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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE.
OPIOID RECEPTOR RHYTHMS AND
THE CONTROL OF SEXUAL MATURATION

WILLIAM JACOBSON
DEPARTMENT OF PHYSIOLOGY AND BIOPHYSICS

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Dalhousie University
September 10, 1986
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ISBN 0-315-33083-0
Wie der lebendige Strom der Zeit aus ebensoviel Nächten wie Tagen besteht, so beruht auch die wirkliche Erkenntniss auf ebensoviel Irrthum wie Wahrheit.

Jung
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In female rats, an afternoon decrease in the ability of naloxone to elicit increases in circulating LH levels was seen with the approach of puberty. The development of a similar diurnal pattern in male rats was not found. The specific binding of $[^3]H$-naloxone to hypothalamic slices from prepubertal females showed a biphase diurnal pattern, with peaks after the light and dark phases had begun. The decrease in $[^3]H$-naloxone binding over the course of the day was determined to be due to a decrease in $B_{max}$ and not to a change in the $K_d$ of the receptor. Cortical binding of $[^3]H$-naloxone did not change over the course of the day. Ontogenetic studies with female rats revealed that the appearance of this afternoon decrease in receptor number paralleled the appearance of the decrease in the ability of naloxone to stimulate LH release in the afternoon.

The diurnal rhythm in hypothalamic $[^3]H$-naloxone was susceptible to interruption with barbiturate anesthesia, and could be modified by infantile gonadectomy, neonatal androgenization, and exposure to constant light or darkness. Furthermore, the rhythm could be induced prematurely with PMSG. The noradrenergic neurotoxin DSP4 was also used in an attempt to disrupt the pubertal process. While these initial experiments were unsuccessful, it was shown that DSP4 had profound effects on basal and naloxone-stimulated LH release. Further investigation of these phenomena revealed, for the first time, a direct interaction of DSP4 with the opioid receptor.

Further investigation of rhythmic variations in neurotransmitter binding may lead to an improved understanding of the regulation of the pubertal process.
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<tr>
<td>CAMP</td>
<td>cyclic adenosine 3', 5' monophosphate</td>
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<td>CL</td>
<td>corpus luteum</td>
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<td>DALA</td>
<td>D-ala²-met-enkephalinamide</td>
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<tr>
<td>DADLE</td>
<td>D-ala²-d-leu⁵-enkephalin</td>
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<td>DPBS</td>
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<td>(D-Ala²-MePhe⁴-Met(ο)⁵-ol)-enkephalin</td>
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GnRH  gonadotropin hormone releasing-hormone
GS    Golden Syrian hamster
HPLC  high performance liquid chromatography
LD    light:dark cycle
LE    Long Evans rats
LH    luteinizing hormone
MBH   mediobasal hypothalamus
NSB   non-specific binding
PBZ   phenoxybenzamine
PCO   polycystic ovarian syndrome
PGF   plerocercoid growth factor
PMSG  pregnant mare serum gonadotropins
QNB   quinuclidinyl benzilate
RIA   radioimmunoassay
s.c.  subcutaneous
SCN   suprachiasmatic nucleus
SD    Sprague Dawley rats
s.e.m standard error of the mean
SW
VO
6-OHDA

Swiss Webster mice
vaginal opening
6-hydroxydopamine
ACKNOWLEDGEMENTS

I am, and shall always be, in the debt of my advisor Dr. Michael Wilkinson. He has been a friend, a teacher and more over the past four years. I shall always value his counsel.

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INTRODUCTION

This Thesis will examine one aspect of the control of puberty in the female rat, viz. the development of a peripubertal diurnal change in hypothalamic opioidergic tone, as expressed through two variables: (a) a variation in the complement of hypothalamic opioid receptors, and (b) a change in the LH secretory response to opioid blockade by naloxone.

Puberty is not the result of a single event; it is, rather, the reflection of an exceedingly complex series of interactions involving many systems and events. While the body of this Thesis is concerned with the effects of discrete changes in only one of these components - the opioidergic system - it is important to remember that such changes do not occur in isolation, but form only a small part of a much larger continuum. A comprehensive examination of all of the interactions involved in the control of puberty is well beyond the scope of this Thesis. However, a review of some of the major theories which aim to explain basic control mechanisms in puberty will be presented in this Introduction. Following this, consideration of the role of endogenous opioid
compounds in the neural control of puberty will be presented. This will be followed by a discussion of some aspects of diurnal and circadian variations in neurotransmitter receptors.
THE NEURAL CONTROL OF PUBERTY

This Introduction will consider possible mechanisms controlling the onset of female puberty. Most of the data cited here deals with puberty in the rat, but of necessity, experiments performed on other species will occasionally be discussed. The word puberty derives from the Latin pubes, or hair. Puberty is most easily detected by the phenotypical changes associated therewith. However, many complex changes occur in the physiological functioning of the organism long before these external markers of sexual maturity are apparent. For the purposes of this Thesis, puberty, in the female, will refer to the first preovulatory LH surge and subsequent ovulation.

While it is now well recognized that the brain plays a crucial role in the control of puberty, this was not always the case. Hypothalamic releasing factors were unknown, and it was the seminal work of Harris and his students that led the way to an acceptance of the role of the CNS in the control of pituitary function. (Harris, 1972). Evidence had, however, been developed indicating that the ovaries did not control the pubertal process. Dunham et al. (1941) demonstrated that neonatal ovaries were
capable of follicular maturation and the development of corpora lutea when transplanted into the anterior chamber of the eyes of mature female rats. Harris and Jacobsohn (1952) also demonstrated that the pituitary was not the controlling factor, by grafting pituitaries from newborn rats into the hypothalamus of hypophysectomized nursing mother rats. They found that those pituitaries grafted into the median eminence (and not the temporal lobe or the emptied pituitary capsule of the hypophysectomized mother) were capable of bringing about a return to normal gonadal function. Within 7 days (i.e. when the pituitary glands were 12 days old) animals with immature pituitary grafts showed normal cycles, with subsequent pregnancy and normal support of the young pups, except for milk letdown. The conclusion drawn from these experiments was that immature pituitary glands were capable of assuming normal adult functioning. Once it had been demonstrated that both immature ovaries and the immature pituitary gland were capable of assuming normal adult functioning, attention was focused on the brain, and how it might control the process of puberty.

The first purported association between an intracranial abnormality and precocious puberty was
that presented by Kussmaul in 1862. Bailey and Jellife (1911), in a discussion of pineal tumors and precocious puberty, wrote "It may be that the precocious sexual development may be an early irritative sign of a purely nervous character acting through the pars nervosa sympathetic system, which is very rich, and that later increase in pressure brings about degeneration or atrophic phenomena". Bing et al. (1937) pointed out the possibility of specific centres in the hypothalamus "regulating with an endocrine organ, most probably the pituitary gland, the development of sex characters. This in turn leads to the thought that the precocious development of sex characters may be the result of some hypothalamic disorder, such as may be provoked by a tumor in the pineal region."

There are many examples in the clinical literature of derangements of the pubertal process in man associated with lesions of the brain, and in particular the hypothalamus (Weinberger and Grant, 1941; Bauer, 1954; Morley, 1954). Bernard Donovan, (a student of G.W. Harris) and his collaborator, J.J. van der Werff ten Bosch first showed that anterior hypothalamic lesions advanced vaginal opening (Donovan and van der Werff ten Bosch, 1956). Vaginal
opening (VO) is a common marker of female puberty; it is usually, but not always associated with first ovulation (Gorski-Firlit and Schwartz, 1977). Other groups, notably Bogdanove and Schoen (1959), Gellert and Ganong (1960), and Ruf et al. (1974) confirmed the findings that certain lesions of the anterior and mediobasal hypothalamus led to precocious sexual development in the female rat. Corbin and Schottelius (1960) also showed that appropriate lesions of the posterior hypothalamus cause a delay of puberty in the female rat, although recent work by Terasawa and her collaborators clearly shows that posterior hypothalamic lesions in monkeys induce precocious puberty (Terasawa et al., 1983;1984). The development of the thinking concerned with the role of the brain in pubertal control, is well reviewed by Donovan and van der Werff ten Bosch (1965), and by Ruf (1973). Most of the early experiments led to the conclusion that the hypothalamus somehow constrained gonadal function during immaturity. The hypothalamic lesions were thought to remove a sustained inhibitory influence, which allowed the animal to progress to sexual maturity. In contrast, and as proposed by Ruf, the lesions could equally well provide a stimulatory influence on brain maturation. Nevertheless, although
the nature of the inhibitory influence is not completely understood, even today, the idea led to the genesis of the major theory of the control of puberty, i.e. the gonadostat hypothesis.

**THE GONADOSTAT THEORY OF THE CONTROL OF PUBERTY**

The gonadostat hypothesis proposes that at a certain stage of development, the hypothalamic restraint on gonadal function diminishes. This would involve the resetting of a hypothetical inhibitory feedback system. Most often proposed has been a system wherein neural sensitivity to the negative feedback effects of estradiol (E2) decreases as sexual maturity approaches. The first proposal of such a mechanism was put forth by Hohlweg and Junkmann in 1932, with their observations that younger rats were much more susceptible to the effects of gonadal steroids on the so-called "castration cells" of the pituitary gland which were prominent after gonadectomy. Subsequently, Ramirez and McCann (1963), were able to show that the immature female rat is exquisitely sensitive to the negative feedback effects of E2, and the adult much less so. Their systematic study of the change in E2
sensitivity (as determined by the LH response to EB following ovariectomy) with maturity in the female rat stands as a landmark in modern reproductive neuroendocrinology, and it was in this study that the phrase "resetting the gonadostat" was coined. They concluded that somehow, between birth and the onset of puberty, the gonadostat is reset, thus allowing LH to escape from tonic negative feedback. The neurochemical substrate for this change in sensitivity is still unknown, although progress has been made recently in this area implicating the endogenous opioids (Wilkinson and Bhanot, 1983b; these data are discussed in the section on the involvement of endogenous opioid peptides in the control of puberty, below). These early experiments are all the more remarkable today, because they were performed without the benefit of radioimmunoassay (RIA) techniques. Instead, they relied on the somewhat cumbersome ovarian ascorbic acid depletion test to determine LH levels. Eldridge et al. (1974) were the first to confirm that the negative feedback effect of estradiol on LH levels lessened as the female rat matured, using the technique of RIA. Ramirez and McCann (1965) reported a similar loss of sensitivity in the LH response to testosterone in
male rats with maturity. However, Odell and Swerdloff (1976) were unable to repeat these observations, although they showed just such an age-related change in the ability of testosterone to inhibit circulating FSH levels. Andrews et al. (1981) reported that in the female rat, the lessening of the negative feedback effect of E2 on circulating LH levels occurred after the first preovulatory LH surge, and interpreted this to mean that the resetting of the gonadostat is a consequence of, and not a trigger of, the pubertal process. However, a close and critical examination of their data reveals that the LH response to E2 was examined at only one age prepubertally. It is entirely conceivable that the change in negative feedback occurred after their test point (i.e. day 29), yet before the preovulatory surge. Obviously further experiments designed to construct a more complete time course of this important phenomenon are necessary. Furthermore, recent findings by Docke et al. (1984), using both subcutaneous as well as hypothalamic implants of EB, but for only two days exposure (cf. Andrews et al., 1981, 4 days exposure), have reasserted that the change in sensitivity to estradiol occurs prior to the first ovulation.
Ojeda's group (e.g., Andrews et al., 1981) was not the first to propose the demise of the gonadostat theory of pubertal control. This hypothesis has not always (and indeed still does not) received universal acceptance. A key point in validating the theory would be the demonstration that as puberty approached, and as the negative feedback influence of estradiol on LH secretion waned, there would be a gradual increase in the circulating levels of LH. With traditional sampling techniques, such a difference has not been apparent (Odell and Swerdloff, 1976; Dohler and Wuttke, 1975; Ojeda and Ramirez, 1972; Ojeda et al., 1976; Meijs-Roelofs et al., 1975). Steele and Weisz (1974) used chronic venous cannulae to infuse estradiol and to sample blood from the same animal over a period of time. They found a decrease in the ability of E2 to inhibit LH with maturation, but they also reported that with prolonged E2 infusion, there was an abrupt secondary increase in the circulating LH concentration which in some animals coincided with the time of VO. In a follow-up study during which the time of ovariectomy was varied with respect to the onset of puberty, Steele (1977) showed that the hypothalamic-pituitary component of the system regulating the development of
the negative feedback action of E2 on serum LH with maturity is able to develop normally in the absence of the ovaries. These experiments led to the conclusion that the hypothalamus itself might be the site of control for the change in sensitivity to gonadal steroids with sexual maturity. This so called "intrinsic control" of gonadotropin release has been further investigated in the lamb.

Foster's group has investigated the development of negative feedback in the E2-LH system as it relates to puberty in the lamb. (Foster and Ryan, 1979a; Ryan and Foster, 1980). They found that untreated ovariectomized animals showed a secondary increase in castrate LH levels to adult castrate levels at the time of first ovulation in intact lambs. Presumably this increase was entirely due to a neural signal, whose nature is unknown. Furthermore, maintenance of chronic low levels of E2, with a subcutaneous silastic implant, suppressed LH levels in prepubertal lambs until the age at which untreated intact lambs normally ovulated for the first time. At this time, circulating LH concentrations spontaneously increased to untreated castrate levels in the face of continued E2 feedback. In this species, then, puberty is associated with a reduction
in the ability of E2 to potently suppress LH secretion.

