

# Open Research Online

---

The Open University's repository of research publications and other research outputs

## Sexual differences in synaptogenesis in the suprachiasmatic nucleus of the rat brain

### Thesis

How to cite:

Leblond, Christine B (1983). Sexual differences in synaptogenesis in the suprachiasmatic nucleus of the rat brain. MPhil thesis The Open University.

For guidance on citations see [FAQs](#).

© 1982 The Author

Version: Version of Record

---

Copyright and Moral Rights for the articles on this site are retained by the individual authors and/or other copyright owners. For more information on Open Research Online's data [policy](#) on reuse of materials please consult the policies page.

---

[oro.open.ac.uk](http://oro.open.ac.uk)

UNRESTRICTED

SEXUAL DIFFERENCES IN SYNAPTOGENESIS  
IN THE SUPRACHIASMATIC NUCLEUS  
OF THE RAT BRAIN

CHRISTINE B. LEBLOND

Submitted for the degree Master of Philosophy  
Biological Sciences  
The Open University

SEPTEMBER 1982

Date of submission: OCT 1982

Date of award: 26.4.83

ProQuest Number: 27777206

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent on the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 27777206

Published by ProQuest LLC (2020). Copyright of the Dissertation is held by the Author.

All Rights Reserved.

This work is protected against unauthorized copying under Title 17, United States Code  
Microform Edition © ProQuest LLC.

ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106 - 1346

## ABSTRACT

1. A literature review of the effects of perinatal sex steroids on the development of the brain is presented. It can be shown that the hormones can modify the morphology of discrete areas of the brain.
2. It was concluded that there was a lack of information about how soon these morphological changes appeared.
3. It was therefore decided to study the effects of sex and perinatal hormonal levels on synaptogenesis in a discrete area of the brain.
4. The Suprachiasmatic Nucleus (SCN) was chosen for anatomical convenience and because of its role in the photocontrol of ovulation.
5. In this thesis I describe synaptogenesis in the suprachiasmatic nucleus of rats of both sexes ranging from prenatal (20 days of gestation) to adult and in neonatally androgenised females. Preliminary results from neonatally castrated males are also included.
6. Synaptogenesis was studied using morphometric analysis of electron micrographs. It was found that the rate of development of synapses was similar in the SCN of both sexes, but that absolute values in males were higher than in females. Male type development cannot be mimicked by neonatal androgenization but initial results suggest that femal type development can be induced by neonatal castration.



7. My results suggest that both prenatal and postnatal androgens are essential to normal male development.

Table

Page

- |   |  |    |
|---|--|----|
| 1 | Statistical differences between male and female and androgenised female and neonatally castrate males.     | 98 |
| 2 | An analysis of sexual dimorphism in the area occupied by synaptic vesicles in normal female and male rats. | 99 |

## Abbreviations

E <sub>2</sub>	17β oestradiol
T	testosterone
DHT	5α dihydrotestosterone
P	progesterone

### ACKNOWLEDGEMENTS

I would like to express special thanks to Dr. P. Thomas, Pharmacology Department, Chelsea College, for his help and encouragement throughout the project and to Dr. M. Stewart, Biology Department, Open University for his guidance.

Thanks to Mr L. Foster and Miss E. Seeds for their help in preparation of the animals and to Mr M. Wineberg and Dr. A. Brain for help in the E.M. Unit.

I am most grateful for the help given by Steven Morris, Computer Studies, Chelsea College, who carried out the statistical analyses on the results.

Thanks to Mrs A. Gregory for typing the manuscript.

## CONTENTS

### INTRODUCTION

Sexual Differentiation - Background	1
Sexual Differentiation - Mechanism	6
Is testosterone the true masculinizer?	9
When does masculinization take place?	10
What are the mechanisms of masculinization?	16
The role of neurotransmitters in sexual differentiation	33
Model	39
The Synapse	44
The Suprachiasmatic Nucleus	45
Functions of the Suprachiasmatic Nucleus	50

### MATERIALS AND METHODS

Animals and Treatments	51
Processing	53
Location of SCN in Epon Blocks	57
Ultramicrotomy	62
Photography	62
Morphometric analysis	62
Phosphotungstic Acid (PTA) staining	67

<u>RESULTS</u> - descriptive	70
Synapses	71
Vesicles	72
Results - quantitative	89
Synapses - normal male, female and castrated male	89
Vesicular Area - normal males, female and castrated male	95
Synapses and Vesicular Area - Androgenised females	96

## DISCUSSION

Processing of samples	100
Location of SCN	101
Stereology	101
Randomness of sampling	101
Statistical	102
Neglect of Biological Rhythmicity	102
Pattern of development of synapses	103
Pattern of vesicle formation	103
Differences apparent from birth	104
Synapses	106
How stable are synapses once formed?	107
Proposed extensions of work and Experiments in hand	108
General comments	109
References	110

## ILLUSTRATIONS AND TABLES

Fig. No.		Page No.
1.	Relationship between Hypothalamus and pituitary gland.	4
2.	Positive and negative feedback mechanisms.	5
3.	Consequences of treatment with testosterone, oestrogen and progesterone on brain development.	7
4.	Classes of hormone which can and cannot masculinise.	11
5.(A)	The major sex steroids.	12
(B)	Enzyme systems capable of metabolizing testosterone.	
6.	Action of testosterone and oestrogen on male and female brains.	14
7.	A model for the action of oestradiol.	17
8.	The distribution of cytoplasmic and nuclear receptors in the brains of newborn females.	19
9.	Distribution of cytoplasmic receptors for oestradiol in the brains of female adult rats.	21
10.	The effect of testosterone on the hypothalamic/amygdaloid area and cortex of the brain.	22
11.	The distribution of cytosol oestrogen receptors in the anterior, middle and posterior hypothalami of adult female, male and castrated rats.	24

Fig. No.		Page No.
12.	The stimulation of adenylate cyclase by neurotransmitters.	27
13.	Adenylate cyclase activity in the hypothalamus of 5 day old rats and it's response to neurotransmitters.	28
14.	The activation of the adenylate cyclase system by a neurotransmitter leads to the formation of cAMP.	29
15.	Adenylate cyclase activity in the anterior hypothalamic of brains of adult rats.	30
16.	Some trophic effects of cAMP in neural tissue.	31
17.	Use of neural circuit leads to its stabilisation.	34
18.	The selective stabilization of synapses.	35
19.	Denervation supersensitivity.	37
20.	A model for the interaction of oestrogen cAMP and neurotransmitters.	40
21.	The synaptic junction.	42
22.	Some examples of neurotransmitters.	46
23.	Neurotransmitter release at the synaptic junction and its consequences.	47
24.	Some different types of synaptic junction.	48
25.	Location of SCN in the hypothalamus.	49



Fig. No.		Page No.
26.	Sections taken for processing of prenatal brains.	54
27.	Slicing of brains for processing for electron- microscopy.	56
28.		59
29.	Examples of light micrographs used for locating	60
30.	the SCN in immature animals.	61
31.	Location of SCN in brain and trimming of block	63
32.	for electronmicroscopy.	
33.	The Holmes Effect.	66
34.		
35.	Light micrographs of SCN.	74
36-49	Selection of electronmicrographs.	75-88
50. (A)	Graphs of synaptic contacts for normal males, females and castrated males.	90
(B)	Numbers of synapses for normal males, females and castrated males.	91
51.	Areas of vesicles for normal males, females and castrated males.	94
52.	Results of areas of vesicles, numbers of synapses and synaptic length in androgenised females.	96

## INTRODUCTION

The sexual differentiation of the brain is not solely and directly under genetic control but is brought about by sex hormones acting in infancy. Neurotransmitter activities also have an effect on brain differentiation and it seems that they, and sex hormones, interact at the second messenger (cAMP) level. What is not clear, however, is the age at which the earliest manifestation of sexual differentiation occurs.

The work in this thesis shows that sexual differences in synaptogenesis are found at a very early age and it seems that prenatal as well as postnatal stimuli are involved.

The sexual differentiation of the brain is of fundamental importance to an understanding of brain development and function in general since:

- 1) It is not solely and directly under genetic control; it is an aspect of brain development which is influenced by the internal environment.
- 2) It involves only a small part of brain function (there are many more similarities than differences between the brains of males and females).
- 3) It distinguishes between two normal states; it is therefore an example of an extremely subtle aspect of brain development.

#### Sexual Differentiation - Background

Differences between adult male and female rats are manifested both behaviourally and physiologically as summarised below:

##### Behavioural



maternal  
lordosis



aggression  
territorial  
mounting

##### Physiological



sexual cycle  
ovulation  
+ve) feedback  
-ve) response  
mechanisms



acyclic  
-ve) feedback  
response only

Under appropriate conditions the female displays lordosis and maternal behaviour and when appropriate the male displays aggression, territorial and mounting behaviour. (for recent reviews see 1,2)

Underlying these are physiological differences, the female has a sexual cycle<sup>3</sup> and ovulates regularly; the male is acyclic. These peripherally manifest endocrine differences are under the control of the anterior pituitary which in turn is controlled by the releasing factors of the hypothalamus. (Fig. 1)

The classical experiments of Harris<sup>4</sup> involving the transplantation of pituitaries in rats of both sexes show that for the pituitary to function properly it must lie adjacent to the hypothalamus; which is itself sexually differentiated. Thus as well as the more obvious peripheral differences there are possible neuroendocrine sexual differences. Later work has shown that one of the main differences is the so-called "feedback mechanism" where both pituitary and peripheral hormones influence hypothalamic function. The pituitary hormones we are concerned with here are the gonadotrophins - follicle stimulating hormone (F.S.H.) and luteinizing hormone (L.H.) The peripheral hormones we are concerned with are the gonadal steroids. The releasing factors, gonadotrophins and gonadal steroids interact in a complex manner influencing each others secretion giving rise to the so-called "feedback mechanisms" as described below. There are two types of feedback mechanism (Fig 2) 5,6,7,8

- 1) Negative feedback - under certain conditions an increase in the concentration of peripheral hormones acts on the pituitary

**HYPOTHALAMUS**  
**MEDIAN EMINENCE**

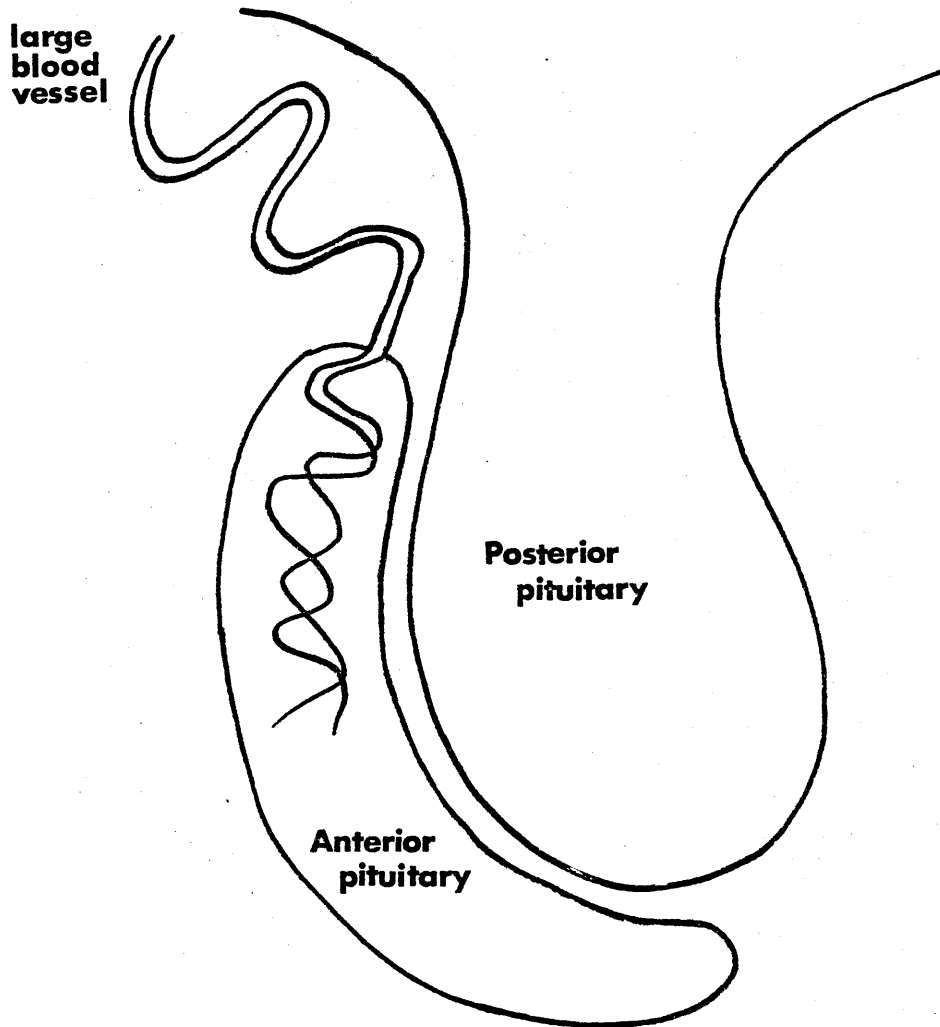


Fig. 1

The releasing factors pass from the median eminence in the hypothalamus via portal blood to the anterior pituitary where they bathe the cells in high concentrations.

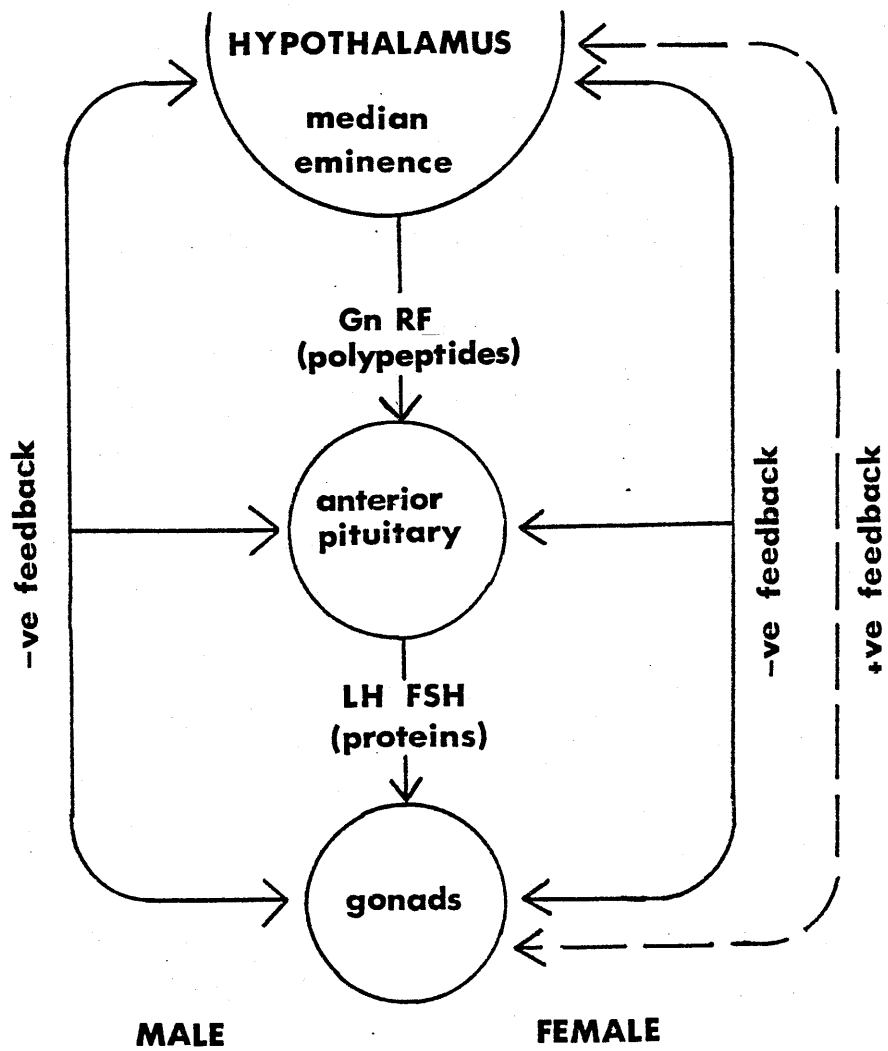


Fig. 2.

The positive and negative feedback control by the hypothalamus in male and female rats. The diagram is described in detail on page 3 in the text and here is concerned primarily with oestrogen regulation.

and hypothalamus and causes a decrease in the concentration of releasing and/or pituitary hormones.

- 2) Positive feedback - under certain conditions an increase in the concentration of peripheral hormones acts on the hypothalamus and leads to an increase in releasing and pituitary hormones.

The negative feedback response alone leads to the stabilising of hormone levels in the periphery. The positive feedback alone leads to an unstable increase of hormones in the periphery.

A combination of both negative and positive responses lead to fluctuations in hormone levels and cyclical changes as seen in the female in ovulation.

Perhaps the most striking difference is that the male has only the negative feedback response whereas the female, under appropriate conditions, has the capacity to respond to oestrogens by both negative and positive mechanisms.

#### Sexual Differentiation - Mechanism

Adult manifestations of male and female ness are dependent on the continued presence of the appropriate sex hormones. Thus a castrated male does not behave like a normal male<sup>9</sup> he will not display mounting or aggressive behaviour, nor does a spayed female behave like a normal female - she will not be responsive to mounting by a male. These behavioural 'losses' can, however, be made good by appropriate hormone replacement therapy.<sup>10,11,12</sup> However the male brain is not the same as the female in that the castrate male

Adult

Newborn

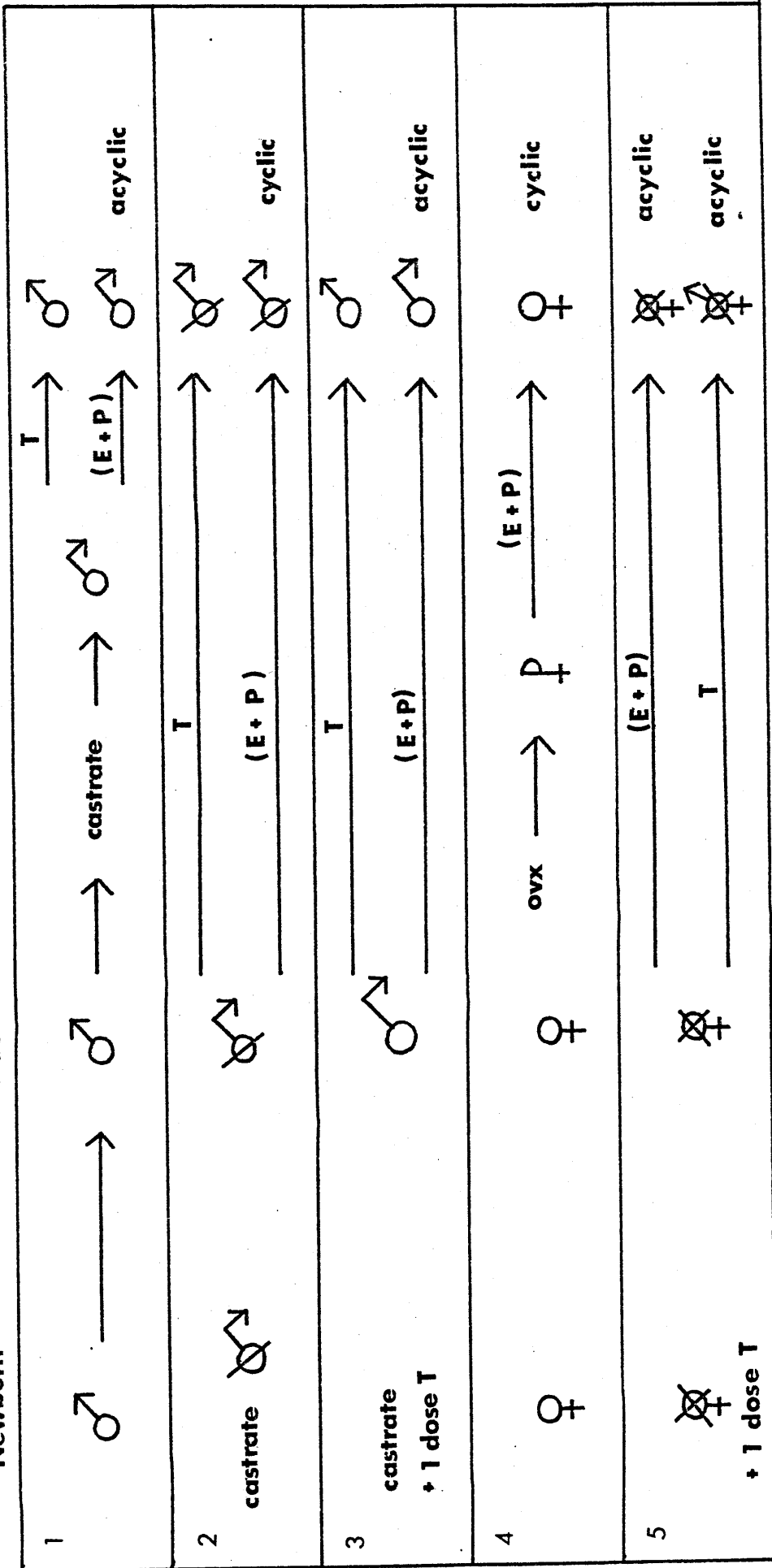




Fig. 3.

The consequences of treatment with testosterone (T) and oestrogen (E) and progesterone (P) on brain development.

- 1) A newborn male is allowed to reach adulthood and is then castrated, if testosterone is administered continually the animal will maintain a male state. If, after the adult castration, E&P are administered the castrated state behaviour persists and the animal has no positive feedback.
- 2) A newborn male is castrated and allowed to grow to adulthood. If T is then administered the animal still displays the castrated state behaviour. If E&P are administered in place of T the animal shows positive feedback and behaves in a female manner.
- 3) A male castrated at birth but given but one dose of T in infancy will grow up as a castrated male, but if a continual dose of T is administered the animal will display normal male sexual behaviour. If E&P are administered instead of the T the animal will maintain castrated state behaviour and will show no positive feedback.
- 4) If the newborn female is allowed to reach adulthood and the ovariectomized and E&P are administered the normal female behaviour and positive feedback will be restored.
- 5) A newborn female is given one dose of T shortly after birth, if on reaching adulthood E&P are administered the animal will fail to show female sexual behaviour or positive feedback. If the animal is given T when adult this leads to bizarre sexual behaviour.

for example will not respond to female sex hormones in a female manner nor will the spayed female respond to male sex hormones in a male manner. Thus it would seem that the brains must have to have an initial stimulus if they are to respond in an appropriate manner when adult.

### Neonatal Androgens

This initial stimulus required for response in the appropriate sexual manner is that of androgens immediately after birth<sup>4</sup>. It can be shown that these androgens masculinize the (potentially female) brain. A rat who is deprived of his endogenous androgens by castration immediately after birth cannot respond (when adult) to testosterone in a male way. If however he is given but a single dose of testosterone immediately after castration he grows up as a potential male. The effects of testosterone are therefore permanent; this poses three questions;

- 1) Is testosterone the true masculinizer?
- 2) When does masculinization take place?
- 3) What is the mechanism for masculinization?

### Is testosterone the true masculinizer?

Although it has been suggested that both oestrogens and progestogens<sup>13,14,15</sup> are necessary for full development of the female type brain I am primarily interested in the masculinizing effects of androgens of which testosterone is the most important. (Fig 3)  
Testosterone can act only as a masculinizer after being converted to oestradiol in the brain.

There is evidence to support this;

- a) oestrogens as well as androgens can act as masculinizers<sup>16</sup>
- b) masculinization (by either oestrogens or androgens) can be prevented by concurrent administration of anti- oestrogens  
17,18,19,20
- c) only those androgens which can be converted to oestradiol in the brain (testosterone for example) can masculinize whereas those which cannot be converted - for example 5  $\alpha$  dihydrotestosterone (5  $\alpha$  DHT) cannot masculinize<sup>21,22,23,24</sup>  
(Figs 4,5)

When does masculinization take place?

The critical period of masculinization is believed to be around the first five days after birth<sup>25</sup> in the rat, although the fifth day is not a rigid cut-off point, heavy and increasing pharmacological doses of hormones can cause masculinization at up to ten days, after then it is too late.

A Protective Mechanism for Females

Why then is the foetal female brain not masculinized by maternal oestrogens or the neonatal female brain masculinized by any oestrogens from the ovaries of newborn rats?

There is in the blood of foetal rats a protein<sup>26,27</sup>  $\alpha$  1 fetoprotein which is capable of binding 17 $\beta$  oestradiol and thereby reducing its concentration, thereby reducing the amount of oestrogen reaching the brain. The  $\alpha$  1 fetoprotein is particularly selective in binding 17 $\beta$  oestradiol but will allow passage of testosterone, (Fig 6) and many artificial oestrogens such as diethylstilboestrol and RU2858 which consequently act as potent masculinizers<sup>16</sup>. It is worth noting that if something analogous occurs in utero in humans there could be a real danger of foetal damage if pregnant women

HORMONE	MASCULINISES
Testosterone	✓
5 $\alpha$ DHT	✗
Oestrogens	✓
Oestrogens and antiestrogens	✗
Testosterone and antiestrogen	✗

Fig. 4.

A summary of the classes of hormone which can or cannot masculinise.

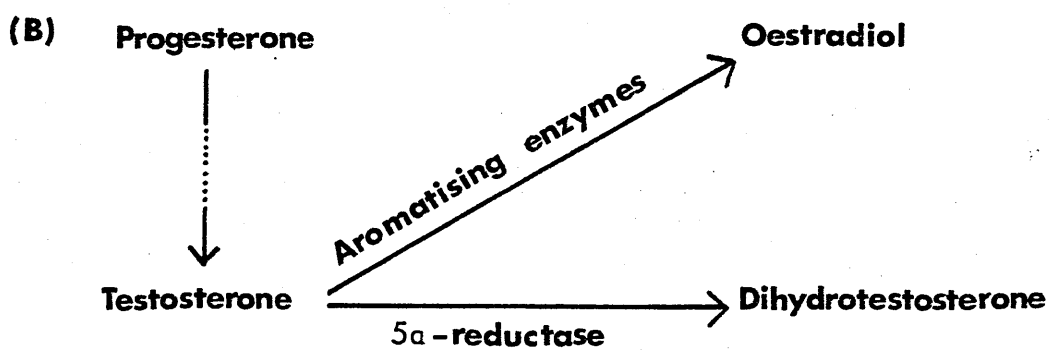
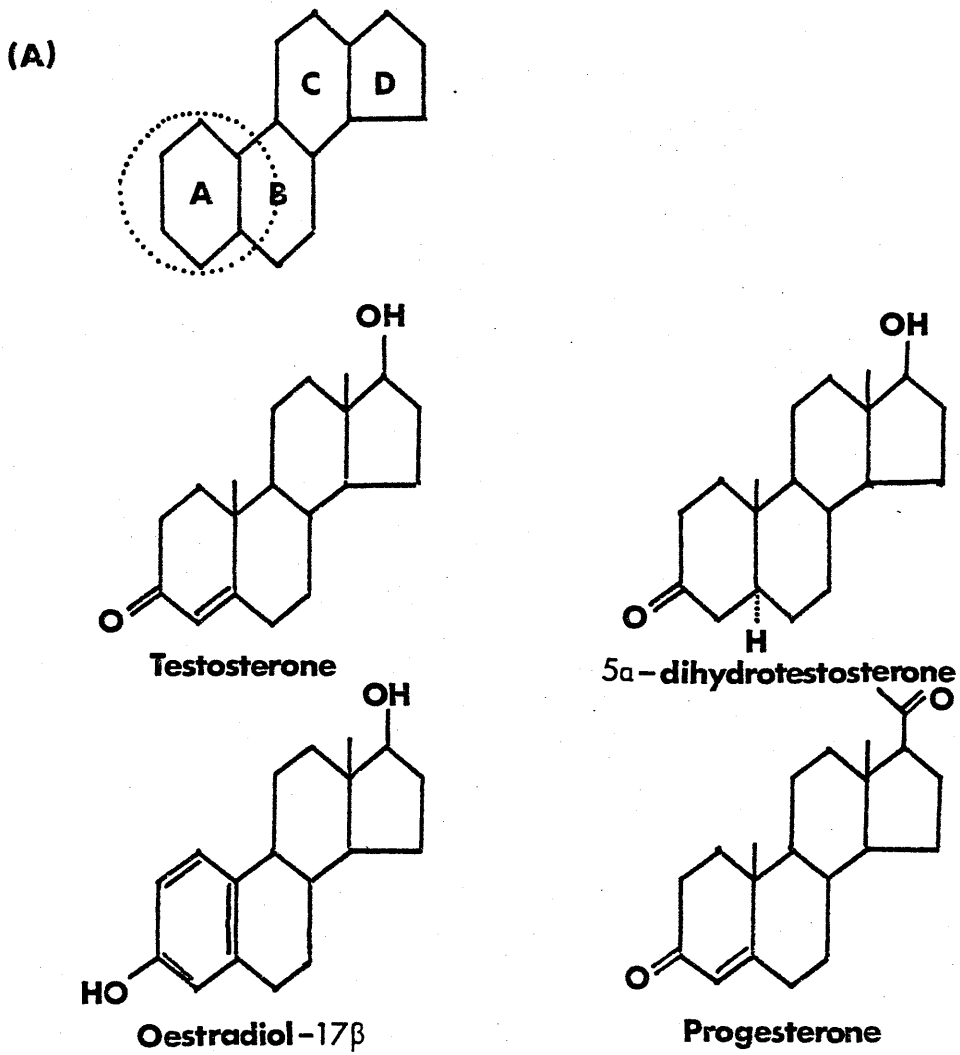


Fig. 5.

Fig. 5.

A) The major sex steroids. The structure of the natural sex hormone is based on the steroid nucleus. The site of aromatization is the ring designated A in the structure.

B) There are two enzyme systems capable of metabolizing testosterone (T) the aromatizing system that converts it to oestradiol and  $5\alpha$ reductase which converts it to  $5\alpha$ dihydrotestosterone (DHT).

DHT cannot be converted to oestrogens in the brain.

The aromatizing system is concentrated in the hypothalamic/limbic region but the  $5\alpha$ reductase is more widely distributed.

