ESTROGENS AS NEUROMODULATORS

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By

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ABSTRACT

Ovulation is the result of the positive feedback action of estrogen, either alone or in combination with progesterone, and the integrated sensory input from the external environment onto the neural circuits controlling gonadotrophin secretion. During dioestrus II in the 4-day cycling rat the ovaries secrete an increasing quantity of estrogen which stimulates neurons in the preoptic area (POA) to trigger the surge of luteinizing hormone (LH) from the pituitary, thereby effecting ovulation. The positive feedback action of estrogen must ultimately be expressed in terms of an increased activity of the neurosecretory cells which secrete luteinizing hormone releasing hormone into the capillaries of the hypophysial portal plexus. However, the mechanisms whereby estrogen stimulates this increased activity are, as yet, unknown.

The binding of estrogens to specific target areas within the brain may induce changes in the metabolic function of neurons or it may directly or indirectly affect the electrical properties of neuronal membranes. The latter possibility has been tested by observing the effects of an intravenous (i.v.) injection of estrogen on the spontaneous activity and responsiveness to iontophoretically applied putative neurotransmitters of identified preoptic neurons.

Experiments I and II were undertaken in order to establish

certain parameters of the experimental design. Experiment I demonstrated that halothane anaesthesia inhibits ovulation in the cycling rat by an action on the neuroendocrine circuits which control gonadotrophin secretion. In experiment II measurements were made of the plasma concentration of estrogen following various doses of an i.v. injection of estradiol benzoate to ovariectomized animals. It was also necessary to establish whether or not an i.v. injection of estrogen or progesterone would stimulate an increased secretion of LH in estrogen primed ovariectomized rats. Measurements of serum LH concentrations one and five hours after the injection showed there was no stimulation of LH under these conditions.

Experiment III was undertaken to identify neurons in the POA which are thought to be implicated in a neuroendocrine circuit controlling gonadotrophin secretion and which may be target neurons for the positive feedback action of estrogen. Cells in the POA which receive an input from the stria terminalis have been identified by stimulating this pathway at its point of convergence in the amygdala. Antidromic stimulation techniques were employed to identify cells in the POA which make a direct connection with the basal hypothalamus.

Experiment IV constitutes the major objective of the research, which was to investigate a) the sensitivity of antidromically identified preoptic neurons to iontophoretically applied putative neurotransmitters; the cells were inhibited by both the catecholamines, dopamine (DA) and norepinephrine (NE) but were unresponsive to acetylcholine applied iontophoretically; b) to test the possible membrane effects of estrogen

on these antidromically identified cells. Two indices of a change of the excitability of preoptic neurons were used (i) the effects of an i.v. injection of estrogen on the rate of spontaneous activity and (ii) changes in the response of a neuron to electrophoretically applied DA or NE following the injection of estrogen or progesterone in primed and unprimed animals. The results of the first series of experiments demonstrated that estrogen can decrease the rate of spontaneous activity in identified preoptic units. Some cells showed a long lasting depression in their spontaneous activity following the steroid injection while others showed a short term decrease in the rate of unit discharge five minutes after estrogen administration. In the second series of experiments no changes were observed in the dose response curves of iontophoretically applied DA or NE thirty minutes after the steroid injection in either group of animals. The negative finding could be due to the technical limitations of this type of experiment or to the fact that estrogens do not affect the response of neurons to synaptic inputs in which these amines may function as neural transmitters.

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
CRO	Cathode Ray Oscilloscope
DA	Dopamine
DOPS	Dihydroxyphenylserine
EB	17β -estradiol-3-benzoate
FSH	Follicle Stimulating Hormone
i.p.	Intraperitoneal
i.v.	Intravenous
LH	Luteinizing Hormone
LRH	Luteinizing Hormone Releasing Hormone
NE	Norepinephrine
POA	Preoptic Area
s.c.	Subcutaneous

The chemical formulae for ACh, DA, EB, NE and glutamate are included in Appendix I.

CHAPTER I: INTRODUCTION

INTRODUCTION

The concept that hormonal signals from the gonads control the secretion rates of gonadotrophins from the pituitary by an action on the central nervous system was first proposed by Hohlweg and Junkmann (1932). Since that time a large amount of research has been directed toward determining the precise mechanisms involved in steroid regulation of gonadotrophin secretion.

1) Estrogen

It is now well documented that estrogen can exert both a positive (stimulatory) and negative (inhibitory) effect on the neural circuits controlling the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), and these actions are responsible for the cyclical release of gonadotrophins observed during the reproductive cycle (Schwartz and McCormack, 1972). The apparent paradox that a single hormone may either inhibit or stimulate a specific neural substrate may be resolved if one considers the possibilities that a) the effects of estrogen could depend on circulating levels of the hormone, b) different populations of neurons may exhibit a differential sensitivity to estrogen and c) other coincidentally circulating steroid hormones may influence the action of estrogen on the central nervous system.

The most obvious change in hormonal levels during the cycle is the sharp rise of LH concentration which precedes ovulation, and this surge is triggered by an increased secretion of estrogen from the

ovaries (Everett, 1969). In the 4-day cycling rat the circulating level of estrogen begins to rise on the afternoon of dioestrus II reaching a peak on the morning of prooestrus (Brown-Grant et. al. 1970). Following this increased secretion of estrogen, plasma levels of LH begin to rise between 1400-1500 h on the afternoon of prooestrus reaching a maximum concentration between 1700-1800 h and falling to a low level around 2000 h (Daane and Parlow, 1971a). This ovulatory surge of LH can be prevented by the administration of an estrogen antagonist, clomiphene (Labhsetwar, 1970) or by an estrogen anti-serum (Niell et al., 1971) on the morning of dioestrus II. However, if estrogen action is inhibited on the morning of prooestrus ovulation occurs, which supports the concept that estrogen secreted by the ovaries during dioestrus II stimulates LH secretion.

2) Progesterone

The role of progesterone in the ovulatory surge of LH is less clear. In many species the increased progesterone secretion occurs after the rise of LH suggesting that this hormone is not an important trigger for ovulation (Schwartz and McCormack, 1972). In the rat conflicting results have been reported for the sequence of LH and progesterone secretion. Some experiments show that the progesterone rise follows the LH rise, although recently Barraclough et. al. (1971) observed that the plasma levels of progesterone had already increased slightly and significantly by the onset of the LH surge. More recent experiments indicate that progesterone secreted by the ovaries acts synergistically with estrogen to facilitate the prooestrous surge of

LH. In the 4-day cycling rat, estrogen administered on dioestrus I advances ovulation 24 hours and induces a surge of LH secretion during the afternoon of dioestrus II (Krey et. al., 1973). Animals ovariectomized within an hour of estrogen treatment show a decreased LH surge compared to the sham operated controls, but this effect could be reversed by a concomitant injection of progesterone. Similarly Mann and Barraclough (1973) showed that progesterone could potentiate the secretion of LH in response to estrogen administration to ovariectomized rats, and Swerdloff et. al. (1972) have reported that progesterone alone induces the secretion of both LH and FSH in estrogen primed ovariectomised rats.

3) Feedback Actions

The negative feedback effects of low levels of circulating estrogen during the oestrous cycle is suggested by the rapid increase of blood FSH and LH after ovariectomy (Yamamoto et. al., 1970). The administration of estrogen can lower pituitary and plasma LH and FSH in gonadectomised rats although progesterone by itself has little effect (Rothchild, 1965).

In order to observe the stimulatory effects of sex steroids on LH release in the ovariectomized animal, it is necessary to first treat the animal with estrogen which lowers the levels of gonadotrophins, and perhaps primes some central receptors, and then to give a second dose of either estrogen (Caligaris et. al., 1971a, Karsch et. al., 1971) or progesterone (Caligaris et. al., 1971b) which results in an increased secretion of LH.

In addition to the positive and negative feedback actions of ovarian steroids there is also evidence for the existence of a 'short'

feedback mechanism for the control of LH secretion (Motta, Fraschini and Martini, 1969). David et. al. (1966) found that implants of small amounts of LH in the median eminence of normal or castrated rats of both sexes results in a decrease of pituitary and plasma LH levels. The participation of the hypothalamus in the 'short' feedback effects of LH is also indicated by the observation that hypophysectomy, which eliminated the inhibiting signal provided by LH, induces hypersecretion of luteinizing hormone releasing hormone (LRH) and this effect can be overcome by the administration of exogenous LH (McCann et. al., 1968).

4) Location of Implicated Neural Circuits Controlling Gonadotrophin Secretion

Lesion and stimulation experiments have led to the idea that the preoptic area (POA) includes the neurons whose activity triggers the ovulatory surge of LH and that neurons in the basal hypothalamus exert a tonic inhibitory control over gonadotrophin secretion. Deafferentation of the POA does not interfer with ovulation, but when the neural connections between the POA and the basal hypothalamus are transected, ovulation is inhibited (Tejasen and Everett, 1967, Koves and Halasz, 1970). On the other hand, stimulation of the POA will overcome the effects of pentobarbital anaesthesia in blocking spontaneous ovulation in the rat (Everett and Radford, 1961). The stimulus they used proved to be 'electrochemical' resulting from the deposition of iron from the stainless steel electrode by pulses of direct current, which caused a small irritative lesion in the brain tissue (Everett and Radford, 1961). More recently, Everett et. al. (1973) have demonstrated that

the amount of LH secreted is significantly related to the intensity of the stimulation current.

The evidence that neurons in the basal hypothalamus inhibit LH secretion arises from experiments in which circulating levels of gonadotrophins are measured following transection of the hypothalamic connections with the anterior pituitary. Lesions of the median eminence not only block ovulation but result in a sustained increased secretion of LH in normal female rats and prevent the inhibitory action of estrogen treatment in ovariectomized rats (Bishop et. al., 1972). Such lesions must, therfore, destroy the synaptic input which inhibits the activity of the neurosecretory cells rather than the LRH secreting neurons themselves. If the latter were the case then there would be no increased secretion of LH from the pituitary.

In addition to the POA and hypothalamus, experimental evidence implicates the amygdala as being also sensitive to the feedback action of estrogen and possibly being important in the control of gonadotrophin secretion (see Introduction, Chapter Va). In reviewing the literature pertaining to the role of the amygdala in the secretion of LH and FSH, Sawyer, (1972) has proposed that there are two functional groups of neurons in the corticomedial amygdala which project to the hypothalamic area via the stria terminalis; cells which inhibit gonadotrophin secretion and cells which facilitate the ovulatory surge of LH.

The evidence which suggests that certain brain regions are sensitive to steroid feedback effects is further supported by the results obtained from uptake studies of labelled estrogen and progesterone. Liquid scintillation counting of bisected brain regions

Kato and Villee, 1967, Mc Ewen and Pfaff, 1970) and autoradiographic studies (Pfaff, 1968, Stumpf, 1970) have shown that the hypothalamus, POA and amygdala concentrate and retain ^{3}H-estradiol by a saturable binding mechanism (McEwen and Pfaff, 1970); similar findings have been reported for progesterone (Madhabananda and Stumpf, 1973). The other areas of the brain which retain labelled steroid include the anterior pituitary, the bed nucleus of the stria terminalis and to a lesser extent the septal area and ventral hippocampus. Thus the correlation between the sites of steroid retention, the sites where estrogen implants affect LH and FSH release and those areas of the brain implicated in controlling gonadotrophin secretion suggest a functional role for the binding of the hormone by specific neurons.

5) Neural Activity and Ovarian Steroids

How is this binding of sex steroid to specific target neurons translated into changes of gonadotrophin secretion or induction of sexual behaviour? One approach to studying this problem has been to record the effects of sex steroid on the spontaneous activity and responsiveness of neurons to certain stimuli in certain brain regions.

Barraclough and Gross (1963) were the first to observe changes during the oestrous cycle of rats in the responsiveness of lateral hypothalamic neurons to pain, cold and vaginal probing. They also showed that an intravenous (i.v.) injection of 40 - 400μg progesterone selectively depressed the excitatory response of these units to cervical probing, this effect being maximal 30 minutes following the injection. This study was continued by Lincoln (1967) and Lincoln

and Cross (1967) who showed that high levels of circulating estrogen in rats with light induced persistent-oestrus or a subcutaneous (s.c.) injection of estrogen to ovariectomized rats decreased the number of units firing spontaneously in the preoptic anterior hypothalamic area, and of those neurons which were active a greater percentage were inhibited by cervical probing compared with the untreated controls. In the ovariectomized deermouse, Zolovich and Eleftheriou (1971) reported that a s.c. injection of estrogen 5 days prior to experimentation increased the inhibitory effect of vaginal stimulation on spontaneously active hypothalamic neurons compared to the untreated controls. In the immature monkey an i.v. injection of estrogen has been shown to reduce the rate of spontaneous activity of anterior hypothalamic units (Chhina and Anand, 1969).

Less consistent results have been reported for the effects of estrogen treatment in ovariectomized cats. Ratner, et. al. (1971) found that a daily s.c. injection of estradiol benzoate, for three days prior to the experiment, had no effect on anterior hypothalamic units but depressed the spontaneous activity and increased the proportion of cells in the posterior hypothalamus which were inhibited by cervical probing. However, using multi-unit recording techniques Alcaraz et. al., (1969) observed that a daily s.c. injection of 20 µg estradiol benzoate 4-6 days prior to experimentation increased the numbers of hypothalamic units which were excited by visual, somatic and vaginal stimulation compared to the untreated ovariectomized controls.

Yagi, (1973) studied the effects of an i.v. injection of

estrogen on the spontaneous firing rate of preoptic and hypothalamic neurons. Almost an equal number of units were accelerated or depressed by the hormone, but even those that were excited subsequently showed a prolonged period of reduced activity.

In general such results suggest that high levels of estrogen depress the responsiveness and activity of neurons which are implicated to be target cells for the feedback action of the steroid hormone. This would correlate with the observations on the change of mean firing rate of preoptic/hypothalamic units during the oestrous cycle of rats. When endogenous levels of estrogen are increasing throughout dioestrus II, the mean firing rate of hypothalamic units is low but a significant increase in spontaneous activity is recorded during prooestrus when plasma estrogen concentrations are decreasing (Moss and Law, 1971, Dyer et. al., 1972).

It is interesting to speculate on whether or not the increased unit activity during prooestrus is causally linked to the preovulatory surge of LH. Teresawa and Sawyer (1969) showed that stimulation of the POA during the afternoon of prooestrus will induce ovulation in rats in which the physiological trigger for the LH surge has been blocked by pentobarbital, and that this stimulation resulted in an increased multi-unit activity in the arcuate nucleus and median eminence. In 4 and 5-day cycling rats with chronically implanted electrodes a similar increase of multi-unit activity occurred for 12-25 minutes in the medial POA, septum, bed nucleus and ventromedial and arcuate nuclei during the afternoon of prooestrus (Kawakami et. al., 1970). However, experiments in which plasma LH assays were performed showed that an

increase in multi-unit activity occurred in the ventromedial arcuate region when LH secretion was increased by stimulating the POA or decreased by stimulating the hippocampus. Furthermore, the prooestrous peak of unit activity is observed in anaesthetised and in unanaesthetised animals with hypothalamic islands (Cross and Dyer, 1971); ovulation is inhibited in both preparations.

Several electrophysiological investigations have supported the concept of a short loop feedback of LH although the results obtained have been inconsistent. Intravenous injections of LH have been shown to both increase and decrease multi-unit activity recorded from the basal hypothalamus (Ramirez et. al., 1967, Teresawa et. al., 1969, Gallo et. al., 1972).

Although there is a great deal of data pertaining to the effects of hormones on the electrical activity of hypothalamic neurons, our understanding of feedback mechanisms and the neural control of gonadotrophin secretion has not been greatly advanced. The hypothalamus is a highly complex regulatory region controlling a variety of autonomic functions such as hunger, thirst, thermo-regulation and output of adrenal steroids. Can one hope to distinguish the different neural circuits involved in such diverse regulatory functions? And to what extent are the observed hormonal effects on neural activity related specifically to the neuroendocrine control of gonadotrophin secretion?

6) Neuroendocrine Pathways and Biogenic Amines

To date, two intrahypothalamic pathways, which may form a link in the neuroendocrine circuits concerned with reproduction, have

been identified. Histofluorescent studies (Fuxe and Hökfelt, 1966) and electrophysiological recordings employing the technique of antidromic stimulation (Markara et. al., 1972) demonstrated the existence of the tuberoinfundibular tract, the neurons of which lie in the arcuate nucleus with their axons projecting down to the median eminence More recently Dyer and Cross (1972) have identified, by antidromic stimulation techniques, neurons in the POA which made direct connection with the basal hypothalamus and have suggested that such neurons may trigger the release of LRH from the neurosecretory cells in the basal hypothalamus or may themselves be LRH containing neurons.

In addition to these two intrahypothalamic pathways, the stria terminalis which projects from the amygdala and terminates in several parts of the hypothalamus (Heimer and Nauta, 1969) forms an important link in the extrahypothalamic control of gonadotrophin secretion (see Introduction to Chapter Va).

Further research, directed toward elucidating steroid feedback mechanisms and pituitary control, must necessarily involve electrical recordings from specific identified neurons before one can begin to understand the complex integrative processes which ultimately control the activity of the releasing factor neurons.

One important aspect of gonadotrophin control is the role of biogenic amines which seem likely to act as transmitters at certain synapses involved in the neuroendocrine circuits under consideration. The neurons of the tuberoinfundibular tract are known to be dopaminergic (Fuxe and Hökfelt, 1966) and these neurons are thought to inhibit the release of LH and FSH. The rate of dopamine (DA) turnover during the

oestrous cycle has been estimated histochemically by observing the decline of DA stores in the median eminence following tyrosine hydroxylase inhibition (Hökfelt and Fuxe, 1972). During prooestrus and early oestrus DA turnover in the median eminence is decreased, implying that the activity of the tuberoinfundibular neurons is decreased at the time when LH and FSH secretion rates are high. In support of this evidence is the finding that ovariectomy also decreases DA turnover of these neurons (Fuxe and Hökfelt, 1969). However, it should be noted that biochemical measurements of hypothalamic catecholamine content during the oestrous cycle have yielded divergent results (Coppola, 1971), although this may reflect different methods for estimating catecholamines.

Apart from this dopaminergic system which may be sensitive to the negative feedback action of estrogen and inhibit the activity of the releasing factor neurons, experimental evidence now indicates that the neural trigger for ovulation may involve an adrenergic synapse (Kalra and McCann, 1973). The increased secretion of LH which results from electrochemical stimulation of the POA can be partially blocked by inhibiting nor-epinephrine (NE) synthesis, but when brain NE levels are selectively restored by dihydroxyphenylserine (DOPS) without restoring brain DA levels, this inhibition is reversed. The involvement of nor-adrenergic synapses in stimulating the pre-ovulatory surge of LH is also suggested by the findings that depletion of brain NE levels inhibits ovulation (Weiner and Ganong, 1971, Myerson and Sawyer, 1968) and that NE levels in the anterior hypothalamus rise significantly during dioestrus reaching a maximum at

prooestrus (Stefano and Donoso, 1967). It is therefore tempting to conclude that the neurons in the POA which trigger ovulation are nor-adrenergic. However, histofluorescent studies do not demonstrate the presence of adrenergic cell bodies in this region but only a dense innervation of nor-epinephrine containing nerve fibres which originate from groups of nerve cells within the brain stem (Ungerstedt, 1971). Perhaps these ascending adrenergic fibre systems are important for modulating the activity of preoptic neurons?

7) Mechanism of Action of Hormones on the Brain

The positive and negative feedback effects of estrogen are now well defined, and the neural sites for this control mechanism have been established. One of the basic questions which remains unanswered is how hormones modulate brain function. Does the binding of steroids alter the genomic expression of the cell which results in an alteration of metabolic function or are the effects more directly related to the electrical properties of the cell membrane?

Studies on the binding of estrogen in certain limbic and hypothalamic structures of the brain have revealed both cytoplasmic and cell nuclear binding sites (McEwen et. al., 1972) indicative that estrogens may act as metabolic inductors. Biochemical changes related to alterations in the level of circulating estrogens have in fact been observed; these include changes in the levels of certain enzymes, changes in incorporation of precursors into RNA and protein, changes in oxidative metabolism and changes in the turnover of the presumptive neurotransmitters such as NE and DA (McEwen and Pfaff, 1973).

Protein inhibitors, such as actinomycin D, have been shown to inhibit the effects of estrogen in decreasing LH and FSH secretion in ovariectomized rats (Schally et. al., 1969) and also to block the stimulatory action of the steroid hormone in estrogen primed rats (Jackson, 1973). These findings support the concept that estrogen induced alterations in brain RNA and protein metabolism which must eventually be understood in terms of enzyme activity and levels of metabolites and presynaptic neurotransmitter substances. This may explain the fact that the positive feedback effects of estrogen or progesterone can only be observed after the animal has been primed with estrogen which induces certain metabolic changes in the steroid sensitive target neurons.

The evidence for hormone dependent metabolic processes does not exclude the possibility that hormones may also have a direct action on cell membranes and there are numerous reports in the literature concerning the effects of hormones on certain properties of cell membranes. For example, antidiuretic hormone (ADH) is well known to increase the permeability of cell membranes (Windhager, 1969) and estrogen has been shown to inhibit the depolarising action of NE on rat pineal cells in vitro (Sakai and Marks, 1972). Iontophoretic studies have demonstrated that certain hormones can directly affect the electrical excitability of neuronal membranes. The synthetic steroid, dexamethasone, which is a potent inhibitor of ACTH, inhibits the spontaneous activity of hypothalamic neurons (Ruf and Steiner, 1967) whereas thyroid hormones increase the spontaneous unit discharge of cortical and hypothalamic neurons (Davidoff and Ruskin, 1972).

Iontophoretically applied oxytocin, in very low concentrations, has also been shown dramatically to excite neurons in the paraventricular nucleus (Moss et. al., 1972). In all of these examples, the time course of the hormone action on the membrane is extremely brief and the effects may be observed within seconds of the hormone reaching the cell membrane.

However, in those experiments in which an i.v. injection of progesterone (Barraclough and Cross, 1963) or estrogen (Yagi, 1973) were shown to alter the excitability of cell membranes the latencies of the responses were between 15-30 minutes suggesting that these effects may not have been the results of a direct action on the neuronal membrane. It is thus worth considering whether or not the feedback action of estrogen can alter the electrical activity of target neurons or their sensitivity to synaptic inputs by altering the electrical properties of the cell membrane; the effect could be either at the level of the releasing hormone neurons themselves or at some other neuron relay in the neuroendocrine circuits.