PULSATILE HORMONE RELEASE AND PUBERTY

Foster and Ryan (1979b) also demonstrated that the pattern of pulsatile LH release in the lamb changes dramatically at some point in the transition from reproductive immaturity through puberty. Prior to puberty, peaks of LH occur approximately once every three hours. The adult pattern is circuloroidal, with approximately one peak per hour. This shift in pulsatility is proposed as a means of explaining how the release of negative feedback of E2 on LH results in an increase in the LH baseline. The experimental data obtained from sheep by Foster's group has done much to further the understanding of the pubertal process. However, the use of the sheep as an experimental model presents logistical problems for many laboratories. By far the most popular species for pubertal studies are the rodents, and in particular the rat (Ojeda et al., 1980b). Until recently, it was extremely difficult to obtain longitudinal data from prepubertal rats, because of their small size and limited blood volume. However, the laboratory of Sergio Ojeda has reported the
development of a new methodology which permits both frequent blood sampling, and constant blood volume replacement in unrestrained conscious prepubertal rats (Urbanski and Ojeda, 1984; 1985). This technique has permitted the detection of a diurnal change in the pattern of LH secretion with the approach of puberty in the female rat. The juvenile-peripubertal transition period is characterized by an increase in the amplitude of the LH pulses, and the appearance of "mini-surges" of LH secretion in the afternoon. Meijs-Roelofs et al. (1983) also detected an afternoon change in LH secretion in female rats approaching puberty. By using extremely large groups of animals they were able to determine that afternoon LH levels rise as puberty approaches. The fact that these observations could only be seen with large groups of animals is compatible with a change in the pulsatile nature of LH release.

Thus we can see that the major criticism of the gonadostat hypothesis of pubertal control - the lack of a detectable increase in basal LH secretion - is overcome by the observation of the appearance of pulses of LH at a specific time in the day. It is not surprising that this was not detected earlier.
The importance of the pattern of pulsatile LH release in pubertal control mechanisms has been further investigated by Foster et al. (1984). They showed that the imposition of an "adult" pattern of circulating LH, achieved by hourly administration of the hormone, is able to induce ovulation in intact prepubertal female sheep. Changes in the patterns of pulsatility have emerged as a critical component of the control of puberty, particularly in the higher primates and man. With an elegant combination of three techniques, Boyar and his associates made a major contribution to our understanding of the pubertal process in humans. The three techniques were polygraphic monitoring of the stages of sleep, the use of indwelling catheters to permit frequent blood sampling without disturbing the subject, and the use of radioimmunoassay to determine the serum levels of gonadotropins. The major finding of their investigations was that in the human, puberty is associated with the development of an episodic pattern of pulsatile LH and FSH release, and that these episodes are associated with a restricted period each day, i.e. during sleep (Boyar, 1978; Boyar et al., 1972; Weitzman et al., 1975). These investigations have been summarized by Chipman
(1980). However, as is the case with many other "explanations" of the pubertal process, the factors and mechanisms controlling these events are not, at this time, understood. Ernst Knobil's group took a similar approach to the study of puberty in the rhesus monkey as Foster's group did with the lamb, except they went one step further. In a classic experiment, they infused GnRH in a pulsatile fashion (as opposed to pulsatile administration of LH) to prepubertal female rhesus monkeys, and were able to induce ovulation in this species, well ahead of the normal time of puberty (Wildt et al., 1980). However, once the pulsatile pattern of GnRH administration was discontinued, the female animals reverted to their normal prepubertal condition. It was concluded from this series of experiments that the immature pituitary and ovary are capable of supporting ovulation, but are unable to sustain the adult pattern of functioning in the absence of an appropriate pattern of GnRH pulses. Therefore, what is necessary for normal puberty is a maturation of the GnRH releasing system in the brain, such that the appropriate pattern of pulsatility is achieved.

It seems clear that one of the key events in the control of puberty is, in fact, the maturation of the
central nervous system to the point that an appropriate pattern of GnRH release is achieved. This pattern will, in turn, act on a competent pituitary gland to cause the release of an appropriate pattern of gonadotropins. These hormones can then act on a competent gonad to release steroids which can act on the brain and pituitary gland to induce a spontaneous LH surge, and ultimately ovulation.

CATECHOLAMINERGIC CONTROL OF PUBERTY

What are the neural circuits, or neurotransmitters, responsible for the maturation of pulsatile GnRH release? It is well established that catecholamines are intimately involved in several aspects of reproductive function. Sawyer et al. (1949) first demonstrated a role for adrenergic systems in the central control of ovulation. The role of catecholamines in the control of LH release has been recently reviewed (Barraclough and Wise, 1982; Kalra and Kalra 1983; Ramirez et al., 1984). A large body of evidence implicates noradrenergic systems in the control of GnRH release (Sarkar and Fink, 1981; Lofstrom et al., 1977a, 1977b; Barraclough and Wise, 1982) and sexual behavior in a number of species (Crowley et al., 1976, 1978b; Nock and Feder, 1978,
Catecholaminergic mechanisms have also been shown to be implicated in the control of puberty (Hyppa, 1974; Advis et al., 1978, Wuttke et al., 1980). One of the means by which the resetting of the hypothalamic gonadostat at the time of puberty could be explained, as put forth by Ruf (1973) is the "growth and functional maturation of steroid sensitive adrenergic systems" in the brain.

The central nervous system of the rat is functionally and structurally immature at birth, and development continues postnatally. As the rat matures, the number of adrenergic neurons and adrenergic activity increases. The crux of the Ruf hypothesis is that the increased adrenergic neural activity could promote GnRH secretion into the hypophyseal portal circulation, which in turn would result in an increased secretion of gonadotropins. The increased gonadotropin levels could act on the gonads to stimulate steroid production and release, and the steroids could then feed back to inhibit the adrenergic activity. According to this line of thinking, the controlling mechanism of the gonadostat theory is not the removal of inhibition but an increase in the stimulatory drive for GnRH release.
Further evidence for the role of central catecholaminergic mechanisms in the control of puberty is found in the observations of Ruf and Holmes (1974) and Hyppa (1974), showing that intraventricular injection of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) delays vaginal opening.

**SYNAPTIC REORGANIZATION AND PUBERTY**

Pubertal development may indeed be dependent on neuronal growth and plasticity. Matsumoto and Arai (1976a) reported that postnatal development of the female rat was accompanied by a marked increase in the number of some specific synapses in the arcuate nuclei of the mediobasal hypothalamus, i.e. axodendritic rather than axo-axonic. In their colony, vaginal opening coincided with the attainment of a plateau in the numerical density of these specific synapses. Anderson (1982) has also reported increases in the dendritic spine density, but in the preoptic area of the female rat at the time of vaginal opening. He postulates that such changes may be involved in the control of puberty. These neuronal changes may in turn be dependent on steroids. Following daily treatment with estradiol benzoate
(EB) for the first 30 days of life, female rats have
twice the number of axodendritic synapses in the
arcuate nucleus as do control rats (Matsumoto and
Arai, 1976b). The number of synapses in the
estrogenized animals closely approximated that seen
in normal postpubertal females. Clough and Rodriguez-
Sierra (1983) have also examined the effect of acute
treatment with EB on synapses in the preoptic area
and the arcuate nucleus of the prepubertal rat. They
found that treatment with EB more than doubled the
density of synapses in the arcuate nucleus, while
there was no synaptogenic effect apparent in the
preoptic area. Estrogens, thus, appear able to induce
synaptogenesis in the arcuate nucleus. Presumably a
maturational effect on neuroendocrine processes which
control phasic release of LH from the pituitary gland
is exerted through estrogenic stimulation, and this
contributes to the sexual development of the animal.
Trophic influences of sex steroids on neural tissue,
as manifested by a selectively accelerated growth and
progressively intense arborization of neuronal
processes have also been demonstrated in vitro
(Toran-Alerand, 1976, 1980; Toran-Alerand et al.,
Treatment of prepubertal female rats with pregnant mare serum gonadotropins (PMSG) induces precocious vaginal opening, the generation of an LH surge, and ovulation (Zarrow and Quinn, 1963; Lunn and Bell, 1968; Kostyk et al., 1978). PMSG activates central catecholaminergic mechanisms (Sarkar and Fink, 1981; Sarkar et al., 1981) and ultimately induces ovulation through a surge of E2 (Wilson et al., 1983; Sarkar and Fink, 1979). This induced precocious sexual maturation is also associated with an increased number of axo-dendritic synapses in the arcuate nucleus (Matsumoto and Arai, 1977). A unilateral electrolytic lesion in the area of the pituitary stalk can also induce precocious puberty in the immature female rat; these lesions are associated with an increased number of synapses in the contralateral arcuate nucleus (Ruf et al., 1980; Ruf, 1982).

Few investigators have concerned themselves with the nature of the new synapses, or with their function. It is unknown if they are inhibitory or stimulatory, adrenergic, peptidergic or cholinergic, etc. Inspection of the data of Matsumoto and Arai has led to the conclusion that while most of the new synapses were excitatory in nature, less than 20%
were adrenergic (Wilkinson et al., 1979). Wilkinson et al. (1979) have examined the effects of PMSG induction of precocious puberty on the adrenergic receptor component of the hypothalamus and the cerebral cortex. They were unable to show an increase in the number of noradrenergic receptors in hypothalamus in PMSG-treated animals, as might be expected from the Ruf hypothesis. Rather they found, an increase in cortical β-receptors, and a decrease in hypothalamic α-adrenergic receptors. While the significance of the change in cortical receptors is unclear, the authors speculate that the decrease in hypothalamic α-adrenergic receptors may represent the removal of an α-adrenergic mediated inhibition, and may itself represent a functional substrate for the resetting of the gonadostat.

ESTROGEN AND PUBERTY

Several other factors have been implicated in the control of puberty. As pointed out above, estrogen appears to have a trophic and organizational effect on the neurons of the arcuate nucleus, an area important in the control of gonadotropin release. It was shown more than 60 years ago that administration of an estrogenic placental extract (an admittedly
impure preparation) induced precocious puberty in the rat (Frank et al., 1925). Both long-term and short-term treatment of prepubertal rats with low doses of EB induced precocious vaginal opening, ovulation and estrous cyclicity in a study by Ramirez and Sawyer (1965), while perinatal EB treatment advanced puberty in the guinea pig (Medero and Dominguez, 1977). However, in a subsequent investigation, Lung and Docke (1981) were unable to demonstrate an estrogen-induced advancement of the pubertal process. Other than the fact that two different strains of rats were used in these studies, Sprague-Dawley in the former and Wistar in the latter, there is no ready explanation for the difference. Smith and Davidson (1968) further examined the question of the effect of estrogen on puberty, and attempted to ascertain the locus of action of the hormone by implanting crystalline EB both chronically and acutely in the brain. They showed that chronic exposure to the hormone resulted in precocious VO but disrupted vaginal cycling, regardless of whether the implant was placed in the hypothalamus, midbrain or cortex, presumably because the steroid hormone was released systemically. However, acute (i.e. 48 hour) exposure to the implant in the anterior hypothalamic-preoptic
area (and only in this area) resulted in advancement of VO with normal cycling and normal appearing reproductive organs. It should be noted that in light of the fact that normal cycling followed the premature VO, this represents true precocious puberty. These investigators concluded that puberty results from an action of estrogen on the brain. Administration of dehydroepiandrosterone (DHEA) to prepubertal female rats has been reported to induce a prompt ovulatory response, and it is posited that the mechanism of action of this steroid involves its aromatization to estrogens (Knudsen and Mahesh, 1975). Some groups have reported a dissociation between the advancement of VO by estrogen, and the initiation of normal ovarian cycles, as reflected by exfoliative vaginal cytology (see for example Gentry and Wade, 1980). Such studies are difficult to reconcile however, because of differences in the dose, mode and timing of administration of steroid.

If estrogen were the controlling factor in the initiation of puberty, one might expect to see a prepubertal rise in circulating E2 levels. Such does not appear to be the case (Dohler and Wuttke, 1975; Kamberi et al., 1980). However, in the rat, estradiol is bound to a-fetoprotein. Germain et al. (1978) have
shown that while there is no increase in the circulating levels of E2 before puberty, α-fetoprotein levels do decrease. Thus, tissue levels of free E2 (i.e. not bound to α-fetoprotein, and therefore biologically active) actually increase in target organs. Subsequently, this was confirmed by Puig-Duran et al. (1979). The decline in α-fetoprotein is proposed as an important controlling mechanism for the initiation of estrogenic events involved in the sexual maturation of the female rat. The relevance of this observation to the case of human puberty is questionable, however, since human α-fetoprotein does not bind estradiol.

**PROLACTIN AND PUBERTY**

Prolactin has also been implicated in the control of puberty in the rat. Voogt et al. (1970), using an RIA, determined that there is a transpubertal increase in the pituitary content of prolactin in female rats. They also found that serum prolactin increases markedly on the day of vaginal opening. They conclude however, that the increase in circulating prolactin levels is due to an action of estrogen secretion. Dohler and Wuttke (1974; 1975) measured serum prolactin levels in a large number of
male and female rats from birth until puberty. They found that in both sexes, circulating prolactin levels were low for the first three weeks of life, following which they increased gradually, reaching peak levels just prior to puberty. There was considerable variation in the prolactin levels seen in female rats just prior to vaginal opening. In girls, a peripubertal increase in prolactin levels has also been reported (Ehara et al., 1975).

Induction of a state of hyperprolactinemia, by either subcutaneous injection or implantation of prolactin into the median eminence was first shown to advance puberty by Voogt et al. (1969). This finding was subsequently confirmed by Ojeda's group (Advis and Ojeda, 1978; Advis et al., 1981a). These investigators determined that the hyperprolactinemia was associated with an increase in the ability of the ovary to respond to gonadotropins with steroidogenesis. They attributed the puberty-advancing effect of prolactin on the ability of this pituitary hormone to accelerate normal follicular maturation, particularly through the induction of LH receptors by granulosa cells. Suppression of prolactin has also been shown to delay the
development of puberty in female rats (Advis et al., 1981b).

