Tempel et al, 1988). Opioid receptor concentrations change in response to exogenous androgens (Hammer, 1985; Martini et al, 1989) and endogenous opioids are known to be involved in the control of adult sexual behaviour in the rodent (Meyerson, 1981; Sirinathsinghji et al, 1983). Thus by altering the receptor concentrations during development, opioid control of behaviour may also be changed resulting in a higher sensitivity to exogenous androgens in adulthood as has been shown in females in this investigation.

While it has been demonstrated that testosterone given concurrently with morphine pre + postnatally and postnatally has reversed the effects of morphine on the defeminization process in male hamsters, the additional effects of prenatal morphine + T enhancing both feminine

and masculine sexual behaviour remain to be explained. The results suggest that morphine exposure during this period might be acting on other systems as well as or instead of endogenous androgen production.

Experiment 2:

Does Testosterone counteract the effects of morphine?

Females

Behaviour		lordosis latency	total lordosis	maximum lordosis	% showing lordosis
treatment	n				
prenatal morphine	19	145±48 **	365±46	264±44	89%
prenatal morphine + testosterone	6	112± 98	350±88	168±51	83%
pre+post morphine	18	13±3	498±15	256±27	100%
pre+post morphine + testosterone	16	98±50	420±45	192±58 *	87%
postnatal morphine	28	73±26	445±35	298±38	96%
postnatal morphine + testosterone	10	238±84 ** ∞∞∞	194±66 ** ∞∞∞	82±33 ** ∞∞∞	70%
control	15	25±4	431±44	322±60	100%
F value		2.14 p<0.04	2.97 p<0.01	2.52: p<0.02	

Table 2:9. Feminine sexual behaviour as displayed by adult female hamsters treated perinatally with morphine or morphine and testosterone.
Scores in seconds. Group mean ± SEM.

* differs from controls p<0.05
** differs from controls p<0.01
∞∞∞ differs from morphine only, over the same period p<0.01

Experiment 2:

Does Testosterone counteract the effects of morphine?

Males

Behaviour		lordosis latency	total lordosis	max. lordosis	% showing lordosis
treatment	n				
prenatal morphine	18	360±61	76±27	26±9	50%
prenatal morphine + testosterone	14	188±60 ∞ **	172±42 * ∞∞∞	64±17 ** ∞∞∞	86%
pre & post morphine	26	123±33 **	165±25 **	47±8	92%
pre & post morphine + testosterone	15	424±60 ∞ ∞	36±18 ∞ ∞	11±4 ∞ ∞	35%
postnatal morphine	25	187±43 **	126±22 *	42±7	88%
postnatal morphine + testosterone	17	469±52 ∞∞∞	39±16 ∞	16±7 ∞	35%
control	19	360±60	76±23	28±8	53%
F value		7.15 p<0.01	4.81 p<0.01	3.70 p<0.01	

Table 2:10. Feminine sexual behaviour as displayed by adult male hamsters treated perinatally with morphine or morphine and testosterone.
Scores in seconds. Group mean ± SEM.

* differs from controls p<0.05
** differs from controls p<0.01
∞ differs from morphine only over the same period p<0.05
∞∞∞ differs from morphine only over the same period p<0.01

Experiment 2:

Does Testosterone counteract the effects of morphine?

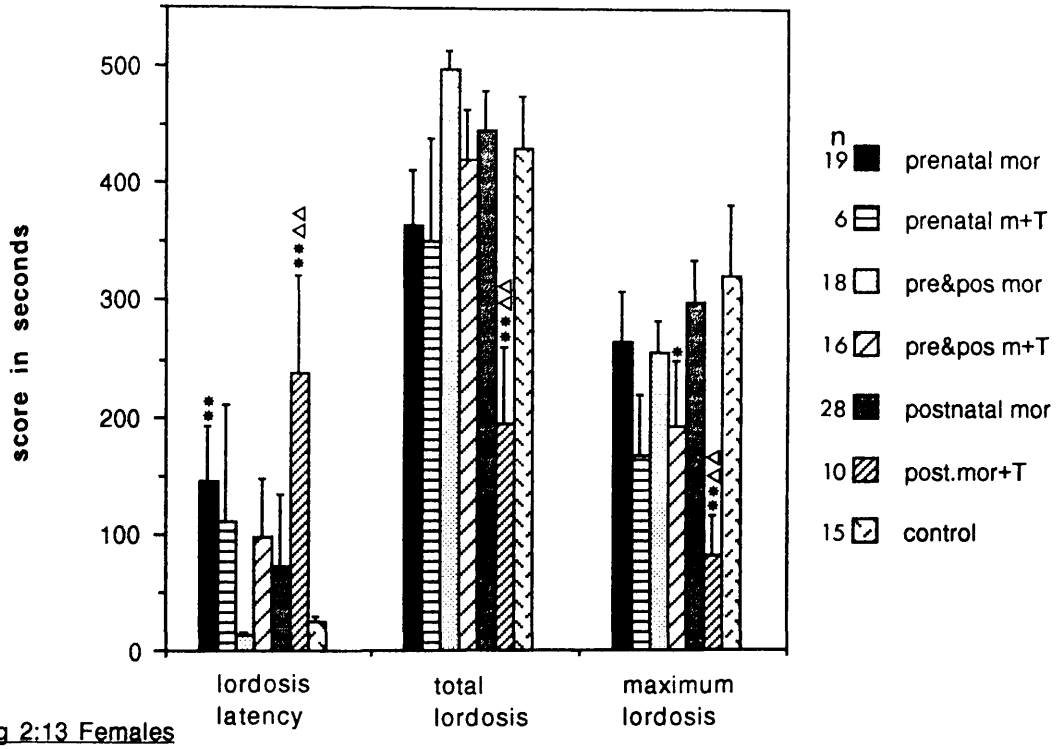


Fig 2:13 Females

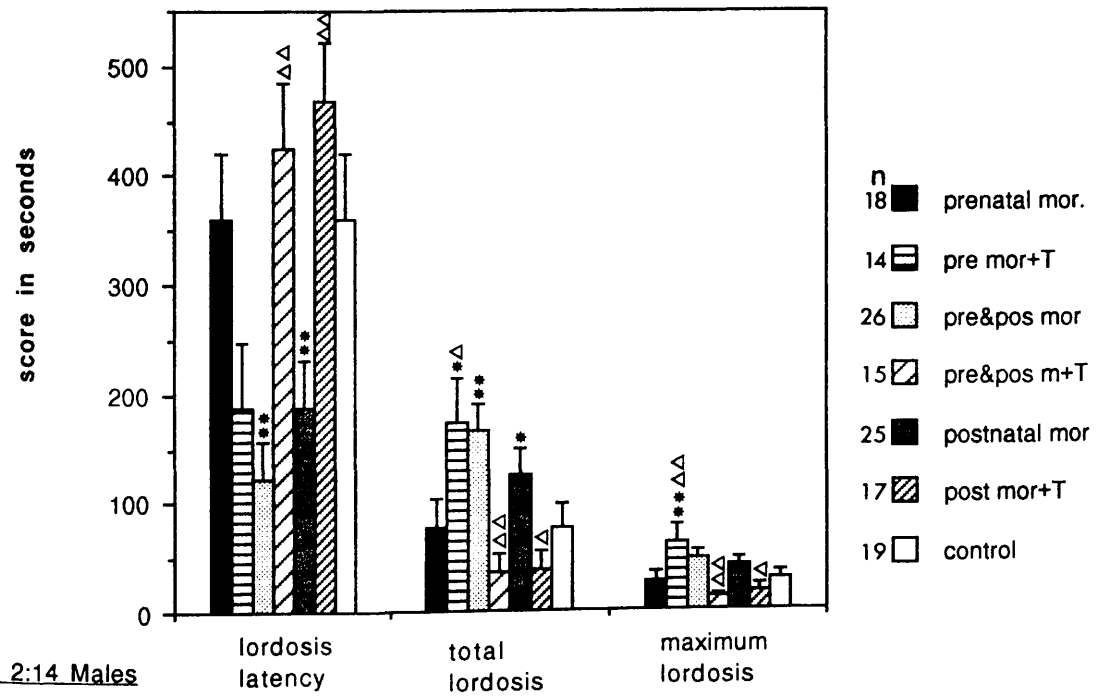


Fig 2:14 Males

*:** differs from controls p<0.05: p<0.01

◁:◁◁ differs from morphine only over same period p<0.05: p<0.01

Experiment 2:

Does Testosterone counteract the effects of morphine.?

Females

Behaviour		mount latency	total mounts	total grooms	total introm	% mounting
treatment	n					
prenatal morphine	19	592±6	0	0	0	11%
prenatal morphine+ testos	6	408±41 ** ∞∞∞	12±4 ** ∞∞∞	10±3 ** ∞∞∞	4±2 * ∞	83%
pre&post morphine	18	597±2	0	0	0	11%
pre&post morphine +testos	18	478±34 ** ∞∞∞	7±3 ** ∞∞∞	5±2 ** ∞∞∞	3±2 * ∞	61%
postnatal morphine	27	586±7	0	0	0	15%
postnatal morphine+ testos	9	360±48 ** ∞∞∞	16±4 ** ∞∞∞	6±2 ** ∞∞∞	1±1	100%
control	12	574±12	1	1	0	50%
F value		18.5 p<0.01	12.4 p<0.01	8.98 p<0.01	4.02 p<0.01	

Table 2:11. Masculine sexual behaviour as displayed by adult female hamsters treated perinatally with morphine or morphine and testosterone.

Latency = average score over three tests (seconds).
Other scores are the total for the three test periods.
Group means ± SEM.

* differs from morphine only over same period $p<0.05$
** differs from morphine only over same period $p<0.01$
∞ differs from controls $p<0.05$
∞∞∞ differs from controls $p<0.01$

Experiment 2:

Does Testosterone counteract the effects of morphine?

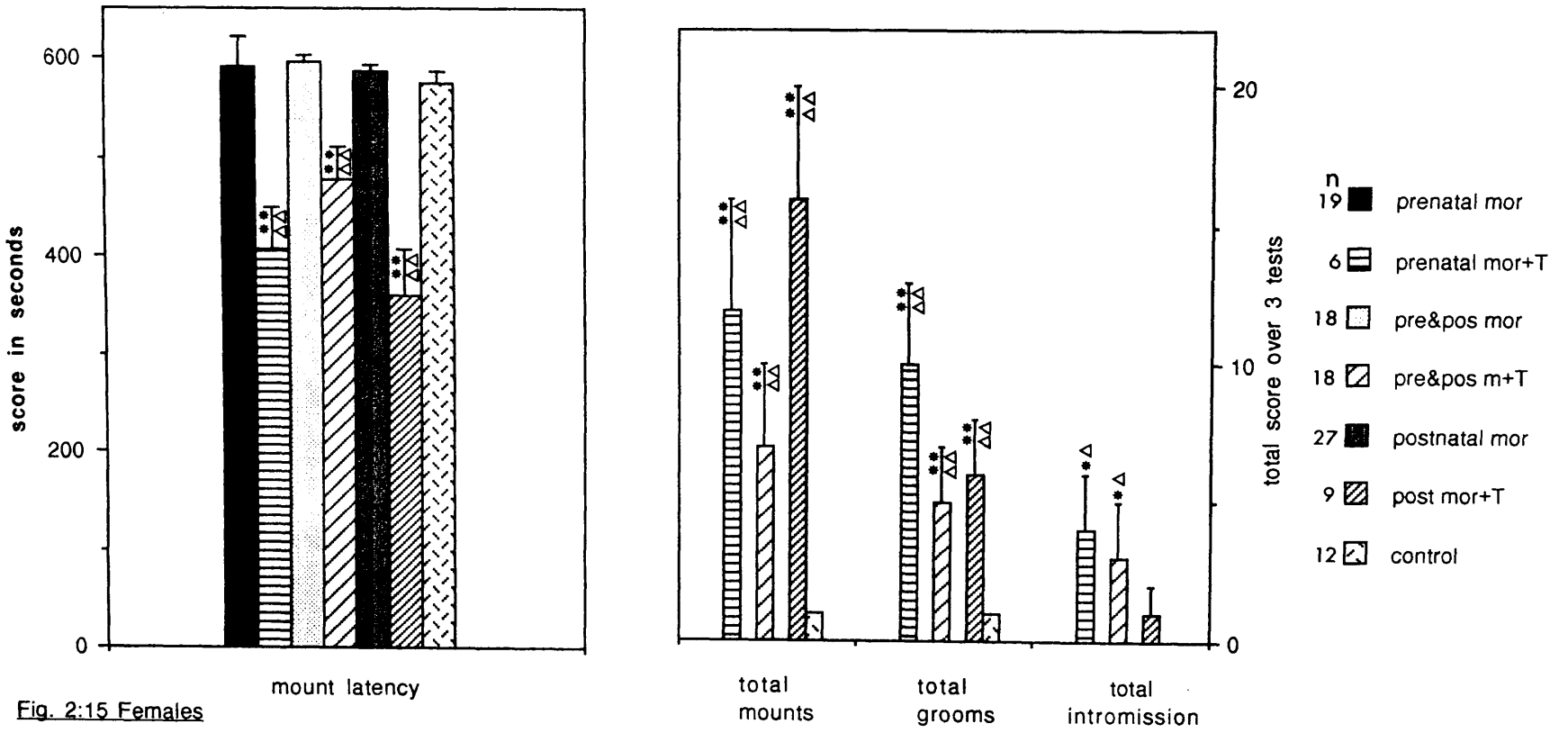


Fig. 2:15 Females

*:** differs from controls p<0.05: p<0.01

△:△△ differs from morphine only over same period p<0.05: p<0.01

Experiment 2:

Does Testosterone counteract the effects of morphine?

Males

Behaviour		mount latency	total mounts	total grooms	total intromit	% mounting
treatment	n					
prenatal morphine	18	352±32	26±5	23±5 *	16±4 **	100%
prenatal morphine+ testos	11	249±45 ∞ *	51±7 ∞∞ **	48±7 ∞∞ **	35±6 ∞∞ **	100%
pre&post morphine	25	434±18	14±3	10±2	3±1	92%
pre&post morphine+ testost	15	404±40	26±5	24±5 *	18±5 **	80%
postnatal morphine	25	381±29	22±4	17±3	9±3 *	92%
postnatal morphine+ testos	17	358±31	28±5 *	24±4 *	12±3 **	88%
control	17	377±26	14±2	10±2	2±1	100%
F value		3.01: p<0.01	6.67: p<0.01	8.60: p<0.01	10.51: p<0.01	

Table 2:12. Masculine sexual behaviour as displayed by adult male hamsters treated perinatally with morphine or morphine and testosterone.

Latency = average score over three test periods.in secs
Other scores are totals for the three test periods.
Group means ± SEM

* differs from controls $p<0.05$
** differs from controls $p<0.01$
∞ differs from morphine only over the same period $p<0.05$
∞∞ differs from morphine only over the same period. $p<0.01$

Experiment 2:

Does Testosterone counteract the effects of morphine?

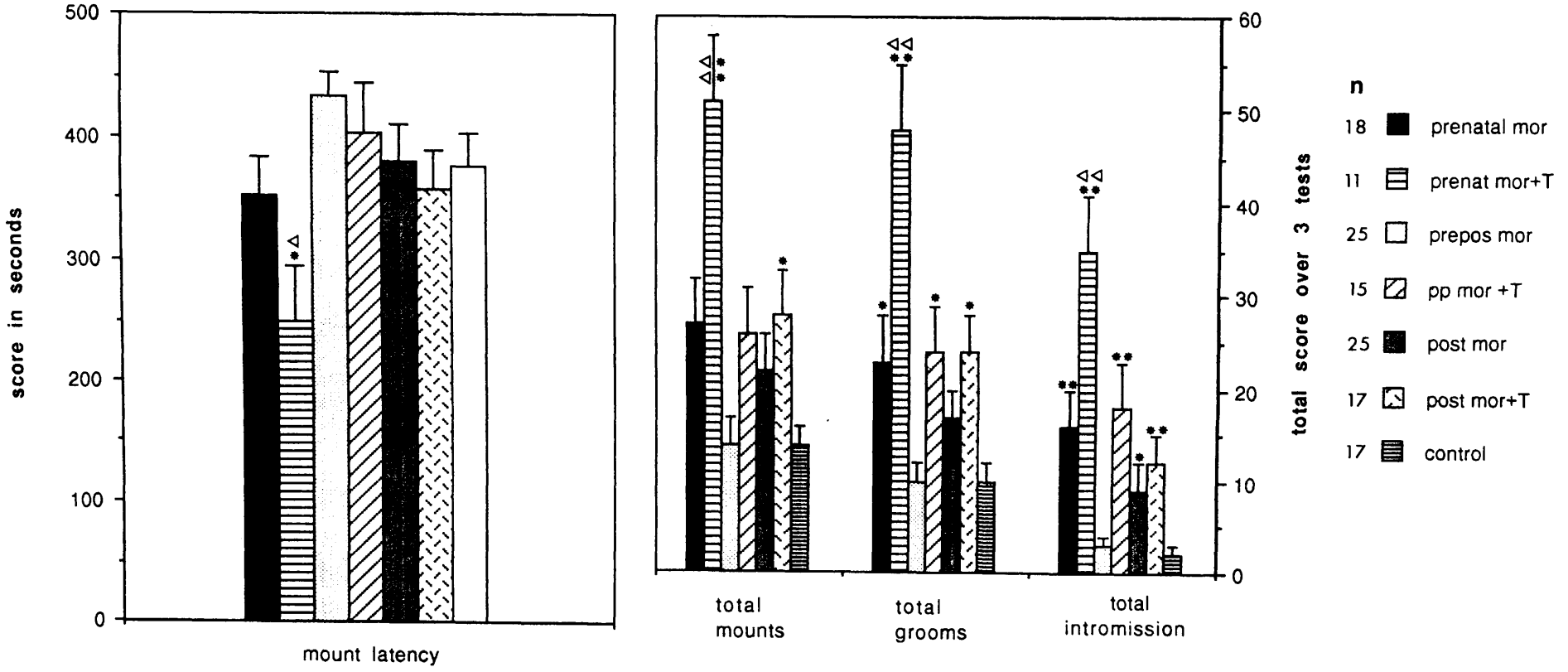


Fig. 2:16 Males

*:** differs from controls p<0.05: p<0.01
◁:◁◁ differs from morphine only over same period p<0.05: p<0.01

Experiment 3

Does Naltrexone counteract the effects of morphine?

From Expt.2 it has been shown that testosterone counteracts most of the effects of perinatal morphine exposure. However there are some unexpected alterations to behaviour as a result of concurrent testosterone administration which are difficult to explain, particularly in males treated prenatally. To try and be more precise in determining the action of morphine a third experiment was set up in which the long-acting opiate antagonist naltrexone (NTX) was administered concurrently with morphine. Since the earlier findings indicated that prenatal morphine alone had little effect on masculine or feminine sexual behaviour in males or females, this treatment period was omitted from this experiment. An additional group treated postnatally with NTX only was however added. Therefore the following groups were set up:-

Pre + Postnatal

1. 10mg/kg Duromorph ip twice daily + 50mg/kg NTX sc once daily was given to pregnant hamsters from day 13 of pregnancy until parturition and to the pups from the day of birth for 4 days (n=9 females, 13 males).

Postnatal

2. 10mg/kg Duromorph ip twice daily + 50mg/kg NTX sc once daily was given to pups from the day of birth for four days (n=21 females, 15 males).