8) The Objective of the Present Studies

The following experiments were undertaken in an attempt to determine whether the positive feedback of estrogen on the POA involves a change of excitability of possible target neurons at the level of the cell membrane. It was necessary first to define certain experimental parameters in order to interpret the results a) Does the anaesthetic interfere with the neural trigger for ovulation? b) What intravenous dose of estrogen will result in a physiological level of the hormone? c) Is there a significant increase in LH secretion during

the period of recording from a specific preoptic neuron? The principal studies were of cells in the POA which are implicated in the control of gonadotrophin secretion; these were identified electrophysiologically, and those which make a direct connection with the basal hypothalamus have been used to study the effects of an i.v. injection of estrogen on their spontaneous activity and sensitivity to iontophoretically applied putative neurotransmitters.

CHAPTER II: MATERIALS AND METHODS

MATERIALS AND METHODS

Animals

All the experiments were performed on adult female rats of the Wistar strain which were purchased from High Oak Ranch in Toronto. The animals were housed in the University animal quarters, kept under a constant temperature of 22°C and fed ad. lib. with Purina Rat Chow.

Daily vaginal smears were taken from those rats which were to be used for the experiments requiring cycling females, and only those animals which displayed at least three consecutive 4-day oestrous cycles were used. They were maintained under a controlled lighting schedule with 14 hours of light centered on midday. At the time of experimentation the rats weighed between 200-280 g. Other experiments were performed on rats which were ovariectomized under ether anaesthesia 3-4 weeks prior to experimentation and kept in constant light conditions. Their weights ranged from 275-310 g.

Operative Proceedures

a) Anaesthesia.

For all experiments the rats were exposed to ether for about two minutes, intubated and anaesthetised with 1.5% halothane (Fluothane Ayerst). The halothane was administered in medical oxygen at a flow rate of 50 ml/min using a Fluotec Mark III vapouriser.

b) Surgical Procedures

In the experiments which required an i.v. administration of

estrogen or progesterone, the left femoral view was cannulated using fine PE 10 tubing and the cannula attached to a 1.0 ml syringe through a 32½ gauge syringe needle.

Electrical recordings necessitated placement of the animal in a stereotaxic head holder and exposure of the dorsal aspect of the brain. The rat stereotaxic holder was custom built in the University Work Shop and was copied from the model made by Harvard Instruments Inc. It was designed for the use of the deGroot stereotaxic co-ordinates of the forebrain and diencephalon (deGroot, 1959). Following cannulation of the femoral vein and placement of the rat in the stereotaxic apparatus, a mid-line incision was made in the skin covering the skull and an appropriate hole was drilled in the bone in order to accommodate both the stimulating and recording electrodes. The dura was then cut and folded back to expose the brain tissue. Throughout the experiment the animal's rectal temperature was constantly monitored and maintained at 37°C using a battery operated heating pad (EKEG Electronics C. Ltd.) which was placed underneath the animal

According to deGroot's stereotaxic atlas the following co-ordinates for stimulation and recording were adopted as follows:-

Stimulation of the amygdala:	A = 4.6, L = 4.0, H = -4.0
Stimulation of the arcuate nucleus:	A = 5.0, L = 0.5, H = -2.0
Recording in the preoptic area:	A = 7.6, L = 0.5, H = -4.0

Hormone Administration

a) Priming

Ovariectomized rats were primed with 20μg β-estradiol-3-

benzoate (EB) three days prior to experimentation (Caligaris et. al., 1971a). The hormone was administered s.c. in 0.8 ml sesame oil.

b) Intravenous hormone administration

The level of plasma estrogen following an i.v. injection of EB was measured in ovariectomized rats. Different doses of EB have been administered ranging from 100 ng - 5 µg and plasma levels of estrogen were measured at various time intervals after the injection (up to one hour). All doses of EB were dissolved in 0.25 ml propylene glycol and infused i.v. over a period of one minute.

For the LH measurements 1 hour and 5 hours following an i.v. injection of either estrogen or progesterone, 2 µg EB dissolved in 0.25 ml propylene glycol or 100 µg progesterone in 0.35 ml propylene glycol was infused i.v. over a period of one minute.

Different types of estrogenic compounds and solvents have been used in the experiments measuring the spontaneous activity of identified cells. In two instances 25 µg Premarin No. 552 (Ayerst Laboratories) in 0.25 ml solvent was injected i.v. Premarin is a prepared solution containing a mixture of the water soluble sodium sulphate esters of estrogen, the main estrogens being estrone and equilin sulphate. Estradiol-17β-hemisuccinate (Ikapharm Ltd.) was used in four successful experiments, and for these 20 µg of the estrogen dissolved in 0.25 ml saline was injected i.v. The remaining four cells were tested using EB dissolved in 0.1 ml propylene glycol. Doses ranged from 10 µg to 100 µg.

Electrodes

a) Stimulating Electrode

A co-axial stainless steel, epoxylite insulated electrode (Rhodes Medical Instruments, SNE-- 200) was used to stimulate either the stria terminalis or the arcuate nucleus. The shaft diameter was 0.25 mm and the distance between the centre positive lead and the outer negative shaft was 0.75 mm; both inner and outer contacts were exposed for 0.25 mm. The electrode was held in a Prior micromanipulator which was mounted on to the stereotaxic instrument.

Rectangular monophasic pulses 0.5 msec duration were delivered via an isolated stimulator (Devices Mk IV), the frequency and pattern of stimuli being controlled by a pulse generator (Digitimer, Devices Type 3290) and a gated pulse generator (Devices Type 2521). The stimulating current was passed through a 1000Ω series resistor; the voltage developed across this resistor was displayed on a Tektronix 5103 storage oscilloscope.

b) Recording Electrodes

Recordings of extracellular unit potentials and evoked potentials were made with single and three barrelled glass microelectrodes. All the micropipettes were made from open ended glass capillaries with an O.D. of 1.75 mm, which had been thoroughly cleaned in a solution of equal parts absolute alcohol and acetone.

Single barrelled electrodes were drawn out to a tip diameter of less than 0.5μ on a David Kopf electrode puller and subsequently, under microscopic examination the tip was broken down to a diameter

of 1.5 - 3.0μ. Three barrelled micropipettes were made by gluing together three glass capillaries at both ends with epoxy resin. A Narishige electrode puller was used to draw out these microelectrodes as it is necessary to twist the glass capillaries in order to fuse the three barrels and form a common tip; during the process of pulling the micropipette, the chuck was rotated by 360°. The barrels were arranged in a triangular shape so that the glass pipettes were in symmetrical contact with each other. The overall diameter of the triple barrelled assemblies was 3.5 - 5.0μ after the tip had been broken down manually under direct microscopic control.

Both single and triple micropipettes were filled by the fibre glass technique as described by Tasaki et. al. (1968). Single barrelled electrodes were filled with a 2% solution of Pontamine Sky Blue 6BX dissolved in 0.5 M sodium acetate with a pH of 6.6. The resistance of these electrodes ranged from 2-4 MΩ dependent upon the tip diameter. The measurement of the electrode resistance was made with a W.P. Instruments probe and amplifier (M-4A) by passing an internal direct current of 1.0 nanoamperes (nA) through the input terminal to ground and the voltage generated was displayed on the Tektronix storage oscilloscope. The recording barrel of the three barrelled micro-electrodes was filled with 3 M sodium chloride at pH 6.8 and its resistance varied between 3 - 8 MΩ. The second barrel was filled with a 1 M solution of L-Glutamic acid dissolved in distilled water, giving a pH of 6.8 - 6.8. The resistance of this channel ranged from 10 - 50 MΩ. The third barrel was filled with one of several

putative neurotransmitters. Three drugs have been used in this study; 0.8 M Dopamine hydrochloride, 0.8 M L-Norepinephrine bitartrate and 1.0 M Acetyl choline chloride. All drugs were dissolved in distilled water and the pH's ranged from 3.8 - 4.2. The resistance varied between 10 - 70 MΩ.

The electrode was held in a microdrive unit (AB Transvertex) which was attached to a Narishige micromanipulator, and it could be advanced in 2μ steps in the vertical plane by means of an electronically controlled stepping motor.

Iontophoretic Techniques

A four channel microelectrophoresis power supply, designed and made in the Department, was used to eject the drugs from the micropipettes. The unit, supplied a constant current, controllable over the range 5×10^{-9} amps to 1.2×10^{-7} amps at either positive or negative polarity. The output could be maintained at a fixed amplitude (usually utilised to provide braking currents) or pulsed by means of a drive signal from the Digitimer or manually operated push button switch. On any one channel the output could be switched from the braking current to the drive current on presentation of an external signal. Current levels were monitored and displayed on a two channel Tektronix 565 oscilloscope.

Braking currents in the order of 10 - 20 nA were applied in order to prevent the free diffusion of drugs from the tip of the pipette. As the retaining current for glutamate (which is an anion) is of the opposite polarity to those required for the cations DA, NE and acetyl choline (ACh), the two channels were set up to give equal

and opposite currents so that there was no net flow into or out of the preparation.

Ejection currents, which ranged from 6 nA - 70 nA, were balanced by passing a current of equal intensity and opposite polarity through the sodium chloride filled recording barrel. Current artifacts were tested by reversing the drive currents applied to the two channels. The maximum current which was used to test the response of any one neuron to iontophoretically applied drugs was also used to test the possibility of current artifacts. Any cell which appeared to 'respond' when the current was reversed was ignored.

Attempts to Apply Estrogens Iontophoretically

Various unsuccessful attempts were made to eject labelled estrogen compounds from multi-barrelled micropipettes. Estrogens are weakly acidic in aqueous solutions and will only form sodium and potassium salts with difficulty. Thus it is difficult to achieve the high concentrations required for the iontophoretic ejection of the hormone. Estradiol 6,7 - $^{3}4$ (40 Ci/mmol) and estrone - 6,7 - H^{3} sulphate (potassium salt, 470 mCi/mmol), obtained from Amersham Searle, were both tried but even after extended periods of free diffusion (≥ 24 h) from the immersed electrode tip, which had a total tip diameter greater than 10μ, or the passage of steady or pulsed direct currents ($\leq$ 150 nA, $\leq$ 2 minutes), of either polarity no measurable amounts of radioactivity could be recovered from the scintillation fluid.

Further attempts were made to eject these labelled estrogen compounds by the addition of an unlabelled steroid in order to increase

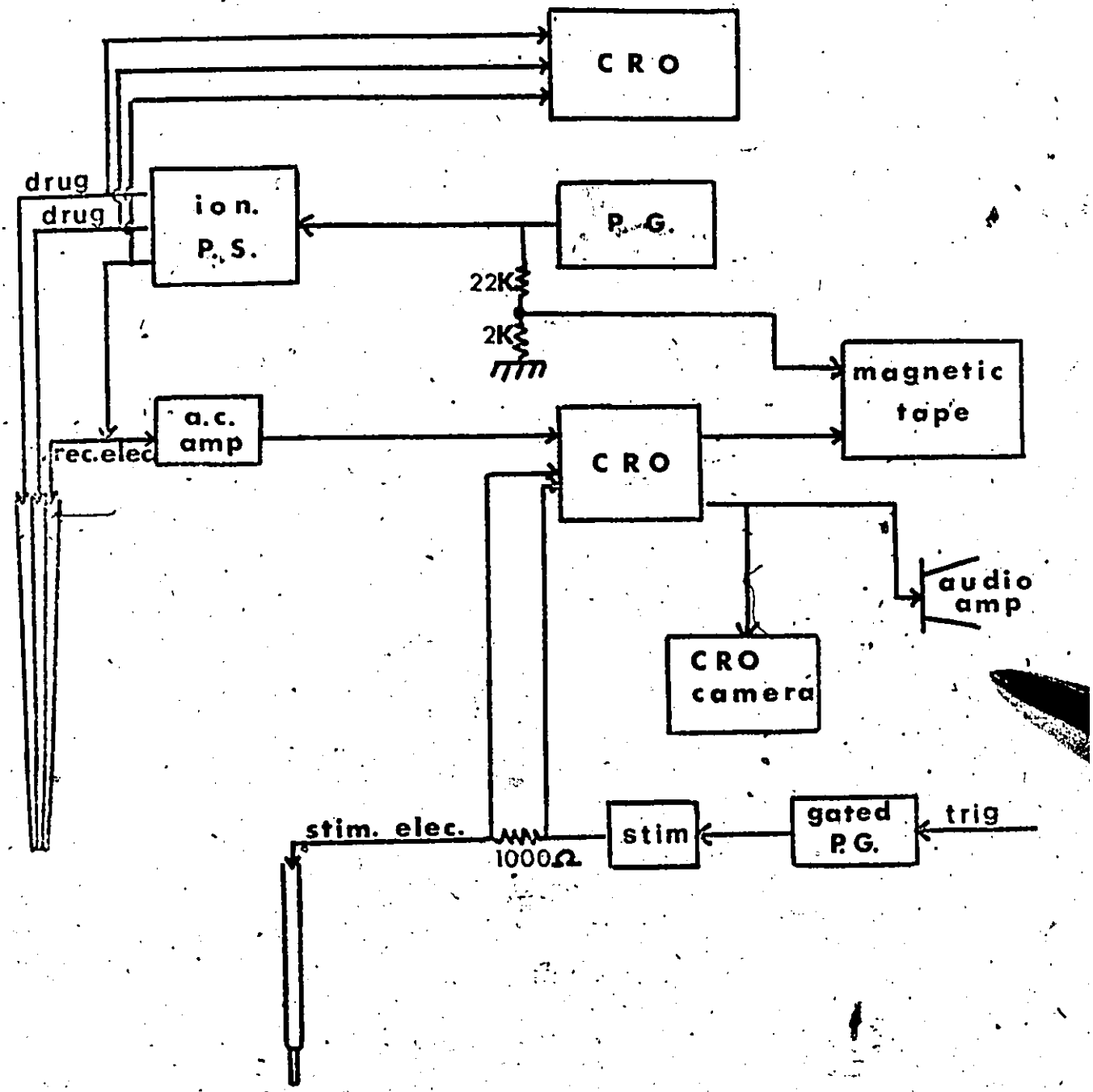
the transport number of the solution. The maximum concentration of Na - 17β estradiol - 3 - monosulphate (0.4 M) or Na - estrone - sulphate (0.1 M) was added to both labelled salts but, as above, no radioactivity could be measured after the passage of steady or pulsed direct currents. The final consideration was whether the labelled estrogen could be ejected from micropipettes by electro-osmosis. For this reason sodium chloride (0.1 M) was added to the solution of labelled estrogens but even with current intensities in excess of 150 nA no measurable quantities of radioactivity were ejected from the micropipette. The method was therefore abandoned.

Recording Techniques and Apparatus

The recording barrel of the microelectrode was connected to a Grass P15 A.C. coupled preamplifier with a gain of 100 and frequency limits set at 300 - 1000 Hz. The amplified signals were displayed simultaneously on two cathode ray oscilloscopes (CRO); a Tektronix 5103 storage oscilloscope and a Tektronix 502 with a camera attachment and recorded on a Hewlett Packard 3960 magnetic tape recorder. An audio amplifier was also connected to monitor those signals. Pulses derived from the crystal controlled pulse generator (Digitimer) which were used to drive the iontophoresis unit were recorded on another channel of the tape. Photographs of orthodromically and antidromically driven neurons were taken directly from the Tektronix 502 oscilloscope and were not recorded on tape.

For the analysis of the spontaneous activity, single unit potentials (spikes) were converted to pulses of constant amplitude

Figure 1. Diagram of the stimulating and recording set up. Extracellular unit potentials were recorded through single and triple barrelled glass micropipettes (rec. elec.), amplified through an A.C. coupled preamplifier (a.c. amp) and displayed on a cathode ray oscilloscope (CRO). The output of the CRO was recorded on a magnetic tape and simultaneously displayed on a second CRO for photographic recording and monitored on an audio amplifier (audio amp). Current was supplied to the drug containing barrels of the microelectrode (drug) from two channels of the iontophoretic power supply (ion. P.S.); a third channel was connected to the recording barrel. The ejection currents delivered from the iontophoretic power supply were triggered by a pulse generator (P. G.) and the trigger pulse was attenuated by a potential divider and recorded on the magnetic tape. Antidromic and orthodromic stimuli were provided by an isolated stimulator (stim.), the frequency and pattern of stimuli being controlled by a pulse generator (trig.) and a gated pulse generator (gated P.G.). Current passing through the stimulating electrode (stim. elec.) was monitored by the voltage drop across a 1000 series resistor.


CRO
drug
drug
ion.
P.S.
P.G.
22K
2K
magnetic
tape
a.c.
amp
rec.elec
CRO
audio
amp
CRO
camera
stim. elec.
1000Ω
stim
gated
P.G.
trig

and width. The spikes were used as a triggering signal for the Tektronix storage oscilloscope, and the level of the trigger circuit was set so that only the spikes of interest were processed. When the sweep was triggered by a spike, a square wave pulse was derived from the horizontal deflection amplifier of the CRO and after suitable amplification this pulse was recorded on magnetic tape. A display of the frequency trend was obtained by playing the recordings made in this way into a Hewlett Packard 5480 Signal Analyser used in the Interval Histogram mode. This instrument displayed on a CRO, a time by frequency histogram, a hard copy of which was subsequently made with the use of a Hewlett Packard X-Y plotter.

The results of the iontophoretic experiments were subsequently transcribed onto a pen recorder. Recordings of the unit potentials were played back and utilised to trigger a Tektronix storage oscilloscope, the trigger circuit of which was used as an amplitude discriminator. On triggering, a square wave pulse was derived from the horizontal deflection amplifier of the CRO and after suitable pulse conditioning this signal was displayed on a Gould Brush 220 pen recorder. The recorded iontophoretic pulses were also transcribed onto the pen recorder by a direct relay from the magnetic tape.

Histological Verification of Electrode Placements

The position of the recording electrode was marked by the iontophoretic application of the dye, Pontamine Sky Blue 6BX (Hellon, 1971). Ten microamps delivered through the electrode for

A) Stimulation of the Stria Terminalis.

ST. Stria Terminalis
ACO. Corticomedial Nucleus of the Amygdala.

B) Stimulation of the Arcuate/Ventromedial Nuclei.

AR. Arcuate Nucleus
VM. Ventromedial Nucleus

C) Recording from the Preoptic Area.

AC. Anterior Commisure
POA. Preoptic Area.

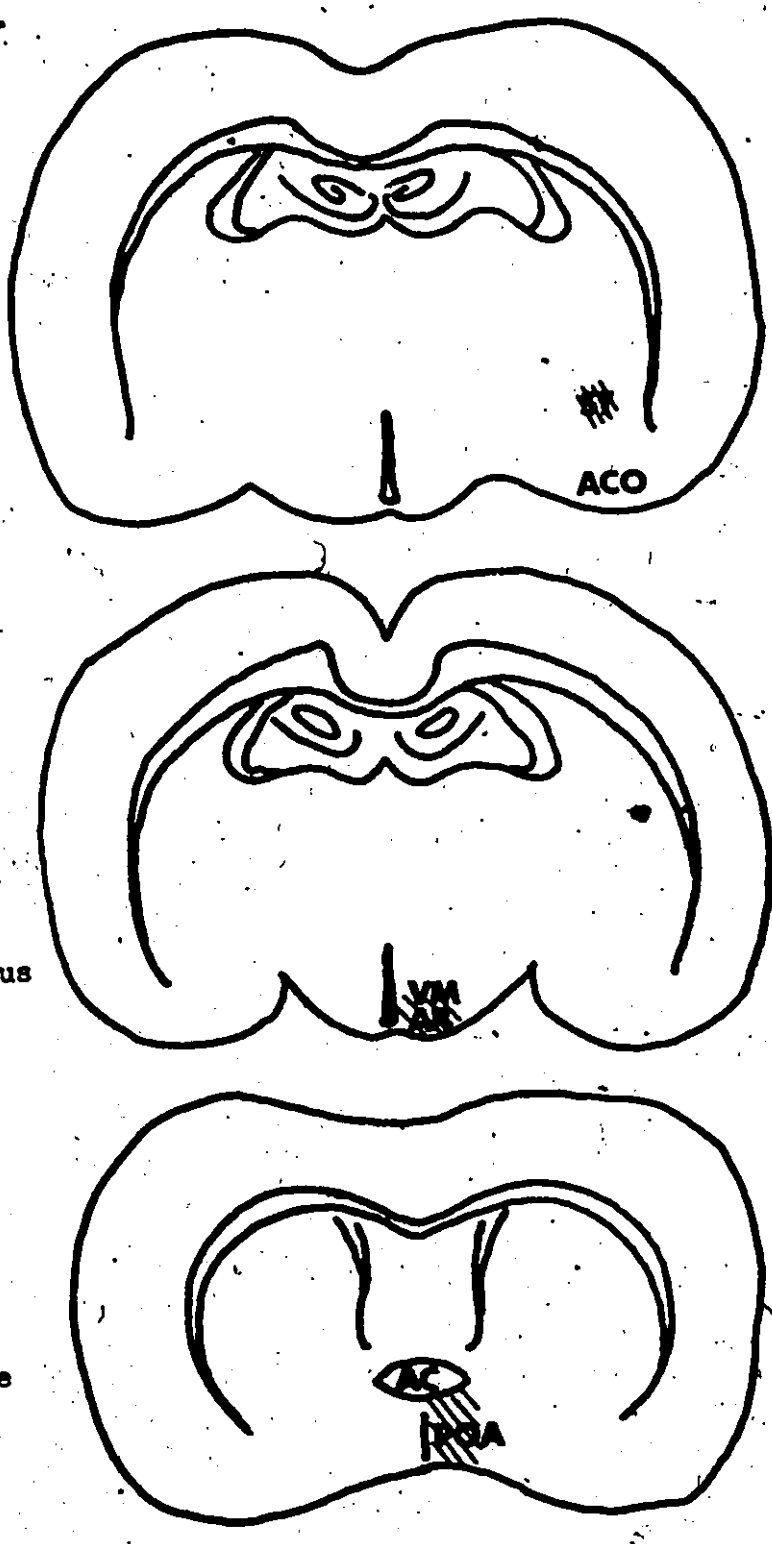


Figure 2. Tracings of brain sections showing positions of stimulating and recording electrodes, denoted by the shaded areas

2 minutes ejected sufficient dye to give a 'blue spot' which could be seen with the naked eye while cutting frozen sections. This eliminated the necessity to mount serial sections of the appropriate area of the brain. For those experiments in which triple barrelled microelectrodes were used; a dye-filled single barrel electrode was inserted into the same location using a common reference point mounted on the stereotaxic head holder and the position was marked accordingly. This indirect method of marking was adopted because unsatisfactory results were obtained when the recording barrel of the three barrelled electrodes was used for ejecting the dye.

