The Role of Testosterone in the Development of Neuroanatomical Lesions associated with Specific Learning Disorders

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For Karen

" Many waters cannot quench love "

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Summary

Developmental learning disorders have attracted interest since they were first described in the late 19th century. An anatomical basis was suggested for acquired dyslexia, dysphasia and other language related syndromes, a view strengthened by the discovery of cerebral asymmetry for language and by detailed cytoarchitectonic investigations which revealed specific cortical abnormalities in the brains of dyslexic individuals. These anomalies consisted mainly of clusters of ectopic neurons in layer I of the cerebral cortex, located principally in the perisylvian and frontal areas. The presence of these abnormalities is considered to be typical of the condition.

The developmental learning disorders are increased in males, certain families, twins and left-handers. In addition, an increased incidence of autoimmune disorders has been reported in dyslexic individuals. It was my purpose in this Thesis to investigate whether the male sex hormone testosterone plays a role in the development of the cortical ectopias which are associated with these disorders.

For this I used an animal model, consisting of mouse strains in which similar ectopias in comparable areas have been demonstrated, together with strains in which they are absent. Mice with ectopias show behavioural disturbances including impaired learning abilities, so that although at first it seems inconceivable that there could be a mouse model for human developmental problems of learning, such as dyslexia, it is nevertheless possible to study the aetiology of related cortical abnormalities.

I started by confirming the reported incidence of cortical ectopias in the BXSB mouse and found that 31% had ectopias, which is similar to the figure of 29% previously reported. The DBA strain was confirmed to have no ectopias.

Ectopias were also demonstrated in 33% of AJ mice, a strain reported to have high adult serum testosterone levels. The incidence of ectopias was confirmed to be more common in males than in females, with 58% of BXSB and 62.5% of AJ mice with ectopias being male. Preliminary results also indicate that the uterine proximity of male fetuses may be important in determining which females develop ectopias.

In light of the finding of ectopias in the AJ strain I examined serum testosterone levels in males and females of other strains known to develop ectopias along with a number that do not, in order to determine if high testosterone levels were a general phenomenon associated with strains that develop ectopias. No such association was found in normal adult animals; however, testosterone levels were found to be increased at late gestation in mothers of two strains that develop ectopias, the BXSB and the NZB, compared to the DBA strain which does not, although the increase was significant only for the BXSB.

Testosterone related effects upon the nervous system were also studied by examining the spinal nucleus of bulbocavernosus (SNB), a sexually dimorphic motor nucleus (L5-S1) which is directly influenced by perinatal testosterone levels. Neuronal numbers in the SNB were compared between strains that developed ectopias and those that did not. A significant correlation was found between the incidence of ectopias and numbers of neurons in the SNB, with the presence of ectopias being associated with increased numbers of neurons. This probably indicates an androgenic inhibition of neuronal cell death in these strains.

Finally, I increased the testosterone levels during pregnancy in the BXSB and DBA strains by daily injections of testosterone propionate, in order to study the effect that this would have on the incidence of ectopias. A

significant increase in the incidence of ectopias (from 30% to 62%) was seen in the BXSB strain after treatment with testosterone; no such increase was seen in the DBA strain. In the latter, however, a significant proportion of animals developed hydrocephalus upon treatment with testosterone.

In conclusion, the results of this work confirm that cortical ectopias in mice are associated with high levels of testosterone in utero and that their incidence can be increased by the prenatal administration of testosterone. It is suggested that testosterone plays an important role in the development of the human cortical ectopias which are characteristic of developmental learning disorders.

Introduction

Historical approach to Dyslexia

A developmental disorder in learning to read and write, "congenital word-blindness" or "dyslexia", was first described more than 90 years ago (Morgan, 1896). The report attracted the attention of a well known ophthalmologist working in the Glasgow Eye Infirmary, Dr. James Hinshelwood, who had devoted many years to the study of the acquired word-blindness which may develop after a stroke (Hinshelwood, 1895; 1896 a,b; 1898; 1899; Behan and Behan, 1989). Other developmental disorders in language and speech i.e. congenital word deafness or congenital aphasia, were described in the 1920's (Worster-Drought and Allen, 1929; Morrison, 1930), but dyslexia has always attracted the most attention, possibly because, as Hinshelwood stated, the children affected are of normal, or often above normal, intelligence (Hinshelwood, 1902; 1904; 1907).

It is now accepted that approximately 5% of the school age population are affected by the disorder, with four times as many boys affected as girls (Finucci and Childs, 1981). This figure, however, does not take into account the frequency of undiagnosed cases of reading difficulty and these are likely to be considerably higher (Sherman et al, 1989). The difficulty occurs in spite of normal intelligence, emotional stability and adequate family and educational opportunities.

The most commonly used definition of dyslexia is that given by Critchley (1970) : " a disorder manifested by difficulty in learning to read, despite conventional instruction, an adequate intelligence and sociocultural

opportunity, dependent on fundamental cognitive disabilities that are frequently of constitutional origin ". Diagnosis is usually made in children of eight or nine years of age, who have a reading age of two years or more below normal. It is more common in males than females (Finucci and Childs, 1981), occurs in families (Hinshelwood, 1907; De Fries et al, 1978; Smith et al, 1983), and is associated with left-handedness and ambidexterity (Geschwind and Behan, 1982). Recently it has also been reported to be associated with dysfunction of the immune system, such as allergies and certain autoimmune conditions (Geschwind and Behan, 1982). While falling into the larger category of developmental disorders of the nervous system, it has attracted special attention because of the insight it offers into the organisation of language in the brain.

Dyslexia itself was originally recognised in the acquired form. The first clinical description was given by Professor Lordat of Montpellier in 1825 of his own, temporary, acquired loss of reading ability (Critchley, 1970). A few more cases were reported from the continent, including those by Charcot and Dejerine (reviewed by Hinshelwood, 1895), and one from Britain by Broadbent (1872).

Credit for the intensive study, investigation and careful delineation of the lesion found in the acquired form must go to James Hinshelwood, who spent considerable time searching the French and German literature, before reporting the first of his life-long series of cases (Hinshelwood, 1895). He attempted to localise the site of the causative lesion. He had an excellent understanding of neuroanatomy and stated that the damage "may be accounted for by a single lesion.....A subcortical lesion in the white matter of the left occipital lobe, so situated as to cut across the fibres passing from both occipital lobes to the left angular gyrus, would render the patient word-blind

but not agraphic."

His best-studied case died after 9 years of observation and a lesion was found at the site he had predicted, on the inferior aspect of the left occipital lobe, a large triangular area limited by the calcarine fissure medially and the third temporal gyrus laterally. The lesion encroached upon the fibres connected to what remained of the occipital cortex and the fibres ascending to the left and right primary visual centres at the calcarine fissures (Hinshelwood, 1904; Behan and Behan, 1989).

Hinshelwood had been alerted to the possibility of congenital cases by a brief case description by Dr. Pringle Morgan, who described a young boy with the syndrome (Morgan, 1896). In 1900, within two months of one another, two boys aged 10 and 11 years, with dyslexia were brought to see Hinshelwood. He studied them over a long period of time, together with a series of similar cases subsequently brought to him, trying to find an anatomical basis for their disorder. He did not have the opportunity to show such a basis and over the ensuing years other theories, mainly of a pyschological nature, were proposed to account for the condition (Critchley, 1970).

A different hypothesis to emerge was that put forward by the American neurologist, Samuel T. Orton (Orton, 1925). Orton believed that there was no anatomical lesion present in developmental dyslexia, and the disorder was caused through inappropriate development of cerebral dominance. This concept of cerebral dominance, its anatomical basis, dyslexia and laterality, has been of great importance in our understanding of the brain and will be discussed briefly below. Orton based his hypothesis of dyslexia on the increased association with left-handedness, and ambidexterity which he observed and his view was accepted widely for many years : developmental

dyslexia was thought to have no anatomical basis, until Galaburda and Kemper published their classic paper on the neuroanatomical abnormalities in the brain of a 20 year old dyslexic male (Galaburda and Kemper, 1979), consisting of symmetry in the normally asymmetric planum temporale and also focal ectopic collections of neurons in layer I of the cerebral cortex.

Orton had commented on the increased frequency of left-handedness in individuals with dyslexia and their families, in his first paper (Orton, 1925) and this has been confirmed in many later studies (Geschwind and Galaburda, 1985). Left-handedness is more common in males (Geschwind and Galaburda, 1985), as is dyslexia (Finucci and Childs, 1981). Geschwind and Behan (1982) were searching for associations with dyslexia other than maleness and left-handedness when they embarked on a study of strongly left-handed (-100 laterality quotient) and strongly right handed individuals (+100 laterality quotient) on the Oldfield Handedness Inventory (Oldfield, 1970). Left-handed subjects reported not only an increased incidence of learning disorders, as expected, but also significantly more immune disorders, in themselves and their family members (Geschwind and Behan, 1982).

Geschwind and Behan formulated a hypothesis to account for these findings, namely that the association between learning disorders, left-handedness and autoimmune disorders reflected the prenatal effect of fetal testosterone on the developing nervous and immune systems. They postulated that, during embryogenesis, testosterone slowed neuronal development in the left hemisphere of the brain predisposing the individual to learning disorders, right cerebral dominance and left-handedness. At the same time, testosterone impaired development of the immune system, leading to the later development of autoimmunity.

The aim of this thesis is to explore the role of testosterone in the

development of learning disorders. As I will go on to show there are strong anatomical correlates of human dyslexia, with specific cerebral cortical lesions associated with the disorder (Galaburda and Kemper, 1979). Similar cortical lesions (ectopias) can be demonstrated in autoimmune mice (Sherman et al, 1987) which show deficits in avoidance learning (Spencer et al, 1986; Schrott et al, 1990)). Thus an experimental model for learning disorders is available.

During my study I examined the effect of testosterone, endogenous and exogenous, on the incidence of cortical ectopias in inbred experimental and control strains of mice. The work was part of a major project on the roles of different factors in the development of dyslexia, supported by the National Institutes of Health, USA. Drs Sherman and Galaburda (Harvard University) have carried out separate anatomical studies confirming and characterising the existence and constitution of the ectopias, Dr Morrison (Institute of Neurological Sciences, Glasgow), has attempted to establish whether or not maternal immune factors were operative in the formation of ectopias and Dr Denenberg (University of Connecticut), has examined related behavioural aspects.

Cerebral Dominance and Laterality in relation to Dyslexia

Cerebral dominance refers to the greater participation of one of the two hemispheres of the brain in certain learned activities, while laterality refers to the processes which lead to the development of an asymmetrical nervous system. The first is a functional concept while the second provides its anatomical basis. The hypothesis of cerebral dominance was championed in the late 19th century by the French physician, Paul Broca, based on his

observations of individuals who were suffering from aphasia and dysphasia, the loss and impairment of the ability to express or comprehend language as a result of damage to the brain. Broca found that in all the patients he examined, loss of speech was always associated with damage to the left frontal lobe (reviewed in Joynt, 1964) and that this was accompanied by the expected right-sided paralysis of the body. Broca went on to argue that many important functions, including handedness, were under the control of the left hemisphere of the brain, which was dominant to the right. He argued that the right hemisphere would be subordinate in most individuals and that in right-handed individuals the regions necessary for acquisition of language would lie on the left side of the brain, while in left-handed individuals language acquisition would be centred in the right side of the brain (reviewed in Dimond, 1972).

It is now apparent that the situation is more complex than Broca's early observations would suggest and that while dysphasia in right-handers is consistently the result of injury to the language-processing region on the left side of the brain, injury to the left hemisphere in a majority of left-handers will also produce dysphasia. Only 15% of left-handers have speech centred in the right hemisphere of the brain, with 15% showing evidence of bilateral speech control, and the remaining 70% showing left hemisphere dominance for speech (Rasmussen and Milner, 1977). Thus it appears that while language skills in the right-hander are controlled almost exclusively by the left side of the brain, this function in the brain of the left-hander is more symmetrical.

Up until the 1960's Broca's view held the field and it was believed that cerebral dominance did not have any anatomical basis, but, in 1968, Geschwind and Levitsky described in detail the difference in size of the planum temporale in the two hemispheres of the human brain. This anatomical asymmetry was present on the upper surface region of the temporal lobe, an

area known to be important for speech and language (Geschwind and Levitsky, 1968). Of 100 brains they examined at post mortem, 65 were found to have a longer planum temporale in the left hemisphere than in the right, 11 were longer in the right than the left, while the remaining 24 showed no difference. Therefore, it appeared that asymmetry was a normal feature of the brain. Geschwind thought that the imbalance was therefore due to greater development of the left side, but it has since been shown that the size of the left planum remains relatively constant, while the right side is larger in symmetrical than in asymmetrical brains (Galaburda et al, 1987). Symmetrical brains tend therefore to have two large plana, whereas asymmetrical brains have a small right and a large left planum.

Other areas of the brain which differ in size between the two hemispheres in subjects with different abilities have also been described. For instance the size of the corpus callosum in right and left handers has been compared (Witelson, 1985). The midsaggital cross-sectional area of the corpus callosum was found to be 11% larger in left handers and ambidextrous people than in right handers. This increase is presumably associated with greater anatomical connections between the two hemispheres, with symmetrical cases containing denser and more extensive projections than asymmetrical cases, as well as increased numbers of neurons (Rosen et al, 1987). The adult corpus callosum contains less fibres than are present in the fetus or neonate. Natural cell death is thought to be responsible for the decrease so that the larger size in left-handers is thought to be due to a slower rate of cell death in these individuals (Witelson, 1985).

A link with other biological factors of importance in the development of dyslexia, is shown here because, although the mechanisms responsible for neuronal cell death are not yet clear, it is obvious that testosterone is

implicated. For instance, treatment of neonatal female rats with testosterone propionate will increase the size of the callosa, suggesting that testosterone may prevent cell death (Fitch et al, 1990). Indeed, corpora callosa may be larger in the male (Fitch et al, 1990) : this is an example of sexual dimorphism.

The Neuroanatomical Lesions in Dyslexia

The careful neuroanatomical study of developmental dyslexia made by Drake (1968) first suggested that there was a structural basis for the functional abnormalities. He reported abnormal gyri in the parietal region, ectopic (displaced) cortical neurons, and thinning of the corpus callosum. Galaburda and Kemper (1979) then studied the brain of a left-handed, male, childhood dyslexic in whom there was a strong family history of the disorder. On microscopic examination, they found an area in the region of the left planum temporale with abnormal organisation of nerve cells (cortical dysplasia) and with polymicrogyria (small convolutions). The right cerebral hemisphere and remainder of the brain appeared normal. These results have since been confirmed and extended to include other dyslexic subjects, with two types of abnormality being identified (Galaburda et al, 1985 a). i.e.

1. Absence of the usual pattern of cerebral asymmetry of the language-related planum temporale.
2. Focal developmental abnormalities of cortical architecture, particularly in the perisylvian region of the left hemisphere.

As described above, the normal human brain is strongly asymmetric in various regions, including the planum temporale, which is a part of Wernicke's language area on the upper surface of the temporal lobe. In 65% of cases, the left planum is larger than the right, in 10% the right planum is larger and in 25%

the plana are symmetrical in size and shape (Galaburda et al, 1987). In all brains from individuals with developmental dyslexia examined, the plana temporale have been symmetrical (Galaburda et al, 1985 a). The total volume of the planum is inversely correlated with the degree of planum asymmetry, so that the greater the amount of asymmetry, the lesser the total volume of the planum (Galaburda et al, 1987). In other words, the planum temporale is larger in the brains of dyslexics, a finding in keeping with an excess of neurons.

This relationship has also been shown to occur in the primary visual cortex of the rat brain where increasing asymmetry appears to be related to a loss of numbers of neurons in the smaller side, with no overall changes in architectonic appearance (Galaburda et al, 1985 b). Thus it appears that asymmetry in bilaterally represented cortical areas is best explained by differences in the numbers of neurons and abnormally symmetrical areas, such as those found in dyslexics, have an excess of neurons.

The reason for the neuronal increase is not known, but various suggestions in regard to development have been made :

1. There might be differences in the germinal zone where neurons are produced, such that there are more neuroblasts in the symmetrical cases.
2. There might be differences in cell production, with an excess amount of neurons produced in symmetrical cases.
3. There might be differences in the amount of neuronal migration.
4. There might be differences in neuronal cell death, with less neurons lost in symmetrical cases.

With regard to the first two hypotheses, the only experimental work is by Rosen who labelled neurons generated in the visual cortex of the rat to look for side differences in cell production, but was unable to demonstrate asymmetry of neurogenesis in any layer or at any time in the visual cortex (Rosen et al,

1987). It seems most likely that the development of asymmetry is a relatively late event and is based on neuronal cell death.

Another type of asymmetry is provided by sexually dimorphic regions of the CNS. These are examples where both sexes have the same number of neurons up to birth, but different rates of cell death lead to the establishment of sexual asymmetries, as in certain of the lumbo sacral neuron groups, which will be discussed at greater length later. Mechanisms such as mitotic differences have, in these cases been discarded (Segelaub, 1989)

Apart from the finding of asymmetry in the brains of dyslexics, the other consistent feature is the presence of focal anomalies (microdysgenesis) in cortical architecture. Focal accumulations of cells (ectopias) occur in the molecular layer of the cerebral cortex, which is normally cell-free and these are often accompanied by underlying distortion of the laminar and columnar cortical architecture (dysplasia). In extreme cases, the anomalies lead to a disruption of the pial surface of the brain forming a brain "wart", or to microgyria. There may be large numbers of ectopias, ranging from 30 to 100 per brain, present predominantly in the left hemisphere, clustering around the sylvian fissures. In normal brains, such focal areas are very rare, limited to no more than two foci per affected brain.

The reason for the development of ectopias is unknown but they have been attributed to agents acting at the time of late neuronal migration, around, or shortly after, the period of mid-gestation (McBride and Kemper, 1982). They have been observed in association with a number of congenital toxic, vascular and infectious insults (Christensen and Melchior,1967 ; Hicks, 1953 ; Slotnick and Brent, 1966) and can be produced experimentally in animals by these mechanisms during the same stages of development (Dvorak et al,1978 ; Sato et al, 1982).

Galaburda and co-workers have proposed that these cortical anomalies are linked causally to the syndrome of developmental dyslexia (Galaburda, 1985 a). As will be shown later, similar anatomical abnormalities can be detected in certain strains of mice (Sherman et al, 1987).

Biological associations of Dyslexia

Since the early studies by Orton (1925) it has been known that there is a higher proportion of left-handedness among dyslexics and persons with related language disorders than among the general population. It is estimated that between 5-10% of the population are left-handed, with more males affected than females (Oldfield, 1970). What determines handedness is unknown but increased numbers of left-handed individuals are found in certain families. Increased numbers are also found in twins, both identical and fraternal. Since not all genetically identical twins show the same handedness, however (Springer and Deutsch, 1989), it has been suggested that left-handedness may result from damage to the brain during difficult births rather than from genetic factors.

In the early 1980's clinical observations by the late Norman Geschwind led him to believe that left-handedness was related not only to disorders of the brain but also to a wider category of diseases, including those of the immune system. He put these observations to the test, in association with Peter Behan of Glasgow University, in a controlled study on strongly left-handed and right-handed groups (Geschwind and Behan, 1982).