In addition to these peripheral sites of action, prolactin also has central effects. Honma et al. (1979) have shown that daily injection of prolactin early in life can increase dopamine turnover in the mediobasal hypothalamus. There is a physiological correlate of this in that hypothalamic dopamine turnover increases between day 15 and day 25 of life, a period during which an increase in the amount of prolactin in the circulation is found (Hohn and Wuttke, 1979). Sustained hyperprolactinemia has been shown to desensitize the dopaminergic inhibition of LH release in ovariectomized female rats (Beck and Wuttke, 1977). It has also been proposed that there is a peripubertal decrease in the ability of dopamine to inhibit GnRH secretion, and that this in turn results in prepubertal increases in gonadotropin release (Hohn and Wuttke, 1978; Wuttke et al., 1980). Recent work by Herdon and Wilson (1985) appears to confirm this hypothesis. They were able to demonstrate a prepubertal decrease in hypothalamic D2-dopamine receptors.
Interestingly, the ability of estradiol to inhibit LH release appears to have a dopamine-mediated component (Lofstrom et al., 1977c), and thus the action of the prolactin-dopamine system may fit in with the concept of the gonadostat.

GROWTH HORMONE AND PUBERTY

Because of the temporal association of the so-called growth spurt with puberty in humans, and because of the close association between body composition (or weight) and puberty (Frisch, 1980; Kennedy and Mitra, 1963), the role of growth hormone (GH) in the pubertal process has been investigated. It has been shown in immature female rats that GH levels rise gradually from low levels at 18-22 days of age to much higher levels peripubertally (Ojeda and Jameson, 1977; Eden et al., 1978). By implanting growth hormone into the median eminence of immature female rats, Advie et al. (1981c) were able to delay puberty. This delay was associated with a significant decrease in circulating growth hormone levels, presumably through the activation of a short loop negative feedback system. A much more ingenious approach was taken by Ramaley and Phares (1980, 1982). The plerocercoid larvae of the tapeworm
Spirometra mansonides elaborates a factor (PGF) which maintains normal growth while inhibiting GH release in intact rats. By infecting immature female rats with S. mansonides they were able to examine the effects of an absence of growth hormone on puberty, without any of the complications which arise from an inhibition of somatic growth. Remarkably, they found that puberty was delayed, while body weights were normal. Thus, it appears that GH is necessary for sexual maturation to take place.

THE ROLE OF DIURNAL SIGNALS IN THE ONSET OF PUBERTY

Because reproductive success in animals is dependent on the coordination of a number of well-timed events, one would naturally expect close interactions between many facets of the reproductive system and the circadian system. As has been discussed above, immature ovaries and pituitary glands are able to function in an adult manner given the appropriate, carefully timed stimuli. Much time and effort has been devoted to the search for a neural trigger which controls the regular cyclic functioning of the hypothalamic-pituitary-ovarian axis. Everett and Sawyer (1950) first demonstrated the importance of a "daily signal" in the control of
reproductive phenomena in the rat. They reported that the depression of neural activity with a barbiturate during a key period of approximately 2 hours duration on the afternoon of proestrus resulted in a 24 hour delay in the generation of an LH surge. The timing of this neural signal is linked to the light:dark (LD) cycle, and can be manipulated by changing the photoperiod (Critchlow, 1963). The suprachiasmatic nuclei (SCN) of the basal hypothalamus are known to be responsible for the control and entrainment of many circadian phenomena (Rusak and Zucker, 1979; Minors and Waterhouse, 1986). The SCN also appears to be necessary for the generation of the daily signal which induces the proestrus LH surge (Bishop et al., 1972). Current thinking on the role of the circadian system in reproductive phenomena has been well reviewed by Turek et al. (1984) although puberty is not discussed.

Because of the importance of circadian signals in normal adult reproductive functioning, it seems reasonable that the establishment of diurnal signals may be important in the control of sexual maturation. MacKinnon et al. (1978) examined peripubertal patterns of hormone release in female rats at several time points over the day. They found "circadian"
variations in circulating levels of FSH, prolactin and corticosterone, but not LH. (Strictly speaking, it is not clear that these variations were in fact circadian, because their persistence in the absence of time cues, e.g. photoperiod, was not demonstrated). They found higher levels of FSH in the morning in 28-day-old rats, with a diminishing amplitude of the rhythm as puberty approached. No difference was found in am/pm LH levels. These investigators conclude that changes in the phase and/or amplitude of "circadian" rhythms of hormone output may be important in the control of the development of ovarian follicles, preovulatory elaboration of estrogen and generation of the first preovulatory LH surge.

Pamela MacKinnon and her coworkers have shown that mild stress prevents the detection of high levels of LH (MacKinnon et al., 1976; MacKinnon and Mattock, 1976). Thus, failure to detect diurnal differences in the secretion of this gonadotropin may merely reflect a stress artifact in the earlier studies. With the advent of improved radioimmunoassays, and the development of techniques which permit the frequent unstressful sampling of blood from immature rats, it has become apparent that
a diurnal pattern of LH secretion does develop prepubertally. Andrews and Ojeda (1981), sampling frequently via indwelling cannulae, first demonstrated the existence of a peripubertal increase in the amplitude of LH pulses in the afternoon, in conjunction with a depression of baseline LH levels. This change in the mode of LH release during the late juvenile period was proposed as an indication that facilitatory neural mechanisms controlling LH release were being activated, in advance of the first preovulatory LH surge. Ojeda's group has subsequently modified the sampling methodology to allow the constant reinfusion of a whole blood substitute (Urbanski and Ojeda, 1984), thus permitting very frequent determination of circulating hormone levels without undue concern over alterations in the relatively small blood volume of prepubertal rats. Detailed examination of the pattern of LH release in female rats during the peripubertal period as determined in this manner has confirmed the existence of large amplitude LH pulses, and has revealed the existence of "minisurges" of LH release in the afternoon (Urbanski and Ojeda, 1985a). This pattern of LH release appears to be of some physiological consequence, as perfusion of immature rat ovaries in
vitro with a pattern of LH corresponding to the afternoon LH release pattern (i.e. either large amplitude LH pulses or "minisurges" results in relatively large increases in ovarian secretion of progesterone and E2. Perfusion with a pattern of LH resembling the morning LH release pattern (i.e. low amplitude LH pulses) was considerably less effective in inducing gonadal steroid release (Urbanski and Ojeda, 1985b). It appears that the diurnal difference in the pattern of LH release may thus be important in the development of adult ovarian functioning by the prepubertal animal. Crucial observations would be the detection of afternoon "pulses" of E2 in prepubertal female rats, in response to the changing pattern of LH release.

As mentioned above, mild stress inhibits LH release. The inability to observe the peripubertal diurnal difference in LH release by previous investigators may indeed be attributable to stressful handling of the animals, and the subsequent obliteration of subtle differences. Additionally, these differences in circulating release are only apparent when the sampling is frequent enough to reveal changes in the pulsatile nature of LH release. Clearly the technique of Urbanski and Ojeda permits
the animals to be handled without undue stress, and provides for an examination of pulsatile patterns of hormone release. Meijs-Roelofs et al. (1983) have also examined the development of a temporal pattern of LH release in the immature female rat. They examined groups of several hundreds of rats in order to get a clearer picture of the changes in hormone release with development. By using such large numbers, they were able to detect subtle differences in circulating hormone levels. Meijs-Roelofs et al. (1983) found that there was a definite increase in circulating LH values in the afternoon, as the animals neared puberty. Such changes, which could represent differences in the pulsatile outflow of LH, might be masked in the examination of small groups of animals. As will be detailed in this Thesis, there also appears to be a diurnal component to the opioidergic control of LH release in the female rat approaching puberty.

An interesting variation of this phenomenon is seen in the hamster. Females of this species exhibit a readily-detectable daily surge of LH and FSH in the late afternoon, beginning around day 15 or 16 of life (Smith and Stetson, 1980; Donham et al., 1984). These daily rhythms persist until normal 4-day ovulatory
cycles begin between days 30 and 40 of life. Recent findings have revealed an absolute dependency of these daily rhythms on the ovaries. They can be prevented from occurring if ovariectomy is performed prior to their onset, and they can be abolished by ovariectomy after their onset (Donham et al., 1985). Furthermore, neonatal androgenization with testosterone propionate prevents the appearance of this neural-clock-dependent rhythm (Donham and Stetson, 1985). The importance of the daily rhythm in the control of puberty has been illustrated recently by Donham et al. (1986). They reported that advancing the initiation of daily gonadotrophin surges with injections of GnRH resulted in an advancement of puberty, as detected by V.O. Similarly, the use of daily injections of phenobarbital sodium, to block the generation of the daily LH surge, resulted in a delay of puberty. It is thought that the appearance of the daily rhythms represents a maturation of those neuroendocrine mechanisms responsible for the control of estrous cyclicity in the adult animal, and that the ovary delivers an essential signal to the hypothalamo-pituitary axis for the initiation and maintenance of these daily surges. (Donham et al., 1985)
Diurnal variations in the responsiveness of the pituitary gland to GnRH may also represent an important factor in the onset of puberty in the rat. Dullart (1977) and Wilkinson and Moger (1981) have shown that the pituitary responsiveness to GnRH (as measured by GnRH-induced LH release in vitro) increases with age up to days 20-25, following which there is a rapid decline. The sensitivity redevelops at the first proestrus (Castro-Vasquez and Ojeda, 1977; Johnson and Davis, 1981). Wilkinson and Moger (1981) further demonstrated the existence of a diurnal difference in pituitary sensitivity in female rats (aged 21, 27, and 30, but not 5 or 13 days old), with more LH being released by GnRH in the late afternoon than in the morning. Such differences were not seen with males. This differential sensitivity is proposed as a means by which hormone output might be synchronized at the first ovulation. It could also be a result of the pulsatile release of GnRH in the afternoon (see above).

In summary then, there does not appear to be a single trigger or a master controller for the transition from reproductive immaturity to competent adult reproductive functioning. The pubertal process, rather, probably results from the integration of
changes in several systems. It does appear certain, however, that major control of the processes regulating the pubertal transition is exerted by the brain. Again, it must be stressed that no one event in the maturation of the central nervous system controls the process, and there is a clear paucity of information on precisely how neurotransmitters are implicated in the critical induction of the pulsatile mode of GnRH secretion.

This Thesis is concerned with changes in a single neurotransmitter system - the opioid control of LH release before puberty. This, of course represents one of only a number of systems which may contribute to the control of gonadotropin release. Before presenting the body of research which makes up this Thesis, a consideration of the role of the endogenous opioid peptides in the control of LH release before puberty is presented.
ENDOGENOUS OPIOID PEPTIDES AND PUBERTY

ACTIONS OF OPIOIDS ON LH SECRETION

Pharmacological manipulation permits the investigation of the role of the endogenous opioid peptides (EOP) in the control of gonadotropin release and on the control of sexual maturation. The actions of the EOP can be revealed by the use of a narcotic antagonist such as naloxone, or the closely-related but longer-acting naltrexone. At the doses used in the experiments presented here naloxone is devoid of intrinsic agonist activity (Sawynok et al., 1979). Opioid antagonists exert their effect by displacing endogenous opioid ligands from their specific receptor. Prolonged action of these antagonists has profound effects on the opioid receptors, as will be discussed below. The pharmacological consequences of receptor occupation by an opioid antagonist is just the opposite of the actions of the endogenous opioid peptide. Similarly, opioid agonists can be administered to mimic and accentuate the effects of the EOP. By careful experimentation with both agonists and antagonists, one can reveal the nature of the opioidergic control over various physiological processes. The investigations presented here are
primarily concerned with the opioidergic control of gonadotropin release in the prepubertal rat. In some cases, because the relatively low levels of both FSH and LH in these immature animals can obscure the inhibitory action of opioids, the gonadectomized animal is used as the experimental model. The postcastration rise in circulating gonadotropin levels provides an amplification of the inhibitory action of opioid peptides on their release.

It is now well recognized that the control of gonadotropin release in immature female rats is under a tonic opioidergic inhibition (Bhanot and Wilkinson; 1983b). The subcutaneous administration of the opioid antagonist naloxone to prepubertal females is followed by a prompt rise in the circulating levels of LH (Blank et al., 1979; Ieiri et al., 1979). There is a frank sexual dimorphism in the prepubertal control of gonadotropin release, as the same dosage of naloxone is without effect on LH release in male rats until approximately 30 days of age (Blank et al., 1979; Ieiri et al., 1979; Maric et al., 1982; Schulz et al., 1982; Cicero et al., 1986). Early experiments with the opioid receptor agonist morphine provided somewhat equivocal results in immature male and female rats, only occasionally lowering basal LH
levels (Ieiri et al., 1979). The difficulty in demonstrating an inhibitory action of this opiate agonist on LH release is most probably due to the fact that basal LH levels in these animals are already quite low. Bhanot and Wilkinson (1983a), using acutely gonadectomized rats (48 hours) have demonstrated that the long-acting met-enkephalin analog FK 33-824 promptly and potently lowers castrate levels of LH.