The position of the stimulating electrode was marked by the Prussian Blue reaction. A small amount of iron was deposited by passing 0.25 mA through the electrode for 15 seconds. At the end of each experiment the animal was perfused with 40 ml of 10% potassium ferrocyanide in 10% buffered formalin, the brain was dissected out and placed in formalin for further fixation.

Frozen sections were cut at 50 μm, stained with cresyl violet and viewed under low power to locate the markings precisely. The position of histologically identified points, together with other recording sites determined from the readings on the micromanipulator carrying the electrode, were then plotted on stereotaxic diagrams of the rat brain. (see Fig. 2)

CHAPTER III: EFFECTS OF HALOTHANE ON OVULATION IN THE RAT

EFFECTS OF HALOTHANE ON OVULATION IN THE RAT

Introduction

One of the major disadvantages of neuroendocrinological experiments carried out under general anaesthesia is the possible effect of the anaesthetic itself on the neural substrate understudy, in particular the preoptic area and the basal hypothalamus.

Urethane, which has been extensively used for electrophysiological investigations of neuroendocrine circuits, is known to block ovulation in the rat when it is administered at appropriate times during the estrous cycle (Lincoln and Kelly, 1972). A similar effect has been described for pentobarbital (Nembutal^{R}) (Everett and Sawyer, 1950); both drugs prevent ovulation by interferring with the neural triggering of pituitary LH release.

The electrophysiological studies, described below, of the action of estrogens on the neural circuits implicated in the control of ovulation have been carried out under halothane anaesthesia. It was therefore of interest to determine whether or not this anaesthetic would also interfere with spontaneous ovulation and if so, to attempt to define the site of the ovulatory blockade. In order to achieve surgical anaesthesia a concentration of 1.5% halothane was required, although sedation could be induced by 1% halothane. The effect of both these dose levels on ovulation has been assessed.

Non-anaesthetic doses of pentobarbital or urethane do not block ovulation. For this reason it became of interest to determine whether a subthreshold dose of either Nembutal or urethane supplemented with a low concentration of halothane (0.5%) would induce a sufficient degree of anaesthesia for sedation, and to observe the effect of these combinations on ovulation.

Methods

On the day of prooestrus, 4-day cycling rats were exposed to ether for about 2 mins between 13.15 - 13.45 h, intubated and anaesthetised with either 1% or 1.5% halothane (see Methods section, Operative Procedures). The halothane was switched off at 16.30 h and the animals allowed to recover. Those animals in which a combination of anaesthetics was used were injected intraperitoneal (i.p.) with either Nembutal (17.5 ng/kg) or urethane (500 mg/kg) at 13.15 h and subsequently anaesthetised with 0.5% halothane as described above. All experimental animals were paired with one control animal injected with 35 mg/kg Nembutal at 13.15 h.

To determine the site of ovulatory blockade rats anaesthetised with 1.5% halothane were treated with 50 or 100 µg ovine Luteinizing Hormone (LH NIH-LH-S17) or synthetic Luteinizing Hormone Releasing Hormone (LRH Beckman Instruments Inc. Spinco Division, Palo, Alto, Calif. USA) at 1600 h on the afternoon of prooestrus. The LH was injected i.p. in 0.5 ml of saline and the LRH was injected subcutaneously in 0.5 ml of 0.0 M acetic acid in saline (Arimura et al, 1971). Each experimental animal was paired with a control rat anaesthetised with 1.5% halothane and sham injected with the appropriate solvent.

For all experiments ovulation was assessed by counting the number of ova contained in the Fallopian tubes during the following morning (oestrus), in the absence of ova the ovaries were preserved for histological examination to confirm the presence of unruptured mature follicles (Fig. 3).

Results

The results have been summarised in Tables I and II. 1% halothane during the 'critical' period of proestrus blocked ovulation in 7 of the 10 experimental rats whereas the use of 1.5% halothane inhibited ovulation in all animals tested (Table I).

Rats anaesthetised with a combination of halothane and either Nembutal or urethane showed a degree of anaesthesia comparable to that obtained with 1.5% halothane. Both combinations of anaesthesia were effective in blocking ovulation (Table I). The ovulatory blockade by 1.5% halothane could be overcome by the administration of 50 or 100 µg LH or by 500 ng LRF (Table II).

Discussion

The results demonstrate that an anaesthetic dose of halothane is effective in blocking ovulation in the female rat when administered during the 'critical period' of prooestrus. This is also true when two subthreshold doses of different anaesthetics are used in combination to produce full surgical anaesthesia. The evidence suggests that the blockade of ovulation is a result of anaesthesia per se, and not a separated associated property of the specific drugs employed.

TABLE I AND II

Table I. Blockade of ovulation by halothane

Anaesthetic	No. of rats*	Ovulation blocked	Partial ovulation < 10 Ova	Full ovulation > 10 Ova
1% Halothane	10	7	1	2
1.5% Halothane	6	6	0	0
0.5% Halothane + 17.5 mg/kg Nembutal	4	4	0	0
0.5% Halothane + 500 mg/kg urethane	4	4	0	0

*Similar numbers of rats were concurrently injected with Nembutal, 35 mg/kg i.p.; ovulation was consistently blocked in these controls.

Table II. Effects of LH and LRH on halothane blockade

Anaesthetic	Hormones	No. of rats*	Full ovulation > 10 Ova	Partial ovulation < 10 Ova	Ovulation blocked
1.5% Halothane	50 μg LH	8	6	0	2
1.5% Halothane	100 μg LH	3	3	0	0
1.5% Halothane	500 ng LRH	10	9	0	1

*21 rats were concurrently anaesthetized with 1.5% Halothane but did not receive any hormone treatment; ovulation was fully blocked in 17 and partially blocked in 3 controls.

Figure 3. Photomicrograph of ovary showing mature unruptured follicles at oestrus; the animal had been anaesthetised with 1.5% halothane during the afternoon of prooestrus. Paraffin section cut at 8 µand stained with haematoxylyn and eosin. Magnification X 40

The injection of either LH or LRH overcame the effects of halothane in blocking ovulation. Since these hormones eliminate the requirement for the neural trigger responsible for the LH surge, the results indicate a neural site of action of the anaesthetic, and rule out the possibility of a direct effect on the ovary.

Recent experiments suggest that halothane acts by causing a decrease in the amount of transmitter released from nerve endings (Richards, 1972, a). Similar findings have been reported for barbiturates (Richards, 1972, b). However, Nicoll (1972) suggested that halothane, urethane and barbiturates anaesthetics may act by prolonging the action of inhibitory synapses, either by increasing the amount of inhibitory transmitter released or by a postsynaptic action.

In invertebrate preparations pentobarbital and urethane were shown to depress selectively the excitatory post-synaptic potentials (Barker and Gainer, 1973). Whether these drugs act on the pre- or post-synaptic element, it is conceivable that anaesthetics interfere with ovulation by reducing the synaptic activation of neurons involved directly or indirectly in the production of LRH, by a mechanism which may be a general property of anaesthetics. Therefore, the exploration of neural mechanisms underlying spontaneous ovulation in the rat, which are carried out under the influence of any general anaesthetic may give results which do not necessarily explain normal physiological processes.

CHAPTER IV: INTRAVENOUS HORMONE ADMINISTRATION, PLASMA ESTROGEN CONCENTRATIONS AND LH SECRETION.

INTRAVENOUS HORMONE ADMINISTRATION

PLASMA ESTROGEN CONCENTRATIONS AND LH SECRETION

Introduction

Many investigations of the feedback action of estrogen in the control of gonadotrophins involve hormone administration of ovariectomized rats with subsequent measurements of circulating LH and FSH. Typically hormones are injected s.c., the doses of estrogen ranging from 0.1 - 50 μg (Barraclough and Haller, 1970) and those of progesterone being in the order of 1 - 2 mg (Mann and Barraclough, 1973). However, there are no reports to date on direct measurements of circulating hormone levels following a s.c. injection, and therefore it is unknown if such doses of estrogen or progesterone are within a physiological range, and what is the time course of the rate of rise of the circulating hormone. Both these factors are of utmost importance when studying feedback action of sex steroids.

In the present studies the stimulatory action of estrogen on the activity of neurons in the POA has been investigated using electrophysiological techniques and the experiments have involved continuous recording from single cells before and after hormone administration. In order to reduce the time to obtain adequately high levels of estrogen in the environment of the preoptic neurons, it was necessary to inject the hormone intravenously.

The consequence of positive feedback action on the POA is an increased secretion of LH from the pituitary, as occurs during the

preovulatory period in cycling rats. In ovariectomized rats this increase can only be observed after the administration of estrogen (Caligaris et. al., 1971a) or progesterone (Caligaris et. al., 1971b) in animals which have been previously injected with estrogen ('primed'). The workers who have demonstrated this stimulatory action of hormones in primed ovariectomized rats used the s.c. method of hormone administration; the experimental animals were unanaesthetised and measurements of LH secretion following estrogen administration were only made five hours following a second injection (Caligaris, et. al., 1971a, Jackson, 1973).

The lack of data on the effects of an i.v. injection of either estrogen or progesterone in anaesthetised rats would make it difficult to interpret results obtained from the kind of electrophysiological experiments like those to be described further in the present work. Thus it was important to establish what i.v. dose of estrogen would produce a level of the circulating hormone which lay within the physiological limits and whether, estrogen or progesterone, injected i.v. was effective in stimulating an increased secretion of LH in estrogen primed ovariectomized rats anaesthetised with halothane.

Methods

a) Measurement of plasma estrogen concentrations.

Ovariectomized rats were anaesthetised with halothane and injected i.v. with 0.25 ml EB dissolved in propylene glycol (see Chapter II, Materials and Methods). The doses of EB used were 100 ng, 500 ng, 1 μg, 5 μg, 25 μg and 50 μg. Blood samples (2 ml) were taken by heart puncture using a 25 g syringe needle, from one group of

animals immediately after the injection (0 minutes) and at 30 minutes and from the other group at 15 minutes and 1 hour following the injection. After each sample the volume of blood removed was replaced by an intraperitoneal (i.p.) injection of saline. Plasma estrogen concentrations were measured by a radioimmunoassay technique (Bell et al., 1971). The limit of sensitivity for the assay was 0.2 pg and the coefficient of variation for replicate analyses was 14.2%.

b) Measurement of serum LH levels.

All ovariectomized rats were primed with 20µg EB injected s.c. at 1200 h three days prior to experimentation and the second dose of either EB or progesterone was injected i.v. between 1130 h and 1200 h on the day of sampling (see Chapter II, Materials and Methods). LH measurements were made from four groups of animals

(i) Rats anaesthetised with halothane and injected with 20 µg EB through the femoral vein.

(ii) Rats anaesthetised with halothane and injected with 100 µg progesterone through the femoral vein

(iii) Unanaesthetised rats injected with 20 µg EB through a tail vein.

(iv) Unanaesthetised rats injected with 100 µg progesterone through a tail vein.

Serum LH concentrations were measured by a radioimmunoassay technique using reagents distributed by NIAMD. For these determinations the limit of sensitivity was 0.2 ng/ml and the coefficient of variation for replicate analyses was 8.1%.

Results

a) Plasma estrogen concentrations.

The plasma concentrations of estrogen following an i.v. injection of different doses of the hormone have been plotted on a logarithmic scale in Figure 4. Each point represents the mean concentration of four blood samples, the vertical bars representing the standard error of the mean. The initial level of plasma estrogen following different doses of EB ranged from 35 ng/ml to 0.14 ng/ml and a gradual decrease of plasma estrogen concentrations, at all dose levels, occurred during the sampling period, which probably reflects the binding of the steroid hormone to specific target tissues and/or its metabolism.

b) Serum LH levels.

The results have been summarised in Table III. There was no significant difference in the serum LH concentrations between the one and five hour samples from estrogen primed ovariectomized rats injected i.v. with 20 μg EB. This was true for both the anaesthetised and unanaesthetised groups of animals, (a and c). Similar findings were observed when 100 μg progesterone was administered to ovariectomized estrogen primed rats (b and d) although one unanaesthetised animal did show a significant increase of serum LH five hours following the injection. The LH concentration increased from 132 ng/ml to 925 ng/ml within the sampling period.

These preliminary observations demonstrate that an i.v. injection of an ovarian steroid was ineffective in stimulating an increased secretion of LH during the sampling period, in both anaesthetised and

Figure 4. Plasma estrogen concentrations, plotted on a logarithmic scale, at 0,15,30 and 60 minutes following an i.v. injection of EB. The doses of EB injected are indicated against each curve. The vertical bars represent the standard error of the mean.

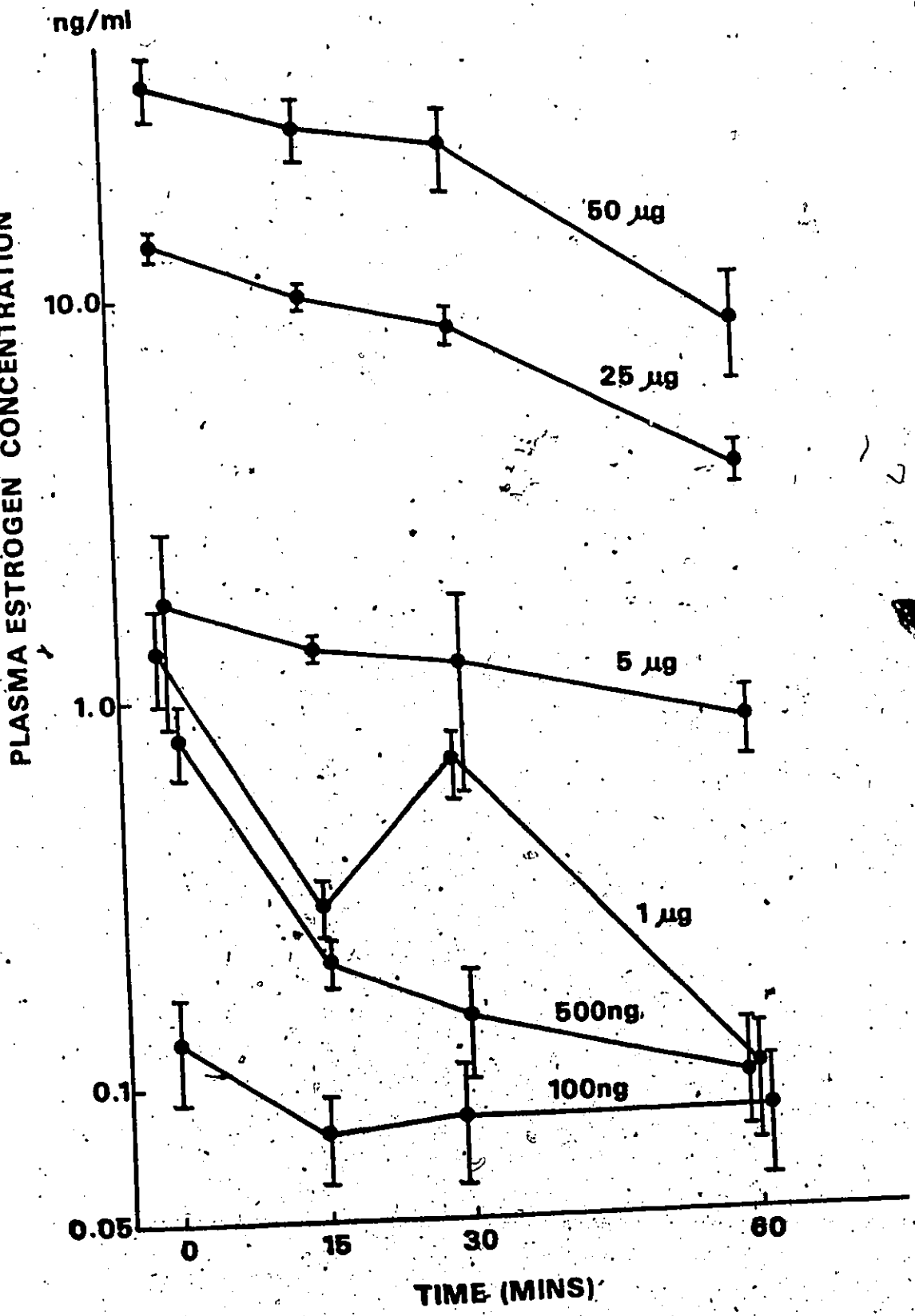

ng/ml
PLASMA ESTROGEN CONCENTRATION
10.0
1.0
0.1
0.05
50 µg
25 µg
5 µg
1 µg
500ng
100ng
0
15
30
60
TIME (MINS)

TABLE III

SERUM LH LEVELS.

Exp. Groups	No. of Samples	1 Hour		5 Hours	
		Mean ng/ml	S.E. ±	Mean ng/ml	S.E. ±
a) Anaesthetised Estrogen treated	4	97.8	13.6	96.8	15.6
b) Anaesthetised Progesterone treated	3	107.6	9.4	74.7	13.0
c) Unanaesthetised Estrogen treated	4	168.5	8.1	148.8	21.2
d) Unanaesthetised Progesterone treated	3	150.6	9.4	401.6	261.7

unanaesthetised groups of animals. Although no measurements of the serum LH concentrations were made prior to the second i.v. injection of either estrogen or progesterone the values of the LH levels of the one hour samples are comparable to the serum LH levels reported by Caligaris et. al., (1971 a,b) in estrogen primed ovariectomized rats. They showed that three days after a s.c. injection of estradiol benzoate, prior to a second dose of a steroid hormone, the serum LH concentrations were in the order of 100 ng/ml but, after a second s.c. injection of either estrogen of progesterone, the peak concentration achieved was, on average, 500 ng/ml. It is therefore unlikely that in these experiments any significant increase of LH secretion occurred between the time of the i.v. injection and the first 1 hour sampling period.

Finally it was of interest to compare whether there was any significant difference in the LH concentrations between the anaesthetised (a and b) and unanaesthetised (c and d) groups of animals at 1 and 5 hours after the i.v. injection of estrogen or progesterone. The results from a paired t-test showed that the LH concentrations were significantly lower ($p < 0.01$) in the two anaesthetised groups of animals at 1 hour after the steroid injection compared with the unanaesthetised animals. At 5 hours no significant difference ($p > 0.2$) was observed in the serum LH concentrations between these two groups of animals. In this regard the high standard error of the mean of the unanaesthetised progesterone treated group (d) should be noted.

Discussion

The lowest dose of EB, 100 ng, resulted in a concentration of estrogen which exceeded the peak concentration of estrogen attained during the oestrous cycle of the 4-day cycling rat. Brown-Grant et. al. (1970) reported that the highest level of peripheral plasma estrogen in the intact rat was on average 30 pg/ml, whereas 100 ng EB injected i.v. resulted in a mean estrogen plasma concentration of approximately 140 pg/ml. Thus even at a dose of 100 ng EB the plasma concentration of estrogen attained exceeded the physiological range required for the positive feedback action of estrogen for stimulating the ovulatory surge of LH. At the end of the one hour sampling period the plasma concentrations, at all dose levels, still exceeded the physiological range.

The findings that both estrogen and progesterone were ineffective in inducing an increased secretion of LH between one and five hours after their i.v. administration in ovariectomized rats may have been due to the method of hormone administration. An i.v. injection results in an instant rise in the level of the circulating hormone as opposed to the relatively slow rise of estrogen levels as occurs during the oestrous cycle or which follows a s.c. injection of the steroid. The rate of rise of plasma estrogen concentration may be an important factor in feedback action. Another factor to consider is the constant light conditions under which the animals were maintained. It is well known that normal adult female rats become persistent-oestrus by exposure to constant illumination and do not secrete a prooestrous

surge of LH (Daane and Parlow, 1971b). In the present experiments the rats were kept in constant light to reduce the possibility of circadian rhythms affecting the results since electrophysiological recordings were made at various times of the day. Finally, the anaesthetic was shown to reduce the secretion of LH which is consistent with the findings that halothane anaesthesia blocks ovulation in the cycling rat by interfering with the activation of those neurons which elaborate and secrete LRH (see Chapter III).

These experiments therefore define certain parameters of the experimental conditions of the electrophysiological studies; a dose of estrogen was used which exceeded the physiological range required for the positive feedback effect of estrogen on the POA and an i.v. injection of either estrogen or progesterone was ineffective in stimulating the release of LH in these estrogen primed ovariectomized rats. In addition the results stressthe importance of establishing the experimental conditions under which electrophysiological investigations into steroid feedback mechanisms and control of gonadotrophin secretion are made.

CHAPTER V: ELECTROPHYSIOLOGICAL IDENTIFICATION OF NEURONS POSSIBLY INVOLVED IN THE CONTROL OF GONADOTROPHIN SECRETION

(A) IDENTIFICATION OF CELLS IN THE PREOPTIC AREA RECEIVING AN ORTHODROMIC INPUT FROM THE STRIA TERMINALIS.

(B) IDENTIFICATION OF CELLS IN THE PREOPTIC AREA RESPONDING ANTIDROMICALLY TO STIMULATION OF THE ARCUATE NUCLEUS.

(A) IDENTIFICATION OF CELLS IN THE PREOPTIC AREA RECEIVING AN ORTHODROMIC INPUT FROM THE STRIA TERMINALIS

Introduction

The stria terminalis which arises from the corticomedial and basolateral nuclei of the amygdala connects this part of the limbic system to a large area of the hypothalamus, and it is thought that the amygdaloid control of gonadotrophin secretion is mediated through this pathway. The most proximal and largest termination of this pathway is in the bed nucleus which lies around the anterior commissure. Continuous with this projection is the so called post-commissural component of the stria terminalis which is distributed to the medial POA and anterior hypothalamus. The more medial fibres of the stria terminalis form the supracommissural component which curves caudally over the rostral aspect of the anterior commissure to terminate in a rather restricted synaptic region surrounding the ventromedial nucleus (Heimer and Nauta, 1969).