3. 50mg/kg NTX sc once daily was given to pups from the day of birth for 4 days (n=14 females, 11 males).

All other conditions and treatments were as in Expt 1.

Results

Feminine behaviour

Females (See Table 2:13 Fig. 2:17)

Females treated pre + postnatally with morphine + NTX showed a significant decrease in the amount of feminine sexual behaviour displayed. Lordosis latency was increased, while total and maximum periods of lordosis were decreased. No other treatment group in this experiment differed significantly from the controls in their ability to display lordosis.

Males (See Table 2:14 Fig 2:18)

NTX given with morphine reversed the effects of opiates on the ability to display feminine sexual behaviour in males. Postnatal NTX given alone had no effect on feminine sexual behaviour in males.

Masculine sexual behaviour

Females (See Table 2:15, Fig 2:19)

Neither NTX injected alone or with morphine had any effect on masculine sexual behaviour in females; no group in this experiment showed any significant mounting behaviour.

Males (See Table 2:16, Fig 2:20)

NTX alone given postnatally resulted in a marked enhancement of mounting behaviour in males; in particular it caused a significant reduction in mount latency while increasing all other aspects of masculine sexual behaviour. NTX given concurrently with morphine postnatally also enhanced the number of mounts and intromissions but did not affect the mount latency.

Discussion

The results from this experiment indicate that the changes noted in feminine sexual behaviour in the male golden hamster exposed to opiates during the perinatal period are opiate mediated. Evidence for this is:-

NTX given concurrently with morphine reverses the opiate effect on defeminization, such that males treated with both morphine and NTX do not differ from controls in the amount of feminine sexual behaviour displayed. NTX alone postnatally had no effect on the males' ability to display lordosis. These results suggest that the endogenous opioids do not participate in the defeminization process in the males of this species. In contrast, when masculine behaviour in males is considered, although morphine alone appeared to have little effect on mounting it did increase the number of intromissions in each test. However the administration of NTX concurrently with morphine, or on its own postnally significantly enhanced both the number of

mounts and intromissions. NTX alone postnatally also markedly reduced the mount latency in males. This would suggest that the endogenous opioids might be actively involved in the patterning of adult masculine behaviour during the postnatal period in the Golden hamster. It is possible that the increased masculine behaviour in these males is due to increased testosterone production from the testes during the postnatal period. Anandalaxmi & Vijayan (1989) reported increased plasma gonadotrophin and testosterone levels in rat pups given naloxone fron day 1-21 postnatally. The fact that there was a marked increase in masculine sexual behaviour in males treated with testosterone concurrently with morphine adds support to this theory.

General Discussion.

In female hamsters, morphine exposure during the perinatal period had little effect on their ability to display normal feminine sexual behaviour except to increase lordosis latency in the group treated prenatally and thus slightly decrease the total amount of lordosis. None of the treatments had any effect on the females' inability to show mounting. That latency to lordosis has been altered suggests that these females have been affected such that their ability to respond to the advances of a stud male has been reduced; once lordosis was initiated, there was no difference between the treated animals and the controls. Vathy et al, (1983) reported that prenatal morphine

sulphate administration partially inhibited feminine behaviour in female rats. Since prenatal androgen administration concurrently with morphine did not alter the females ability to show lordosis this would suggest that the changes noted in this group were not mediated via androgen responsive mechanisms. Surprisingly, females treated pre and postnatally with morphine showed no change whatsoever in their adult sexual behaviour, either feminine or masculine. One might have expected, since prenatal morphine exposure had affected the females response to male advances, that a continuation of the opiate treatment into the postnatal period would have had a more marked effect. Nor is the change in response likely to be as a result of stress due to handling, as has been reported in the rat (Herrenkohl, 1983) since both prenatally and pre + postnatally groups were handled equally prior to parturition and only the prenatally-treated females have shown any changes in behaviour.

Androgen administration concurrently with morphine prenatally and pre + postnatally did not significantly reduce feminine sexual behaviour in females except that the maximum single episode of lordosis in females treated pre + postnatally was reduced when compared with controls. In contrast, androgen + morphine postnatally markedly reduced the quantity of lordosis in females. Since postnatal morphine alone had no effect on lordosis, it must be assumed that the changes observed in this group were caused by the steroid administration. This is in

agreement with previous findings in hamsters (Debold & Whalen, 1975; Whalen & Etgen, 1978), mice (Manning & McGill, 1974) and rats (Whalen & Edwards, 1967).

Perinatal androgen + morphine had a more marked effect on the female hamsters' ability to display mounting behaviour. Females in all three treatment groups mounted and displayed intromission-like behaviour. This is surprising since investigations to determine the critical period for masculinization of the golden hamster have suggested that androgen administration prenatally would have little significant effect on masculinization of behaviour in the female of this species (Nucci & Beach, 1971). Therefore, the present findings would suggest that the opiates have had an effect in facilitating masculinization of females prenatally exposed to androgens. Perhaps this has been mediated by alterations to the neural substrate involved in establishing masculine behaviour. It has been shown that perinatal morphine exposure alters the developmental patterns of the endogenous opioid systems (Di Guilio et al, 1988) and that the endogenous opioids are involved in control of sexual behaviour (Murphy, 1981).

When the opiate antagonist NTX is given alone or in conjunction with morphine postnatally, there is no change in either feminine or masculine behaviour in females. However extending the treatment into the prenatal period results in a marked reduction of feminine sexual behaviour. How these changes are mediated is unclear since morphine

alone had no significant effect during either of these treatment periods nor did NTX alone postnatally have any significant effect.

NTX alone given postnatally is reported to enhance lordosis behaviour in female rats (Meyerson et al, 1988). Morphine binds to both μ and δ receptors in the brain, but binds preferentially to μ receptors. Prenatal exposure to morphine has been shown to down-regulate opiate receptors in the neonatal rat brain (Tempel et al, 1988) while naloxone (a morphine antagonist) increases μ-receptor numbers (Bardo et al, 1982). Naloxone binds to all three opioid receptor-types, thus it is possible that the changes noted in this investigation were mediated via the k receptors. To test this, a specific k receptor ligand such as dynorphin should be employed with morphine and naltrexone. If the changes observed are mediated via k receptors, blocking of these receptor with a specific ligand may reverse the effects.

In contrast, morphine exposure during the perinatal period has a marked effect on the adult sexual behaviour of male hamsters which is completely reversed by NTX. Males administered morphine over the pre and postnatal period or just postnatally, showed a significant increase in feminine sexual behaviour. Prenatal morphine, on the other hand, did not facilitate the expression of lordosis in male hamsters. Defeminization takes place during the early postnatal period in the golden hamster (Carter et al, 1972; Payne, 1976). Testosterone administered concurrently with

morphine reversed the effects of morphine alone in the pre + postnatal and postnatally treated males. However, testosterone given in addition to morphine prenatally had the reverse effect in that these males displayed significantly more feminine sexual behaviour than did controls. How this action is mediated is unclear. However one could speculate that a high dose of steroid prenatally may desensitise the CNS such that it is unable to respond to endogenous androgens present during the early postnatal period and so undergo the normal process of defeminization. Thus this action may have been mediated via a down-regulation of androgen receptors in the areas of the CNS involved in sexual differentiation. However when the masculine sexual behaviour of the same group of animals is considered it will be noted that prenatal morphine + testosterone markedly enhanced masculine sexual behaviour. Prenatal morphine alone also increased the number of intromissions in this group. Thus it would seem that males exposed to both opiates and steroids prenatally have become bisexual, they will readily respond to a receptive female with mounting behaviour, but are equally capable of adopting the lordotic posture after the appropriate priming when approached by other males. It must be pointed out that, while these males showed increased feminine sexual behaviour, no group spent as much time in lordosis as normal females. Since masculine sexual behaviour was increased after this treatment it seems unlikely that the

effects were mediated via a down-regulation of androgen receptor numbers. If the action is by receptor reductions, it would imply that while the neural substrate mediating defeminization has become less sensitive to androgen postnatally, the substrate mediating masculinization has become more sensitive. From Appendix 1:3 it can be seen that morphine administered prenatally accumulates in relatively high concentrations in the fetal brain. Acute prenatal opiate administration has been reported to have many effects on the developing CNS, including alterations in postnatal neurotransmitter levels (Rech et al , 1980) and synaptogenesis (Seidler et al, 1987). In culture, opiates inhibit serotonin uptake (Davila-Garcia & Azmitia, 1989) and in vivo reduce androgen production (Singh et al, 1980; Anandalaxmi & Vijayan, 1989). The multiple effects of opiates on the developing organism makes it very difficult to determine by which methods the drug has caused the changes in behaviour reported here.

When masculine behaviour in treated males is examined it can be seen that all treatments, except continuous opiate treatment pre + postnatally, significantly increased intromission scores. This is normally a particularly androgen-sensitive behaviour (only females treated neonatally with androgens displayed any intromission-like behaviour). However, it would seem that the changes in behaviour were not as a result of reduction in endogenous androgens, since radioimmunoassay of circulating plasma androgens in 2 day old male hamsters treated with opiates

shows no reduction (see appendix 2)

During late gestation and in the early postnatal period a considerable amount of neuronal growth, cell death and synaptogenesis is occuring in the mammalian neonate. It has been shown both in vivo and in vitro that opiates can interfere with these processes, and indeed that the endogenous opioid peptides are involved in the regulation of some of them. The hypothalamus, which is deeply involved in control of reproductive behaviour, has a high concentration of opiate receptors (Simon & Hiller, 1978) and of opioid neural pathways (Simerly et al,1988). Thus, it is not surprising that changes in reproductive behaviour would become apparent, as in this investigation, when acute opiate administration is applied to the developing animal during the perinatal period.

In this investigation postnatal NTX markedly enhanced mounting behaviour in male hamsters. This is in contrast to findings in the rat (Meyerson et al, 1988) where 10μg NTX s.c. on days 1-10 postnatally had no effect on masculine behaviour in males. However, the dose used in this experiment was higher, 250μg s.c. on days 1-4 postnatally, and may have had a more pronounced effect. It is possible that the enhanced masculine behaviour may have been caused by an increase in endogenous testosterone under NTX stimulation. It is unlikely though that endogenous production would match the levels of the exogenous androgen administered in expt 2 which did enhance

male behaviour, but not to the same extent as did NTX. In fact, the levels of masculine behaviour in the NTX-treated males are comparable to those treated prenatally with morphine and testosterone. Here are two dissimilar treatment regimes, given over different periods, yet having a similar effect on mounting behaviour. However here the comparison ends as, prenatal morphine + testosterone also enhanced feminine sexual behaviour in males while NTX postnatally did not. Thus while the end results are similar, it is most unlikely that they were reached in the same manner.

The long-acting opiate antagonist, NTX blocks opiate receptors thus preventing the endogenous opioids from influencing neural development. It is believed that the endogenous opioids have an inhibitory influence on neural growth and development, mainly in regulating dendritic outgrowth and synaptogenesis. Opioid antagonists enhance dendritic arborisation in the developing rat (Zagon & McLaughlin, 1984, 1986; Hauser et al, 1987). Therefore it is feasible that NTX - treated animals may have developed neuronal networks specifically involved in male sexual behaviour which are exquisitely sensitive to androgens in adulthood. When such animals are primed with androgen and encounter a receptive female, they respond faster (as is noted in the reduced mount latency) and with more vigour than do normal males.

In conclusion, acute morphine exposure over the perinatal period has little effect on the female golden

hamsters' ability to show normal sexual behaviour patterns but has a profound effect on the male. Males so - treated show pronounced bisexual behaviour, in that they respond to encounters with stud males by adopting the lordotic stance, while they are equally responsive when placed with an oestrous female and will readily mount and intromit.

In the second part of experiment 1, animals which had been exposed to morphine throughout gestation and into the postnatal period, were tested for sexual behaviour. As with the acutely -treated females, those treated chronically with morphine showed no significant difference in either feminine or masculine sexual behaviour. However, the males again showed altered adult behaviour. Lordosis latency was reduced and total lordosis increased [although not quite significantly] indicating that chronic morphine treatment has altered the males' responsiveness to stud males. However a surprising aspect of this part of the investigation was that masculine sexual behaviour was also significantly increased. This finding suggests that long-term exposure to opiates is less harmful than is short-term, and is consistent with proposals by Lichtblau & Sparber (1983). These authors reviewed several studies on prenatal opiate exposure and concluded that many of the detrimental changes reported (e.g. low birth weight and early postnatal death) may have been caused by the fact that the drug was given acutely and that the animals had not had time to build up tolerance to it.

Experiment 3:

Does Naltrexone counteract the effects of morphine?

Females

Behaviour		lordosis latency	total lordosis	maximum lordosis	% showing lordosis
treatment	n				
postnatal naltrexone	12	125±46	409±45	237±46	92%
pre&post morphine	18	13±3	498±15	256+27	100%
pre&post morphine+ naltrexone	9	226±94 ** ∞∞∞	281±85 * ∞	143±58 *	67%
postnatal morphine	28	73±26	445±35	298±38	96%
postnatal morphine+ naltrexone	21	111±43	362±45	218±40	90%
control	15	25±4	431±44	322±60	100%
F value		3.16 p<0.01	2.42 p<0.04	1.57 n.s.	

Table 2:13 Feminine sexual behaviour as displayed by adult female hamsters treated perinatally with morphine or morphine and naltrexone.

Scores in seconds. Group mean ± SEM

* differs from controls $p<0.05$
** differs from controls $p<0.01$
∞ differs from morphine only over same period $p<0.05$
∞∞∞ differs from morphine only over same period $p<0.01$

Experiment 3

Does Naltrexone counteract the effects of morphine?

Males

Behaviour		lordosis latency	total lordosis	maximum lordosis	% showing lordosis
treatment	n				
pre&post morphine	26	123±33 * *	165±25 *	47±8	92%
pre&post morphine+ naltrexone	13	394±75 ∞ ∞	83±33 ∞	22±10 ∞	38%
postnatal morphine	25	187±43 * *	126±22 *	42±7	88%
postnatal morphine+ naltrexone	15	445±61 ∞ ∞	72±32	33±14	40%
control	19	360±60	76±23	28±8	53%
postnatal naltrexone	7	378+95	95+61	36+22	43%
F value		6.33 p<0.01	2.10 n.s.	0.92 n.s.	

Table 2:14 Feminine sexual behaviour as displayed by adult male hamsters treated perinatally with morphine or morphine and naltrexone.

Mean scores in seconds ± SEM.

* differs from controls $p<0.05$
** differs from controls $p<0.01$
∞ differs from morphine only over same period $p<0.05$
∞∞ differs from morphine only over the same period $p<0.01$

Experiment 3:

Does Naltrexone counteract the effects of morphine?

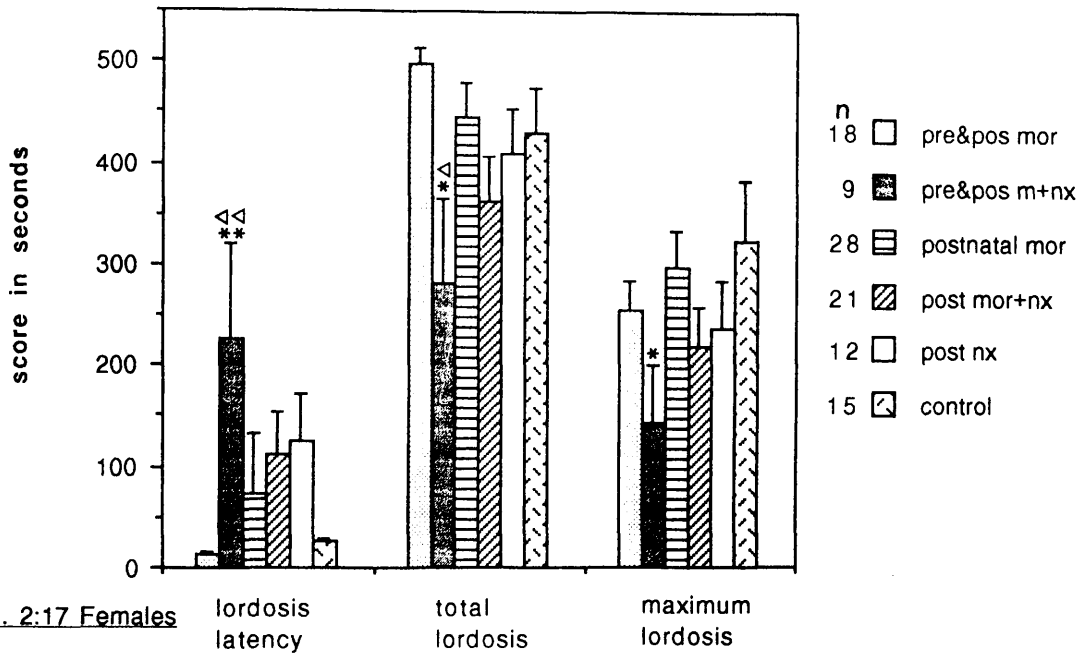


Fig. 2:17 Females

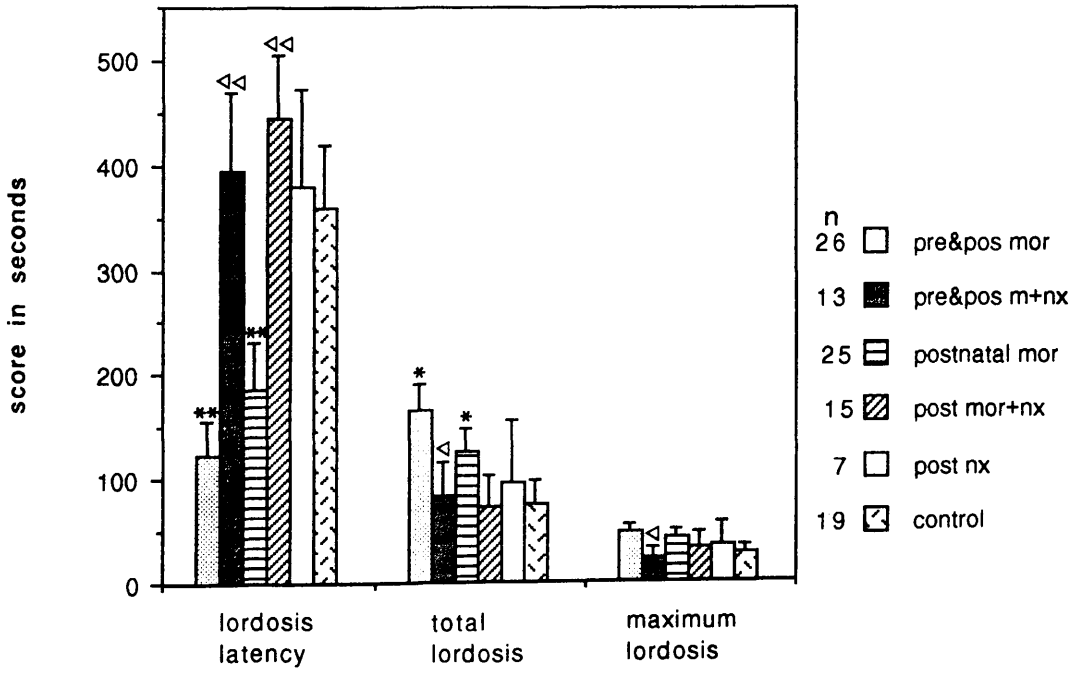


Fig. 2:18 Males

*: **differs from controls p<0.05: p<0.01
◁: ◁◁ differs from morphine only over same period p<0.05: p<0.01

Experiment 3:

Does Naltrexone counteract the effects of morphine?