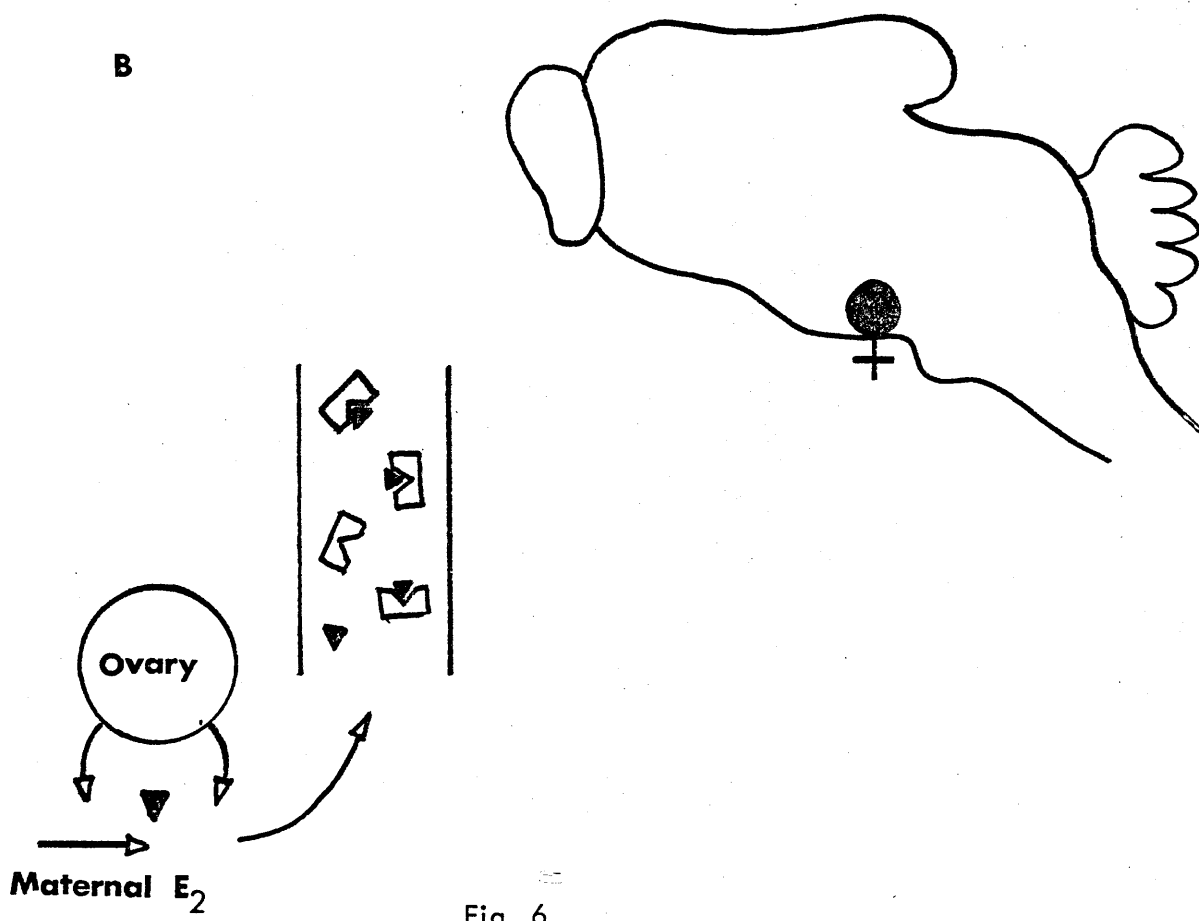
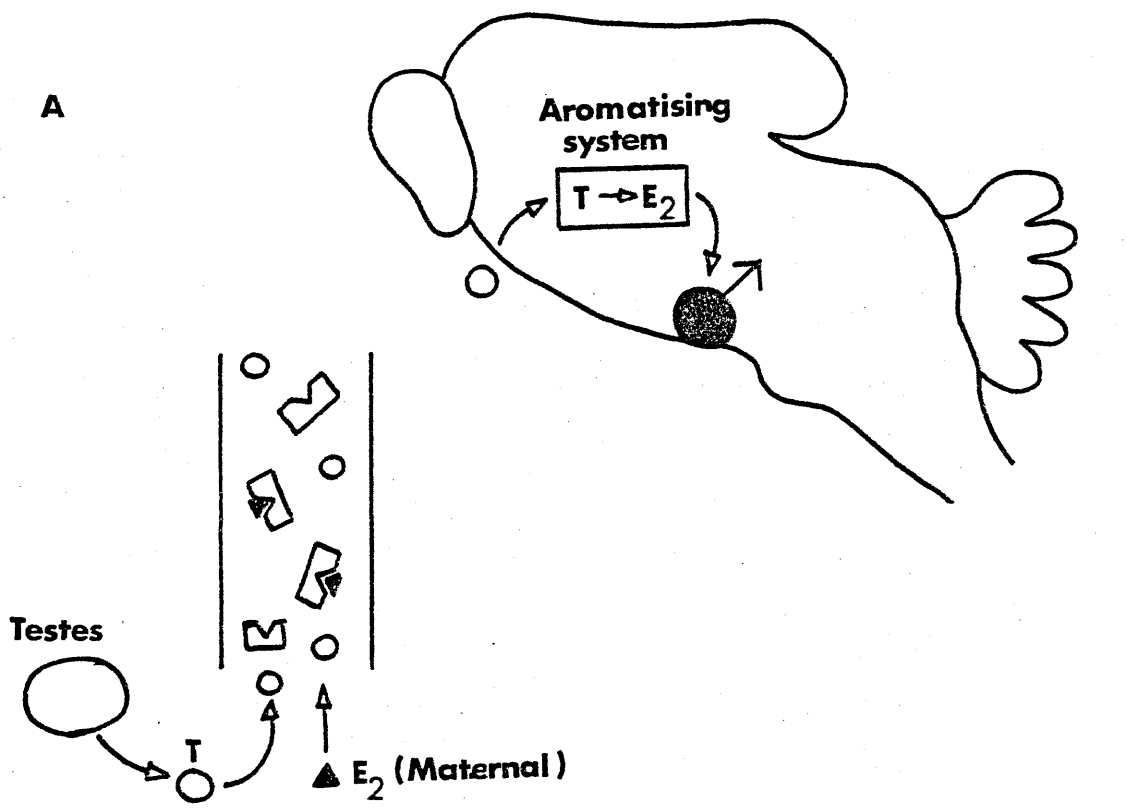


Fig. 6. - 14 -

Fig. 6.

A) MALES

Testosterone secreted from the testes is not bound by the  $\alpha_1$  fetoprotein  $\Sigma$  and reaches certain areas of the brain where it is converted to oestradiol by the action of aromatizing enzymes. The oestradiol then produced masculinises the brain.

B) FEMALES

The  $\alpha_1$  fetoprotein  $\Sigma$  binds any oestradiol so protecting the brain from maternal and any of its own oestradiol. The brain is then unaffected and develops in the female manner.



were exposed to artificial oestrogens<sup>28</sup> for example from oral contraceptives or from hormones used in meat production. Although production of  $\alpha 1$  fetoprotein ceases at birth, masculinization is unlikely to occur in the newborn female as their ovaries do not produce oestrogens in any quantity during the critical period, so that the level of oestrogens in the neonatal blood will be very low.

Therefore we have established that for a (potentially female) brain to be masculinized during the critical period, androgens must be available to pass from the blood stream to the brain and be converted to oestrogen in the presence of aromatising enzymes.

#### What are the mechanisms of masculinization?

It is well known that oestrogens act in the periphery<sup>29</sup> (see Thomas 1973) binding to specific cytoplasmic receptors and that the ensuing complex moves to the nucleus where it associates with the chromatin (where it is commonly called the nuclear receptor) leading to the synthesis of messenger RNA (mRNA), proteins and thence metabolic effects<sup>30,31,32,33,34</sup> (Fig 7.)

It has been shown that there are both nuclear and cytoplasmic receptors<sup>35,36,37,38</sup> capable of responding to oestrogen in the brains of immature rats. These receptor sites are distributed in the hypothalamic/amygdaloid area and the cortex. The cytoplasmic receptors are found in both areas whilst the nuclear receptors are concentrated in the hypothalamus (Fig 8.) Presumably the cortex lacks acceptor sites on the chromatin.

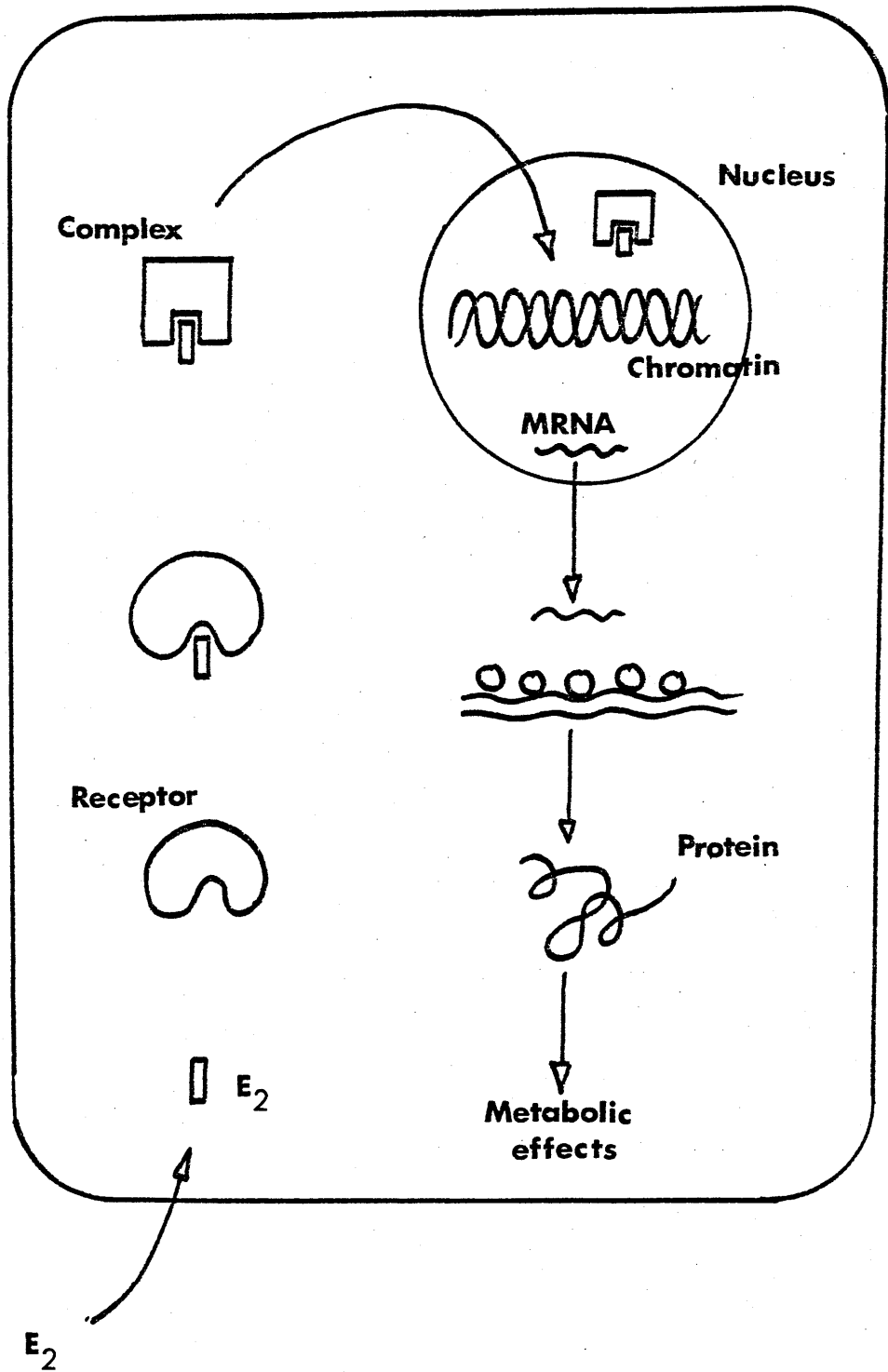


Fig. 7.

A model for the action of oestradiol. See legend overleaf.

Legend Fig. 7

The hormone passes to a target cell and combines with a receptor to form a hormone-receptor complex. The complex changes shape, moves to the nucleus where it associates with the chromatin at specific acceptor sites, this leads to the transcription of messenger RNA (mRNA). The mRNA is released from the nucleus and moves towards the ribosomes on the endoplasmic reticulum; a specific protein(s) is/are formed which may affect the cell metabolism.

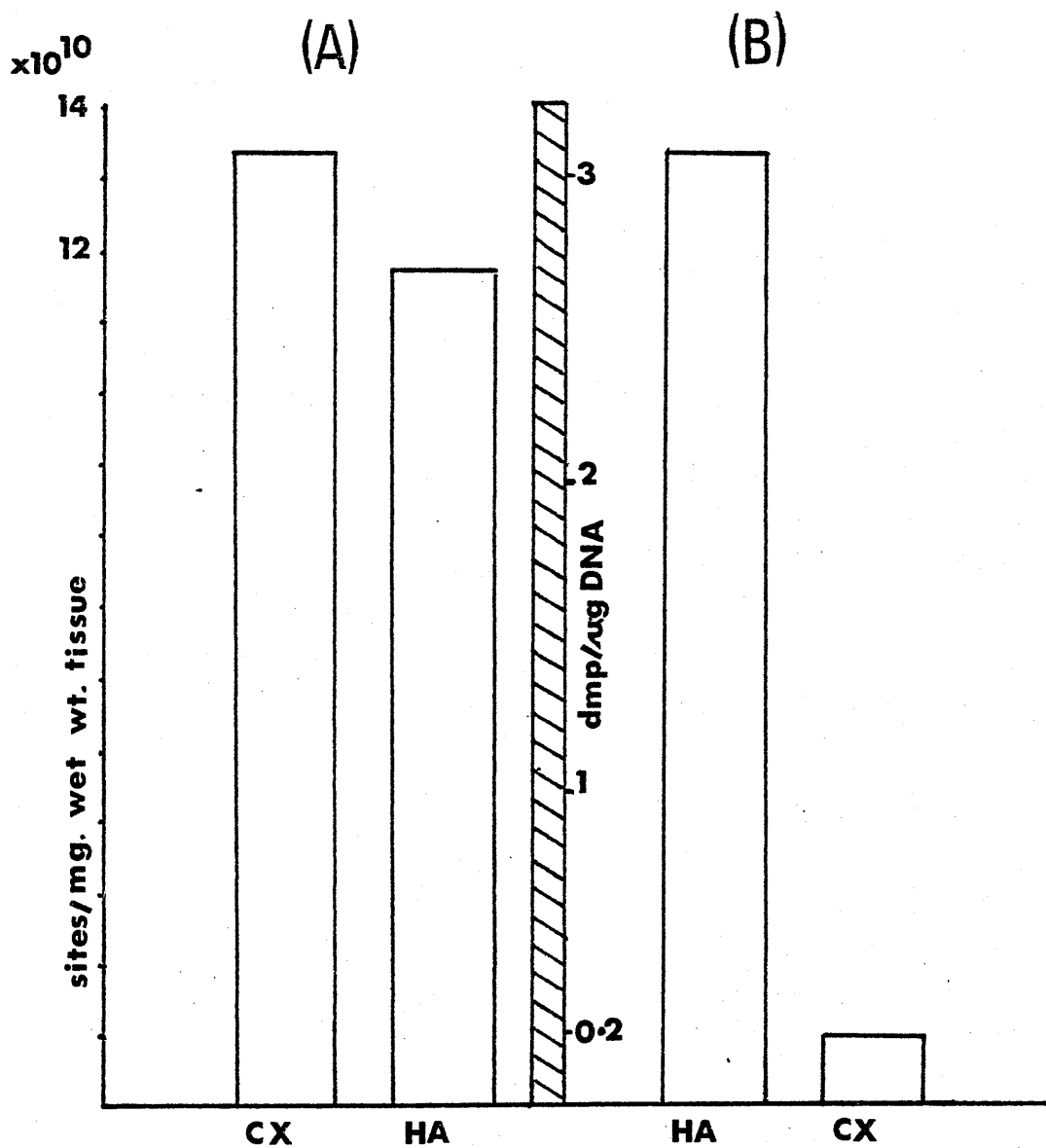


Fig. 8.

The distribution of A) cytoplasmic<sup>35</sup> and B) nuclear<sup>36</sup> receptors in the brains of newborn females. CX = cortex, HA = hypothalamus.

Note that the axes of A) and B) are different.

Similar results were obtained in the male.

In the adult male and female rats however the distribution of cytosol receptor sites has been shown to be greater in the hypothalamus and relatively sparse in the cortex. (Fig 9)

Although the distribution of oestrogen receptors in the newborn is not restricted to the hypothalamic limbic region, the aromatising enzymes, which aid the conversion of testosterone to oestrogen in the immature rat brain, are concentrated in the hypothalamic/limbic region; so testosterone cannot be converted to oestrogen elsewhere<sup>22</sup> so conferring some anatomical specificity. (Fig 10)

Presumably in the normal animal, oestrogen acts as a masculinizer via these receptors in a way analogous to that in the periphery, causing RNA and protein synthesis in the hypothalamus.

Does oestrogen affect RNA and protein biosynthesis in the brain of newborn rats?

It has been shown that oestrogen stimulates the biosynthesis of a specific protein in infant brain slices in vitro and that when testosterone is injected in vivo into newborn female rats it induces the synthesis of a protein in the hypothalamus/amygdaloid area (only this area contains the aromatising enzymes capable of converting testosterone to oestrogen) and that this protein is concentrated in the nuclear fraction<sup>39</sup>. It has been suggested that this protein may have a regulating effect. Furthermore it has been shown that if either the RNA or protein synthesis is inhibited then masculinization can be blocked<sup>40,41</sup>:

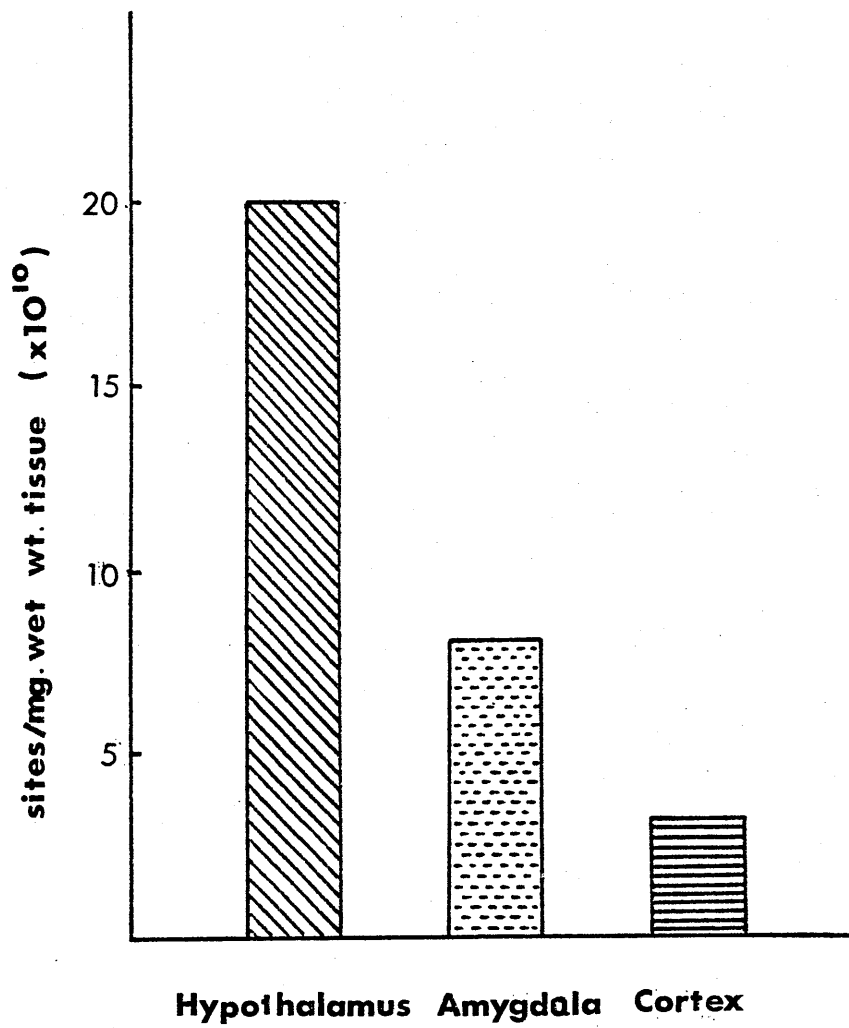


Fig. 9.

Distribution of cytoplasmic receptors for oestradiol in the brains of female adult rats. Similar results were found in castrate males.

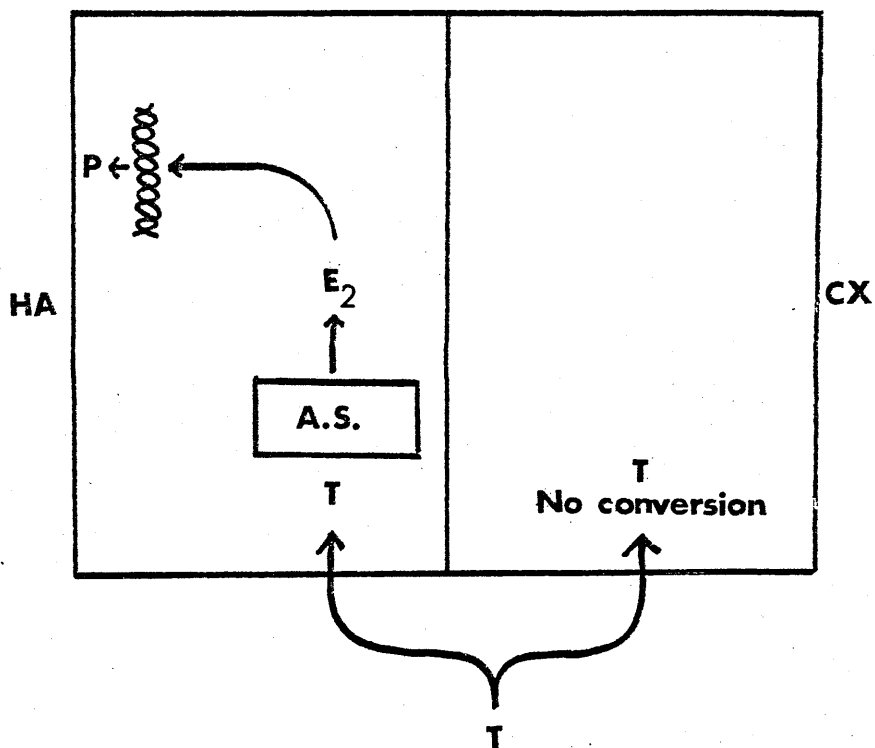


Fig. 10.

The effect of testosterone (T) on the hypothalamic/amygdaloid area and cortex of the rat brain. Both have cytoplasmic and nuclear oestradiol receptors.

Testosterone can only affect the hypothalamic region, it cannot affect the cortex because the aromatizing enzymes which convert the T to oestradiol (E<sub>2</sub>) are not present in this area.

How then could the action of oestrogen and the formation of specific protein(s) lead to a sexual differentiation in the brain?

- 1) Does it alter the sensitivity of the hypothalamus to oestrogen?

This is unlikely because it has been shown that the sexual difference in oestrogen receptors is not necessarily permanent. Though adult male rats have fewer receptors than adult female rats in the hypothalamus, this difference can be lost after castration and so the situation can be reversed<sup>42</sup>, (Fig 11.)

- 2) Does it alter the capacity of the hypothalamus to produce gonadotrophin releasing hormones (G.n.R.H.)?

This also seems unlikely as there is no evidence of sexual dimorphism in the concentration of GnRH in rats or in man<sup>43,44</sup> so;

- 3) Does it then effect neural pathways involved in GnRH release, which impinge on oestrogen sensitive neurones?

This seems the most likely possibility, indeed Raisman and Field<sup>45</sup> have shown<sup>46</sup> a sexual dimorphism in the number of synapses of non amygdaloid origin on dendritic spines in the preoptic area of rats. They



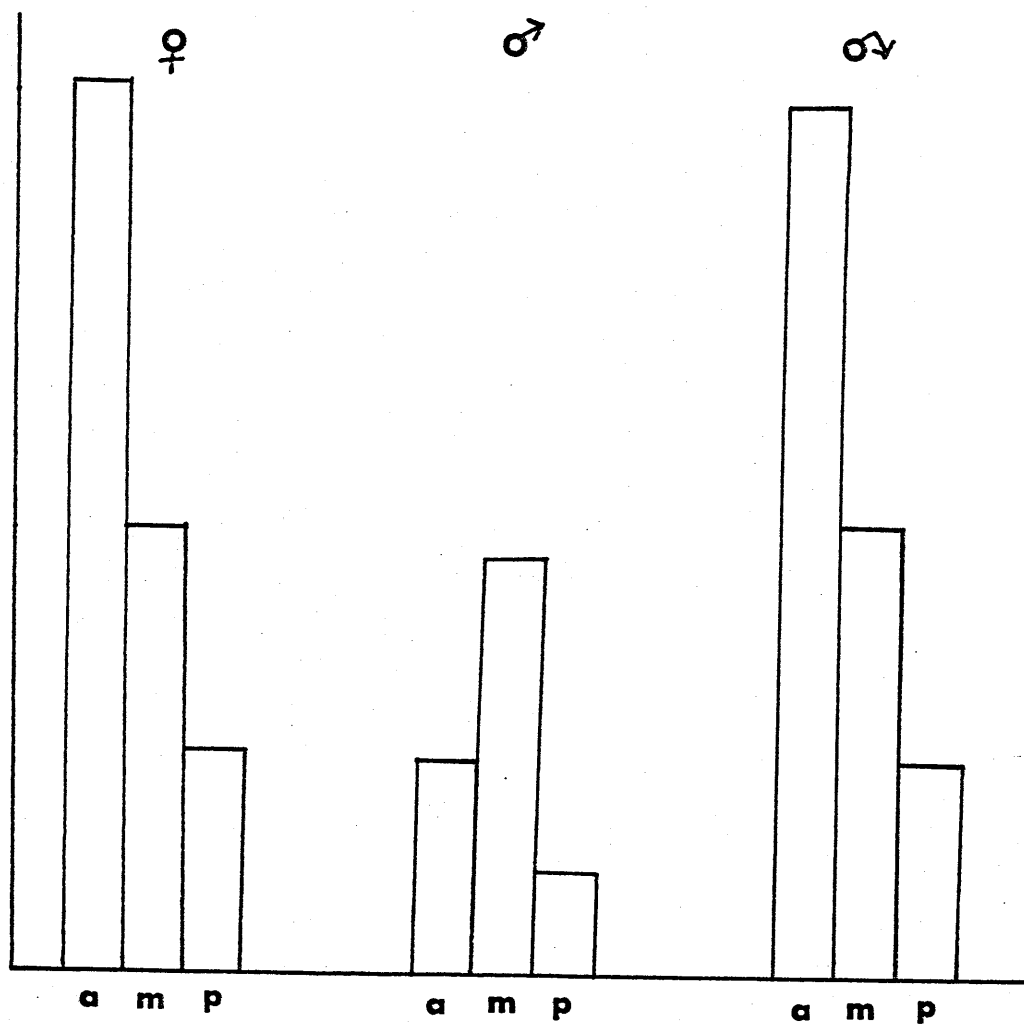


Fig. 11.

The distribution of cytosol oestrogen receptors in the anterior (a), middle (m) and posterior (p) hypothalami of adult female, male, and castrated rats. Castrated and female are similar. Normal males have lower levels particularly in (a) where aromatizing enzymes are present.

showed a higher number in adult female rats than in adult males. They also showed that castration within twelve hours of birth caused an increase in the number of synapses whilst neonatal androgenisation of female rats caused a decrease in the number of synapses to within male levels. Therefore oestrogen and the subsequent production of the protein are probably involved in neural organisation leading to sexual differentiation in the brain.

How can the oestrogen induced protein present in a great many cells have long term effects on but a few? - the role of cAMP.

The timing (critical period?) and amount of protein produced could be the key factors as could be the responsiveness of individual cells to it. It has been suggested<sup>39</sup> that only those cells which are being actively stimulated by neurotransmitters during the critical period are responsive to the protein (and thence to oestradiol). If this is so there should be a common factor to connect neurotransmitters and oestrogen. It appears that this could be cAMP<sup>47,48,49</sup>.

In order to discuss this we have to first consider :

- a) the effects of neurotransmitters on adenylate cyclase
- b) the effects of oestrogen on adenylate cyclase and the trophic effects of cAMP.

a) The effects of neurotransmitters on adenylate cyclase.

There is evidence that adenylate cyclase activity and thence the formation of cAMP is higher in neurones than in any other mammalian tissue, therefore it is possible that cAMP is involved in neural

function. Indeed it has been shown that stimulation of many neurones is accompanied by an increase in cAMP levels in the post synaptic region and that neurotransmitters released from the presynaptic nerve endings act on the post synaptic region leading to adenylate cyclase activation, (Fig 12,) and the subsequent formation of cAMP<sup>50</sup>.

It is therefore postulated that<sup>50</sup> cAMP is involved in the transference of messages from neurone to neurone.

b) The effects of oestrogen on adenylate cyclase.

It has been shown that<sup>48,49</sup> the basal level of adenylate cyclase activity in the hypothalamus of 5 day old female rats is greater than that of males. The adenylate cyclase activity in females is also sensitive to relatively low doses of dopamine, but not to two other neurotransmitters tested, noradrenaline and 5HT, (Fig 13.)

*This could be a consequence of the early development of dopamine receptors - they are present at the time of birth, whereas noradrenaline and 5 hydroxytryptamine (5HT) receptors develop later.*

A possible link between oestrogen and these sexual differences in adenylate cyclase activities could be formulated as follows; (Fig 14) As shown earlier oestradiol is present in the hypothalamus of male rats during the critical period for sexual differentiation having been converted from testosterone. The female hypothalamus remains unaffected.