A questionnaire was devised, based on the Oldfield Handedness Inventory (Oldfield, 1970), to test for laterality. In addition, questions were asked about a family history of learning disorders, and the presence of

migraine and any autoimmune illnesses. Only individuals with a laterality quotient of -100 or +100, corresponding to those who were either completely left-handed or completely right-handed, were selected for study. It was found that developmental dyslexia was 12 times as common in left-handers as in right-handers and an increased frequency of immune disorders (2.7 times) was also detected in left-handers.

Geschwind and Behan (1982) therefore proposed that, in addition to left-handedness, there was an association between learning disorders and autoimmune disease. This could be due to a common factor operating in utero and affecting the development of both the brain and the immune system and leading to abnormalities of function. They postulated that testosterone was this factor. It was this hypothesis that I wished to test in my study.

The effect of testosterone on the development of the nervous system

It has been established for some time that sex hormones play a major role in establishing the anatomical differentiation between the developing male and female phenotype but, until recently it was not recognised that these hormones also have a critical effect on the developing nervous system.

The first investigations were carried out in the rat, on structures in the limbic system and hypothalamus which control sexual behaviour. It is now realised, however that there are receptors for the gonadal hormones at many different locations in the nervous system, some of which are present only at a particular time of development (Geschwind and Galaburda, 1985). Perinatal exposure to androgens can modify neuronal growth and differentiation permanently, and influence cell death. In humans, early exposure to androgens appears to have less dramatic, but nevertheless far-reaching

effects, than in the rat.

Sex hormones affect the cortex and underlying structures. For instance, it has been reported that the cortex in several areas of the posterior right hemisphere of the newborn male rat is significantly thicker than in corresponding areas on the left. In contrast the female has several areas which are thicker on the left hemisphere at the same period (Diamond et al,1981). If the male rat is castrated at birth, many of the differences observed are not detectable when the brains are examined at 90 days of age (Diamond, 1984).

Thus exposure to sex hormones at critical periods of neurological development can produce permanent structural alterations. This leads to sexual dimorphism i.e. anatomical and functional differences between the nervous systems of males and females.

In mammals the pattern for sexual differentiation of the CNS, like the body as a whole, is believed to be in the direction of the female, with masculine patterns being primarily established as a result of exposure to fetal testicular hormones during development (Gorski, 1971). The placenta has also been reported to synthesise testosterone and its metabolites (Soares and Talamantes, 1983). Maternal serum androgen levels may rise at midpregnancy. Placental androgens may play a role in mediating the androgen-dependent maturational events which occur before testicular secretions are established in the individual. The main testicular factor responsible for sexual differentiation of the mammalian CNS, however is believed to be testosterone, produced by the developing testis and acting either directly or after conversion to oestrogens.

The X chromosome plays an important role in the effects of testosterone as it contains the Tfm locus (Geschwind and Galaburda, 1985), one of the

genes that controls sensitivity to testosterone. The female embryo may be more sensitive to placentally-produced testosterone than the male, but this higher sensitivity is lost before embryonic gonadal secretions begin. The Y chromosome carries few genes, although it does code for the H-Y antigen, which is located very close to the testes determining-gene (Tdy) and may be necessary for normal spermatogenesis (Burgoyne et al,1986).

In humans, the male and female gonads begin to develop during the 5th week of gestation. Testicular differentiation in humans occurs between the 6th and 7th week of gestation with the specialised Leydig cells starting to produce androgens (Wilson et al, 1981). Proliferation of the Leydig cells proceeds rapidly, and the subsequent rise in testosterone production plays a key organisational role in male sexual development. Leydig cell numbers then decrease to reach a low by week 21 of gestation, and this low level is maintained in the postnatal period. Plasma testosterone levels have been shown to be highest on days 18 to 19 postconception in male rat fetuses and to decline thereafter (Weisz and Ward, 1980).

There is an optimum period when the organisational effect of androgens upon the developing nervous system is at a peak. This time, referred to as the critical period, varies between species. In species that are born in a relatively immature state, such as the mouse and rat, it encompasses the late prenatal to the early postnatal, period whereas in those species that are more fully developed at birth, such as the rhesus monkey, this period is predominantly or entirely prenatal (MacClusky and Naftolin, 1981). Androgens circulate in females as well as males during the critical period; indeed, in one study on rats it was shown that only on day 18 of pregnancy (from a study of days 17 to 23), were the levels of plasma testosterone in males consistently higher than those in females (Weisz and Ward, 1980).

Current understanding on the role of testosterone on the developing CNS dates back to a series of experiments performed by Pfeiffer (1936). He demonstrated that the expression of masculine patterns of pituitary gonadotropin secretion in adult rats was dependent on factors released from the testes during early postnatal life, and that masculine patterns of gonadotropin secretion could be induced in females by transplantation of a testis into the neck shortly after birth. Castration of males at birth resulted in development of female patterns of secretion in adulthood. When it was shown later that the functions of the pituitary are regulated by the hypothalamus, it became clear that factors released from the testes must have influenced the development of centres within the brain (Green and Harris, 1947). This androgen effect is time-dependent, as demonstrated by Barraclough (1961), who showed that a single dose of testosterone propionate given to females on postnatal days 2 to 5 resulted in 100% anovulatory sterility. The same dose given at day 20 had no effect while intermediate effects were seen on day 10, with only some females becoming sterile. Subsequent experiments have shown that complete masculinisation of gonadotropin release is regulated by oestradiol - 17 β , a metabolite of testosterone, as opposed to testosterone itself (McDonald and Doughty, 1972).

Testosterone enters a target cell by diffusion, where it may act directly or through one of its metabolites. Testosterone and all other steroid hormones share a common mechanism of action. They bind to a cytosolic receptor which has a specific hormone-binding domain and a DNA-binding domain. On binding the hormone, the receptor transforms to the nuclear form with its DNA-binding domain exposed. The latter has a high affinity for nuclear chromatin and binds to specific nucleotide sequences present in the promoter regions of target genes so that it regulates the expression of the genes that

contain receptor-binding domains (McEwen, 1981).

Males exhibit significantly higher nuclear androgen-receptor concentrations than females, but females have higher concentrations of cytosolic receptors (Roselli et al, 1989). This difference is due to circulating levels of testosterone, with the receptors in males being predominantly transformed by high testosterone levels and tightly bound in the nucleus. The low concentrations of nuclear androgen receptors in females are due to insufficient levels of the androgens required to transform the receptor. In the brains of male rats, the levels of serum testosterone, nuclear androgen receptor and aromatase activity have been shown to decrease with age (Chambers et al, 1991). This decrease is associated with a fall in testosterone levels and can be partially restored on treatment with testosterone.

Androgen receptors show significant differences in concentration in different regions of the brain, with the highest concentrations being found in anatomical areas known to be implicated in androgen-dependent responses, such as sexual and other social behaviours and gonadotropin regulation. These areas include the ventral hypothalamus, the medial preoptic area, the bed nucleus of the stria terminalis, the medial amygdala and the lateral septum (McGinnis et al, 1983).

In addition to these regions of high androgen-receptor density, it has recently been shown in monkeys that androgen receptors may vary in distribution between the right and left cerebral hemispheres (Sholl and Kim, 1990). Androgen-receptors in the cerebral cortex of male and female fetal rhesus monkey brains were compared : in males, androgen-receptor levels in the right frontal lobes were higher than those in the left frontal lobe, while the levels were lower in the right temporal lobe than in the left. No significant differences were found for females. The authors hypothesised that the

unequal distribution of androgen receptors could favour the formation of synapses on the side which had the higher concentration of androgen receptors with, in contrast, loss of neurons on the side which failed to establish connections. This demonstration of a different degree of androgen effect on the two sides of the cortex lends further support to the Geschwind hypothesis that androgens play a central role in the establishment of asymmetries, with the delay in maturation of the left hemisphere dependent on androgens.

Many of the effects of testosterone on the CNS are mediated through aromatisation of androgen to oestrogen (MacClusky and Naftolin, 1981), with the minimum dosage required to produce defeminisation of behaviour and gonadotropin secretion in neonatal female rats being significantly lower for oestrogens than for testosterone. There is, however compelling evidence that androgens also act directly upon the CNS, for example, specific anti-androgens have been shown to block the masculinisation of the sexually dimorphic spinal nucleus of the bulbocavernosus (Fishman and Breedlove, 1988), also, dihydrotestosterone, a non-aromatisable androgen, can cause masculine behaviour in females, if given prenatally (Goy and McEwen, 1980).

Sexual Dimorphism in the CNS

There is sexual dimorphism in the nervous system i.e. anatomical and functional differences are seen between males and females. These differences are further reflected in behavioural responses associated with reproduction e.g. courtship, copulation and maternal behaviour and in those not directly associated e.g aggression and learning ability. As would be expected from the discussion above, sexual dimorphism depends in part on the individual's hormonal milieu during the critical period of nervous system

development.

Exhibition of masculine sexual behaviour in adult rats requires exposure to testicular androgens during the first five days of life (Whalen and Edwards, 1967 ; Paup et al, 1972). Castrated male rats that have not been exposed to testicular androgens postnatally, will fail to respond to them in adulthood and instead, will respond to priming with oestrogen and progesterone by displaying feminine sexual behaviour. If the testosterone is replaced in the castrated rats during this early period normal male responses will develop. Similarly, if female rats are treated postnatally with androgens, they will fail to display normal female sexual behavior in adulthood (Whalen and Nadler, 1963) and express more male typical play patterns as pups (Olioff and Stewart, 1978 ; Tonjes et al, 1987).

Infanticide (the killing of young), is a sexually dimorphic trait in mice, dependent on androgen stimulation during perinatal life and adulthood. One third to one half of random bred Rockland-Swiss (R-S) albino adult male mice kill pups compared to only 5% or less of adult females (Gandelman, 1972). Sex differences are influenced by exposure to gonadal hormone levels during the critical period of CNS development as well as the activational period of adulthood (Gandelman, 1982). For instance, while castration of newborn male mice increases the proportion of animals that will display infanticide, it decreases the time required for androgen activation of the behaviour in adulthood and similarly, treatment of neonatal female mice with testosterone propionate lowers the proportion of adult animals that will display infanticide in response to adult stimulation with testosterone (Samuels et al, 1981). On the other hand, the onset of pup killing behaviour in male R-S mice coincides with the androgen surge associated with puberty, at about 35 days (Gandelman, 1973 ; Svare et al, 1978). If the adult males are castrated, the level of

infanticide is reduced. It can be restored in a dose-dependent way with testosterone propionate replacement therapy (Gandelman and Vom Saal, 1975). Virgin female mice, normally maternal to newborns, will exhibit killing behaviour towards them if they are given testosterone in adulthood (Davis and Gandelman, 1972).

It has become apparent during the past 20 years that many of the dimorphic physiological and behavioural responses correspond to morphological differences in CNS structure between the sexes. There is now convincing evidence that sex steroids, particularly testosterone, can alter CNS structure as well as function. Early studies by Dorner and Staudt (1968), on the preoptic area of the rat hypothalamus, an area important in the establishment of adult sexual behaviour patterns, demonstrated a sexual dimorphism in cell nuclear and nucleoli size in the medial part, with the volume being greater in females than in males. Raisman and Field (1973) found differences in the numbers of dendritic spine synapses of non-strial origin in the same area, with more non-strial derived synapses in females and neonatally castrated males than in males or in neonatally testosterone-treated females.

Greenough et al (1977) reported that, in the hamster brain, neurons of the dorsomedial preoptic area had significantly more total dendritic volume in males than females, with increased dendritic density in a circumscribed region ventral to the anterior commissure. Gorski et al (1978), similarly reported a pronounced sex difference in a densely staining region of the rat brain medial preoptic nucleus, with the region being larger in males than in females. These workers stated that the difference was so striking that the sex of the animal could be predicted solely by examining histological sections of brain with the naked eye. The region has now been named the sexually dimorphic nucleus

of the preoptic area (SDN-POA).

Not surprisingly, hormonal manipulation in adulthood by a variety of methods including oestrogen, progesterone and testosterone administration, did not alter the extent of the dimorphism in either sex. Subsequent research has shown that the difference in volume is under the influence of testicular hormones acting during the perinatal period (Jacobson et al, 1980).

The Spinal Nucleus of Bulbocavernosus

One of the most striking examples of sexual dimorphism within the CNS is the spinal nucleus of bulbocavernosus (SNB), a motor nucleus first identified in the rat lumbar spinal cord (Breedlove and Arnold, 1980) and subsequently in mice (Wee and Clemens, 1987). In the rat it consists of approximately 200 neurons in the male but only 40 in the female. The cells of the SNB form a discrete nucleus within the dorsomedial aspect of the ventral grey horn between segments L5 and S1 in the spinal cord. The SNB cells are large, multipolar, motor neurons 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 reflexes in the rat penis (Sachs, 1982).

These motor neurons and their target muscles have been shown both to accumulate androgens and to depend on testosterone or its analogue dihydrotestosterone (DHT) for their maintenance. Autoradiographic studies have shown that all motor neurons in the lumbar regions of the rat spinal cord are capable of accumulating testosterone and DHT, but not oestrogen and that those areas, such as the SNB, involved in specifically masculine functions take up significantly more label than those involved in non-sexual functions

(Breedlove and Arnold, 1983). There is also a significant difference in levels of uptake between males and females, with motor neurons in the male accumulating more label than those in the female. Breedlove and Arnold (1983) have also demonstrated that castration of adult male rats results in a decrease in the size of the soma of motor neurons and in a reduction of muscle mass, while not affecting the actual number of neurons.

During the perinatal period, both sexes develop the perineal muscles and the motor neurons which innervate them (Cihak et al, 1970 ; Nordeen et al, 1985). At birth, substantial cell death occurs in both sexes but is less extreme in the male, or in females treated postnatally with testosterone or DHT (Nordeen et al, 1985). Prenatal exposure of males to the antiandrogen flutamide, plus neonatal castration, causes a complete sex reversal of the SNB, with neuron numbers falling to around one third that of normal control males (Breedlove and Arnold 1983).

The testicular feminisation (Tfm) strain of rat, which is androgen-insensitive, shows an early embryonic development equivalent to that of a normal male, suggesting that androgens are not essential for the initial rise in motor neuron number, but thereafter, SNB motor neuron numbers decline to the level typical of the normal female.

Finally, androgens have been shown to enhance neurite outgrowth from spinal cord explants in murine fetal lumbar spinal cord (Hauser and Toral-Allerand, 1989). Cell numbers were increased at all spinal cord levels, whether from sexually dimorphic regions or not.

It is clear that both the number and size of the motor neurons in the SNB is consistently related to androgen exposure, especially in the critical period of CNS development. Anything interfering with androgen synthesis, release or uptake, can be expected to cause a difference in the motor neuron population

of this area. Thus this nucleus provides an ideal record of testosterone effect during the period we wished to study and was selected for this reason.

The Animal Model : Mice with Neuroanatomical Lesions and Autoimmune Diseases.

Geschwind and Behan (1982) reported an association between learning disorders, left-handedness and a group of immune diseases. The finding of an association between disorders of the nervous system and disorders of the immune system led investigators to examine genetically-inbred strains of mice with well documented immune abnormalities, for the presence of congenital brain anomalies (Sherman et al, 1985 ; Sherman et al, 1987).

Initial explorations were carried out in the New Zealand Black (NZB) and New Zealand White (NZW) strains and in their F1 crosses (NZB/NZW). The NZB strain develops a well documented autoimmune disease (Theofilopulus and Dixon, 1981; Talal, 1983) similar to the human disorder systemic lupus erythromatosus (SLE), with autoantibodies and abnormalities of stem cells, macrophages and T and B lymphocytes. It develops haemolytic anaemia and dies prematurely at about 16-17 months of age. The NZW does not develop severe immune problems, although in later life autoantibodies and mild nephritis occur. The NZB/NZW develops an autoimmune condition which is more severe than that in the NZB, with antibodies to nucleic acids, lymphoproliferation of B cells and deposition of immune complexes in the kidney. The latter leads to renal insufficiency and death in females at 11 months (Talal, 1983), and in males at 15 months, the difference in severity being under the influence of sex hormones (Roubinian et al, 1978). Other inbred strains studied, to determine whether autoimmunity was related to brain

abnormalities, include the BXSB, MRL/Lpr and DBA2 mice.

The BXSB strain develops severe autoimmune disease, manifested by the production of autoantibodies, proliferation of B-cells, and immune complex glomerulonephritis (Andrews et al, 1978 ; Talal, 1983 ; Theofilopulus and Dixon,1981). BXSB males die at 5 months of age, while females die at 15 months. This is thought to be due to the presence of the Yaa gene in the Y chromosome which accelerates the immune disease. Similarly, the MRL/lpr strain develops autoimmune disease early, with massive T-cell proliferation and enlargement of the lymph nodes and spleen (Andrews et al, 1978 ; Talal, 1983 ; Theofilopulus and Dixon, 1981)). Death occurs between 4 to 6 months, from immune complex glomerulonephritis, with females being more severely affected than males. Unlike the above, the DBA2 strain has no autoimmune feature and does not develop autoimmune disease.

The initial studies were most interesting since ten out of 56 NZB mice examined showed disordered cortical architecture similar to that found in the brains of humans with developmental dyslexia (Sherman et al, 1985) i.e. ectopic collections of neurons in layer I with dysplasia in the underlying layers. Males were affected twice as often as females. The ectopias were primarily unilateral, and located mainly in the somatosensory cortices.

Later studies showed similar cortical lesions were present in 26% of the NZB, 29% of the BXSB and in 2% of the MRL/lpr brains. No ectopias were found in the brains of the DBA2 mice. In the NZB strain 86% of the anomalies were located in the somatosensory cortex, whereas in the BXSB, 81% of the anomalies were in the frontal motor cortices (Caviness, 1975).

Behavioural disturbances have also been reported in these mice (Nandy et al, 1983; Denenberg et al, 1991a).

Although these cortical anomalies were first demonstrated in

autoimmune strains of mice, autoimmunity is not the sole factor in determining their appearance, indeed recent findings provide strong evidence that, at least in BXSB mice, the occurrence of ectopias is independent of immune parameters (Schrott et al, 1993).

Studies also suggest that the maternal enviroment does not play a major role in their development since transferring fertilised ova between autoimmune and normal control mice does not affect the incidence of ectopias (Denenberg et al, 1991 b). In contrast, similar transfers of fertilised ova did affect the later appearance of autoimmune disease, and of behavioural changes. This dissociation of ectopias from behaviour and autoimmunity indicates the probability of separate mechanisms being involved in their establishment.

Finally, there was an increased incidence of affected males in the experimental animals, similar to that associated with dyslexia in humans. Possible explanations for this are that females are affected more severely, die and are reabsorbed in utero, or that susceptibility differs between the sexes. Both these suggestions can be tested in the experimental animal.

It was the existence of this animal model which made possible the study of the effects of testosterone on the development of ectopias.