Females

Behaviour		mount latency	total mounts	total grooms	total introm	% showing mounting
treatment	n					
pre&post morphine	18	597±2	0	0	0	11%
pre&post morphine+ NTX	9	596±3	0	0	0	22%
postnatal morphine	27	586±7	0	0	0	15%
postnatal morphine+ NTX	21	565±17	2±1	3±2	0	5%
control	12	574±12	1	1	0	50%
postnatal NTX	14	567±29	3±2	3±2	3±2	21%
F value		1.36 n.s.	1.79 n.s.	1.81 n.s.	0.00 n.s.	

Table 2:15. Masculine sexual behaviour as displayed by adult females treated perinatally with morphine, or morphine and naltrexone, (NTX) or naltrexone alone.

Latency = average over three tests. in seconds
Other scores are total counts over three test periods.
Group means ± SEM.

Experiment 3:

Does Naltrexone counteract the effects of morphine?

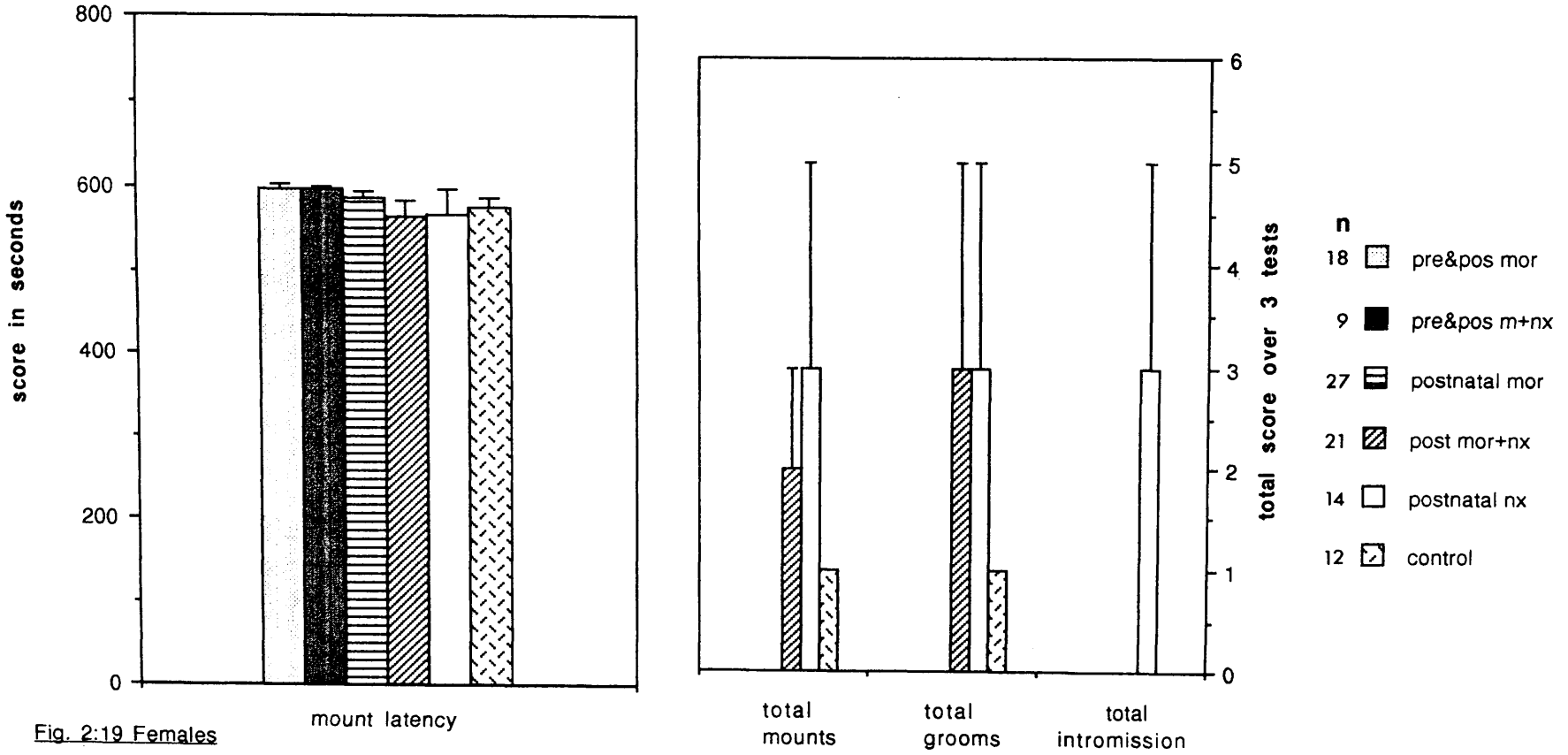


Fig. 2:19 Females

Experiment 3:

Does Naltrexone counteract the effects of morphine?

Males

Behaviour		mount latency	total mounts	total grooms	total introm	% showing mounting
treatment	n					
postnatal naltrexone	11	230±47 ** ∞∞∞	53±8 ** ∞∞∞	50+8 ** ∞∞∞	31±6 ** ∞∞∞	91%
pre&post morphine	25	434±18	14±3	10±2	3±1	92%
pre&post morphine+ naltrexone	13	385±24	23±3	21±3 * ∞	7±2	100%
postnatal morphine	25	381±29	22±4	17±3	9±3 *	92%
postnatal morphine+ naltrexone	15	364±28	34±6 ** ∞	31±6 ** ∞∞∞	17±4 ** ∞	100%
control	17	369±26	15±2	10±2	3±1	100%
F value		4.60: p<0.01	10.28: p<0.01	13.18: p<0.01	12.81: p<0.01	

Table 2:16 Masculine sexual behaviour as displayed by adult male hamsters treated perinatally with morphine, or morphine and naltrexone, or naltrexone.

Latency = average over three tests in seconds
Other scores are totals over three test periods.
Group means ± SEM.

* differs from controls $p<0.05$
** differs from controls $p<0.01$
∞ differs from morphine only over same period $p<0.05$
∞∞∞ differs from morphine only over same period $p<0.01$

Experiment 3:

Does Naltrexone counteract the effects of morphine?

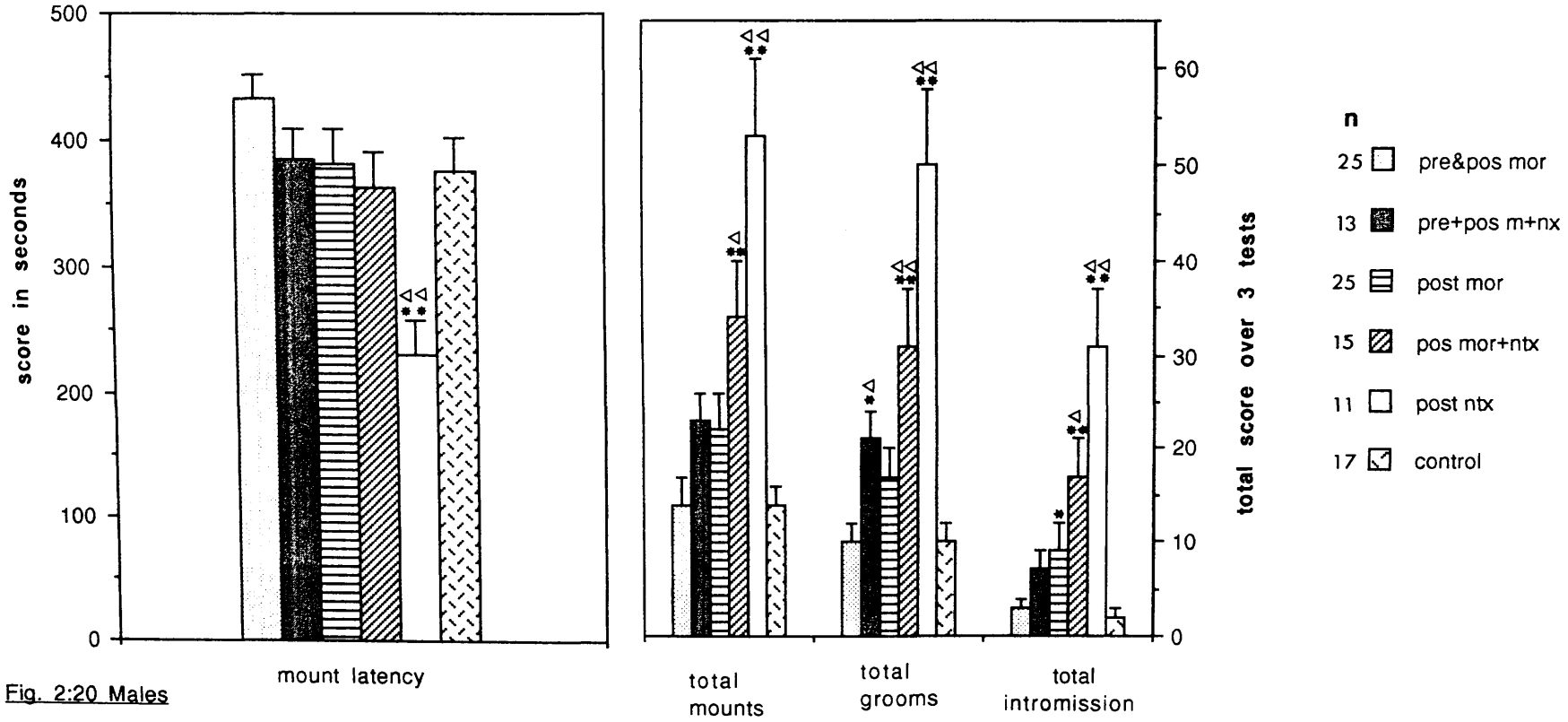


Fig. 2:20 Males

*: ** differs from controls p<0.05: p<0.01
◁: ◁◁ differs from morphine only over same period p<0.05: p<0.01

Appendix 2

Research into the sexual differentiation of adult sexual behaviour in the rodent has shown that castration within 24 hours of birth results in males which display significantly more feminine sexual behaviour as adults after appropriate priming, than do intact males or males castrated in adulthood.

Androgens acting postnatally have been shown to be important in the sexual differentiation of gonadotropin release patterns, morphology of dimorphic structures in the CNS and differences in hypothalamic monoamine levels during the critical period.

In the adult rat, opiate administration causes a reduction in gonadotropin release from the anterior pituitary resulting in a decline incirculating androgens. Similar results have been reported in neonatal rats. Thus it was important to determine whether opiate administration, as used in this investigation, lowers circulating testosterone levels in the neonatal male hamster. Two groups of animals were established:-

1) saline-treated male pups (controls)

2) morphine-treated male pups

Procedure

Treatment: Male pups were given 10mg/kg Duromorph i.p. or saline twice daily on the day of birth (day 1) and on day 2.

Two to three hours after the final injection, pups were sacrificed by cervical dislocation and trunk blood was

collected in heparinised tubes. Since testosterone levels are very low in the neonatal hamster (Pang & Tang, 1983) blood from 18-20 pups was pooled for each sample. Control pups were treated with saline.

The blood samples were centrifuged at 3200 rpm for 3 minutes and the separated plasma stored at -20°C until assayed.

The testosterone radioimmunoassay was carried out in the Department of Pathological Biochemistry at the Royal Infirmary, Glasgow by Mr Stuart Keir.

Testosterone levels were measured by using a ^{125}I-tracer and double antibody separation. Data were analysed using Student's unpaired t-test.

Results

Control n = 4: testosterone levels = 0.445 ± 0.09 nM/l

Morphine-treated n = 4: testosterone levels = 1.21 ± 0.52 nM/l

DF = 6: t =1.44: $p < 0.2$; not significant.

These results indicate that morphine-treatment postnatally has had no effect on the levels of circulating androgens in the male hamster. Plasma from pre-natally treated animals was not analysed at this stage. Further studies are required to determine whether prenatal exposure to opiates would lower plasma androgens. In-vitro studies of testosterone output from hamster testes from E14 and E15 fetuses and postnatal days 1 and 2 have shown that androgen output is higher prenatally and more responsive to hCG than

testes from day 1 or 2 pups (Vomachka & DiMario, 1987). Masculinization of behaviour is believed to occur in the postnatal period in the hamster since prenatal androgen does not masculinization female hamsters (Nucci & Beach, 1971).

In addition, preliminary studies of androgen levels in postnatally morphine-treated rats did not reveal any change; Control levels = 2.78nM/l
Morphine treated pups = 3.4nM/l.
However, only two pooled samples were used in this study and sample numbers need to be increased before any conclusions can be drawn.

Chapter 3

The effects of perinatal opiate administration on the concentrations of hypothalalmic neurotransmitters at postnatal day 4 or 12.

The monoaminergic neurotransmitter systems are amongst the earliest to develop in the mammalian brain. This early ontogeny suggests that these systems may be active in regulation of brain development . Indeed, serotonin (5HT) has been reported to act as a signal for neural differentiation *in vitro* and *in vivo* (Lauder and Krebs, 1978; Lauder *et al*, 1982).

Dopaminergic (DA), noradrenergic (NA) and serotonergic (5HT) neurones are located in various regions of the CNS; all three monoamines occur within the hypothalamus and are known to be involved in the control of sexual behaviour (Agmo & Fernandez, 1989; Vathy & Etgen, 1989) and gonadotrophin release (Negro-Vilar *et al*, 1984; Clayton, 1988).

Serotonin in particular is reported to show a transitory sexual difference in levels in the rat (Ladosky & Gaziri, 1970) and hamster (Johnston *et al*, 1990) hypothalamus, being higher in females than in males at 12 days after birth. This difference in serotonin concentration is dependent on postnatal androgen levels, as castration before day 5 increases concentrations in males to that found in females (Ladosky & Gaziri, 1970).

Conversely androgen administration to female pups postnatally, reduces levels to that of males (Giulian et al, 1973).

Neonatal reduction of 5HT levels by treatment with pCPA results in both behavioural (Hyyppa et al, 1972; Wilson et al, 1986), and morphological changes in the rat CNS (Handa et al, 1986), although the latter is reported to be temporary (Hines & Gorski, 1986). Nonetheless, these alterations may be important during the development and differation of the CNS. Since the monoamines are believed to have trophic effects during morphogenesis (McMahon, 1974; Lanier et al, 1976) it is important to consider the possible effects that alterations to these levels might have on sexual differentiation.

In the previous set of experiments it was shown that opiate administration during the perinatal period altered the ability to display feminine sexual behaviour. These behavioural changes may have been mediated via decreased synaptogenesis, or neural migration, since opiates can inhibit these processes. On the other hand they possibly may have resulted from alterations in monoamine levels during this critical period of sexual differentiation. There is no compelling evidence that they relate to reduced androgen levels (see appendix 2) even though they are reversed by testosterone administration, as well as by naltrexone.

While changes in behaviour can be effected in

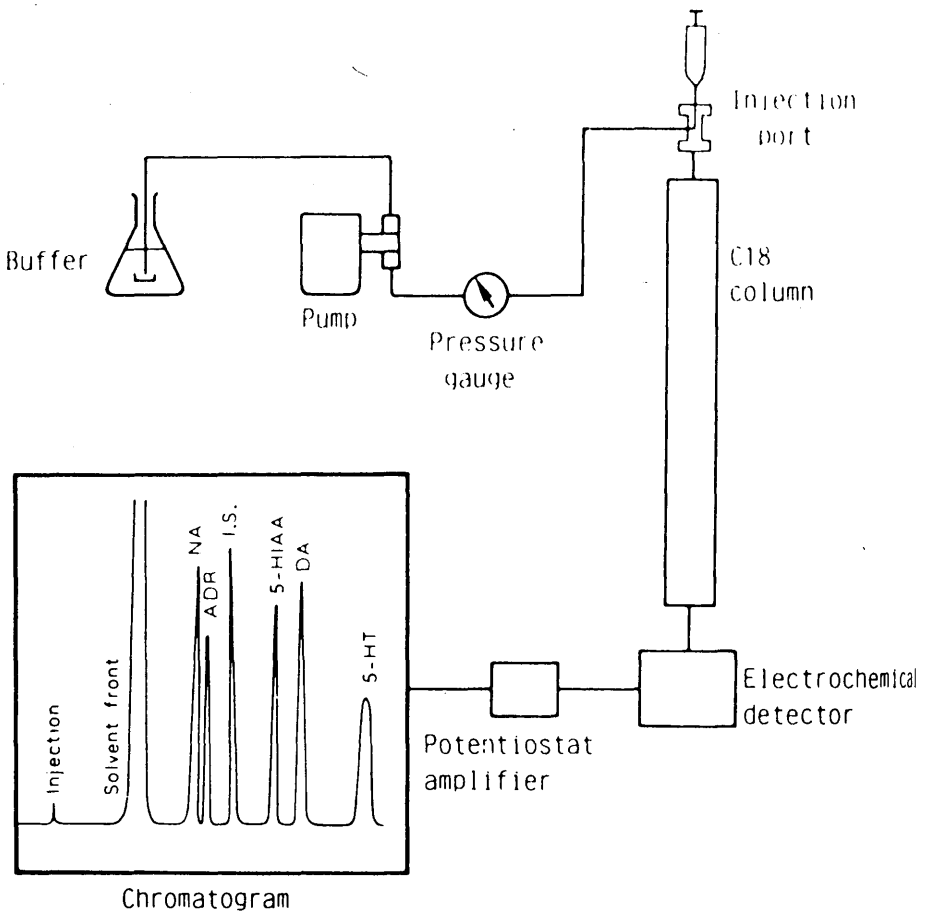


Fig.3:1. Schematic diagram of HPLC apparatus.

adulthood by manipulating monoamine concentrations and turnover, it would be unwise to assume that similar changes in the perinatal period would have the same effect. However, behavioural patterns are being established during the perinatal period and it has been shown that 5HT manipulation during this time does affect behaviour in adults. Thus, if opiates affect the concentrations of the amines during this period, it could be speculated that these changes have contributed to the behavioural alterations.

In the present experiment amines were measured by excising the hypothalamic tissue, homogenising it and after centrifugation injecting the supernate into a chromatographic column in which the components of the mixture were separated and finally measured. A brief description of the system used follows .(Also see Fig. 3:1)

Separation of components by HPLC.

The apparatus used for the analysis of biogenic amines in this experiment was a High Performance Liquid Chromatography (HPLC) system coupled to an electrochemical detector.

HPLC is a method of separating different components of a mixture by a successive series of equilibrium stages. These equilibria depend on the differential adsorption of the components between two phases; a mobile phase, the

eluant, and a stationary phase. The sample mix is introduced into the mobile phase and carried through the chromatographic column, undergoing a series of partition or adsorption interactions between two phases as it goes. The components of the sample mixture pass through the column at different rates according to their physical and chemical properties, such that those with the greatest affinity for the stationary phase will take longest to move through the system.

The eluent is continually monitored by the detector system. As each component leaves the column, it passes over the electrode and is oxidised; the resulting current generated in the flow cell is displayed as a peak on the integrator. Ideally, each component should be eluted such that the peaks do not overlap, and should show maximum height and minimum width. Thereby resulting in good resolution, efficiency and selectivity. The time between injection of the sample and the elution of each component in the mixture is referred to as the retention time and is dependent on the molecular interactions between the two phases. It is also subject to changes in the environment such as temperature, pH and solvent concentration. To allow for these changes, and to determine the identity of each peak as it is eluted, a standard mixture of the components of interest is analysed at the start of each day and at various times throughout the day as the retention times change.