Adenylate cyclase activity in the hypothalamus varies through the

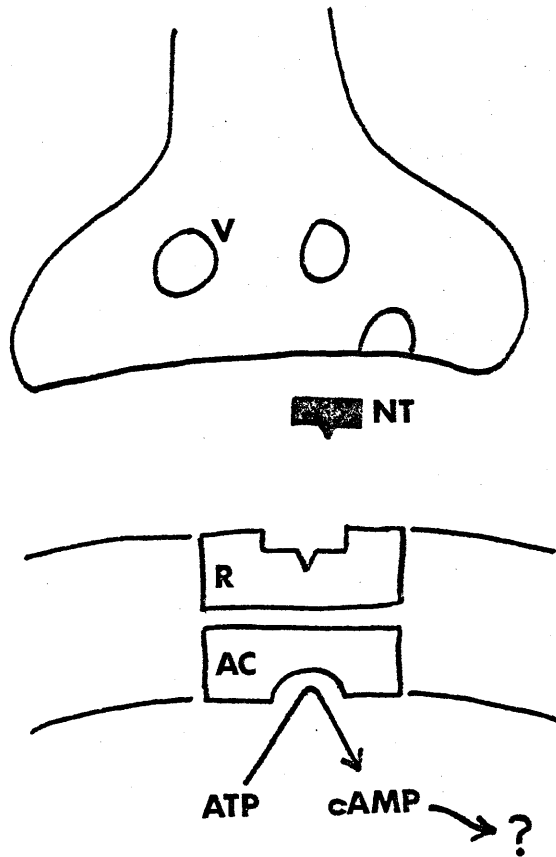


Fig. 12.

Shows the stimulation of adenylate cyclase (AC) by neurotransmitters (NT). NT, released by exocytosis from the vesicles (V) in the presynaptic area, binds to a receptor (R). This activates AC, leading to the formation of cAMP. The resulting cAMP affects the intracellular metabolism.

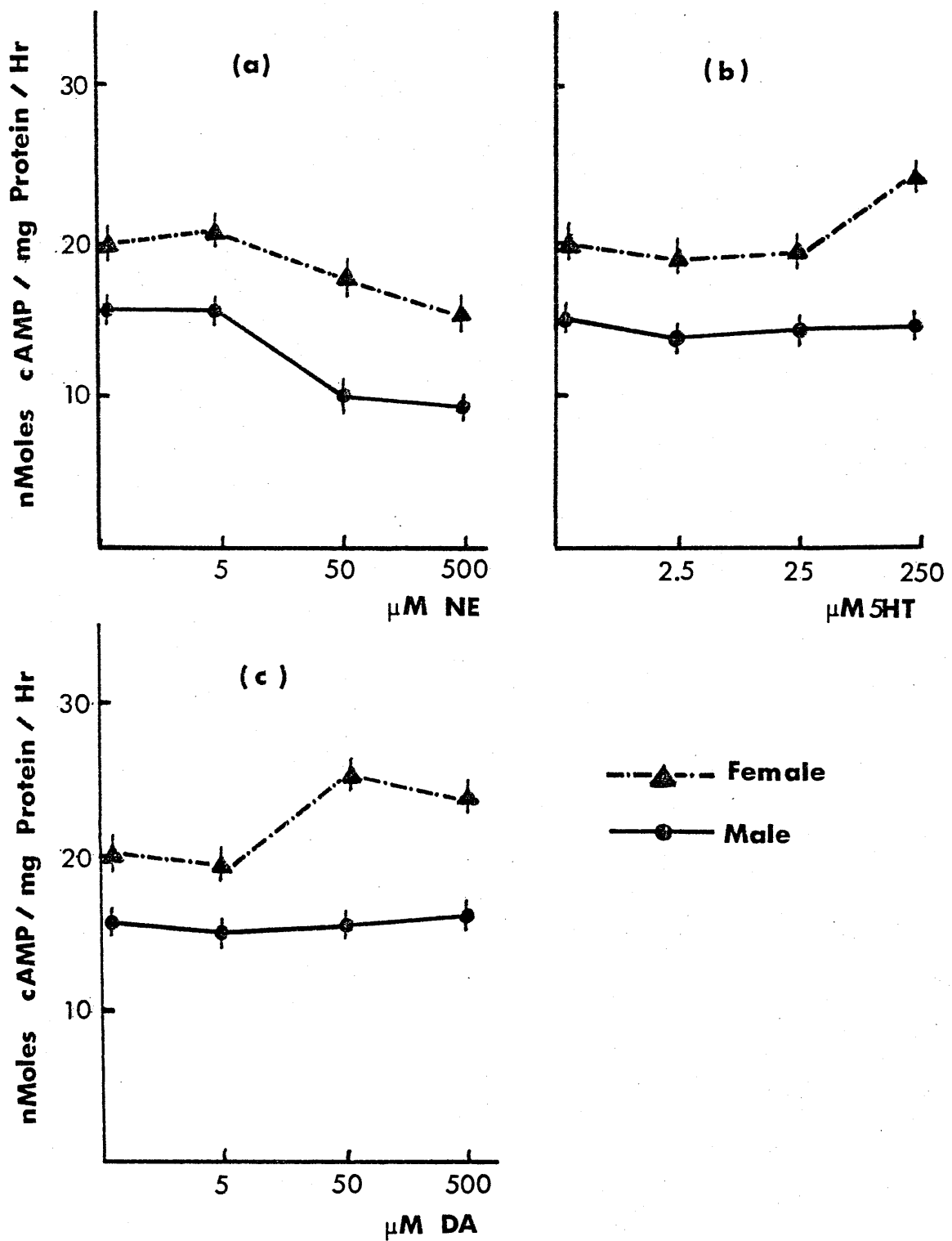


Fig. 13.

Adenylate cyclase activity in the hypothalamus of 5 day old rat and its response to neurotransmitters, norepinephrine (NE), 5 hydroxytryptamine (5HT) and dopamine (DA). Note that at relatively low concentrations only DA activates adenylate cyclase. Furthermore, DA activates the enzyme in females but not males. The enzyme activity in males is always lower than in females.<sup>48,49</sup>

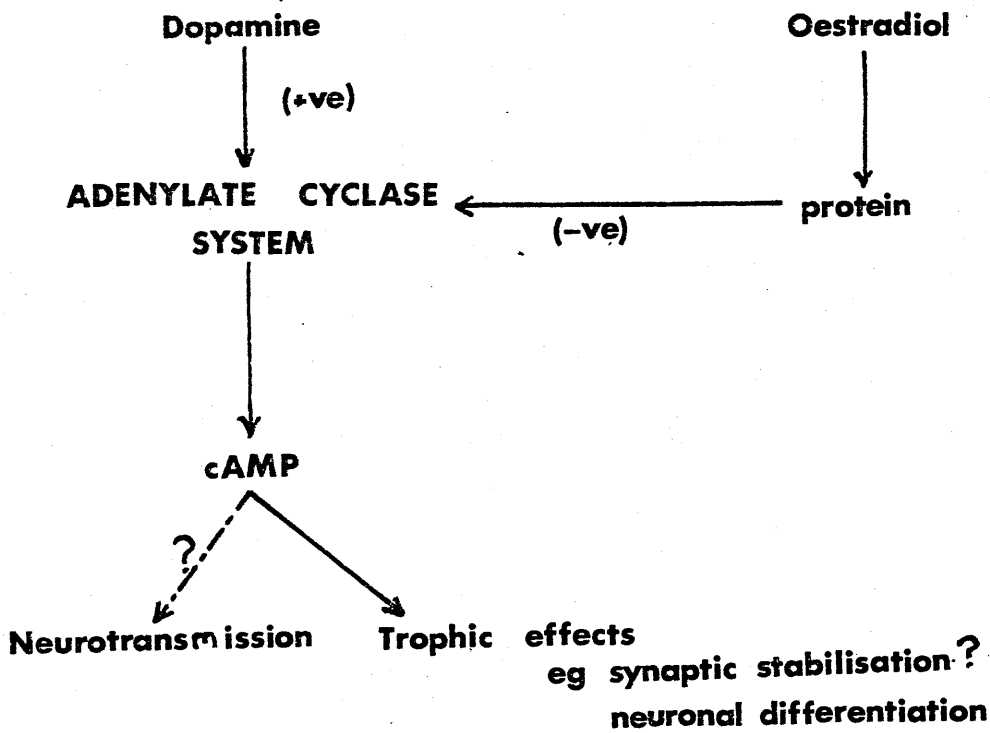


Fig. 14.

The activation of the cyclase enzyme by a neurotransmitter e.g. dopamine, leads to the formation of cAMP. cAMP, as well as having a possible role in neurotransmission, may have some trophic effects in nervous tissue.  $E_2$  reduces adenylate cyclase activity thence the actions of cAMP. <sup>49</sup>

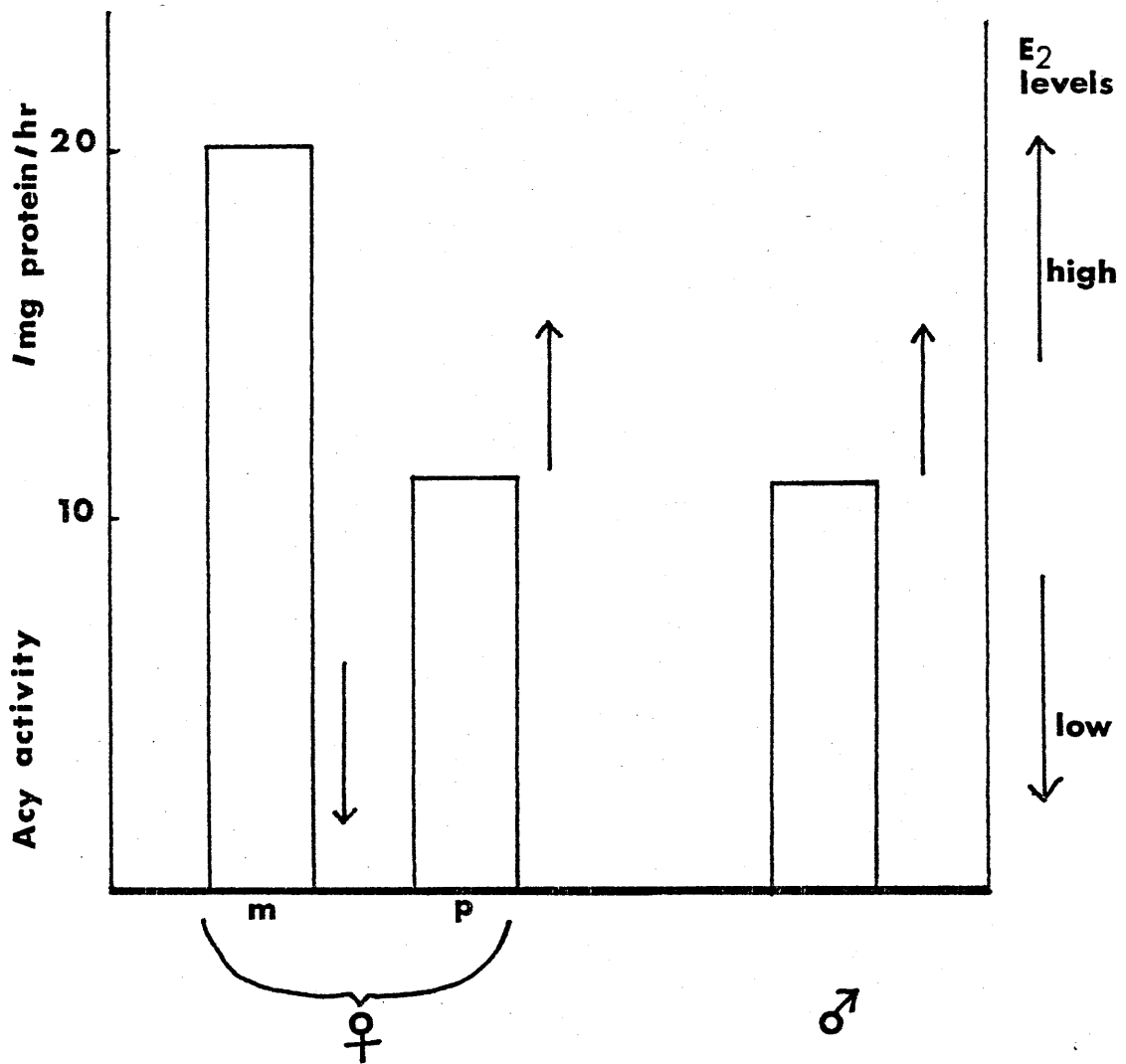


Fig. 15.

Adenylate cyclase activity in the anterior hypothalami of brains of adult rats. Note that when E<sub>2</sub> levels are low (metoestrus- m) adenylate cyclase activity is high.

When E<sub>2</sub> levels are high (♂ due to aromatization) and (proestrus- p) adenylate cyclase activity is low.

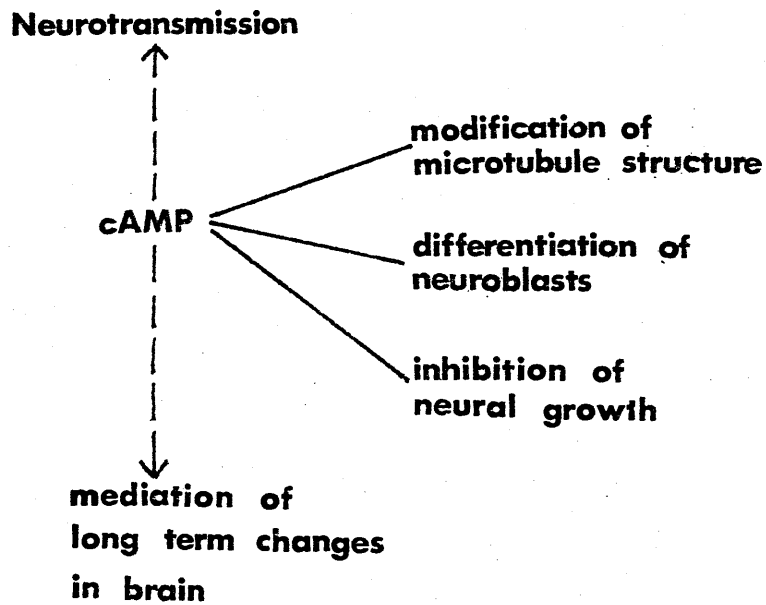


Fig. 16.

Some trophic effects of cAMP in neural tissue.

\* It should be noted that not all neurotransmitters act via cAMP.  
 $\text{Ca}^{2+}$  and cGMP are other candidates for second messengers. <sup>101,102</sup>



adult female oestrus cycle<sup>49</sup>, activity being higher in the metestrus period than in the proestrus where the activity is similar to that found in the hypothalamus of the acyclic male.

Oestrogen levels in the female hypothalamus are low in metestrus but high in proestrus and high in the male hypothalamus (where oestradiol is converted from testosterone) (Fig 15.)

It has also been shown<sup>49</sup> that when hypothalamic slices from 5 day old female rat brains are incubated with or without oestrogen and assayed for adenylate cyclase activity the tissue with oestrogen present has lower levels of activity. The effects of oestrogens on adenylate cyclase activity is dependent upon an oestrogen induced protein (described earlier p16). The trophic effects of cAMP are reasonably well documented and are set out in Fig 16. cAMP is known to cause differentiation of neuroblasts in culture<sup>51</sup> at the expense of growth. It also modifies microtubule structure<sup>52</sup> in adult nerve cells and leads to ultrastructural changes in the cell and nucleus.<sup>53-55</sup> It has been shown<sup>56</sup> that nerve cells contain specific receptor proteins for cAMP and that these proteins carry the cAMP to the nucleus where it binds to the chromatin and may affect genetic expression.<sup>57</sup>

Thus as with the oestrogen receptor system it could be that cAMP causes a protein biosynthesis which affects permanent changes on the cell.

#### What is the cAMP doing?

It could be acting as a stabilising agent. It has been proposed that use of a neural circuit leads to its stabilisation<sup>58</sup> - if a circuit was used in the past it is more likely to be used in the

future. Evidence for this can be obtained by preparing slices of hippocampus in tissue culture medium and stimulating circuits as shown in Fig 17. It is interesting to note that in 1975 Libet et al<sup>59</sup> produced evidence that the long term changes induced by dopamine in mammalian sympathetic ganglion were mediated by cAMP. They have further shown that cGMP disrupts the retention of these changes in a time dependent manner that is similar in principle to the well known disruption that can occur in the memory storage process<sup>59</sup>. The fact that use leads to the selective stabilization of synapses has been used to formulate a model for brain development<sup>60</sup>, which, it has been suggested could be extended to memory<sup>39,49</sup>. Therefore it seems probable that cAMP is involved in the selective stabilization of synapses in the brain, (Fig 18)

Thus it appears that there is a common pathway, involving cAMP, by which both neurotransmitters and oestrogens could affect brain development, (Fig 14.) This leads to the question; -  
Can modification of neurotransmitter activity affect sexual differentiation?

#### The role of neurotransmitters in sexual differentiation

Neurotransmitters have the ability to modify synaptogenesis and this can be illustrated by the well known phenomenon of denervation supersensitivity<sup>61,62</sup>. If a nerve to a peripheral target is severed that organ becomes hypersensitive to the neurotransmitter normally secreted by that nerve, (Fig 19.) It can be shown that the increase in sensitivity involves an increase in receptor

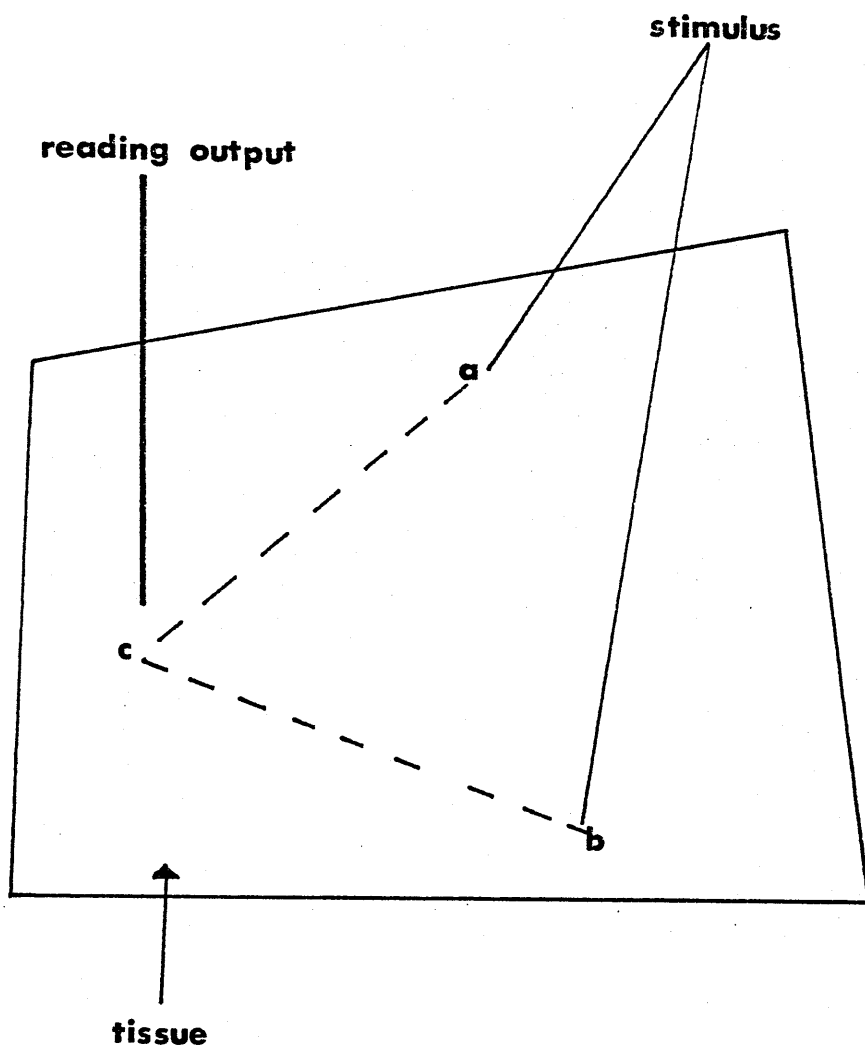


Fig. 17.

In the piece of tissue (a) and (b) are stimulated equally to give the same output at (c).

If (a) is stimulated excessively and (b) not at all, then if (a) is stimulated later it is found that a much smaller amount of power is required to produce the same output at (c). If (b) is then stimulated after a long delay much more power is required to give the same response as initially.

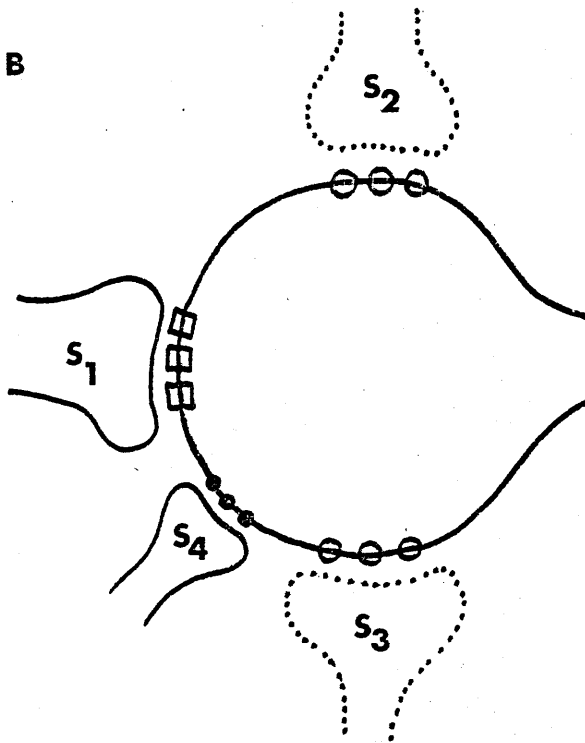
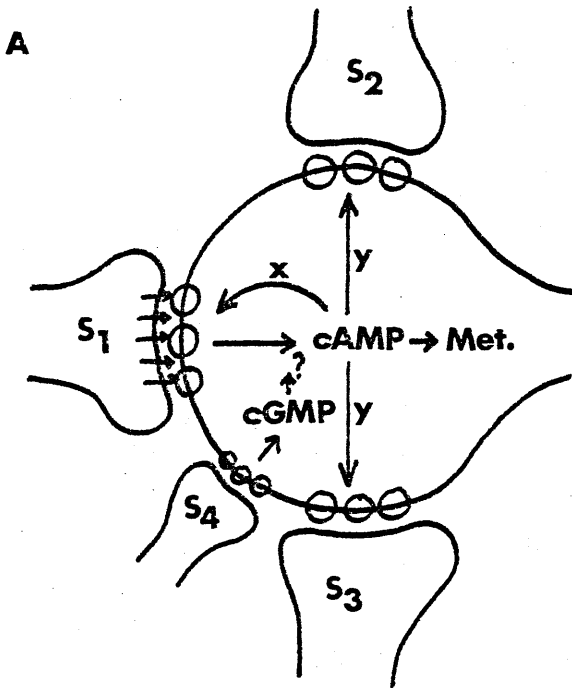


Figure 18. See legend overleaf.

Fig. 18.

A) It is proposed that receptors at synapses S1 S3 are initially labile (ooo). Stimulation of the receptor at S1 by a neurotransmitter (NT) produces a substance (x) which stabilises S1 at the expense of S2 & S3. <sup>60</sup>

B) (x) has been suggested to be cAMP <sup>49</sup>. cGMP may also be involved at a different synapse S4.  $\gamma$  is an unknown factor which, it is suggested destabilises S2 & S3 <sup>60</sup>.

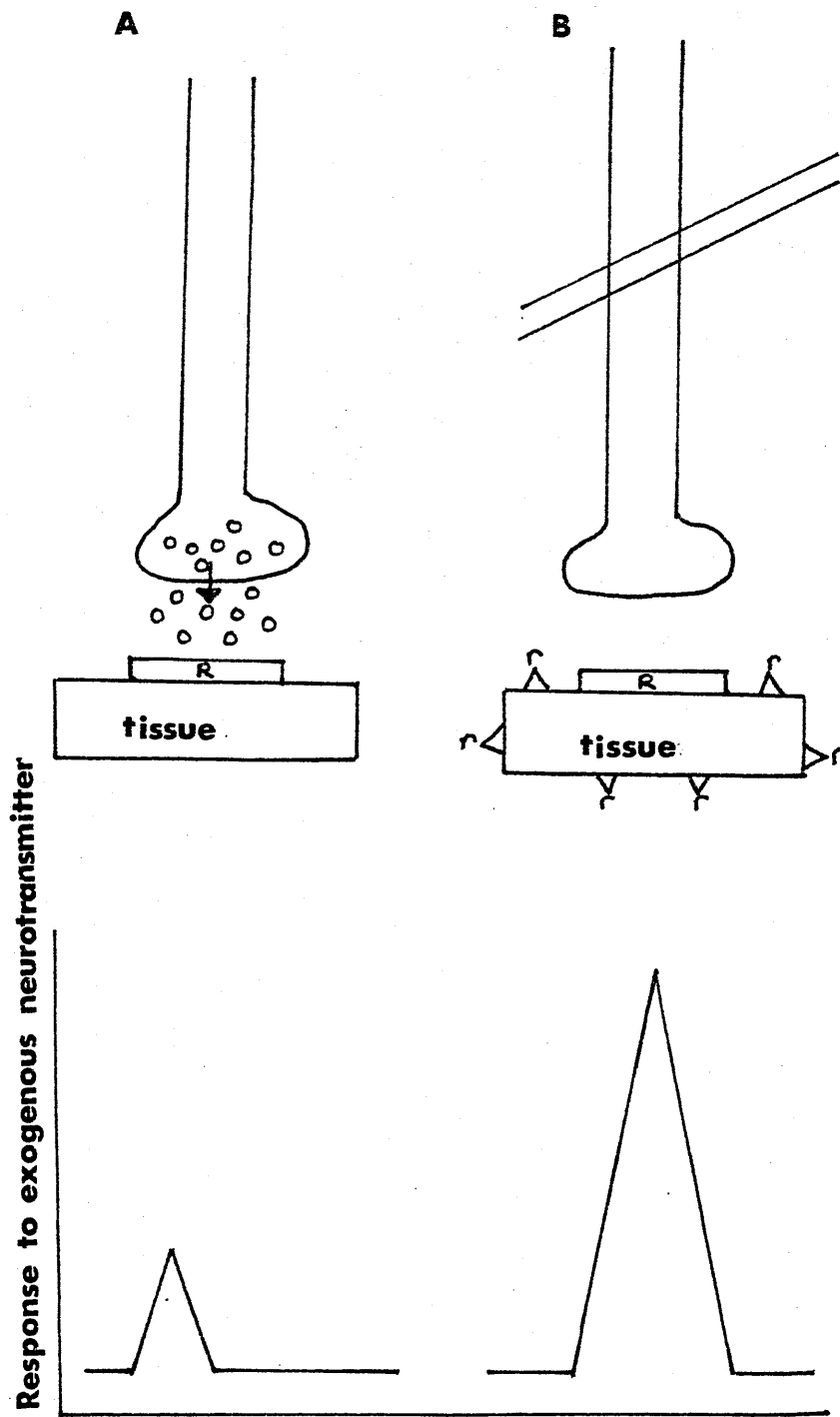


Fig. 19. see legend overleaf.

Fig. 19.

Denervation Supersensitivity.

(A) Normal state: the tissue is stimulated by neurotransmitters released from a nerve ending, which bind to their receptors (R) on the surface of the tissue.

(B) If the nerve is severed, no neurotransmitters are released but the tissue becomes supersensitive to exogenous neurotransmitters which may indicate that additional receptors (r) are formed. The magnitude of the response of the tissue to neurotransmitters is also shown. <sup>61, 62</sup>

number. Similar effects occur in the brain<sup>63,64,65,66,67</sup>

Can neurotransmitters (N.T.) and drugs which modify their levels affect sexual differentiation?

Indeed there is evidence albeit somewhat conflicting that a number of substances which affect NT levels - for example barbiturates reserpine, chlorpromazine, parachlorophenylalanine (P.C.P.A.) when administered during the critical period modify sexual differentiation, (see Booth<sup>1</sup> for details). Furthermore it has been shown that neurotransmitters are neurogenic and stimulate tract growth.<sup>68,69,70</sup>

Model

If therefore seems that although oestrogen, cAMP and neurotransmitters can effect brain development independently, in real life they are interdependent. A model for their possible interaction can be drawn up as follows:

On the stimulation of a neurone a neurotransmitter (e.g.dopamine) is released. This NT binds to its receptor (R) on the postsynaptic membrane leading to activation of the adenylate cyclase system and production of cAMP. Oestrogen induces the biosynthesis of a specific protein ( via binding to the cytoplasmic receptors (CR)) which then moves to the nucleus and results in the formation of mRNA and further protein biosynthesis. A protein so produced has the effect of reducing the activity of the adenylate cyclase system, (Fig 20)thence preventing selective stabilisation of synapses.

Most of the work cited above about the sexual differentiation of the brain has been biochemical or physiological, though some



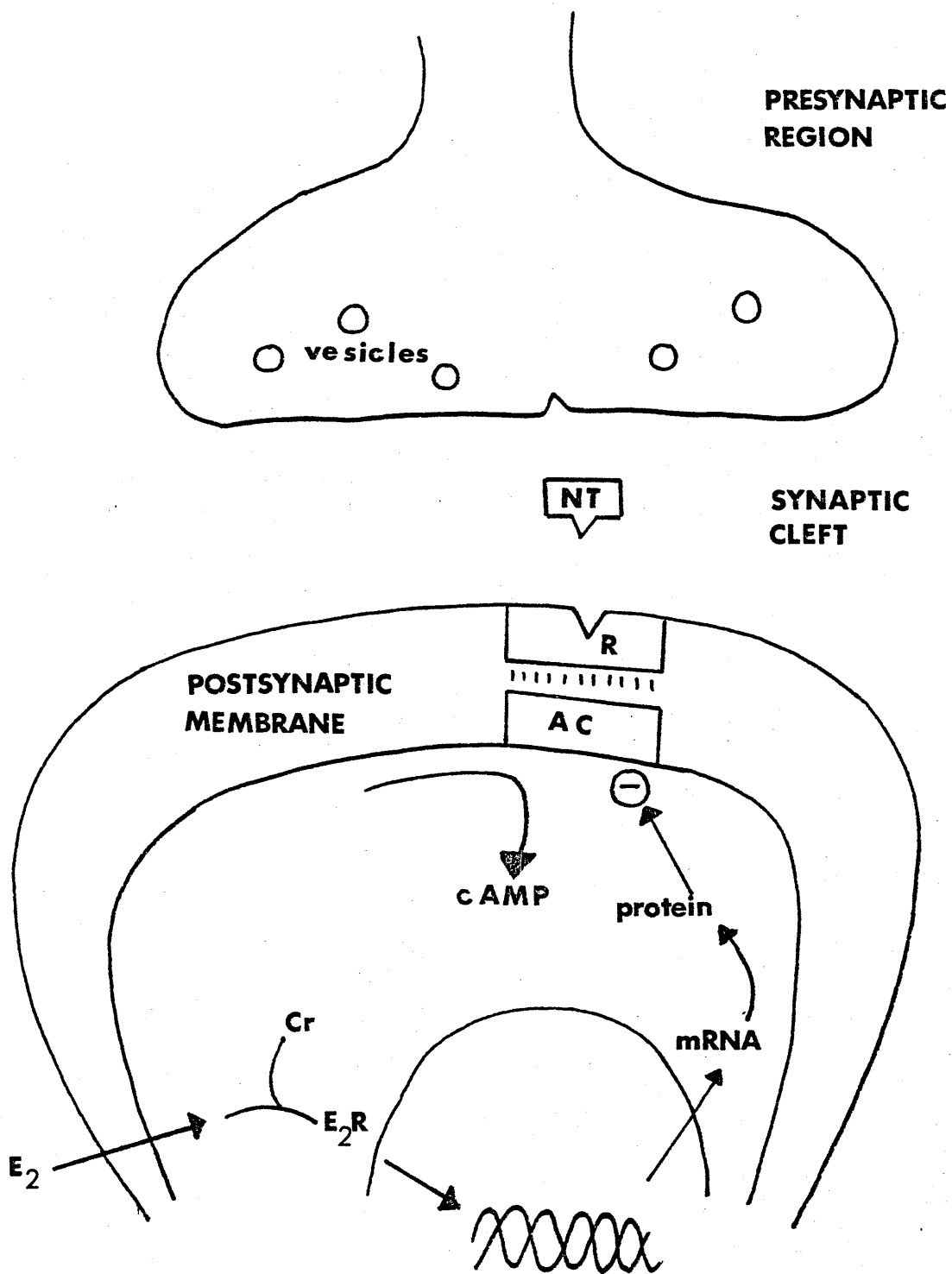


Fig. 20. see legend overleaf.