The approach to the Study

Specific neuroanatomical lesions can be identified in the brains of humans with developmental dyslexia. The aetiology and/or pathogenesis of these anomalies is unknown but a central role has been suggested for the male hormone, testosterone (Geschwind and Behan, 1982 ; Sherman et al, 1985). The fact that similar lesions can be identified in the brains of certain strains of inbred mice with autoimmune abnormalities (Sherman et al, 1987), allows us to investigate the effects of testosterone on their development.

The problem was approached in the following way :

1. The neuroanatomical lesions were identified and previous work extended to confirm their incidence in the different autoimmune strains. In addition, an autoimmune strain with high serum testosterone levels was studied for the presence of ectopias.
2. An assay system, based on a radiolabelled method used for human assays, was used to measure circulating levels of testosterone in adult mice, in order to see if the presence of ectopias was associated with a life long increase in serum testosterone. Testosterone levels in adult male and female mice of the different strains were measured.
3. Testosterone levels were next determined in pregnant mice of various strains at late gestation, when the ectopias are believed to form.
4. Following this, attempts were made to estimate serum testosterone levels in fetal and neonatal mice. Unfortunately the minute amounts of serum which could be obtained from the pups were too small for analysis. Therefore I decided to use an indirect measurement of the effect of testosterone on the nervous system, namely the number of neurons within the nucleus

bulbocavernosus. As outlined already, in the introduction, this nucleus is a striking example of sexual dimorphism. Its neuronal content is five times higher in the adult male rat than in the female, dependent entirely on the effect of testosterone in late prenatal and early postnatal life i.e. the time which includes the critical period of CNS development. The SNB was examined in adult animals that were 6-10 weeks old.

5. The position of male and female fetuses in the uterus was determined at sacrifice on day 18, in order to see if the incidence of ectopias varied in relation to the sex of the adjacent pup. For instance, whether a female between two males (2M) was more likely to develop ectopias than one between two females (0M). This experiment was designed to evaluate the testosterone effect of sibling males in utero.

6. Finally, the intrauterine milieu was manipulated to increase testosterone, by injecting pregnant mice with testosterone propionate each day from day 10 to day 18 of gestation. This time span covers the period most sensitive to the effects of androgen, and includes the time when ectopias develop.

Materials and Methods

Animals

The following inbred strains of mice, of different genetic origins were used ; DBA2, NZB/Binj, BXSB, MRL/Lpr (Jackson Labs), AJ/Ola (Harlan Olac Labs). All animals were housed in sex matched groups of 10 animals / cage and maintained on 12:12 hour light dark cycle, with water and chow available ad libitum. Of these the NZB, BXSB, MRL and AJ are autoimmune strains, in addition the AJ strain shows elevated levels of serum testosterone (Hampl et al, 1971).

Time Matings

Female mice, aged 6-10 weeks, were time-mated with age-matched males of the same strain. Females were left overnight in a group cage with a stud male. If a sperm plug was observed the following day, this was designated as being day 1 of gestation. Pregnant females were housed separately and maintained on a light/dark cycle with water and chow available ad libitum. Animals were handled daily 1 week prior to mating, and then daily until the point of sacrifice, in order to reduce stress-related changes in serum testosterone.

Collection of Serum

Animals were handled daily for a period of at least one week prior to sacrifice, in order to reduce stress-related changes in testosterone from handling prior to sacrifice. They were killed under a schedule 1 method (i.e. by a rising concentration of CO2 leading to respiratory depression) and the blood was removed by cardiac puncture and then allowed to clot on ice for

1 hour. After clotting, the blood was spun at 4°c for 15 minutes at 1,000 rpm and the serum was collected and stored at - 70°c until assayed.

Histological Examination of Fetuses including their Brains

All examinations of brains were conducted on fetal mice, sacrificed on day 18 of gestation. Ectopias can be readily identified in the brain at this period (Sherman, personal communication).

At day 18 pregnant females were sacrificed and exsanguinated. They were then perfused intracardially with saline, followed by 10% buffered formalin. The entire uterus was removed and fixed in 10% formal saline for at least one week. It was then opened and the number of fetuses recorded. Individual fetuses were removed and their sex was determined by microscopic dissection and examination for the presence of testes or ovaries. The sex of each fetus and its position in the uterus was recorded.

The heads were then removed and dehydrated in 70%, 90%, and 100% ethanol, before processing and embedding in paraffin. The entire head was cut into 5μm sections on a microtome, with evey fifth section mounted on glass slides and stained with toluidine blue. The slides were examined under a light microscope for the presence of cortical ectopias, with the topography of any anomalies being noted. All observations were made on an Olympus BH 2 light microscope.

Histological Examination of Spinal Cords

Adult male and female mice, aged 6-10 weeks were sacrificed by anaesthetic overdose, with CO2. Blood was removed by cardiac puncture and then the animals were perfused intracardially with saline, followed by 10%

buffered formalin. The spinal cords from approximately spinal levels L4-S1 were removed and post-fixed in 10% buffered formalin for at least 1 week, until sectioned. The pieces of cord were embedded in 7% agar and sectioned at 50 μm on a vibratome. Every 5th section was mounted and stained with 0.2% thionine in 70% alcohol, until the the first motor neuron of the SNB was visualised, whereupon every previous section within the field and all subsequent sections were mounted and stained with thionine.

Thionine stains the cell bodies of neurons dark blue, while the nuclei remain pale with dense blue/black nucleoli. The motor neurons are very large and are readily identified by virtue of their size and position.

Assay System for Serum Testosterone

A competitive binding radioimmunoassay was used to measure serum testosterone. The method used was a modification of the human diagnostic assay, employed by the Clinical Biochemistry Dept. of Glasgow Royal Infirmary. The primary antibody detected testosterone and a small amount of 5 α - dihydrotestosterone (10 % cross reaction).

A known amount of ^{125}Iodine-labelled testosterone was added to a test tube along with the serum sample. The addition of a sheep anti-testosterone and a donkey anti-sheep antibody forms a complex with the testosterone, the radioactive value of which can be measured. Due to the competitive nature of the assay, a low radioactive count is obtained if the serum sample has a high testosterone content and has subsequently bound little of the labelled testosterone.

Sample values can be determined by incorporating testosterone standards of known concentration into the assay. This enables a standard

curve to be drawn, of concentration against counts, from which sample concentration can be determined from counts obtained.

All tubes were set up in duplicate with 100 µl of serum sample added. Three ml of diethyl ether was added and the samples then vortexed for 4 minutes to extract the testosterone. The samples were allowed to stand for 5 minutes at room temperature, during which time phase separation occured. The lower phase was then frozen in a bath of solid CO2 and methanol and the solvent phase, which contained the testosterone, decanted into glass assay tubes and dried under vacuum. Basal serum was spiked at two levels (6 and 12 n mol/litre) with standard testosterone and was extracted in each assay. These, in addition to assessing the reproducibility of the assay, acted as recovery pools for estimation of the efficiency of extraction. Two hundred µl of the primary antibody, a sheep anti-testosterone polyclonal (kindly supplied by Dr. Christina Gray, of the Dept. Biochemistry, Glasgow Royal Infirmary), was added to the dried extraction residue (at a final titre of 1: 80,000) along with 200µl of the ^{125}Iodine-labelled testosterone tracer, and incubated for 2 hours at room temperature. Two hundred µl of a donkey anti-sheep/goat antibody (at a final titre of 1: 80) was then added to the primary reaction mixture, along with 200µl of normal goat serum (at a final titre of 1: 2,000), and incubated overnight at 4^{o}c, during which time the precipitate forms.

After overnight incubation the assay tubes were centrifuged at 2,500 rpm for 25 minutes at 4^{o}c. The supernatant was then aspirated off taking care not to damage the pellet. The total count tubes were not aspirated.

Assay tubes were counted on a NEN 1600 gamma counter, linked to a Commodore 4032 and printer 4022. The total count tubes were counted until

10,000 counts were accumulated. A standard curve from the known testosterone levels of the standards was drawn and the concentration of testosterone in the sample tubes was calculated from the curve.

Statistical Analysis

Differences in serum testosterone levels and SNB cell counts were analysed by one way analysis of variance. The variance was calculated according to the following equation.

$$s^2 = \frac{\Sigma (x1 - \bar{x}1)^2 + \Sigma (x2 - \bar{x}2)^2}{n1 + n2 - 2.}$$

Where; s = The estimation of variance.

x = The individual value.

$\bar{x}$ = The mean value.

n = The number of observations (10).

The significance of the variance was calculated by a student's t-test where;

$$t = \frac{\bar{x}1 - \bar{x}2}{s\sqrt{1/n1 + 1/n2.}}$$

The degrees of freedom = n1 + n2 - 2 (10 +10 - 2 = 18).

The standard deviation was calculated by the following equation.

$$s^2 = \frac{\Sigma x^2 - (\Sigma x)^2/n}{n-1.}$$

The standard error was then calculated by dividing the standard deviation by the square root of n - 1.

$$S.E. = \frac{s}{\sqrt{n - 1.}}$$

The statistical significance of differences observed in levels of ectopias between groups of mice was analysed with a x^2 test according to the following equation.

$$x^2 = \frac{n\,(\,ad - bc - 1/2\,n\,)^2}{(a+b)\,(c+d)\,(a+c)\,(b+d).}$$

Where;

a = The number of animals in group 1 with ectopias.

b = The number of animals in group 1 without ectopias.

c = The number of animals in group 2 with ectopias.

d = The number of animals in gruop 2 without ectopias.

n = The total number of animals examined.

The degree of freedom = 1.

Experiment 1

The brains of fetal DBA (litters, n =6 ; pups, n = 50), BXSB (litters, n = 5 ; pups, n = 38) and AJ (litters, n = 3 ; pups, n = 24), mice were examined for the presence of neuroanatomical anomalies. The sex and uterine placement of each pup was noted and examined in relation to the presence of ectopias.

Experiment 2

The circulating levels of serum testosterone were compared in adult male and female mice, of the above strains (n = 10 males and 10 females of each strain).

Experiment 3

The circulating levels of serum testosterone were compared at day 18 of gestation, in pregnant females of the DBA, NZB, and BXSB strains of mice (n = 10 of each strain).

Experiment 4

The spinal nucleus of the bulbocavernosus was examined and compared in all the experimental groups (n = 10 males and 5 females of each strain). To determine the total number of cells in the SNB, only those cells containing a distinct nucleolus were included. Nucleoli have diameters of approximately 1.5-2.0 μm and since sections were cut at 50μm, this meant that it was virtually impossible to count the same cell twice. All observations and counts were made on an Olympus BH 2 light microscope.

Experiment 5

Female mice of the DBA and BXSB strains, were time mated. From day 10 of gestation, until sacrifice at day 18 pregnant females received a daily intra-peritoneal injection of either 100 μg of testosterone propionate (T.P.) in 0.05 ml of peanut oil (DBA litters, n = 5, pups, n = 34 ; BXSB litters, n =5, pups, n = 34), or 0.05 ml of vehicle alone (DBA litters, n = 5, pups, n = 38 ; BXSB litters, n = 5, pups, n =36). The brains of the fetuses were then examined for the presence of neocortical anomalies.

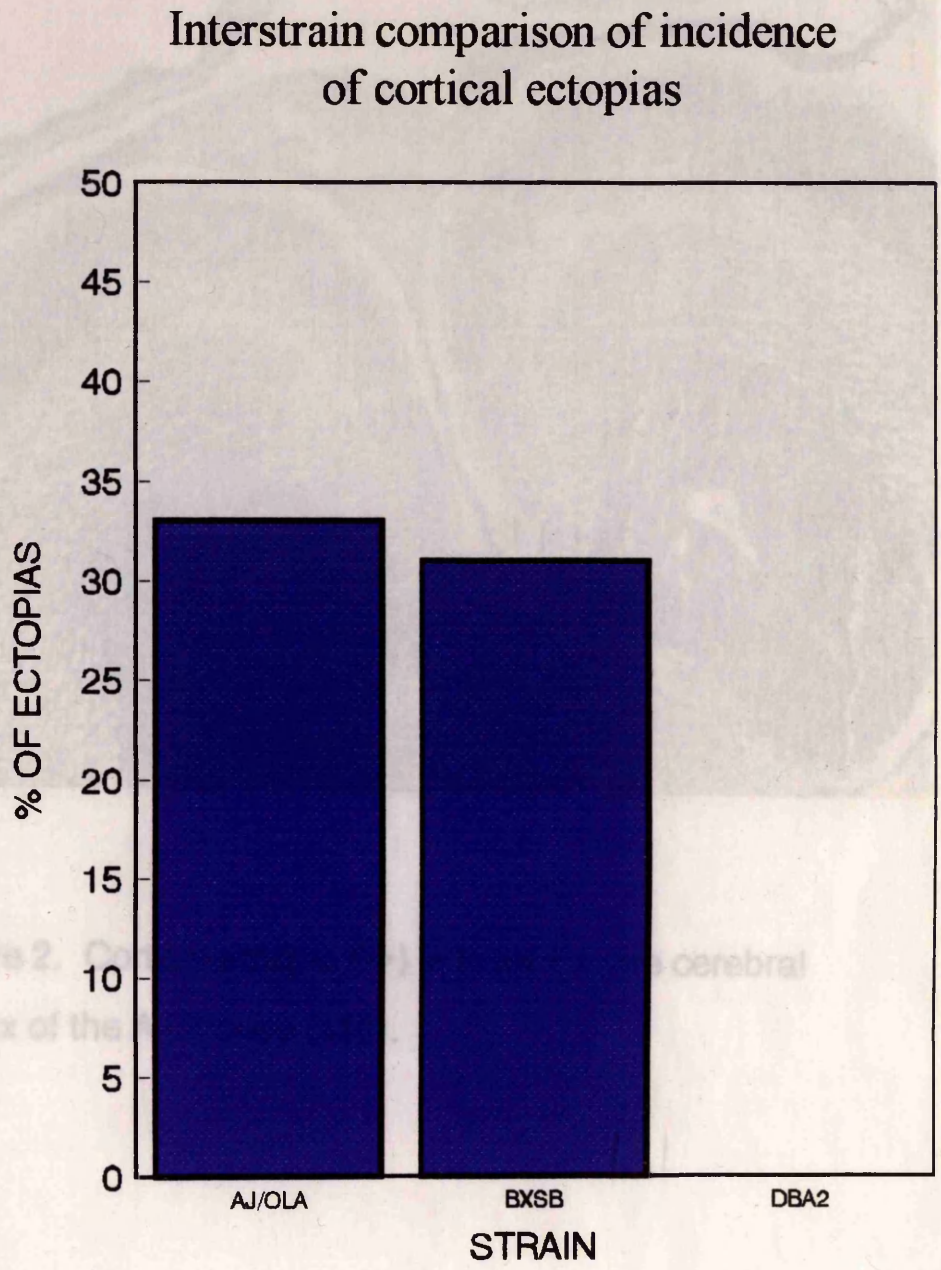
Results

Experiment 1

Neuroanatomical abnormalities were detected in 8 out of 24 (33%, x^2 = 15.3, df = 1, $p < 0.001$), of the AJ mice, and 12 out of 38 (31%, x^2 = 15.7, df = 1, $p < 0.001$), of the BXSB mice, but in none of the DBA mice (Fig. 1). These were predominately unilateral and in both cases were located primarily in the frontal motor cortices, as can be seen from Figs. 2, 3 & 4, showing abnormalities in the brain of the AJ and in Figs 5 & 6, showing abnormalities of a similar size and location in the BXSB. The abnormalities consisted of ectopic collections of neurons in the normally acellular layer I of the cerebral cortex, whose morphology was largely indistinguishable from the underlying cells in layer II, as is seen most clearly in the high powered magnifications in Figs 4 & 6. The ectopias varied in incidence from 1-4 per brain and were composed of 30-60 cells/section.

Of the AJ mice that developed ectopias 5/8 (62.5%) were male, and 3/8 (37.5%) were female. Seven out of 12 BXSB mice (58%), with ectopias were male and 5/12 (42%), were female. Neither of these differences reached levels of significance within the sample size. All of the female AJ mice (3/3) which had ectopias developed between 2 males (2M females), while 3/5 BXSB with ectopias were 2M females , while one 1M and one 0M female also had ectopias. No statistical significance however, could be shown for a relationship between ectopias and uterine placement.

Fig. 1


Interstrain comparison of incidence of cortical ectopias
% OF ECTOPIAS
50
45
40
35
30
25
20
15
10
5
0
AJ/OLA
BXSB
DBA2
STRAIN

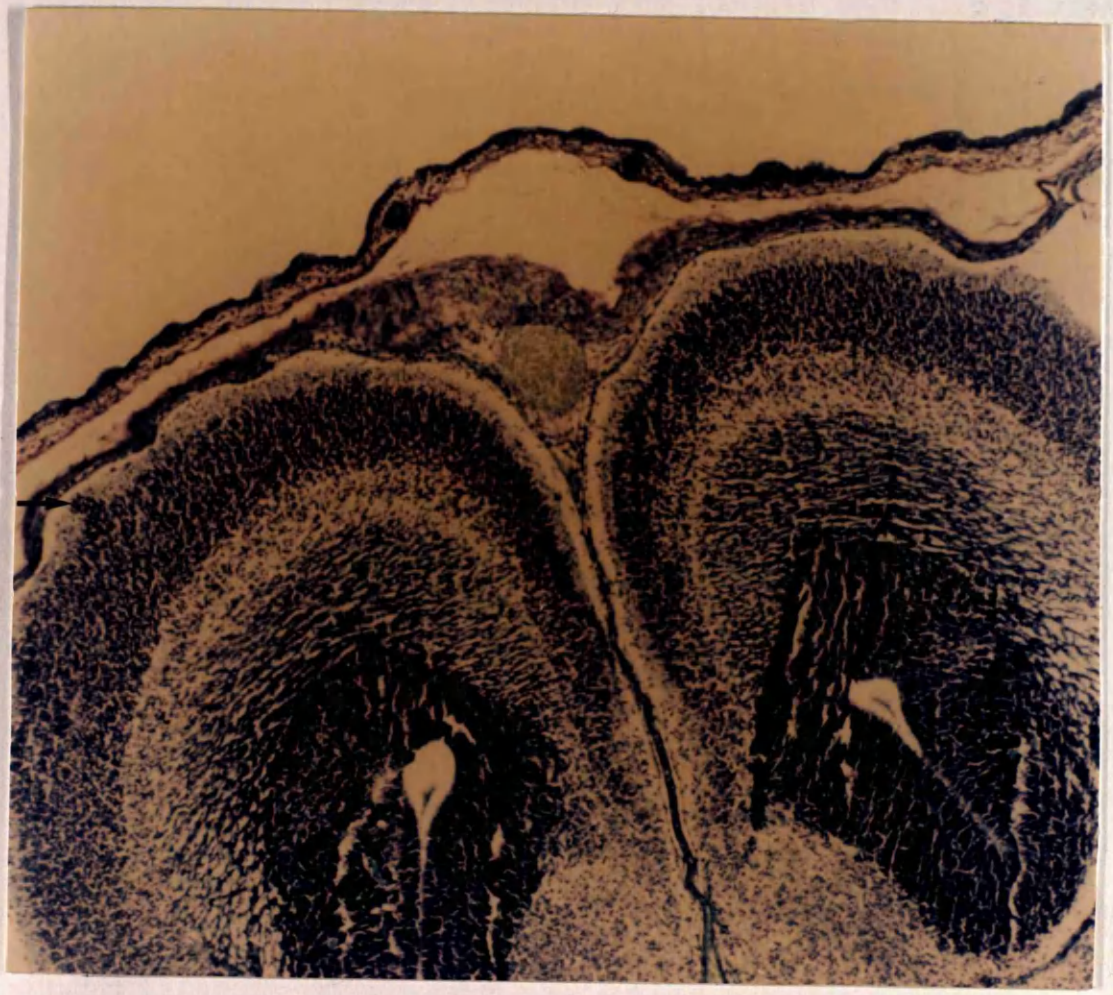


Figure 2. Cortical ectopia (->) in layer I of the cerebral cortex of the AJ mouse (x40).