The ability of naloxone to release LH in immature rats changes as the animal matures. Schulz et al. (1982) showed a progressive increase in naloxone-induced LH release in female rats from birth; maximal release was achieved from day 10 to day 16 of life. Following this, there was a sudden decrease which was maintained through approximately day 40 of life. Male rats did not respond significantly to naloxone until 12 days of age, and following that there was only a minimal response. Cicero et al. (1986) have carefully reinvestigated the ontogeny of the opioid-mediated control of LH release in male and female rats. They report that female rats 10 or 25 days of age responded to naloxone with prompt increases in circulating LH, while 15 and 20 day old animals did not respond
significantly. They also found that male rats did not have a significant LH response to naloxone from 10 to 30 days of age. However, a sudden increase in the sensitivity of LH to naloxone in male rats was noted between 30 and 35 days of life (coinciding with the onset of puberty in their colony), and continuing until 60 days of age. These investigators also examined the effects of morphine on circulating levels of LH in both males and females. They found that morphine was most effective in males in reducing LH levels from days 15 to 60 and only moderately effective in females from day 30 on. The differences in the reports of various laboratories may be explained on the basis of dose- and time-response considerations, and the difference in sample sizes. Cicero et al. (1986) used large groups of animals, and repeated their experiments several times to minimize biological variation. One fact which clearly emerges from the different studies is that there are major sex differences in the opioidergic control of gonadotropin release, and in the development and expression of this control.

Opioids act through specific receptors in the central nervous system. A number of receptor subtypes have been identified, and recently some progress has
been made in identifying which receptor subtypes are involved in the control of LH release. The best evidence available indicates that LH release is under the influence of u opioid receptors. Schulz et al. (1981) have shown that injection of B-endorphin antiserum into the mediobasal hypothalamus was effective in increasing the amount of LH released into the general circulation in prepubertal female rats, while an antiserum to met-enkephalin was ineffective. Anti-dynorphin had some LH-releasing ability, but was less potent than anti-B-endorphin. This was the only investigation examining the question of which EOP were involved in the control of LH release specifically in prepubertal animals. However, Forman et al. (1983) demonstrated that systemic administration of an antiserum to B-endorphin to adult male rats was able to induce a prompt increase in the amount of circulating LH. B-endorphin interacts primarily with the u-opioid receptor, although there is some interaction with the d-site (Holaday and Tortella, 1984; Herz, 1984). This peptide is also found in high concentration in the hypothalamus, and of the endogenous opioids, is the most potent in inhibiting LH release (Forman et al., 1983). Using specific opioid receptor antagonists,
Panerai et al. (1985) determined that both u and k receptors were involved in the control of LH release, but that the u receptor was approximately 10 times as sensitive as the k receptor. This study complements earlier work (Schulz et al., 1981) in which antibodies to the putative k-agonist dynorphin were not as effective as β-endorphin antiserum in elevating LH secretion.

**EFFECTS OF OPIOIDS ON SEXUAL MATURATION**

The experiments discussed so far have clearly demonstrated that LH secretion in the prepubertal female rat is under opioidergic control. The fact that opioidergic inhibition appears to change as the animal matures does not necessarily mean that the pubertal process itself is under such control. Experiments to investigate the effects of manipulations of the EOP systems on the development of sexual maturity must be examined. Sirinathsinghji et al. (1985) have shown that the injection of naloxone every 6 hours into female rats from day 1 through day 10 of life significantly advanced puberty, as determined by the day of VO and of first estrus. Administration of naloxone at times other than this neonatal period had no effect on the onset
of puberty. These results suggest that chronic neonatal treatment with the opioid antagonist naloxone has somehow modified the CNS mechanisms controlling GnRH secretion later in life. The authors further suggest that there is a central opioidergic inhibition of hypothalamic function which is lifted in the normal course of sexual development, and that neonatal treatment with naloxone was able to prematurely release this opioid "brake on prepubertal gonadotropin secretion." Sarkar and Lira (1984) have reported that daily treatment of female rats with a single injection of naloxone from birth to day 7 of life (i.e. through the so-called "critical period" during which sexual differentiation of the brain occurs) resulted in a delay of vaginal opening, and in a loss or a marked diminution of the ability of estradiol benzoate (EB; 10 μg/kg on days 21 and 23) to induce a surge of LH. It has also been shown that chronic opioid receptor blockade is able to significantly affect growth in infant rats. Zagon and McLaughlin (1983a) reported that once daily treatment of infant rats, from birth to day 21 of life, with the long-acting opioid antagonist naltrexone, in a dosage sufficient to block opioid receptors (mediating analgesia) for 24 hours a day, resulted in
a greater brain size and body weight than in control animals. Treated animals also had a thicker somatosensory cortex and a larger number of cerebellar glial cells and granule neurons. The stimulatory effect of opioid receptor blockade on brain and body growth supports a role for endogenous opioid peptides in the modulation of prepubertal growth. While the results of this initial study would suggest an inhibitory role of the endogenous opioids on prepubertal growth, a second investigation by this same group showed that the naltrexone-induced acceleration of growth was a dose dependent phenomenon. Decreasing the dosage of naltrexone from 50mg/kg to 1 mg/kg, (i.e. a dose sufficient to block opioid analgesia for only 4 hours per day), had the opposite effect. Somatic growth, as reflected by body and organ weight, was slowed relative to the control group, as were the attainment of certain developmental milestones, such as eye opening, walking and hair covering (Zagon and McLaughlin, 1983b).

The effects of treatment with opioid agonists on sexual development have also been studied; Zimmerman et al. (1974) were unable to show any effect of a daily injection of morphine from birth to day 21 of
life on vaginal opening. Weiner and Scapagnini (1974), on the other hand showed that treatment with morphine significantly advanced vaginal opening. These latter two studies differed in the dosages of morphine used and in the ages at which the drug was administered (birth to day 21 in the former study, from day 20 forward in the latter). As Sirinathsinghji et al. (1985) have shown, interference with the EOP system is only effective in disrupting normal sexual maturation within a narrow window. Furthermore, coadministration of the opioid antagonist nalorphine (which had no effect in and of itself on puberty) with morphine had no effect on the ability of morphine to advance vaginal opening in the study by Weiner and Scapagnini (1974), indicating that the morphine-induced advancement of VO was of a non-specific origin. Advis et al. (1982) used osmotic minipumps to provide a continuous supply of morphine to female rats starting at day 23 and continuing for 14 days. Such treatment resulted in a 6 day delay in VO and age at first diestrus. Lintern-Moore et al. (1979) have shown that a single injection of morphine sulphate to 21 day old female rats decreased the number of ovarian follicles already recruited to grow, within 24 hours. Chronic daily treatment with
morphine (7 days) sustained the decrease in the initiation of follicular growth. It is unclear whether or not this alteration of ovarian morphology can be attributed to a direct action of morphine on the prepubertal ovary, or is mediated through an effect on hypothalamic-control of pituitary gonadotropin release, although the latter is more probable.

Rabbi's group have shown that prenatal exposure to morphine has profound effects on neuroendocrine function later in life (Litto et al., 1983). The female offspring of mothers treated with morphine sulphate during days 5-12 of pregnancy showed a significant delay in vaginal opening when compared to offspring from saline treated controls. Serious derangements in the negative feedback sensitivity to estrogen was also seen in the female offspring exposed to morphine in utero. In the first instance, these animals did not respond to ovariectomy with an increase in circulating LH levels 24 hours later, as did the controls. Secondly, treatment with 10ug/kg of EB was without effect on the circulating LH levels, while it suppressed the castration-induced LH levels in the control animals. Male pups exposed in utero to morphine responded to orchidectomy and steroid
(testosterone propionate) replacement in a manner similar to the females.

It is clear from these experiments that our understanding of the role of the endogenous opioid peptides in the control of sexual maturation is incomplete. Many questions are raised by the provocative work of Sirinathsinghji et al. (1985). Why, for instance, is chronic treatment with naloxone effective in disrupting pubertal progression only when treatment is begun in the neonatal period? What would be the effect of constant exposure to opioid antagonists, e.g. with an osmotic minipump, on sexual development? Is it not possible that the repeated exposure to GnRH seen by the pituitary gland is the actual determinant of precocious puberty, and that it is not the "release of the opioid brake mechanism" which is advancing sexual maturation? Numerous experimental approaches are suggested to help answer some of these questions. Both endogenous opioid levels and opioid receptors could be measured throughout development with and without chronic opioid receptor blockade. The use of specific antisera to block the action of selected endogenous opioid peptides would do much to further our understanding of precisely which receptors and which
EOP systems are involved in the regulation of this developmental process. While much remains to be done, in the last several years, some progress has been made in our understanding of the means by which opioid peptides are able to exert some of their actions on the developing reproductive system.

MODE OF ACTION OF OPIOIDS ON SEXUAL MATURATION

It is clear from the experiments presented above, that the endogenous opioid system is important in the regulation of gonadotropin release in the prepubertal female. Evidence has also been developed which indicates that the EOP system may play an important role in the control of sexual maturation, at least in the female rat. Further evidence has been developed recently, particularly in this laboratory, which provides a possible explanation for the means by which opioid peptides may act to control gonadotropin release.

While neither group of investigators took particular notice of the phenomenon, the data of both Blank et al. (1979) and Schulz et al. (1982) reveals that naloxone is less able to release LH in older prepubertal females than in younger ones. Schulz et al. (1982) report that the peak sensitivity to
naloxone is seen in female rats between 10-16 days of age. Bhanot and Wilkinson (1983a) also demonstrated the existence of a puberty related attenuation of the ability of the opioid peptide FK 33-824 (FK) to reduce LH secretion in the acutely (48 hour) gonadectomized rat. They found a 4-fold decrease in the responsiveness of circulating levels of LH to the peptide in females in the 10 day interval preceding first ovulation. Similarly, in males a 50% decrease was seen transpubertally. Since the effect of FK on serum LH could be blocked by coinjection of the opioid antagonist naloxone HCl, FK presumably exerts its effects through an opioid receptor. Further experiments indicated that GnRH-stimulated release of gonadotropins from prepubertal pituitary glands (both male and female) in vitro was unaffected by the presence of either naloxone or FK, indicating that these substances were exerting their effect through a central, and presumably hypothalamic, site. It was proposed that the peripubertal decrease in opioid inhibition of LH could represent a neurochemical substrate for the loss of negative feedback sensitivity to gonadal steroids which occurs as sexual maturation nears.
In view of the earlier discussion on diurnal signals, it is interesting that Blank and Mann (1981) were able to detect diurnal differences in the ability of naloxone to release LH in prepubertal (30 day old) female rats, with greater release occurring in the morning than in the afternoon. They did not examine the development of this rhythm, or its role in the generation of a signal which might be involved in the initiation of puberty in the female rat. That is one of the major thrusts of this Thesis.

How, then, are opioids involved in the well described steroid feedback control of LH release? Bhanot and Wilkinson (1984) reported that the inhibitory influence of endogenous opiates is dependent upon the presence of gonadal steroids. Forty-eight hours following ovariectomy, prepubertal rats showed significant increases in circulating levels of LH following administration of naloxone, an opiate antagonist. However, 1 week after ovariectomy, naloxone was no longer able to induce an increase in circulating LH levels. Careful priming of the ovariectomized animals with EB resulted in a prompt reinstatement of the LH response to naloxone. The subsequent findings of Petraglia et al. (1984) confirm a role for steroids in the modulation of
endogenous opiate systems. Using the adult rat as a model, they observed a time-dependent disappearance of the ability of endogenous opioid peptides to inhibit LH secretion following gonadectomy, in agreement with the findings of Bhanot and Wilkinson (1984) in prepubertal animals. In some cases, Petraglia et al. (1984) were able to reinstate the LH response to naloxone with careful priming of the long-term ovariectomized animals with EB.

The observation that the presence or absence of gonadal steroids can so broadly affect an action of opioid agonists and antagonists leads one to suspect that gonadal steroids might influence opioid receptors. The effects of both short term and long term exposure to gonadal steroids, as well as the effects of castration on opioid binding sites have been examined. In addition, some studies have undertaken the examination of sex differences in opioid binding. These findings are discussed in detail in APPENDIX III. There have been no studies of changes in opioid receptors through sexual maturation.

With the recent development of the appropriate techniques, a number of reports have appeared in the
literature concerning changes in the processing of endogenous opioid peptides in the brain and the pituitary gland with the approach of puberty. Barden et al. (1981b) first studied changes in the level of β-endorphin in various brain nuclei with aging in the male rat. While they did not examine these changes trans-pubertally, they showed that as the rats aged from 3 to 24 months, there was a decrease in the β-endorphin content of virtually all areas examined with the exception of the median eminence, which was unchanged. Genazzani et al. (1983) reported that in humans, there is a high degree of correlation between age and serum β-endorphin levels from infancy through puberty. Based upon age-related increases in the circulating β-endorphin and β-lipotropin levels in the absence of changes in circulating ACTH during prepuberty, they suggest that there is either a change in the processing of the parent proopiomelanocortin molecule (POMC), or in the secretion of the processed peptides from the anterior pituitary gland as puberty approaches. Seizinger et al. (1984) have demonstrated that the newborn rat is equipped with all the machinery necessary to process POMC to β-endorphin and β-lipotropin, and therefore changes in the processing of the parent compound may
well have implications for sexual maturity. More recently, Martensz (1985) has examined the processing of β-endorphin in the hypothalamus, anterior and neurointermediate pituitary gland in female rats during sexual maturation. It was found that there are marked differences in the processing of β-endorphin in both lobes of the pituitary gland and the hypothalamus as sexual maturity is reached. The hypothalamus is probably much more important than the pituitary gland with respect to the opioidergic control of LH release in prepubertal females. With the approach of puberty, there was a significant increase in the C-terminal proteolysis of this peptide in the hypothalamus. Note that the resulting metabolites do not have opioid activity. This increase in the inactivation of hypothalamic β-endorphin could possibly explain some of the decrease in opioid inhibition of LH release as puberty approaches in the female rat.