Fig. 20.

The neurotransmitter (NT) is released from the vesicles in the presynaptic region into the synaptic cleft. It binds to specific receptor sites on the postsynaptic membrane. This causes activation of adenylate cyclase and thence production of cAMP (the proteins which are associated with cAMP and which are necessary for its activation and linkage to the receptor are not shown<sup>12, 103</sup>). Oestradiol binds to cytoplasmic receptors and through the mechanism described in the text on page 16 leads to the production of protein(s) which inhibit the adenylate cyclase activity.

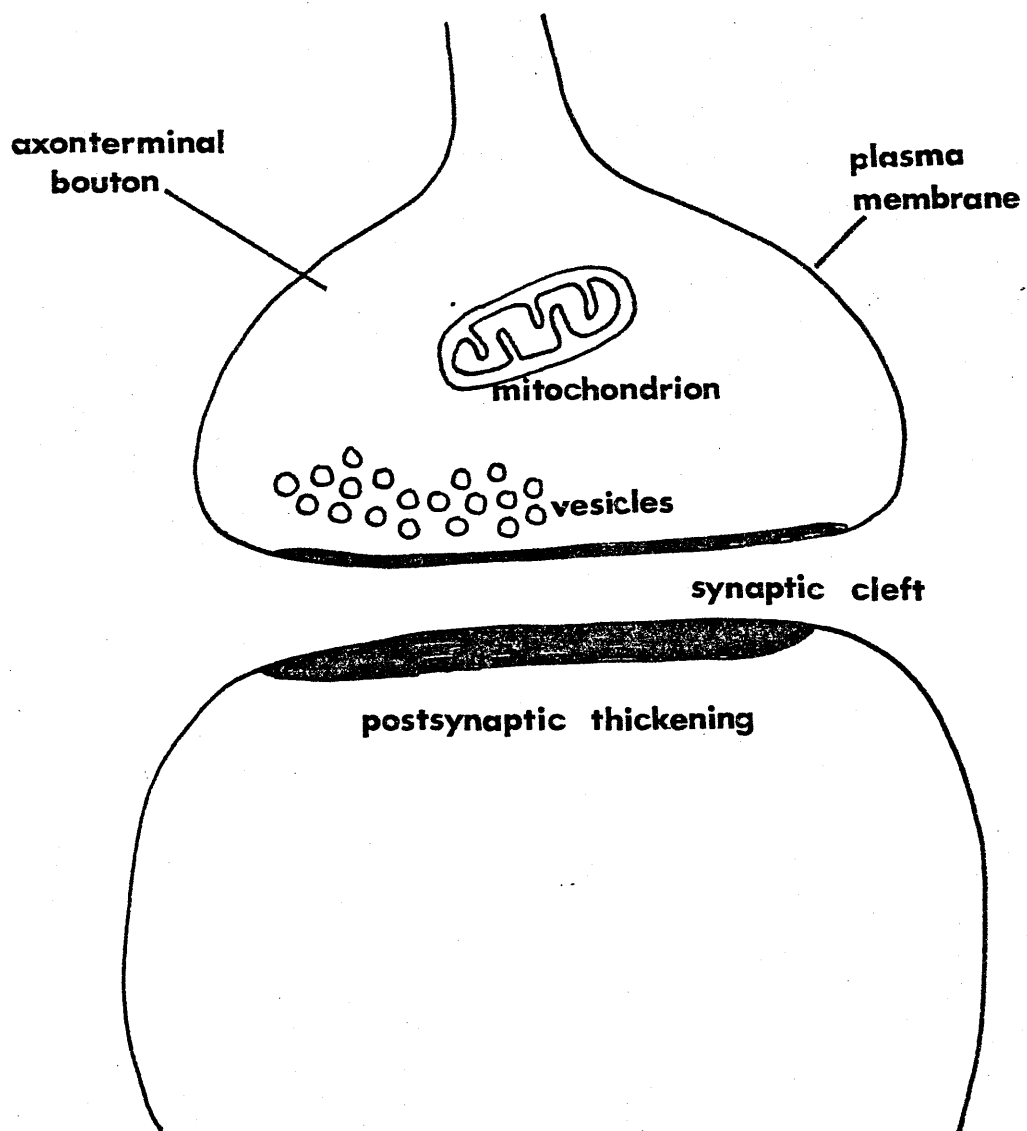


Fig. 21. see legend overleaf.

Fig. 21.

The Presynaptic side of the synapse is characterised by features which include a number of mitochondria and clusters of small spherical organelles— the synaptic vesicles. These vesicles are 60-90 nm in diameter and contain the neurotransmitter substance.

The presynaptic and postsynaptic membranes lie close to each other and are separated by a narrow cleft of 20-30 nm . this is the 'synaptic cleft' .

Waves of depolarisation travel down the axon to the terminal bouton and the vesicles containing the neurotransmitter substance fuse with the presynaptic membrane. The neurotransmitter substance is released by exocytosis into the synaptic cleft in quantum amounts, it diffuses to the postsynaptic membrane where it binds with receptors specific to that neurotransmitter. This leads to rapid changes in membrane conductance of the postsynaptic neurone membrane and continuation of transmission of impulse. The neurotransmitter which was released is degraded by enzyme action and/or taken up again by the presynaptic element to be recycled.

histological work, showing hormonally induced sexual dimorphism in various hypothalamic areas has also been carried out. Moreover, most of the histological work has been on adult brains except for the work of Reier et al 1977<sup>71</sup> who showed that there were no sexual differences in the synapses in the medial preoptic area (m.P.O.A.) of rats of various ages.

It is obviously important to know how soon after birth histological changes occur and as no information is available on these early changes it is necessary to make good these omissions.

The connections between individual nerve processes are the single most important aspect of brain development, therefore I decided to study synaptogenesis in normal male and female rats and the effects of hormone treatments on synaptogenesis.

Before embarking on my own work I will illustrate briefly the function of the synapse and its role in the brain and then describe the area of the brain I chose, and the reasons for choosing this particular area.

### The Synapse

The synapse is a junction between two neurones, (Fig 21), and has the function of transmitting the nerve impulses travelling along one neurone, which are in the form of waves of depolarisation, by converting these to chemical action at the junction and back to waves of depolarisation in the second neurone.

The most important feature is that the electrical transmission has the potential to travel two ways, whereas chemical transmission at the synapse is unidirectional.

There are two distinct parts to the synaptic junction - the

Presynaptic area which contains the synaptic vesicles filled with neurotransmitters (for examples see Fig 22) which form the basis of chemical transmission. The vesicles move towards the presynaptic membrane and release the neurotransmitter into the synaptic cleft. The neurotransmitters bind to specific receptor sites on the post synaptic part of the junction and transmission is then reconverted into waves of depolarisation (Fig 23).

The forms of synaptic endings vary but usually the axons expand into rounded terminal boutons close to other neurones. A common form of synapse in the central nervous system (CNS) is where the presynaptic element is a terminal bouton and the post synaptic element is a dendrite. Some different junctions are shown in (Fig 24)

#### The Suprachiasmatic Nucleus

The term 'nucleus' in this context refers to a discrete cluster or collection of cells coming together to form a spherical structure within the neuropil of the brain. The cells of the SCN have characteristic large cell nuclei which have an affinity for some light microscope stains e.g. Haematoxylin, Tolluidine Blue and Alcian Blue.

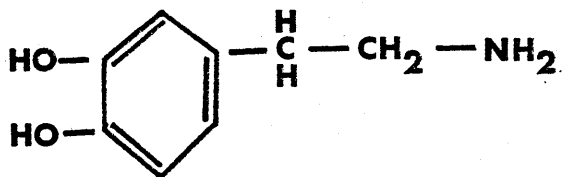
#### Anatomical location

There are two suprachiasmatic nuclei (left and right) which, as the name suggests lie upon the optic chiasm making a slight indentation in it. The left and the right nuclei are separated from each other by the median tubero-infundibular tract and the bottom of the third ventricle (Fig 25). In an adult rat the transverse diameter of a whole SCN would be in the order of 300  $\mu$  M.

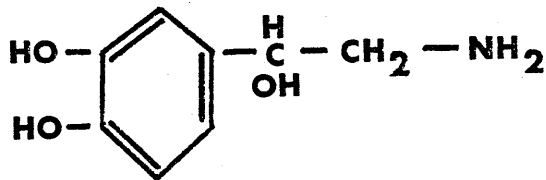
Fig. 22.

Some examples of neurotransmitter structures.

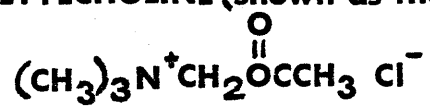
**DOPAMINE**



**norADRENALINE**



**ACETYLCHOLINE (shown as the chloride)**



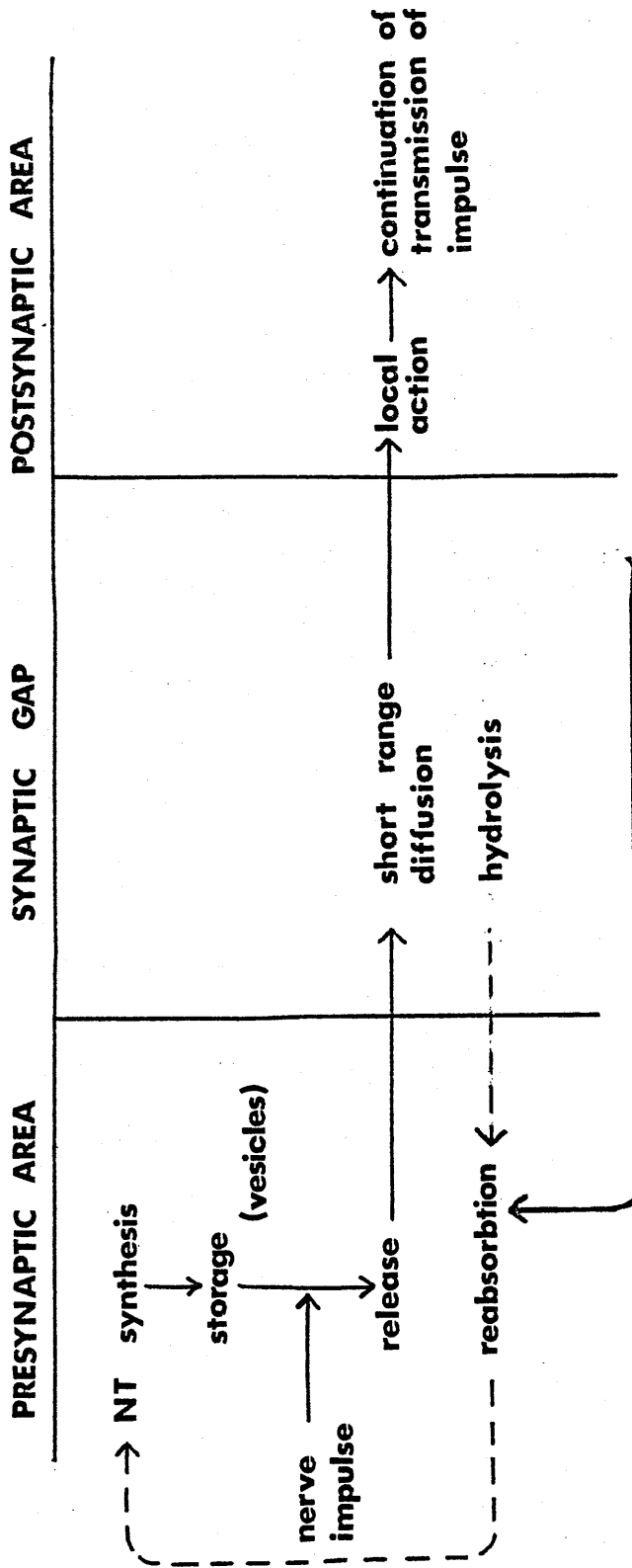


Fig. 23.



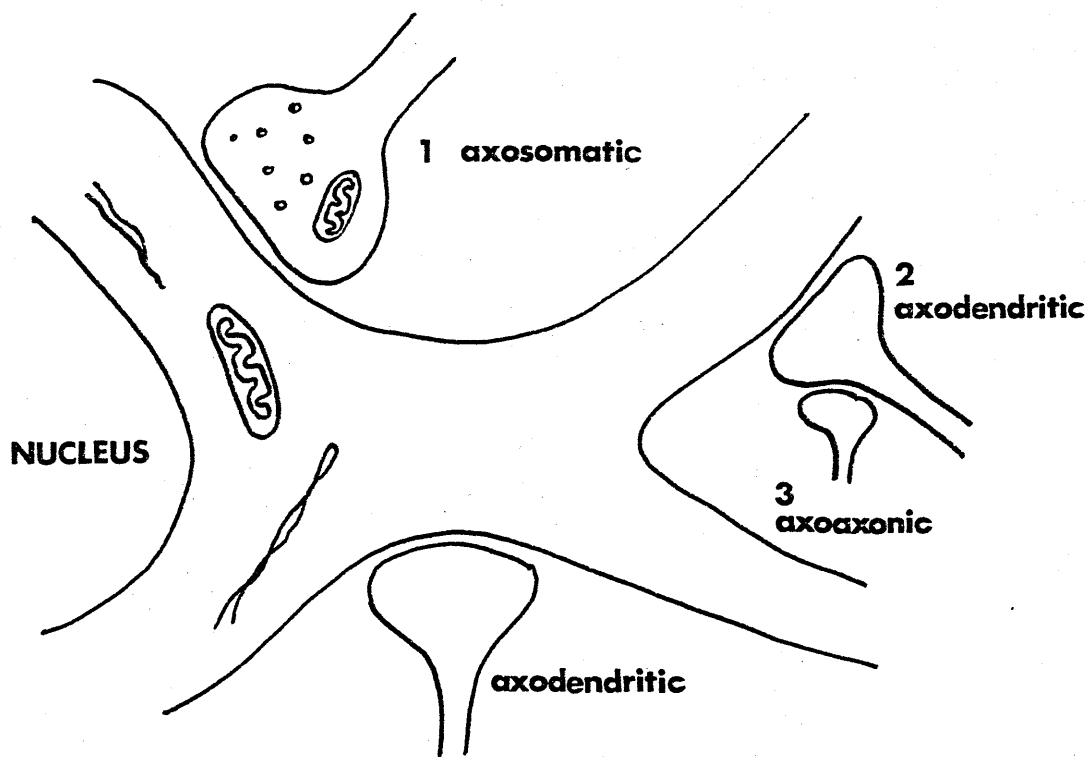
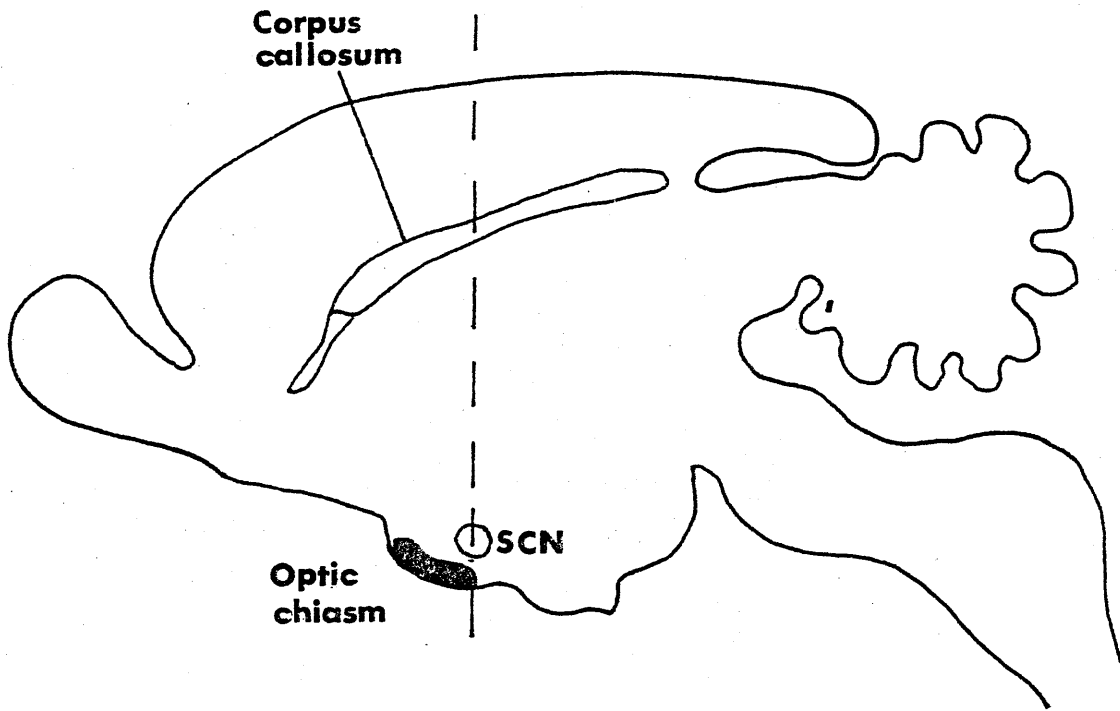


Fig. 24.

A diagram illustrating the different types of synaptic junctions:-

- 1) Axosomatic- from axon to perikaryon.
- 2) Axodendritic- from axon to dendrite.
- 3) Axoaxonic- From axon to axon.

**SAGITTAL SECTION**



**CORONAL SECTION**

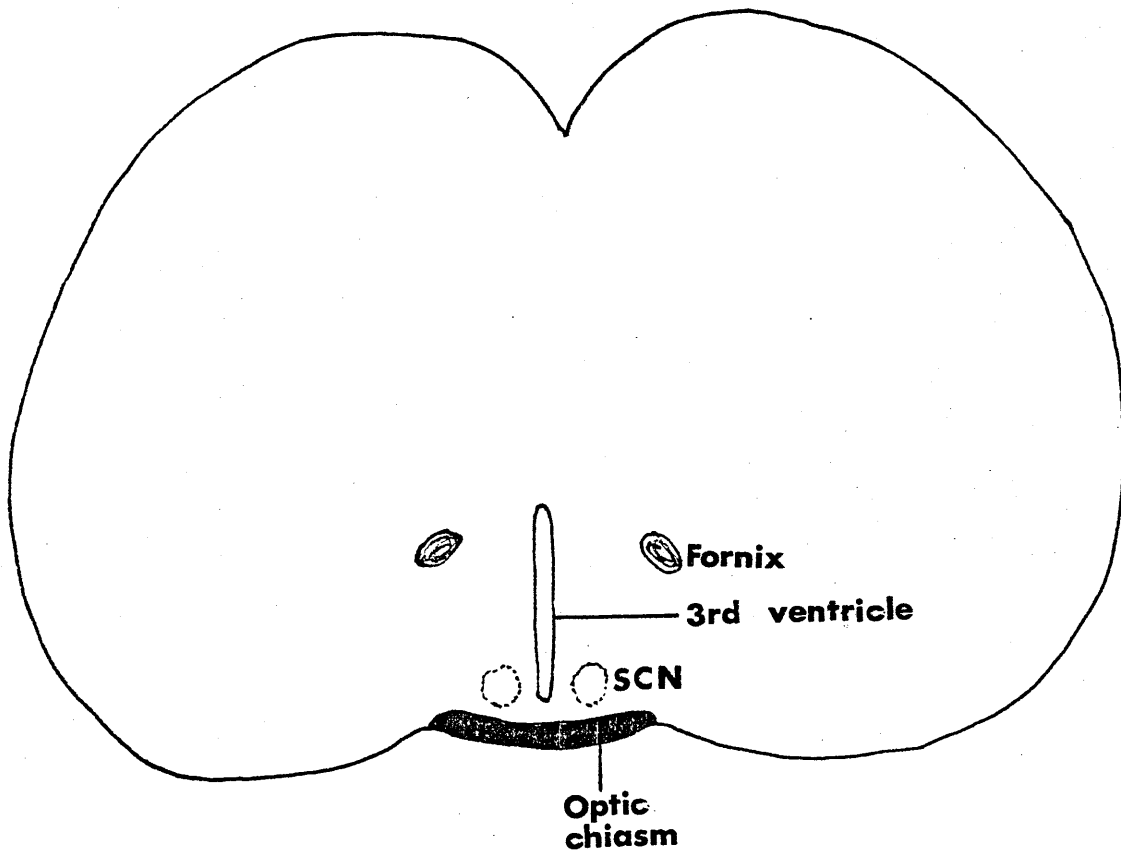


Fig. 25.

A diagram showing the location of the Suprachiasmatic Nucleus in the hypothalamus.

The chiasm and third ventricle provide prominent landmarks around the SCN and with practice and by using these landmarks and slicing carefully through the hypothalamus it is possible to reproduce sections of the area surrounding and including the SCN even in the newborn animal, so making it a suitable choice for investigation at the microscopic level.

#### Functions of the Suprachiasmatic Nucleus.

The retinohypothalamic tract (RHT) provides a direct connection between the retina and the SCN<sup>72</sup> in the hypothalamus. This RHT is probably necessary for the entrainment of circadian rhythms.<sup>72,73</sup> It has been reported that there is a loss of circadian rhythmicity when rats are exposed to light/dark cycles, and constant light after SCN lesions.<sup>74</sup> It appears though that the location of the lesion must be very precise to cause disruption to the circadian rhythm as damage to structures immediately adjacent to the SCN (e.g. optic chiasm, anterior portion of the arcuate nucleus or preoptic area) is neither necessary nor in itself sufficient to cause failure of ovulation whilst lesions within the SCN destroying at least half of it will result in anovulation.<sup>75</sup>

Therefore it would appear that the SCN is a critical area involved in the regulation of circadian rhythms and it has been implicated in the control of oestrus cyclicity and in the control of daily surges of luteinizing hormone and timing of ovulation.<sup>76</sup>

Because of its involvement in sexual function and its relative ease of location even in the young, the SCN was chosen for a study of synaptogenesis and the effects of sex hormones thereon.

## HYPOTHESIS

The experiments described in this thesis were designed to test the hypothesis that the exposure of certain areas of the brain to testosterone which may be converted to oestradiol leads to morphological changes which should become evident during the critical period. To test this synaptogenesis was measured in the suprachiasmatic nucleus of rats of both sexes (and in those who had been subjected to abnormal <sup>normal</sup> treatment) from birth to maturity.

## MATERIALS AND METHODS

### Animals

The rats used in the experiments were Wistar (Carworth, Europe) remote strain bred in our own colony.

The groups of animals used fall into the following categories:

<u>Animals</u>	<u>Treatment</u>	<u>Sacrificed</u>	<u>Processing</u>
Normal males	-	Age 5 hrs to adult	Perfuse fixation
Normal females	-	"	"
Neonatally castrated males	castration day 1	"	"
Neonatally androgenised females	TP administration days 1-5*	"	"
Normal prenatal females	-	20 days gestation	Immersion fixation
Normal prenatal males	-	"	"

\*date of birth = day 1

### Treatments

Neonatal Castration - This was carried out on day of birth after the animals had been anaesthetised by hypothermia.

Neonatal Androgenisation - This was carried out by administering testosterone proprionate (TP) 0.5µg per rat s/c daily for the first five days of life, date of birth = day 1.

## Solutions

Stock solutions stored at 4° C

Glutaraldehyde 25%                      Osmium tetroxide ( $\text{OsO}_4$ ) 2%

Buffer 0.14M sodium cacodylate pH 7.4 (adjusted by addition of  
0.1N HCL)

## Perfusion Solutions

Perfusate made up fresh before use.

6.5% glutaraldehyde in sodium cacodylate buffer pH 7.4 and allowed  
to warm to room temperature before use.

## Post fixative

1% osmium tetroxide in sodium cacodylate buffer - used at 4° C

Immersion fixation solutions - made up fresh before use

3% glutaraldehyde made up in sodium cacodylate buffer pH 7.4 -  
used at 4° C

## Post fixative

1% osmium tetroxide in sodium cacodylate buffer - used at 4° C

Perfusion fixation - Immature animals aged up to 10 days

The animals were anaesthetised by hypothermia and up to 0.5 ml of  
heparin (1000 units/ml) were injected into the left ventricle.

The perfusate was introduced by passing a blunt hypodermic needle  
(25 gauge) through a slit in the left ventricle until the tip  
just reached the aorta. The left ventricle was clamped and  
perfusion was started and as soon as the right auricle began to  
swell it was cut to allow the perfusate to flow. Perfusion was  
for 30 minutes at not less than 160mm Hg. The animal was then  
decapitated and the head placed in fresh buffered glutaraldehyde for  
24 hours before the brain was removed.

### Perfusion - older animals

The animals were anaesthetised with ether and about 0.5ml (1000 units/ml) heparin was injected into the left ventricle to prevent blood clotting. The perfusate was introduced by passing a blunt hypodermic needle (23 gauge) up into the aorta where it was tied. The right auricle was cut on swelling to allow the perfusate to flow. Perfusion was continued for 30 minutes at not less than 160 mmHg before the animal was decapitated. The head was placed in fresh buffered glutaraldehyde for 2 hours before the brain was removed.

In both cases perfusion was abandoned if the animals' nose and chin had not turned hard and yellowish within 2 - 3 minutes.

The brains were cut slowly from the skull, taking extreme care not to cause mechanical stress. The cranial nerves were cut free rather than being pulled or torn.

### Immersion fixation - foetal animals

Perfusion fixation was impractical for anatomical reasons therefore immersion fixation was carried out on foetal brains. The brains of these prenatal animals were removed from the skulls and immersion fixed in buffered glutaraldehyde for at least four hours. As the prenatal brains were very small they were sliced horizontally Fig 26, for processing as below.

### Processing

The brains were left in glutaraldehyde for up to 2 days, then rinsed thoroughly in sodium cacodylate buffer and sliced coronally

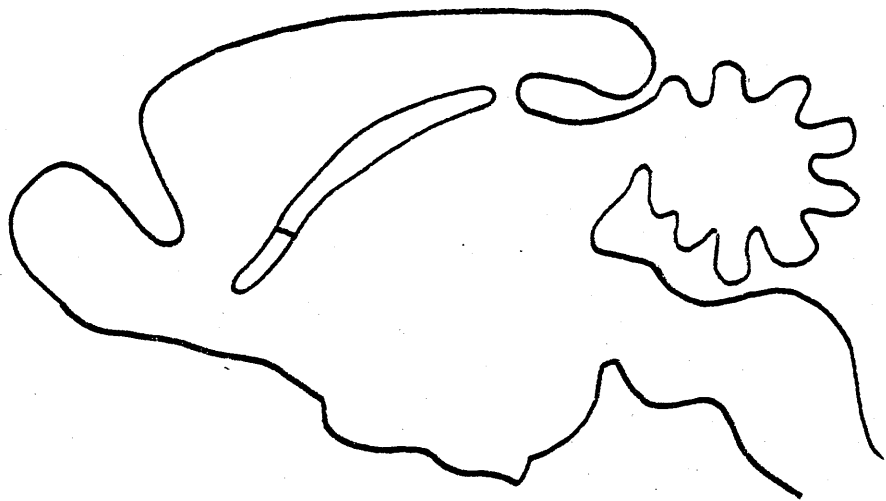
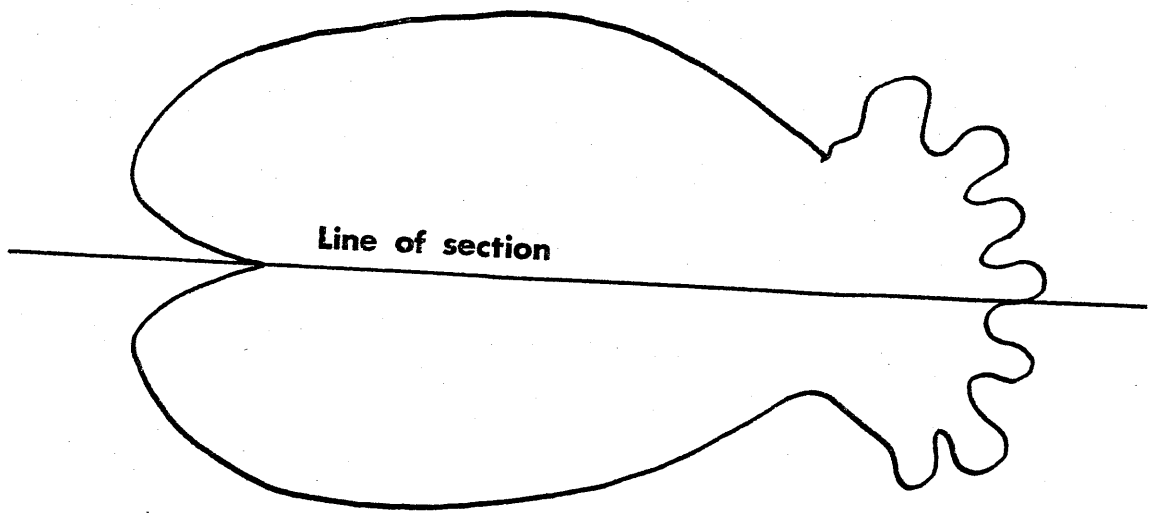


Figure 26. Diagrams to show lines of section taken for processing of prenatal brains.



into sections of approximately 1mm thick, using a long razor blade and a glass template (Fig 27). Drawings were made of each slice, showing gross features and landmarks, from the level of the pineal gland onwards. The slices were quartered (Fig 27) and transferred to vessels containing fresh sodium cacodylate buffer at 4°C. Extreme care was taken during the handling of the brains so as not to deform them in any way and to keep the tissue moistened with buffer during all stages.