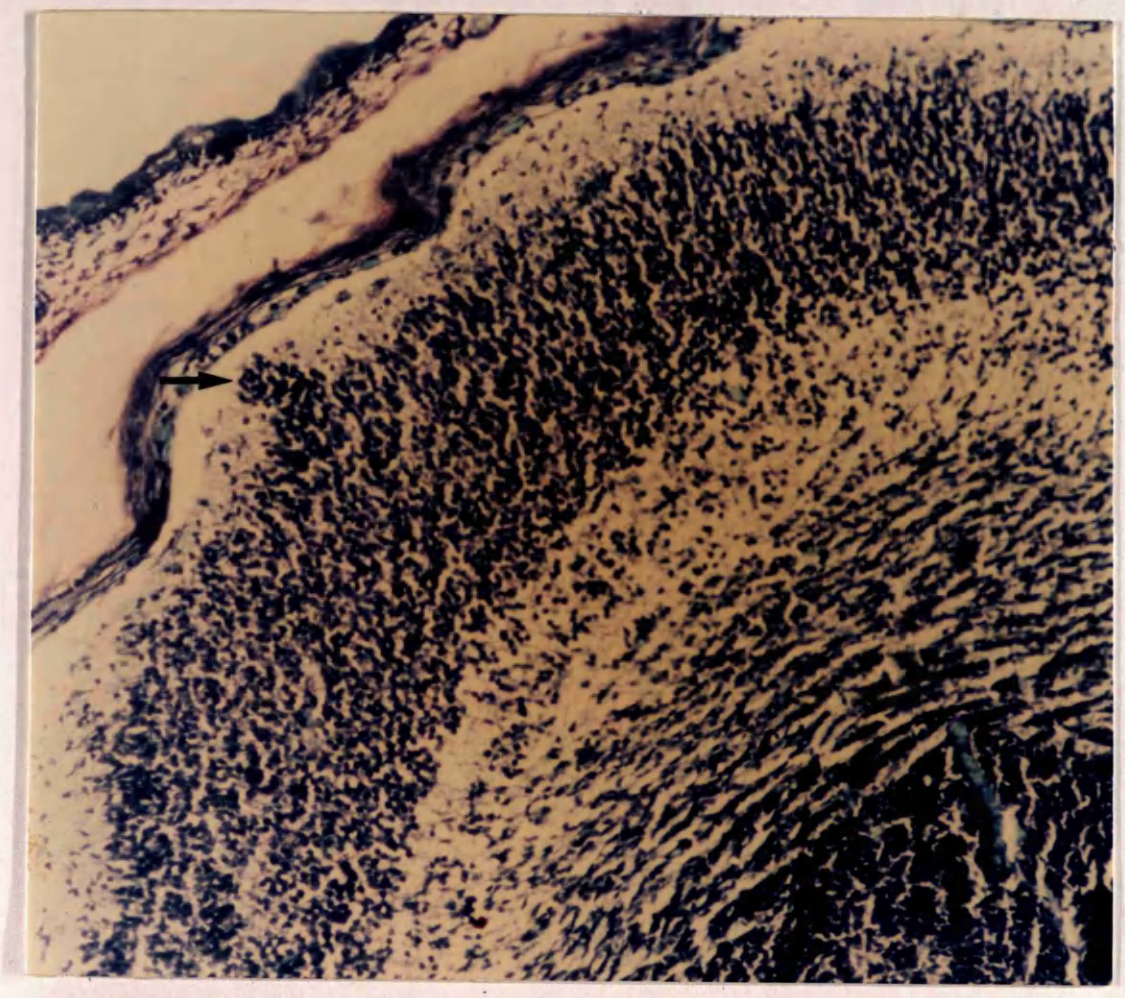


Figure 3. Cortical ectopia (->) in layer I of the cerebral cortex of the AJ mouse (x100).

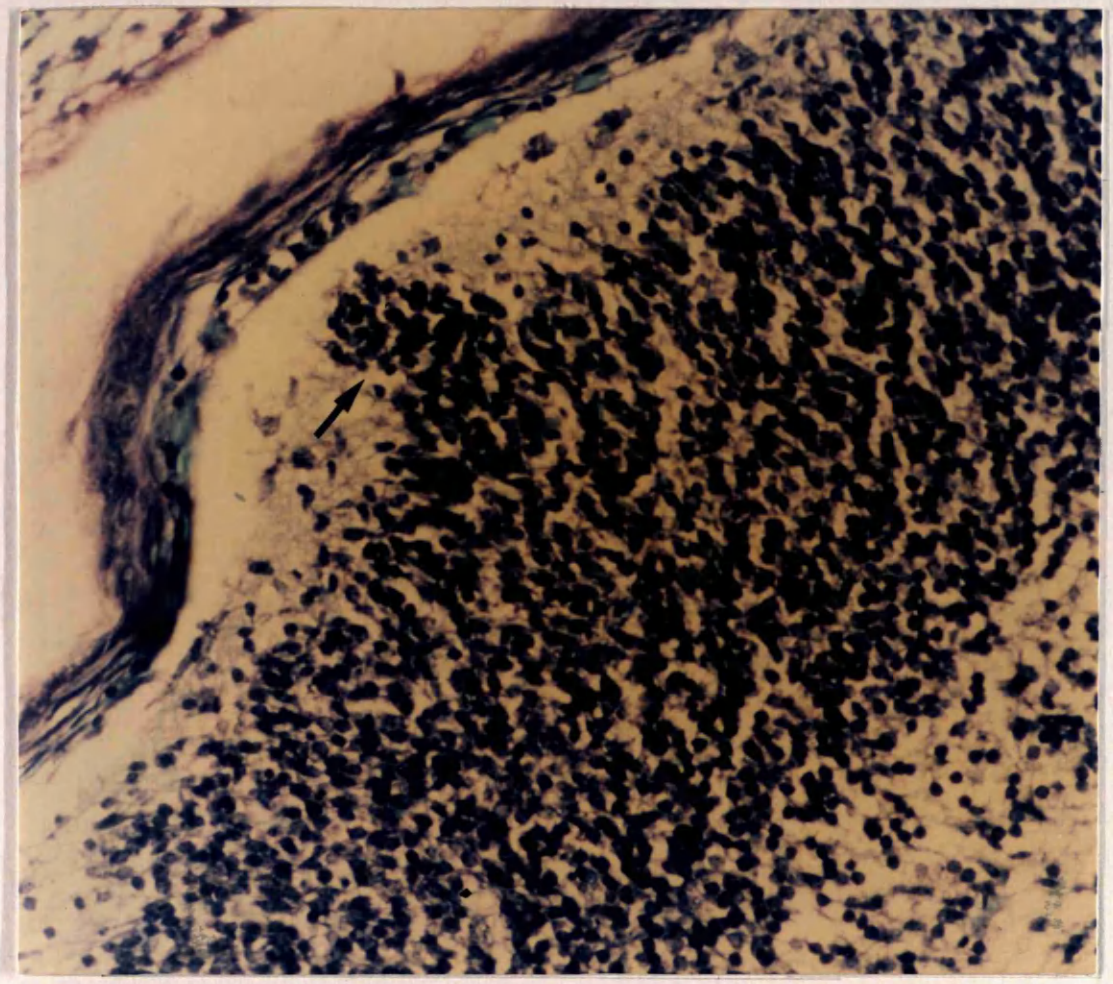


Figure 4. Cortical ectopia (->) in layer I of the cerebral cortex of the AJ mouse (x200).

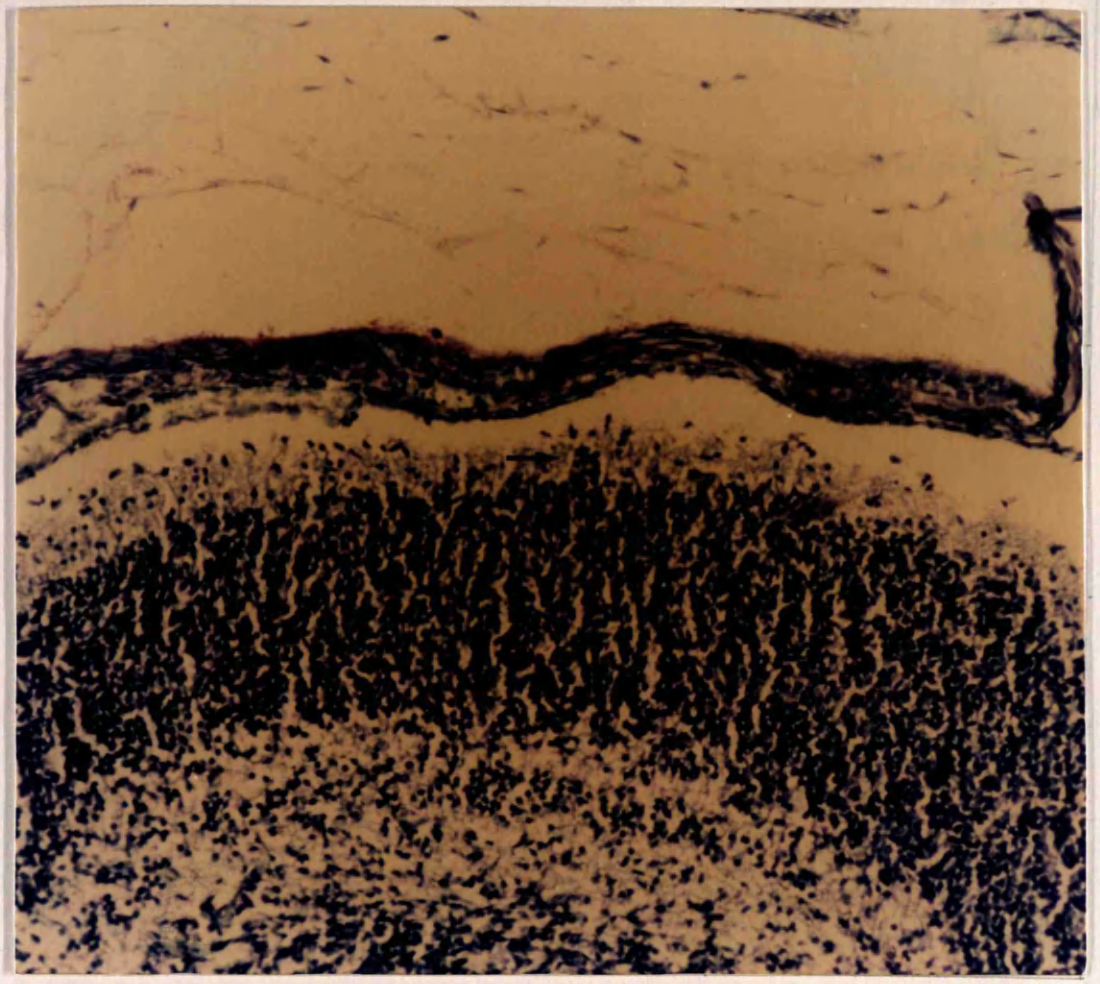


Figure 5. Cortical ectopia (->) in layer I of the cerebral cortex of the BXSB mouse (x100).

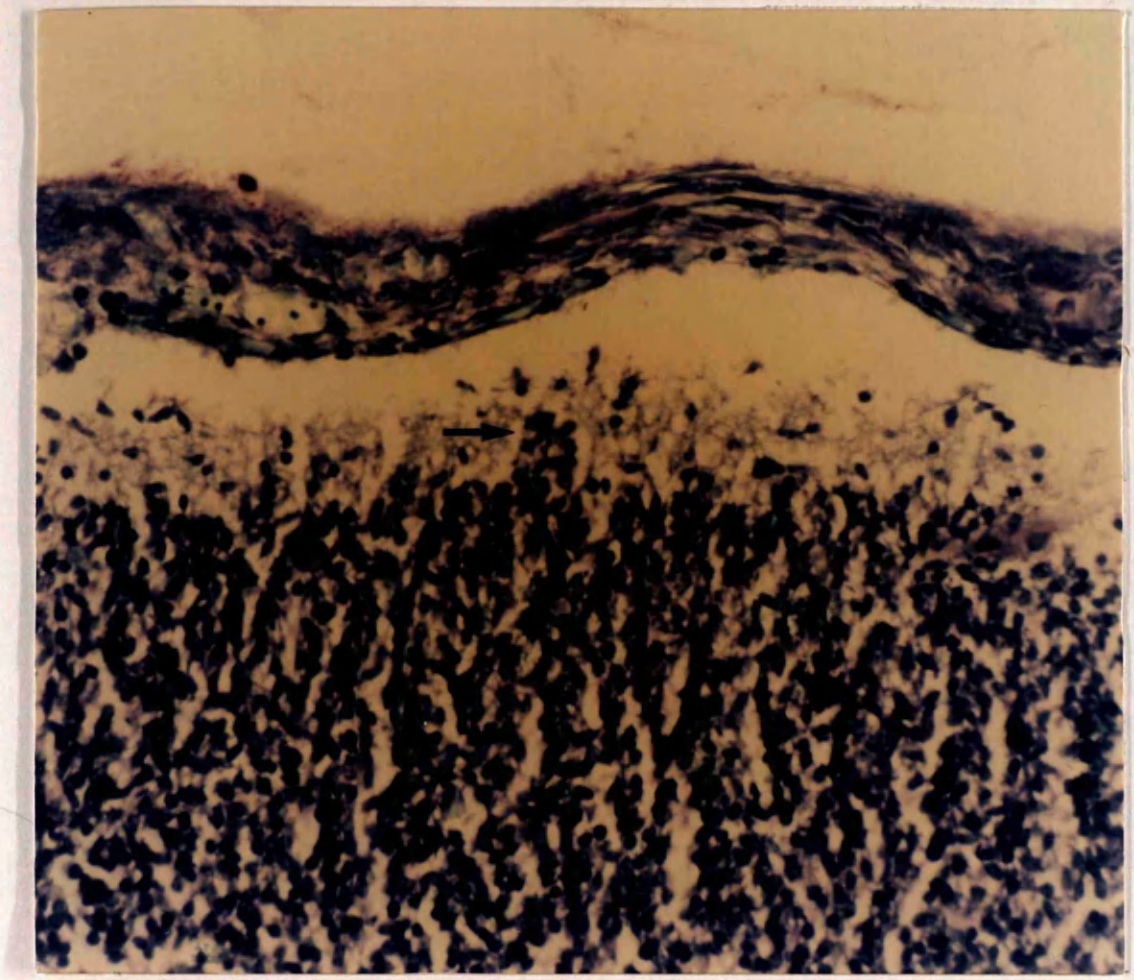


Figure 6. Cortical ectopia (->) in layer I of the cerebral cortex of the BXSB mouse (x200).

Experiment 2

The levels of circulating serum testosterone obtained from adult mice of different inbred strains are presented in Fig. 7a. Testosterone assay characteristics can be seen in Fig. 7b. This shows a typical dose response curve. The precision profile compiled from all the duplicate analyses performed during this study is superimposed upon the standard curve. The sensitivity of the assay was 0.7 n mol/litre (CV 22%) and good precision (CV < 10%) was achieved over the concentration range 2 - 35 n mol/litre. Spiked recovery pools (basal serum, 6 and 12 n mol/litre) extracted similarly to the mouse sera demonstrated good agreement between batches (CV = 3%), albeit the number of assays was small, and acceptable recovery was achieved (77.2% and 95.8%).

It can be seen from Fig 7a. that testosterone values varied considerably between the strains. The NZB strain showed the highest mean testosterone levels (12.3), while the BXSB strain showed the lowest (4.62), although no significant difference was found between the strains.

Testosterone values obtained from the males were as follows;

Strain	Testosterone concentration (n mol/litre)	Standard Error
DBA2	10.89	1.25
NZB	12.30	3.84
BXSB	4.62	4.32
MRL/LPR	6.50	2.28
AJ/OLA	12.10	3.72

Considerable variation in testosterone levels was also observed within

Fig.7a

Interstrain comparison of serum testosterone levels in sexually mature mice.

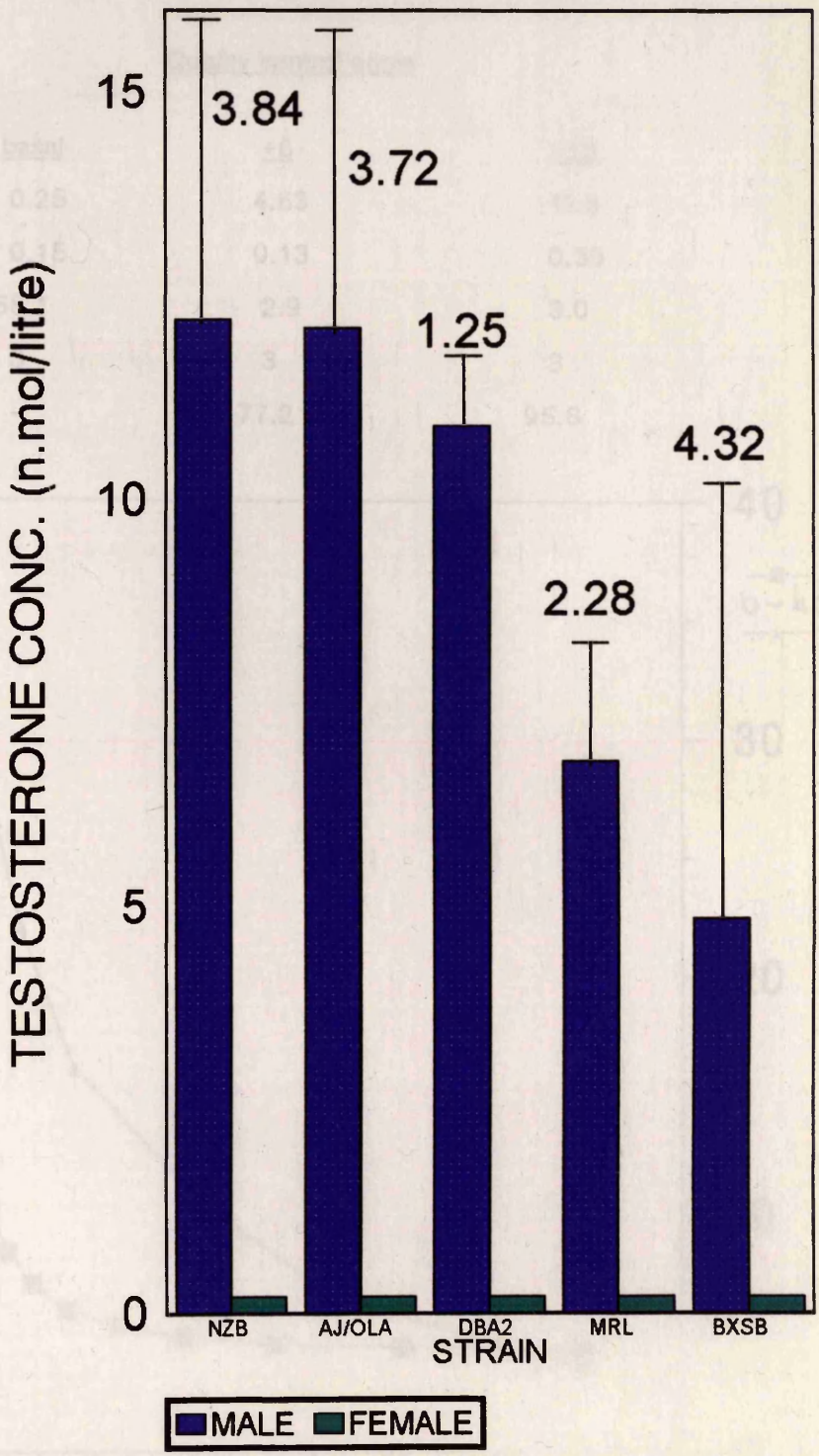


Fig. 7b. Standard dose response curve and precision profile of testosterone assay.