The EDP are also known to control, to some degree, prolactin release, and may exert control over the pubertal process through this mechanism. In the rat, circulating levels of prolactin are increased following administration of opioid agonists such as morphine and β-endorphin, and depressed following
opioid blockade with naloxone (Guidotti and Grandison, 1978; Van Vugt et al., 1979; Blank, 1980). As has been shown by Van Vugt et al. (1979) these actions are mediated through a dopaminergic mechanism. The involvement of prolactin in the pubertal process has been described above (Voogt et al., 1969; Advis and Ojeda, 1978; Advis et al., 1981b). As Advis et al. (1981b) have shown, suppression of prolactin delays the onset of puberty in female rats. A prepubertal increase in opioidergic influence would result in an increase in circulating prolactin levels. Negro-Villar et al., (1973) have found a clear rise in peripheral prolactin levels in the male rat with sexual maturation. Furthermore, prolactin release in male rats is markedly diminished by naloxone treatment at 44 or 51 days of age, but is unaffected at 37 days (Maric et al., 1982). It is unclear what the age-related responses are in the female rat. This apparent increase in opioidergic tone with sexual maturation is not necessarily in conflict with the notion that opioidergic tone decreases with puberty as evidenced by the work of Bhanot and Wilkinson, (1983a), or by the findings of this Thesis. The presence of different opioid receptor subtypes in the hypothalamus allows for the
independent opioidergic control of gonadotropin and prolactin release. Note also that although gonadectomy ultimately uncouples the GnRH system from opioid control (see above, Bhanot and Wilkinson, 1984) it has no effect on the prolactin release mechanism.

It is the contention of this Thesis that a lessening of the opioidergic inhibitory tone exerted on LH release in the female rat occurs prepubertally. The opioidergic influence appears to wane in a diurnal manner, and it is proposed that this is important in the ability of the brain to generate a daily signal which in turn is necessary for the initiation of cyclic gonadotropin release. It is further proposed that the daily lessening of opioid inhibition of LH release is effected through changes in the hypothalamic opioid receptor complement. Diurnal and circadian changes in neurotransmitter receptors have been reported in a number of other systems. Some of this work will be reviewed here.
DIURNAL AND CIRCADIAN CHANGES IN NEUROTRANSMITTER RECEPTORS:

Circadian rhythms are those with a period of approximately 24 hours (circa = about; dies = day). These rhythms are intrinsic to the organism displaying them, and are set by an internal clock. In the absence of external time cues or zeitgebers such as an imposed photoperiod, these rhythms persist. In nature, these rhythms are often entrained to the length of the solar day, but when the external time cues are removed, the natural period of the circadian phenomena is seen to differ slightly from 24 hours, and the rhythm may "free run". Circadian rhythms are by no means the only rhythms of biologic interest. To cite just a few, there are also diurnal (day time), nocturnal (night time), ultradian (periodicity of less than a day), circchoral (periodicity of approximately one hour) and a host of other rhythms which are important in biological control. Diurnal phenomena are often incorrectly classed as circadian, but this is a misnomer unless the rhythm can be shown to persist in the absence of any external time cues. Rhythmic phenomena are not restricted to the entire organism. They can be found at the organismal, tissue and cellular levels.
neural regulation of circadian rhythms and their role in the control of physiological phenomena has been the subject of several reviews (Rusak and Zucker 1979; Takahashi and Zatz, 1982; Moore-Ede et al. 1983a,b; Turek, 1983). The central pacemakers for the generation of circadian rhythms are the suprachiasmatic nuclei (SCN), found in the anterior hypothalamus. Destruction of these nuclei results in the disruption of a wide variety of rhythmic phenomena, including locomotor, eating and drinking behavior (Raisman and Brown-Grant, 1977; Van Den Pol, 1980). Perinatal ablation of the SCN prevents the development of a number of circadian rhythms (Mosko and Moore, 1978). Moore (1983) has summarized the evidence pointing to the SCN as the generator of endogenous circadian rhythms.

Many endocrine and physiological phenomena are characterized by a strong rhythmic component. Since these phenomena are in part, regulated by neurotransmitters, it is not surprising that rhythmic variations in neurotransmitter levels and metabolism have been detected (e.g. Owasoyo et al., 1979; Lemmer and Berger, 1978; Saito et al., 1975; Perlow et al., 1978; Scheving et al., 1978) in the central nervous system. Furthermore, since neurotransmitters exert
their effects through interactions with specific membrane bound receptors, one might logically expect to see rhythmic variations in the population or binding characteristics of these receptors. Many drugs also exert their effects through an interaction with a specific receptor. It is well known that the effect of various drugs is not constant throughout the day (Reinberg and Smolensky, 1983). While diurnal or circadian variations in a number of factors, such as absorption, metabolism, and elimination may be responsible for these rhythmic variations in the efficacy of the pharmacologic agent in question, changes in the receptor complement may also play a role. Such thinking has led to the evolution of a rapidly increasing body of data which shows that neurotransmitter receptors do undergo rhythmic changes, and that these changes are often correlated with physiological effects.

Perhaps the most studied and best known example of a rhythmic change in a receptor population is that of the β-adrenergic receptor of the pineal gland. Under the influence of darkness, noradrenaline is released by the terminals of sympathetic neurons which originate in the superior cervical ganglia. The noradrenaline acts on β-adrenergic receptors in the
pineal gland, to increase cyclic AMP (cAMP) concentrations; this in turn is followed by the release of the pineal neurohormone melatonin. (Kebabian et al., 1977). There are well described circadian rhythms in the complement of pineal β-adrenergic receptors, the concentration of cAMP, and adrenergic agonist-induced increases of cAMP (Romero et al., 1975; Wilkinson, 1978; Mikuni et al., 1981); i.e. at the onset of darkness, the sympathetically released noradrenaline binds to pineal β-adrenergic receptors which have reached maximal levels at this time. This complement of receptors quickly declines to a minimum and then recovers, during the light period, to its maximum to coincide with further noradrenaline release. Such clear changes are not apparent in the pineal population of γ-adrenergic receptors (Sugden and Klein, 1985). This system is a model for the rapid regulation of neurotransmitter receptor populations.

The work of Axelrod's group (e.g. Romero et al., 1975) led Marian Kafka, Dieter Naber, and Anna Wirz-Justice to initiate a series of investigations which conclusively demonstrated the existence of daily rhythms in binding to a number of brain neurotransmitter receptors. They demonstrated changes
in $B_{\text{max}}$, but not $K_d$, for $\alpha$- and $\beta$-adrenergic (Kafka et al., 1981a), cholinergic (Kafka et al., 1981b), opiate (Naber et al., 1981), dopaminergic (Naber et al., 1980) and benzodiazepine (Kafka et al., 1983) receptors over the course of a 24 hour period. The binding to each class of receptor had its own characteristic wave form, with maxima and minima occurring at different times. Pharmacological and physiological manipulations were effective in modifying the rhythm with respect to phase, wave form, etc., but in all cases examined the differences were due to changes in receptor density ($B_{\text{max}}$) and not affinity ($K_d$) (Kafka et al., 1981b; Kafka et al., 1982; Naber et al., 1980; Wirz-Justice et al., 1980; Wirz-Justice et al., 1981; Wirz-Justice et al., 1982).

It was revealed that these rhythms were truly circadian, i.e. they persisted when the animals were placed into constant darkness for 2-3 days (Kafka et al., 1983). Kafka et al. (1985) have reported that ablation of the SCN abolishes the circadian rhythm in the number of $\alpha$-adrenergic and benzodiazepine receptors present in forebrain homogenates. However, the authors appear to have used very subjective criteria in their determination of differences in rhythms, as inspection of the data presented fails to
reveal any clear changes in the wave form of benzodiazepine binding following SCN destruction. Furthermore, while the α-adrenergic binding was disrupted with SCN ablation, it appears that a new circadian pattern of binding was imposed. A second interpretation of these data is that the circadian rhythms in neurotransmitter binding are controlled or at least modified by a second neural centre, in addition to the SCN. There is good evidence, at least in primates, of a second circadian oscillator (Moore-Ede, 1983; Fuller et al., 1981; Reppert et al., 1981). These experiments should be re-examined to clarify the connection between the SCN and the control of endogenous neurotransmitter rhythms.

The experiments cited above represent an important new step in the investigation of neurotransmitter binding sites. However, they involved the determination of receptor binding patterns in homogenates prepared from forebrain, i.e. whole brain less cerebellum. It is well known that circadian variations in neurotransmitter metabolism and content vary widely in their geographic distribution within the brain (see for example Koulu et al., 1985; Rosenwasser et al., 1985; Semba et al., 1984). Similarly, there is wide variation in the
geographic distribution of each specific neurotransmitter receptor (see for example Herkenham and Pert, 1982; Dohanich et al., 1985; Sutin and Minneman, 1985). It is apparent that the receptor rhythms referred to above represent the sum total of individual receptor rhythms in a number of geographically discrete brain regions. As such, variations in the receptor complement of a relatively small nucleus could well be masked in such a preparation. A large number of studies have been performed on cerebral cortex. While virtually all of the studies performed on whole forebrain homogenate revealed discernible diurnal variations in receptor binding, only approximately 40% of the studies performed with cortex revealed such differences (see for example Mogilnicka et al., 1986, Kafka et al., 1986a; Wesenmann et al., 1986a, b). Cortical binding patterns did not vary significantly over the course of the day, in fully 60% of the investigations undertaken (e.g. DiLauro et al., 1986; Fowler et al., 1985; Jenni-Eiermann et al., 1985). This discrepancy occurs independent of strain or ligand (Wirz-Justice et al., 1986). Approximately 60% of the studies which have used hypothalamic tissue have revealed a diurnal pattern of receptor binding (Wirz-Justice et al.,
1986). Of late, attempts have been made to quantify receptor rhythms in discrete brain nuclei, usually obtained with the Palkovits punch technique (Palkovits, 1973; Palkovits and Brownstein, 1983). For instance, a diurnal pattern of binding has been reported for imipramine in the suprachiasmatic nuclei of the SCN, while a smaller amplitude ultradian pattern was seen in the occipital cortex, and no rhythm at all in the caudate putamen (Wirz-Justice et al., 1983a; Krauchi et al., 1986). Tissue from less well-defined brain regions, containing several important brain nuclei (e.g. lateral hypothalamus) has also been shown to have significant temporal variations in receptor systems which are well correlated with physiological function (Krauchi et al., 1984).

Several investigators have been unable to replicate the findings of circadian variations in neurotransmitter receptors. (Fowler et al., 1985; Di Lauro et al., 1986; Campbell et al., 1986; Watanabe and Seeman, 1984). This fact in itself does not refute the findings of those laboratories which have seen the patterns. Neurotransmitter receptor rhythms in the CNS are somewhat fickle, and are quite sensitive to disruption. Moreover, there are a number
of purely technical issues which may account for the variation in binding patterns seen over time in different laboratories. In the first place, strain differences are being recognized as playing an important role in the determination of patterns of receptor binding. Strain differences in the wave form of adrenoceptor binding in hypothalamus were seen in SHR rats and their WKY controls. Other regional differences in receptor binding rhythms were seen in the cerebellum and pons-medulla (Wirz-Justice et al., 1983b). Besides the potential for discrepant results obtained from experiments performed on different strains of rats, there is a report of differences in rhythmic patterns of neurotransmitter binding between different lines of the same strain of rats. Jenni-Eiermann et al. (1986) have found differences in a number of neurotransmitter receptor rhythms in different regions of the brain between Kfm:Wist and Wistar-Fuellinsdorf strains of the Wistar rat. Thus, merely repeating an experiment with the same generic strain of animal may not assure duplication of results. Other variables which may account for differences in the rhythmic pattern of neurotransmitter receptor binding are the age and the sex of the animals. Many neurobiological laboratories
routinely use adult male rats for their experiments. Besides the present investigations, no other investigators have systematically looked for sex differences in the patterns of receptor binding. As has been reported, significant sex differences in the temporal pattern of opioid binding to mediobasal hypothalamus have been found (this Thesis, Jacobson and Wilkinson, 1985). Bruinink et al. (1983) have examined the ontogeny of diurnal variations of spiperone binding to forebrain homogenates. While no diurnal pattern of binding was apparent on day 15 of life, a definite pattern of both dopaminergic and serotonergic binding had developed by day 30. The pattern of spiperone binding was different yet again in 90 day old rats, although these latter experiments were performed in a different laboratory (Wirz-Justice et al., 1981). Jenni-Eiermann et al. (1985) examined the change in binding patterns of several ligands in male rats with age. While the results were highly variable, certain trends are apparent. In general, aging was accompanied by a decrease in the amplitude of the receptor binding rhythm. This was not a hard and fast rule, as high amplitude rhythms were seen for certain ligands. Furthermore, there
appeared to be a wide dispersion in the phase of the receptor binding rhythms with age.

Of course, the ultimate test of the physiological relevance of these temporal variations in receptor binding is to relate them to physiological processes which are known to be receptor-mediated, and which can be shown to vary in a similar manner. This thesis attempts to establish such a relationship between diurnal changes in hypothalamic opioid ([\( \text{H} \)-naloxone]-binding sites and the opioid inhibition of LH release in peripubertal female rats. Other physiological phenomena have been correlated with changes in receptor populations. Feeding behavior in the rat is under the control of two reciprocal systems. Feeding is stimulated by an \( \alpha_2 \)-adrenergic receptor-coupled center in the medial hypothalamus; presumably the paraventricular nucleus (Leibowits, 1970; Leibowitz, 1981; Marino et al., 1983), while it is inhibited through a \( \beta \)-adrenergic mechanism in the lateral hypothalamus, especially the perifornical area (Leibowitz, 1970; Leibowitz, 1981; Leibowitz and Rossakis, 1978). Krauchi et al. (1984) have demonstrated the coupling of feeding behavior to a bimodal pattern of \( \beta \)-adrenergic receptor binding to the lateral hypothalamus and a unimodal pattern of
a₂-adrenergic receptors in the medial hypothalamus.