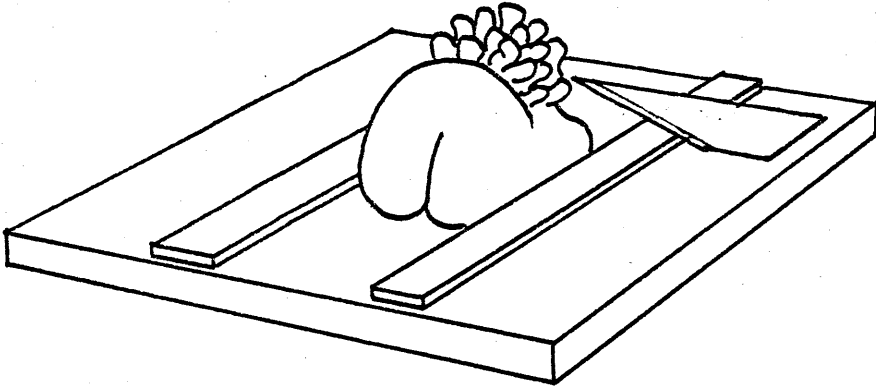
The slices were left in buffer overnight at 4°C and transferred to 1% osmium tetroxide in sodium cacodylate buffer, for 18 hours at 4°C. The osmicated slices were dehydrated in a graded series of ethanols and propylene oxide, and infiltrated with Epon as shown below:

<u>Solution</u>	<u>Time</u>	<u>Temperature</u>
50% ethanol	20 mins	Room temp
70% "	20 "	" "
95% "	20 "	" "
Absolute ethanol	30 "	" "
Absolute ethanol	30 "	" "
Propylene oxide I	15 mins	" "
Propylene oxide II	15 "	" "

The slices were infiltrated with:

Propylene oxide : Epon 3 : 1 overnight 4°C in capped bottles.  
 Propylene oxide : Epon 2 : 1 3 hours 4°C  
 Propylene oxide : Epon 1 : 1 3 hours room temperature  
 Pure Epon 3 hours room temperature

A



B

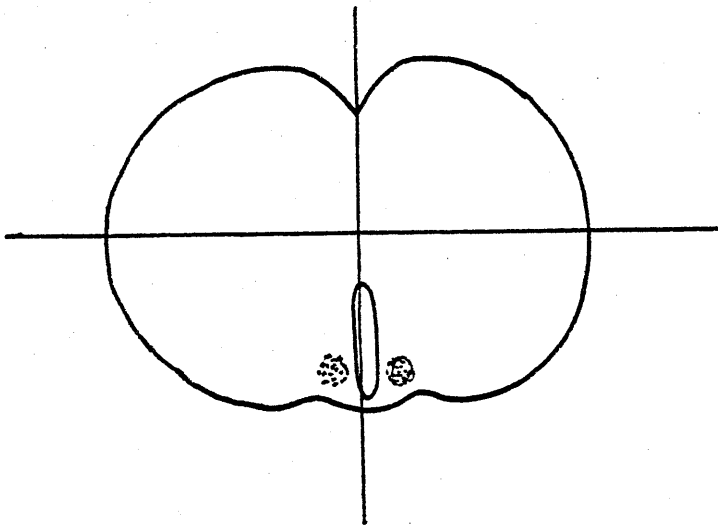


Figure 27. Diagram to show slicing of brain for processing for E.M.

#### Epon Resin Mixture

Resin Epon 812	16 gms
D.D.S.A.	10 gms
M.N.A.	10 gms
B.D.M.A.	0.6 mls.

The above chemicals were obtained from Polaron Equipment Limited.

The slices were taken from the pure Epon carefully orientated and embedded in fresh Epon in flat embedding moulds. The blocks were polymerised at 60°C for 3 days.

#### Location of SCN in Epon blocks

In order to become familiar with the structure of the areas of the brain surrounding and including the SCN a small 'atlas' of light microscope sections, based on the atlas of Timaras and Vaccari<sup>77</sup> (which could not be used accurately as it does not deal with animals younger than 10 days of age) was compiled from the hypothalamus of a 4 day old rat, taking sections of 1.5  $\mu$ m thickness at 50  $\mu$ m intervals. The sections were stained with tolluidine blue, mounted in DPX and photographed using a Reichart Microstat light microscope (Figs 28, 29, 30.).

The SCN was located in individual animals by cutting sections 1.5  $\mu$ m thick and staining with the tolluidine blue for examination under the light microscope. By referring to the 'atlas' it was possible to follow the landmarks and features in the hypothalamus and accurately locate the SCN. Having located the nucleus in the light section the block was carefully trimmed for sectioning for electronmicroscopy. (Figs. 31a, b., 32).

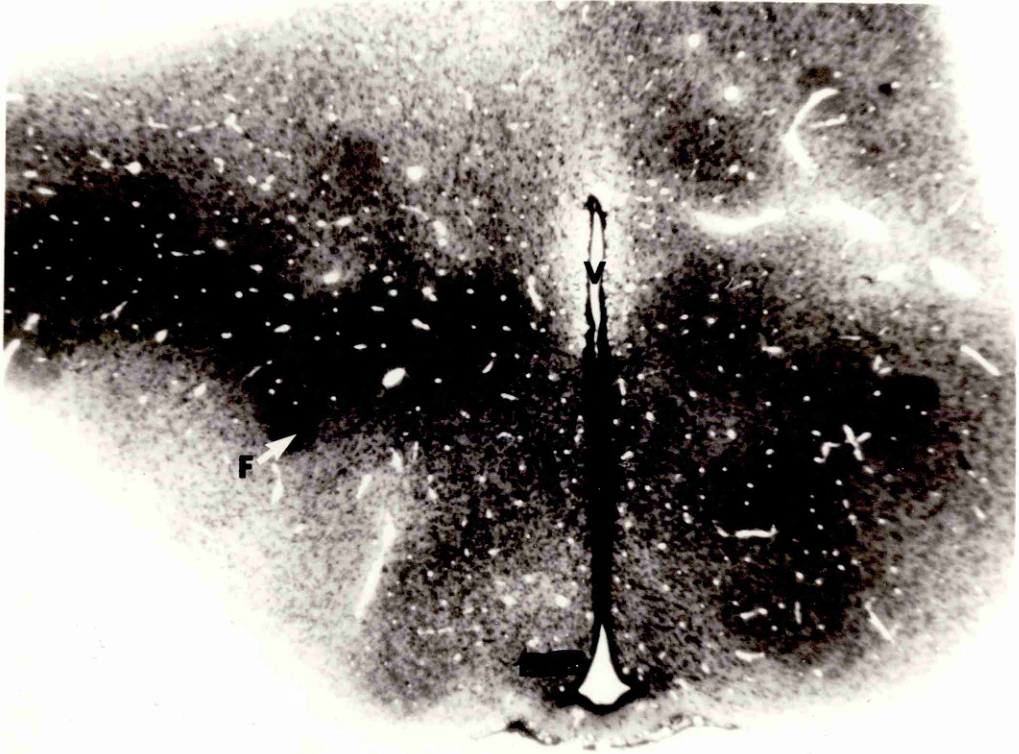
The final block included the area of the SCN together with a portion

Figs. 28, 29, 30.

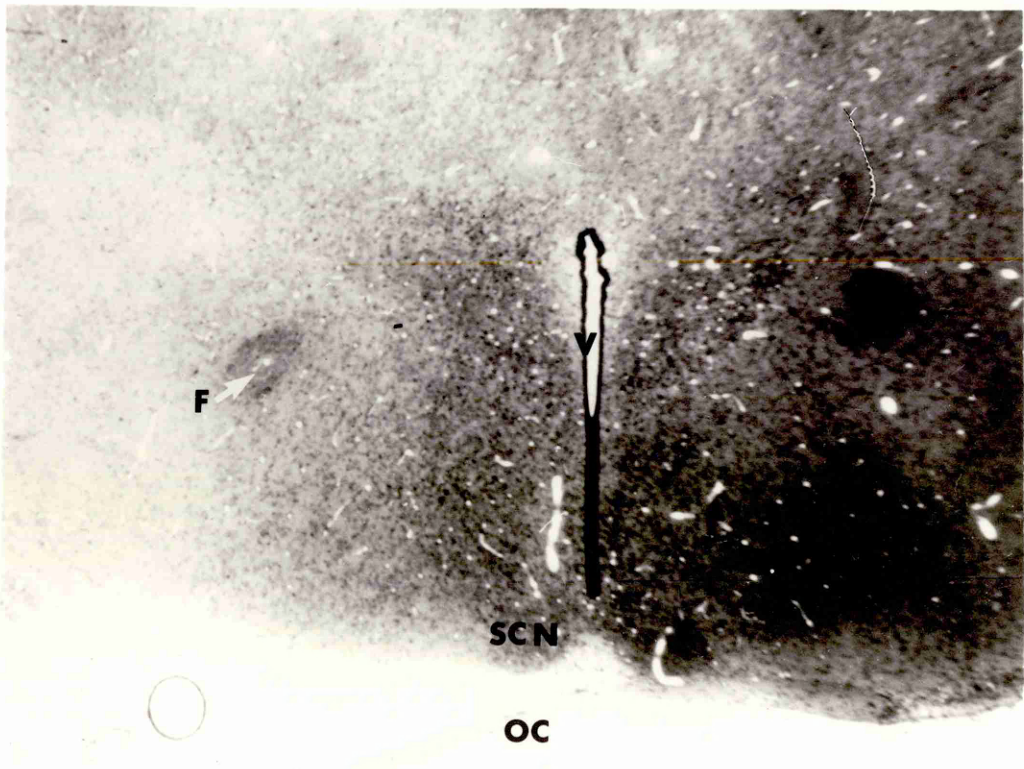
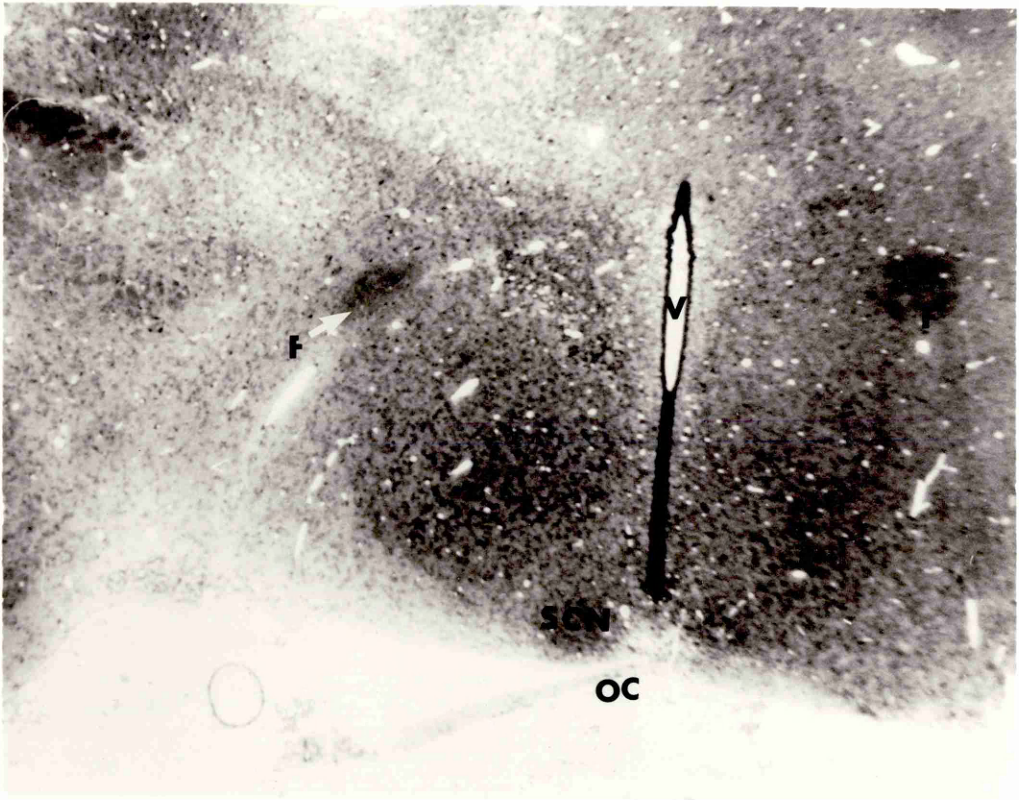
Show examples of the light micrographs used in the ' atlas' for location of the S.C.N. in young animals. The landmarks used for location and features of particular interest are abbreviated as follows:-

F     Fornix  
M E    Median Eminence  
O C    Optic Chiasm  
V       Ventricle  
S C N   Suprachiasmatic Nucleus  
O N     Optic Nerve  
A H N   Arcuate Nucleus  
  
P M N   Premammillary Nucleus

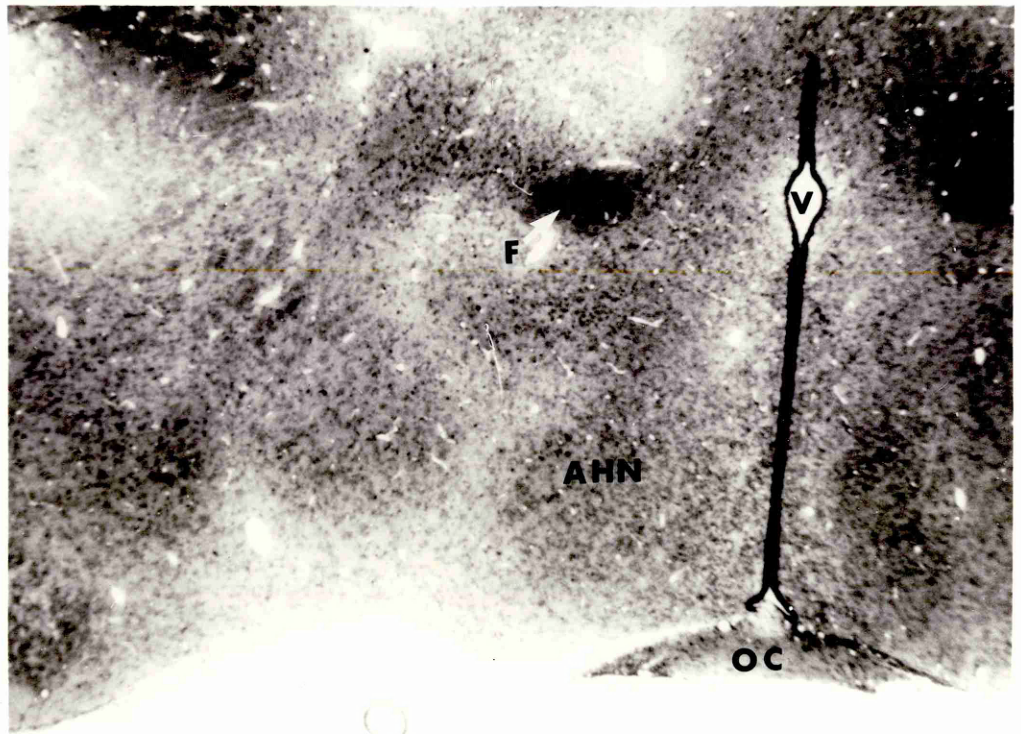
Magns. **x 70**











of the third ventricle and part of the optic chiasm, which were useful for orientation purposes, (Fig 32.)

#### Ultramicrotomy

The trimmed block was sectioned on a Reichart OM4 ultramicrotome and sections of thickness 60 - 70 nm were collected on G400 (Gilder) hexagonal copper support grids.

Sections were stained in a saturated solution of uranyl acetate in 70% ethanol for 15 minutes, washed in distilled water and counterstained in 0.4% lead citrate in 0.1N sodium hydroxide (carbonate free) for 3 - 5 minutes. The sections were rinsed in distilled water and dried before examination in the Philips 301G electron microscope.

#### Photography

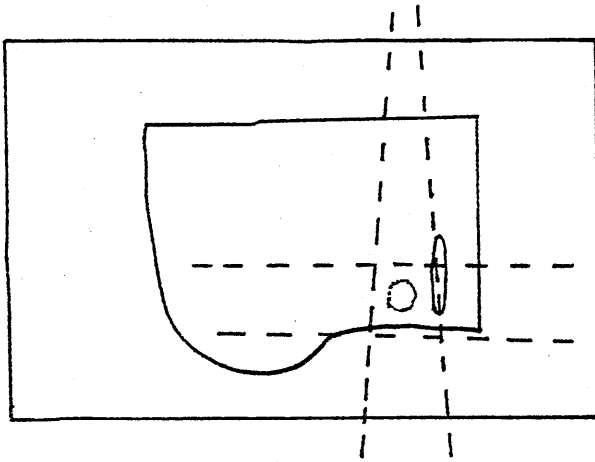
The optimum magnification chosen for taking the micrographs was 13000 times - this magnification being sufficiently high to identify the features in question whilst not being too high as to drastically reduce the area of sample being examined. At least 23 negatives were taken at random over the whole of the section of the SCN. Any blocks which showed any evidence of poor fixation, for example, presence of blood cells (in perfusion fixed animals), evidence of swelling or shrinkage of sub-cellular organelles, ill-defined cell membranes or tears in the tissue, were rejected at either the light or electron microscope stage.

#### Morphometric Analysis

The negatives for each sample were contact printed on Agfa resin-coated paper grade 3 and a piece of clear acetate sheet placed

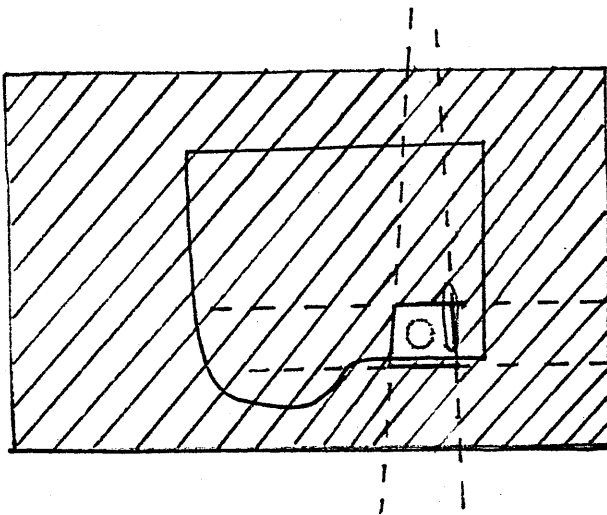


FIG.31a



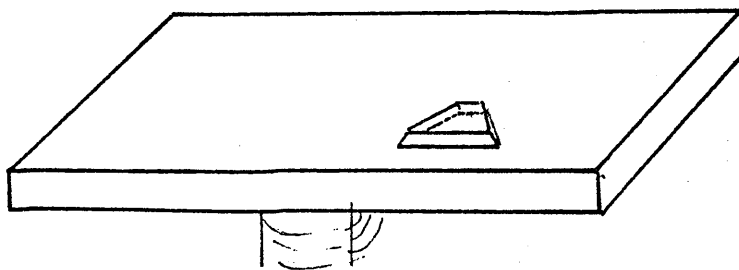
**Light  
microscope  
section**

FIG.31b



**block**

FIG.32.



**block**

Figures 31 a, b.

Show the location of the SCN and trimming  
of blocks for E.M.

over the photographs. Coloured marking pens were used to outline the following features

- 1) Areas other than neuropil; e.g. cell bodies, cell nuclei, extra-cellular space.
- 2) Areas covered by synaptic vesicles.
- 3) Numbers of synapses.
- 4) Length of synaptic contact.

A grid made up of squares of 0.585 cm sides was superimposed onto the marked acetate sheet and the following were counted;

- a) points (each intersection between a vertical and a horizontal line on the grid being defined as a point) overlying areas other than neuropil (Px)
- b) points overlying areas of synaptic vesicles (P Ves)
- c) length of synaptic junctions were calculated by counting the intersections between them and the horizontal and vertical lines on the grid (Ni)
- d) points on areas of extracellular space (Psp)

The results for each sample were tabulated and calculated as described below.

#### Computation

After correction for magnification the following formulae were used:

$$\text{Vesicular area} = (Pv/Pn) \times C_2$$

$$\text{Areas of space} = (Psp/Pn) \times C_2$$

$$\text{Synaptic length} = \frac{\sum Ni}{2 L} \times C_1$$

$$C_1 = 1 + (3h/2d)$$

$$C_2 = 4r (4r + 3h)$$

$$P_n = P_t - P_x$$

where	Pv	=	no. of points on areas of vesicles
	Pt	=	total no. of points on photograph
	Px	=	no. of points <u>not</u> on neuropil
	Pn	=	no. of points on neuropil
	Psp	=	no. of points on extracellular space
	Ni	=	no. of intersections between synaptic junctions and grid lines
	L	=	total length of vertical and horizontal lines on grid
	h	=	section thickness
	d	=	average length of individual synapses
	r	=	average radius of vesicle clusters

Formulae derived from Weibel (1963)<sup>78</sup> and Vrensen and de Groot (1973)<sup>79</sup>.  $C_1$  and  $C_2$  were correction factors for the Holmes Effect and were calculated to be 1.16 and 1.23 respectively.

#### Holmes Effect

Morphometric analyses are based on the assumption that the section is of infinite thinness.<sup>78</sup> This is not true and structures whose dimensions are of the similar order of the section thickness will give distorted values, Fig 33.

#### Calculations

A computer program was written to calculate the values for vesicular area, synaptic length and extracellular space from the figures derived from the grid point counting. (Table 1).

FIG. 33.

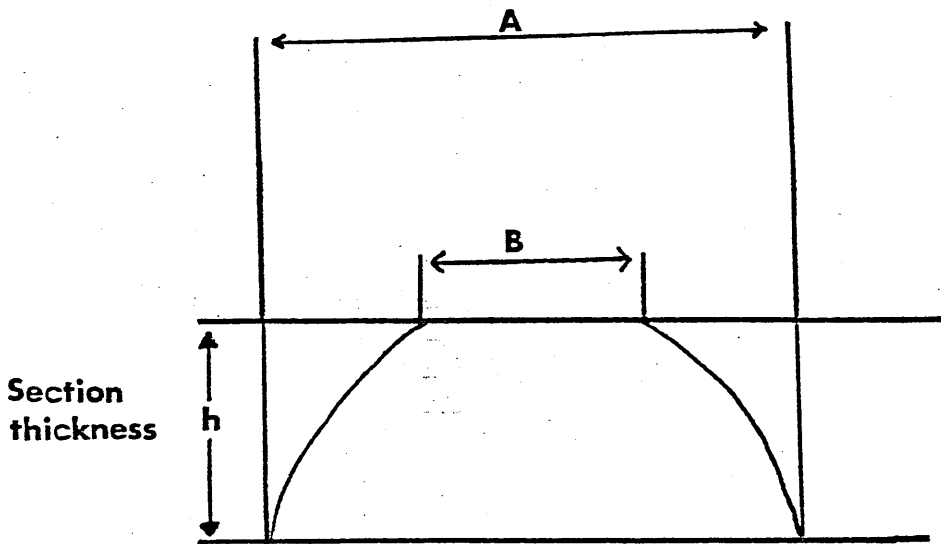


Figure 33.

A diagram illustrating the Holmes effect. Morphometric measurements are made assuming the section is of infinite thinness (B). As this is not possible in practice subcellular structures will actually appear larger (A). The degree of distortion depends on the size of the organelle in relation to the thickness of the section. The thinner the section the lesser the degree of distortion. Corrections for the Holmes effect are made on the assumption that organelles are spherical.

### Phosphotungstic Acid (P.T.A.) staining

As PTA is a stain which binds to proteins, making them electron opaque, it is often used on brain tissue to demonstrate synaptic junctions using the electron microscope.

By omitting the stage of post fixation with osmium tetroxide (this stain would compete with PTA for the protein binding sites) in the processing schedule, the block of tissue can be stained with PTA solution in ethanol during the dehydration stages.<sup>80</sup>

I tried several different methods of PTA staining on blocks of tissue, see page 68, from perfuse fixed brains but decided not to pursue the technique for the following reasons:

The results achieved after many trials were not reproducible.

In all eight series of experiments were carried out on blocks of tissue taken from the hypothalami of perfuse fixed brains.

Concentration of PTA, times of infiltration, were varied systematically with stains obtained from several different sources, but results were inconsistent.

It seems possible that the inability to produce consistently well-stained blocks could be partly due to the different chemical properties in the immature brain from those of the adult. Indeed, immature brains may often show varying affinities for some histological stains.<sup>81</sup>

Even so, this does not account for the variation in staining between blocks of tissue from adult animals and it seems that here, consistency of results is very much dependent on the amount of water present in the stain and/or the ethanol. Nevertheless, when the PTA staining was successful the synaptic junctions and cell membranes

can be demonstrated very well (Figs 44,45). It can be seen from the photograph that the technique has limited use for this project as only the synapses are stained not the synaptic vesicles. As other parts of the tissue do not show up very clearly it would not be easy to locate a discrete area such as the SCN using this technique. Furthermore such lack of definition of cellular tissue other than membranes and synapses makes it extremely difficult to assess the quality of the fixation.

Method of PTA staining

Pieces of hypothalamus from perfuse fixed brains were rinsed in sodium cacodylate buffer pH 7.4 several times over a period of 2 hours at room temperature, then dehydrated as follows:

50% ethanol	15 mins	room temperature
70% ethanol	15 "	" "
95% ethanol	15 "	" "

Samples were then stained with PTA as follows, dividing the pieces into 4 groups, varying the amount of water added, and time of infiltration 1% PTA dissolved in absolute ethanol.

	Staining Time	
1)	1 hour	No H <sub>2</sub> O
	2 hours	No H <sub>2</sub> O
2)	1 hour	1 drop H <sub>2</sub> O/100ml Abs EtOH PTA stain
	2 hours	1 drop H <sub>2</sub> O/100ml Abs EtOH PTA stain

	Staining Time	
3)	1 hour	2.5 drops H <sub>2</sub> O/100ml Abs EtOH PTA stain
	2 hours	2.5 drops H <sub>2</sub> O/100ml Abs EtOH PTA stain
4)	1 hour	5.0 drops H <sub>2</sub> O/100ml Abs EtOH PTA stain
	2 hours	5.0 drops H <sub>2</sub> O/100ml Abs EtOH PTA stain

Dehydration was continued as shown below:

Absolute ethanol	2 x 10 minutes	room temperature
Propylene oxide	2 x 15 minutes	

Samples were infiltrated with Epon and embedded as described on

Page 55.

## RESULTS - Descriptive

From the light microscope investigations no qualitative differences were seen in the suprachiasmatic nuclei of male or female rats.

Fixation was assessed to be good if there were no blood cells present in the capillaries and there was a clear differentiation between cells. The information obtained by viewing the sections under the light microscope was limited by the choice of toluidine blue staining (Fig. 34 ) which suited the needs for SCN location (Fig. 35 ) staining equally well in both mature and immature, although the latter appearing paler as a whole due to the extra-cellular space in the immature.

By selectively staining with other histological reagents other features would have been brought out but this was not the object of the exercise. Examination of the tissue under the electron microscope showed the SCN of males and females to be morphologically similar. Confirmation of the light microscope assessment of good fixation was seen by the quality of preservation of individual cell nuclei, mitochondria, cell membranes, nissl bodies and endoplasmic reticulum, shown in Figs 36 and 37 and also the ciliated ependymal cells lining the third ventricle.

In animals older than 20 days the preservation of the myelinated nerve fibres in the optic chiasm and scattered throughout the nucleus was indicative of good fixation (Fig. 38 ) Once again the lack of blood cells in the capillaries also served to indicate good perfusion technique.



There were striking qualitative differences between the SCN of newborn and adult animals of both sexes. As well as the difference in SCN size, there was a lack of myelination of nerve processes (Fig 41 ) and presence of growth cones (Fig. 40 ) in the immature. There were also large amounts of extracellular space in the immature which decreased as the animals matured and disappeared at 10 days of age. It is presumably through this extracellular space in the immature that nerve processes grow and cells migrate<sup>82</sup>. No space was seen within the SCN of adult animals but was obvious in the region between the SCN and the third ventricle, referred to as the cell poor zone (CPZ) by Van den Pol<sup>83</sup> in both immature and mature (Fig. 43 ).

#### Synapses

In 1959, Gray<sup>89</sup> described two different types of synapse, these being Type I and II. The synapses which were measured in this study proved mostly to be type I or asymmetric. The postsynaptic side is a continuous plaque of dense material containing protein and is filamentous in structure. The individual filaments are often not discernible because they overlap in the section thickness. The presynaptic side is not continuous but is made up of pyramidal mounds of dense material set up in an array or 'grid'. The extent of this 'grid' varies but is not so clearly defined in material fixed with glutaraldehyde and  $O_2O_4$  but can be clearly demonstrated using the PTA staining technique (Fig. 44 and 45) Note that whilst the PTA stained synapses were shown in great detail, because of the limits of the method, this was at the expense

of the clarity of the surrounding tissue.

Only axo-axonic and axo-dendritic (spine and shaft) synapses were counted and measured as photographs were limited to areas of neuropil and not cell bodies. Figs 39,46 and 47 show typical types of synaptic junctions present in the SCN. Note that while a few photographs show the synaptic junctions clearly, with double membranes, marked synaptic cleft and dense postsynaptic thickenings, more often pictures showed the junctions cut obliquely and thus not so clearly defined (Figs 48).

### Vesicles

The vesicle clusters contained mostly clear spherical vesicles as normally associated with Type I synaptic junctions as described by Gray<sup>84</sup>.

Synaptic vesicles occurred for the most part in the presynaptic region, but in a very few they were also apparently present in the postsynaptic region (presumably forming the presynaptic part of a synapse with another nerve process). Most of the vesicular clusters were made up of clear vesicles ranging in size from 60 - 90 nm but very occasionally one or two dense cored vesicles were present in a cluster (Fig. 49)

In the immature animals there were fewer vesicles in total but of these a high proportion were not visually associated with synapses (Fig. 42 ). On the whole these vesicles tended to be of slightly larger diameter (90-100 nm) than in more mature animals.