Quality control pools

	basal	+6	+12
mean	0.25	4.63	11.5
SD	0.15	0.13	0.35
CV	58.1	2.9	3.0
n	3	3	3
% recovery	-	77.2	95.8

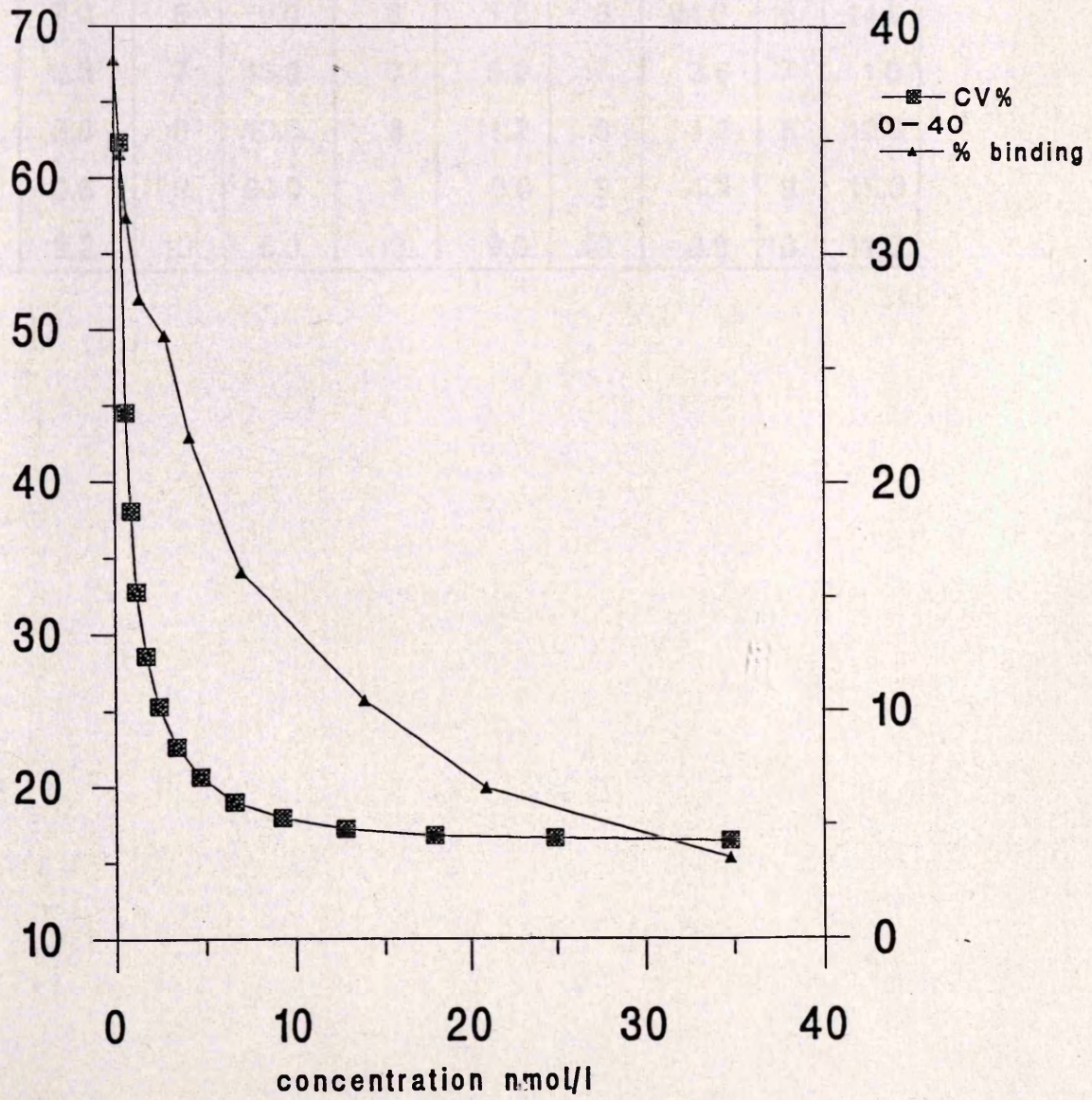


Table 1. Testosterone values (n mol/litre) for adult male mice.

DBA	Concn	NZB	Concn	BXSB	Concn	MRL	Concn	AJ	Concn
1	4.2	1	1.2	1	2.5	1	8.4	1	34.8
2	34.8	2	27.0	2	0.7	2	3.8	2	12.0
3	7.0	3	35.0	3	1.4	3	7.2	3	0.7
4	2.2	4	0.7	4	2.0	4	1.5	4	11.0
5	34.8	5	4.2	5	3.6	5	1.7	5	10.0
6	7.0	6	6.0	6	1.8	6	24.0	6	14.0
7	0.3	7	13.0	7	5.0	7	3.6	7	1.0
8	8.8	8	10.0	8	11.2	8	4.2	8	12.0
9	0.6	9	20.0	9	9.0	9	4.3	9	15.0
10	9.2	10	6.0	10	9.0	10	6.3	10	11.0

all the strains, as can be seen from table 1. with, for example values ranging from 2 - 20 n mol/litre for the BXSB.

All female testosterone levels fell below the sensitivity range of the assay (0.7 n mol/litre), making any strain comparison impossible. It is clear however that no females amongst the strains examined had noticeably high testosterone values.

Experiment 3

The levels of circulating serum testosterone, obtained from pregnant mice on day 18 of gestation are presented in Fig. 8.

The mean results were as follows;

Strain	Testosterone concentration (n mol/litre)	Standard Error
DBA2	3.6	0.21
NZB	5.8	2.6
BXSB	7.0	0.78

The values for the BXSB strain were significantly higher than the DBA2 ($p < 0.025$). The NZB strain, while also having a higher overall level of testosterone than the DBA2, did not reach levels of significance. Levels observed in individual animals are documented in table 2.

Fig.8

Interstrain comparison of serum testosterone levels in pregnant mice at day 18 of gestation.

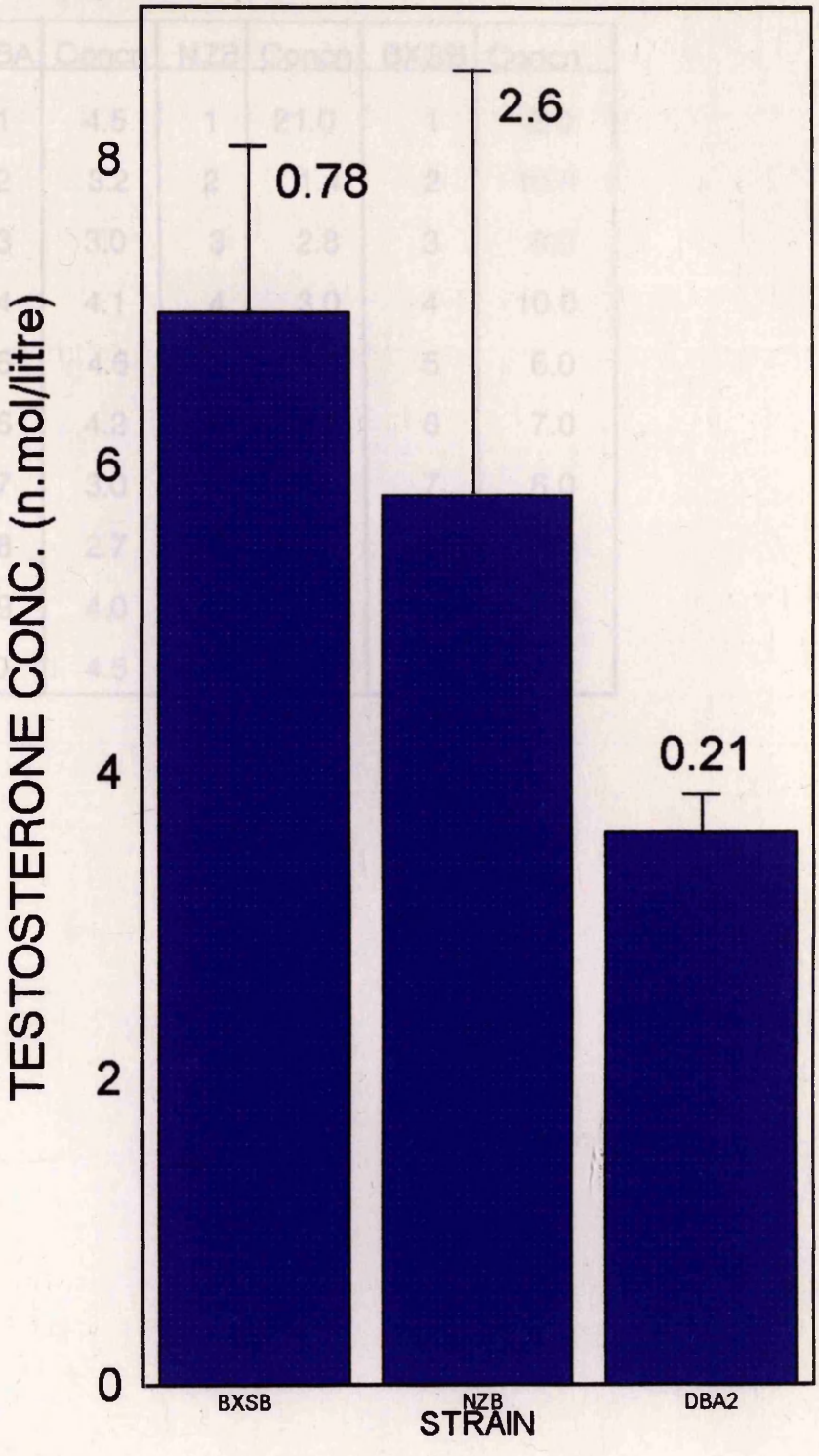


Table 2. Testosterone values (n mol/litre) for female mice on day 18 of pregnancy.

DBA	Concn	NZB	Concn	BXSB	Concn
1	4.5	1	21.0	1	2.0
2	3.2	2	1.4	2	10.0
3	3.0	3	2.8	3	8.0
4	4.1	4	3.0	4	10.0
5	4.6	5	1.4	5	6.0
6	4.2	6	2.8	6	7.0
7	3.0	7	1.4	7	6.0
8	2.7	8	19.5	8	9.0
9	4.0	9	1.3	9	6.0
10	4.5	10	3.0	10	6.0

Experiment 4

The neurons of the Spinal Nucleus of Bulbocavernosus (SNB), were distinguished by their large somata, prominent Nissl substance, and anatomical location, between spinal cord levels L4 - S1 (Fig. 9.) This region corresponds to the position of the SNB as described for the rat (Breedlove and Arnold, 1980).

The sexually dimorphic nature of the SNB was confirmed by parallel examination of female mice at corresponding spinal cord levels to the males (Fig. 10) In each case no discernible nucleus was observed for any of the females examined, making any strain comparison between females impossible.

Cell counts for males of the different strains are presented in Fig. 11. The mean number of SNB neurons counted were as follows;

Strain	Number of SNB neurons	Standard Error
DBA2	80	0.68
NZB	110	1.4
BXSB	100	0.83
MRL/LPR	135	1.06
AJ/OLA	110	0.76

The DBA's had significantly fewer SNB neurons than any of the other strains ($p < 0.001$).

The BXSB had significantly fewer SNB neurons than the NZB, MRL, or AJ/OLA ($p < 0.001$), although significantly higher than the DBA2.

The NZB and AJ/OLA did not differ significantly from each other, but were both lower than the MRL strain.

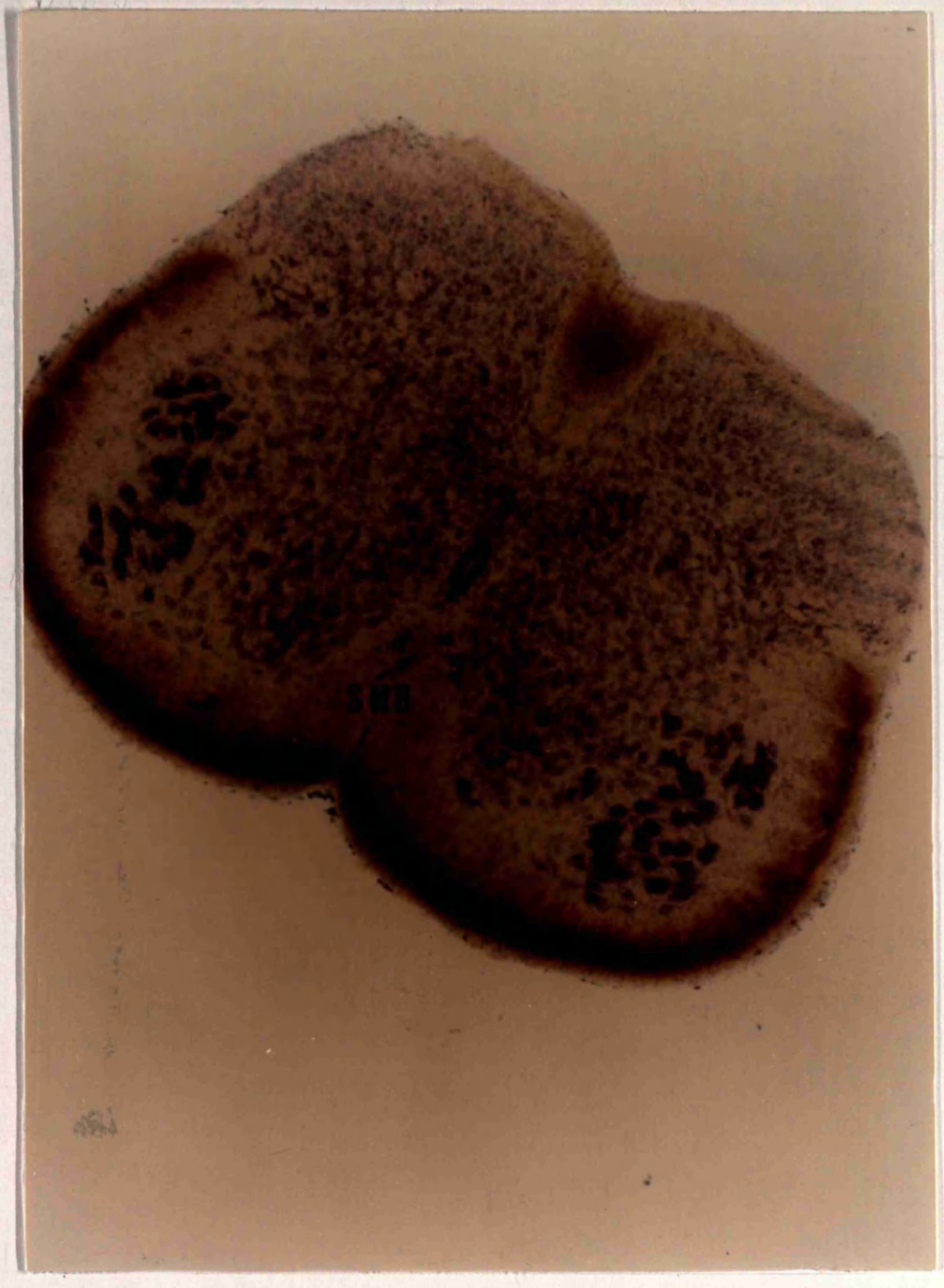


Figure 9. A section of spinal cord from an intact, male AJ mouse at level L5, showing a prominent SNB (x40).