Steroid Uptake Studies. Uptake studies of radioactive estrogen have shown that the pattern of uptake in the hypothalamus closely follows the distribution of the stria terminalis (Stumpf, 1972) and that the amygdala also retains the labelled steroid (Pfaff, 1972). These findings suggest that both the neurons in the amygdala and the neurons in the hypothalamus to which they project are sites of feedback action. Estrogen implants into the corticomedial amygdala results in an increased secretion of LH in ovariectomized rats (Lawton and Sawyer, 1970) supporting the concept that the amygdala is sensitive to the feedback action of estrogen.

Physiological Studies. Stimulation and lesion experiments have provided evidence for a functional role of the amygdala, in the control of gonadotrophin secretion. Stimulation of the amygdala induces ovulation with a concomitant rise in serum LH and FSH, and this response is blocked by transection of the stria terminalis (Velasco and Taleisnik, 1969). Kawakami et. al., (1973) also demonstrated an increase in the level of serum LH and FSH following stimulation of the amygdala during prooestrus and further demonstrated that an increase in multi unit activity could be recorded in the POA for 2-2.5 hours following this stimulation. These results suggest a stimulatory role of the amygdala in the ovulatory surge of LH.

In the ovariectomized rat, lesions of the cortical amygdala result in a significant increase of plasma LH compared to the controls despite the fact the LH levels are already elevated due to ovariectomy (Lawton and Sawyer, 1970), suggesting that the amygdala may be important in tonically inhibiting the release of LH. Further support of this suggestion is provided by the experiments of Smith and Lawton (1972) in which they showed that ovarian compensatory hypertrophy following unilateral ovariectomy could be blocked by lesioning the corticomedial nucleus of the amygdala or the stria terminalis...

Sawyer, (1971) reviewing the evidence for the role of the amygdala in the control of gonadotrophins has proposed that the corticomedial amygdala of the female rat contains two functional groups of neurons; a) cells which exert a tonic inhibitory effect on gonadotrophin secretion and b) cells which facilitate the ovulatory surge of LH in response to the increased levels of estrogen. Both groups of neurons appear to

project to the hypothalamus via the stria terminalis. However, it is interesting to note that lesions in the corticomedial amygdala either prepuberally or after maturation does not affect the normal cycling of a female rat (Critchlow and Bar-Sela, 1967).

On the basis of such experimental evidence it was of interest to attempt to identify cells in the POA receiving an input from the stria terminalis, the rationale being that such cells would be part of a neuroendocrine circuit related to the control of gonadotrophin secretion, and possibly therefore sensitive to estrogen feedback mechanisms.

Methods

A full description of the operative procedures, electrodes and recording techniques is given in Chapter II, Materials and Methods. The stria terminalis was stimulated at a point just caudal to its point of emergence from the amygdala before it courses dorsally and rostrally round towards the bed nucleus. This point of stimulation was chosen to avoid additional stimulation of the fimbria which lies adjacent to the stria terminalis in its dorsal course. Stimulation thresholds ranged from 200 μA to 1mA and cells which could only be excited by higher current intensities were disregarded due to the possibility of stimulus spread involving adjacent brain areas. The recording electrode was placed stereotaxically in the strial part of the POA which lies in a localised region just ventral to the anterior commissure. Extracellular unit potentials and evoked potentials were recorded through a single barrel glass micropipette filled with Pontamine Sky Blue. The correct position of both the stimulating and recording electrode

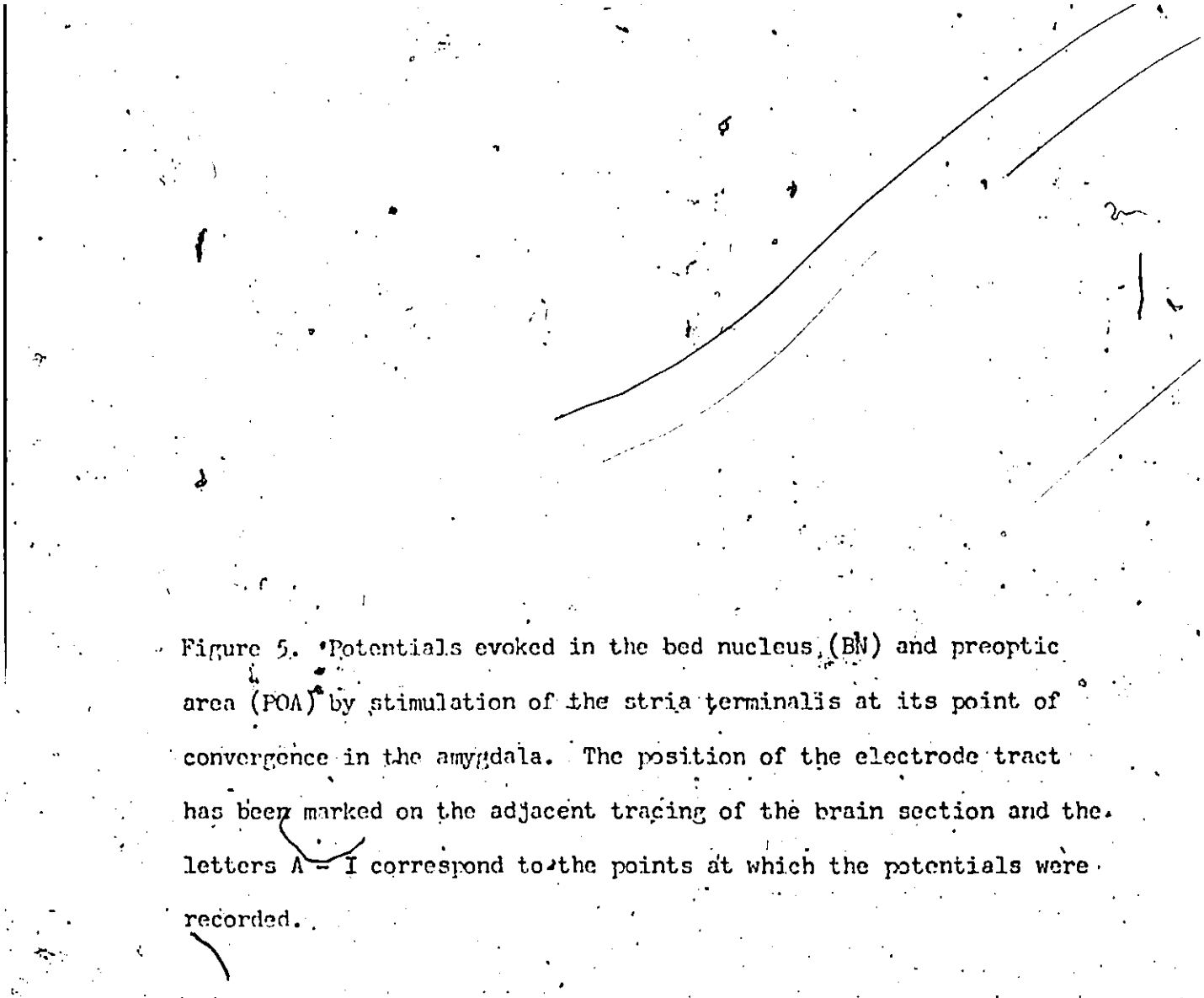
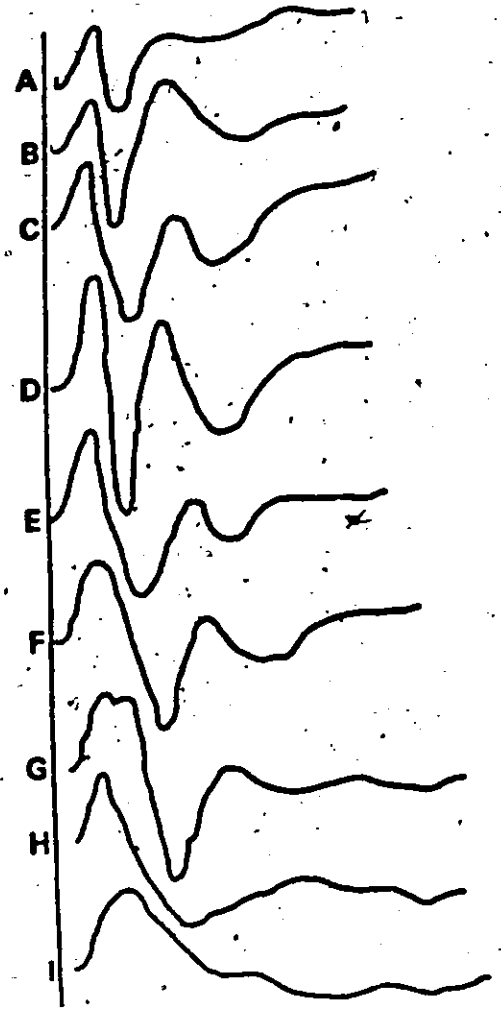


Figure 5. Potentials evoked in the bed nucleus (BN) and preoptic area (POA) by stimulation of the stria terminalis at its point of convergence in the amygdala. The position of the electrode tract has been marked on the adjacent tracing of the brain section and the letters A - I correspond to the points at which the potentials were recorded.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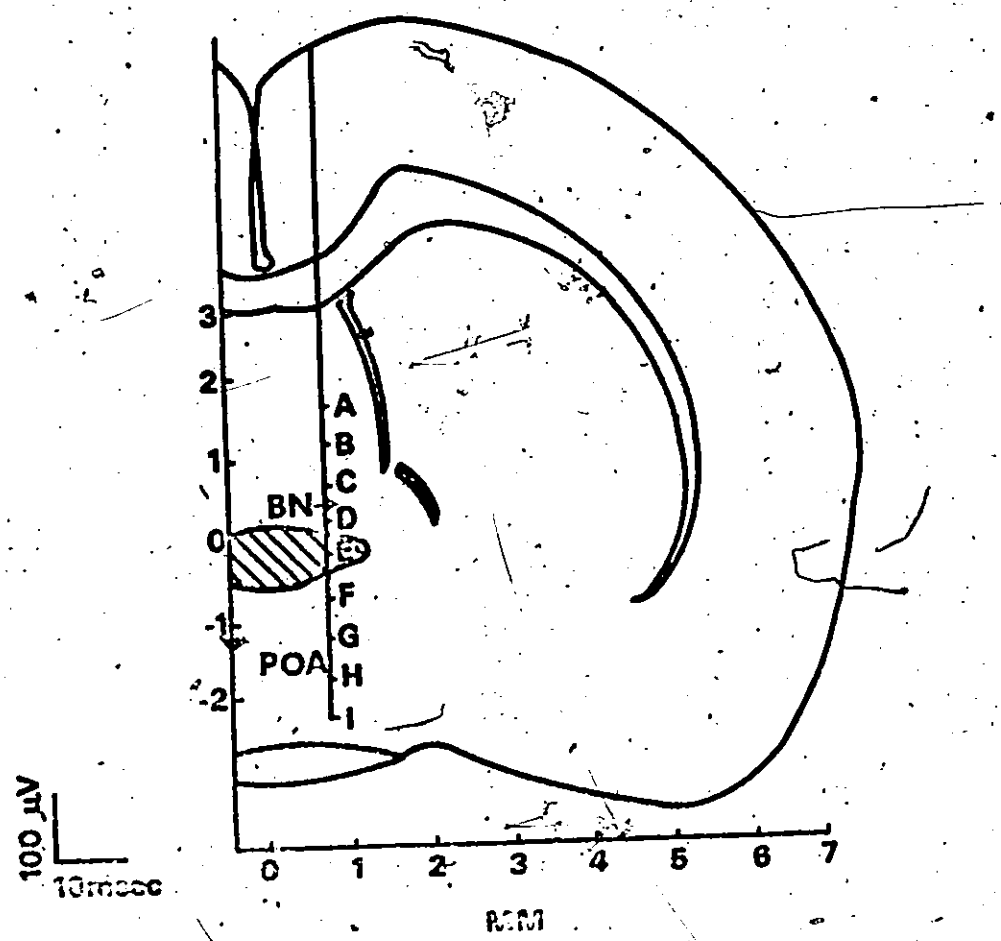
3
2
1
0
-1
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A
B
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E
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G
H
I
BN
POA
100 μV
10msec
0
1
2
3
4
5
6
7
MM

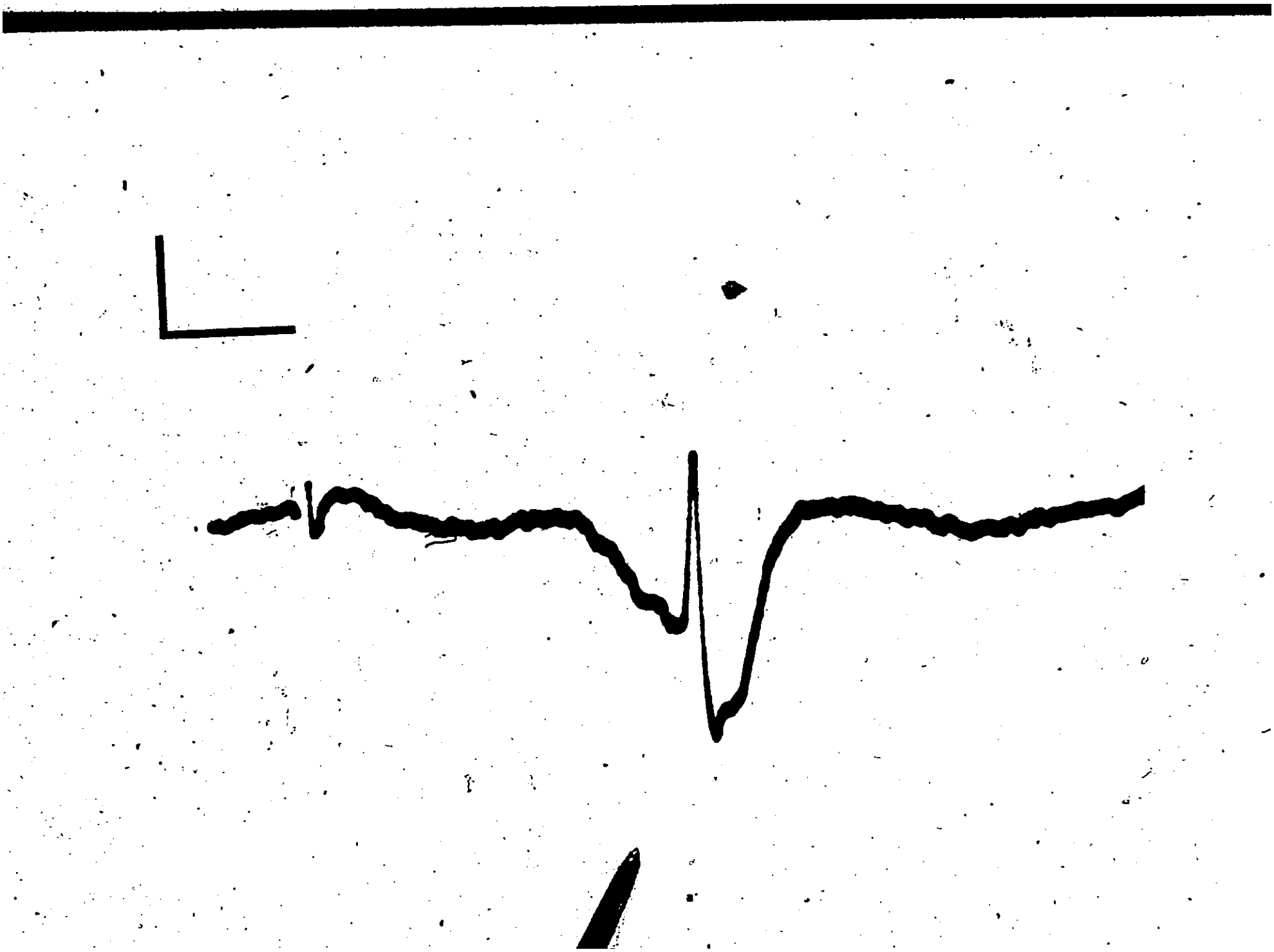
were confirmed by subsequent histological examination (see Chapter II, Histological Verification).

In order to have some check on the correct placement of both the stimulation and recording electrodes during the experiments a number of studies were undertaken in which a series of evoked potentials were recorded along several vertical electrode tracts. The potentials were traced directly from the screen of a storage oscilloscope. Three points within the bed nucleus and the POA were marked by ejection of the dye from the recording electrode. Following histological identification of the marks, the electrode tracts could be reconstructed and the potentials recorded at specific points could be plotted on tracings of brain sections, (see Fig. 5).

Results

Figure 5 shows an example of a series of potentials recorded along one electrode tract and a diagram of the brain showing the corresponding points of the potentials. These mapping experiments showed that the potentials of maximum amplitude and shortest latency could be recorded in the bed nucleus. In the POA the potentials were dispersed and of lower amplitude suggesting the potential recorded in this area was merely a result of passive electrotonic spread of the current generated in the bed nucleus. Typically the response in the bed nucleus contained two distinct negative waves; the latency of the first negative peak varying between 6 - 12 msec and that of the second peak ranging between 14 - 24 msec.

Figure 6. Oscilloscope trace showing an orthodromic action potential recorded in the medial POA following a single stimulus to the stria terminalis at its point of convergence in the amygdala. (calibration 5 msec, 300 μV, positive upwards.



The response to strial stimulation has been recorded from 34 neurons in the POA and 4 neurons in the bed nucleus. The latency of the responses in the POA ranged from 7 - 25 msec, the mean latency being 14.3 msec. In the bed nucleus the range was 5 - 22 msec with a mean latency of 12.8 msec. Sixty percent of the total number of units which could be driven by stimulating the stria terminalis were spontaneously active; typically the firing rate was slow and varied between 10 spikes/sec to 0.05 spikes/sec, the average being 4 spikes/sec.

Discussion

The recordings of the evoked potentials and single units in the bed nucleus and POA confirm the anatomical findings that the stria terminalis is distributed to both these brain regions.

Wave forms with double negative peaks characterised the evoked potentials recorded in the bed nucleus suggesting that stimulation of the amygdala activated two separate components of the stria terminalis. This finding is in agreement with the results of Sherwood (1968) who demonstrated that there were two components of the stria terminalis with different conduction velocities. Thus it is unlikely that the second negative peak of the evoked response recorded in the bed nucleus represents activity generated by recurrent collaterals or interneuronal links.

The distance from the area of convergence of the stria terminalis in the amygdala to the bed nucleus can be estimated from the atlas of deGroot (1959) to be 9.6 mm. Therefore, if in the present experiments the two negative peaks recorded in the bed nucleus represent the strial

components, the conduction velocities (estimated from the mean latency of the evoked response following strial stimulation) are calculated to be 1.0 m/sec and 0.5 m/sec. This finding suggests that the stria fibres are C fibres with little myelination.

In the present study the evoked potentials recorded in the POA appeared to be the result of passive electronic spread of current generated in the bed nucleus and not a direct result of the activity in this area which was evoked by stimulating the stria. However, anatomically stria terminalis fibres have been traced to the POA (Knook, 1965). This histological evidence is supported by the results of these experiments demonstrating that cells in the POA could be driven orthodromically by stimulating the stria terminalis at its point of convergence in the amygdala. The range of latencies of the responses to strial stimulation suggest that the POA receives an input from both components of this pathway.

Dreifuss et. al. (1968) have shown that stimulation of the amygdala accelerated the firing pattern of some units and inhibited others in the hypothalamus. Perhaps the two components of the stria terminalis represent the two groups of neurons in the amygdala which either inhibit or facilitate the secretion of gonadotrophins as suggested by Sawyer (1972).

(B) IDENTIFICATION OF CELLS IN THE PREOPTIC AREA RESPONDING ANTIDROMICALLY TO STIMULATION OF THE ARCUATE NUCLEUS

Introduction

The evidence that has accumulated from stimulation and lesion experiments implies that impulses from the POA arriving at the basal hypothalamus are responsible for triggering the preovulatory LH surge. Electrical or electrochemical stimulation of the POA induces ovulation in the pentobarbital-blocked rat (Everett et. al. 1964, Cramer and Barraclough, 1971) by stimulating the release of LH. The pattern of change in plasma LH concentrations following preoptic stimulation in the pentobarbital anaesthetised animal are comparable to those observed in the normal prooestrous rat (Cramer and Barraclough, 1971).

Terasawa and Sawyer (1969) showed that activation of the POA resulted in an increased multi unit activity in the median eminence region which could be correlated with the occurrence of ovulation in Nembutal blocked rats. This finding is consistent with the idea that the afferent input to the basal hypothalamus from the POA stimulates either directly or indirectly the activity of the neurons which secrete LRH.

Lesions placed between the POA and the basal hypothalamus or complete deafferentation of the basal hypothalamus blocks ovulation in the female rat and leads to a persistence of vaginal cornification

(Halasz and Gorski, 1967). However, deafferentation of the preoptic-hypothalamic area does not inhibit ovulation, suggesting that the neurogenic stimulus triggering ovulation arises in part from the POA (Koves and Halasz, 1970). Stimulation of the POA following unilateral lesions of the preoptic-tuberal pathway has shown that the majority of fibres in this tract are uncrossed and that activity in even one half on the pathway conveying impulses from the POA to the hypophysiotrophic area is sufficient to induce ovulation (Cramer and Barraclough, 1973).

Dyer and Cross, (1972) have electrophysiologically identified a direct pathway between the POA and the basal hypothalamus by antidromic activation of cells in the POA following stimulation of the ventromedial/arcuate nucleus. In view of the evidence that the POA concentrates estradiol-H^3 (Lisk, 1971) and that it triggers the preovulatory surge of LH, it is tempting to speculate that such antidromically identified cells are sensitive to the stimulatory feedback action of estrogen which controls ovulation. For these reasons it was decided to repeat the experiments of Dyer and Cross and subsequently to use such antidromically identified neurons to study the effects of estrogen on their electrical activity and sensitivity to iontophoretically applied putative neurotransmitters.

Methods

The operative procedures, recording techniques and a description of both the stimulating and recording electrodes have been fully outlined in the Material and Methods, Chapter II.

The stimulating and recording electrodes were stereotaxically positioned in the arcuate nucleus and medial POA respectively (see Chapter II, Operative Procedures) and their positions verified by subsequent histological examination (see Chapter II, Histological Verification). Extracellular recordings have been made using single and three barrelled glass microelectrodes and all cells which were either spontaneously active or were activated by the iontophoretic application of glutamate were tested for an antidromic response to stimulation of the arcuate nucleus. Stimulation thresholds ranged from 80μA to 1mA, cells showing a higher threshold being disregarded due to the possibility of stimulus spread involving adjacent brain areas.