Fig. 34

Light micrograph showing the suprachiasmatic nucleus (SCN) in the hypothalamus of an adult male rat. The lumen of the third ventricle (V) is wide and lined with ependymal cells (e). The myelinated nerve fibres in the optic chiasm (O.C.) are darkly stained.

Magn. **X 300**

Fig. 35

Light micrograph of the suprachiasmatic nucleus (SCN) of an immature male, 4 days old. The lumen of the third ventricle (V) is much narrower than in the adult and the nerve fibres of the optic chiasm (O.C.) are unmyelinated.

Magn. **X300**

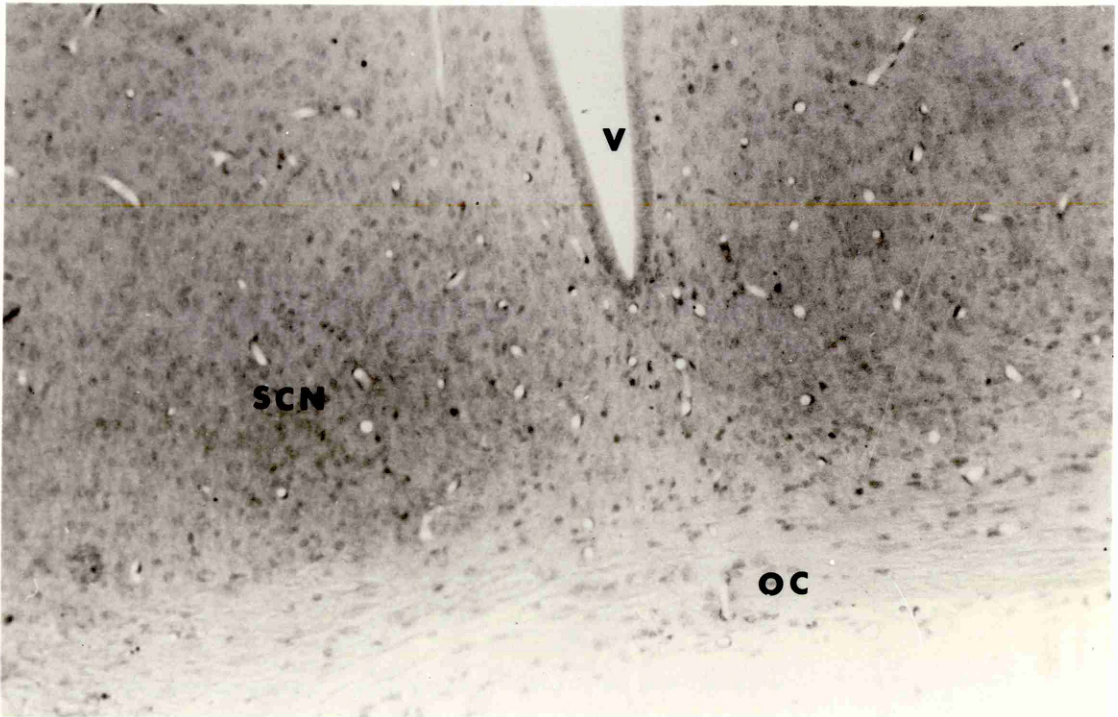
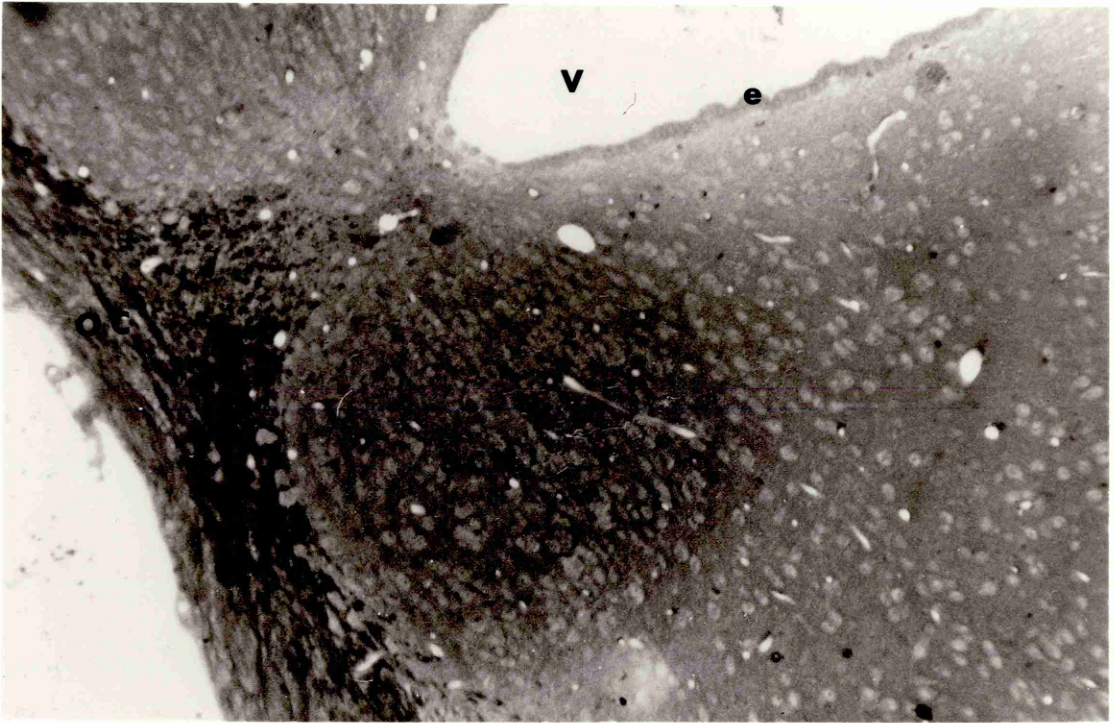


Fig. 36

Electronmicrograph showing the neuropil from a mature male and a portion of a cell body on the right. Within the cell body the nucleus (n) shows a clear nuclear membrane (nm). The cytoplasm shows rough endoplasmic reticulum (rer) and clusters of ribosomes (r). The perikaryon is separated from the neuropil by the cell membrane (cm) and the neuropil is full of nerve processes with neurotubules (nt), many also have large mitochondria (m). One synaptic junction (sj) can be seen between two processes.

Magn. **X36000**

Fig. 37

Another example of the neuropil on the left and perikaryon on the right of an adult male rat, symbols as for Fig. 36.

Magn. **X36000**



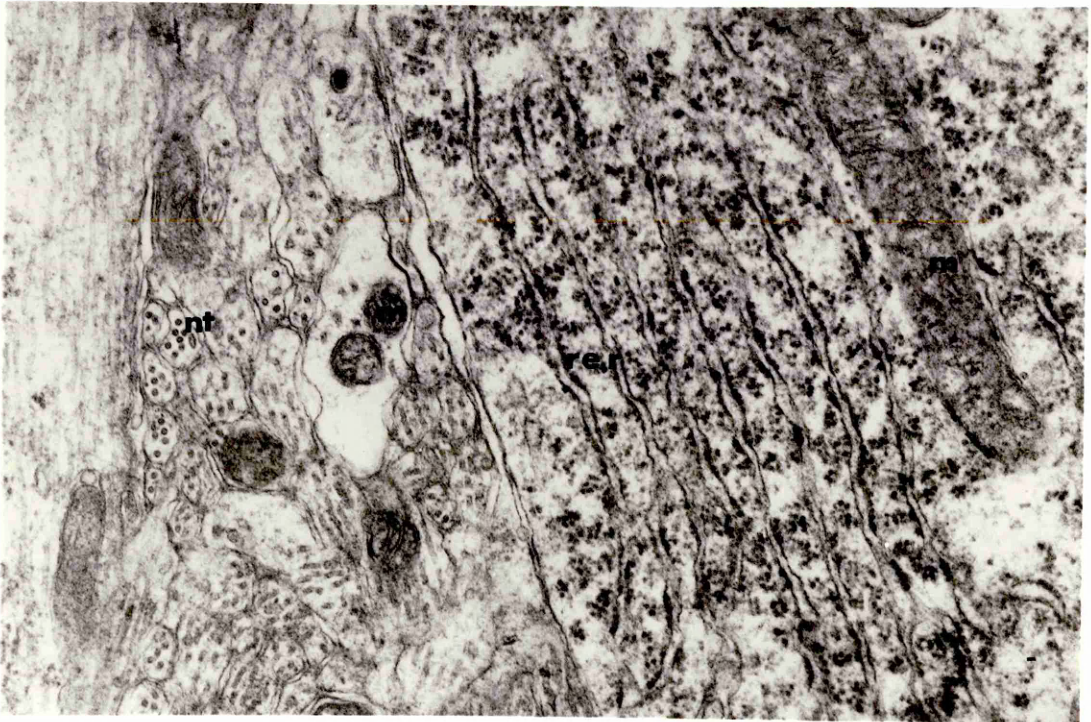
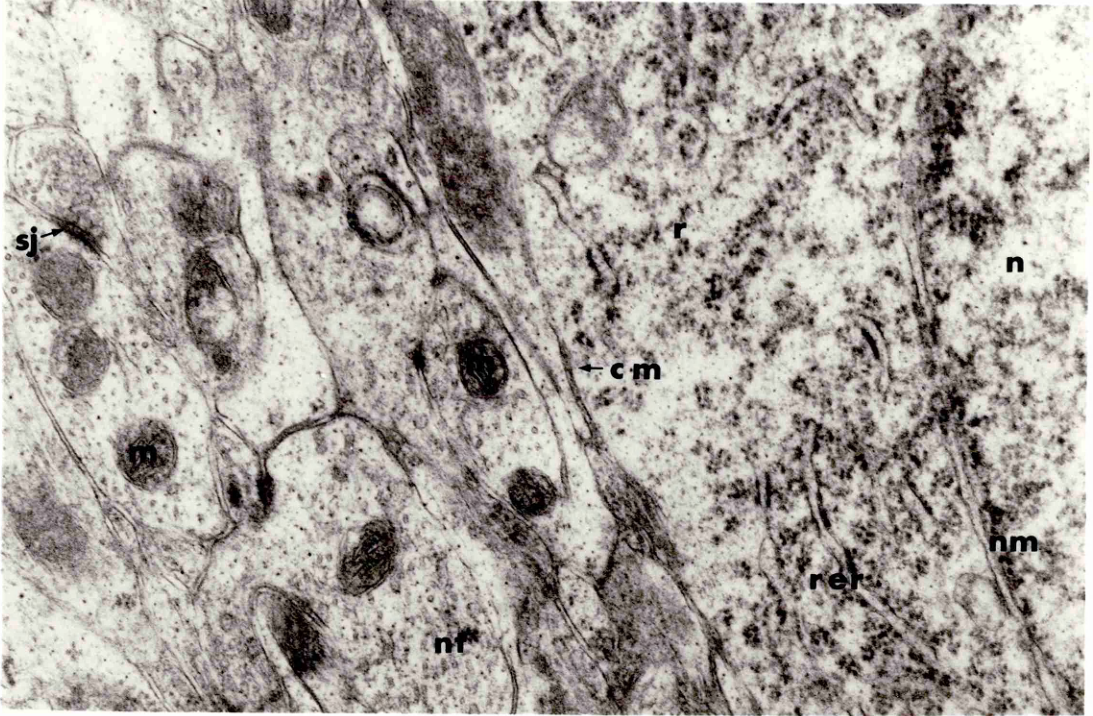


Fig. 38

Lower power electron micrograph showing part of a cell body and neuropil of an adult female. The neuropil is packed with nerve processes, one myelinated nerve (my) can be seen. A cell nucleus (n) surrounded by its perikaryon can be seen on the left showing rough endoplasmic reticulum (rer) and mitochondria (m).

Magn. X**19600**

Fig. 39

Portion of neuropil from an adult male animal. Two synaptic junctions (sj) are impinging onto a long dendritic spine (d) containing a long mitochondrion (m). Clusters of vesicles (v) can be seen in many nerve processes. There is one myelinated nerve (my). A third synaptic junction (sj) can be seen at the bottom of the photograph.

Magn. X**26000**



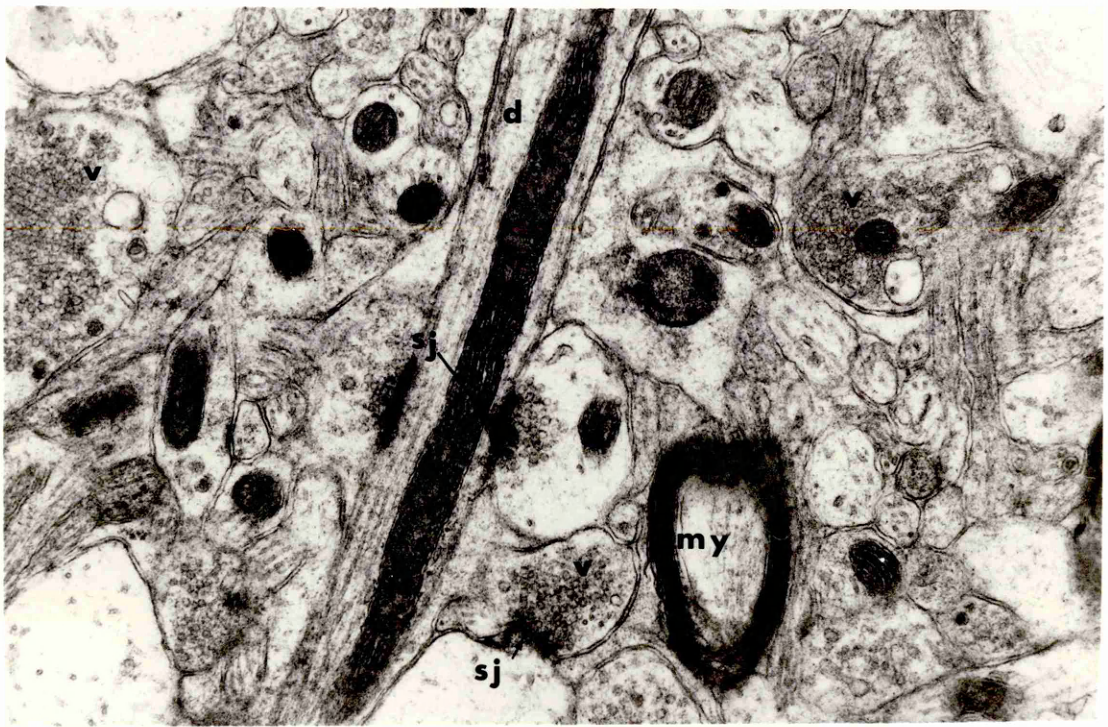
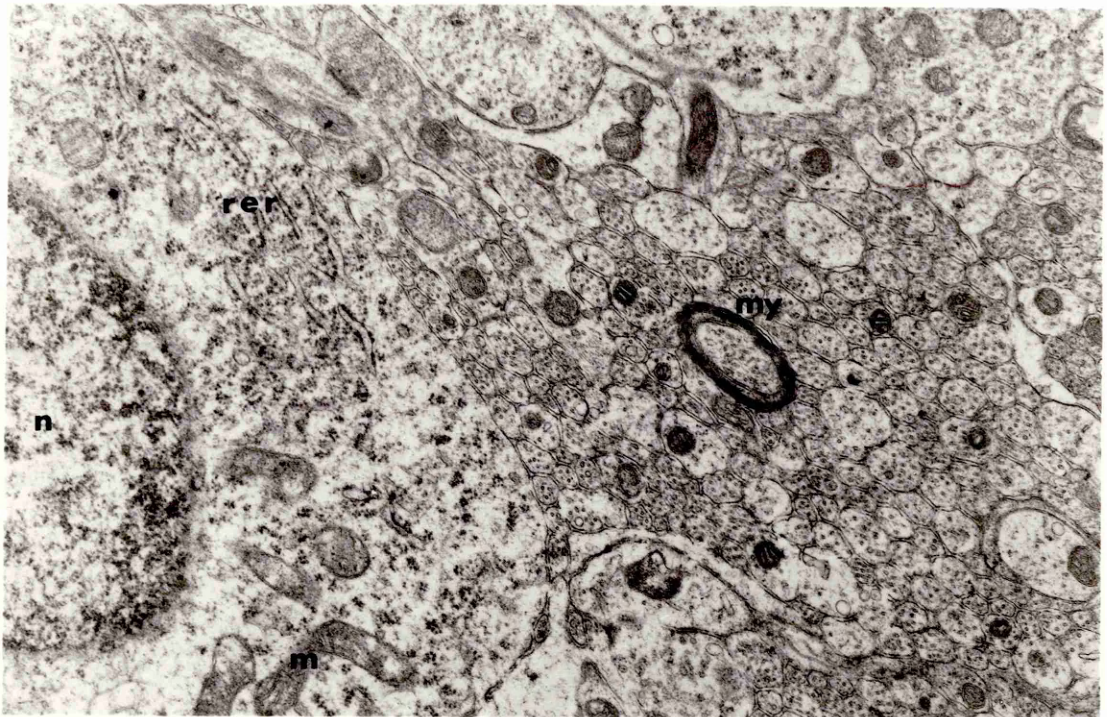




Fig. 40

Electron micrograph of the neuropil of a young male of eight days of age showing a synaptic junction (sj) with associated vesicles (v). A portion of perikaryon (p) can be seen in the top left hand corner.  
Magn. X **36000**

Fig. 41

Portion of neuropil from a very young (2 days of age) male showing areas of extracellular space (sp) between the nerve processes. There are neurotubules (nt) within the nerve processes and one or two mitochondria (m).  
Magn. X **45000**





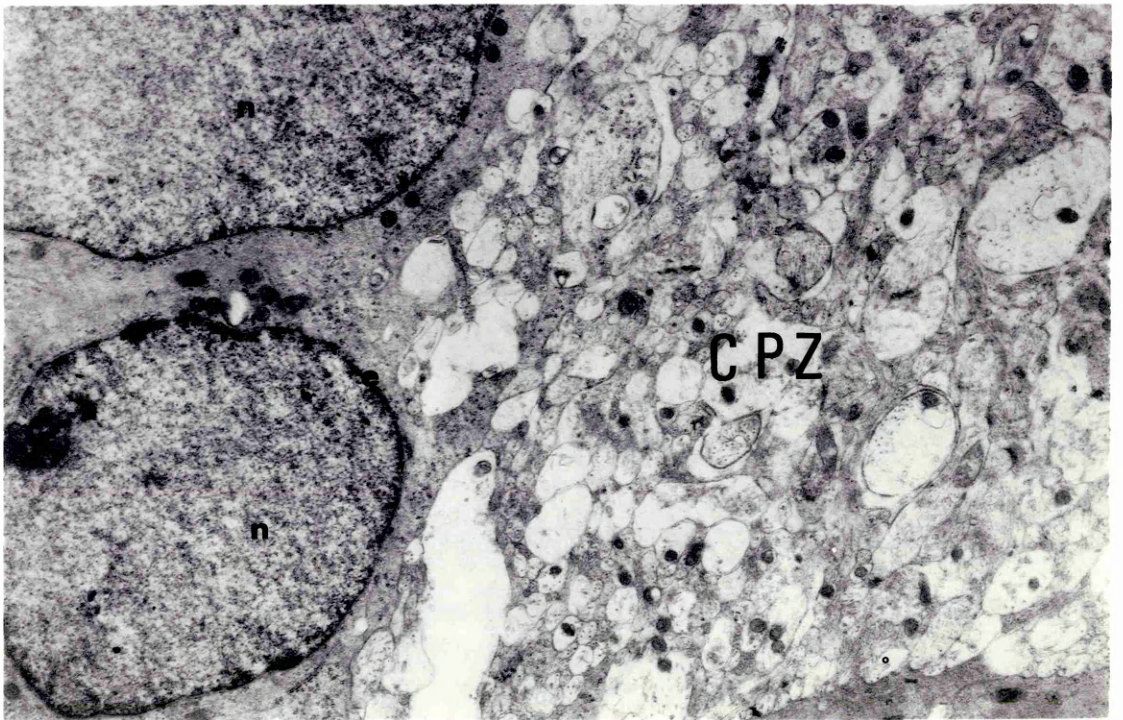
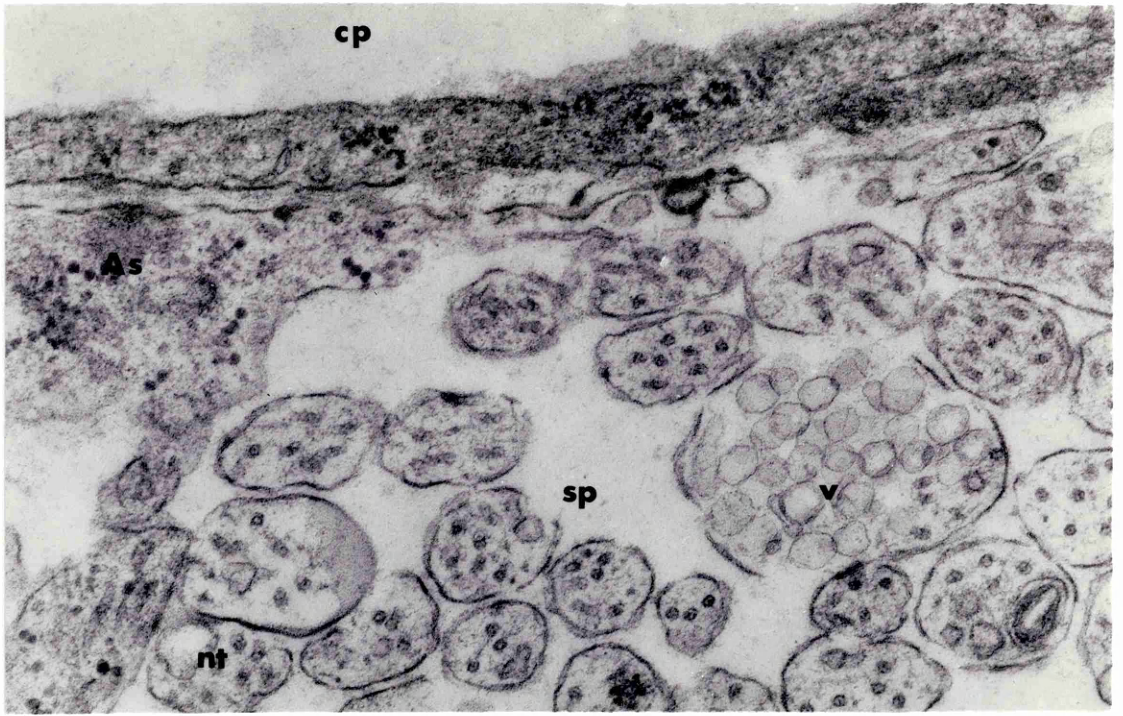


Fig. 42

Portion of neuropil from a 2 day old male with the lumen of a capillary (cp) in the top of the photograph. A portion of the endfoot of an astrocyte (As) is seen in close association with the capillary wall. There are areas of extracellular space (sp) between the nerve processes and a cluster of large vesicles (v) can be seen, these have no apparent association with a synapse.

Magn. X**70000**

Fig. 43

Electronmicrograph showing the cell poor zone (CPZ) between the ependymal cells (e) with their large nuclei (n) lining the third ventricle and the suprachiasmatic nucleus.

Magn. X**10000**

Figs. 44, 45

Electromicrographs of tissue prepared using the Phosphotungstic acid (PTA) method.

Each photograph shows one synaptic junction. Note the clarity of synaptic clefts (c) and the clear distinction between the presynaptic side (PrS) and the post synaptic side (PoS). Because of the limitations of the method, the rest of the tissue is ill defined.

Magn. 44 } **X 108000**  
Magn. 45 }



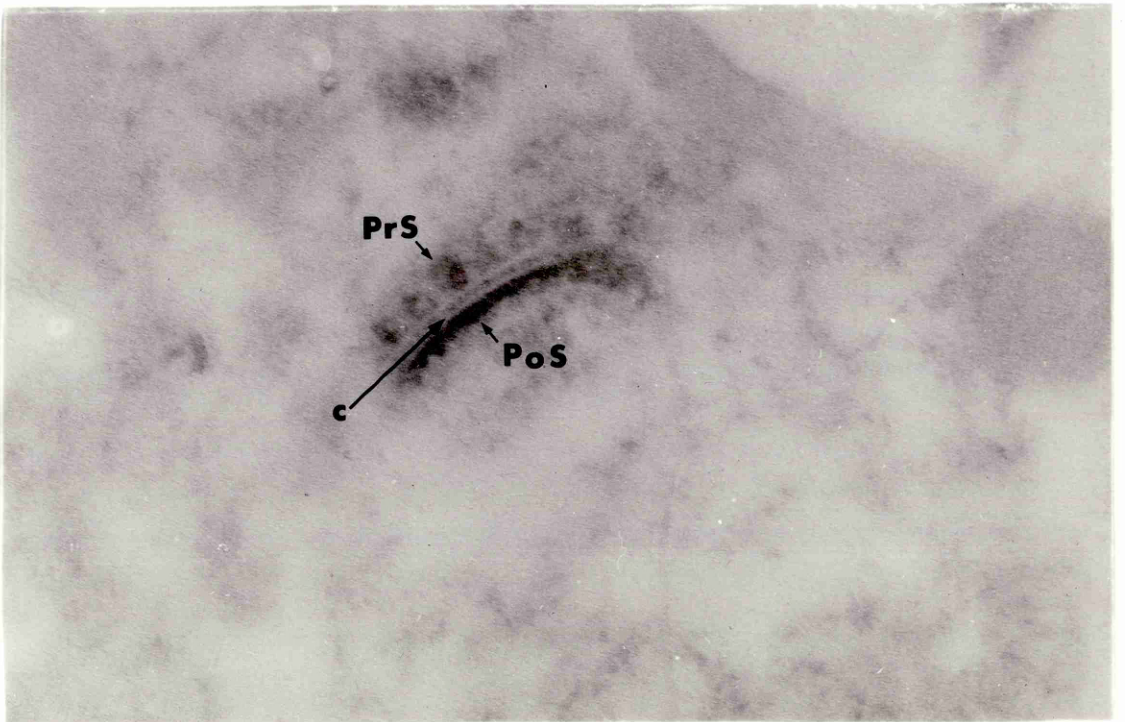
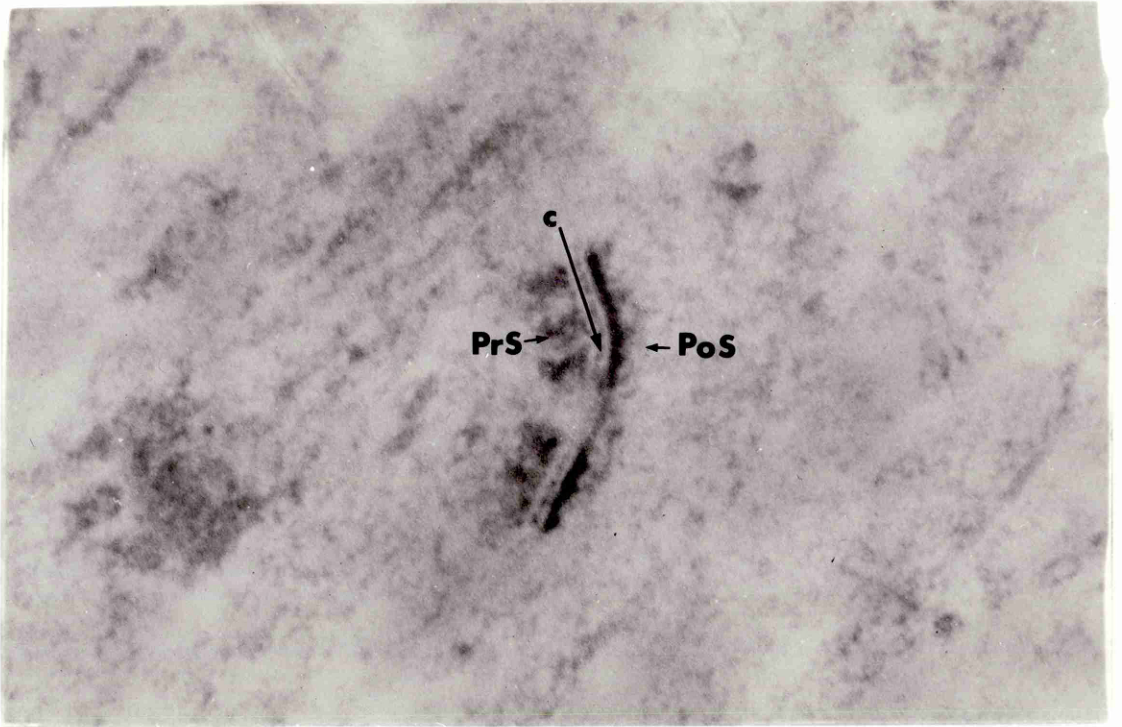


Fig. 46

Electronmicrograph showing synaptic junction (sj) in the neuropil of an immature male 2 days old. There is a cluster of vesicles (v) in the presynaptic process. Extracellular space (sp) is abundant. Other nerve processes show neurotubules (nt).

Magn. **X 72000**

Fig. 47

Portion of neuropil from a mature male showing a synaptic junction (sj) and its associated vesicles (v). The post synaptic portion shows neurotubules (nt) and two mitochondria (m). Note that no extracellular space is visible.