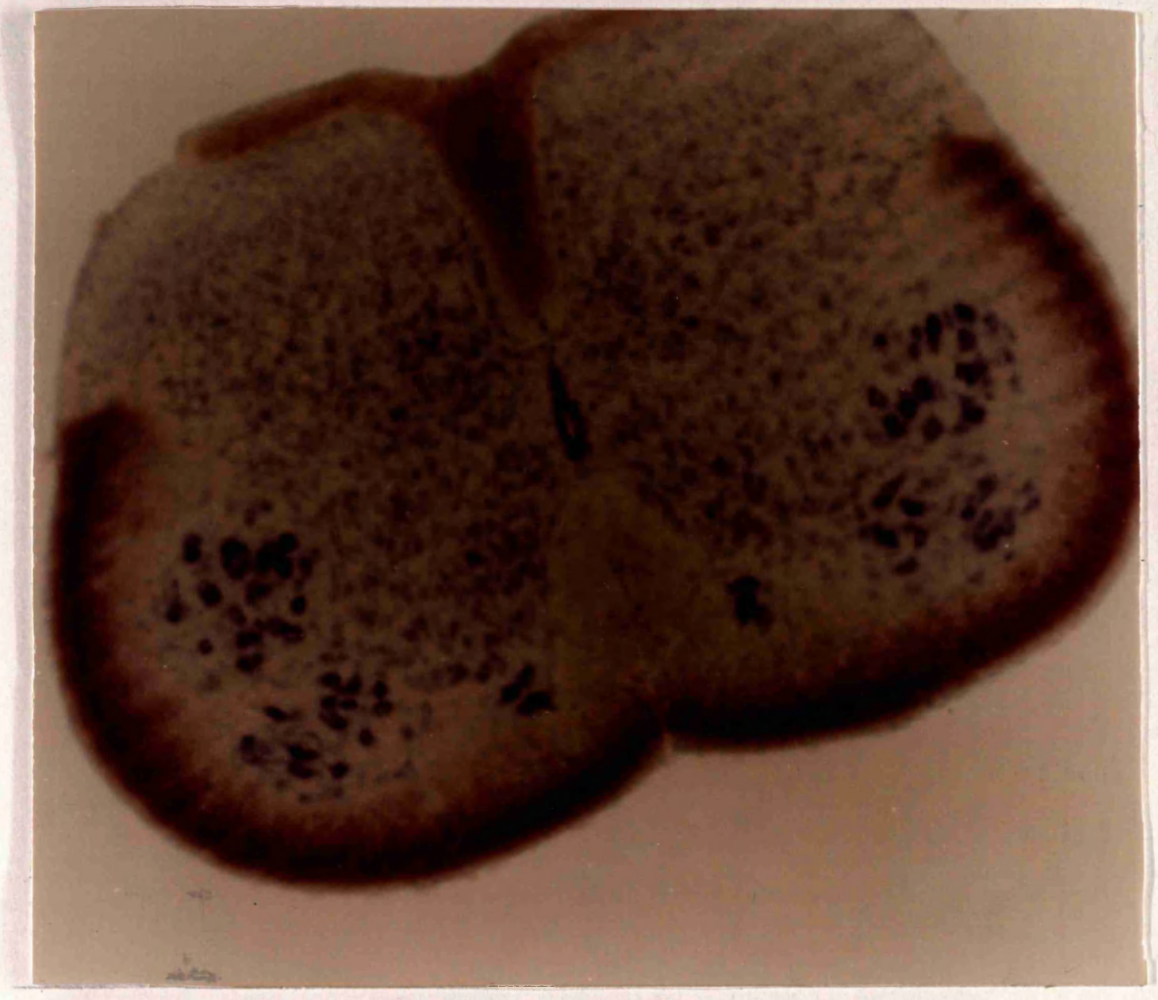
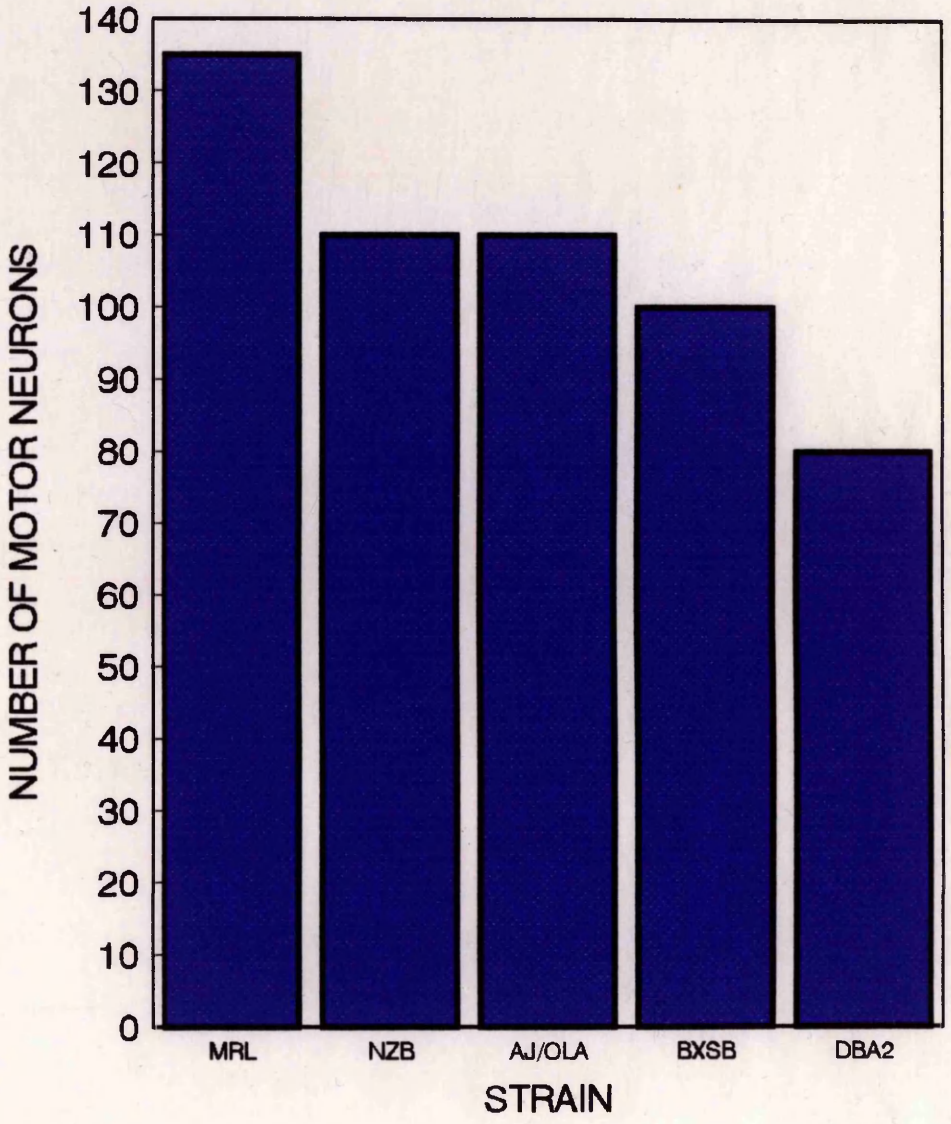


Figure 10. A section of spinal cord from an intact, female AJ mouse at level L5, with no obvious SNB nuclei present (x40).

Fig. 11

Interstrain comparison of SNB motor neuron number



In addition to the differences observed in neuronal number, differences in proximity to the central canal were also observed. This difference was at it's most striking between the DBA and the MRL strain. In the DBA, the SNB forms a discrete nucleus just ventral to the central canal and within approximately 200μm of the midline (Figs. 12 &13). In the MRL the SNB is more spread out, with neurons streaming towards the dorsolateral nucleus and at points running into it (Figs. 14 &15).

Figure 12. A section of spinal cord from an intact, male DBA mouse at level S1 (x40).

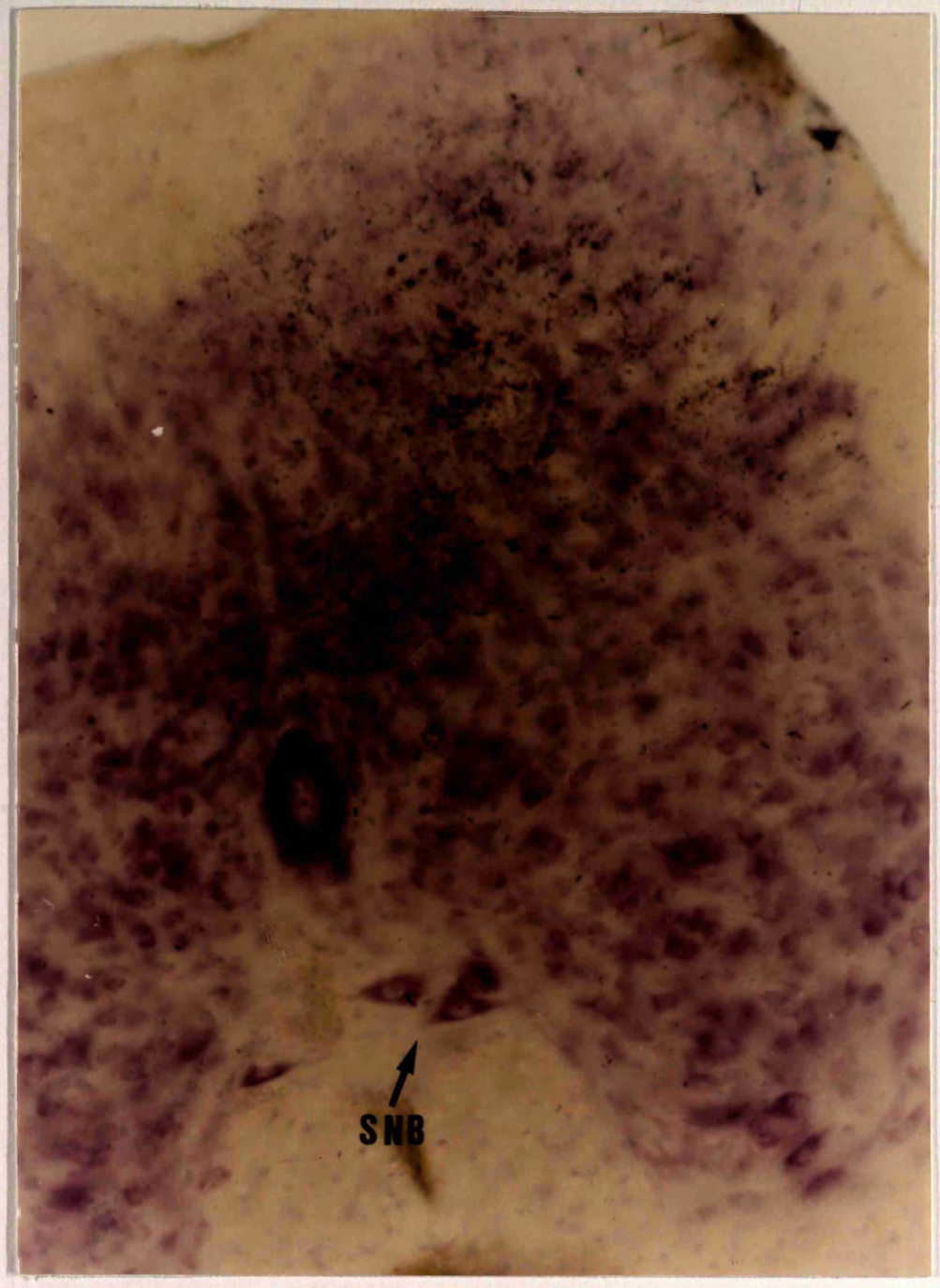


Figure 13. A section of spinal cord from an intact, male DBA mouse at level S1 (x100).

Figure 14. A section of spinal cord from an intact, male MRL mouse at level L5 (x40).

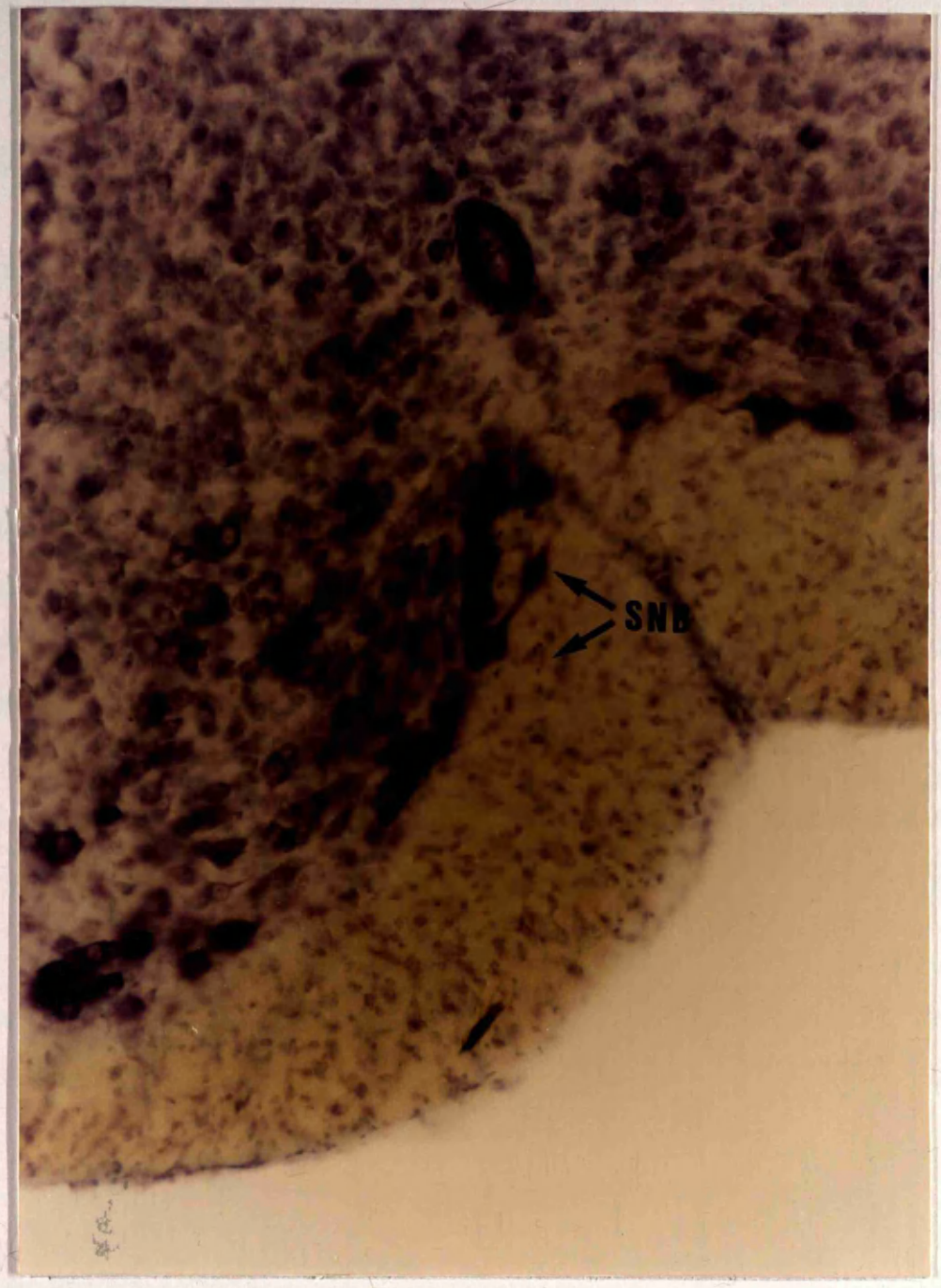


Figure 15. A section of spinal cord from an intact, male MRL mouse at level L5 (x100).

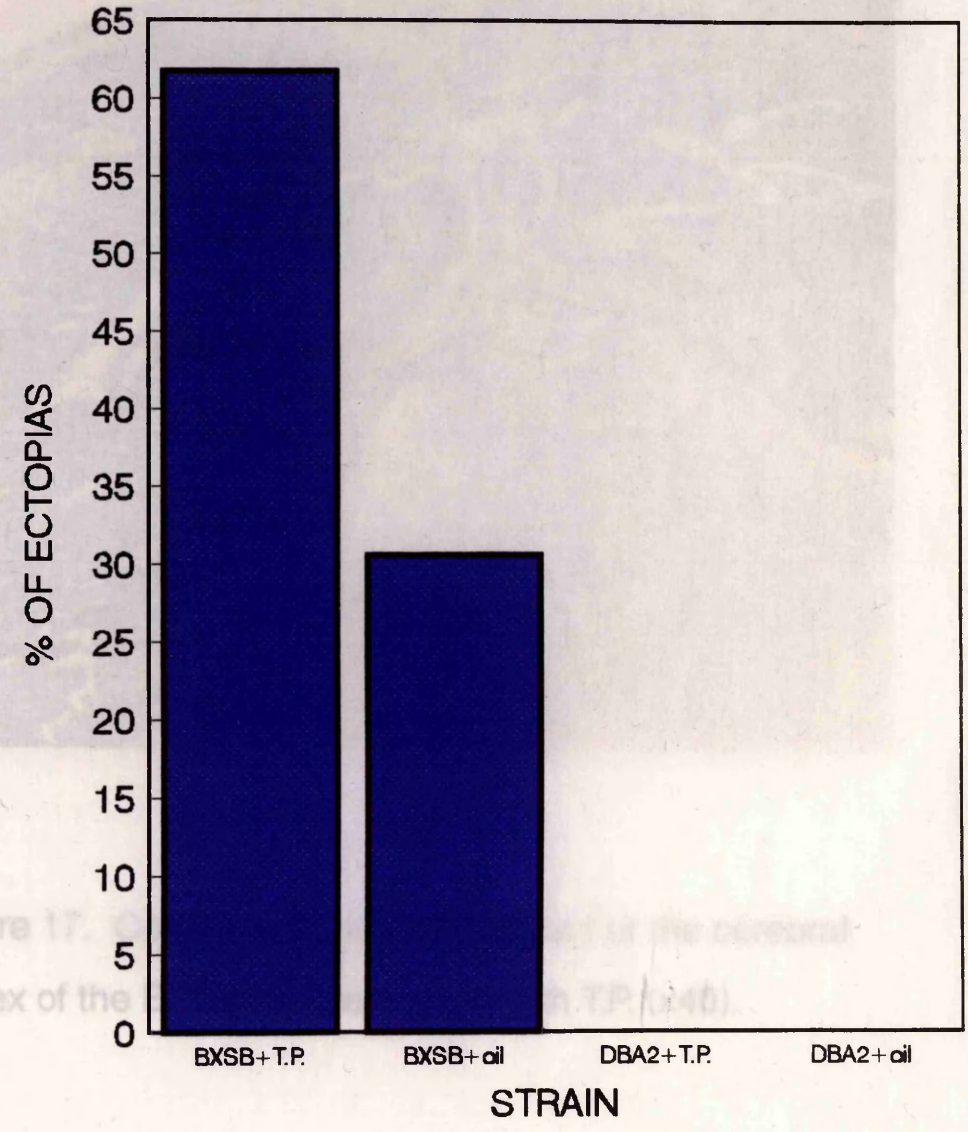
Experiment 5

Eleven out of 36 BXSB mice, plus oil vehicle (30.5%), and 21/34 BXSB mice plus testosterone propionate (61.7%), showed ectopic collections of neurons in layer I of the cerebral cortex (Fig. 16. $x^2 = 8.18$, d.f. =1, $p < 0.01$). The ectopias identified in the testosterone treated group, while differing in incidence, were similar in both size and location to those found in the control group, being predominately unilateral and located in the frontal motor cortices as can be seen from Figs 17 &18. Like the control group the ectopias consisted of abnormal accumulations of neurons in layer I of the cerebral cortex, as is seen from Fig. 19.

No ectopias were seen in the DBA mice regardless of treatment (Fig. 16). However, 25/34 (73.5%) of the DBA mice treated with testosterone propionate developed hydrocephalus (Fig. 20. $x^2 = 39.6$, d.f. = 1, $p < 0.001$), with moderate to severe dilation of the lateral ventricles (Figs. 21 & 22). Five out of 34 (14.7%) of BXSB mice treated with testosterone propionate, also developed hydrocephalus, although this did not reach levels of significance (Fig. 20).