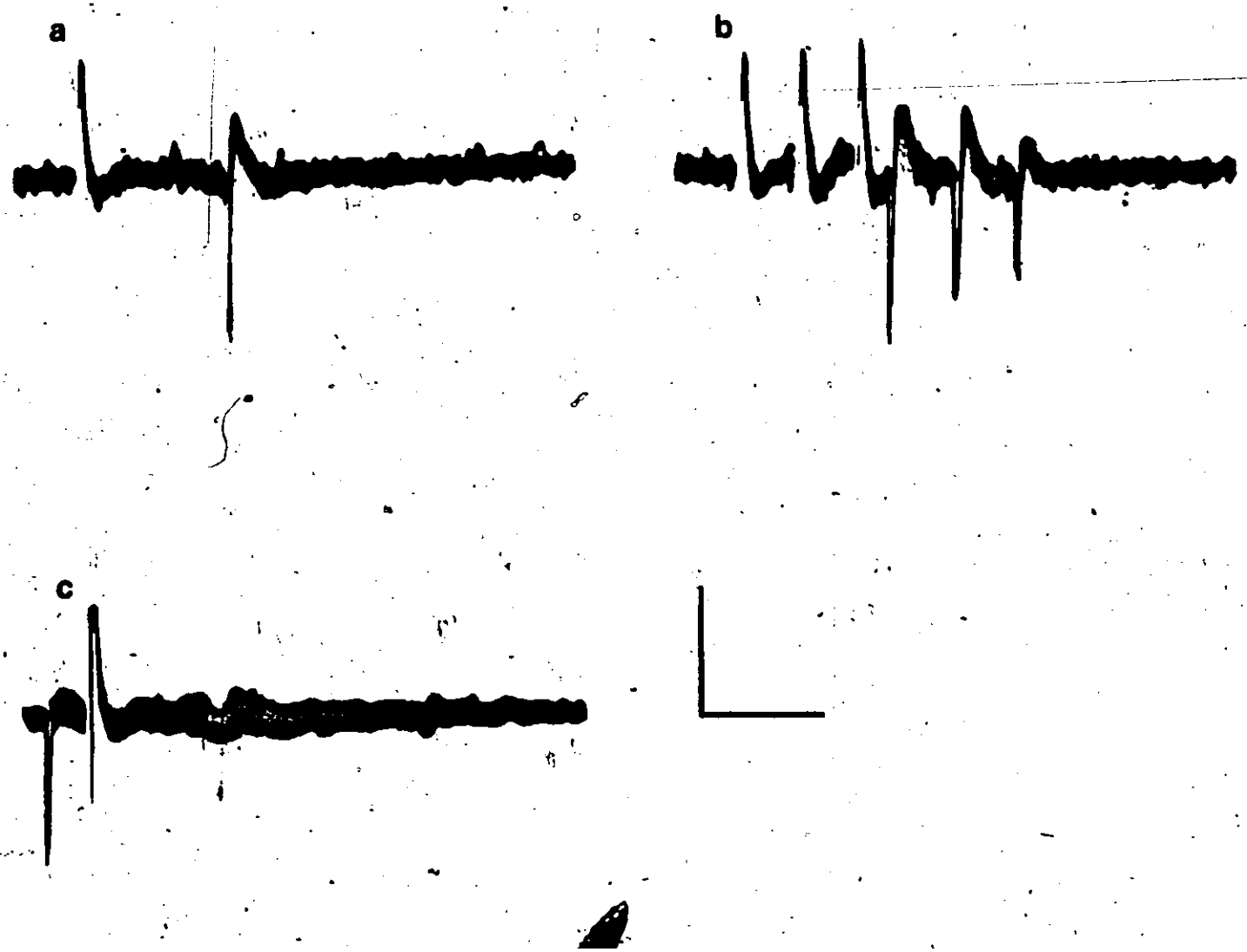
Three criteria were used to determine the antidromic nature of the response to stimulation (see Fig 7).

(i) Constant latency of the response at any fixed stimulation intensity

(ii) Triggering of an equal number of responses at the same frequency by a train of three stimuli delivered in excess of 180 Hz.

(iii) Cancellation of the antidromic spike by collision with an orthodromic spike (occurring either spontaneously or as a result of glutamate application).

Figure 7. Oscilloscope traces of antidromic action potentials recorded from cells in the preoptic area following stimulation of the ventromedial/arcuate region of the basal hypothalamus. In (a) five superimposed oscilloscope traces of the antidromic action potential demonstrate the constant latency of the responses. Three spikes following an equal number of stimuli administered at a rate of 200/sec are shown in (b). In (c) a glutamate induced spike 3 msec prior to the stimulus collided with the antidromically evoked action potential (calibration marks; 10 msec. 300 μV, positive upwards.


a
b
c

Results

A total of 179 neurons in the POA have been identified by antidromic stimulation from the arcuate nucleus (for example see Fig 7). Extracellular potentials have been recorded from both normal cycling rats and ovariectomized rats. There have been 59 antidromically driven cells recorded from the cycling rats, 45 of which were recorded from dioestrous I females and the remaining 14 from oestrous females. The mean latency of the response from the dioestrus I and oestrus group of females was 8.02 msec. and 8.07 msec respectively with the mean for the entire group being 8.03 msec. The latencies ranged from 3 msec. to 17 msec. On the assumption that the average separation between the stimulating and recording electrode was 3.0 mm one can calculate a mean conduction velocity of 0.37 m/sec.

In the ovariectomized group of animals, 120 cells have been antidromically identified. The latencies ranged from 3 msec. to 20 msec with a mean latency of 8.4 msec, giving a conduction velocity of 0.36 m/sec. Of the total number of cells only 12.5% were spontaneously active and these displayed comparatively slow firing rates. The number of spikes per second ranged from 1 spike/10 sec. to 15 spikes/sec., the average being 1/3 secs.

Discussion

The experiments demonstrate that cells in the POA have axons which terminate in the medial basal hypothalamus. The technique of antidromic activation has been previously used to identify neural

pathways which had already been described by other techniques. The neurosecretory cells in the paraventricular (Sundsten et. al., 1970) and supraoptic (Dreiffus and Kelly, 1972) nuclei and the tubero-infundibular neurons of the median eminence (Markara et. al., 1972) were antidromically identified after their identification by histological techniques. However, by this technique, Dyer and Cross (1972) have established the existence of a direct connection between the POA and the basal hypothalamus, a pathway previously unknown.

The average conduction rate of this pathway was calculated in the present experiments, to be 0.36 m/sec. which is similar to the 0.32 m/sec. observed by Dyer (1973) and compares with the slow conduction velocities of the axons of the magnocellular neurosecretory cells (Dyball and Koizumi, 1969) and the tubero-infundibular fibres (Markara et. al., 1972).

A distinguishing feature of these preoptic neurons was the frequent lack of spontaneous activity in them (over 50%); those that were active showed very slow firing rates. It is therefore likely that previous recordings from unidentified cells in the POA which were spontaneously active and showed relatively higher firing rates (Cross and Silver, 1966) were not made from these cells making direct connection with the basal hypothalamus.

The neural inputs from the POA to the basal hypothalamus are essential for the preovulatory surge of LH (Halasz, 1969). Crighton et. al. (1970) have shown that neurons in the POA contain LRH and it is thus possible that some of these preoptic neurons which make

direct contact with the basal hypothalamus are neurosecretory cells. They may also synapse on the LRH secreting cells in the basal hypothalamus either directly or via an interneuron. Further evidence is required to determine the exact anatomical connections within the hypothalamus.

CHAPTER VI: IONTOPHORETIC STUDIES ON ANTIDROMICALLY IDENTIFIED PREOPTIC NEURONS.

IONTOPHORETIC STUDIES ON IDENTIFIED PREOPTIC NEURONS

Introduction

Histofluorescent and histochemical studies have demonstrated the existence of both adrenergic (Hökfelt and Fuxe, 1972) and cholinergic (Schute and Lewis, 1966) terminals within the POA of the rat brain. Thus it is not surprising that there is evidence that the stimulatory action of the POA on the secretion of gonadotrophins may involve both aminergic and cholinergic synapses.

Catecholamines. Experimental evidence indicates that the preovulatory surge of LH is mediated via nor-adrenergic pathways. Typically drugs which reduce nor-adrenergic neural activity suppress the release of LH and FSH (Coppola, 1971). For example, treatment with reserpine or α-methyl-p-tyrosine prior to the rise of LH secretion on the afternoon of proestrus blocks ovulation in the female rat (Myerson and Sawyer, 1968, Coppola et. al., 1966). A similar effect can be observed with adrenergic blocking agents (Zarrow and Brown-Grant, 1964). In addition recent evidence implicates a role of catecholamines in the post-castration rise in plasma gonadotrophins (Ojeda and McCann, 1973). Fuxe et. al., (1970) have demonstrated an increased turnover of NE in the hypothalamic area during proestrus, indicative of an increased activity of nor-adrenergic terminals during this period.

Kalra and McCann (1973) have suggested that nor-adrenergic synapses exist between the POA and the LRH neurosecretory cells and

that these synapses are involved in the stimulatory action of estrogen on LH release. They showed that the rise of LH following stimulation of the POA may be inhibited by blocking the synthesis of DA or NE and this effect could be reversed by selectively restoring brain NE levels using DOPS. From such results one could conclude that the preoptic neurons which trigger ovulation are nor-adrenergic. However, histo-fluorescent studies (Hökfelt and Fuxe, 1972) do not demonstrate the presence of adrenergic cell bodies in the POA but only an extensive innervation of nor-adrenergic terminals from the medial forebrain bundle.

It is now well established that the intrahypothalamic dopaminergic pathway from the tuberal region to the median eminence inhibits the release of LRH (Hokfelt and Fuxe, 1972). However, the possibility of other DA containing fibres terminating within the preoptic-hypothalamic area has not been excluded. In vitro experiments of Schneider and McCann (1969) indicate that the addition of DA to the medium enhances the release of LH when pituitary glands are incubated with median eminence tissue and an intraventricular infusion of DA will trigger ovulation in prooestrus pentobarbital-blocked rats although this catecholamine was only effective in high doses (Rubenstein and Sawyer, 1970).

Finally epinephrine has also been implicated in the control of gonadotrophin secretion. Rubenstein and Sawyer (1970) showed that epinephrine was the most effective catecholamine in triggering ovulation when infused into the third ventricle of prooestrus

pentobarbital-blocked rats, and the possibility of an intrahypothalamic adrenergic pathway is suggested by the immunohistochemical studies of Hökfelt et, al. (1972) which demonstrated the presence of the enzyme which converts norepinephrine to epinephrine in the rat hypothalamus.

Acetylcholine. Although little research has been directed toward determining a role of ACh in the control of gonadotrophin secretion it appears that hypothalamic regulation may be related to the activity of afferent cholinergic fibres converging from other areas of the central nervous system. Cholinergic blocking drugs, such as atropine, block ovulation when administered to female rats prior to the surge of LH (Markee et. al., 1952) and will prevent the post-castration rise of LH and FSH (Libertum and McCann, 1973). The inhibition of ovulation by atropine appears to be mediated at the level of the central nervous system for the effect can be abolished by administering LRH during the afternoon of prooestrus (Libertum and McCann, 1973).

Libertum et. al., (1973) have recently observed cyclical changes during the oestrous cycle of rats, in the activity of the enzymes which synthesise and hydrolyse ACh. The activity of acetylcholine esterase and choline acetylase is high during dioestrus and low during oestrus and this change in the level of these enzymes during the oestrous cycle may only be observed in the preoptic-suprachiasmatic area of the brain. These findings are consistent with a possible cholinergic input involved in the control ofgonadotrophin secretion. In light of this kind of evidence for the involvement of

aminergic and cholinergic neurons in the control of gonadotrophin secretion, the sensitivity of antidromically identified cells to iontophoretically applied DA, NE and ACh has been tested.

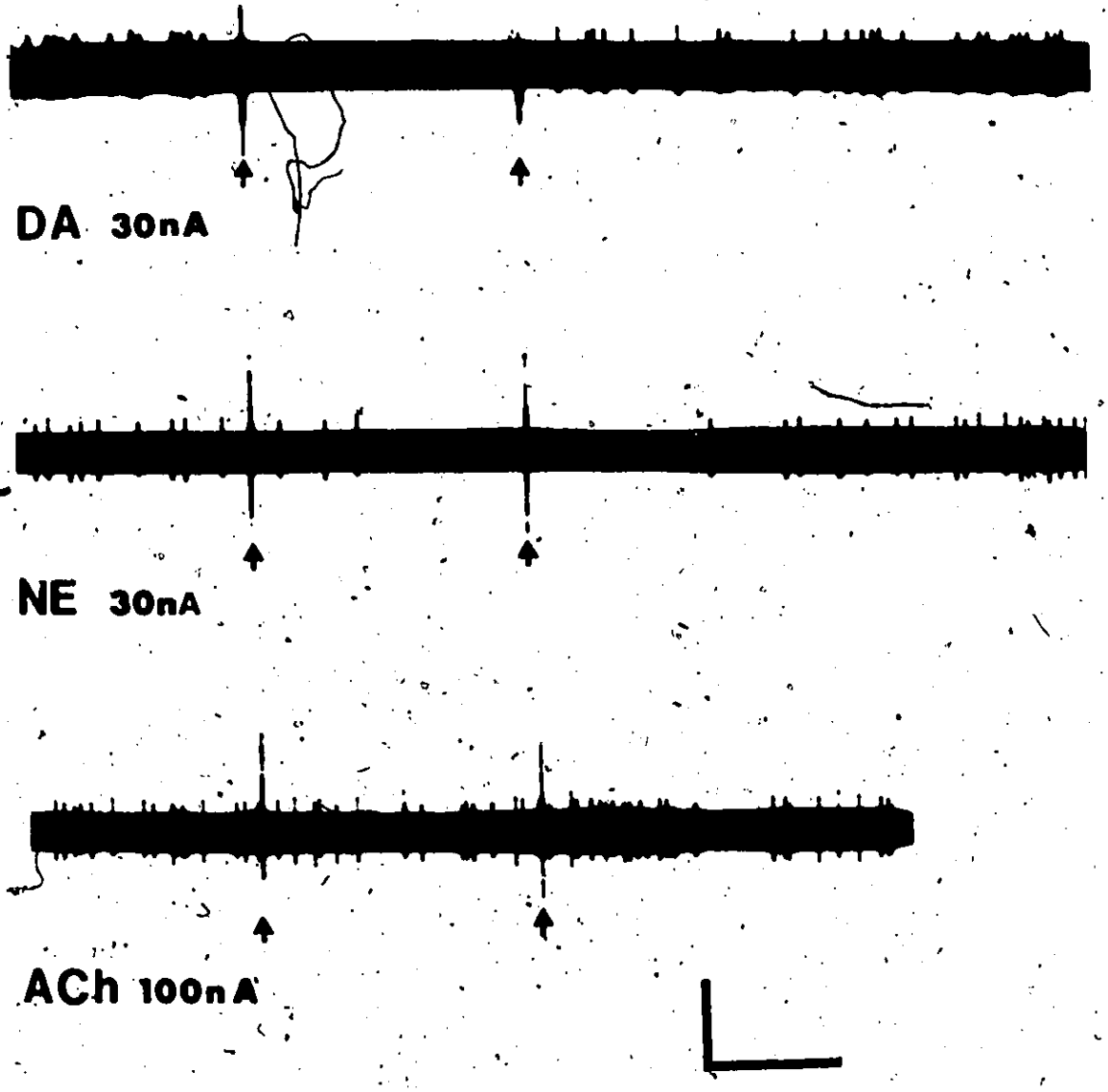
Methods

Ovariectomized and ovariectomized estrogen primed rats were used for these observations. The preparation of the three barrelled micropipettes and the iontophoretic and recording techniques employed have been fully described in Chapter II, Materials and Methods.

In many units the response to DA or NE was tested against activity of the cell which was induced by the iontophoretic application of glutamate. For some recordings free diffusion of glutamate from the tip of the micropipette induced sufficient activity to allow the testing of drug responses; in other cases ejection currents of between 6–20 nA were required.

Cationic currents were used to expell the positively charged putative neurotransmitters from the microelectrodes and generally current intensities ranged from 6 - 40 nA. In all experiments constant current pulses, triggered by the pulse generator, were delivered from the iontophoretic power supply for 10 seconds. The correct placement of the stimulating and recording electrode were subsequently confirmed by histological examination (see Chapter II, Histological Verification).

Figure 8. Oscilloscope traces of the action of iontophoretically applied dopamine (DA), norepinephrine (NE) and acetylcholine (ACh) on antidromically identified preoptic neurons. The upper two traces show typical inhibitory effects of DA and NE, the lowest trace shows the unresponsiveness of preoptic neurons to ACh. Arrows indicate the onset and termination of the ejection current pulses and the intensity of the current pulses is indicated in nanoamperes (nA). (calibration marks; 5 sec, 300 μV positive upwards.)


DA 30nA
NE 30nA
ACh 100nA

Results

The basic results of these iontophoretic studies are shown in Figure 8. The activity of antidromically identified cells in the POA was inhibited by the iontophoretic application of DA and NE but was unaffected by ACh applied iontophoretically.

In any one single identified unit, it was only possible to test the effects of one of these three drugs applied iontophoretically as the second drug containing barrel of the triple barrelled microelectrodes was filled with glutamate, the third being the recording barrel. Although there is no direct evidence that glutamate acts as a neurotransmitter in the mammalian central nervous system, it has been shown to exert apparently non-specific excitatory actions on most, or indeed all, neurons when applied iontophoretically. In view of the fact that many of the antidromically identified neurons were not spontaneously active or showed only very low levels of spontaneous activity, (< 2 spikes/sec) the inhibitory responses to the iontophoretic application of the catecholamines could only be observed against a background of glutamate induced activity. For this reason it was important to fill the second drug containing barrel with this amino acid.

The characteristics of the responses of identified neurons to the electrophoretic application of these drugs and the time course of the drug action will be discussed separately.

Glutamate

Glutamate increased the unit discharge rate of all spontaneously active cells and induced activity in all silent preoptic units tested.

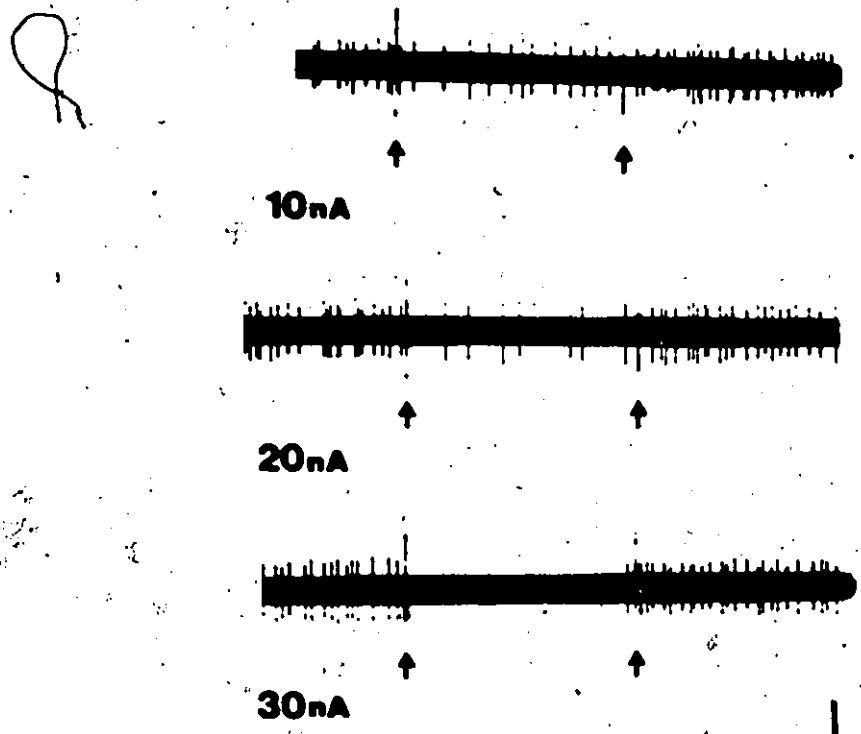
When a dose of glutamate was iontophoretically applied, sufficient to induce a maximum firing rate greater than 3 spikes/sec, the latency of the first spike was between 1 and 2 seconds after the ejection current pulse had been switched on. The maximum firing rate induced by glutamate was attained between 4 - 6 seconds after the onset of the current pulse and the termination of the drug action was usually coincident with the termination of the current pulse. This time course of the drug action is a function of the concentration of glutamate ejected from the pipette. Thus at higher current intensities the concentration of glutamate required to excite the cell membrane will be achieved in a shorter period of time.

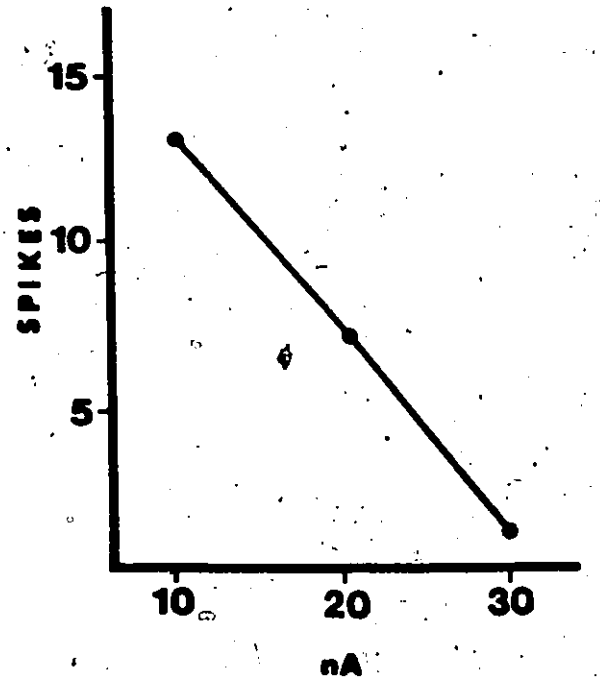
Dopamine

The response to DA has been tested on 39 identified preoptic neurons, 27 of which were inhibited and the rest were unaffected. Typically the cells responded to DA with a short latency depression in firing rate, the mean latency following the onset of the current pulse being 0.5 seconds. The inhibitory action of DA did not outlast the termination of the current pulse by longer than 2 seconds.