Magn. **X 100000**



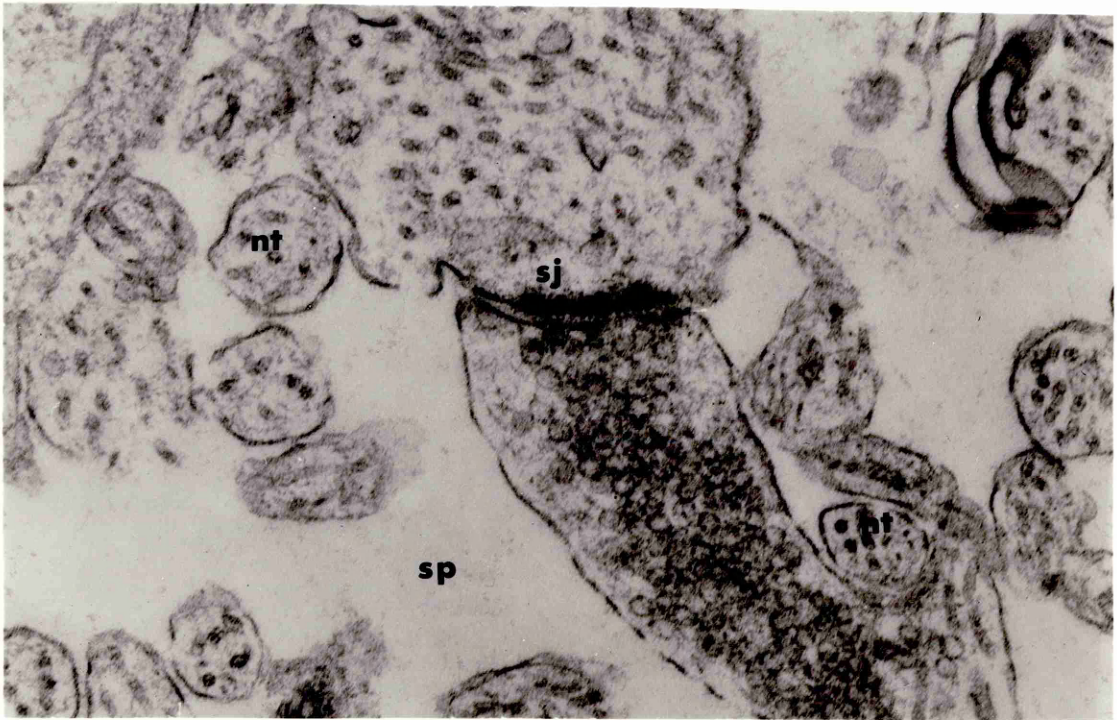




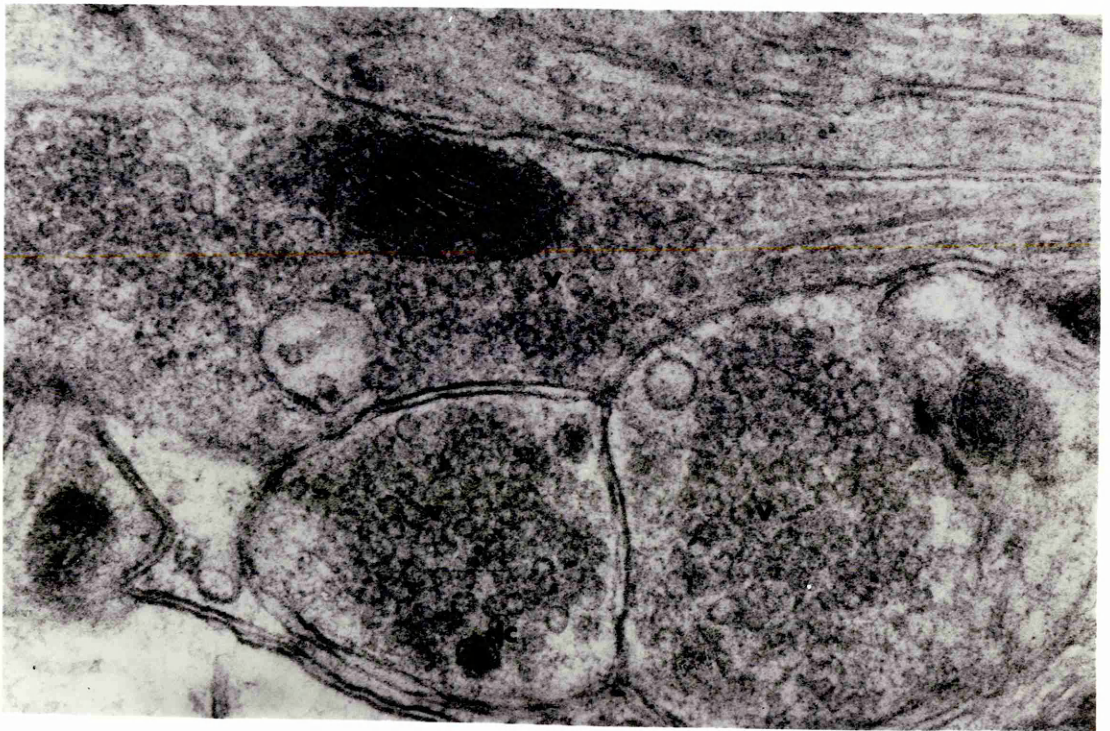
Fig. 48

Neuropil of adult female showing an obliquely cut synaptic junction (sj 1) and one cut in cross section (sj 2) showing the synaptic cleft (c), both impinging on the same process. Clusters of vesicles (v) are associated with both synapses. Other nerve processes show neurotubules (nt) and mitochondria (m).  
Magn. **X56000**

Fig. 49

A high magnification electromicrograph of clusters of vesicles (v) some dense cored (dc) with no apparent associated synapses, from a mature male. Mitochondria (m) are also seen.

Magn. **X70000**



## RESULTS - Quantitative

### Synapses - normal male, female and castrated male.

The dimensions of individual synapses are similar in rats of all ages, and of all groups tested, mean values + or - SEM for lengths of individual synapses being male  $0.385 \pm$  um (d.f. = 8), female  $0.396 \pm 0.022$  um (d.f. = 7), androgenised females  $0.365 \pm 0.0196$  um (d.f. = 8), neonatally castrated males,  $0.538 \pm 0.025$  um (although the value in neonatally castrate males is greater than others I make no further comment as n is only 4) (d.f. = 3). These values are similar to those described elsewhere in the brain<sup>79</sup>.

Numbers of synaptic contacts do, however, change with age and vary with sex. In Figure 50 the development of the synaptic contacts with respect to age, sex and neonatal castration is expressed in two ways:

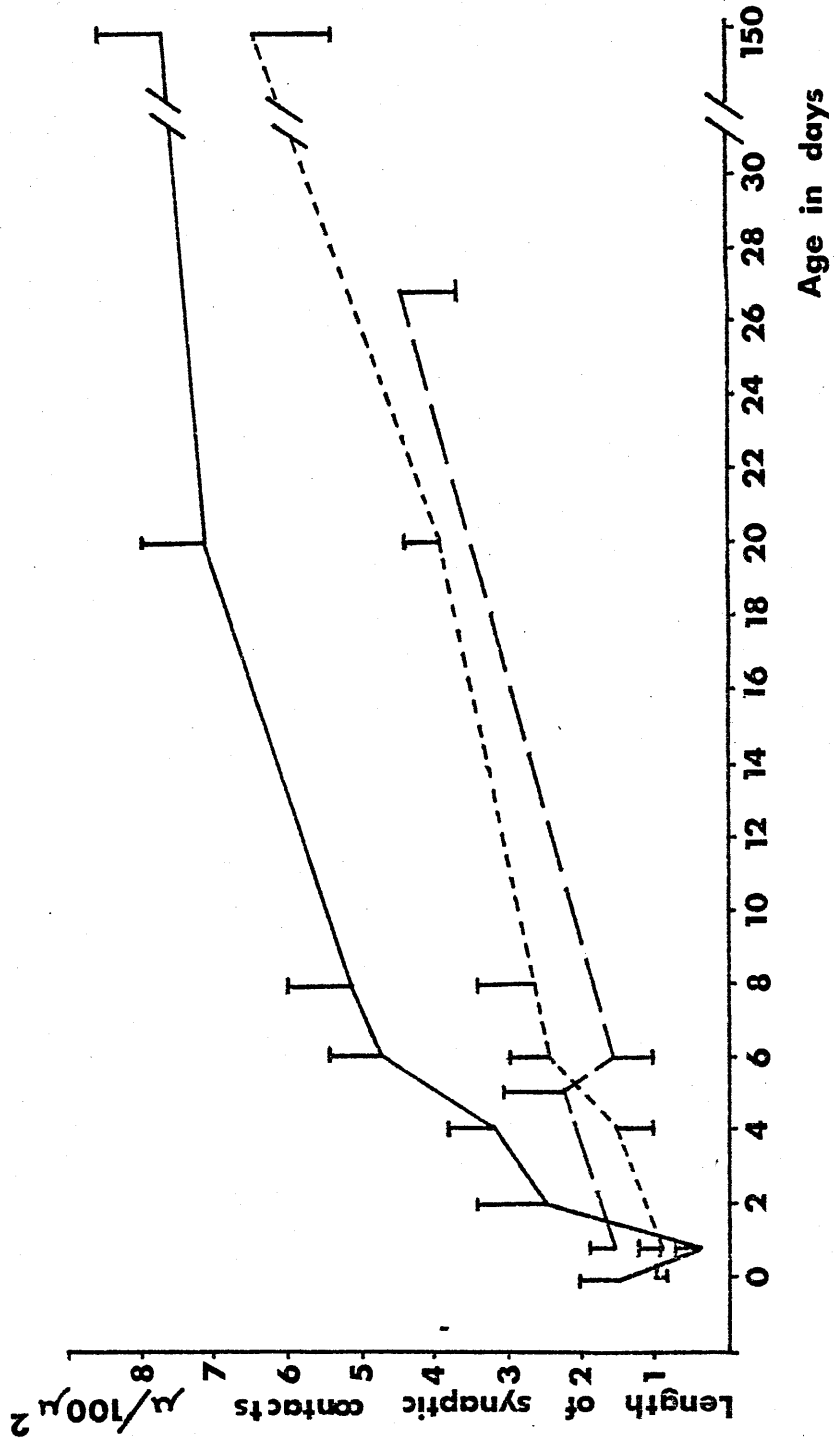
- (A) as the total length of synaptic contact (the product of numbers of synapses and of their individual lengths, and
- (B) as numbers of synapses

Male values, expressed in either way, are higher than those for females, and values for neonatally castrate males are similar to those for females.

The three curves in Figure 50a are similar to those in Figure 50b consistent with the hypothesis that the dependent variable is synaptic number rather than the dimensions of individual synapses.

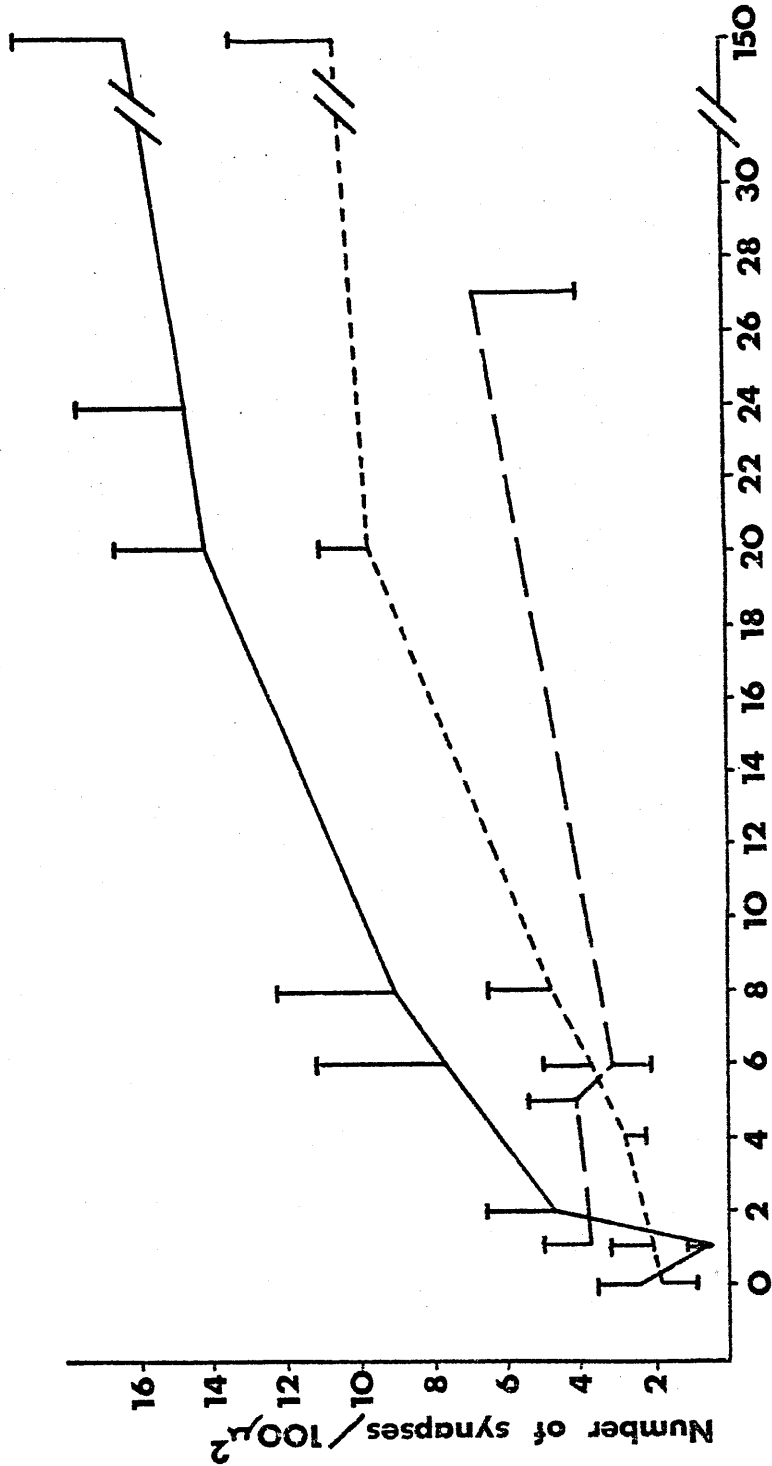
Data were analysed on the assumption that the curves describing all groups were identical but differed in position by a constant

Figure 50 (A)



Relationship between age in days and the total length of synaptic contacts ( $\mu\text{m}$ ). Both values were expressed per  $100\mu\text{m}^2$  of neuropil. Male(—), female(····) and neonatally castrated(---) values are shown. The vertical lines represent S.E.M. of results from the means of at least 23 micrographs per rat (for clarity only one half of each S.E.M. line is shown). Each point represents one animal except for 4-day-old females where two animals were used.

Figure 50(B)



**Age in days**

Relationship between age in days and the total number of synapses. Both values were expressed per 100µm<sup>2</sup> of neuropil. Male (—), female (---) and neonatally castrated (---) values are shown. The vertical lines represent S.E.M. of results from the means of at least 23 micrographs per rat (for clarity only one half of each S.E.M. line is shown). Each point represents one animal except for 4-day-old females where two animals were used.

amount. An analysis of covariance was used to estimate the difference and subsequently to test it. The model:

$$Y = \mu + \beta_0 Z + \beta_1 X + \beta_2 X^2 + \text{error}^*$$

where

Y is the dependent variable (i.e. total synaptic length or synaptic number).

Z is a defined variable (0 for females, 1 for males)

X is the age in days

assumes that the pattern of response is quadratic and differs only by the value of  $\beta_0$ , which is to be estimated.

Results of these analyses are shown in Table 1.

In the case of total lengths of synaptic contact (male vs female) the regression coefficient was significant at the 0.01% level, but more importantly, the  $R^2$  score of 0.90268 implies that the model accounts for more than 90% of the total variation and thus is successful in describing the data. The value of  $\beta_0$  was significantly different from zero at the 0.7% level. Thus we can conclude that there is a real difference between male and female scores.

Statistical analyses of male vs female synaptic number (Table 2) were similar to those for total synaptic length, reinforcing the view that the variable is number of synapses rather than their

\* This model was adopted because of the impracticability of defining an appropriate time "zero".

individual dimensions.

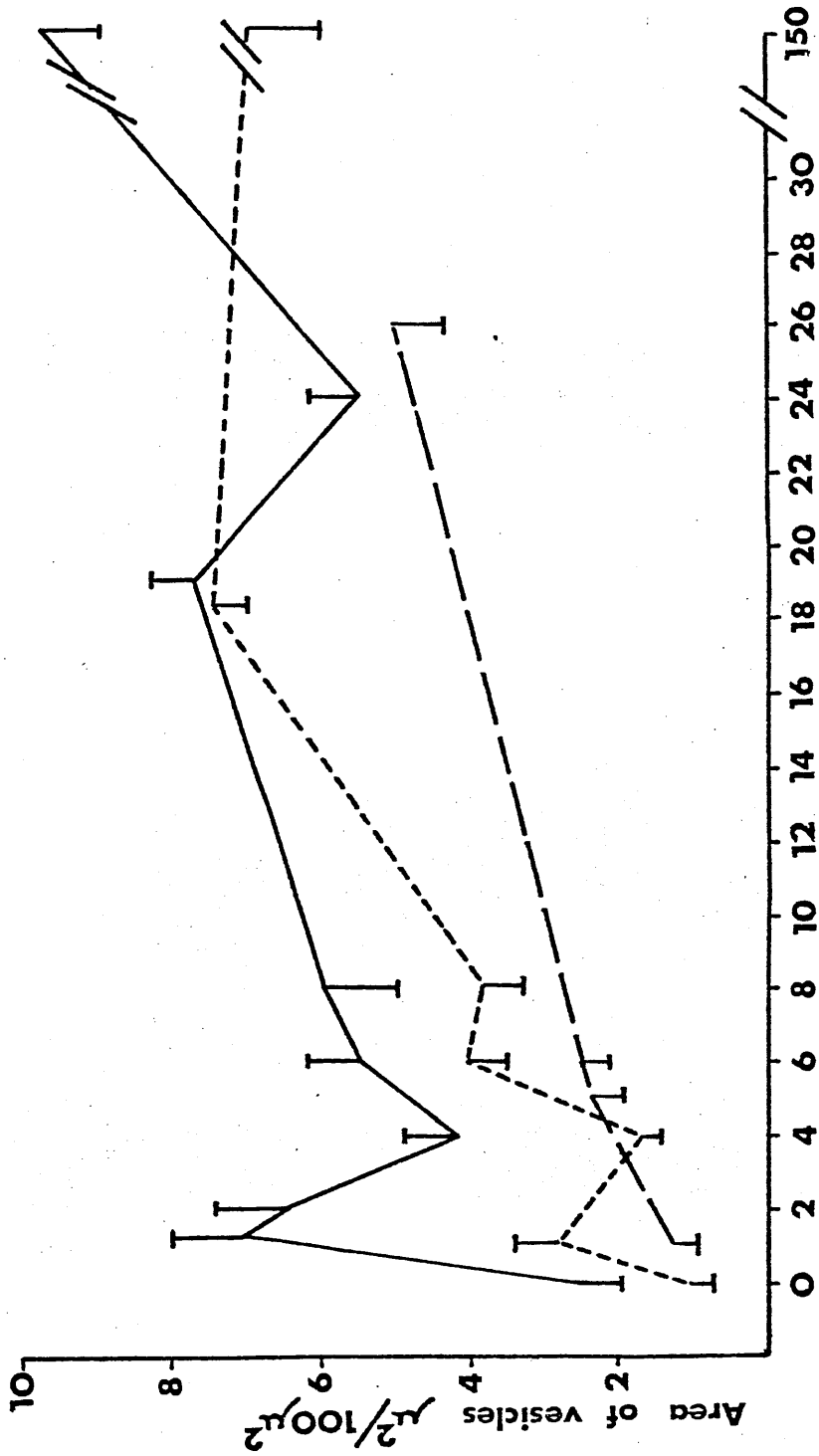
Preliminary results from four neonatally castrate males Table 1 ) show that values both for total synaptic length and for synaptic number are lower than for the normal male and do not differ significantly from those of the normal female.

Vesicular Area - normal male, female and castrated male

Areas occupied by synaptic vesicles in male, female and neonatally castrated males are shown in Fig 51 . As the pattern of responses though similar in shape between groups (including the striking dip at day 4) was more erratic than that for the synaptic contact measurements, we felt it unwise to try to put these results to the quadratic model. A more direct approach, which takes advantage of the 'pairing' of male and female animals is to carry out a related sample T test on the differences between the means of male and female values.

To do this the data were arranged into pairs so that each pair consisted of a male and a female value observed at similar ages. This leads to the seven pairs shown in Table 2. The difference between the means was 2.133 and the associated T statistic was 4.345 which was significant at the 0.39% level. Thus, on average the male response is 2.133 higher than the female. Neonatally castrate male values were lower than those of the male, and similar to those of the female, but as it was not possible to pair them with either sex, the statistical analysis was not performed.

Figure 51



**Age in days**

Relationship between age in days and the proportion of neuropil occupied by synaptic vesicles ( $\mu^2/100\mu^2$ ). Male (—), female (---) and neonatally castrated (---) values are shown. The vertical lines represent S.E.M. of results from the means of at least 23 micrographs per rat (for clarity only half of the S.E.M. bar is shown). Each point represents one animal except for 4-day-old females where two animals were used.

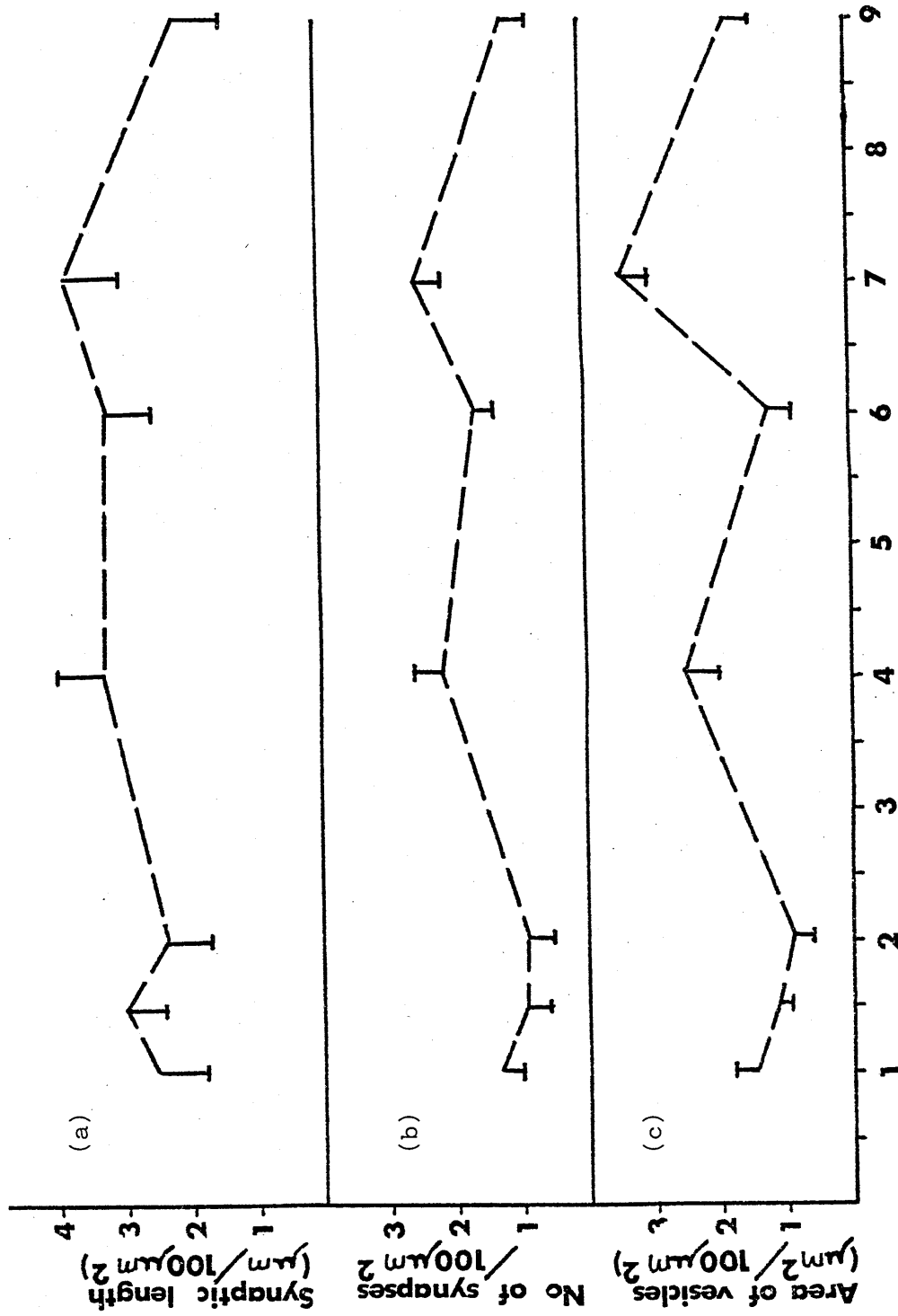


### Synapses and Vesicular Area - Androgenized Females

In Figure 52 results (total synaptic length, synaptic number and vesicular area) obtained from androgenized females of up to 9 days of age are shown. The curve describing total synaptic length is similar to that describing synaptic number, but both curves are different in form from those of normal animals, values for the dependent variables being lower than in the normal and not increasing so markedly with age. An attempt to use the quadratic model (Table 1) was made to analyse these data compared with those obtained from the normal males of up to 9 days old, but the model was found to account for only 67% (total length) and 46% (number) of the variation. Nevertheless, a value for  $\beta_0$  of 1.476 (significant at the 0.5% level) was obtained in the case of total synaptic length. No attempt was made to estimate  $\beta_0$  in the case of number as the value for  $R^2$  indicated that the model did not adequately describe the data.

The curve describing vesicular area in androgenised females is similar in form to those describing total synaptic length and number and once again is different from those describing vesicular area in normal animals, values being consistently lower and not increasing with age. No attempt was made to compare the data for vesicular area in androgenised females with those of normal animals since the distribution of points did not allow use of a paired sample T test and the quadratic model was not applicable. No data are quoted in Figure 52 for animals of over 9 days of age, as the nucleus was not identifiable as a discrete cluster

Figure 52.



Age in days

Synaptic parameters in neonatally androgenized female rats, expressed as functions of age. (a) The total synaptic length in  $\mu\text{m}/100\mu\text{m}^2$  are shown, (b) the number of synapses/ $100\mu\text{m}^2$  and (c) the area occupied by synaptic vesicles in  $\mu\text{m}^2/100\mu\text{m}^2$  are shown. The vertical lines represent S.E.M. of results from the means of at least 23 micrographs per rat (for clarity only one half of the S.E.M. line is shown). Two animals were used at 1 and 4 days of age, otherwise each point represents one animal.

of cell bodies in any of the 5 such animals (ranging in age from 15 - 150 days) examined. This was despite the fact that we examined semi-thin sections, taken throughout the suprachiasmatic region at 5 - 10  $\mu$ m intervals in each animal and was in marked contrast to our ability to locate the nucleus in other groups. Considered in conjunction with the low numbers of synapses in the younger androgenised animals and their failure to increase with age, this suggests that neonatal TP treatment inhibits normal development of the nucleus.

No data are quoted for prenatal animals of either sex. This is because the nucleus was not yet identifiable as a discrete cluster of cells in any of the 10 animals tested, although we searched the appropriate area at 5 - 10  $\mu$  intervals, as in the case of the older androgenized females.

#### Summary

In summary synapses develop at similar rates in the suprachiasmatic nucleus of rats of both sexes, but values are higher in male than in female animals from birth to maturity. Male-type development cannot be mimicked by neonatal androgenization but results suggest that female-type development can be induced by neonatal castration of males. The results suggested that both prenatal and postnatal androgens are essential to normal male development.

TABLE 1

The statistical differences between male and female, androgenised female and neonatally castrate male data values are shown. The states of the animals used are shown in the first column and the parameters measured in the second column; L is the total synaptic length ( $\mu\text{m}/100 \mu\text{m}^2$ ); N is the number of synapses per  $100 \mu\text{m}^2$ ;  $\beta_0$  is the difference between groups, its dimensions being  $\text{m}/100 \mu\text{m}^2$  for L and number of synapses/ $100 \mu\text{m}^2$  for N.  $R^2$  is the proportion of variation accounted for by the model; a value of one indicates a perfect fit.

State	Parameter	$\beta_0$	S.E.M.	Significance	$R^2$	Degrees of Freedom (residual)
Males v females	(L)	1.334	0.412	0.007	0.903	13 <sup>#</sup>
	(N)	2.86	0.675	0.001	0.935	13 <sup>#</sup>
Males v androgenised females	(L)	1.476	0.487	0.005	0.674	11*
	(N)	2.35	0.087	0.001	0.578	11*
Neonatal castrates v females	(L)	0.42	0.624	NS	0.72	9 <sup>†</sup>
	(N)	0.027	0.81	NS	0.80	9 <sup>†</sup>

<sup>#</sup>Two 4 day old females are represented by one point

\*Data from normal animals over 10 days of age were excluded from this analysis

<sup>†</sup>Only 4 castrated rats were analysed

TABLE 2

An analysis of sexual dimorphism in the area occupied by synaptic vesicles in normal female and male rats.

Age (days)	Vesicular area ( $\mu\text{m}^2/100\mu\text{m}^2$ )		
	Females	Males	
0.25	0.95	2.6	
1	2.94	7.01	
4	1.85	4.3	
6	4.2	5.7	
8	3.86	6.1	
20	7.6	7.7	
150	7.01	9.93	
	Mean	4.06	6.19

Difference in mean = 2.133

As the pattern of response was more erratic here, it was unwise to try to fit a linear model. A more direct approach which takes advantage of the natural 'pairing' of the data is to carry out a related sample t-test on the difference in means (between males and females). To do this the data was first arranged into pairs so that each pair consisted of a male and female result observed at the same age.

The associated t statistic is 4.545 which is significant at the 0.39% level.

## DISCUSSION

Before discussing the implications of this work it is worth noting the following as possible methodological limitations;

### PROCESSING OF SAMPLES

Apart from the immersion fixation technique described on page 53, only one method was used for fixation and processing tissue throughout the experiments. Any single method is almost certain to introduce artefacts, but it is hoped that by careful preparation of solutions, monitoring their pH and temperature, and by adhering closely to the same protocol, that the artefacts introduced are consistent from one batch of samples to the next. Furthermore it is unlikely that fixation artefacts would show a systematic sexual bias.

*In this context it is worth noting that the presence of visible extracellular space in some immature animals is unlikely to be an artefact of perfusion fixation. Such space has been demonstrated by Caley et al<sup>82</sup> in their studies on development of rat cerebral cortex using different fixation techniques. I have found the extracellular space in both perfusion and immersion fixed tissues. It is usually found next to the third ventricle, in the so-called 'cell poor zone' (CPZ) described by Van den Pol<sup>83</sup> within the SCN. The extracellular space occurs in immature animals of both sexes, disappearing at about 15 - 20 days of age, concomittant with the maturation of the neuropil.*

#### LOCATION OF SCN

As coronal sections were used the samples are less well defined with respect to the coronal than the sagittal plane. It seems unlikely that this would introduce any systematic sexual bias.