Methamphetamine is known to have a powerful anorexigenic effect (Panskepp and Booth, 1973; Levitsky et al., 1981) and to affect adrenergic receptor populations as well (Banerjee et al., 1979; Reisine et al., 1982). Chronic methamphetamine treatment resulted in temporally separated effects on the two disparate adrenergic systems. A decrease in nocturnal food intake at dusk was correlated with diminished β-adrenergic binding in the lateral hypothalamus at that time, while an increase in the medial hypothalamic a₂-adrenergic receptor population at dawn was correlated with delayed termination of feeding. These results support the observation that feeding behavior is inhibited by local injection of amphetamine into the lateral hypothalamus, while it is stimulated by injection into the medial hypothalamus (Leibowitz, 1970). Follow-up experiments have shown that clonidine, an a₂-adrenergic agonist, stimulates feeding at dusk but not dawn, the times at which the medial hypothalamic populations of a₂-adrenergic receptors are at their maximum and minimum respectively (Krauchi et al., 1985).

Several studies have examined diurnal variations in pain sensitivity. Using the techniques of paw lick
latency (Oliverio et al., 1982; Puglisi-Allegra et al., 1982) and tail flick latency (Hendrie et al., 1983), somewhat different patterns of analgesia are revealed. However, these three investigations all showed an increase in analgesia during the dark phase, although the rhythms were somewhat out of phase. A reciprocal pattern in the binding of the opioid antagonist naloxone has been reported in whole forebrain homogenate (Naber et al., 1981) and in hypothalamus obtained from immature female rats (Jacobson and Wilkinson, 1985), i.e. the amount of naloxone bound decreases during the period in which analgesia increases. This reduction in available opioid binding sites may represent occupation of the receptor by endogenous ligand. Investigations of the diurnal pattern of β-endorphin content in the brain support this supposition, as highest levels are generally found in the dark phase (Simantov et al., 1980; Kerdelhue et al., 1983; Jung et al., 1984). Only one study has examined both opioid binding and analgesia concurrently. Hendrie et al., (1983; personal communication) examined pain latency over 24 hours and found that the maximum (0900 hours) and the minimum (2100 hrs) were accompanied by significant differences in opioid binding in the predicted
Additional studies are needed to directly compare opioid binding rhythms to patterns of opioid analgesia. Furthermore, these binding studies would be best performed on those regions of the CNS known to be involved in the mediation of analgesia, rather than whole forebrain.

Testing open field behavior has revealed the presence of circadian rhythms in the rat. Such behavior is partly under the control of adrenergic mechanisms; exploration is enhanced by α-adrenergic receptor stimulation, and inhibited by β-adrenergic stimulation at the onset of the dark phase, but not at the onset of the light phase (Gentsch et al., 1982; Vassout et al., 1982). A recent investigation has confirmed that both α- and β-adrenergic receptors are highest at the onset of the dark phase (Mogilnicka et al., 1986).

Because external light cues are of such importance in entraining circadian rhythms to the solar day, some attention has been directed to the question of circadian rhythmicity in the retina. There is a direct retinal-hypothalamic pathway, which links external time cues directly to the hypothalamic pacemaker, the SCN (Conrad and Stumpf, 1975; Rusak
and Zucker, 1979). The eye itself displays a daily rhythm in the shedding of photoreceptive disks from the outer rod segments (LaVail, 1976). This rhythm persists in the absence of external light cues (Goldman et al., 1980), and is not interrupted by either optic nerve transection (Teirstein et al., 1980) or pinealectomy (LaVail and Ward, 1978). These rhythms are thus truly circadian, and point to the existence of a circadian pacemaking mechanism contained wholly within the eye, and operating independently of the SCN. Terman and Terman (1985) have provided strong support for the existence of a visual system pacemaker with their recent finding of a circadian pattern in visual signal detectability which free runs in constant conditions, is entrained to the LD cycle, and persists after SCN ablation. The anatomical locus of this pacemaker is not known.

Concentrations of dopamine and melatonin, neurotransmitters important in the regulation of retinal function are also known to undergo circadian variations. (Pang et al., 1980; Besharse, 1982; Dubocovich, 1983; Wirz-Justice et al., 1984). However, to date a circadian rhythm in the pattern of retinal benzodiazepine binding has not been detected (Wirz-Justice et al., 1985). Dubocovich et al. (1985)
were unable to detect any significant variation in binding to dopamine D₂-receptors over 24 hours. However, following one week in constant light, there was a reduction in D₂-receptor binding, coincident with an increase in retinal dopamine. The first report of a diurnal, and possibly circadian rhythm in neurotransmitter binding in the retina has recently appeared (Jacobson et al., 1986). There is a significant decrease in retinal β-adrenergic binding when rats enter the dark phase (Jacobson et al., 1986; Wilkinson et al., 1986). This may be related to a dark-induced increase in retinal noradrenaline levels (Hadjiconstantinou and Neff, 1984). These results are based on preliminary experiments involving only a single point on the binding curve, and as such cannot be attributed to changes in either the B_{max} or the K_{d} of the binding site.

Circadian rhythms in neurotransmitter binding are obviously the reflection of fairly rapid (hours) changes in receptor number. Regulation of neurotransmitter receptor populations has traditionally been thought to be a slower (days-weeks), long-term process, as, for instance, that following denervation or chronic pharmacological treatment (Müller and Seeman, 1978; Friedhof and
Miller, 1983). The mechanism(s) behind the rapid change in receptor populations necessary for the appearance of a circadian rhythm are not clear. However, several possibilities exist. Agonist-induced desensitization of β-adrenergic receptors provides a model system for the study of rapid receptor regulation. Two distinct mechanisms are invoked to explain the phenomenon. In the first case, chronic occupation of the receptors by agonists results in a reduction in binding which is slow (days) to recover. However, brief exposure to agonist causes a rapid conversion of the β-adrenergic receptors to a form which is unable to couple to the N protein of the adenylate cyclase complex. This uncoupled receptor is internalized into the cytoplasm. Internalized receptors are not available for interaction with ligand, but the fate of the internalized receptors is such that they may be degraded, and lost permanently, or they may be recycled to the plasma membrane (Lefkowitz et al., 1984; 1985; Chuang et al., 1980). This sequence of internalization/recycling can be seen in a matter of minutes (see Wilkinson and Wilkinson, 1985, and references therein). Several polypeptide hormones are also known to be internalized through receptor-mediated endocytosis
(Carpentier et al., 1984; Hazum et al., 1980), and there is a suggestion that desensitization to these hormones may involve the phenomenon of clustering (Amsterdam et al., 1980). The rapidity and reversibility of this mode of receptor regulation make this a suitable model for the control of receptor populations in a circadian manner.

A circadian pattern in muscarinic binding to brain tissue was recently shown to be a function of changes in receptor occupancy over the course of the day (Mash et al., 1985). When the receptors are occupied by endogenous ligand, they are unavailable for binding to exogenous labeled antagonists. As the number of occupied receptors changed over the course of the day, so did the number of binding sites available for detection with conventional binding techniques. However, by incubating the tissue in such a manner as to encourage dissociation of the endogenous ligand-receptor complexes, Mash et al. (1985) were able to demonstrate that the actual number of receptors did not change over a 24 hour period. Diurnal or circadian rhythms may thus be due to changes in the use of the receptors which are present in the cell membrane, rather than to changes.
In the synthesis, degradation, or recycling of the receptors themselves.

In summary, circadian and diurnal rhythms in neurotransmitter binding have been described for a number of receptors. Some investigations have described an association between these rhythms and similar rhythms in the effector systems. These rhythms have been shown to be reproducible, but are exquisitely sensitive to methodological variations, and are affected by the sex, age, strain, housing conditions, etc., of the experimental animals. While the mechanisms through which these rhythmic changes take place are unclear, as yet, they may have profound implications for the homeostasis of the organism. While much of the research in this area has been purely descriptive, the emphasis is shifting to an understanding of the physiological consequences of these rhythms and their disruptions. Finally, and as outlined elsewhere in this Thesis (see Discussion), the study of receptor binding rhythms in intact tissue may lead to a greater understanding of the subtleties, and physiological consequences of variations in receptor numbers.
METHODS AND MATERIALS

ANIMALS:

Sprague-Dawley rats were obtained from either Canadian Hybrid Farms (Hall Harbour, N.S.) or from Canadian Breeding Farms and Laboratories (St. Constant, Quebec). Long Evans Rats were obtained from Canadian Breeding Farms and Laboratories. Swiss Webster mice were obtained from High Oak Ranch, Goodwood, Ontario. C57BL/6J mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. Golden Syrian Hamsters (GS) ($\textit{Mesocricetus auratus}$), as well as cardiomyopathic (CHF 147) and noncardiomyopathic albino control (CHF 148) strains of this species were obtained from Canadian Hybrid Farms. All animals were housed in the University Animal Care Centre. The room temperature was maintained at 21±1°C. Food (Purina rat chow) and filtered tap water were freely available at all times. Nursing litters consisted of 8 animals, all of the same sex. Litters were weaned at 21 days of age. Upon weaning, animals were housed in groups of 4-6 animals of the same sex. Unless otherwise noted, animals were maintained under a light:dark photoperiod (LD) of 14:10 hours, with the lights on at 0700 hours and off at 2100 hours. With the
exception of neonates, animals were present in the animal care colony for at least 7 days before being used in an experiment.

**DRUGS:**

Phenoxybenzamine, cocaine, and dibenamine were gifts from Dr. M. Vohra, Dalhousie University. Levorphanol, dextrophan, and clonazepam were gifts from Hoffman-LaRoche, Vaudreuil, Quebec. DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride) was a gift from Dr. S. Agurell, Astra Lakemedal AB, Sodertalje, Sweden. Morphine sulphate was donated by British Drug Houses (BDH), Toronto, and ouabain by Dr. M. Horackova, Dalhousie University. Naloxone hydrochloride was provided by E.I. Dupont de Nemours, Garden City, N.Y. Xylamine hydrochloride (N-(2-chloroethyl)-N-ethyl-2-methylbenzylamine hydrochloride), AF-64 (acetylcholine mustard hydrochloride), and chloroethyl clonidine were purchased from Research Biochemicals Incorporated, Wayland, MA. 2-dimethylaminoethyl chloride was obtained from Aldrich Chemicals, Milwaukee, WI. Sodium pentobarbital (Somnotol) was purchased from M.T.C. Pharmaceuticals. Estradiol valerate was obtained from Research Plus
Steroid Laboratories, Denville, N.J. Gonadotropin releasing hormone (GnRH) was obtained from Dr. A.F. Parlow, NIH/NIAMDDK, Bethesda, Md. 3H-naloxone and 3H-flunitrazepam were purchased from Amersham Canada, Mississauga, Ontario. D-al\textsubscript{3}-d-leu\textsubscript{5}-enkephalin (DADLE) was obtained from Sigma chemicals, St. Louis, Mo, as were U-14624 (1-phenyl-3-(2-thiazolyl)-2-thiourea), isoproterenol, desipramine hydrochloride, phentolamine, yohimbine, and clonidine. All other drugs were obtained commercially.

EXPERIMENTAL PROCEDURES

Unless specifically indicated in the methods section, experimental details (e.g. sex and age of animals, incubation conditions, concentrations and dosages of drugs, etc.) are presented in the figure legends.

ADMINISTRATION OF DRUGS:

Unless otherwise noted, all drugs were administered by subcutaneous (s.c.) injection in a volume of 0.1 ml of physiological saline.
COLLECTION AND PROCESSING OF BLOOD:

Animals were sacrificed by decapitation. Trunk blood was rapidly collected into 12 x 75 mm glass test tubes. The blood was stored for 24 hours at 4°C, following which it was centrifuged (600 x g, 4°C, 20 minutes) and the supernatant serum aspirated with a clean pasteur pipette. The serum was transferred to small plastic beakers and capped. Serum was stored at -20°C or at -80°C until assayed.

ANIMAL SURGERY:

Animals older than 9 days of age were anesthetized with diethyl ether. Anesthesia was maintained by nose cone inhalation of ether. Animals aged 9 days or less, including neonates, were anesthetized by placing in a -20°C freezer for 15 minutes. Neonates of both sexes were gonadectomized through an abdominal incision, under binocular magnification if necessary. Wounds were closed with fine silk surgical sutures. Older females had both ovaries exteriorized through a single lateral flank incision. The uteri were clamped and the gonads removed. The abdominal wall was closed with silk sutures, and the skin wounds closed with stainless steel wound clips.
**LH RESPONSE TO NALOXONE**

Naloxone HCl was dissolved in physiological saline and administered subcutaneously to rats at a dosage of 2.5 mg/kg, and in a volume of 0.1 ml. Control animals received a s.c. injection of saline. Fifteen minutes after the administration of drug or vehicle, the animals were sacrificed by decapitation and trunk blood was collected. The blood was processed as described above, and the LH concentration in the serum was determined. Unless otherwise specified, the naloxone was administered at 1400 hours.
RADIOIMMUNOASSAY OF LH

The concentration of LH in rat serum and in Medium 199 in which pituitary glands had been incubated was determined with a double antibody radioimmunoassay. Reagents were provided by Dr. A.F. Parlow of the NIADDK, Bethesda, Md.

BUFFERS:

LH assay buffer was a phosphate buffered saline. Each litre contained 0.189 g NaH2PO4·H2O, 1.225 g Na2HPO4, 1.0 g gelatin, and 0.1 g thimerosol (as a preservative). The pH of the LH assay buffer was 7.6.