Unlike the time course of the glutamate action on these preoptic neurons, the degree in inhibition of DA was constant throughout the period of the drug action (see Fig 9). As described above, in many instances the response of the preoptic neurons to iontophoretically applied DA had to be tested against glutamate induced activity. A constant amount of glutamate was allowed to pass out of the tip of the

Figure 9. Oscilloscope traces of graded inhibitory effects to increasing quantities of dopamine applied iontophoretically. Arrows indicate the onset and termination of the current pulse, the intensity of which is given in nanoamperes (nA). Note that the degree of inhibition is constant throughout the period of the drug action. (calibration marks, 5 sec, 300μV, positive upwards). Below, a graph showing the total number of spikes which occurred during the period of inhibition plotted against the intensity of the current pulse (nA).


10nA
20nA
30nA


15
10
5
SPIKES
10
20
30
nA

electrode, either by free diffusion or by a sustained drive current, so that the rate of unit discharge (2 - 8 spikes/sec) evoked in the cells was comparable to the firing frequencies observed in other spontaneously active identified preoptic neurons. The responses of glutamate activated cells to the iontophoretic application of DA were qualitatively and quantitatively identical to the responses observed when DA was applied to spontaneously active neurons.

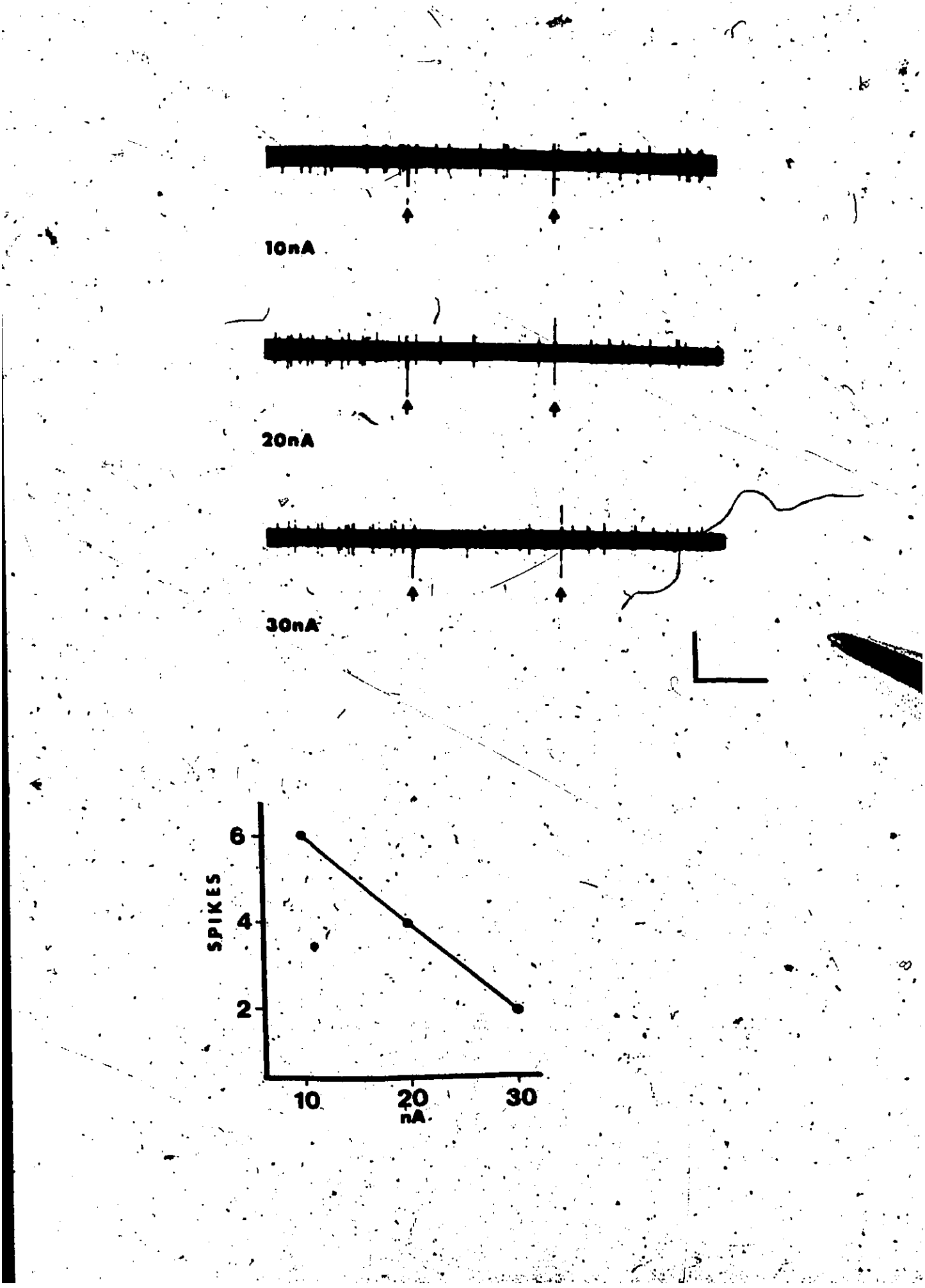
Two cells responded to the iontophoretic application of DA with an entirely different time course. The latency in depression of firing rate following the onset of the current pulse was between 4 - 7 seconds and drug effects outlasted the termination of the iontophoretic current pulse by an average of 30 seconds.

The degree of inhibition was dependent upon the quantity of DA ejected and therefore graded inhibitory responses were obtained by increasing the intensity of the ejection current (see Fig. 9).

Norepinephrine

Iontophoretic application of NE inhibited the unit activity in 29 of the 30 antidromically identified cells tested with this drug. There was a greater variation in the latency of the inhibitory effect of preoptic neurons following the onset of the current pulse compared with the DA effects; the latencies ranged from 0.5 to 4.0 seconds. There was also a large variation in the length of time the drug effects outlasted the termination of the current pulse, and in some cases the inhibition of the activity was observed up to 8 seconds after the ejection current pulse.

Figure 10. Oscilloscope traces of graded inhibitory effects to increasing quantities of norepinephrine applied iontophoretically. Arrows indicate the onset and termination of the current pulse, the intensity of which is given in nanoamperes (nA); (calibration marks, 5 sec, 300 μV positive upwards). Below a graph showing that the degree of inhibition is proportional to the quantity of norepinephrine ejected from the micropipette; the number of spikes which occurred during the current pulse is plotted against the intensity of the pulse (nA).


10nA
20nA
30nA
SPIKES
6
4
2
10
20
30
nA

As with the DA responses no apparent difference could be observed between the inhibitory responses to iontophoretically applied NE in the preoptic neurons that were either spontaneously active and those activated by glutamate. The degree of inhibition was found to be roughly proportional to the amount of NE ejected from the tip of the electrode which was dependent upon the intensity of the driving current (Fig. 10). These currents usually ranged from 10 - 40 nA.

Acetylcholine

The response to ACh was tested in 15 antidromically identified cells in the POA. All neurons were insensitive to this drug even when iontophoretic currents in excess of 100 nA were applied (Fig. 8). In order to check that the drug was infact being ejected through the micropipette, at the end of each experiment the electrode was stereotaxically positioned in the zona incerta where long latency excitatory responses to ACh applied iontophoretically were observed from a small percentage of the spontaneously active cells. This confirmed that the insensitivity of identified preoptic neurons to ACh probably was not a result of inadequacy of the iontophoretic technique for this substance. Indeed, since it required positive current pulses (as did NE and DA) there are no special problems in its ejection as are sometimes encountered with substances requiring pulses of the opposite polarity.

Current Effects and pH Effects

After testing the sensitivity of each identified neuron to glutamate and one of the three putative neurotransmitters, careful

controls were made to eliminate the possibility that the response was a result of current artifacts (see Chapter II, Iontophoretic Techniques). Therefore a current pulse of the same polarity and of the same intensity which was used to test the response of a neuron to one of the drugs applied electrophoretically was passed through the sodium chloride filled recording barrel. Any cell which appeared to 'respond' to this current pulse was ignored. Finally it was of interest to determine whether the acidity of these three drug solutions (around pH 4.0) was affecting the response of the preoptic neurons. In several experiments the recording barrel was filled with acidified sodium chloride (pH 3.5-4.0) and when positive ejection currents, with intensities greater than 20 nA were applied to this barrel an increased rate of firing of the unit understudy was commonly observed. This control eliminated the possibility that the inhibitory responses to DA and NE were secondary to the possible local pH changes produced by the drug solutions.

Discussion

The results show clearly that the activity of antidromically identified neurons in the POA is inhibited by iontophoretically applied DA and NE but is unaffected by similar applications of ACh. Of the total number of cells tested with DA, 69% were inhibited as were 96% of those tested with NE. That a higher proportion of cells were affected by NE is not surprising in view of the histochemical studies which demonstrate that the POA is extensively innervated by ascending nor-adrenergic terminals, the cell bodies of which orig-

inate in the brain stem (Ungerstedt, 1971). However, although the above results, considered together with the histochemical observations, are suggestive that preoptic neurons which make a direct connection with the basal hypothalamus are innervated by nor-adrenergic and possibly dopaminergic synapses, they do not constitute proof that catecholamines are in fact transmitters at synapses on these neurons.

To prove that a chemical substance acts as a neurotransmitter required the demonstration of its presence in the relevant axon terminals, its release by nerve stimulation and that its actions and those of naturally released transmitter are identical both qualitatively and quantitatively, and have similar pharmacological characteristics. The technique of microelectrophoresis to some extent simplifies the analysis of the potential sites of drug action, since only those sites near the electrode tip (e.g. within about 100μ at the most, in all probability) need to be considered. (Curtis et. al., 1960, Diamond, 1968).

However, it is difficult to establish whether the drug is acting on the membrane of the preoptic cell understudy or on the endings which synapse with it deriving from distant cell bodies, or even on adjacent neurons with short axons ending on the recorded unit. In order to distinguish among these possibilities one would need to be able, for example, to stimulate at least one neural pathway making monosynaptic connections with the identified preoptic neuron, in order both to compare the effects of the drug with those of nerve stimulation, and to test the effects of agonists and antagonists on the synaptic and drug responses. Such conditions were not possible to realise in

these studies and thus the exact site of action of the iontophoretically applied monoamines cannot be stated. Nevertheless, since the pipette tips must have been close to the recorded unit (in order to be able to detect the electrical response) it seems probable that the unit itself (soma or dendrites) was affected and/or the nerve endings synapsing on it.

The other possible sites of action of the drugs are the glia cells and blood capillaries. However, in the present experiments, it is unlikely that the inhibitory responses to iontophoretically applied catecholamines was caused by a non-neuronal site of action of the drugs. In a few instances, the activity of two neurons with different spike amplitudes, was recorded simultaneously and only the antidromically identified unit was inhibited by DA or NE while the activity of the second unit was unaffected. If the drug's action were secondary to local non-neuronal effects the activity of both cells would have been expected to change similarly (Salmoiraghi and Stefanis, 1967).

The differences observed in the magnitude and time course of the drug action of either DA or NE may be explained by varying the distances between the micropipette tips and the neurons and the differences in size and shape of individual neurons; all these will affect the amount of the drug reaching receptor sites on the neuronal membrane. In addition possible variations in the location and concentrations of receptive sites over the neuron surface will also affect the speed and magnitude of the cell response.

The basic differences observed in the time course of typical responses to DA compared with those to NE must reflect differences in the local mechanisms available for the reuptake or for the enzymatic breakdown of the drug and/or differences in drug receptor activation time (Diamond and Roper, 1973).

In view of the evidence outlined in the Introduction to this chapter, that catecholamines play an important role in stimulating the ovulatory surge of LH and that neurons in the POA are responsible for triggering this increased secretion of LH, it was interesting to observe that the iontophoretic application of both catecholamines decreased the activity of neurons in the POA which connect with the basal hypothalamus. Possible interpretations of these results are discussed in the General Discussion.

CHAPTER VII: EFFECTS OF ESTROGEN ON THE RESPONSE OF ANTIDROMICALLY IDENTIFIED PREOPTIC NEURONS TO DOPAMINE AND NOREPINEPHRINE APPLIED IONTOPHORETICALLY.

THE EFFECTS OF ESTROGEN ON THE RESPONSE OF ANTIDROMICALLY IDENTIFIED PREOPTIC NEURONS TO DOPAMINE AND NOREPINEPHRINE APPLIED IONTOPHORETICALLY.

Introduction

One of the most challenging questions in neuroendocrinology is how steroid hormones may act as neuromodulators, this action being ultimately expressed as an alteration of impulse activity in specific neurons involved in a particular neuroendocrine circuit. When radioisotopic steroids are injected systemically it has been found that certain 'target' neurons in the hypothalamic and limbic areas of the brain retain and concentrate the steroid for several hours after the plasma concentration of the steroid hormone has declined to a low level (McEwen and Pfaff, 1971). The existence of such estrogen concentrating neurons, outside of the area of the hypothalamus which contains the neurons directly implicated in the synthesis of releasing hormones, requires one to consider other modes of estrogenic control of gonadotrophin secretion apart from a direct action on the releasing factor neurons themselves. It seems likely that the preoptic neurons which trigger the preovulatory surge of LH are the same cells which concentrate estrogen (Pfaff, 1968) and it is feasible, therefore, that the hormone may alter the sensitivity or the response of these neurons to afferent inputs.

Sensory information from the external environment, such as that relating to light or sexual and olfactory stimuli play an impor-

tant role in inducing or inhibiting ovulation (Harris and Cambell, 1966). These are reflex effects and there seems little doubt that they involve preoptic neural mechanisms responsible for triggering LH secretion. The kind of sensory information associated with such reflex effects is likely not to vary greatly throughout the oestrous cycle. If such afferent neural signals to the POA are unchanged throughout the oestrous cycle, why is the activity of neurons in this area, which trigger the ovulatory surge of LH, increased during proestrus following the rise of circulating levels of estrogen? As indicated, in Chapter VIII, during the peak concentration of circulating estrogen the neuronal activity in this area is likely to be diminished. However, it is well established that at the time when estrogen levels are declining, the activity, of what may be the same neurons is increased.

It is not inconceivable that estrogen plays a part in both situations, the inhibitory one at the peak concentrations masking an excitatory one revealed later. If this were so, one or other of the estrogen actions would need to be a presynaptic one (indeed, both could be so) - but it is most improbable that both actions would be exerted directly on the POA neurons themselves. Of special interest in this context are the aminergic inputs to the POA (Ungerstedt, 1971) which may play an important role in the control of gonadotrophin secretion (Coppola, 1971). The results presented in this thesis show that the cells in the POA that make a direct connection with the basal hypothalamus are sensitive to both DA and NE. It seemed therefore worth

studying the effect of the ovarian hormones on the response of these cells to DA and NE applied iontophoretically in an endeavour to show whether or not this could be responsible for the change in activity of the preoptic neurons observed during proœstrus.

In order to eliminate possible variations in the level of endogenous estrogen between different experimental animals, ovariectomized rats have been used. Numerous studies have demonstrated that certain physiological manifestations of hormone administration to ovariectomized rats e.g. the lordosis response, can only be observed if the animal is primed with estrogen 2–3 days prior to a second injection of steroid hormones (Arai and Gorski, 1968, Komisaruk and Diakow, 1973). Similarly, estrogen or progesterone will only stimulate an increased secretion of LH in primed ovariectomized rats (Caligaris et. al., 1971, a,b). For these reasons the responsiveness of preoptic neurons has been tested in two groups of animals;

a) following a single injection of estrogen to unprimed ovariectomized rats.

b) following a single injection of either estrogen or progesterone to estrogen primed ovariectomized rats.

Methods

A full description of the operative procedures, recording and iontophoretic techniques is given in Chapter II, Materials and Methods. Female ovariectomized rats were primed with a s.c. injection of 20 µg EB three days prior to experimentation. Recordings were

made with three barrelled microelectrodes, a full description of which is given in Chapter II, Electrodes.

Following antidromic identification of a preoptic neuron, dose response curves for both glutamate and either DA or NE were obtained. A minimum of three points for each dose response curve was obtained by recording the response of a neuron to different doses of the drug, achieved by changing the intensity of the iontophoretic current. The length of the current pulse (10 sec) remained constant. In many cases the variation of the response was tested by making two or more recordings of the drug response at a fixed iontophoretic dose level.

After the initial recording 1 or 2 μg EB or 100 μg progesterone (see Chapter II, Hormone Administration) was infused i.v. over a period of 1 minute. Thirty minutes after the injection a second dose response curve was obtained for both glutamate and the catecholamine. Further recordings were made at subsequent thirty minute intervals depending on how long recordings coudl be made from the same cell.

Many experiments failed because the particular neuron under investigation was 'lost' prior to obtaining the second dose response curve but after the administration of the hormone. Once the rat had been injected it was obviously not possible to test the effect of the hormone on the responsiveness of any other identified cell in the same preparation. Each cell successfully investigated was also the only one which could be investigated in that animal.

The location of the stimulating electrode and the site of the

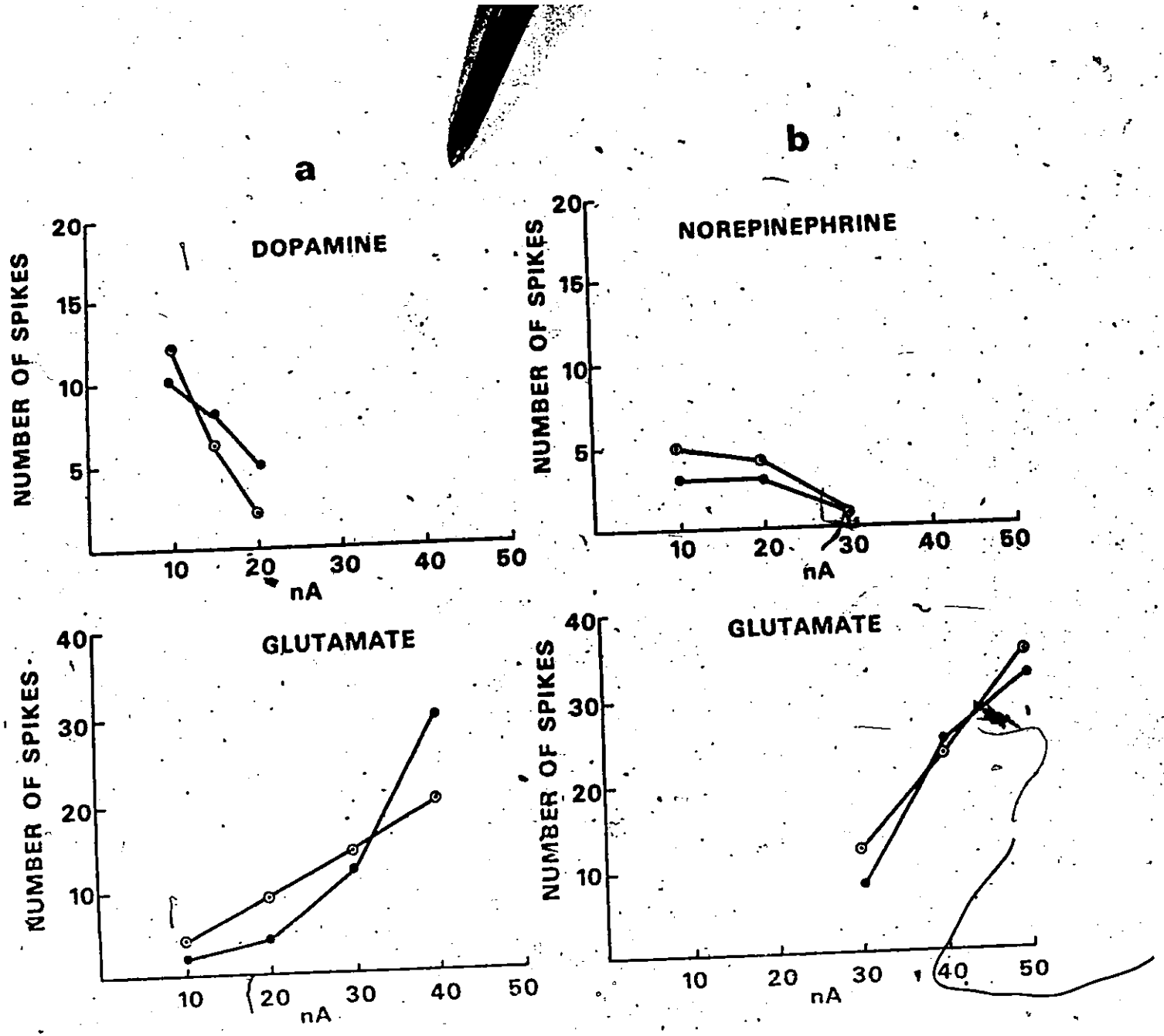
antidromically activated cell was confirmed by histological examination (see Chapter II, Histological Verification).

Results

Dose-response relations were obtained by measuring the total number of spikes which occurred during the period of iontophoretic application of the drug, and plotting these against the ejection current (Fig. 11, also Figs. 9 and 10). The glutamate dose response curves were used as a standard; if any shift in the dose response curve of the catecholamine after the steroid injection was paralleled by a similar shift of the glutamate curve, it would indicate that the effect was non-specific and merely due, for example, to a change in the spatial relationship between the neuron and the micropipette tip. Because the whole dose response relation was used rather than individual responses to a given dose of the drug (see below), it did not affect the results that glutamate caused responses which were excitatory i.e. of the opposite direction to those of the catecholamines. It was essential to employ glutamate 'driving' as a large proportion of cells were not spontaneously active and thus inhibitory effects could only be tested against cells which were firing impulses (see Chapter VI).

The effects of an i.v. injection of estrogen in unprimed ovariectomized rats, on the sensitivity of neurons to iontophoretically applied monoamines has been tested on eight antidromically identified units. Similar experiments were made from five estrogen primed rats

Figure 11. Dose response curves of identified preoptic neurons obtained before and 30 minutes after an i.v. injection of estradiol benzoate. (a) the dose response curves of the iontophoretic application of both dopamine and glutamate recorded from one identified preoptic neuron and (b) the response of another cell to the iontophoretic application of norepinephrine and glutamate. Open circles represent the dose response curves obtained before the estrogen injection, the closed circles, the curves obtained 30 minutes after estrogen.


a
b
DOPAMINE
NOREPINEPHRINE
GLUTAMATE
GLUTAMATE
NUMBER OF SPIKES
nA
20
15
10
5
30
40
50

i.v. injected with either estrogen (three animals) or progesterone (two animals).