#### STEREOLOGY

The application of stereological methods in histology implies the use of randomly orientated sections of tissue.<sup>78</sup> As it was necessary to search for the SCN using coronal sections this condition was not fulfilled. However, once again, this is unlikely to cause any systematic sexual bias.

#### RANDOMNESS OF SAMPLING

Though micrographs were not taken in a strictly correct fashion (by plotting randomly generated positions on the sections, and taking micrographs accordingly) it seem unlikely that this would lead to any systematic bias towards any one area within the nucleus or to any sexual bias as the orientation of the section on the grid would be different for each sample

The photographs were taken by firstly counting the number of grid 'squares' overlying the area of SCN and dividing this number by 23. This factor was then used to count along the 'squares' for choice of neuropil area for photography. A micrograph was taken from the centre of the appropriate grid 'square'.

### STATISTICAL

One other apparent limitation is that while I used up to 9 animals per group I was generally restricted to using but one animal of each age. The statistical analyses therefore distinguish between the trends taken by groups as a whole, and the degrees of significance shown should not be interpreted as necessarily showing differences between groups at any one given age. However, inspection of the data plots indicate that such an interpretation is probably justified (i.e. there is a roughly constant difference, whatever the age).

### NEGLECT OF BIOLOGICAL RHYTHMICITY

Ideally, because of the possible interference by circadian rhythms animals should be killed at exactly the same time each day. This was not possible for technical reasons, (I had to use animals which were killed sometime between 11 am and 3 pm) but once again this is unlikely to cause any sexual bias. The stage of the sexual cycle of the adult female was not determined. This however, would not be relevant to animals of less than 30 days of age, i.e. the bulk of the data.

Although there are, in principle, many methodological limitations to my work they are unlikely to have any important effect on its outcome. Taking the above into consideration the main findings of this work are;

- 1) That synapses develop at similar rates in the suprachiasmatic nucleus of rats of both sexes.



- 2) That from birth to maturity values are higher in males than in females.
- 3) Male development cannot be mimicked by neonatal androgenization of female rats.
- 4) The preliminary results suggest that female type development can be induced by neonatal castration of males.

No numerical results are quoted from the immersion fixed foetal animals as the SCN had not developed as a recognisable discrete area in the hypothalami of these animals.

#### PATTERN OF DEVELOPMENT OF SYNAPSES

Although the pattern of synaptic development (males > females) is the reverse of that one would have predicted from the results of Raisman and Field (1973)<sup>46</sup> who found that the preoptic area of adult female rats had more spine synapses of non-amygdaloid origin than did those of males and that these differences could be mimicked by appropriate neonatal hormonal manipulation, it is consistent with and extends the observations of Greengough et al<sup>85</sup> who found that dendritic length is greater in the preoptic area of adult male than of adult female hamsters.

My results show that there is little difference in the length of individual synapses in any of the groups, this suggests that the difference is in the number of synapses.

#### PATTERN OF VESICLE FORMATION

Sexual differences in vesicular cluster area are seen, and once again male levels are higher than females. It is interesting to note the relatively early onset of vesicular as opposed to synaptic development.

Clusters of vesicles were found in abundance in growing axons in the neuropil of immature rats. Some of these vesicles were larger than those in more mature animals.

The ratio of vesicular area to synaptic contact is much greater in the newborn than the adult, although the sizes of individual clusters of vesicles is much the same throughout the age range. Considering this and the fact that in the immature animals many of the clusters of vesicles are concentrated in growth cones which apparently had not yet made contact with any other axons, cell bodies or dendrites, suggests that neurotransmitters may have a synaptotrophic as well as subsequent neurotransmitter function. Indeed Changeux and Danchin<sup>60</sup> have discussed the role of neurotransmitters as synaptotrophic agents. The direction of sexual dimorphism is also consistent with work by Ani et al (1980)<sup>49</sup> who conclude that neonatal androgenisation inhibits the selective stabilization of synapses in the brain, leading to levels of synapses in the male being higher than those in the female. This is also consistent with some of my other work, which does not form part of this thesis, in which my colleagues and I showed a sexual dimorphism ( $\sigma^7 > \text{♀}$ ) in putative receptors for dopamine, in various areas of rat brain.<sup>86</sup>

#### Differences apparent from birth

Since the statistical analyses indicate a difference between grouped values for males as against females and since the shape of the graphs indicates that this difference applies to animals of all ages, the work suggests that difference in number of synapses are apparent from birth. This, taken together with the evidence outlined in the introduction indicating that male type development is dependent upon postnatal hormones would suggest that in addition there is a prenatal

differentiating stimulus which is essential for the full development of the male type brain. This is consistent with the recent observation that adult male rats have higher concentrations of nor-adrenaline in their SCN than do adult females and that these sex differences cannot be altered by neonatal hormonal manipulations.<sup>87</sup>

These observations are in keeping with the fact that there is a surge of testosterone in 18 day old male fetuses (Rats usually have 21 day pregnancies)<sup>88</sup> which, it is believed is necessary to 'prepare' the brain for the postnatal action of the hormone.

The suggestion that part of the stimulus necessary for masculinization is prenatal is reinforced by the failure of my experiments with neonatal androgenization of females to produce male type differentiation in the SCN. Initial experiments with neonatally castrate males indicate that their development is similar to that of normal females, suggesting that post-natal exposure to testosterone (as well as the surge at 18 day gestation) is necessary for full male development.

Thus it appears that the relative abundance of both pre and post natal sex steroids is critical if the SCN is to develop in its normal sexually dimorphic manner. Pre and post natal androgens are essential for normal male development

Exposure to post natal androgens alone, as in the neonatally androgenised females, leads to sexually abnormal development lowered rates of synaptogenesis followed after 10 days by a loss

of definition of the SCN. As mentioned in the introduction the SCN is located in an area of the brain rich in aromatising enzymes capable of converting testosterone to oestradiol. It is also proposed that adenylate cyclase could be involved in the process of sexual differentiation, indeed cAMP has been shown to promote differentiation at the expense of growth in neuroblastoma cultures<sup>54</sup>. Furthermore, Ani et al<sup>48</sup> have shown that adenylate cyclase activity (and thence cAMP formation) is lower in the neonatal male hypothalamus than in the female. Thus the sexual dimorphism seen in synaptogenesis in the SCN is presumably a result of the action of testosterone, and its metabolite  $17\beta$  oestradiol, in the male. The fact that the number of synapses is greater in the male than in the female is consistent with the hypothesis of Ani et al - that  $17\beta$  oestradiol lowers adenylate cyclase activity and thence cAMP levels and this in turn inhibits the selective stabilisation of synapses.

#### Synapse formation

The neuromuscular <sup>junction</sup> can be considered as a model for synapse formation. In 1975, Jansen et al<sup>89</sup> showed that in the formation of a neuromuscular junction the growing nerve terminal dictates where a synaptic junction is going to be on another neurone, this implies that it is not necessary to have a specific receptor site already in existence on the second neurone.

A cell prepares to receive a synaptic contact by developing a non-uniform sensitivity to a neurotransmitter over its entire surface. The contact with the presynaptic cell is made at sites with no

obvious signs of specialisation. Gradually a synaptic junction develops with a very specialized structure - with a high density of junctional receptors for the neurotransmitter but with low extrajunctional sensitivity to that neurotransmitter. Thus it appears that in the periphery at least the growing nerve terminal or presynaptic cell is the one which organises the formation of synaptic junctions. This is consistent with my observation that nerve terminals contain vesicles (which presumably contain neurotransmitters) which develop before synapses.

#### How stable are synapses once formed?

I have spoken earlier of "stabilization of synapses" but perhaps this phrase, though convenient, is too strong. In 1976, Blakemore et al<sup>90</sup>, when working on cat visual cortex has demonstrated clearly the plasticity of developing synapses and similar conclusions have been reached by Stewart and Rose<sup>91</sup> who showed a transient increase in muscarinic acetylcholine receptors in visual cortex on first exposure of dark reared rats to light. In 1978, Rose and Stewart<sup>66</sup> also showed that this may be due to a modification of axonal and dendritic flow. These observations of course take place in the developing brain but there is also evidence of plasticity of synapses in the adult. There is for example a reduction in the amount of 5HT binding (possibly a difference in the number of receptors) in the brain after long term administration of anti-depressants<sup>92,93</sup> and a phenomenon similar to denervation supersensitivity can be seen after pharmacological lesions of dopaminergic pathways in the brains of mice.<sup>94</sup> Furthermore,

concurrent administration of imipramine and oestradiol leads to short term modifications in 5HT<sub>2</sub> binding in the brains of rats<sup>95</sup> and oestradiol alone has short term effects on the numbers of muscarinic receptors<sup>96,97</sup>.

Whether or not these changes represent a difference in the number of synapses as would have been shown by electronmicroscopy or activation or inactivation of existing receptors<sup>98</sup> remains to be seen. Thus it would appear that synapses are functionally plastic in the brains of adult as well as developing animals and can be modified not only by neurotransmitters but also by hormones.

#### Proposed extensions of work and Experiments in hand.

It would be interesting to continue these studies by administering testosterone propionate (TP) both prenatally and postnatally and examining the SC Nuclei of female rats to check whether their patterns of synapse formation were following the male trends, I predict that they would. There are however practical limitations to these experiments, not least that a high maternal dose of testosterone propionate will cause abortion.

As discussed earlier, it has been suggested that adenylate cyclase is a key mediator in sexual differentiation<sup>49</sup>. Furthermore, perinatal administration of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) inhibits sexual differentiation in dopamine receptors in the cortex<sup>99</sup>. It would be interesting to see if this drug modulates sexual differentiation in the SCN.

### General Comments

Studies on the synaptogenesis of the SCN provide ultrastructural detail of some of the sexual differences in the brain. The results obtained in this study were in general consistent with the behavioural and biochemical works mentioned elsewhere in this thesis. The SCN is of particular interest because of its recently shown link with the pineal gland and the relationship with light, melatonin secretion and mood disorder (Winfree, 1982)<sup>100</sup>.

Sexual differentiation of the brain is a convenient tool to study ways in which environment can have profound and long term influence on brain development, and might, in itself, be applicable to humans.

## REFERENCES

1. Booth, J.E. 1979  
Sexual Differentiation of the Brain  
Oxford Review in Reproductive Biology Vol. 1 58 - 158  
Edited C.A. Finn
2. Goy, W.R. and McEwan, B.S. 1980  
Sexual Differentiation of the Brain  
The M.I.T. Press Cambridge, Mass. London, Eng.
3. Donovan, B.T. 1970  
Mammalian Neuroendocrinology  
MacGraw-Hill London
4. Harris, G.W. 1904  
Sex hormones, brain development and brain function  
Endocrinology 75 627 - 648
5. Motta, M., Fraschini, F and Martini, L. 1969  
"Short" Feedback Mechanisms in the control of the  
Pituitary Function  
Frontiers in Neuroendocrinology 211 - 253  
Oxford Univ. Press
6. Chowers, I and Macann, S.M. 1967  
Comparison of the effect of hypothalamic and pituitary  
implants of estrogen and testosterone on reproductive  
system and adrenal  
Proc. Soc. Exp. Biology Med. 124 260 - 266
7. Martini, L., Fraschini, F. and Motta, M. 1968  
Neural control of anterior pituitary functions  
Recent Prog. Hormone Res. 24 439 - 496



8. Motta, M., Piva, P., Tima, L., Zanisi, M. and  
Martini, L. 1970  
Feedback mechanisms and the control of hypothalamic  
releasing factors  
Mem. Soc. Endoc. 18 407 - 422
9. Stone, C.P. 1939  
Copulatory activity in adult male rats following castration  
and injections of testosterone propionate  
Endocrinology 24 165 -174
10. Grady, K.L., Phoenix, H and Young, W.C. 1965  
Role of the developing rat testis in differentiation of  
the neural tissues mediating mating behaviour  
J. Comp. Physiol. Psychol. 59 176 - 182
11. Gorski, R.A. 1973  
Hormones and Brain Function 27 - 45  
Plenum Press New York.
12. Thomas, P.J., and Ani, M. 1982  
The sexual differentiation of the brain  
Molecular Aspects of Medicine 15 1 - 61  
Pergamon Press, Oxford, New York, Frankfurt
13. Döhler, K.D. and Mancke, J.L. 1978  
Thoughts on the mechanism of sexual brain development  
in "Hormones and Brain Development" 153 - 158  
Edited G. Dorner, M. Kawakami  
Biomedical Press Elsevier, N. Holland
14. Döhler, K.D. 1978  
Is female sexual differentiation hormone mediated?  
Trends in Neurosciences 1 138 - 140

15. Shapiro, B.H., Golmann, A.S., Bongiovanni, M. and  
Marino, J.M. 1976  
Neonatal Progesterone and female sexual development  
Nature (London) 241 795 - 796
16. Doughty, C., Booth J.E., McDonald, P.G. and  
Parrot, R.F. 1975  
Effects of oestradiol, 17 $\beta$  oestradiol benzoate and the  
synthetic oestrogen RU 2858 on the sexual differentiation  
in the female rat  
J. Endocr. 67 419 - 425
17. McDonald, P.G. and Doughty, C. 1972  
Comparison of the effect of neonatal administration of  
testosterone and dihydrotestosterone in the female rat.  
J. Reprod. Fert. 30 55 - 62
18. McDonald, P.G. and Doughty, C. 1972  
Inhibition of androgen sterilization in the female by  
administration of an anti-oestrogen  
J. Endocr. 55 455 - 456
19. McDonald, P.G. and Doughty, C. 1973  
Androgen sterilisation in the neonatal female rat and its  
inhibition by an estrogen antagonist  
Neuroendocrinology 13. 182 - 188
20. McDonald, P.G. and Doughty, C. 1974  
Neonatal administration of different androgens in the female  
rat. Correlation between aromatization and the degree of  
sterilization  
J. Endocr. 64 97 - 103

21. McDonald, P.G. and Doughty, C. 1974  
 Effect of administration of different androgens in the  
 female rat, correlation between aromatization and the  
 induction of sterilization  
 J. Endocr. 61 95 - 103
22. Reddy, V.V.R., Naftolin, F. and Ryan, K.J. 1974  
 Conversion of androstenedione to estrone by neural tissue  
 of foetal and neonatal rats  
 Endoc. 94 117 - 121
23. Luttge, W.G. and Whalen, R.E. 1970  
 Hormones and Behaviour 1 265
24. Brown-Grant, K., Munck, A., Naftolin, F. and  
 Sherwood, M.K. 1971  
 The effects of the administration of testa propionate  
 alone or with phenobarbitone and of testos metabolites  
 to neonate female rats  
 Hormones and Behaviour 2 173 - 184
25. Arnold, A.P. 1980  
 Sexual differences in the Brain  
 Am. Sci. 68 165 - 173
26. Nunez, E.A., Benassayg, C., Sanu, L., Valleta, G. and  
 Jayle, M.F. 1976  
 Serum binding of some steroid hormones during development  
 in different animal species, discussion of the biological  
 significance of the binding.  
 Ann. Biol. Anim. Bioch. Biophys. 16 (3) 491
27. Raynard, J.P., Mercier-Bodard, C. and Baulieu, E.E. 1971  
 Rat estradiol binding plasma protein (E.P.P.)  
 Steroids 18 767 - 788

28. Reinisch, J.M. 1981  
Prenatal exposure to synthetic progestrins increases  
potential for aggression in humans  
Science 211 1171 - 1173
29. Thomas, P.J. 1973  
Steroid hormones and their receptors  
J. Endocr. 57 333 - 359
30. O'Malley, B.W. and Means, A.R. 1974  
Female steroid hormones and target cell nuclei  
Science 183 610 - 620
31. Muller, G.C. 1965  
in "Mechanism of hormone action" p223  
Academic Press New York
32. Muller, G.C. 1971  
Estrogen Action: A study of the influence of steroid  
hormones on genetic expression  
in "The biochemistry of steroid hormone action" 1 - 29  
Edit. R.M.S. Smellie  
Academic Press London and New York
33. Hamilton, T.H. 1968  
Control by estrogens of genetic transcription and  
translation  
Science 161 649 - 661
34. Hamilton, T.H. 1971  
Steroid hormones, RNA synthesis and transport and the  
regulation of cytoplasmic translation  
in "The biochemistry of steroid hormone action" 49 - 84  
Edit. R.M.S. Smellie  
Academic Press London and New York

35. Barley, J., Ginsburg, M., Greenstein, B.D., MacKlusky, N.J. and Thomas, P.J. 1974  
A receptor mediating sexual differentiation?  
Nature 252 259 - 261
36. Salaman, D.F., Thomas, P.J. and Westley, B.R. 1976  
Characteristics and Properties of a high affinity oestradiol receptor in the neonatal rat brain  
Annls. Biol. Anim. Bioch. Biophys. 16 479 - 490
37. Westley, B.R. and Salaman, D.F. 1976  
Role of oestradiol receptors in androgen induced sexual differentiation of the brain  
Nature 262 407 - 409
38. Ginsburg, M., Greenstein, B.D., MacKlusky, N.J., Morris, I.D. and Thomas, P.J. 1975  
Occurrence and properties of gonadal receptors in the rat brain  
J. Steroid. Bioch. 6 989 - 991
39. Thomas, P.J. and Knight, A. 1978  
Sexual differentiation of the brain  
in "Current Studies of Hypothalamic Function" 1 192-203  
Eds. Lederis, Veal, Karger, A.G.
40. Salaman, D.F. and Birkett, S. 1974  
Androgen induced sexual differentiation of the brain is blocked by inhibitors of DNA and RNA synthesis  
Nature 247 109 - 112
41. Salaman, D.F. 1974  
The role of DNA, RNA and protein synthesis in sexual differentiation of the brain  
Progress in Brain Res. 41 349 - 361

42. Barley, J., Ginsburg, M., MacKlusky, N.J., Morris, I.D.  
and Thomas, P.J. 1977  
Sexual differences in the distribution of cytoplasmic  
oestrogen receptors in rat brain and pituitary effect of  
gonadectomy and neonatal androgen treatment  
Brain Res. 129 309 - 318
43. Wheaton, J.E., Kruuch, L. and McCann, S.M. 1975  
Localisation of luteinizing hormone releasing hormone in  
the P.O.A. and hypothalamus of the rat using radioimmunoassay  
Endoc. 97 30 - 37
44. Okon, E. and Koch, Y. 1976  
Localisation of gonadotrophin and thyrotrophin releasing  
hormones in human brain by radioimmunoassay  
Nature 263 345 - 347
45. Raisman, G. and Field, P.M. 1971  
Sexual dimorphism in the Preoptic Area of the rat  
Science 173 731 - 733
46. Raisman, G. and Field, P.M. 1973  
Sexual dimorphism in the neuropil of the P.O.A. of the  
rat and its dependence on neonatal androgen  
Brain Res. 54 1 - 29
47. Ani, M., Butterworth, P.J. and Thomas, P.J. 1978  
Role of dopamine sensitive adenylate cyclase in the  
sexual differentiation of the brain  
J. Endocr. 79 31 - 32
48. Ani, M., Butterworth, P.J. and Thomas, P.J. 1978  
Sexual dimorphism in the adenylate cyclase activity in  
the hypothalamus of newborn rats  
Brain Res. 151 615 - 618

49. Ani, M., Butterworth, P.J. and Thomas, P.J. 1980  
Effects of oestradiol on dopamine sensitive adenylate  
cyclase in the brain, a possible role in sexual  
differentiation  
Brain Res. 183 341 - 353
50. Greengard, P. 1976  
Possible role for cyclic nucleotides and phosphorylated  
membrane proteins in post synaptic actions of neuro-  
transmitters  
Nature 260 101 - 108
51. Ehrlich, Y.H., Brungraber, E.G., Sinha, K. and  
Prasad, K.N. 1977  
Specific alteration in phosphorylation of cytosol proteins  
from differentiation neuroblastoma cells grown in culture  
Nature 265 238 - 240
52. Sloboda, R.D., Rudolph, S.A., Rosenbaum, J.L. and  
Greengard, P. 1975  
cAMP dependent phosphorylation of microtubule associated  
protein  
Proc. Nat. Acad. Sci. U.S.A. 72 177 - 181
53. Seite, R., Leonetti, J., Luciano-Vuillet, J. and  
Vio, M. 1977  
cAMP and ultrastructural organisation of the nerve cell  
nucleus  
Brain Res. 124 41 - 51
54. Shapiro, D.L. 1973  
Morphological and biochemical alteration in foetal rat  
brain cells cultured in the presence of MB cAMP  
Nature 241 203

55. Lim, R., Mitsunobu, K. and Li, W.K.P. 1973  
 Maturation-stimulating effect of brain extract and DB-cAMP  
 on dissociated embryonic brain cells in culture  
 Exp. Cell Research 79 243
56. Walter, U., Kanof, P., Schulman, H. and Greengard, P. 1978  
 Adenosine 3'5' monophosphate receptor protein in  
 mammalian brain  
 J. Biol. Chem. 253 6275 - 6280
57. Schumm, D.E. and Webb, T.E. 1978  
 Effect of cAMP and cGMP on RNA release from isolated nuclei  
 J. Biol. Chem. 253 8513 - 8517
58. Lynch, G.S., Dunwiddie, T. and Gribkoff, V. 1977  
 Heterosynaptic depression; a post synaptic correlate of  
 long term potentiation  
 Nature 266 737 - 739
59. Libet, B., Kabayashi, H. and Tanaka, T. 1975  
 Synaptic coupling into the production and storage of a  
 neuronal memory trace.  
 Nature 258 155 - 157
60. Changeux, J.P. and Danchin, A. 1976  
 Selective stabilization of developing synapses as a  
 mechanism for the specification of a neural network  
 Nature 264 705 - 712
61. Cannon, W.B. and Rosenbleuth, A. 1949  
 The supersensitivity of damaged structures  
 MacMillan N.Y.
62. Drachman, D.B. 1974  
 The role of acetylcholine as a neurotrophic transmitter  
 Ann. N.Y. Acad. Sci. 228 160 - 176



63. Creese, I., Snyder, S.H. and Burt, D.R. 1977  
Dopamine receptor binding enhancement accompanies lesion  
induced behavioural supersensitivity  
Science 197 596 - 598
64. Degnichi, T. and Axelrod, J. 1973  
Supersensitivity and subsensitivity of the  $\beta$ -adrenergic  
receptor in the pineal gland regulated by the catecholamine  
neurotransmitters  
Proc. Nat. Acad. Sci. USA 70 2411 - 2414
65. Lugiiani, Y.A., Bradford, H.F., Birdsall, N.J.M. and  
Hulme, E.C. 1979  
Dopolarisation induced changes in muscarinic cholinergic  
receptors in synaptosomes  
Nature 277 481 - 482
66. Rose, S.P.R. and Stewart, M.G. 1978  
Transient increases in muscarinic acetylcholine receptor  
and achetylcholinesterase in visual cortex on first  
exposure of dark reared rats to light  
Nature 271 169 - 170
67. Nelson, D.L., Bourgojn, H.A., Brownstein, S. and  
Hamon, M. 1978  
Characteristics of central 5HT receptors and their  
adaptive changes following intracerebral 5-7 dihydroxy-  
tryptamine administration in rats  
Molec. Pharmacol 14 983 - 995
68. Lauder, J.M. and Bloom, F.E. 1974  
Ontogeny of monoamine neurons in the locus coeruleus,  
Raphe nuclei and substantia nigra of the rat  
I cell differentiation  
J. Comp. Neurol 155 469 - 482

69. Lauder, J.M. and Bloom, F.E. 1975  
 Ontogeny of monoamine neurons in the locus coeruleus,  
 Raphe nuclei and substantia nigra of the rat.  
 II synaptogenesis  
 J. Comp. Neurol. 163 251 - 264
70. Baker, P.C. and Quay, W.B. 1969  
 5-Hydroxytryptamine metabolism in early embryogenesis and  
 the development of the brain and retinal tissues. A Review  
 Brain Res. 12 273 - 295
71. Reier, P.J., Cullen, M.J., Froelich, J.S. and Rothchild  
 Rothchild, R. 1977  
 The ultrastructure of the developing medial preoptic  
 nucleus in the postnatal rat  
 Brain Res. 122 415 - 436
72. Hendrickson, A.C., Wagoner, N. and Cowan, W.M. 1972  
 An autoradiographic and E.M. study of retinohypothalamic  
 connections  
 Zeitschrift für Zellforschung 135 1 - 26
73. Milhouse, O.E. 1977  
 Optic chiasm collaterals afferent to the S.C.N.  
 Brain Res. 137 351 - 355
74. Brown-Grant, K. and Raisman, G. 1977  
 Abnormalities of reproductive function associated with  
 destruction of the SCN in female rats  
 Proc. Roy. Soc. (B) 198 279 - 296
75. Raisman, G. and Brown-Grant, K. 1977  
 The SCN syndrome; endocrine and behavioural abnormalities  
 following SCN lesions in female rats.  
 Proc. Roy. Soc. (B) 198 297 - 314

76. Kawakami, M., Arita, J., and Yoshioko, E. 1980  
Loss of oestrogen induced daily surges of prolactin and  
gonadotrophins by SCN lesions in ovariectomised rats.  
Endocrinology 106 1087 - 1092
77. Timaras, P.S. and Vaccari, A. 1978  
An atlas of the developing rat brain  
California Univ. Press
78. Weibel, E.R. 1963  
Morphometry of human lung  
Springer Verlag New York
79. Vrenson, G. and de Groot, D. 1973  
Quantitative stereology of synapses; a critical investigation  
Brain Res. 58 23 - 25
80. Mercer, E.H. and Birbeck, M.S.C. 1972  
Electron microscopy - A handbook for biologists  
Blackwell
81. Brown, A. 1978  
Personal communication
82. Calley, D.W. and Maxwell, D.S.  
Development of the blood vessels and extracellular spaces  
during postnatal maturation of rat cerebral cortex.  
J. Comp. Neurol. 138 31 - 48
83. Van den Pol, A.N. 1980  
The Hypothalamic Suprachiasmatic nucleus of rat-intrinsic  
anatomy  
J. Comp. Neurol. 191 661 - 702

84. Gray, E.G. 1959  
Axo-somatic and axo-dendritic synapses of the cerebral cortex  
J. Anat. 93 420
85. Greengough, W.T., Carter, C.S., Steerman, C. and De Voogt, T.J. 1977  
Sex differences in the dendritic patterns in hamster preoptic area  
Brain Res 126 63 - 72
86. Jalilian-Tehrani, M., Karakiulakis, G., LeBlond, C. Powell, R and Thomas, P.J. 1982  
Androgen induced sexual dimorphism in high affinity dopamine binding in the brain transcends the hypothalamic-  
limbic region  
British Journal Pharmacology 75 37 - 48
87. Crowley, W.K., O'Donohue, T.L., and Jacobowitz, D.M. 1978  
Sex differences in catecholamine content in discrete brain nuclei of the rat: effects of neonatal castration or testosterone treatment  
Acta Endocrinologica 89 20 - 28
88. Weisz, J. and Ward, K. 1980  
Plasma testosterone and progesterone of pregnant rats, then male and female fetuses and neonatal offspring  
Endocrinology 106 306 - 310
89. Jansen, J.K.S., Van Essen, D.C. and Brown, M.C.  
Formation and elimination of synapses in skeletal muscles of the rat  
Cold Spring Harbour Symposia on Quantitative Biology  
Vol XL 425 - 431

90. Blakemore, C., Van Sluyters, R.C. and Movshon, J.A. 1976  
 Synaptic competition in the kittens visual cortex  
 Cold Spring Harbour Symposia on Quantitative Biology  
 Vol XL 601 - 609
91. Stewart, M.G. and Rose, S.P.R. 1978  
 Increased binding of <sup>3</sup>H Colchicine to visual cortex  
 proteins of dark reared rats on first exposure to light  
 J. Neurochem 30 595 - 599
92. Segawa, T., Midzuta, T. and Nemura, Y. 1979  
 Modifications of central 5HT binding sites in synaptic  
 membranes from rat brain after long term administration  
 of tricyclic anti-depressants  
 Eur. J. Pharmac. 58 75 - 83
93. Childers, S.R. and Snyder, S.H. 1980  
 Characterization of <sup>3</sup>H guanine nucleotide binding sites  
 in brain membranes  
 J. Neurochem 35 183 - 192
94. Matre, M.P., Costentin, J., Baudry, M., Marcais, H.,  
 Protais, P. and Schwartz, J.C. 1977  
 Long term changes in the sensitivity of pre and post  
 synaptic dopamine receptors in mouse striatum evidenced  
 by behaviour and biochemistry  
 Brain Res. 136 319
95. Kendall, D.A., Stancel, G.M. and Enna, S.J. 1981  
 Imipramine: effect of ovarian steroids on modification in  
 serotonin receptor binding  
 Science 211 1183 - 1185
96. Rainbow, T.C., Degroft, V., Luine, V.N. and McEwen, B.S. 1980  
 Estradiol 17 $\beta$  increases the number of muscarinic receptors  
 in hypothalamic nuclei  
 Brain Res. 198 239 - 243

97. Al-Dahan, M. and Thomas, P.J. 1981  
 The binding of [<sup>3</sup>H] scopolamine to the brains of male and  
 female rats: the effects of ovariectomy versus the proestrus  
 stage of the cycle  
 Proc. Brit. Journal Pharmacology 74
98. Fillion, G. 1982  
 5HT Receptors in the brain  
 Handbook of Psychopharmacology 779  
 Eds. Ivesen, L.L., Ivesen, S.D. and Snyder, S.H.  
 (In the Press)
99. Jalilian-Tehrani, M. and Thomas, P.J. 1982  
 Perinatal inhibition of phosphodiesterases alter the number  
 and affinity of high affinity dopamine binding sites in  
 the adult brain  
 Presented to; Biochemical Society Group Symposium on  
 'Membrane Receptors' Cambridge
100. Winfree, A. 1982  
 Human body clocks and the timing of sleep  
 Nature 297 23 - 27
101. Goldberg, N.D., Haddock, M.K., Nicol, S.E., Glass, D.B.  
 Sanford, C.H., Kuehl, F.A. and Estensen, R. 1975  
 Biologic regulation through opposing influences of cyclic  
 GMP and cyclic AMP : the Yin Yang hypothesis  
 Adv. Cyclic Nucleotide Res. 5 307 - 330
102. Greengard, P. 1978  
 Phosphorylated Proteins as Physiological Effectors  
 Science 199 146 - 152
103. Cheung, W.Y. 1982  
 Calmodulin  
 Scientific American 246 6 48 - 56