Electrochemical Detection:

As an electrically active molecule passes through the flow cell it is oxidised, and the current generated is displayed as a peak on the integrator. The detector cell consists of 3 electrodes, a working electrode (in this case glossy carbon), a reference electrode (Ag/AgCl) and a stainless steel auxilliary electrode. The eluant from the column enters a very low volume thin-layer cell, where the flow is reduced to a thin film passing over a planar electrode held at a fixed potential. If the potential is greater than that required for the electrolysis of the analyte, then a measurable charge passes between the electrode and analyte. The resulting current is directly proportional to the concentration of solute passing through the cell.

Analysis of hypothalamic samples;

Fig 3:2 is a typical trace recorded when a mixture of standards is injected onto the column. As can be seen from this trace, there are eight peaks. However, during the course of a day, as the ambient temperature of the room and the column rises, the elution times of each amine is brought forward and peaks appearing close together on the trace begin to overlap. In the present study this frequently occurred with the MHPG and DOPAC peaks. Because of this overlap it was not always possible to determine the areas of the two peaks and in these instances the concentration of the amines could not be calculated. For this reason, concentrations of MHPG and DOPAC were not analysed.

Similarly, although the NA peak was easily identified when a standard mixture was run through the system it was often engulfed by the solvent front (which in a sample trace is much larger than that of the standard) (see Fig 3:3). NA can be analysed readily when conditions are suitable, but this system was set up to analyse both catecholamines and indoleamines. The optimal condition for analysis of the indoles is not the best for analysis of the catecholes. Since the concentrations of 5HT were of particular interest, NA could not always be accurately measured in these samples. This was unfortunate, as NA is known to be involved in the control of gonadatrophin release (Rance et al, 1981; Wise et al, 1981) and sexual

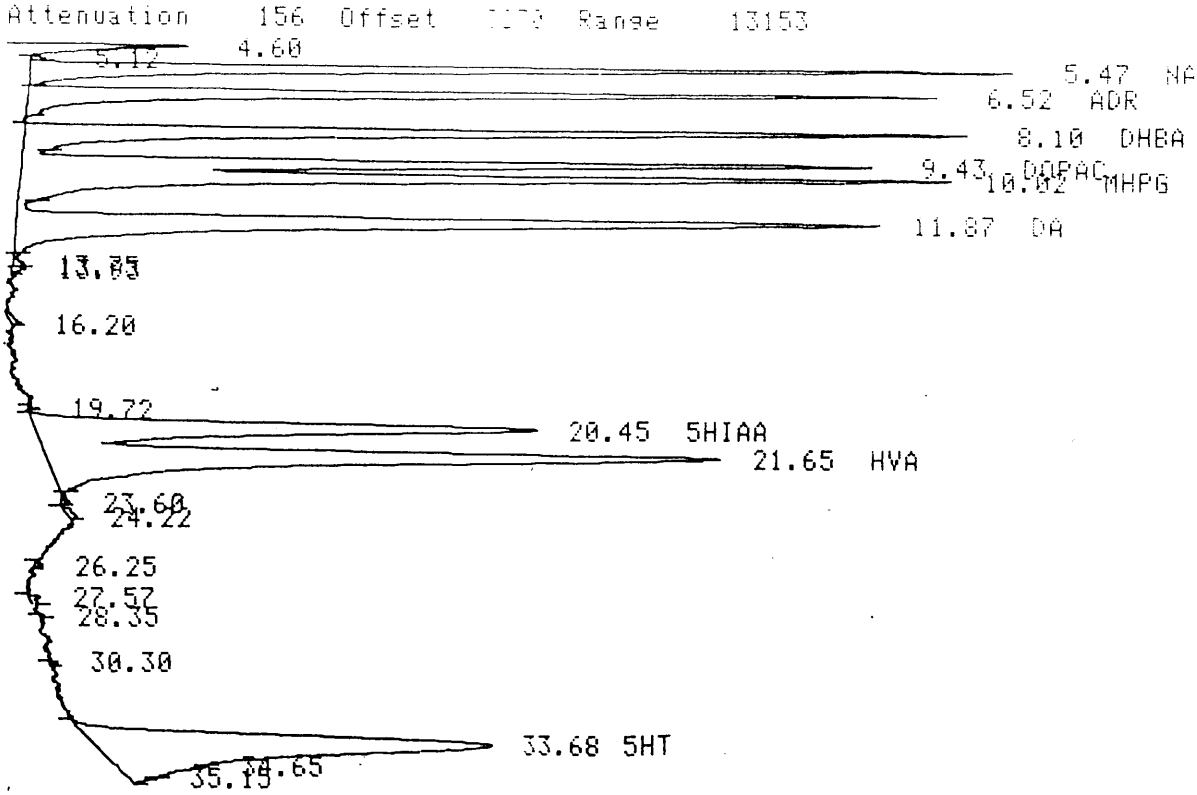


Fig.3:2. Trace produced when 20 μl of a mixture of standards is injected into the HPLC system. Each peak represents a concentration of 1ng.

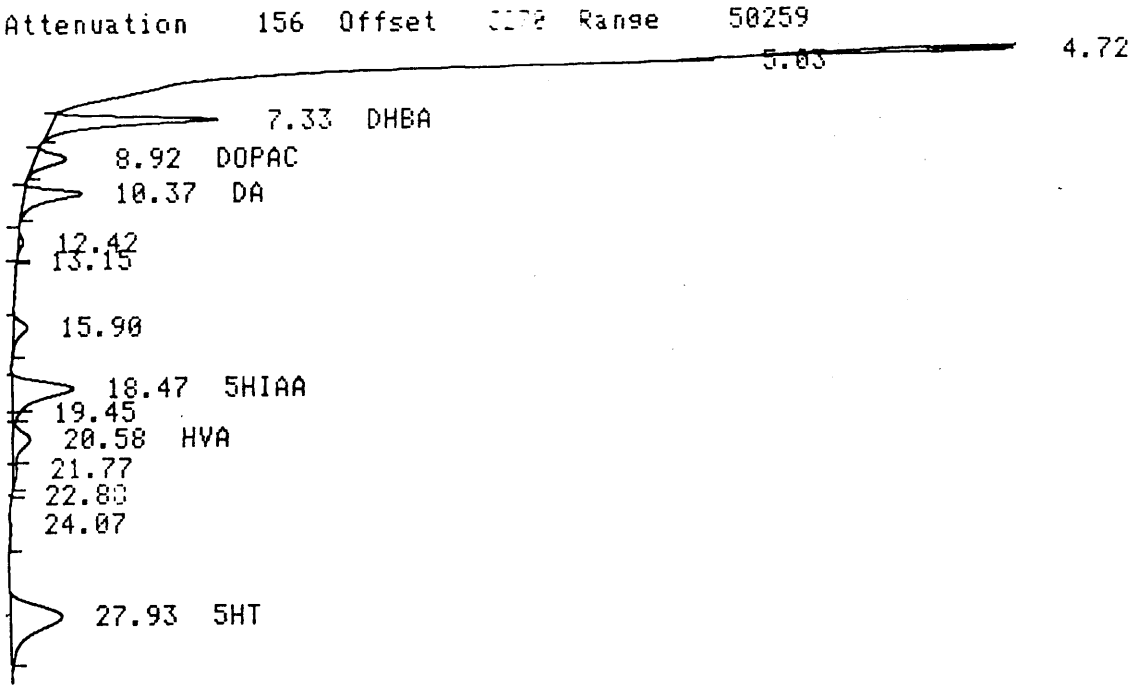


Fig 3:3. Trace produced when a sample of hypothalamic tissue is prepared and the supernatant injected into the HPLC system. Peak labelled DOPAC is DOPAC + MHPG which overlap. The NA peak has been overlapped by the solvent front.

behaviour in the rodent.

Materials and Methods

High Performance Liquid Chromatography analysis of hypothalamic biogenic amines in postnatal hamster pups.

Two separate experiments were performed to detect:

1 Delayed effects of opiates; here the pups were culled at 12 days postnatally while the morphine administration terminated on day 4.

2 Short-term effects of opiates here pups were culled at 4 days postnatally (i.e. on the final day of morphine administration).

Experiment 1

Treatments:

All perinatal drug treatments in this experiment were the same as those described in the behavioural experiments (see chapter 2). Groups were as follows:-

1) Duromorph given prenatally (n=8 females, 6 males)
2) Duromorph pre- and postnatally (n=4 females, 9 males)
3) Duromorph postnatally (n=7 females, 7 males)
4) Duromorph + testosterone prenatally (n=4 females, 6 males)
5) Duromorph + testosterone pre- and postnatally (n=7 females, 7 males)
6) Duromorph + testosterone postnatally (n=6 females, 5 males)

7) Duromorph + NTX pre- and postnatally (n=5 females, 6 males)

8) Duromorph + NTX postnatally (n=7 females, 6 males)

9) NTX postnatally (n=7 females, 5 males)

10) Saline treated controls (n=7 females, 7 males)

Tissue preparation.

The brain was extracted and the hypothalamus was removed by making sagittal cuts on either side of the median eminence followed by coronal cuts (1) immediately rostral to the optic chiasm and 2) immediately rostral to the mammillary bodies (incorporating levels A48-A34 as described Knigge & Joseph, 1968). The excised block of tissue produced by these cuts was divided horizontally 2-3mm above its ventral surface and stored at -80°C until processed. Hypothalami were weighed and homogenised in 600μl 0.1M HCl to which 100μg/l of internal standard, dihydroxybenzylamine (DHBA) had been added. The homogenate was centrifuged at 3000 r.p.m. for 10 minutes and the supernatant was injected into the chromatographic column in quantities of 20μl; at least 2 injections per sample were analysed and any unused supernatant was stored at -80°C until further required. The HPLC apparatus consisted of a Gilson pump and manometric unit (models 302 and 802 respectively), a Rheodyne 7125 injection valve, a 15 x 0.46cm Ultrasphere ion-pair reversed-phase column preceded by a 2 x 0.46cm guard column (both manufactured

by Beckman), a BioAnalytical Systems flow cell TL-5 and a LC-3A electrochemical detector. The potential was set at 0.70V vs an Ag/AgCl reference electrode (sensitivity 1 nA, time constant 5 sec). The output signal was recorded and analysed by a Trivector Trio integrator. The mobile phase consisted of :-

6.8g/l sodium acetate; 5.75g/l citric acid; 2.4g/l sodium hydroxide; 0.037g/l Na EDTA; 0.125g/l heptane sulphonic acid (Na salt). The pH was adjusted to 4.8 using glacial acetic acid; the methanol content was 10% and the flow rate was 0.6ml/min. (modified from the method of Siddiqui & Gilmore, (1986).

Statistics:

All statistical analysis was computer aided. Groups were compared using one-way ANOVA followed by inter-group comparisons using Least Significant Difference Computations. Individual groups were compared where necessary using Student's t test.

Long-term effect of opiates (12 Days)

Results

1) Dopamine

Females: (see Table 3:1)

None of the groups treated with morphine showed hypothalamic DA levels which differed from those in the controls. However females treated postnatally with both morphine + testosterone had significantly lower levels of DA than did controls or those treated with morphine only. In contrast, morphine + testosterone given pre and postnatally increased DA levels above those of controls as did morphine + naltrexone postnatally.

Males: (see Table 3:2)

Levels of DA were significantly higher in males treated pre- and postnatally with morphine or with morphine + testosterone when compared to controls. Concurrent administration of naltrexone reversed this effect. On the other hand, morphine + testosterone treatment postnatally significantly reduced DA levels below those found in controls or in males treated with morphine only postnatally.

2)Homovanillic acid

Females (see Table 3:3)

None of the perinatal treatments altered hypothalamic

HVA levels when compared with those of controls. However morphine + testosterone administration prenatally significantly increased HVA levels beyond those of females treated with morphine only over the same period.

Males (see Table 3:4)

There was no significant difference in hypothalamic levels of HVA between any of the treatment groups.

3) Serotonin

Females (see Table 3:5)

Morphine administered prenatally significantly reduced hypothalamic 5HT levels in females, while concurrent testosterone administration reversed this effect. 5HT levels in postnatal morphine + testosterone-treated females were significantly lower than those in controls or those given morphine only postnatally.

Males (see Table 3:6)

Males treated prenatally with morphine + testosterone had significantly elevated 5HT levels in comparison to controls, or to those treated prenatally with morphine only. Males treated with morphine pre- and postnatally also had significantly higher 5HT levels than did controls, as did males given morphine + naltrexone pre- and postnatally.

4) Hydroxyindoleacetic acid

Females (see Table 3:7)

Morphine treatment prenatally and pre + postnatally reduced levels of 5HIAA significantly below those of controls. In contrast testosterone given in conjunction with the opiate over the same period reversed this effect, as did naltrexone. Postnatally administered morphine did not alter 5HIAA levels in females, but the opiate antagonist naltrexone significantly reduced 5HIAA concentrations below those of controls and other postnatally-treated groups.

Males (see Table 3:8)

None of the perinatal opiate/antagonist treatments had any significant effect on hypothalamic 5HIAA levels measured in 12-day old male hamsters.

Hypothalamic dopamine levels in neonatal Golden Hamster treated perinatally with morphine and testosterone or naltrexone.

Females

Treatment	DA levels	n
prenatal morphine	362 ± 33	8
prenatal mor + T	413 ± 69	7
pre + post morphine	453 ± 46	4
pre + post mor + T	524 ± 59 *	7
pre + post mor + NTX	331 ± 82	5
postnatal morphine	326 ± 27	7
postnatal mor + T	163 ± 30 *§	6
postnatal mor + NTX	517 ± 39 *§	7
postnatal naltrexone	430 ± 44	7
control	329 ± 56	7
F value	4.46: $p<0.01$	

Table 3:1 Hypothalamic DA levels in 12 day old female hamsters . Group means ± SEM

Males

Treatment	DA levels	n
prenatal morphine	366 ± 39	6
prenatal mor + T	412 ± 67	6
pre + post morphine	564 ± 39 *	9
pre + post mor + T	472 ± 37 *	7
pre + post mor + NTX	413 ± 62	6
postnatal morphine	340 ± 44	7
post morphine + T	144 ± 36 * §	5
postnatal mor + NTX	372 ± 37	6
postnatal naltrexone	434 ± 28	5
control	318 ± 35	7
F value	6.15 $p<0.01$	

Table 3:2 Hypothalamic DA levels in 12 day male hamsters
* differs from controls $p<0.05$
§ differs from morphine only over same period.

Hypothalamic homovanillic acid levels in neonatal Golden Hamsters treated perinatally with morphine and testosterone or naltrexone.

Females

Treatment	HVA levels	n
prenatal morphine	216 ± 16	8
prenatal mor + T	337 ± 28 §	7
pre + post morphine	434 ± 143	4
pre + post mor + T	388 ± 78	7
pre + post mor + NTX	419 ± 41	5
postnatal morphine	357 ± 19	7
postnatal mor + T	272 ± 32	6
postnatal mor + NTX	358 ± 26	7
postnatal naltrexone	264 ± 22	7
control	323 ± 32	7
F value	F=2.33 $p<0.03$	

Table 3:3 Hypothalamic HVA levels in 12 day female hamsters.
Group means ± SEM
§ differs from morphine only over same time period: $p<0.05$

Males

Treatment	HVA levels	n
prenatal morphine	432 ± 101	6
prenatal mor + T	414 ± 76	6
pre + post morphine	382 ± 20	9
pre + post mor + T	362 ± 44	7
pre + post mor + NTX	285 ± 32	6
postnatal morphine	357 ± 35	7
postnatal mor + T	338 ± 42	5
postnatal mor + NTX	366 ± 40	6
postnatal naltrexone	304 ± 49	5
control	295 ± 16	7
F value	0.98 n.s.	

Table 3:4 Hypothalamic HVA levels in 12 day male hamsters.
Group means ± SEM.

Hypothalamic serotonin levels in neonatal Golden Hamsters treated perinatally with morphine and testosterone or naltrexone.

Females:

Treatment	5HT levels	n
prenatal morphine	803 ± 43 **	8
prenatal mor + T	1251± 78 §§	4
pre + post morphine	1194 ± 219	4
pre + post mor + T	1303 ± 112	7
pre + post mor + NTX	1240 ± 56	5
postnatal morphine	1208 ± 56	7
postnatal mor + T	892 ± 53 * §	6
postnatal mor + NTX	1268 ± 79	7
postnatal naltrexone	1065 ± 41	7
control	1156 ± 67	7
F value	4.87: p<0.01	

Table 3: 5 Hypothalamic 5HT levels in 12 day female hamsters. Group means ± SEM
*: ** differs from controls p<0.05: p<0.01
§: §§ differs from morphine only over same period.

Males:

Treatment	5HT levels	n
prenatal morphine	1020 ± 58	6
prenatal mor + T	1443 ± 175**§	6
pre + post morphine	1508 ± 94 **	9
pre + post mor + T	1166 ± 68	7
pre + post mor + NTX	1363 ± 63 *	6
postnatal morphine	1223 ± 87	7
postnatal mor + T	941 ± 88	5
postnatal mor + NTX	1253 ± 185	6
postnatal naltrexone	1206 ± 100	5
control	964 ± 29	7
F value	3.65 p<0.01	

Table 3:6 Hypothalamic levels of 5HT in 12 day male hamsters. Group means ± SEM.

Hypothalamic 5-Hydroxyindoleacetic acid levels in neonatal Golden Hamsters treated perinatally with morphine and testosterone or naltrexone.

Females

Treatment	5HIAA levels	n
prenatal morphine	382 ± 13 **	8
prenatal mor + T	603 ± 48 §§	7
pre + post morphine	430 ± 70 *	4
pre + post mor + T	592 ± 41 §	7
pre + post mor + NTX	621 ± 52	5
postnatal morphine	601 ± 20	7
postnatal mor + T	521 ± 56	6
postnatal mor + NTX	650 ± 64	7
postnatal naltrexone	361 ± 13**§§	7
control	582 ± 57	7
F value	5.61 $p<0.01$	

Table 3: 7 Hypothalamic 5HIAA levels in 12 day female hamsters. Group means ± SEM
* :** differs from controls $p<0.05$: $p<0.01$
§ §§ differs from morphine only over the same period.

Males

Treatment	5HIAA levels	n
prenatal morphine	513 ± 58	6
prenatal mor + T	525 ± 98	6
pre + post morphine	608 ± 28	9
pre + post mor + T	513 ± 53	7
pre + post mor + NTX	593 ± 26	6
postnatal morphine	593 ± 49	7
postnatal mor + T	594 ± 40	5
postnatal mor + NTX	611 ± 77	6
postnatal naltrexone	459 ± 63	5
control	576 ± 62	7
F value	0.75 n.s.	

Table 3:8 Hypothalamic 5HIAA levels in 12 day male hamsters. Group mean ± SEM

Expt 2

Short-term effects of opiates.

Materials and methods

1) Pups treated with 10mg/kg Duromorph ip twice daily from the day of birth till culling on Day 4 (n=6 females, 6 males).
2) Controls which received saline were culled on day 4 postnatally (n=5 females, 5 males).

Tissue extraction and processing were carried out in a similar manner to that described in experiment 1. Since the tissue weight from 4 day-old pups was approximately 50% of that in the 12 day pups, 300μl of HCl were used for homogenisation.