The dose response curvesto glutamate and DA or NE obtained in each experiment were plotted as shown in Fig. 11. Because of the wide variation in the rate of discharge of spontaneously active or glutamate driven units it was decided that the drug sensitivity could be best assessed by establishing the slope of the dose response curves of iontophoretically applied catecholamines, on the assumption that the range of doses was well below "saturation" and above threshold. The results of all such experiments were entirely consistent.

No significant difference ($p > 0.2$) in the best fitting slopes of the dose response curves obtained before, and thirty minutes after the steroid injection were observed in the following groups of animals:

a) unprimed rats tested with DA and glutamate

b) unprimed rats tested with NE and glutamate

c) primed rats injected with estrogen and tested with NE and glutamate

d) primed rats injected with progesterone and tested with NE and glutamate.

In most cases the dose response curves remained remarkably constant throughout the period of recording (see Fig. 11). In four cases shifts in the dose response curve of one or other of the catecholamines was observed which were paralleled by similar shifts in the glutamate dose response curve for the same cell. This can be explained by changes in neuron/pipette distance as discussed in Chapter VI. It was interesting to note, however, that in these four experiments in

which there was an apparent change in the responsiveness to both the catecholamine and glutamate that the direction of the changes differed. Glutamate is thought to excite neurons largely by depolarising the membrane of the soma itself. If the receptors for the catecholamines were situated out on the dendrites, then a movement of the electrode tip away from the soma (as was indicated by the fact that the amplitude of the units was decreased at the end of the recording period) would put the catecholamine receptors nearer and the glutamate receptors further from the tip of the micropipette thus accounting for the observed changes of apparent sensitivity in these particular experiments.

It is important to note that the results of all of these individual experiments, whilst of interest with respect to explanations involving the location of the pipette tip were not significant with regard to possible effects of steroids; this is indicated by the analysis of all the results described above and referred to again below.

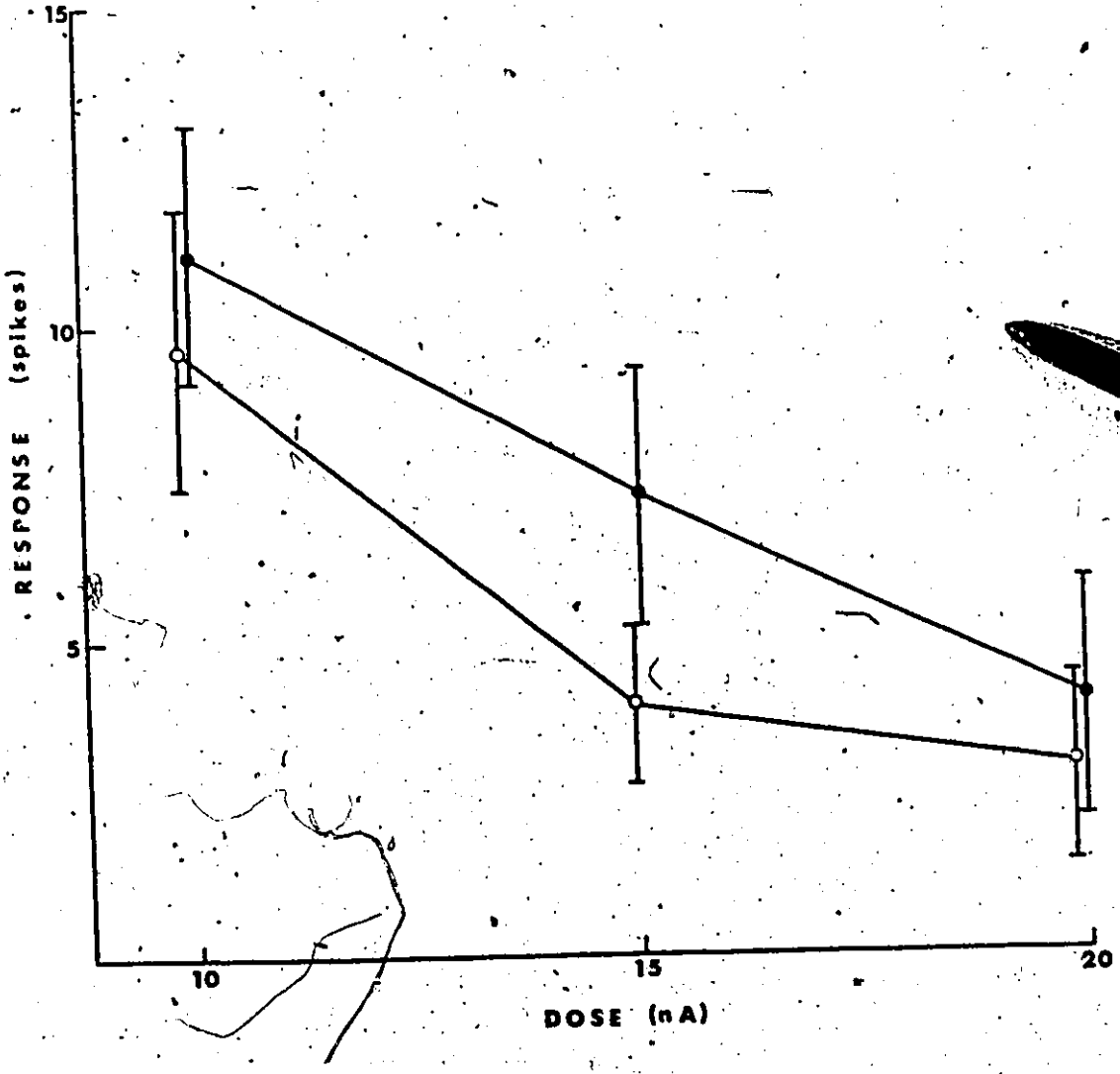
Following the analysis of the slopes of the dose response curves, the assistance was sought of the statisticians in the Department of Clinical Epidemiology and Biostatistics so that the dose response curves could be further analysed and in particular to see whether or not steroids caused significant "shifts" in the mean responses as opposed to changes in their slopes. A statistical analysis was made on the pooled results of the 5 dose response curves to iontophoretically applied DA obtained before and 30 minutes after an i.v. injection of EB in unprimed ovariectomized rats. Table IV shows the layout of the data for the five cells

TABLE IV

STATISTICAL ANALYSIS OF DOPAMINE RESPONSES

	DOSE LEVELS	DOPAMINE RESPONSES					NO. OF OBSERVATIONS	MEAN	STANDARD DEVIATION
Before	10 nA	13	12,5	4	5	13,15	7	9.6	4.7
Estrogen	15 nA	3	6	4	2	5	5	4.0	1.6
Injection	20 nA	2,6	2	5,4	0,1	4	8	3.0	2.1
30 Minutes	10 nA	17,16	10	8	6	11,10	7	11.1	4.0
After Estrogen	15 nA	12	8	6	0	9,9	6	7.3	4.1
Injection	20 nA	11	6,5	3,4	0,0	—	7	4.1	3.8

Figure 12. Graph showing the mean responses of five identified preoptic neurons at different dose levels of dopamine applied iontophoretically. The curve with the open circles is the dose response curve recorded before an i.v. injection of estradiol benzoate and the closed circles represent the dose response curve recorded 30 minutes after the estrogen injection. Vertical bars represent the standard error of the mean response.


15
10
5
RESPONSE (spikes)
10
15
20
DOSE (nA)

considered and some descriptive statistics of the dose levels. Only those frequently used dose levels (i.e. 10nA, 15nA and 20nA) have been included in the analysis because of the large number of instances in which higher or lower doses were not tested. The mean responses of these 5 cells to the dose levels of DA applied are shown in Figure 12. The results were fitted to a statistical model and an analysis of variance showed that the estrogen effect on the cell responses at 30 minutes after the injection was not significant at the 0.05% level. Two other points emerged from the statistical analysis; a) that there are significant differences ($p<0.01$) in the responses between individual neurons for a specific dose of DA and b) that there is a significant difference ($p<0.01$) in the response of a neuron to different doses of iontophoretically applied DA.

In view of the fact that this type of statistical analysis of an almost complete set of results showed that there was no significant effect of the estrogen on the response of neurons to DA applied iontophoretically, it was felt it was of no value to analyse further the other incomplete sets of data, in which, due to technical limitations only a few observations had been made for either DA or NE under specific hormone regimes.

Discussion

A quantitative approach to the iontophoretic studies has been adopted in order to establish whether steroid hormones may modulate neural activity by altering the sensitivity of the cell membrane to transmitter substances. One of the major problems of the experiment was the requirement to hold one particular cell up to thirty minutes after the hormone injection. This proved to be extremely difficult

and, even in successful experiments there was no way of determining to what extent the distance between the electrode tip and the cell had changed during the recording period. In view of the steep concentration gradients which exist when a drug, released from a point source, diffuses through the extracellular space (del Castillo and Katz, 1955) a change of a few microns in this distance from the pipette tip to the cell would markedly alter the effective concentration of a specific dose of the drug at the sensitive membrane. This factor could be sufficient to mask any action of the steroid hormone on the apparent sensitivity of the cell membrane.

Another consideration is whether this technique is sensitive enough to detect small changes in the excitability of the cell membrane. At any one fixed dose (ejection current) the response of a neuron to two or more applications of DA, NE or glutamate is not constant and there are large standard deviations of the responses at each dose level (see Table IV). Any predicted change in the sensitivity of the cell membrane may lie within the limits of the response variation to iontophoretically applied catecholamines and therefore the possible membrane effects of steroid hormones may not be detected by this experimental technique.

The ideal solution toward defining steroid action on the membrane properties of the neuron would be to apply the steroid itself onto the cell iontophoretically and to observe any direct effect on transmembrane potential and spontaneous activity and on the response to iontophoretically applied catecholamines. In preliminary experiments

we were unsuccessful in attempts to apply various estrogenic compounds iontophoretically (see Materials and Methods, Attempts to Apply Estrogens Iontophoretically); consequently the present experimental design was adopted.

Aside from the technical limitations of quantifying this type of iontophoretic study, one has also to consider other experimental factors such as the anaesthesia and the ineffectiveness of an i.v. injection of a steroid hormone in stimulating LH secretion in these primed ovariectomized rats (see Chapters III and IV) which may also explain the present somewhat negative results.

There is substantial evidence that the positive feedback action of estrogen results in a modulation of neural activity at the level of the LRH containing neurosecretory cells. However, this effect is only observed several hours following the rise of circulating estrogen levels in both the intact and estrogen primed ovariectomized rats (see General Discussion). Therefore, the effects of estrogen on the neural circuits which control gonadotrophin secretion may not be detected within the period of recording.

However, although the negative results of the present experiments may by due to such technical limitations, the fact that estrogens did not affect the response of neurons to iontophoretically applied catecholamines contrasts with the observed estrogen effects on the rate of spontaneous activity of certain identified preoptic units as described below in Chapter VIII. For this reason, the writer inclines to the conclusion that the results are of significance (see General Discussion).

Steroids appear to affect neither the sensitivity of the preoptic neurons to the iontophoretic application of catecholamines, nor, (since there was no significant shift in the position of the 'mean' dose response curve) was there any evidence of 'competitive inhibition' of aminergic effects by estrogen at the time when the sensitivity was measured. The results may be of interest in attempting to define the exact site of action of steroid hormones on the central nervous system. This is discussed in the General Discussion

CHAPTER VIII: EFFECTS OF AN INTRAVENOUS INJECTION OF ESTROGEN ON THE SPONTANEOUS ACTIVITY OF ANTIDROMICALLY IDENTIFIED NEURONS IN THE PREOPTIC AREA.

EFFECTS OF AN INTRAVENOUS INJECTION OF ESTROGEN ON THE SPONTANEOUS ACTIVITY OF ANTIDROMICALLY IDENTIFIED NEURONS IN THE PREOPTIC AREA

Introduction

It is now generally accepted that neurosecretion is the result of the discharge of impulses in the neurosecretory cell (Wakerley and Lincoln, 1973). Thus the feedback effects of estrogen on the neural circuits which control the release of the gonadotrophin releasing hormones into the capillaries of the hypophysial portal plexus must necessarily involve an alteration of the electrical activity of certain neurons at some point within this neuroendocrine relay.

Attempts have been made to define the action of estrogen on the activity of central neurons by recording the spontaneous rate of discharge of cells in brain regions considered to be sensitive to estrogen feedback mechanisms. Lincoln (1967) demonstrated that high levels of estrogen depressed the activity of neurons in certain limbic hypothalamic areas. Consistent decreases in the number of units firing spontaneously in the hypothalamus, POA and septum were found in the light-induced persistent oestrous and ovariectomized estrogen-treated groups compared with the control spayed group; the decrease was greater in the persistent oestrous animals. In contrast, Kawakami and Kubo (1971) recorded higher levels of multi-unit activity in the anterior hypothalamus, POA and septum in ovariectomized estrogen treated rats

compared to the untreated ovariectomized controls.

More recently, Moss and Law (1971) recorded the spontaneous activity from neurons in the lateral septum, POA and anterior hypothalamus in the 4-day cycling rat. The mean firing-rate was low during dioestrus I and II but high during prooestrus. This finding was confirmed by Dyer et. al. (1972) although they found that the increased activity during prooestrus was restricted to neurons in the ventral portion of the anterior hypothalamus. Recording from chronically implanted electrodes in the 4 and 5-day cycling rat Kawakami et. al. (1970) showed that an increased multi-unit activity in the arcuate nucleus, ventromedial nucleus, medial POA, septum, amygdala and bed nucleus of the stria terminalis occurred for 12-25 minutes during the surge of LH on the afternoon of prooestrus. In addition a gradual increase in multi-unit activity could be recorded from the basal hypothalamus during dioestrus II but no changes were observed in the forebrain limbic areas.

Two reports describe the effects of an i.v. injection of estrogen on the spontaneous unit activity in the POA and hypothalamus. Yagi (1973) found that an i.v. injection of 50 μg 17 β-estradiol to ovariectomized rats resulted in a prolonged decrease in the firing rate of some preoptic and hypothalamic units while other units showed a transitory increase followed by periods of depressed activity. In the conscious freely moving rabbit an i.v. injection of estrone sulphate slowed units in the posterolateral area of the hypothalamus, accelerated those in the ventromedial area and caused only a transient

arousal change in the anterior area (Faure and Vincent, 1971).

These results give no clear picture of estrogen effects on neural activity. Some of the evidence supports the concept that rising levels of estrogen depress neural activity, and that maybe the increased rate of firing observed during prooestrus reflects the activation of the LRH secreting neurons during the preovulatory LH surge and not a direct effect of positive estrogen feedback on the preoptic neurons which trigger the LH surge. However, in all the experiments cited the electrical recordings were made from unidentified neurons, and it is likely that a large proportion of these cells were not involved in the neuroendocrine circuits controlling gonadotrophin secretion. The present study was therfore undertaken to determine whether or not an i.v. injection of estrogen could affect the spontaneous activity of identified cells likely to be involved in triggering the preovulatory LH surge and sensitive to estrogen feedback action.

Methods

A full description of the operative procedures, stimulating and recording electrodes and the recording techniques is given in Chapter II, Material and Methods. The spontaneous activity of antidromically identified cells in the POA was recorded through single barrelled glass micropipettes filled with Pontamine Sky Blue. After a period of 30 minutes control recording, estrogen was infused i.v. over a period of 1 minute. The doses of the hormone, the different

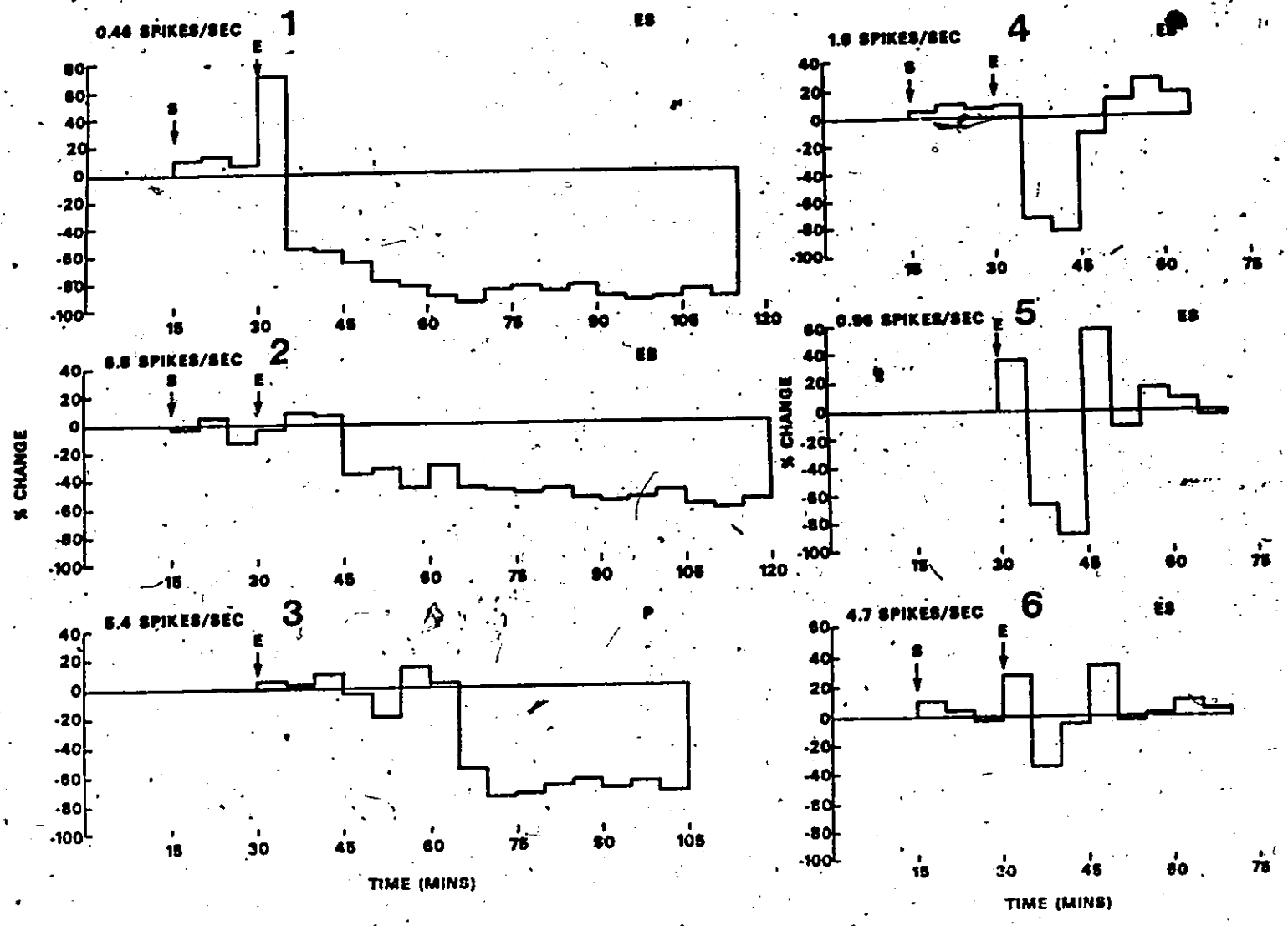
types of estrogenic compounds injected and the types of solvents have been described in Chapter II, Hormone Administration. The period of recording following the estrogen administration was dependent upon how long the cell could be held. In some experiments the solvent alone was injected after the first fifteen minutes of recording to investigate the possibility of solvent effects.

Results

Successful experiments were performed on ten antidromically identified neurons in the POA. The spontaneous rate of firing was averaged over five minute periods and the average frequency of different cells ranged from less than 0.1 spikes to 8.0 spikes/sec. In order to be able to compare the possible effects of estrogen on cells with such a wide range of firing rates it was decided to normalise the results. The first 30 minutes of control recording (15 minutes in those cases in which the solvent alone was injected, Fig. 13, 1,2,4 and 6) was averaged and designated 100%. The frequency averaged over five minute periods following the control recordings was then expressed as a percentage change of firing rate.

A depression in the spontaneous unit discharge was observed in six of the ten antidromically identified neurons following an i.v. injection of estrogen (Fig. 13). Three of these cells (Fig. 13, 1,2,and 3) showed a long lasting inhibition of the spontaneous activity and this depression lasted throughout the period of recording from any one cell (up to 1 hour 25 minutes after the steroid injection). The

Figure 13. Histograms of the percent change in the spontaneous firing rate of antidromically identified preoptic neurons following an i.v. injection of various estrogenic compounds. Zero denotes the average rate of unit discharge calculated during the control period of recording and the absolute values are given above each histogram. Arrows indicate the time at which the solvent (S) and estrogen (E) was administered. Abbreviations of the different types of estrogenic compounds injected; ES – Estrogen Succinate, EB – Estradiol Benzoate, P – Premarin.


0.46 SPIKES/SEC
1
ES
S
E
6.8 SPIKES/SEC
2
5.4 SPIKES/SEC
3
1.9 SPIKES/SEC
4
0.96 SPIKES/SEC
5
4.7 SPIKES/SEC
6
% CHANGE
TIME (MINS)

percent decreases in the spontaneous activity varied between 50 - 90% The latency of this depression after the estrogen administration ranged from 10 - 30 minutes, although in one cell (Fig. 13, 1) there was an initial period of increased activity immediately after the injection followed by the long lasting depression. A short lasting decrease in the rate of unit discharge was observed from three identified preoptic neurons (Fig. 13, 4,5, and 6); the latency of these effects was on average 5 minutes and the depression of spontaneous activity lasted for approximately 10 minutes. In two of these cells (5 and 6) this depression was preceded by an initial increase in the average rate of firing, and following the period of depression there was again a five minute period of increased activity before the spontaneous firing returned to the control level of unit discharge.

Excitatory effects were observed from two spontaneously active neurons immediately after the estrogen administration. The spontaneous activity of the first unit reached a peak frequency (+ 70%) ten minutes after an injection of Premarin, falling to the control rate of unit discharge within 20 minutes of the injection. Similar effects following an injection of estrogen succinate were observed from the second cell although the period of excitation only lasted 10 minutes after the estrogen administration. In neither case did the spontaneous activity fall below the control values. Recordings were also made from one silent neuron which was detected by antidromic stimulation and its presence confirmed by subsequent stimulation of the arcuate nucleus. There was no spontaneous activity during the 15 minute

control period or after injection of propylene glycol. However, immediately after estrogen was injected the cell began to fire spontaneously. The peak frequency of 0.5 spikes/sec was reached during the first five minutes of activity and was followed by a period of slower firing of 0.15 spikes/sec which continued throughout the period of recording (30 minutes after the injection). One identified cell was unaffected by the hormone administration.