Fig.16

Effect of testosterone propionate (T.P.) on incidence of cortical ectopias in the two strains



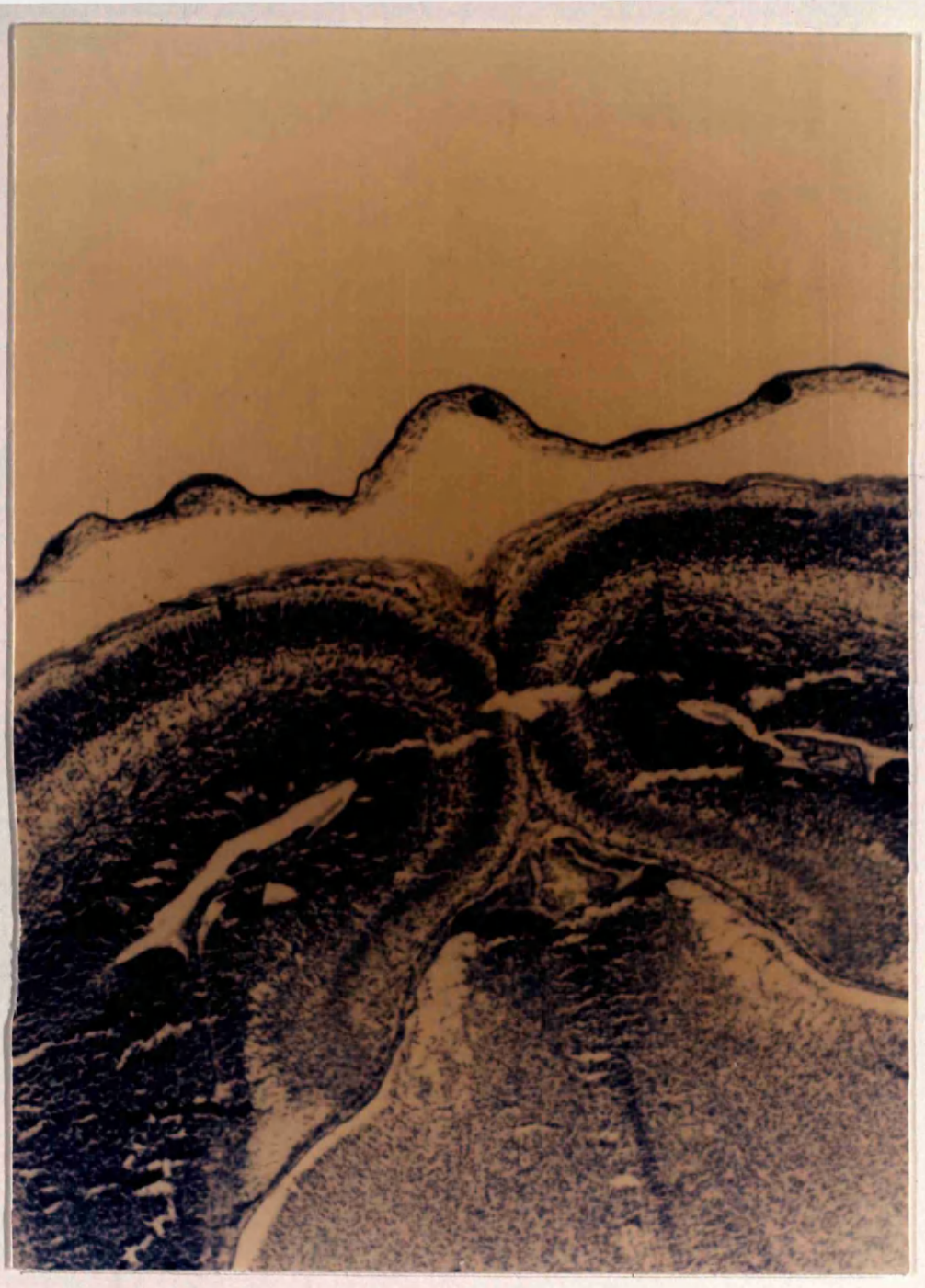


Figure 17. Cortical ectopia (->) in layer I of the cerebral cortex of the BXSB mouse treated with T.P. (x40).

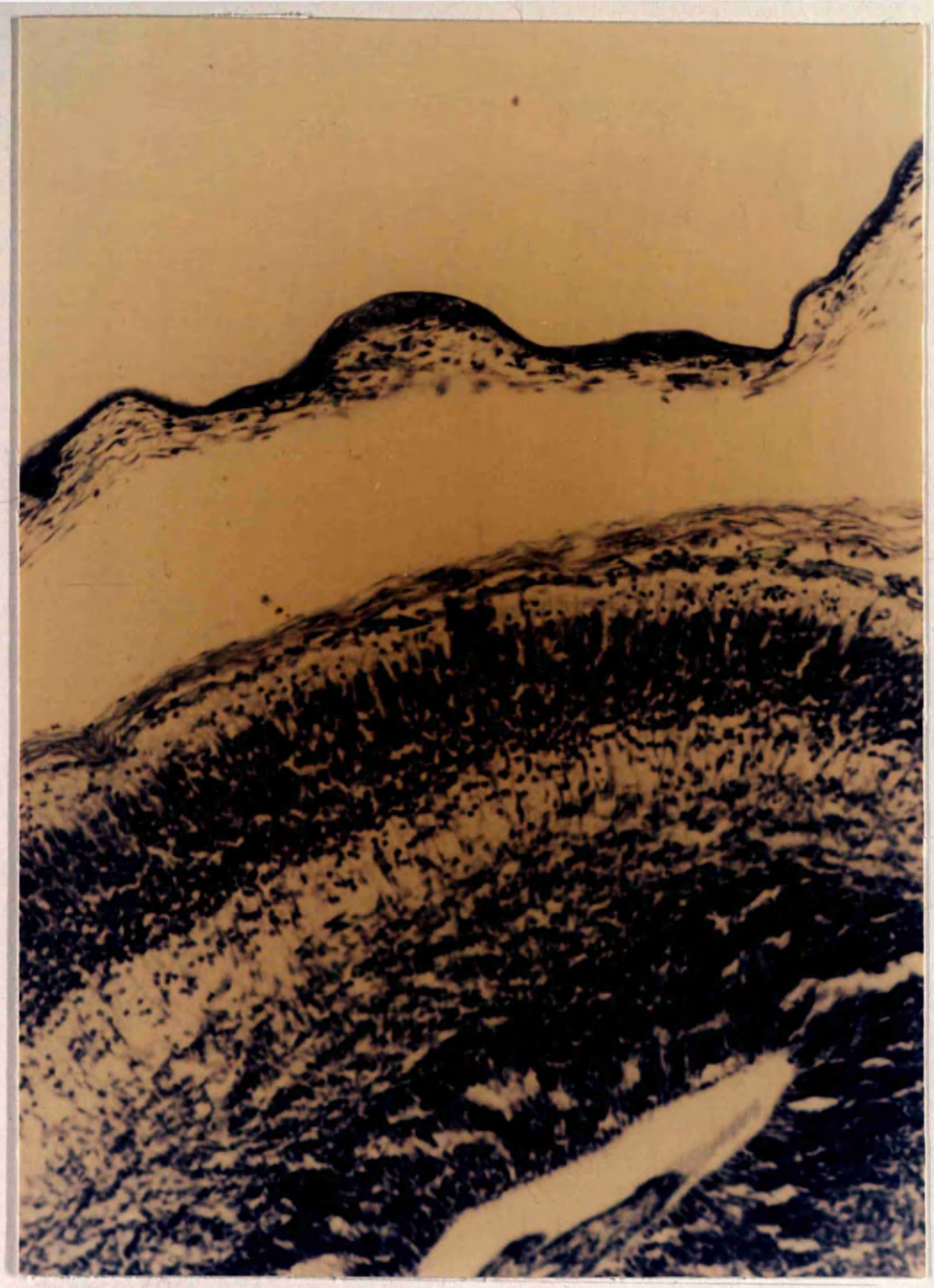
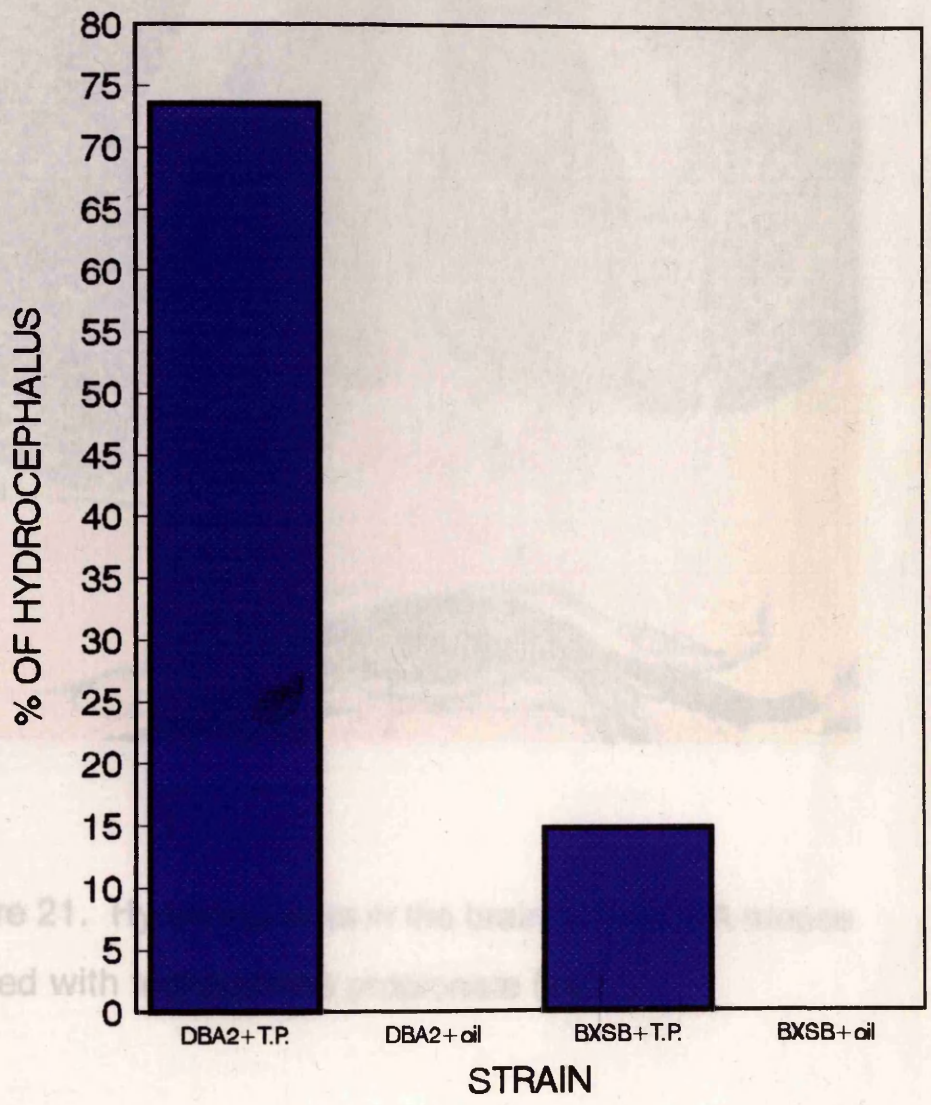


Figure 18. Cortical ectopia (->) in layer I of the cerebral cortex of the BXSB mouse treated with T.P. (x100).

Figure 19. Cortical ectopia (->) in layer I of the cerebral cortex of the BXSB mouse treated with T.P. (x200).

Fig.20

Effect of testosterone propionate (T.P.) on incidence of hydrocephalus



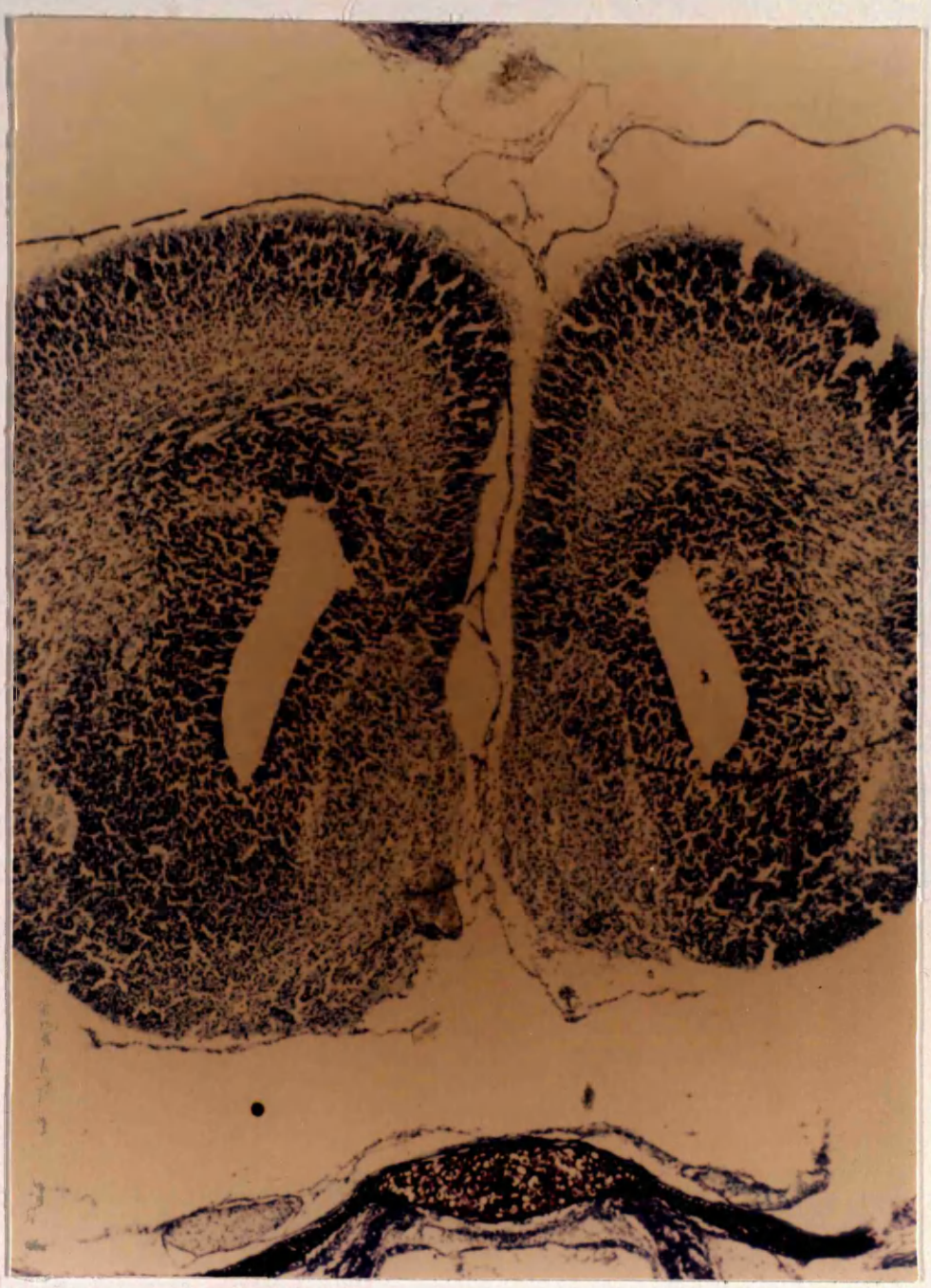


Figure 21. Hydrocephalus in the brain of the DBA mouse treated with testosterone propionate (x40).

Figure 22. Hydrocephalus in the brain of the DBA mouse treated with testosterone propionate (x40).

Discussion

Cerebral cortical ectopias, consisting of abnormal collections of neurons stretching from layer II into layer I of the cortex and up to the pial surface of the brain, are a feature of many congenital, toxic, vascular and infectious insults, including cerebral palsy (Christensen and Melchior, 1967) and primary generalised epilepsy (Meencke and Janz, 1984). Ectopias can be produced in animals by a variety of methods such as toxic and infectious insults (Dvorack et al, 1978 ; Sato et al 1982), that act during the period of mid to late gestation. There is however, one unusual circumstance in which ectopias are believed to be associated with;

1. Disorders of learning, such as developmental dyslexia.
2. The hormonal environment during development (Geschwind and Behan, 1982).

The concept that learning disorders of the brain have an anatomical basis, whilst being relatively recent, is now generally accepted (Geschwind and Galaburda, 1985). At first, it seems ridiculous to suggest that there might be an animal model for human developmental problems of learning, such as dyslexia, but there may be an animal model for the developmental anomalies which are associated with such disorders. The brains of all childhood dyslexics examined so far contain cortical regions whose structure is abnormal with two types of abnormality consistently identified. These consist of ;

1. Symmetry in the size of a language related region, which is normally larger on the left side.
2. The existence of ectopic collections of neurons in layer I of the cortex.

The symmetry observed in the brains of dyslexics is the result of an

increase in the volume of the planum temporale in the right hemisphere, rather than a reduction in the size of the left planum (Galaburda et al, 1987). In rats the volume of the visual cortex shows considerable variation which is dependent on the numbers of neurons present (Galaburda et al, 1985b).

In addition to the finding of symmetry in the brains of dyslexics, ectopic collections of cells, with associated distortion of the underlying cortical layers, have been identified in the normally acellular layer I of the cerebral cortex (Galaburda et al, 1985a). These ectopias are located primarily in the left hemisphere in the language-related area of the perisylvian cortex and vary in number from 20-100 ectopias/brain. Similar ectopic collections of neurons have also been reported in 15% of routine autopsy studies (Kaufmann and Galaburda, 1987); however, these are few in number and located in different areas to those seen in the dyslexic brain.

Sherman et al (1985; 1987) have identified a number of strains of mice in which unilateral cortical abnormalities are formed during development, which are similar in their location and morphology to the ectopias found in the brains of dyslexics. These include the New Zealand Black (NZB) and BXSB strains, where up to 30% of animals are affected, with more males affected than females (Sherman et al, 1987; Schrott et al, 1993). The ectopias in mice have been carefully studied and it was for this reason that this animal model was chosen for the present study. In the NZB, the ectopias are located primarily in the somatosensory cortex while in the BXSB they are found mainly in the frontal motor cortices, as outlined in the architectonic map of the neocortex of the normal mouse described by Caviness (1975). Normally only one or two ectopias are found per mouse brain and these have been shown by Golgi staining techniques and by immunocytochemical staining for vasoactive intestinal polypeptide (VIP) (Sherman et al, 1988), to contain both neurons

and glial cells. The number of cells comprising the ectopias varies from 20-50/section, and they stretch from layer II into layer I of the cortex and up to the pial surface of the brain in the most dramatic cases. Most of the neurons are normal and pyramidal in appearance, belonging to the class usually present in layer II and the upper part of layer III.

It has been shown that the presence of ectopias is linked to a wider disorganisation of the cerebral cortex, characterised by an increase in the number of neurons present in the hemisphere that contains the ectopia (Sherman et al, 1988 ; Sherman et al, 1990). A role for testosterone has been suggested in this due to ;

1. The known effects of testosterone on preventing neuronal cell-death during development (Breedlove and Arnold, 1980 ; 1983 ; Hauser and Toral-Allerand, 1989).
2. The predominance of ectopias in males (Sherman et al, 1987 ; Schrott et al, 1993).

It has also been suggested, due to the initial observation by Geschwind and Behan (1982) of an association between learning disorders and autoimmune disease, and the subsequent finding of ectopias in the brains of autoimmune mice (Sherman et al, 1987), that ectopias are caused by maternal autoantibodies crossing the placenta and damaging the developing brain. This seems unlikely since, despite extensive investigation, no significant correlation has been found between any immune factor and the incidence of ectopias (Schrott et al, 1993). In addition to this, ectopias have been reported in the brains of at least two strains of mice which do not develop autoimmune disease; the C57Bl/6 (Sherman et al, 1987) and the BXSB Yaa+ (Schrott et al, 1993), a strain related to the autoimmune BXSB but without the genes on the Y chromosome that cause autoimmunity (Roths, 1987; Roths and Murphy, 1984)

Genetic mechanisms seem unlikely explanations since inbred strains of genetically identical mice show marked differences in the development of ectopias (Sherman et al 1987). It seems likely, therefore, that a factor independent of the genotype of the animal is exerting influence upon the development of the brain at the critical period, and that this factor affects some animals more than others. This suggestion led naturally to the investigation of testosterone.