Results

Dopamine

Females: (see Table 3:9)

Hypothalamic DA levels were increased in the opiate-treated animals when compared to those of controls but this did not reach significance.

Males (see Table 3:10)

Hypothalamic DA levels were increased in the morphine-treated males when compared to those of controls but this did not reach significance.

Homovanillic acid

Females (see Table 3:9)

No significant changes were recorded in HVA levels in

opiate-treated females.

Males (see Table 3: 10)

Morphine treatment did not significantly alter hypothalamic HVA levels in male hamsters.

Serotonin

Females (see Table 3:9)

5HT levels were significantly elevated in the females treated postnatally with morphine.

Males (see Table 3:10)

5HT levels were significantly elevated in males treated postnatally with morphine.

5Hydroxyindoleacetic acid

Females (see Table 3:9)

5HIAA levels were not altered in females treated postnatally with morphine.

Males (see Table 3:10)

5HIAA levels were significantly increased in males treated postnatally with morphine.

Discussion

Because of the great variation in the hypothalamic amine levels between the different treatment groups, it is difficult to correlate the changes recorded here in pups culled at day 12 with the alterations in behaviour

reported in Chapter 2. In particular, from the present investigation, it would appear that amine levels in females are more susceptible to change than those in males. Significant changes were noted in some groups of females in each of the amines measured, while only 5HT and DA levels were significantly altered in the males.

Within each sex, treatments had opposite effects when given over different periods. In females, morphine alone had no effect on DA orits metabolite HVA yet testosterone given concurrently with morphine pre + postnatally increased DA levels, and when given postnatally decreased these. The two treatments had similar effects in males. It would appear therefore, that long-term exposure to both morphine and androgen has enhanced the development of the dopaminergic neuronal systems or increased the hypothalamic dopaminergic content, while short-term postnatal exposure has had an inhibitory effect on these. Why there should be such a difference in results is unclear, particularly since the former treatment overlaps with the latter. Further investigations into the effects of androgens alone over the same periods would perhaps help to clarify these data. The concentrations of the DA metabolite HVA differed little between the treatments, suggesting that the reduction in DA levels after morphine + testosterone treatment is due to synthesis inhibition rather than to increased turnover.

In contrast to DA, 5HT seems much more sensitive to perinatal manipulation in both sexes, although again there

is considerable variation between the treatment groups. Prenatal morphine administration significantly reduced hypothalamic 5HT and 5HIAA levels in females at postnatal day 12, while concurrent administration of androgen reversed this effect. On the other hand, neither of the other morphine-only treatments significantly affected 5HT levels,although testosterone treatment in conjunction with morphine postnatally significantly lowered 5HT but not 5HIAA. Postnatal androgen administration has been shown to lower day 12 hypothalamic 5HT levels in the rat (Ladosky & Gaziri, 1972; Giulian et al, 1973) or have no effect (Siddiqui et al, 1989)

Day 12 5HIAA levels were significantly decreased by postnatal naltrexone administration in females while 5HT remained unaltered, suggesting an increased clearance of the metabolite from the system, or perhaps a reduction in 5HT synthesis.

5HT levels were increased in 12 day old males as a result of prenatal morphine + testosterone treatment, without a concurrent rise being seen in the hypothalamic 5HIAA content. This treatment resulted in increased feminine sexual behaviour in males, as also did pre and postnatal morphine administration, (see Chapter 2 Expt 2) which also significantly increased 5HT levels at 12 days postnatally. The increase in 5HT caused by morphine treatment pre + postnatally was reversed by concurrent testosterone administration which caused a corresponding change in sexual behaviour.

These changes in 5HT levels in males, coupled to the alterations previously reported in behaviour, suggest a role for the serotonergic system in the defeminization process. Control levels of 5HT are higher in the female rat and hamster hypothalamus at day 12 than in the male (Ladosky & Gaziri,1972; Giulian et al, 1973, Siddiqui et al, 1989; Johnston et al, 1990). Manipulation of these levels results in alterations to adult sexual behaviour (Wilson et al,1986; Johnston et al, 1990). This suggests therefore, that some of the behavioural changes reported in the present investigation may have been mediated via alterations to the hypothalamic serotonergic system at a time when neurotransmitter concentrations are sexually dimorphic.

Investigations into the ontogeny of the serotonergic system in the rodent reveal that 5HT neurones are detectable as early as E11 in the medial raphe of the rat (Lauder et al, 1982). 5HT occurs in high concentrations prior to assuming its role as a neurotransmitter and is thought to act as a developmental signal prior to acting as a neurotransmitter. Inhibition of 5HT synthesis in vivo results in retardation of cell differentiation in those areas innervated by 5HT neurones or in the regions where 5HT is found in high concentrations (Lauder & Krebs, 1978). pCPA administration from day E12 to birth increases 5HT receptor numbers, while 5HT antagonist administration down-regulates receptor numbers in the

newborn rat pup (Whitaker-Azmitia et al, 1987). Although this change in receptor numbers is temporary (they match control levels by postnatal day 15), they are reduced during a period of rapid neuronal change in the young rodent when neuronal maturation, growth and synaptogenesis are occuring and when adult patterns of sexual behaviour are being established. Thus, treatments which alter 5HT concentrations [and possibly receptor numbers] during this period may also cause changes in adult sexual behaviour patterns as a result.

In the second experiment, pups were culled at day 4 postnatally within 3-4 hours of morphine administration and the hypothalamic amine levels were measured. The results from this experiment indicate that, in the short-term, 5HT levels are significantly increased by opiate treatment. Similar findings were recorded in the adult female rat after morphine administration (Gopalan et al, 1989). Whether these effects could be reversed by antagonists or steroid administration is not known. Further studies utilising both NTX and testosterone would be required to establish this.

From these two sets of experiments it has been shown that transient changes in hypothalamic monoamine levels occur as a result of perinatal administration of opiates. Some of the effects are longer-lasting than others and the administration of androgen along with the opiate appears, in some cases, to prolong the effect. While the variability in results makes their interpretation

difficult, it is possible that the short-term changes in 5HT levels in males may be correlated to the alterations recorded in adult sexual behaviour.

Hypothalamic amine levels in 4-day old hamsters treated perinatally with morphine.

Females

Treatment	n	DA	HVA	5HT	5HIAA
control	5	71 ± 23	130 ± 61	159 ± 74	334 ± 105
morphine	6	129 ± 30	127 ± 21	438 ± 66**	393 ± 45
t values		1.76 n.s.	0.05 n.s.	3.30 p<0.01	0.62n.s.

Table 3:9 Hypothalamic amine levels in 4 day female hamsters. Group means ± SEM
** differs from controls p<0.01

Males

Treatment	n	DA	HVA	5HT	5HIAA
control	5	53 ± 17	76 ± 22	112 ± 14	216 ± 35
morphine	6	150 ± 56	120 ± 22	373 ± 48**	369 ± 26**
t values		1.88 n.s.	1.63 n.s.	5.88 p<0.01	4.15 p<0.01

Table 3:10 Hypothalamic amine levels in 4-day male hamsters. Group means ± SEM
** differs from controls p<0.01

Chapter 4

The effect of opiate exposure on two separate sexually dimorphic areas of the CNS

1) The Spinal Nucleus of Bulbocavernosus

The spinal nucleus of bulbocavernosus (SNB) is located in the dorsomedial aspect of the ventral horn of the spinal cord between segments L5 and S1; its motor neurones innervate the perineal muscles bulbocavernosus (BC) and levator ani (LA). These muscles are involved in copulatory reflexes in the male (Breedlove & Arnold, 1980; Sachs, 1982; Hart & Melese d'Hospital, 1983). At birth, the muscles and their motor neurones are present in both sexes, but in the absence of androgens in females during the early postnatal period the muscles atrophy, and the neurone numbers fall from around 300 to less than 50 (Cihak, 1970; Breedlove & Arnold, 1980). There is also a slight decrease in neurone numbers in the male, from 300 to approximately 250 (Breedlove & Arnold, 1980).

The survival of this nucleus and its target muscles is dependent on androgens around the time of birth. This is indicated by the regression of both in neonatally castrated males, but their retention in females treated with testosterone on day two postnatally (Breedlove & Arnold, 1983). Interestingly, in Sprague-Dawley rats testosterone aromatization is not required for the retention of this nucleus and its muscles. Testosterone and DHT have both

been found to be equally effective in preventing the degeneration of the SNB nucleus and its muscles, but they do not survive after oestrogen administration (Breedlove et al, 1982). However in the Albino-Swiss rat (the strain used in this experiment), oestradiol benzoate administered postnatally reduced cell death in females, but did not maintain the muscles (Currie et al, 1990).

Since opiates are known to have an adverse affect on the survival and growth of neurones in culture (Davila-Garcia & Azmitia, 1989), it seems possible that exposure to morphine in the early postnatal period at the time when cell numbers are very labile, could be detrimental. Furthermore, in vivo, opiates suppress LH release which is needed to stimulate androgen production. This effect is a second and much more specific reason why morphine might act on the SNB. Thus rat pups were used to study the effects opiates might have at this time.

Materials and methods

Albino-Swiss male rat pups were given one of the following three morphine treatments at birth:

1) Duromorph 5mg/kg s.c. twice daily from day of birth for 4 days n = 10 males
2) Duromorph 10mg/kg s.c. twice daily from day of birth for 4 days n = 6 males
3) Duromorph 15mg/kg s.c. twice daily from day of birth for 4 days n = 6 males

Controls were given saline n = 7 males, 6 females

Pups were weaned at 21 days and housed with litter mates, 3-4 per cage, with water and rat pellets available ad libitum. At three months of age the animals were culled by anaesthetic overdose and the tissues fixed by cardiac perfusion. 100 ml of Mammalian Ringer solution containing lignocaine was first infused and this was followed by 500ml of Millonigs buffer (pH 7.4) containing 10% formalin. Carcases were pinned out onto cork boards and the testes, epididymides, seminal vesicles, penis and its musculature dissected out and weighed. The spinal cord was then removed and stored in buffered formalin until sectioned.

Pieces of cord were cut such that levels L5 to S1 (the area where the SNB neurones are located) were in the centre of the tissue to be sectioned. The pieces of cord were embedded in 7% agar and sectioned at 100μm on a Vibratome. Sections were stained with 0.033% thionine in 70% alcohol. Thionine stains the cell bodies of neurones dark blue, but the nuclei remain pale with dense blue-black nucleoli. The motor neurones are very large and can easily be identified by their size (see figures 4:1-4:6). The individual cells can be readily counted using a light microscope. Only those cells were counted in which the nucleolus could be seen and no correction was made for cell size.

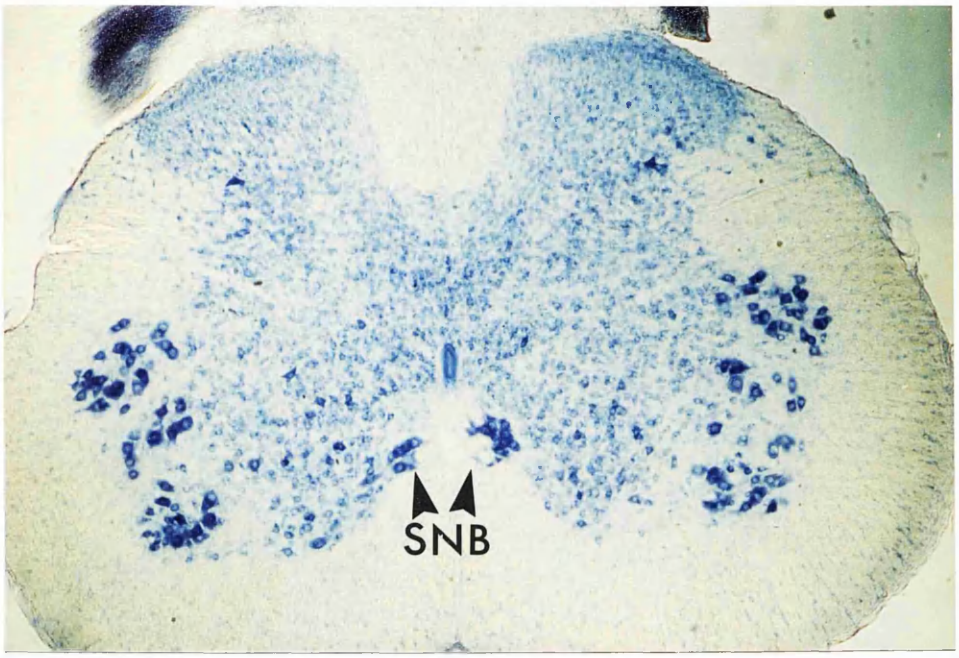


Fig.4:1 Photomicrograph of a section of spinal cord from an intact male Albino Swiss rat at level L45. Stained with Thionine. (x58)

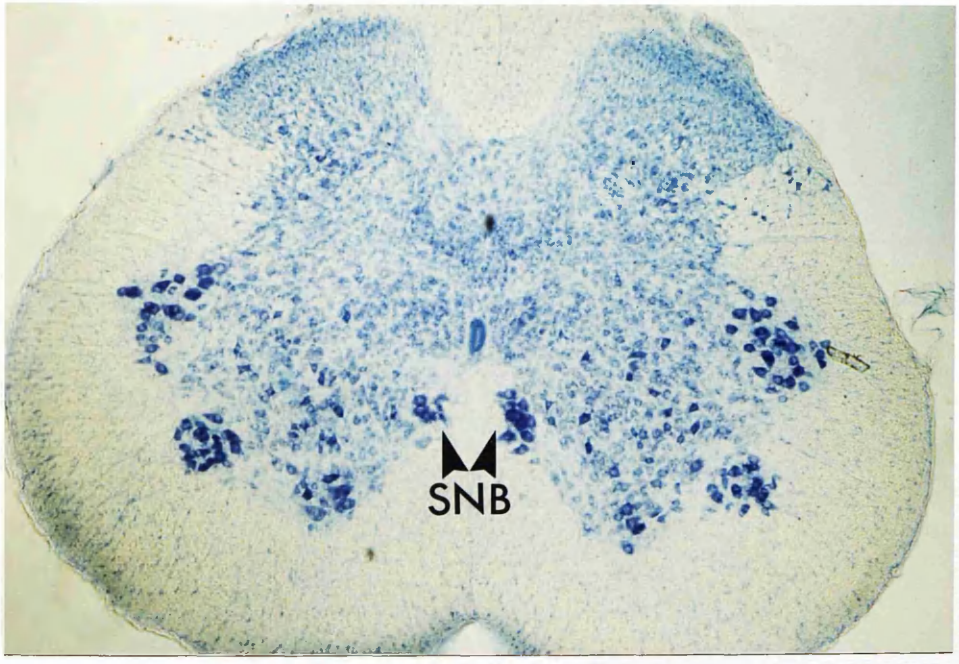


Fig.4:2 Photomicrograph of a section of spinal cord from an intact male Albino Swiss rat at level L5. Stained with Thionine. (x58)

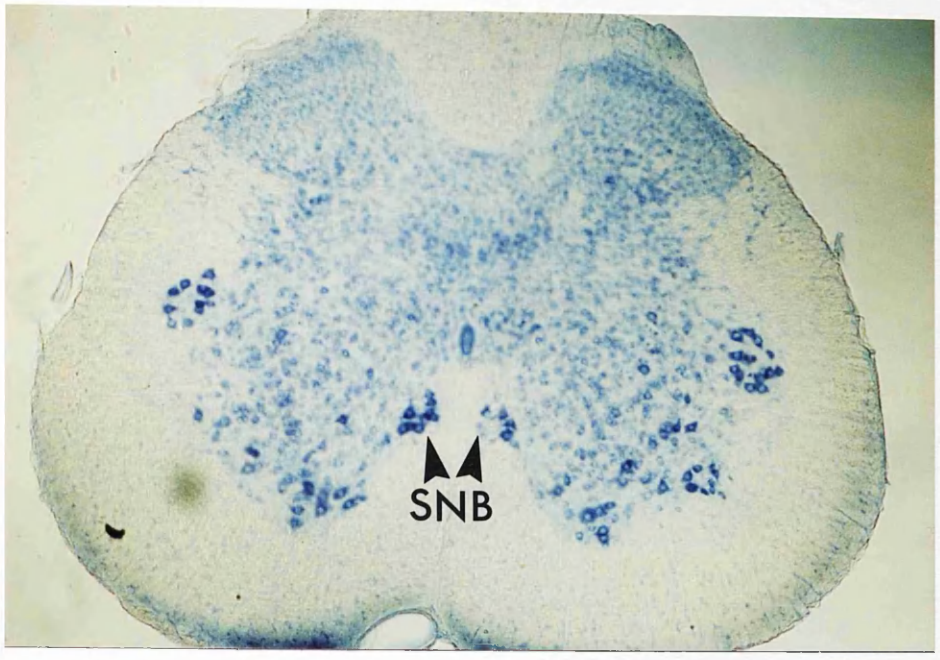


Fig.4:3 Photomicrograph of a section of spinal cord from an intact male Abino Swiss rat at level L6.
Stained with Thionine. (x58)