Normal rabbit serum (NRS) buffer was similar to the LH assay buffer, except that it contained no gelatin, and was made up in distilled water in place of normal saline. Furthermore 18.3 g of disodium EDTA and 5.0 ml of NRS were added per litre of buffer. The pH of this buffer was 7.6.

ASSAY REAGENTS:

The LH standard used was LH RP-1. The first antibody was anti-rat LH S-8. LH I-6 was iodinated and used as the labeled hormone in the assay. The "second" or precipitating antibody was a sheep anti-
rabbit IgG, and was obtained from Daymar Laboratories, Toronto, Ont.

**RADIOIODINATION OF LH:**

LH was radioiodinated with a slight modification of the procedure of Greenwood et al. (1963). 20 \( \mu \)l of LH I-6 and 50 \( \mu \)l of 0.5 M phosphate buffer (pH 7.6; prepared without NaCl) were added directly to a vial containing 1 mCi of Na\(^{25}I\). A 25 \( \mu \)l aliquot of freshly prepared chloramine-T (2 mg/ml of 0.05 M phosphate buffer) was added to the reaction vial. The reaction was terminated after 90 seconds by the addition of a 50 \( \mu \)l aliquot of sodium metabisulfite (2.4 mg/ml of 0.05 M phosphate buffer) directly to the reaction vial. After further dilution with a 200 \( \mu \)l aliquot of LH assay buffer, the reaction mixture was transferred with a Pasteur pipette to a disposable 18.5 cm x 0.9 cm chromatography column packed with Sephadex G-75 superfine. Fifteen 0.5 ml fractions were manually collected while the column was eluted with LH assay buffer. 5 \( \mu \)l aliquots of each fraction were counted on an LKB Clinigamma 1272 solid crystal scintillation gamma counter. Two fractions were collected, the peak of radioactivity and the first fraction following the peak. These
Fig. 1 Elution profile of 125I-labelled LH from an 18.5 x 0.9 cm column of Sephadex G-75 superfine. Fractions marked with an arrow were collected and pooled.
fractions were pooled and used as the "tracer" in the radioimmunoassay (see Fig. 1).

**LH RADIOIMMUNOASSAY PROTOCOL**

The LH assay was performed as has been described by Wilkinson et al. (1980). All samples were assayed in duplicate. A series of standards was prepared by serial dilution. The concentrations of these standards ranged from 0 to 500 ng of LH-RP-1 per 100 ul. Samples consisting of 100 ul of standard or 75-150 ul of rat serum were brought to a final volume of 500 ul with LH assay buffer. First antibody (LH S-8) diluted 1:120 with NRS buffer was added in a volume of 200 ul. Tubes were vortexed and allowed to remain at 4°C for 24 hours. On the second day, 20,000-30,000 cpm of 125I-LH were added to the tubes in a volume of 100 ul of LH assay buffer. The tubes were again vortexed and allowed to remain at 4°C. On day 4 of the assay, a 200 ul aliquot of a 10 fold dilution (in LH assay buffer) of second antibody was added, and the tubes were vortexed and replaced at 4°C. On day 7 of the assay, 800 ul of ice cold LH assay buffer were added to each tube (except the total

1 The final in tube dilution of the first antibody was 1:120000.
count tubes which contained only $^{125}$I-LH. The tubes were centrifuged (600 x g, 4 °C, 20 minutes) and decanted. The tubes were allowed to dry, and the pellets were counted in an LKB Clinigamma 1272 solid crystal scintillation gamma counter. Non specific binding was ascertained by incubating a set of tubes in the absence of sample and first antibody.

CALCULATION OF LH CONCENTRATION IN SAMPLES:

The LKB 1272 Clinigamma solid crystal scintillation gamma counter is equipped with a microprocessor designed to automatically determine concentrations from radioimmunoassay data. The method used for the calculation of concentrations is the log-logit method. Briefly, a linear regression is performed on the data obtained when the logit of the concentration of the standards is plotted against logit $B/B_0$. This last factor is defined as:

$$\text{logit} \left( \frac{B}{B_0} \right) = \ln \left[ \frac{B}{(B_0-B)} \right]$$

where:

$B_0$ = the number of cpm in the absence of LH standard

$B$ = the number of cpm in the presence of increasing amounts of LH
The regression line is used to calculate LH concentrations from cpm. The sensitivity of the assay was in all cases less than 2 ng. Interassay and intra-assay coefficients of variation were 9.4% and 6.8% respectively. Values are expressed as ng NIADDKD rat LH RP-1/ml ± standard error of the mean (s.e.m.).

**RADIOIMMUNOASSAY OF PROLACTIN**

The radioimmunoassay for prolactin is identical to the radioimmunoassay for LH, with the following exceptions. The prolactin used for radiiodination with the chloramine-T method was prolactin 1-5. The standard used was prolactin RP-2. The standard curve was constructed to contain 0.039 – 10 ng of prolactin RP-2 per 0.1 ml. The first antibody used was anti-rat prolactin S-8. The intra-assay coefficient of variation was < 6%. Results are expressed as ng NIADDK rat prolactin RP-2/ml of serum ± s.e.m.
PREPARATION OF BRAIN SLICES

Following sacrifice by decapitation, the brains were rapidly removed and placed into ice cold buffer. For experiments involving mouse tissue, the buffer was 50 mM TRIS (pH 7.4 @ 25° C). For those involving rat and hamster tissue, the buffer was Dulbecco's phosphate buffered saline (DPBS) (Gibco Laboratories, Burlington Ont.). All subsequent manipulations of brain tissue were carried out on ice, unless otherwise noted. Blocks of mediobasal hypothalamus (MBH) were removed by longitudinal cuts medial to the temporal lobes, and by coronal cuts rostral to the mammillary bodies and caudal to the optic chiasm. The depth of the tissue block extended to the level of the anterior commissure. Prisms of cerebral cortex measuring approximately 5 x 2 x 2 mm, trimmed of white matter, were prepared at the same time. The tissue blocks were cut into 300 (rat, hamster) or 400 (mouse) thick coronal slices with a McIlwain tissue chopper, and transferred to fresh ice-cold buffer. The slices were carefully separated with fine jewellers forceps under binocular magnification, and transferred to individual wells of a Linbro multi-well tissue culture plate (24 wells, catalog # 76-
063-05; Flow Laboratories) containing buffer, ligand, and displacer (see below).

**3H-NALOXONE BINDING ASSAY:**

The incubation volume was 500 l. 3H-naloxone was introduced in a volume of 20 l to give the appropriate in-well concentration. Non-specific binding was determined in the presence of 10^{-5} M naloxone hydrochloride, introduced as a volume of 20 l. Saturation curves were constructed by incubating tissue slices in the presence of increasing concentrations of radioligand. Competition curves were prepared by incubating tissue slices in the presence of a single concentration of radioligand with increasing concentrations of unlabelled displacer. The times and temperatures of incubation are provided in the description of the individual experiments.

At the end of the incubation period, a 20 l aliquot of the incubation medium was removed for counting and determination of the actual in-well "free" equilibrium radioligand concentration, using the following formula:
\[
\frac{\text{nM}}{\text{eff}} = \frac{1000}{\text{V}} \cdot \frac{1}{(2.22 \times 10^3)} \cdot \frac{1}{\text{SA} \cdot \text{fac}}
\]

where:
- \( \text{nM} \) = free concentration of radioligand in nanomoles/litre
- \( \text{eff} \) = efficiency of counting in %
- \( \text{V} \) = assay volume in ml
- \( \text{SA} \) = specific activity of radioligand in \( \text{Ci/mmol} \)
- \( \text{fac} = \frac{(\text{V} \times 1000)}{\text{aliq}} \), where \( \text{aliq} = \) volume of aliquot removed in ml

The remainder of the buffer was quickly removed with a Pasteur pipette, and the slices were washed with ice cold buffer (2 x 0.5 ml, 2 x 5 minutes). The slices were picked up with small circles of Whatman GF/B glass microfibre filter and transferred directly to plastic scintillation vials containing 1.8 ml of Aquasol II scintillation fluid (New England Nuclear Corp, Montreal, Quebec). Following vigorous shaking, the vials were allowed to stand for several hours before being counted on an LKB Rackbeta liquid scintillation counter. Counting efficiency varied from 38% to 51%. A minimum of 5 slices per concentration were used for the determination of total binding, and a minimum of 3 slices were used for the determination of non-specific binding. Five to ten representative tissue slices were removed before the start of the assay, and weighed to obtain a mean wet slice weight. The results of the binding
experiments are expressed as fmoles bound per mg of wet tissue weight.

CHARACTERIZATION OF OPIOID BINDING TO BRAIN SLICES:

It is a basic tenet in performing binding studies, that the nature of the binding site must be characterized (Burt, 1985). To this end, a number of experiments were performed to characterize the nature of the binding site, and to determine optimal conditions for the performance of this novel assay. These studies are listed below, and will be described in detail:

- Time course of binding
- Demonstration of reversibility of binding
- Demonstration of penetration of ligand into slices
- Competition studies
- Demonstration of stereospecificity of binding site
- Demonstration of saturability of binding
- Regional distribution of binding sites
- Effect of tissue concentration on binding
- Effect of number of washes on binding
- Effect of metabolic inhibitors on binding
- Thermolability of binding
- Effect of various drugs on binding
- Effect of chronic exposure to naloxone on $^3$H-naloxone binding
TIME COURSE OF BINDING:

Slices were incubated with a free concentration of $^3$H-naloxone estimated to be close to the $K_i$ (2nM). The incubations were terminated at a variety of time intervals. For incubations carried out at 30°C, the time intervals were 5, 15, 30, 60, 120, 180 and 240 minutes. For incubations carried out at 4°C, the time course was extended up to 24 hours.

DEMONSTRATION OF REVERSIBILITY OF BINDING:

Slices were incubated with a free concentration of $^3$H-naloxone estimated to be close to the $K_i$ (2nM) until equilibrium (as determined from time course experiments) was attained. At this point, naloxone HCl was added to the assay wells to achieve a concentration of $10^{-5}$ M, known to maximally displace $^3$H-naloxone from brain slices. The incubation was terminated and slices removed 1, 2, 5, 10, 20, and 30 minutes after the addition of the cold naloxone. The amount of $[^3$H]-naloxone bound to the tissue slices was determined and expressed as a percent of the maximal (equilibrium) binding in the absence of displacer.
DEMONSTRATION OF PENETRATION OF LIGAND

The penetration of the $^3$H-naloxone into the slice was checked autoradiographically, using a modification of the method of Robertson and Leslie (1985). Slices of cerebral cortex (300μ) were prepared from a 30 day old female SD rat, and incubated with $^3$H-naloxone (2 nM) in the normal manner. A 1 cm square mound of minced rat liver was applied to a precooled microtome chuck and was frozen at -20° C. Immediately following the second wash, the coronal sections of cerebral cortex were carefully applied to the liver mince and allowed to freeze at -20° C. Horizontal sections of the cortical slice were cut at 14 μ for a cryostat, and were thaw mounted onto slides previously subbed with 0.25% gelatin. The slides were dried overnight in a refrigerator at 4° C.

LKB $[^3]$H-Ultrofilm was applied to the labeled sections of cerebral cortex under a Kodak Wratten 6B safelight. These were then taped into X-ray cassettes and exposed for 50 days at an ambient temperature of 4° C. The film was then developed under safelight illumination in Kodak D-19 high contrast developer for 5 minutes at 20° C, rinsed in tap water for 30
seconds, placed in Kodak fixer for 5 minutes, and washed in running tap water for 20 minutes. The film was air dried, and relative autoradiographic density was measured with a Frederick Haer Quandens IDEAS image analysis system.

**COMPETITION STUDIES**

Slices were incubated with a free concentration of \( ^{3}H \)-naloxone estimated to be close to the \( K \) (2nM), in the presence of increasing concentrations of a variety of competing non-radioactive ligands. Incubations were carried out for 2 or 3 hours as indicated in the results section. Competition studies were performed using the following competing ligands: naloxone HCl, morphine sulphate, and DADLE.

**DEMONSTRATION OF STEREOSPECIFICITY**

Stereospecificity studies were carried out by constructing competition curves using the biologically active opioid levorphanol and its inactive stereoisomer dextrophan.
DEMONSTRATION OF SATURABILITY OF BINDING:

Saturation curves were constructed by incubating brain slices with increasing concentrations of $^3$H-naloxone. Non-specific binding (NSB) was determined in the presence of $10^{-5}$M of naloxone HCl, a concentration determined to maximally displace $^3$H-naloxone from brain slices (see results). Specific binding was defined as the difference between total binding (that performed in the absence of any competing displacer) and non-specific binding (that determined in the presence of displacer). The tissue was incubated until equilibrium binding, as determined from the time course experiments, had occurred.

REGIONAL DISTRIBUTION OF $^3$H-NALOXONE BINDING SITES:

Medial basal hypothalamic and cerebral cortical slices were prepared as previously described. Brain slices were prepared from cerebellum as follows. The cerebellum was removed from the brainstem, and freed of the cerebellar hemispheres. The vermis was sliced coronally into 400 slices. Binding studies were performed on the slices obtained from these three distinct brain regions.
EFFECT OF TISSUE CONCENTRATION ON BINDING

In order to assess whether or not the radioligand was being degraded by tissue enzymes, increasing numbers of mediobasal hypothalamic slices were incubated with a free concentration of $^3$H-naloxone estimated to be close to the $K_d$ (2nM). The amount of $^3$H-naloxone specifically bound to 1, 2, 3 or 4 brain slices per well was determined.