In the work described here , I carried out a series of experiments to investigate the effect of testosterone on the development of cortical ectopias in mice. It was hoped that the results might suggest comparisons with the development of similar lesions in humans which are associated with learning disorders.

I therefore used a radio-immunoassay, to determine serum levels of testosterone in adult males and females of strains which show cortical lesions (ectopias) and strains which do not. I then investigated the maternal serum levels at the critical period of neuronal development in mice.

In order to detect the neural effects of testosterone, I studied the development of a sexually dimorphic spinal cord nucleus, the spinal nucleus of bulbocavernosus (SNB), which is known to be dependent on androgen levels for its maintenance and survival. This quantitative anatomical approach was also used as an indirect measure of fetal testosterone exposure, because the small volume of serum obtainable from fetal mice proved difficult to assay accurately. Previous reports measuring fetal testosterone levels have used pooled samples (e.g. Weisz and Ward, 1980); this approach was not possible in the present study, since it would be essential to obtain testosterone values for individual animals in order to correlate with the incidence of ectopias.

The proximity in the uterus of a male pup to a female may also result in

increased delivery of testosterone to the female (Vom Saal and Bronson, 1980a) so that the placement of females in the uterus may have testosterone-related effects on the development of the brain. For this reason the sex and placement in the uterus of all pups was examined in relation to the development of ectopias.

Finally, I modified the uterine environment of the pups by administering testosterone propionate to the mother during the prenatal critical period for neurological development.

The end point of all the experiments was to identify whether testosterone could cause cortical ectopias in layer I of the frontal cortex of the mouse.

Fetal mice of both sexes were examined for the presence of cortical ectopias, amounting to a total, for experiments 1 and 5, exceeding 250 animals. Each brain was cut into 5 μm sections with every 10th section stained and examined. This led to the preparation and study of over 40,000 sections. The examination of the spinal cord was similarly very laborious and time consuming. A total of 75 cords were taken with over 3,000 sections prepared, stained and examined.

The inbred autoimmune strains of mice in which ectopias had first been described by Sherman et al (1987) were examined. They had reported that 29% of BXSB mice developed cortical lesions compared to none of the DBA mice, a nonautoimmune control strain. My results are very similar. Ectopic collections of neurons were present in layer I of the cerebral cortex in 31% of the BXSB mice, while no noticeable disturbance of cortical architecture was observed in the DBA mice, confirming the findings of Sherman et al (1987). The ectopias identified were located primarily in the frontal motor cortices, varied in incidence from 1-4 ectopias/brain and were composed of approximately 30-60 cells in the section examined. Under microscopic

examination the morphology of the cells was largely indistinguishable from the underlying cells in layer II of the cortex.

I then investigated the AJ strain, which has been reported to have naturally elevated adult levels of serum testosterone (Hampl et al, 1971) and which develops mild autoimmune phenomena with ageing (Yunis et al, 1972), in order to determine whether or not there was an association between life-long high levels of testosterone and the development of ectopias. Thirty three percent of AJ mice showed ectopic collections of neurons in the frontal motor cortices, similar in both size and morphology to those seen previously in the BXSB strain. My first study suggests therefore, that ectopias occur in strains characterised by both autoimmune features and/or increased levels of testosterone.

I then turned to study the incidence of ectopias in the two sexes of the different strains. I found that, as reported by Sherman et al (1987) and Schrott et al (1993), the incidence of ectopias was higher in males, with 58% of the BXSB and 62.5% of the AJ mice with ectopias being male. It has been suggested that this difference may be due to a much more severe effect on the female brain so that females with ectopias are spontaneously aborted or die before reaching adulthood (Schrott et al, 1993). My data do not support this hypothesis. In the present study pregnancy was terminated at day 18 of gestation. The entire uterus was removed and the pups were sexed prior to examination, by dissection and microscopic examination. Examination of the uterus revealed no evidence of resorptions or spontaneous abortions. The sex ratios of the litters was roughly 50:50 as would be expected. For my study, I examined the entire litter and confirmed that males were affected more often than females. These results suggest that the sex difference is real and that the data are not confused by resorption.

Although the hormones produced by the fetal gonads exert the major effect on sexual differentiation and dimorphism, the position of the pups in relation to siblings of the opposite sex is important since the intrauterine placement of pups is known to influence the degree of androgenisation of the brain. Female mice that develop in utero between two males (2M females) are, as adults, more aggressive and less sexually attractive to males than females that develop adjacent to other females (0M females; Vom Saal and Bronson, 1980a). Also, 2M females have a significantly longer oestrous cycle than 0M females if housed individually, but, initially a shorter cycle if housed in groups with a stud male (Vom Saal and Bronson, 1980b). Such differences are almost certainly due to the direct diffusion of testosterone from male to female fetuses at late gestation and subsequent androgenisation of the female brain (Vom Saal and Bronson, 1980a).

In the present study, fourteen litters were examined, containing an average of 8 pups. Over 100 fetal brains were therefore studied, with the entire brain cut into 5 μm sections, resulting in over 150 sections/animal and a total for the study exceeding 15,000 sections, each of which required individual examination. It is not possible to predict in advance which mice will possess ectopias, so it is necessary to screen the entire litter in this way. Indeed, in order to prove a statistical relationship between uterine placement and the incidence of ectopias, a much larger study would have to be carried out, possibly focussing on only one strain. Despite this problem preliminary data do suggest that there might be a relationship between the incidence of ectopias and uterine placement because all three AJ females that developed ectopias were 2M females, as were three out of five BXSB females. The remaining BXSB females were a1M and a 0M female. The sample size, however is too small for statistical analysis.

The finding of ectopias in the AJ strain suggested a possible relationship between naturally elevated levels of testosterone (characteristic of this strain) and the incidence of ectopias. To test this, an assay system was used, based on one used routinely for measuring human testosterone levels. Serum testosterone levels were measured in male and female NZB, BXSB and AJ mice, all of which are known to develop ectopias. Serum testosterone levels were also measured in the MRL/lpr, an autoimmune strain that does not develop ectopias but does show hydrocephalus, and in the DBA control strain.

In males the two highest mean levels of testosterone were found in NZB and AJ strains, with testosterone concentrations of 12.3 and 12.1 n mol/litre. The lowest mean testosterone level was observed in the BXSB strain, with a testosterone concentration of 4.62 n mol/litre. Hampl et al (1971) assaying pooled plasma samples from males of a comparable age to those used in this study, have reported testosterone concentrations ranging from 7.1 n mol/litre for the B10 strain, to 12.3 n mol/litre for the AJ strain. Considerable variation in testosterone levels was observed between individual mice of the same strain (see Table. 1), making any differences between the strains nonsignificant. The reasons for the wide intrastrain variation are unclear, some of the values (e.g. 1.2 n mol/litre) are more in keeping with pre-pubertal levels of testosterone and yet all the animals examined were of a similar age and sexual maturity. In retrospect it is unfortunate that at the time of sacrifice the reproductive organs were not fixed and removed. This would have allowed histological examination of the reproductive tracts of the male mice and would have determined whether or not active spermatogenesis was in progress. Furthermore this would have revealed whether interstitial Leydig cells, which are responsible for testosterone production, were present and/or active in the

testes of those animals with very low testosterone values. Other reasons for intrastrain variation include the pulsatile release mechanism of testosterone and the presence of dominant males which may have higher levels of testosterone than their litter mates (Goy and McEwan, 1980), although the differences observed in this study exceed changes normally attributable to these mechanisms. From the testosterone assay data it can be seen that the extraction procedure was accurate and reproducible. If the testosterone values are reanalysed, omitting very low testosterone levels (those < 2 n mol/litre), then a slight significant difference is seen between the AJ and the BXSB strain, with the AJ strain being significantly higher ($p < 0.05$). All other differences between groups remain non-significant. Since both the AJ and the BXSB strain develop cortical ectopias it appears that, even with making adjustments for unexpectedly low testosterone values, there is no correlation between androgen levels in adult males and the incidence of cortical ectopias. None of the non-pregnant females from any of the strains examined had testosterone levels that were high enough to fall within the sensitivity range of this assay system (all < 0.7 n mol/litre).

It appears, therefore, that there is no correlation between naturally elevated levels of testosterone in the adult and the formation of ectopias. Such a finding is perhaps not unexpected since the formation of ectopias is believed to be a late gestational event. Thus to affect development of the brain testosterone would need to act at the period of late gestation when ectopias are believed to form. I concentrated therefore on the level of testosterone in pregnant adult females. Testosterone levels were measured at late gestation, on day 18 of pregnancy, in NZB and BXSB mice as well as in the DBA control strain. My results indicate a significant difference in testosterone levels between the BXSB and DBA strains ($p < 0.025$), with testosterone levels

higher in the BXSB. The difference between the NZB, which showed considerable intrastrain variation with two unexpectedly high values (see Table 2), and the DBA did not prove significant.

The testosterone values observed (3.6 - 7.0 n mol/litre) are comparable to those reported by vom Saal and Bronson (1980a), who observed maternal plasma testosterone levels of between 1.47 - 1.55 ng/ml (5.1 - 5.3 n mol/litre), on day 17 of pregnancy. Similarly Humphreys et al (1985) have detected maternal levels of testosterone as high as 3 n mol/litre on day 17. Other investigators have, however, reported lower levels for plasma testosterone at this period. These include, Barkley et al (1977), who observed levels of 130 pg/ml (0.45 n mol/litre) and Soares and Talamantes (1981) who observed levels of 300 pg/ml (1.04 n mol/litre) on day 17 of pregnancy. Jones and Chevins (1993) have recently reported plasma testosterone concentrations in pregnant mice of 1.8 n mol/litre on day 17 of pregnancy. The reasons for these discrepancies are unclear. Humphreys et al (1985) have shown that maternal plasma testosterone concentration increases relative to fetal number, so that values as low as 0.6 n mol/litre were detected in the plasma of a dam with only 1 fetus, while in dams carrying 10 or more fetuses the value is nearer 3 n mol /litre. This mechanism is, however, unlikely to account for the low levels reported by Barkley et al (1977) and does not explain the differences observed between the strains I studied, which had litters of similar size, containing 8-12 fetuses. More likely explanations to account for the differences between the groups, include differences in assay sensitivities and specificities and strain differences in testosterone production. Different groups have used different antisera with potentially varying ligand binding capacities and hence differing sensitivities. Likewise different groups have studied different strains of mice. Vom Saal and Bronson (1980 a) carried out their

studies on the CF-1 strain, whereas Soares and Talamantes (1981) examined the C3H strain and Jones and Chevins (1993) examined the CD -1 strain. Such strain differences could conceivably be the result of unequal sex ratios between strains, with those strains that have higher testosterone levels producing more male fetuses. Such a mechanism does not however account for the strain differences in my study as the sex ratio in each of the strains examined was roughly 50:50.

My results indicate that strain differences in testosterone levels can be detected in dams on day 18 of pregnancy, with the BXSB strain having significantly higher levels than the DBA. It appears for this experiment, therefore, that at least for BXSB females there is a correlation between elevated levels of serum testosterone at late gestation and the development of ectopias. It is important to note that the assay system employed detects both bound and free testosterone. Since, only the free form is active, elevated levels of testosterone are not necessarily a measure of increased testosterone bio-activity. Whether the source of the elevated levels of testosterone was maternal or fetal is not shown by this experiment. Embryo transfer experiments, however, where fetuses that develop ectopias are transferred at an early stage of gestation to a maternal host that does not, or vice versa, have helped to elucidate this point. The incidence of ectopias is unaltered by the change of uterine milieu, indicating that factors independent of the maternal host are responsible for their formation (Denenberg et al,1991 b). Secretions from the fetal testes are likely to be the most important source of testosterone. It may be that the elevated levels of testosterone reported for the AJ mouse are an indicator of elevated levels throughout the lifespan of this strain, including intrauterine life, with either the mother or male embryos producing high levels of testosterone during gestation. A more extensive study, in which

testosterone levels were monitored throughout pregnancy would give valuable insight into the likely uterine environment.

Of course, an association between elevated testosterone levels and strains that develop ectopias does not directly demonstrate that any effect of testosterone upon the nervous system of these strains has occurred. In order to establish this, the spinal nucleus of bulbocavernosus (SNB), a sexually dimorphic motor nucleus which is directly influenced by testosterone levels, was examined to determine if differences in neuronal numbers were detectable between strains. Firstly, this would suggest differences in androgen effect upon the developing nervous system and secondly this could be correlated with the incidence of cortical ectopias.

My experiment confirmed the sexually dimorphic nature of the SNB in the mouse, which was first reported by Wee and Clemens (1987). In addition to the differences seen between male and female mice, striking differences were observed in the number of motor neurons in the SNB between the different strains. The DBA had significantly fewer motor neurons than any of the strains that developed ectopias, indicating a lesser effect of testosterone upon the nervous system of this strain. Interestingly, the MRL strain, which does not develop ectopias but does develop hydrocephalus and associated behavioural disturbances such as poor performance in a nonspatial discrimination task (Denenberg et al, 1992), had significantly higher numbers of motor neurons than any of the other strains examined.

In addition to the differences in neuronal number within the SNB between the strains, differences were also seen in the configuration of the SNB. Strains with the highest numbers of neurons showed a divergence from the normal descriptions of the SNB as a discrete grouping of motor neurons reported for both the rat and the house mouse (Breedlove and Arnold, 1980;

Wee and Clemens, 1987), with an increasingly diffuse spread of neurons away from the central canal, and towards the ventromedial nucleus. The reasons for this are unclear, but it may represent excessive migration of neurons due to high androgen levels which fail to complete the migration path, or a failure of inappropriately placed neurons to undergo apoptosis.

Interstrain differences of motor neuron number in the SNB have been reported previously for the mouse (Wee and Clemens, 1987). The DBA strain male was reported as having 80 motor neurons, a figure directly comparable to that observed in this study, while the C57BL6 strain male was reported as having 115. It is interesting to note that Sherman has reported an 11% incidence of cortical ectopias in the C57 strain (Sherman et al, 1987), further suggesting that levels of perinatal androgens are important in the establishment of ectopias. The differences in the SNB observed between strains could be due to either increased sensitivity of some strains to the effects of testosterone, possibly mediated at the receptor level, or to differences in the relative amounts of testosterone that the fetuses are exposed to during the "critical period" of neurological development. The results obtained from assaying testosterone levels at late gestation would indicate that the latter mechanism is the more likely explanation, at least for the NZB, BXSB and DBA strains.

It seems, from the above results, that there is an association between the presence of cortical ectopias, increased levels of testosterone and a testosterone-mediated effect upon the nervous system, such as formation of the SNB. While the demonstration of such associations would suggest that testosterone may be involved in the formation of the ectopias, they fail to provide any direct evidence of a cause and effect relationship.

For my final series of experiments, therefore testosterone propionate

(T.P.) was administered to pregnant mice of the BXSB and the DBA strains, during the prenatal "critical period" of central nervous system development, to study the effect on the brain of artificially increasing the testosterone concentration at this time. Ectopias were detected in 61.7% of BXSB mice treated with T.P. as opposed to only 30.5% of BXSB given placebo. No ectopias were detected in DBA mice regardless of treatment.

This is the first direct evidence to show that increased levels of testosterone circulating in the uterus at the critical period can lead to the formation of cortical ectopias. The mechanism by which testosterone causes these lesions is still unclear. In the light of the above work and that done by others showing an increase in the number of vasoactive intestinal polypeptide (VIP) neurons occuring in the hemisphere containing the ectopia (Sherman et al, 1988), with extensive underlying cortical disorganisation in layers II and III and dense radially oriented fibre bundles (Sherman et al, 1990), it seems that increased testosterone levels can lead to an excess of neurons. This neuronal excess in turn, may result in the inappropriate placement of neurons which is seen in the ectopias and underlying cortical disorganisation. Whether this increase is due to increased migration of neurons, or to an inhibition of neuronal cell death, remains to be resolved.

The most obvious changes are likely to be seen in sexually dimorphic areas of the nervous system that have the highest concentation of androgen receptors, such as the SNB and other areas invoved in reproductive activity, while more subtle changes, such as the development of ectopias, may occur in areas with relatively fewer androgen receptors. Such disturbances of architecture may well result in the failure of the cortex to make the appropriate connections necessary for normal function and so lead to a variety of abnormalities, depending on the location of the ectopias.

The mechanism by which testosterone prevents cell death remains unresolved. It is clear that phenotypically different strains produce different amounts of testosterone throughout life, so that it is also likely that different strains respond differently to the same amount of testosterone stimulus, stressing the importance of genetic susceptibility coupled to a testosterone effect in producing ectopias. This is exemplified by the different results obtained from administering testosterone to prenatal BXSB and DBA mice. Administration of testosterone to BXSB mice increases the incidence of ectopias, while the same hormonal treatment of DBA mice resulted in no ectopias, but in a large percentage of the animals developing hydrocephalus. This is particularly interesting in view of the demonstration of a high degree of hydrocephalus in the MRL strain, with 46% having severe dilatation of the lateral and third ventricles, and a further 30% showing moderate dilatation. Examination of the SNB in MRL mice shows evidence of high exposure or high sensitivity to testosterone, indicating a possible strong testosterone effect on the nervous system. How testosterone could promote the development of hydrocephalus remains unclear, but there was no evidence of inflammation. It may be therefore that this was a developmental anomaly, similar but more severe than the appearance of cortical ectopias. Prenatal treatment of rats with specific anti-androgens, such as flutamide, coupled with neonatal castration has been shown to completely demasculinise the SNB (Breedlove and Arnold, 1983). It would be of great interest in a future study to determine what effect prenatal treatment with anti-androgens would have on the incidence of cortical ectopias.

The presence of ectopias in the frontal cortex appears to have behavioural implications. In humans, the presence of ectopias, together with symmetry in a normally asymmetric region of the planum temporale is

associated with developmental dyslexia. The two together are believed to be responsible for the learning difficulties associated with it. Disturbances in behaviour have also been reported in strains of mice that develop ectopias. NZB mice show difficulty in learning a conditional avoidance response (Nandy et al, 1983), with only 1/33 NZB mice able to learn to avoid shock in an active avoidance paradigm, as opposed to 33/35 mice of another strain. NZB mice also perform poorly on a passive avoidance task and are initially less active in the open field (Spencer and Lal, 1983). BXSB and DBA mice perform differently on a variety of behavioural tasks, including water escape, water discrimination and paw preference. More importantly, differences in the water escape and water discrimination tasks are seen between BXSB mice with ectopias and those without (Denenberg et al, 1988). However the behavioural disturbances observed in the BXSB are not solely linked to the presence of ectopias; thus two different, genetically inbred sub-strains of BXSB mice, one autoimmune and one non-autoimmune, have similar incidences of ectopias but differ markedly in response to a variety of behavioural tests, including water escape and discrimination learning (Schrott et al, 1993). Thus it appears that the behavioural disturbances observed in BXSB mice are likely to be caused by at least two different mechanisms, one of which is linked to differences in autosomal genes and the other linked to the presence of ectopias .

In conclusion, the results of this work confirm that cortical ectopias in mice are associated with high levels of testosterone in utero and that their incidence can be increased by the prenatal administration of testosterone. It is suggested that testosterone plays an important role in the development of the human cortical ectopias which are characteristic of developmental learning disorders.

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