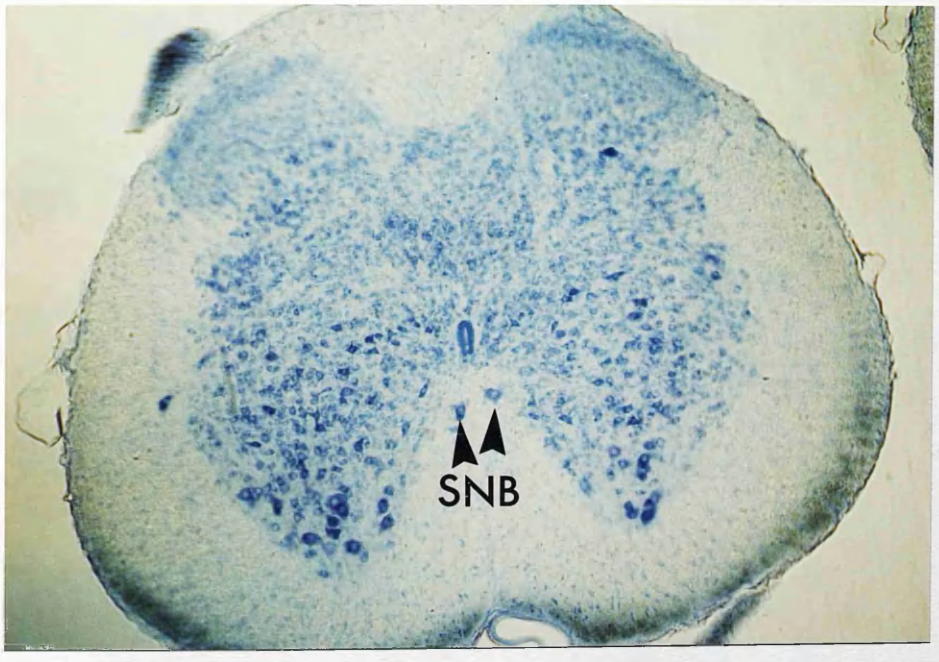
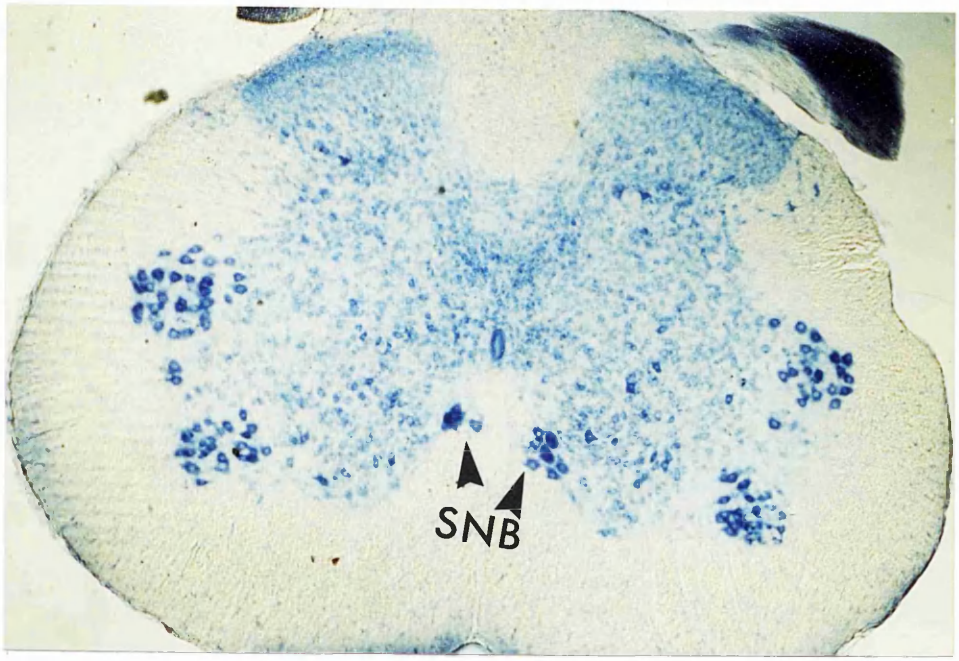
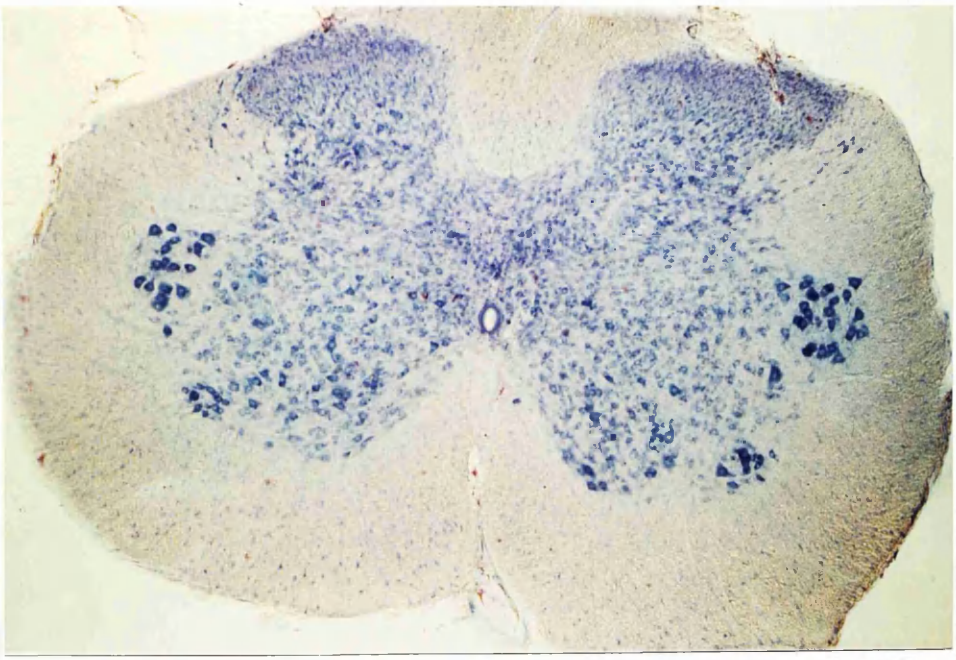


Fig.4:4 Photomicrograph of a section of spinal cord from a male Albino Swiss rat at level S1.
Stained with Thionine. (x58)



g.4:5 Photomicrograph of a section of spinal cord from an intact male Albino Swiss rat at level L5.
Stained with Thionine. (x58)



ig.4:6 Photomicrograph of a section of spinal cord from an intact female Albino Swiss rat at level L5.
Note: no obvious SNB nuclei are present.
Stained with Thionine. (x58)

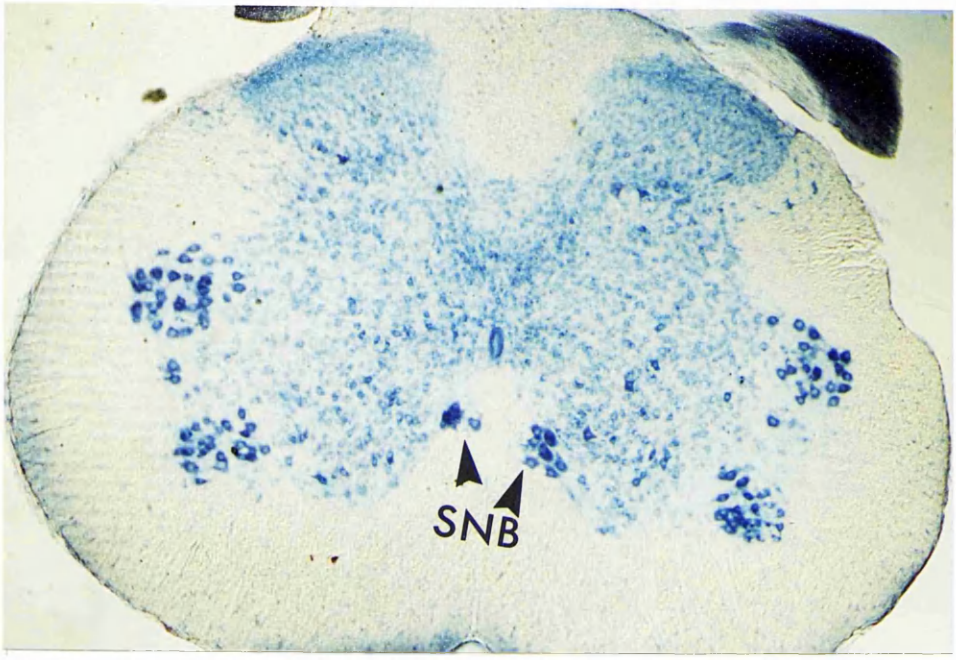


Fig.4:5 Photomicrograph of a section of spinal cord from an intact male Albino Swiss rat at level L5.
Stained with Thionine. (x58)

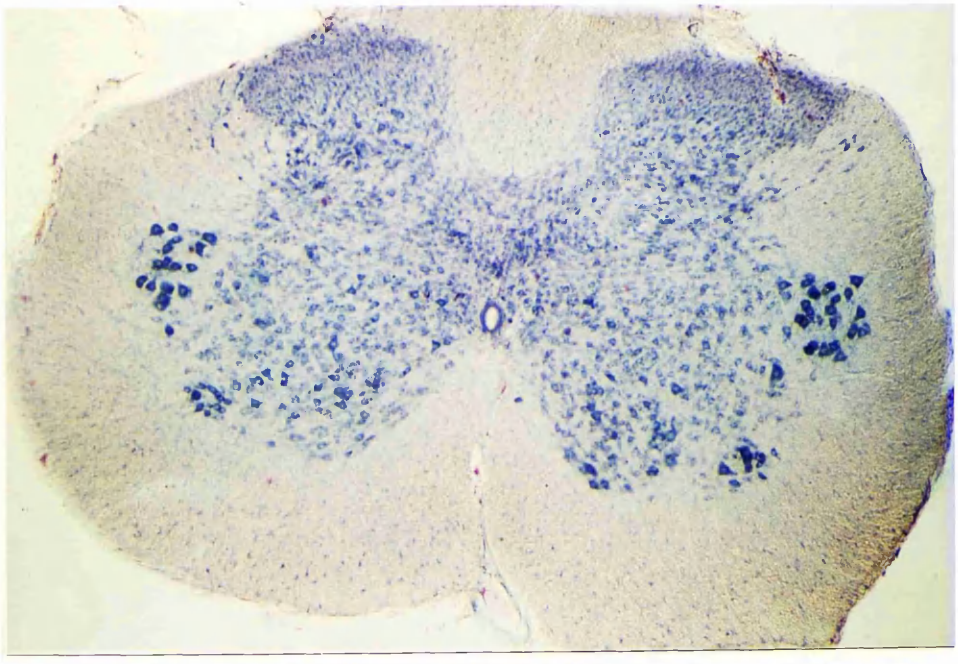


Fig.4:6 Photomicrograph of a section of spinal cord from an intact female Albino Swiss rat at level L5.
Note: no obvious SNB nuclei are present.
Stained with Thionine. (x58)

Results

Control males had 212 ± 8 SNB motor neurones while those in the control females averaged 40 ± 2. Those males in Group 1 averaged 222 ± 7; those in Group 2 201 ± 4 and those in Group 3 averaged 208 ± 6 motor neurones. None of these values differed significantly from those in the controls demonstrating that postnatal opiate exposure did not alter motor neurone numbers. Furthermore there were no significant differences between the weights of the genital organs in any of the treatment groups when compared with those in the controls. (See Table 4:1).

Discussion

This experiment indicates that motor neurone numbers in the SNB are unaffected by postnatal exposure to morphine. In adulthood, morphine suppresses LH output and consequently lowers serum testosterone concentrations (Cicero et al, 1976). If this also occurs in neonates, then it might have been expected that some loss of motor neurones would result from the reduced testosterone concentrations. Although no changes in neurone numbers were found it is possible that, a reduction in testosterone did occur, but was insufficient to reduce the survival of these neurones.

A second consideration that must be taken into account, is the timing of the injections. Treatment did not begin until all pups in a litter were born, and some litters were

born during the night prior to treatment and were therefore not treated until several hours after birth. Analysis of testoterone levels on the day of birth in the rat, mouse and hamster pups by Pang et al, 1979; and by Slob et al, 1980) indicate that testosterone levels rise from the time of birth and reach a peak at around 6 hours post-partum. In experiments on adult rats, testosterone levels begin to fall approximately 1 hour after opiate administration. When these two factors are taken into consideration it may well be the case that in this experiment the morphine was given too late to have any appreciable effect on the androgen levels and consequently on SNB motor neurone numbers. However, castration on the day of birth does result in a decrease in numbers of motor neurones. Therefore, since castration results in a rapid and almost complete loss of androgens, while opiate administration produces a reduction in androgen levels, it would appear from these results, that either levels have not decreased enough to have an effect, or that normal, albeit low levels in the days after treatment are sufficient to prevent cell loss.

In vitro experiments on neurone culture have indicated that opiates have an inhibitory action on neurite growth (Davila-Garcia & Azmitia, 1989). However, by the time of birth in the rat, neurogenesis of the SNB motor neurones is complete (Breedlove, Jordan & Arnold, 1983) and androgens are only required to prevent their regression due to

controlled cell death (Breedlove & Arnold, 1983). It is thus not surprising, that morphine when administered postnatally has no direct affect on the neurone numbers. It might be of interest in future studies to investigate the effects of prenatally - administered morphine on SNB motor neurone numbers, particularly by giving the drug from the early stages of neurogenesis. If such treatments did affect the numbers of SNB cells, one would need to determine by which means did it do so;

a) Did it inhibit neurogenesis? or

b) Did it reduce androgens such that greater then normal cell death occured in males. Using females as controls may help to distinguish between the two effects, since the SNB motor neurones develop similarly in both sexes, and only at the late stages of development does sexual differentiation occur, in that there is greater cell death in females due to the absence of androgens. If the treatment affects neurogenesis then, by culling the animals at the stage prior to differentiation, one should see a reduction in SNB neurone numbers at this stage in both sexes. One unexpected finding in this experiment was that the BC muscle weights in the 5mg/kg morphine-treated males were significantly higher than those in the other experimental groups when analysed using Least Significant Differences tests in analysis of variance. This observation suggests that at this dosage opiates have enhanced the development of the muscle or had the least detrimental effect. The group treated with 5mg/kg of morphine also had the highest

number of SNB motorneurons although they did not differ significantly from other groups. The BC is the target muscle for the SNB thus it may be as a result of the increased muscle mass that there is a concurrent rise in SNB motoneurone numbers.

In conclusion, postnatal administration of morphine at a dose similar to and higher than one which has significantly altered adult sexual behaviour in another rodent species (see Chapter 2) had no effect on the number of motor neurones of the SNB in the Albino-Swiss rat.

Comparison of Motor neurone numbers in Spinal nucleus of bulbocavernosus, muscle and organ weights in female, male and neonatally morphine-treated males.

Rats

treatment	n	testes weight (g)	BC weight (g)	LA weight (g)	IC weight (g)	no. of SNB motor neurones
control females	6	-------	-------	-------	-------	40±2 **
control males	7	2.5±0.04	0.70±0.0	0.34±0.02	0.25±0.01	212±7
5mg/kg Durom	10	2.6±0.04	0.8±0.01 §	0.35±0.01	0.23±0.01	222±7
10mg/kg Durom	4	2.5±0.07	0.6±0.02	0.33±0.00	0.23±0.01	201±4
15mg/kg Durom	3	2.5±0.03	0.6±0.07	0.32±0.02	0.20±0.02	208±6
F value		0.51 n.s.	12.9 p<0.01	1.11 n.s.	1.44 n.s.	141.8 p<0.01

Table 4:1 Testes and muscle weights of adult rats treated postnatally with morphine.
Group means + SEM.

** differs from all other groups $p<0.01$

§ differs from 10mg/kg and 15mg/kg-treated groups $p<0.05$

2) The sexually Dimorphic Nucleus of the Preoptic Area.

In 1978 Gorski and colleagues described a region of the rat preoptic area so strikingly different in size between the sexes, that when the brain is sectioned the difference is visible with the naked eye. This region, termed the sexually dimorphic nucleus of the preoptic area (SDN-POA), is many times larger in males than in females (see figs 4: & 4:) and has also been identified in the guinea-pig, mouse and hamster, (Byne & Bleier,1987; Byne et al, 1987) Bleier et al, 1982), the ferret, (Tobet et al, 1986) and in man (Swaab & Hofman, 1988). Its size is determined by the hormone mileu during the perinatal period. Gorski et al (1978) reported that male castration on the day of birth reduced the volume of the adult male SDN-POA by approximately half, while a single injection of (TP) to female pups during the early postnatal period doubled its size; neither treatment wholly sex-reversed the volume of the nucleus. Jacobson et al (1981) produced further evidence that the SDN-POA volume is androgen-dependent by showing that TP given to male rat pups on day 2 after birth reversed the effects of castration on day 1. Döhler et al (1982) reported that TP administered throughout the last trimester of gestation and up to day 10 postnatally completely sex-reversed the SDN-POA volume in females. The same authors stated that

1) both TP and diethylstilboestrol (DES) were equally effective in sex-reversing the size of the SDN (Döhler

et al,1984a) and

2) that a potent oestrogen antagonist (Tamoxifen) given postnatally, significantly reduced the volume of the SDN-POA in both sexes (Döhler et al, 1984a). This indicates that oestrogen plays an active role in both the masculinization of the SDN-POA in males and in the development of this nucleus in females.

In the rat, the SDN-POA consists of 3 major subdivisions. The cell dense central (MPNc) and medial (MPNm) subdivisions are larger in the male, while the lateral subdivision (MPNl), with few cells, is the major division of the female nucleus. Neurones containing a variety of neurotransmitters have fibres passing through the nucleus or have their cell bodies located within it. These neurotransmitters are not homogeneously distributed within the nucleus (Simerly et al, 1986) and include serotonin (5HT) oxytocin (OXY) and leucine-enkephalin (L-ENK). Simerly et al (1984) investigated the 5HT input to the SDN-POA and reported a sexual dimorphism in serotonin distribution within the nucleus, there being significantly more 5HT fibres per unit area in females than in males. Hypothalamic 5HT levels are reported to be sexually dimorphic on day 12 postnatally in the rat (Ladosky & Gaziri, 1970), and in the hamster (Johnston et al, 1990). Experimental reductions in 5HT levels during the perinatal period sex reversed the volume of the SDN-POA in female rat pups (Handa et al, 1986), but it is not clear if the

effects are lasting (Hines & Gorski, 1986). Postnatal reduction of 5HT in hamsters increases the amount of feminine sexual behaviour displayed in adulthood by males while reducing it in females (Johnston et al, 1990). It has been shown that 5HT may be involved in directing the genesis and/or migration of neurones which receive a 5HT innervation in adulthood. (Lauder & Krebs, 1976; Lauder & Krebs, 1978; Lauder et al, 1982).

Hammer (1984) reported that the SDN-POA in the rat contains denser opiate binding sites in females than in males. Since opiates are known to inhibit neural outgrowth in vitro (Davila-Garcia & Azmitia, 1989) and in vivo (Hammer et al, 1989) this difference in receptor density may be involved during the perinatal period in the control of development. Furthermore as opiates have also been reported to alter levels of 5HT in adulthood (Gopalan et al, 1989), it is therefore possible that exposure to exogenous opiates during the period of sexual differentiation may be detrimental to SDN-POA masculinization. To investigate this possibility rat pups were treated postnatally with morphine and their brains examined for morphological changes in adulthood.

Materials and Methods.

Male rat pups were treated postnatally from the day of birth as follows:

1) Saline-treated controls (n=3)
2) 10mg/kg Duromorph subcutaneously twice daily from day

of birth for 4 days (n=5)

3) 15mg/kg Duromorph s.c. twice daily from day of birth for 4 days (n=5).

At 21 days animals were weaned and housed in groups of 2-3 littermates of the same sex which had received the same treatment.

At 3 months of age each animal was culled by a lethal dose of sodium pentobarbitone (Sagatal) and perfused intracardially with mammalian Ringer solution with added Lignocaine, followed by 10% buffered formalin. The brain was rapidly removed and fixed for a minimum of 2 weeks in 10% formalin before being processed. Following fixation brains were embedded in agar and sectioned in the DeGroot (1959) plane on a Vibratome. Sections were 100μm thick and were stained with thionine (0.2% in 70% alcohol pH 4.5), dehydrated through an alcohol series and mounted on glass slides. Each section in which the SDN-POA was identified, was photographed using an Ultraphot microscope and camera, at a magnification of x 32. The area of the SDN-POA was outlined on the photograph and measured using a MOP AMO2 planimeter.

The volume was calculated as follows:

$$\frac{\text{Area of nucleus}}{\text{Magnification}^2} \times \text{thickness of section}$$

In the first and last section in each group, the SDN-POA was assumed to occupy only half the thickness of the sections.

The volumes of the SDN-POA in each group were analysed

statistically using one-way analysis of variance, followed by intergroup comparisons based on Confidence Intervals; a difference of $p<0.05$ was taken as significant.

Results

Control males had an average SDN-POA volume of 0.0339 ± 0.003mm^3 while males treated with 10mg/kg morphine had a SDN-POA volume which averaged 0.0525 ± 0.004mm^3 and those treated with 15mg/kg had a SDN-POA volume that averaged 0.0442 ±0.007mm^3.

One way ANOVA gave an F value of 1.08 (not significant).

Discussion

The results obtained indicate that postnatal exposure to exogenous opiates had no significant effect on the size of the SDN-POA. This is interesting since there are two reasons why one might expect an effect to occur.

1) Since opiates are known to lower the levels of testicular androgens by suppressing LH secretion (Singh et al, 1980; Genazzi & Petraglia, 1989), and castration on day of birth reduces the volume of the SDN-POA, it might have been expected that opiate exposure during this period would reduce the volume of the SDN-POA.