No marked changes of firing rate were observed after injection of the solvent alone and no correlation could be made between the different effects of estrogen and the different estrogenic compounds administered (see Fig. 10).

Discussion

The preovulatory surge of LH is dependent upon the estrogen secreted from the ovaries during dioestrus II (Labhsetwar, 1970) and this phasic release of LH will only occur if the neural inputs from the POA to the basal hypothalamus are intact (Halasz, 1969). It is therefore possible that estrogens may modulate the activity of such neurons in such a way as to cause triggering of an increased secretion of LH. The effects of an i.v. injection of estrogen on antidromically identified neurons on the POA was tested on a small number of cells and thus only a few tentative conclusions may be drawn from the results. The majority of cells showed a depression in firing rate in response to the hormone administration, although in some of these there may have been a genuine initial excitation preceding the

depression. In those cells which showed only an increased rate of unit discharge the excitation occurred immediately after the injection of estrogen resembling the initial excitation just mentioned. Although such excitations may have been the results of blood pressure changes or other secondary effects, it is possible that estrogen does have a biphasic action on some cells, with the later depression being the more obvious and possibly more important phenomenon. Yagi (1973), working with unidentified hypothalamic units in ovariectomised rats similarly found excitatory and inhibitory effects of an i.v. injection of estrogen but the latencies of the responses observed were generally longer.

Where is the estrogen acting to cause the apparent inhibition of the preoptic cells? One possibility, of course, is that it is a direct action on the cell itself. This seems unlikely in view of the relatively long latency for the depression to be manifest; most drugs which affect membranes directly have extremely short latencies, often indeed a matter of milliseconds rather than minutes. By the same argument, it seems unlikely that the estrogens are acting by a membrane effect on the nerve endings impinging on the preoptic neurons themselves or the cells of origin of such endings. It seems therefore that the estrogen must be taken up into the cells or its presynaptic elements and by a delayed metabolic action modify the response of the cells or for example the output of transmitter from its endings during normal neural activity.

The finding that high levels of estrogen in general depress the spontaneous activity of identified preoptic neurons is consistent

with the evidence that the mean firing rate of anterior hypothalamic units is low during dioestrus and high in prooestrus (Moss and Law, 1971, Dyer et. al., 1972). It is also consistent with the observations that an i.v. injection of progesterone depresses the response of hypothalamic units to cervical probing, the maximum depression being reached between 15 and 40 minutes after the hormone administration. However, Dyer (1973) has recently reported that similar antidromically identified cells in the POA show no significant changes in the rate of spontaneous activity throughout the oestrous cycle.

The present observations, which suggest that estrogens may modulate the activity of preoptic neurons cannot be accepted as indicating a physiological significance of these results in view of the high plasma estrogen concentrations attained after the i.v. injection (see Chapter IV). The experiments would need to be repeated at lower estrogen levels to allow a proper comparison with Dyer's observations.

It is difficult to evaluate the significance of hormone effects on the spontaneous rate of discharge of neurons. Necessarily many experiments are carried out under general anaesthesia and such anaesthetics are known to block ovulation in the rat (see Chapter III). Therefore, do the observed hormonally related changes in the spontaneous firing rate represent functional significance? Do rising levels of estrogen during dioestrus depress preoptic unit activity and so release from tonic inhibition the neural trigger for ovulation? Or does estrogen act by inducing specific metabolic changes in the target-neurons, the subtle effects of which are not readily detected by electrophysiological

recordings, but are ultimately responsible for the increased activity of the neurosecretory cells during prooestrus? Before one can evaluate the possible effects of steroid hormones on neural activity it may be necessary to identify neuroendocrine circuits more precisely than exists at the present time, and to record from appropriate neurons in the unanaesthetised intact female rat.

CHAPTER IX: GENERAL DISCUSSION

GENERAL DISCUSSION

Electrophysiological investigations have been accepted as a necessary approach toward elucidating the action of steroid hormones on brain function. However, the results from such experiments have not greatly advanced our understanding of the neural mechanisms which control the secretion of gonadotrophins from the anterior pituitary when we compare this system with those for example concerned in sensory processing (e.g. Hubel and Wiesel, 1962) or cerebellar functioning (Eccles, 1966). I feel that the principal reason for this is that too little attention has been paid toward defining exactly the particular systems under study. Too many investigations have involved "blind recording" from unidentified hypothalamic cells in both intact and ovariectomized rats under general anaesthesia, and in only a few experiments (e.g. Gallo et. al., 1971, Kawakami et. al., 1970) have changes in neural activity been correlated unambiguously with physiological events i.e. an increased secretion of LH.

In order to interpret better the kind of results obtained from the study of hormone effects on the activity and sensitivity of preoptic neurons, the present studies were designed to provide a good definition of the various physiological and experimental conditions under which observations were made.

1) Chapter III. The anaesthetics, urethane and Nembutal, have been shown to inhibit the neural trigger for the ovulatory surge of LH

(Lincoln and Kelly, 1972, Everett and Sawyer, 1950). In the experiments described above electrical recordings were made under halothane anaesthesia and it was shown that this anaesthetic also inhibits ovulation in the 4-day cycling rat. This inhibition of the neural circuits which stimulate LH secretion appears to be a function of the anaesthesia per se, for when two subthreshold doses of different anaesthetics were used in combination to produce a comparable level of surgical anaesthesia, ovulation was also inhibited. A general property of anaesthetics may be to reduce synaptic activation either pre or post-synaptically (see Chapter III) and therefore anaesthetics may inhibit the prooestrous surge of LH by reducing the activity of neurons involved directly or indirectly in the production of LRH. By a similar action anaesthetics may also reduce or inhibit any effects that steroid hormones may have on the electrical activity of hypothalamic neurons. However, it should be noted that under anaesthesia an increase in the spontaneous firing rate of hypothalamic units is observed during prooestrus even though ovulation is blocked (Dyer et. al., 1972).

2) Chapter IV. The second experimental parameter which required definition was the concentration of plasma estrogen following an i.v. injection of various doses of EB. Measurements of the circulating levels of estrogen following the injection demonstrated that the doses of hormone used throughout the present studies exceeded the peak physiological concentration reached during the oestrous cycle. Estrogen is known to be concentrated in specific target neurons by a saturable binding mechanism, and doses of estrogen were almost certainly sufficient to saturate

these mechanisms.

3) Chapter IV. A third consideration was to determine whether or not an i.v. injection of a steroid hormone, under the present experimental design, would stimulate an increased secretion of LH in ovariectomized estrogen primed rats. A single dose of estrogen administered to ovariectomized rats inhibits the secretion of LH and FSH but the administration of a second s.c. dose of either estrogen or progesterone three days later will stimulate an increased secretion of LH within five hours of the injection (Caligaris, et. al., 1971, a,b). In the present studies measurements of serum LH concentrations one and five hours following an i.v. injection of estrogen or progesterone showed that no significant increase of LH secretion occurred within that period, in both the unanaesthetised and anaesthetised groups of animals. As discussed in Chapter IV, these negative results may be explained by the i.v. method of hormone administration or because of the constant illumination under which the animals were maintained. However, even though a lack of increased LH secretion may genuinely indicate that the positive feedback action of estrogen could be interfered with, the interference may not be at the level of the action of steroids on the POA or on neurons synapsing with this region but rather on the subsequent activation of neurons which secrete LRH.

4) Chapter V. One of the major problems in neuroendocrinology is the paucity of information concerning the neural connections and pharmacological properties of hypothalamic neurons. A large number of

electrophysiological observations have been made within the hypothalamic area from unidentified cells, which may or may not be related to the control of the anterior pituitary; thus the interpretation of these experiments is necessarily limited. In order to study possible 'membrane' sites of action of the stimulatory feedback action of estrogens on the POA it was felt necessary to identify a system of neurons which could be implicated in such feedback and/or which could be involved in the triggering of the ovulatory surge of LH.

In view of the evidence that the stria terminalis plays an important role in the extrahypothalamic control of gonadotrophin secretion (see Chapter IV A) and that the uptake of ^{3}H-estradiol in the hypothalamus corresponds well with the distribution of the strial inputs to this area (Stumpf, 1972), cells in the POA which receive an input from the stria terminalis were electrophysiologically identified by orthodromic stimulation techniques. Observations on the evoked potentials in the bed nucleus suggested that there are two separate components of the stria terminalis with different conduction velocities (see Chapter IV A), and the range of latencies of the response of preoptic neurons to strial stimulation (7 - 25 msec) indicates that this area receives inputs from both components. These two components of the stria terminalis may represent the two groups of neurons in the amygdala which either inhibit or facilitate the secretion of gonadotrophins (Sawyer, 1972).

5. Chapter V B. Stimulation and lesion experiments have shown that the neural input to the basal hypothalamus from the POA is essential for stimulating the preovulatory surge of LH, Therefore following

the initial report of Dyer and Cross, (1972) that cells in the POA which make a direct connection with the basal hypothalmus could be identified by antidromic stimulation techniques, it was decided to repeat their experiments and study the possible effects of steroid hormones on such identified neurons. Although it is likely that such cells may be involved in triggering ovulation there is no definitive proof that these neurons are in any way related to the control of gonadotrophin secretion. Are they themselves the cells which secrete LRH into the portal vessels, or could they be interneurons which control the activity of the neurosecretory cells in the basal hypothalamus? In either case, are they sensitive to the stimulatory feedback action of estrogen?

When considering the positive feedback control of estrogen it is necessary to think of the hormone inducing some change in the specific neuroendocrine circuits which is later expressed in terms of an increased LH secretion. In the 4-day cycling rat the peak estrogen concentration is reached 12 hours prior to the ovulatory surge of LH secretion (Brown-Grant et. al., 1970). Thus it is likely that the higher rate of spontaneous activity in the hypothalamic area observed during proöestrus may represent an increased activity of the neurosecretory cells. But what precise changes in the involved neuroendocrine circuits are brought about by the increased secretion of estrogen? These could be changes in the sensitivity or excitability of neuronal membranes, or metabolic changes which may not be detected by short-term electrophysiological recordings.

6) Chapters VII and VIII. The present experiments were designed to test whether there was any short latency effects of an i.v. injection of a steroid hormone on the activity of antidromically identified cells in the POA which could trigger the ovulatory surge of LH. There are two possible sites of action of steroids which could affect the excitability of preoptic neurons. Firstly, the steroid could act directly on the neurons, so modifying their responsiveness to synaptic activation, or secondly, the steroid could act presynaptically either on the nerve endings themselves or on their cell bodies of origin, so altering the synaptic drive of the POA neurons. One way of helping to distinguish between these two possibilities is to compare the response of the neurons to pharmacological agents applied directly, with their response to the natural synaptic activation; the latter is the cause of 'spontaneous firing'.

7) Chapter VI. To test whether steroid hormones act directly on identified preoptic neurons it was decided to establish their sensitivity to iontophoretically applied putative neurotransmitters, and then measure changes in this sensitivity caused by i.v. steroid injections. Although this is not an unambiguous test of direct steroid action, it was the only one available since direct application of steroids themselves was not practicable (see Materials and Methods, Attempts to Apply Estrogens Iontophoretically)

As shown by the results obtained, identified preoptic neurons were inhibited by DA and NE but were unresponsive to ACh. This is interesting in the context of biochemical and pharmacological studies

on the role of DA and NE in controlling gonadotrophin secretion, which have indicated that both DA and NE sensitive cells in the hypothalamus are involved in the inhibition of LH and FSH, and in stimulating LH and FSH secretion. The adrenergic α-receptor blocking agents, phentolamine and phenoxybenzamine but not β-blockers, will inhibit the post castration increase of LH secretion within minutes of a systemic injection of the drug to ovariectomized rhesus monkey (Bhattacharya et. al., 1972). The responses of the cells to these pharmacological agents are essentially indistinguishable from those observed following the intravascular administration of single pulses of 17 β estradiol (Yamaji et. al., 1972), suggesting that the negative feedback effects of estrogen may be mediated by activation of α-adrener receptors. The evidence that NE stimulates the secretion of LH has been discussed in the General Introduction and in the Introduction of Chapter VI. Thus it is not surprising that chlorpromazine, the potent adrenergic receptor blocker, will inhibit pregnant mare serum (PMS) - induced release of LH in immature rats (Zarrow and Brown-Grant, 1964) and phenoxybenzamine will inhibit the facilitatory effect of progesterone in stimulating ovulation in PMS primed immature rats (Zolovich and Labhsetwar, 1973).

DA has been shown to inhibit the release of LH and FSH (Fuxe and Hökfelt, 1972, Ahren, 1971) although there are reports that DA can stimulate their secretion both in vitro (Schneider and McCann, 1969) and in vivo (Kamberi et. al., 1970). However, it is possible that DA added to the incubation medium or infused intraventricularly is rapidly converted to NE which then stimulates the NE receptors. The evidence

thus suggests that noradrenergic synapses are involved in both the inhibition and stimulation of gonadotrophin secretion. If therefore neurons in the POA are responsible for stimulating LH release why are they inhibited by the iontophoretic application of NE and DA, seemingly contradicting the results of the pharmacological experiments?

Agahanian and Bunney (1974) have suggested that DA released from the nerve terminals in the striatum may not only stimulate the post-synaptic site but may also feedback and inhibit the membrane of the entire dopaminergic neuron, including that of the soma and the terminals. Such a control system may also exist in the identified preoptic neurons; thus the iontophoretic application of catecholamines could have stimulated the 'inhibitory feedback' receptors rather than those activated by other synaptic inputs. Another interpretation of the results is dependent upon the histochemical observations that only NE containing nerve terminals are found in the POA, and not NE containing cell bodies. The ascending NE fibres which terminate in this area could exert a tonic inhibitory effect on these preoptic neurons, and this input is modulated by steroid hormones. When brain NE levels are increased by a systemic injection or an intraventricular infusion of the catecholamine the postulated inhibitory input to the preoptic cells may be reduced by an action on the presynaptic element, in accordance with Agahanian and Bunney's proposal, resulting in an increased activity of the preoptic neurons. However, this hypothesis does not agree with the findings that reserpine (Coppola, et. al., 1966) and adrenergic α-blockers (Bhattacharya et. al., 1972) inhibit LH secretion or the observations that the rate of turnover

of hypothalamic NE increases during proœstrus (Stefano and Donoso, 1967). A further possibility is that these antidromically identified cells may be involved in the secretion of ACTH. Ganong (1972) has postulated that noradrenergic synapses within the hypothalamus may inhibit the secretion of corticotrophin releasing hormone. This hypothesis may also explain the findings that similar identified preoptic neurons show no increase in the mean level of spontaneous activity during proœstrus compared to dioestrus in the intact 4-day cycling rat (Dyer, 1973).

8) Chapter VII. The possibility that the positive feedback of estrogen involves a modulation of the responsiveness of preoptic neurons to "aminergic" inputs was then tested by comparing the slopes of the dose response curve to iontophoretically applied catecholamines before and after an i.v. injection of ovarian steroids. The results showed that under the chosen experimental conditions neither estrogen nor progesterone altered the effectiveness of either DA or NE applied electrophoretically. It therefore seems unlikely that the steroid hormones modulate the activity of these preoptic neurons by ultimately causing changes in their surface membrane properties (e.g. ionic permeability).

9) Chapter VIII. To obtain further information as to whether estrogen could be affecting the activity of preoptic neurons by a pre-synaptic action, the effects of an i.v. injection of the steroid hormone on the rate of spontaneous unit activity has been observed. The results from this study show that under the present experimental conditions estrogens may depress the activity of most of the identified preoptic units. Although both inhibitory and excitatory responses were observed

the majority of cells showed a decrease in the rate of spontaneous activity following an i.v. injection of estrogen. Of those cells which showed an increase in the spontaneous rate of unit discharge the latency of the response was surprisingly short. Although this could indicate a direct excitatory action of estrogens on neural membranes, the effects could also have been secondary to local blood flow changes; in any event the paucity of the experimental observations on this relatively inconstant phenomenon does not warrant further speculation.

The latencies of the observed inhibitory responses (up to 30 minutes) suggest that the effects of estrogen on the spontaneous activity was not the result of a direct action of the steroid hormone on neuronal membranes, but was perhaps secondary to the binding of the steroid hormone to the target neuron, causing subsequent metabolic changes in the cell body or its presynaptic elements. Since estrogen induced a reduction in the rate of spontaneous discharge, in at least some of the examined units, at a time when no effects of the hormone on the sensitivity of similar cells to iontophoretically applied catecholamines was observed, it seems likely that steroids do not act directly on the neuron whose activity is being recorded but rather on a presynaptic site. Both the excitatory action of glutamate and the inhibitory one of the catecholamines would be expected to change in predictable ways if the steroids directly reduced the overall responsiveness of such a neuron, i.e. by a change either in the slope of the dose response curve and/or its position along the abscissa. Although it must be considered that the sensitivity of the iontophoretic technique may not have been

adequate for the detection of subtle changes in the neuronal sensitivity the assumption of a presynaptic site of action (nerve endings or somata of presynaptic inputs) seems more compatible with the findings.

It could be that the changes that are brought about by the stimulatory feedback action of estrogens were not detected within the period of recording in the present experiments because such an action may only be expressed in terms of changes of electrical activity of the LRH secreting neurons during prooestrus. There is evidence that estrogen induced alterations in RNA and protein synthesis is related to gonadotrophin secretion (Pfaff, 1973) and therefore estrogen feedback action may involve metabolic changes which are only later expressed for example in terms of increased levels of transmitter substances and/or releasing factors during prooestrus.

The ultimate solution toward defining steroid hormone actions on the central nervous system would require the ability to record from an identified neuron throughout the oestrous cycle in an unanaesthetised preparation. Even then each individual neuron may be unique in its sensitivity and synaptic connections, and the pattern of its impulse discharge would be related to the temporal and spatial relationship of presynaptic signals impinging upon it. These signals may be modulated by a direct effect on cell membranes by steroid hormones or by a metabolically mediated control of the quantity of transmitter released at the presynaptic terminals. However, despite the innumerable difficulties involved in such electrophysiological studies there can be no true understanding of neuroendocrine mechanisms without the study of the neuron itself.

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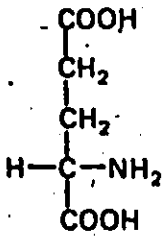
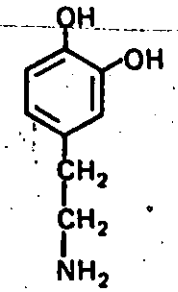
APPENDICES

APPENDIX I

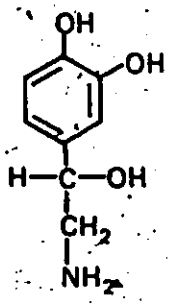
Glutamic acid
(Glutamate)



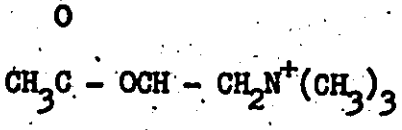
Dopamine
(DA)



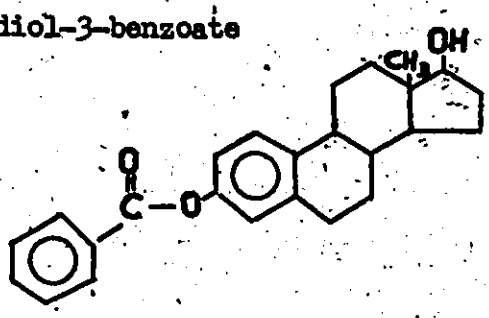
Norepinephrine (noradrenaline)
(NE)

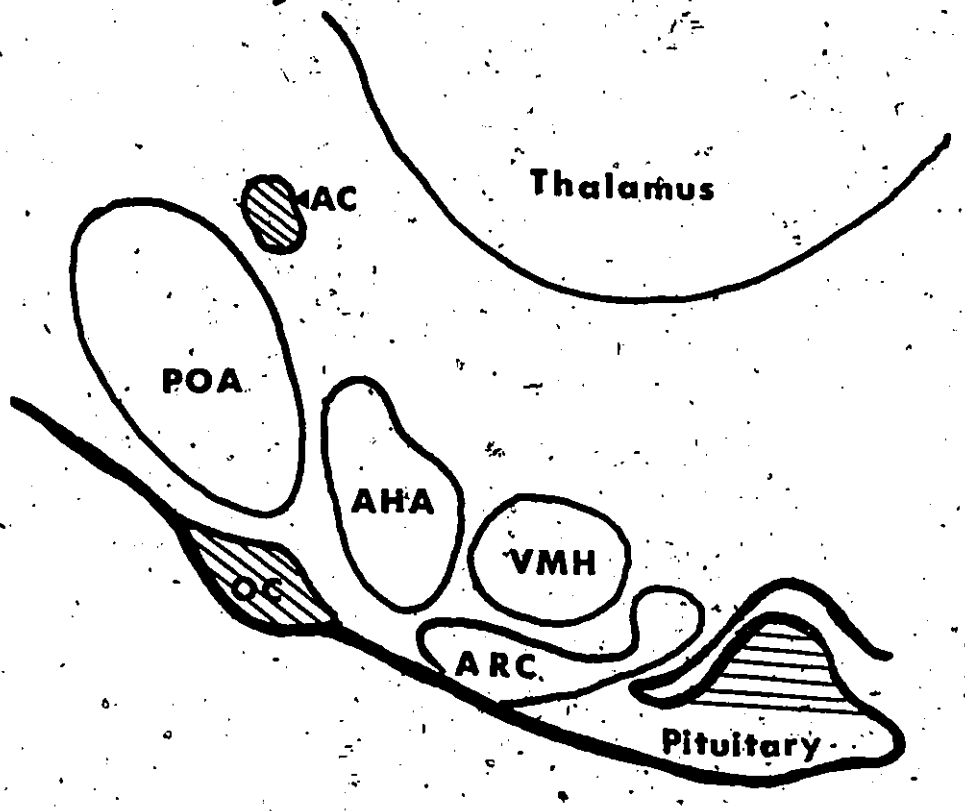


Acetylcholine
(ACh)



17β -Estradiol-3-benzoate
(EB)





APPENDIX II.

Mid-sagittal diagram of rat brain showing location of the preoptic area (POA), anterior hypothalamic area (AHA), arcuate nucleus (ARC), ventromeidal nucleus (VMH) and the pituitary. Other abbreviations; AC, anterior commissure; OC, optic chiasma.