In male rats a surge of testosterone, reaching a peak approximately 6 hours postnatally and falling again to basal levels within 24hrs of birth, is believed to contribute to the masculinization of the SDN-POA. However,

Handa et al, (1985) reported that there was no difference in volume of the SDN between pups castrated at 0, 6 or 24 hrs postnatally. All three groups showed a similar reduction in volume, indicating that the postnatal surge of testosterone is not critical to the differentiation of this nucleus. Therefore, if we assume that the treatment regime described in this experiment does reduce or delay this testosterone surge, it might still not affect the SDN-POA volume.

2) Conversely, if opiates inhibit growth and synaptogenesis, then again we might expect to see an effect on SDN-POA volume. However, neither dose used here appears to have any influence on the SDN-POA, although it is possible that the timing of treatment was later than that around when the drug would have any effect i.e. the drug may have been given after the postnatal surge of androgen had been produced. Investigations into the effect of prenatal stress on sexual differentiation of the rodent CNS, have indicated that stress induces a variety of changes in the animals' behaviour in adulthood (Ward, 1983; Herrenkohl, 1983). In general the changes reflect feminization of behaviour. The preoptic area is known to be involved in the control of sexual behaviour and lesions to this area increase the amount of lordosis displayed by males (Hennessey et al, 1986). This has led to the proposal that the additional volume in males may be inhibiting feminine behaviour, since when the SDN-POA is

ablated this inhibition is removed. Prenatal stress also results in:-

1) a reduction in mounting and an increase in feminine sexual behaviour in male rats (Ward, 1983), and
2) a reduction in the volume of the SDN-POA (Anderson et al,; Anderson et al, 1986).

The latter authors were also able to demonstrate a positive correlation between the volume of the SDN and the amount of mounting displayed by male rats.

Prenatal stress upsets the timing of a particular androgen surge in male rat fetuses. There is a dramatic rise in testosterone on day 18 which begins to fall on day 19 and has reached basal levels by day 20 (Weisz & Ward, 1980). Prenatal stress advances the timing of this rise to day 16, and by embryonic day 18 levels are significantly lower than in control fetuses (Ward & Weisz, 1980). A similar pattern occurs in response to prenatal exposure to morphine (Ward, Orth & Weisz, 1983). The effects of both stress and morphine exposure on testosterone levels during the fetal period can be prevented by prior administration of NTX (Ward, Orth & Weisz, 1983). The above mentioned investigations have shown that

1) prenatal exposure to morphine mimics the effects of prenatal stress on adult sexual behaviour. The effects of both can be prevented by NTX treatment.
2) prenatal stress reduces the volume of the SDN-POA.

It would therefore be interesting to look at the effects of prenatal (rather than postnatal as in this

experiment) morphine on the volume of the SDN-POA. It seems quite feasible that opiate exposure during the last trimester of pregnancy might well reduce the SDN-POA volume.

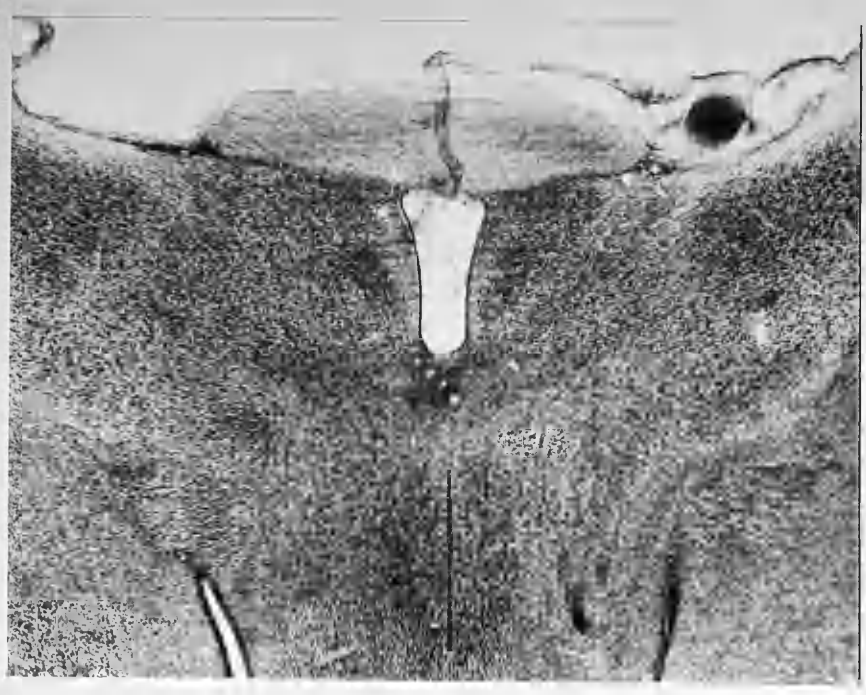
Fig. 4:7 Serial photomicrographs through the medial Preoptic area of a normal male Albino Swiss rat. The sections run in rostral-caudal sequence from top to bottom. This shows the extent of the male SDN-POA (**red asterisk**). Various other structures labelled serve as topographical markers and have been indicated as they emerge.

This represents an example of the photomicrograph series of serial sections from which the volume of the SDN-POA was calculated.

Mag. x 32

Och - Optic chiasm
AC - Anterior commisure
SCh - Suprachiasmatic nucleus
SO - Supraoptic nucleus
MePO - Median preoptic nucleus

1



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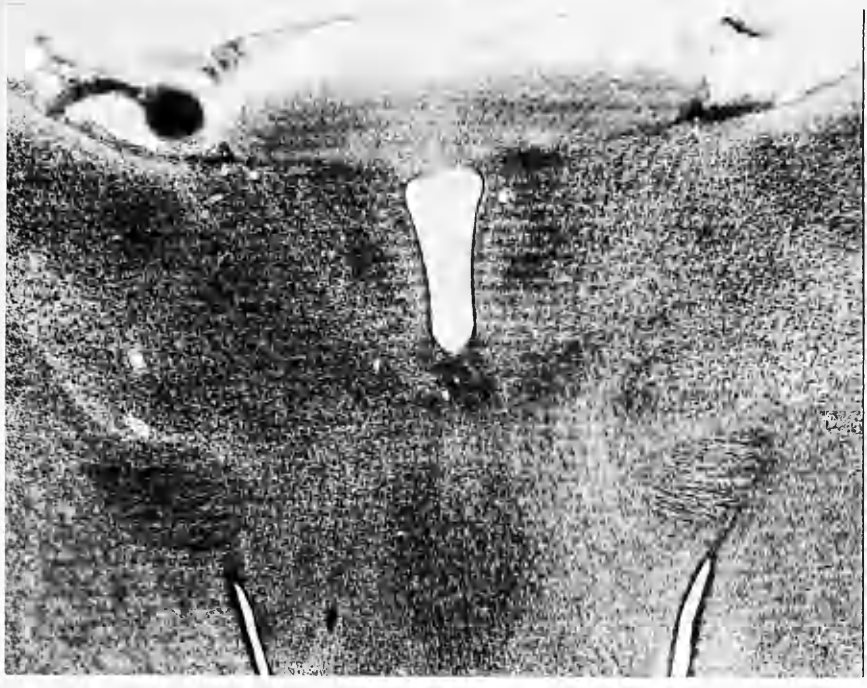

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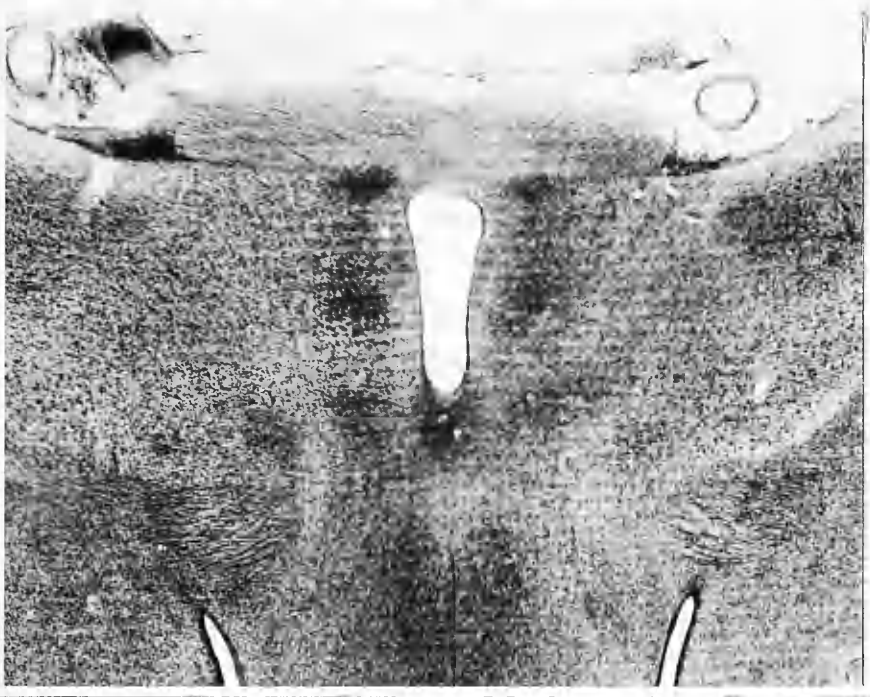

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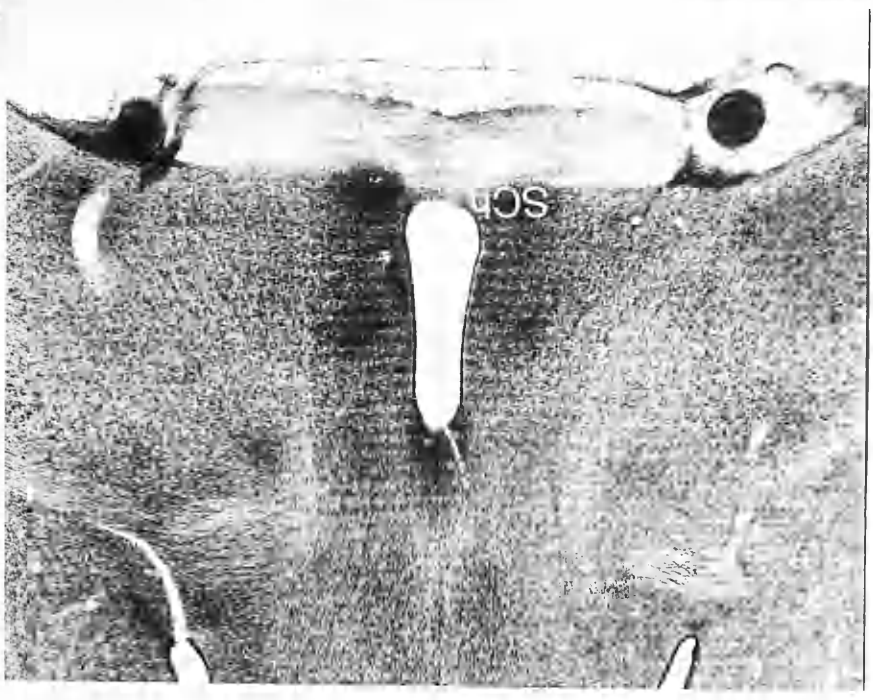

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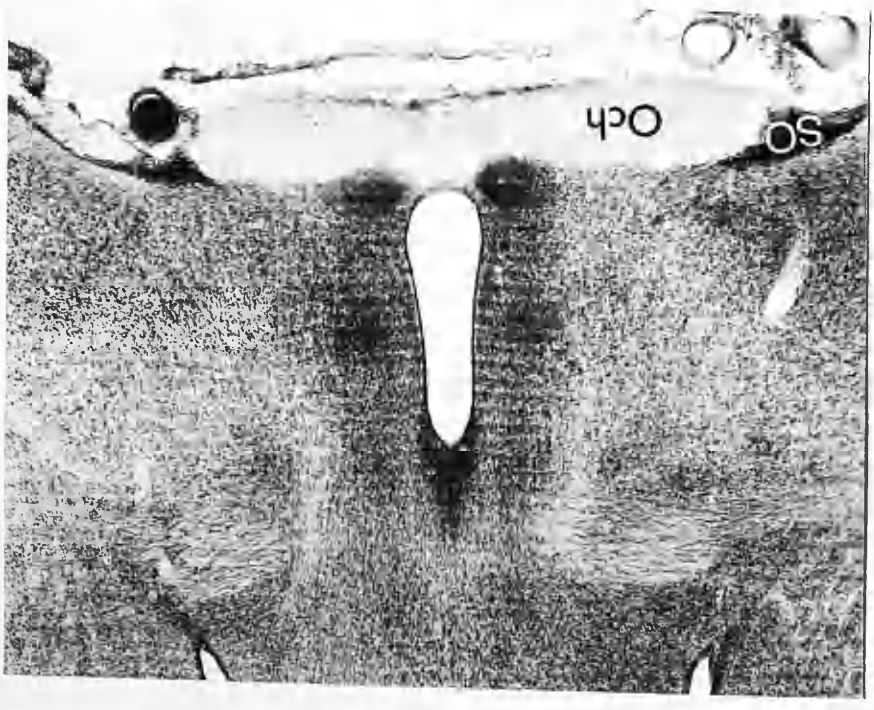

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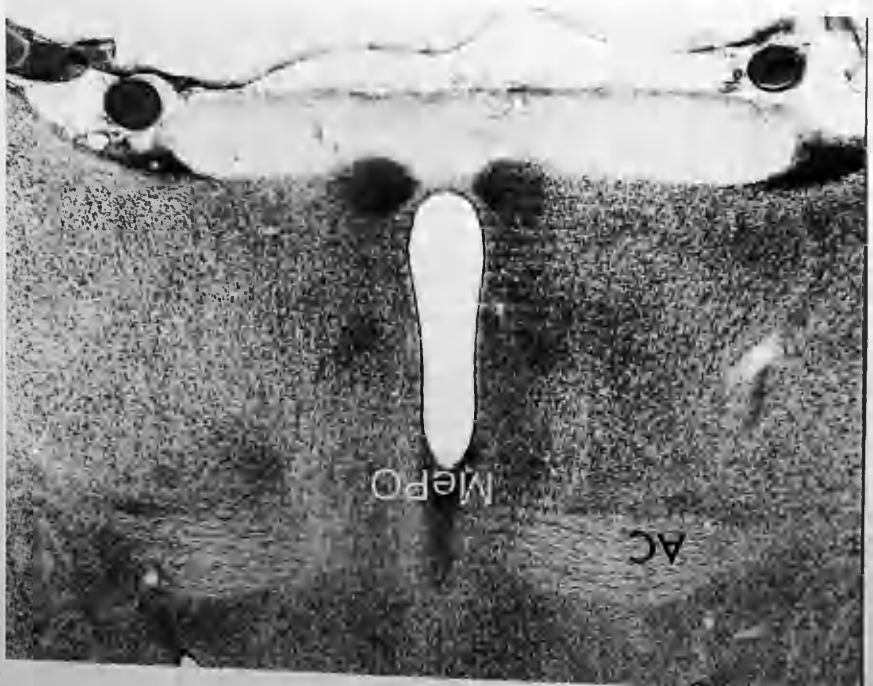
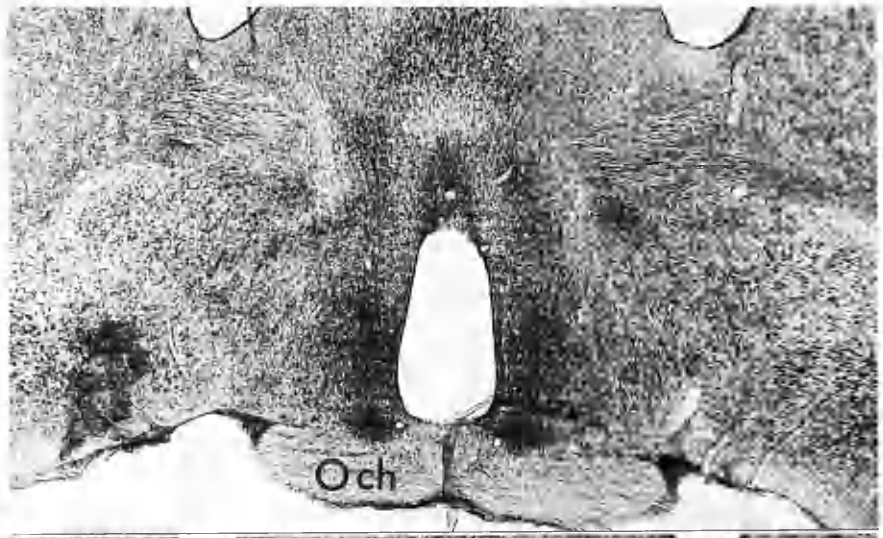


Fig. 4:8 Serial photomicrographs through the MPOA of a normal female Albino Swiss rat. The sections run in rostral-caudal sequence from top to bottom. This shows the limited extent of the female SDN-POA (**red asterisk**). Various other structures labelled serve as topographical markers and have been indicated as they emerge.
This should be directly compared with Fig. 4:6.
Mag. x 32

Och - Optic chiasm
AC - Anterior commisure
Sch - Suprachiasmatic nucleus
SO - Supraoptic nucleus.
MePO - Median preoptic nucleus



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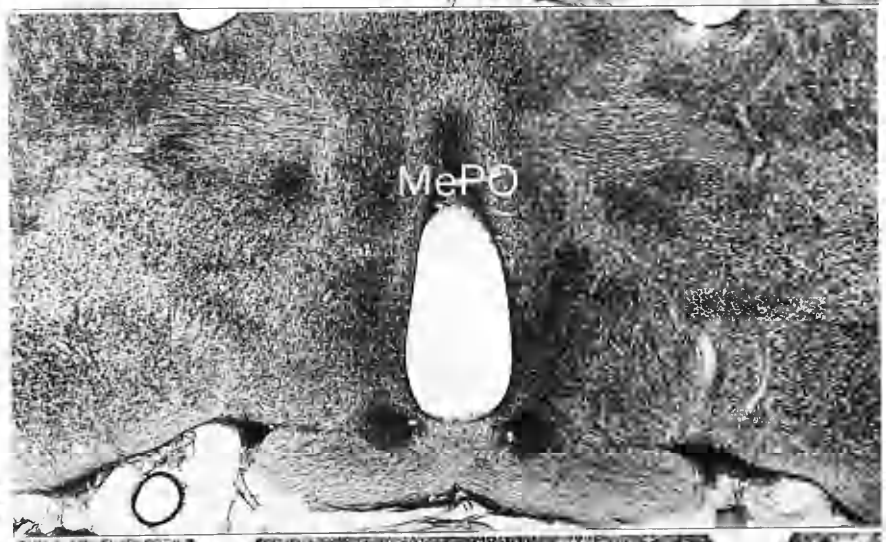

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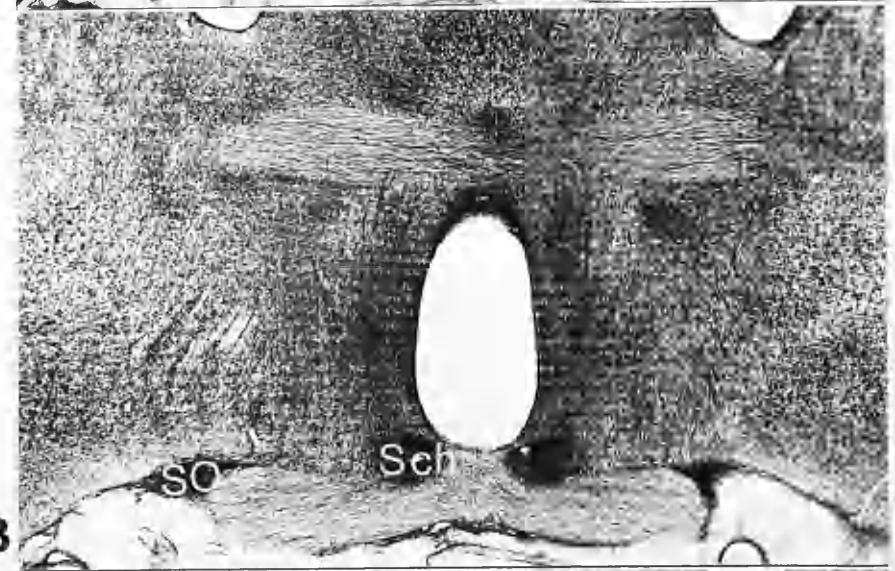

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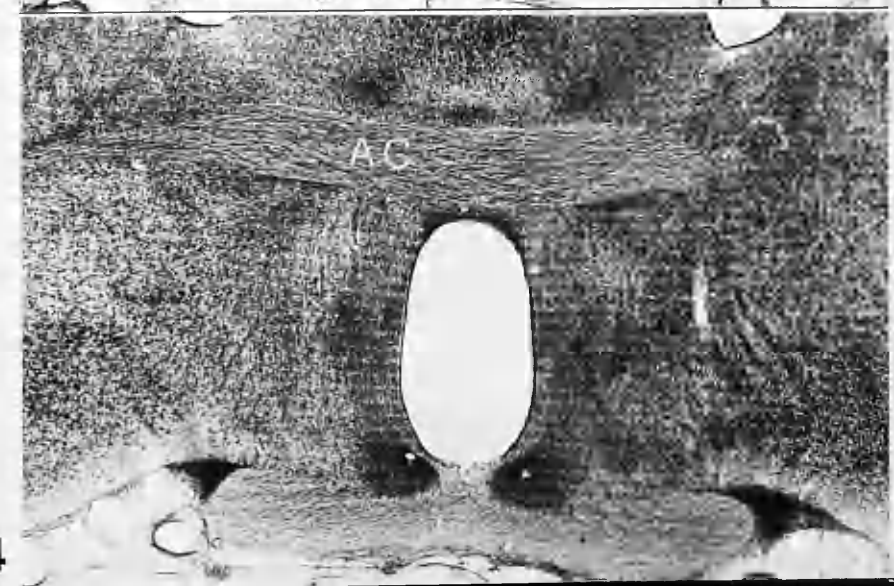

4

Chapter 5

General Discussion

Perinatal exposure to opiates affects different aspects of behaviour.

Opiate administration during the perinatal period has been been reported to affect behaviour in different ways and in varying degrees. Grove et al (1979) found that exposure to methadone prenatally, postnatally or both resulted in pups which were hypoactive in open field tests at postnatal day 28, but were hyperactive and less emotional in the same test as adults. Zagon et al (1979) reported similar findings in rats treated prenatally with methadone. In contrast, Davis & Lin (1972) noted that rats were hyperactive as pups and as adults in open-field tests after prenatal exposure to morphine.

In competition with controls for access to drinking water, rats exposed to methadone pre- and postnatally spent less time at the water-bottle; this was interpreted as reduced social dominance in these animals, (Thompson & Zagon, 1983).

Rats exposed prenatally to methadone were hypersensitive to aversive stimuli and acquired avoidance and escape behaviours more rapidly than controls (Rech et al, 1980). Conversely pre- and postnatal exposure to morphine at several doses did not affect conditioned learning in rats when tested as adults (Banerjee, 1975). Despite the apparent contradictions in results (which may

be due to the type of drug, method of treatment and/or dosage), it seems likely that opiate exposure during the developmental period has adverse effects on the behaviour patterns of rodents later in life.

Perinatal opiates and adult sexual behaviour.

Adult sexual behaviour is also affected by opiate treatment perinatally. Meyerson (1982) treated male rats with the endogenous opioid β-endorphin on days 1-5 postnatally and noted a reduction in mounting behaviour as adults. Ward et al (1983) also found that prenatal morphine reduced masculine sexual behaviour in males rats and increased their ability to display lordosis. Vathy et al (1983) reported a partial inhibition of feminine sexual behaviour in female rats exposed prenatally to morphine.

Patterns of sexual behaviour are established during the perinatal period in rodents and are dependent on testicular androgens for masculinization and defeminization of behaviour in males. In the rat, the critical period for differentiation of sexual behaviour occupies the late prenatal and early postnatal period whereas, in the hamster, the critical period was initially believed to be wholly postnatal (Nucci & Beach,1971). However, treatment with antiandrogens during gestation reduces the males ability to display masculine sexual behaviour as adults (Swanson, 1971). Studies on androgen levels in the fetal rat have shown that the elimination of a prenatal surge of testosterone on days E18.5-19.5 results in males which show

increased feminine patterns of behaviour and decreased mounting (Ward & Weisz, 1980). A more recent investigation of plasma androgen levels in the fetal hamster at E14 & E15 (gestation period 16 days) revealed a significantly higher level of testosterone in the plasma of males compared with females (Vomachka & Lisk, 1986). Fetal androgen levels in the male hamster were found to be 10-fold lower than in the fetal male rat at an equivalent stage, and the authors have suggested that sexual behavioural differences between these two species as adults may be related to this. Thus, male rats, which are exposed to relatively high levels of androgens prenatally, show masculine sexual behaviour as adults but do not display lordosis. Female rats and male hamsters can show either lordosis or mounting depending on the hormone priming given to them in adulthood; these animals are exposed to intermediate levels of androgens prenatally. Female hamsters, which seldom display masculine behaviour even after priming, have very low perinatal plasma androgen levels. If this is so, then reduction of androgen levels during the perinatal period, or a change in the ability of the CNS to respond to them, might result in a shift of adult sexual behaviour capacities to that of the opposite sex. In the present investigation, just such a shift occured in male hamsters exposed both before and after birth to morphine. This might not be considered surprising given that opiates suppress LH and, therefore, androgens (Cicero, 1980).

However, although the amount of feminine sexual behaviour was significantly increased, masculine sexual behaviour was unaffected by opiate treatment although as expected the changes could be reversed by androgens and naltrexone. A second paradox is that, in male hamsters chronically exposed to morphine, both feminine and masculine sexual behaviour were significantly enhanced. If the opiate action were mediated via androgen reduction, one would have expected this group to show more feminine and less masculine behaviour. However it is possible that long-term exposure to opiates produces less effect than short-term exposure or that treated males are more sensitive to androgens during the critical period.

How then do opiates mediate this reported change in behaviour?

Having shown that morphine does alter sexual behaviour, attempts were made to elucidate whether the drug mediated the changes through reduced androgen levels or by some other mechanism. Reports on plasma androgen levels in the golden hamster indicate that levels in male pups are significantly higher than in females on the day of birth and for up 5 days postnatally (Pang & Tang, 1984; Vomachka & Greenwald, 1979). Therefore androgen levels in male pups treated postnatally with morphine were compared to those of untreated 2 day-old males. No significant difference in testosterone levels was noted. This suggests that the alterations in sexual behaviour in the males were unlikely

to have been caused by postnatal androgen reduction. However, since behavioural changes were more marked in males to which opiate exposure had been extended into the prenatal period, the possibility that androgen levels may have been reduced during this prolonged exposure cannot be fully excluded. Further work is required to determine if this is so. In addition, since perinatal levels of androgens in the golden hamster are much lower than in the rat (Vomachka & List, 1986) it may be that subtle changes in concentrations go undetected in this species, whereas relatively small changes in rat testosterone levels would be quite obvious. In a further attempt to determine whether the opiate-induced changes in behaviour were mediated via reductions in androgens, it was decided to look at two different androgen-dependent structural dimorphisms in the CNS. In this study a different species was employed, the Albino-Swiss rat, since in this species the structures in question, the SDN-POA and the SNB, have received considerable attention and require perinatal exposure to testosterone to be masculinized. The SDN-POA is many times greater in volume in the male than in the female (Gorski et al, 1978). Postnatal castration reduces the volume and androgenisation of females on day 1 postnatally increases it, but neither treatment wholly sex-reverses it. Oestrogen is equally effective in increasing the size of the SDN-POA in females. However extending the treatment time into the prenatal period does masculinize

the female SDN-POA volume completely, while treatment with the antioestrogen tamoxifen will feminize the male SDN-POA (Dohler et al, 1983). If opiates reduce postnatal androgens then one would expect to see a reduction in SDN-POA volume in opiate-treated males. In this investigation no reduction in volume was recorded. As with hamster pups (see above) a preliminary investigation into androgen levels in opiate-treated rats at 2 days postnatally did not show any difference between morphine-treated pups and controls.

The second morphological structure, the SNB is dependent on postnatal androgens for neuronal survival. SNB motor neurone numbers in both males and females rats are similar before birth. However, the presence of testicular androgens in the postnatal male inhibits the neuronal cell death which occurs in females, resulting in a nucleus with some 250 neurones in the male and approximately 40 of reduced size in the female (Breedlove & Arnold, 1981). Spinal cords from rats treated postnatally with different doses of morphine were sectioned, stained and the numbers of SNB motor neurones recorded. There was no significant difference in motor neurone numbers between the treatment groups and control males.

These two experiments give further evidence that the alterations in adult sexual behaviour reported in hamsters treated perinatally with opiates were unlikely to have been caused by a reduction in androgen levels during this period.

The monoamines arise very early on in development in

the mammal (Coyle & Henry, 1973; Schlumpf et al, 1980; Lauder et al, 1982) and at this stage are thought to be involved in trophic events in development, possibly acting as a signal for differentiation and migration of neurones (see reviews Lanier et al, 1976; McMahon, 1974). In this manner, they may play an active role in the establishment of behaviour patterns by regulating migration and synaptogenesis of neurones. However opiates have been shown to inhibit neuronal growth when administered prenatally, which could result in the differentiation signal occuring too late or in the wrong place. Therefore, the hypothalamic levels of neurotransmitters on day 4 and day 12 were measured. Unfortunately it was not possible to determine concentrations of NA levels in this study but DA, 5HT and their metabolites HVA and 5HIAA respectively were analysed. Female hypothalamic DA and HVA levels were not altered by opiate treatment. Some changes were noted when testosterone or naltrexone was given with the opiate but it is difficult to relate these changes to alterations in behaviour since similar treatments over different time periods had opposite effects on transmitter concentrations. Similar results were recorded in the males.

Is 5HT the key?

On day 12 postnatally, the hypothalamic concentrations of 5HT are significantly higher in female rats (Ladosky & Gaziri, 1970; Siddiqui et al, 1989) and

hamsters (Johnston et al, 1990) than in males. Manipulations of 5HT levels during the neonatal period results in altered behavioural patterns in adulthood (Wilson et al, 1986; Johnston et al, 1990). Furthermore, Lauder & Krebs (1978) have shown that changes in 5HT levels during development cause alterations in 5HT receptor numbers which, although transient, are sufficiently long lasting to encompass the critical period of sexual differentiation. Changes in 5HT concentrations can also be induced by perinatal androgen manipulation; castration on postnatal day 1 increases day 12 5HT levels to those of females, while androgenization of females postnatally lowers them (Ladosky & Gaziri, 1970).

The results from this study were very variable, but it was evident that 5HT was particularly susceptible to alteration over this period. The only morphine-treated group of females to show a reduction in feminine sexual behaviour were those treated prenatally with morphine; this group also showed a significant reduction in 5HT levels on day 12. Females treated postnatally with both morphine and testosterone also had a marked reduction in both feminine sexual behaviour and 5HT concentrations on day 12. Postnatal androgen treatment of female rat pups resulted in similar changes in amine levels (Giulian et al, 1973) and behaviour (Whalen & Edwards, 1967).

Prenatal morphine + testosterone treatment had the most significant effect on sexual behaviour in males and this group also had significantly elevated levels of 5HT on

day 12. Morphine treatment pre and postnatally raised 5HT levels and increased feminine sexual behaviour in males. Short-term effects of postnatal opiate exposure were most pronounced in that levels of 5HT were significantly increased in both sexes, although this appears to be transient since by day 12 these had returned to normal in many cases. However, these immediate alterations in 5HT concentrations may be sufficient at this time to interfere with the neural substrate which is involved in the defeminization process and could lead to males treated postnatally with morphine showing an increase in feminine sexual behaviour. It would appear therefore that 5HT is involved in the defeminization process in the golden hamster and that opiate administration mediates some of the altered sexual behaviour patterns reported in this study via changes in the serotonergic neural pathways. However, since not all of the changes in adult sexual behaviour were reflected in changes in monoamine levels on day 12 postnatally, it cannot be assumed that these were <u>all</u> mediated via this system, nor indeed can it be assumed that those treatments which did alter both amines and behaviour were only mediated via this system. It is possible that the alterations in 5HT concentrations are merely consequential to actions occuring elsewhere as a result of opiate adminstration rather than being part of the process of change.

In which other ways could morphine have mediated the changes reported here?

The endogenous opioid peptides and their receptors become apparent relatively early in the developing CNS, and have been detected in the fetal rat brain by days E12-E14 (Kent et al, 1982; Khachaturian et al, 1983). This suggests that the endogenous opioids may play a regulatory function in neuronal development. The finding that chronic treatment with the opiate antagonist naltrexone results in neonatal rats which have larger brains than controls (Zagon & McLaughlin,1983) and increased dendritic arborization (Hauser et al, 1987) lends support to this hypothesis. Investigations into the ontogeny of opiate receptor development have revealed regional and temporal development of the different receptor subtypes (Tavani et al, 1985; Petrillo et al, 1987). Alterations to the distribution and timing of receptor differentiation may therefore result in changes in the neural substrates which they were destined to control. Opiate administration during the perinatal period has been shown to have many adverse effects on neuronal development, including inhibition of DNA synthesis, (Vértes et al, 1982; Kornblum et al, 1987) reduced neuronal density and dendritic growth (Hammer et al, 1989) and defective synaptic development (Slotkin et al, 1979). Also of importance is the finding that perinatal morphine administration down-regulates the number of μ receptors and pattern of met-enkephalin development (Tempel et al, 1988; Di Guilio et al, 1988). In this

regard it is hardly surprising that patterns of sexual behaviour which are being laid down during this time were altered. However this does not tell us how the changes were made but merely suggests possible mechanisms of action. Of particular interest is the report by Slotkin et al (1979) that the development and synaptogenesis of monoaminergic systems were defective after perinatal methadone treatment; in the present investigation it was found that 5HT levels were altered by morphine in many cases.

In conclusion, opiate administration during the perinatal period markedly reduces defeminization in the male golden hamster. The changes recorded do not appear to be mediated via androgen reductions, but may be mediated via alterations to serotonergic neural pathways which have been shown to be involved in sexual differentiation of behaviour. However since the CNS is still undergoing considerable growth, maturation and synaptogenesis during this period, opiate-induced alterations to these processes cannot be ruled out.

What next?

During this investigation it was found that androgen administration with morphine prenatally resulted in males which showed significantly enhanced feminine and masculine sexual behaviour suggesting that defeminization may begin earlier than has been reported. Further studies giving

androgens only may help to confirm this. In addition analysis of LH and androgen levels on the day of birth in males treated prenatally with opiates would be useful to determine whether these are suppressed by morphine in this species.

The possibility that prenatal opiates might alter the volume of the SDN-POA and motor neurone numbers in the SNB requires investigation. This should be coupled to androgen analysis to help to elucidate by which mechanism the changes, (if any) are mediated. This is particularly important since opiates have been shown to retard neuronal growth and may delay migration of the neurones into the two nuclei.

Abstract

The primary purpose of this investigation was to study the effects that perinatal exposure to opiates might have on adult sexual behaviour in rodents (using the golden hamster as a model).

Subsequent aims were to elucidate the mechanisms by which such actions might occur. These included the effects of perinatal opiate exposure on

1) Hypothalamic biogenic amine levels during the perinatal period.
2) Sexually dimorphic structures in the CNS of the Albino-Swiss rat, especially
   a) the spinal nucleus of bulbocavernosus
   b) the sexually dimorphic nucleus of the preoptic area
3) Plasma testosterone levels during the neonatal period in male hamster.

Opiate treatment had little effect on the females' ability to display sexual behaviour. By contrast, perinatal opiate administration resulted in alterations to the adult sexual behaviour of male golden hamsters. Masculine sexual behaviour was unaffected but the ability to display feminine sexual behaviour was markedly increased after pre + postnatal or postnatal treatment with morphine. These changes in behaviour were reversed by both androgen and naltrexone treatment. However prenatal morphine and androgen administration markedly enhanced both feminine and masculine sexual behaviour in males, suggesting that the

morphine-induced alterations to feminine behaviour may not have been mediated via an androgen-dependent mechanism. In addition, when the behaviour of males chronically exposed to morphine throughout gestation and during the early postnatal period was examined it was found that both masculine and feminine sexual behaviour was significantly enhanced. The following studies were undertaken with a view to determine how the opiate-induced alterations in behaviour were mediated.

1) Hypothalamic concentrations of DA, HVA, 5HT and 5HIAA were measured using an HPLC-ED system. Levels of 5HT in the rat and hamster are reported to show sex differences at this time (higher in females than in males) and manipulation of 5HT levels during the early postnatal period is known to interfere with sexual behaviour in adulthood. Prenatal administration of morphine significantly reduced female levels of 5HT on day 12 postnatally, this treatment also reduced feminine sexual behaviour. Postnatal morphine + testosterone reduced female 5HT levels and their lordosis behaviour. In contrast, levels of 5HT in males were increased by opiate treatments which also increased their ability to display lordosis. These results suggest that some of the changes reported in adult sexual behaviour may have been caused by alterations to the hypothalamic levels of 5HT in the golden hamster. When the levels of hypothalamic amines were measured in pups culled on the final day of morphine treatment, 5HT and 5HIAA concentrations were significantly

increased in males and 5HT concentrations were raised in females.

2a) Rats treated with 5, 10 or 15mg/kg morphine for 4 days postnatally were culled as adults and the number of motor neurones in the spinal nucleus of bulbocavernosus counted. This nucleus is dependent upon postnatal testicular androgens to promote the survival of its neurones (around 250 in males). The absence of androgens in females at this time results in the neurone numbers falling to around 40. None of the doses of morphine used had any significant effect upon motor neurone numbers in the male rats.

2b) The volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA), which is significantly larger in the adult male than in the female rat, was measured in adult male rats treated postnatally with either 10 or 15mg/kg morphine. No difference was noted in either group of males when compared to controls.

3) Testosterone levels in male hamster pups treated on days 1 and 2 with morphine and culled 2-3 hours after the final injection showed no significant change when compared to those of saline-treated males.

These studies have revealed that perinatal morphine administration alters the sexual differentiation of copulatory behaviour in the golden hamster. These alterations are apparently not mediated via a reduction in androgens. Neither androgen levels, as determined by RIA, nor either of the androgen dependent structures in the

CNS (SNB and SDN-POA) were altered by opiates.

It is possible that some of the alterations in sexual behaviour may have been mediated via alterations to hypothalamic levels of 5HT during postnatal development since some morphine treatments altered both these variables.

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