EFFECT OF NUMBER OF WASHES ON BINDING

Slices of mediobasal hypothalamus were incubated with a single concentration of $^3$H-naloxone estimated to be close to the $K_d$ (2nM). Upon termination of the incubation, the slices were washed with ice cold buffer 1, 2, 3, 4 or 5 times, and the change in specific binding was assessed.

EFFECT OF METABOLIC INHIBITORS ON BINDING

Slices of mediobasal hypothalamus were incubated with a single concentration of $^3$H-naloxone estimated to be close to the $K_d$ (2nM) in the presence of either $10^{-2}$ M sodium azide or $10^{-3}$ M Ouabain. The assays were run to equilibrium, and the specific binding of $^3$H-naloxone to the slices of MBH determined. Control slices were incubated in the absence of metabolic
inhibitors. Because these initial experiments were performed in TRIS buffer, they were repeated in a sodium containing buffer (DPBS).

**THERMOLABILITY OF BINDING**

Slices of mediobasal hypothalamus were placed in buffer and heated to 70° C, at which temperature they were maintained for 20 or 45 minutes. Following this treatment, the slices were incubated with 2 nM of $^3$H-naloxone and specific binding was assessed.

**EFFECT OF VARIOUS DRUGS ON BINDING:**

The effect of the presence of several drugs known to displace or disrupt opiate binding was examined. Slices were incubated with a single concentration of $^3$H-naloxone in the presence of one of the following: phentolamine ($10^{-5}$ M), phenoxybenzamine ($10^{-5}$ M), ascorbic acid ($10^{-3}$ M). The effects of clonidine ($10^{-5}$ M), yohimbine ($10^{-5}$ M) and clonazepam ($10^{-5}$ M) were also investigated in a similar manner.
EFFECT OF CHRONIC EXPOSURE TO NALOXONE ON
$^3$H-NALOXONE BINDING:

Polymeric silicone elastomer pellets containing 30 mg of naloxone each were prepared according to the method of Isom et al. (1978). A small plexiglass mold containing 5 hemicylindrical grooves (radius: 0.35 cm; length: 30 cm) was sprayed with IMS silicone spray parting agent (IMS Company, Auburn, Ohio). Naloxone hydrochloride was well mixed with a commercially available dimethylpolysiloxane silicone compound (Silastic 382 medical grade elastomer, Dow Corning, Midland, Mi.) with a metal spatula. Mineral oil was incorporated into this mixture until a homogeneous preparation was obtained. The ingredients were present in the following proportions (by weight): naloxone hydrochloride: silastic 382: mineral oil (1:1:5:1). Vulcanization was induced by the addition of stannous octanoate (Dow Corning Catalyst M). The uncured naloxone-containing silastic was rapidly transferred to the plexiglass mold, and smoothed into the grooves. Curing was allowed to proceed at room temperature for 24 hours, following which the silastic strips were removed, weighed, and cut with a razor blade into appropriate sized pellets, each containing 30 mg of naloxone.
hydrochloride. Control pellets were prepared by incorporating crystalline α-lactose (Sigma Chemicals, St. Louis, Mo.) in place of naloxone hydrochloride.

An infrascapular subcutaneous tunnel was prepared under light ether anesthesia, and pellets were implanted into adult male Swiss Webster mice. The skin was closed with stainless steel wound clips, and the pellets were allowed to remain in situ for 7 days. Following this, mediobasal hypothalamic slices were prepared, and saturation curves were constructed for $^3$H-naloxone binding. To determine whether or not residual naloxone affected the binding, $^3$H-naloxone binding to mediobasal hypothalamic slices was determined 24 hours after the pellets had been removed.

ANALYSIS OF RECEPTOR BINDING DATA

Saturation curves were analyzed, and binding parameters (receptor density: $B_{max}$; receptor affinity $K_d$; standard error of the raw data: $S_{D_{e rad}}$) determined, using the method of Zivin and Waud (1982), as adapted for use with an Apple IIe or an Apple Macintosh microcomputer. For illustrative purposes, lines fitting the data were drawn freehand. Unless specifically indicated on a figure, the $S_{D_{e rad}}$
for all saturation curves presented in the results section was less than or equal to 0.12. Hill coefficients for the saturation curves were always close to 1.0.

The competition curves were analyzed, and IC₅₀ values calculated using a triad of programs obtained from the Biomedical Computing Technology Information Center, Vanderbilt University, Nashville, TN. This triad of programs is composed of PREFIT, a data preparation program, ALLFIT, a four parameter logistic dose-response analysis program, and GRAFIT, a program designed to plot the fitted four parameter logistic dose response curve. All three of these programs were written in Applesoft BASIC by M.H. Teicher, and the first two of these are adaptations of programs by the same name originally written by DeLean, Munson and Rodbard (1977).
RETINAL BINDING EXPERIMENTS

PREPARATION OF RETINAL QUADRANTS

Animals were sacrificed by decapitation. In most cases, experiments were performed during the light phase of the LD cycle. However, for those experiments which were performed during the dark phase, animals were decapitated and retinas dissected in dim red light. The retinas were removed by incising the cornea and gently expressing the lens and the vitreous humor with gentle pressure on the sclera from the posterior aspect of the globe. Further gentle pressure caused the intact retina to be extruded. The retina was gently placed into ice cold buffer (DPBS). Under binocular microscopic magnification, the retinas were trimmed and any adherent vitreous humor was removed. The retinas were carefully cut into four equal quadrants. A red filter was placed over the microscope illuminator for when dissecting retinas removed during the dark phase.
RETINAL $^3$H-NALOXONE BINDING ASSAY

The retinal $^3$H-naloxone binding assay is very similar to the brain slice $^3$H-naloxone binding assay. The incubation volume was 500 l. $^3$H-naloxone was introduced in a volume of 20 l to give the appropriate in-well concentration. Non-specific binding was determined in the presence of $10^{-6}$ M naloxone hydrochloride, introduced as a volume of 20 l. Saturation curves were constructed by incubating tissue slices in the presence of increasing concentrations of radioligand. Competition curves were prepared by incubating tissue slices in the presence of a single concentration of radioligand with increasing concentrations of unlabelled displacer. The times and temperatures of incubation are provided in the description of the individual experiments.

At the end of the incubation period, a 20 l aliquot of the incubation medium was removed for counting and determination of the actual in-well "free" radioligand concentration, in the same manner as described for the brain slice assay. The remainder of the buffer was quickly removed with a Pasteur pipette, and the quadrants were washed with ice cold...
buffer (2 x 0.5 ml, 2 x 5 minutes). The fragments of retina were picked up with small circles of Whatman GF/B glass microfibre filter and transferred directly to plastic scintillation vials containing 1.8 ml of Aquasol II scintillation fluid (New England Nuclear Corp., Montreal, Quebec). Following vigorous shaking, the vials were allowed to stand for several hours before being counted in an LKB Rackbeta liquid scintillation counter. Counting efficiency varied from 42% to 51%. A minimum of 5 pieces per concentration were used for the determination of total binding, and a minimum of 3 were used for the determination of non-specific binding. Five to six representative retinal quadrants were removed before the start of the assay, and weighed to obtain a mean wet fragment weight. The results of the binding experiments are expressed as fmoles bound per mg of retinal wet weight.
CHARACTERIZATION OF OPIOID BINDING TO RETINAL FRAGMENTS

The binding of \( ^3H \)-naloxone to retinal tissue was characterized in a manner similar to that for the brain slices. The time course of the binding and its reversibility were examined, as were the ability of various compounds to compete with \( ^3H \)-naloxone for binding sites in this preparation. Included in the competition experiments was a demonstration of the stereospecificity of the binding site. Finally the effect of increasing the amount of tissue present during the binding assay was examined.

**TIME COURSE OF BINDING:**

Retinal quadrants were incubated with a single concentration of \( ^3H \)-naloxone (1 nM). The incubations were terminated at 0.17, 0.5, 0.75, 1, 1.5, 2, 3 and 4 hours.

**DEMONSTRATION OF REVERSIBILITY OF BINDING:**

Retinal quadrants were incubated with a single concentration of \( ^3H \)-naloxone estimated to be close to the \( K_d \) (1nM), until equilibrium (as determined from time course experiments) was attained. At this point, naloxone HCl was added to the assay wells to achieve
a concentration of $10^{-5}$ M. The incubation was terminated and slices removed 1, 5, 10, 20, and 40 minutes after the addition of the cold naloxone. The amount of $^3$H-naloxone bound to the retinal fragments was determined and expressed as a percent of the maximal (equilibrium) binding in the absence of displacer.

COMPETITION STUDIES AND DEMONSTRATION OF STEREOSELECTIVITY:

Competition curves were constructed by incubating retinal quadrants with a free concentration of $^3$H-naloxone estimated to be close to the $K_d$ (1 nM), in the presence of increasing concentrations of competing non-radiactive ligands. Incubations were carried out for 2 hours. Stereospecificity studies were carried out by constructing competition curves using the biologically active opioid levorphanol and its inactive stereoisomer dextrorphan. The amount of radioligand bound at a given concentration of competitor was expressed as the percent of radioligand bound in the absence of competitor. Competition curves were analyzed and $IC_{50}$ values calculated using the PREFIT, ALLFIT and GRAFIT programs as described above.
DEMONSTRATION OF SATURABILITY OF BINDING:

Saturation curves were constructed by incubating retinal quadrants with increasing concentrations of \(^3\text{H}\)-naloxone. Non-specific binding (NSB) was determined in the presence of \(10^{-5}\)M of naloxone HCl, a concentration determined through competition experiments to maximally displace \(^3\text{H}\)-naloxone from retina (see results). Specific binding was defined as the difference between total binding (that performed in the absence of any competing displacer) and non-specific binding (that determined in the presence of displacer). The tissue was incubated until equilibrium binding, as determined from the time course experiments, had occurred. The saturation curves were analysed, and the binding parameters \((B_{\text{max}}, K_d, \text{and } S_D_{\text{erad}})\) determined using the method of Zivin and Waud (1982), as adapted for an Apple IIe microcomputer.

EFFECT OF TISSUE CONCENTRATION ON BINDING

Increasing numbers of retinal quadrants were incubated with a free concentration of \(^3\text{H}\)-naloxone estimated to be close to the \(K_d\) (1 nM). The amount of \(^3\text{H}\)-naloxone specifically bound to 1, 2, 3 or 4 retinal fragments per well was determined.
SEX DIFFERENCES IN RETINAL OPIOID BINDING

Sex differences in retinal opioid binding were studied by examining the binding of $^3$H-naloxone to retinal quadrants prepared from male and female animals. Preliminary experiments were performed, at one or two concentrations of $^3$H-naloxone, following which saturation curves were constructed, and binding parameters determined.

STRAIN DIFFERENCES IN RETINAL OPIOID BINDING

The effect of retinal pigmentation on opioid binding was examined by studying $^3$H-naloxone binding to retinal quadrants obtained from different strains of animals. Pigmented retinae were obtained from Long Evans rats, while non pigmented retinae were obtained from Wistar and Sprague Dawley rats. Preliminary experiments were performed with one or two concentrations of $^3$H-naloxone, following which full saturation curves were constructed and binding parameters determined. Some experiments were also performed examining the binding of $^3$H-naloxone to retinae obtained from GS hamsters, as well as CHF 147 cardiomyopathic hamsters and CHF 148 noncardiomyopathic albino control hamsters.
30 day old female rats were sacrificed at 0500 hrs under dim red illumination. Retinae were obtained and dissected under binocular magnification with dim red illumination. The retinal fragments were transferred to culture plates which were wrapped in aluminum foil to exclude ambient light, and the specific binding of a single concentration of $^3$H-naloxone was determined. A similar group of retinal fragments obtained within 30 minutes, under normal room illumination, was incubated at the same time, but without protection from ambient light and the binding of $^3$H-naloxone likewise determined.
DIURNAL RHYTHMS IN THE RAT

ONTOGONY OF DIURNAL VARIATIONS IN THE ABILITY OF NALOXONE HC1 TO RELEASE LH IN PREPUBERTAL RATS

Male and female rats (n=7 per group) aged 2, 9, 23, 26, 28, 30 and 32 days were injected with either naloxone HC1 (2.5 mg/kg, s.c in 0.1 ml saline) or with normal saline (0.1 ml, s.c.). After 15 minutes, the animals were decapitated and trunk blood was collected for determination of LH concentration. These experiments were carried out at 0800 and 1700 hrs.

24 HOUR PATTERN OF $^{3}$H-NALOXONE BINDING TO HYPOTHALAMIC SLICES FROM PREPUBERTAL FEMALE RATS

30 day old female rats were sacrificed at 0900, 1300, 1700, 2100, 0100 and 0500 hours in groups of 5. The 2100, 0100 and 0500 hr groups were sacrificed under dim red light. The brains were rapidly removed and treated as described above. Slices of mediobasal hypothalamus were incubated with a single concentration (10 nM) of $^{3}$H-naloxone, and the 24 hour binding pattern of this ligand was determined.
SATURATION ANALYSIS OF DIURNAL CHANGES IN \(^{3}H\)NALOXONE BINDING TO BRAIN TISSUE FROM PREPUBERTAL RATS

Mediobasal hypothalamic tissue from 26 day old female rats, obtained at both 0800 and at 1700 hrs was incubated with increasing concentrations of \(^{3}H\)naloxone, and saturation binding curves were constructed. Binding parameters \(K_d\) and \(B_{max}\) were calculated from these curves.

EFFECT OF DISSOCIATION OF ENDOGENOUS LIGAND FROM BRAIN TISSUE ON THE DIURNAL PATTERN OF \(^{3}H\)NALOXONE BINDING TO BRAIN TISSUE

In an attempt to remove endogenous ligand from brain tissue, slices of cerebral cortex and of mediobasal hypothalamus obtained in the early morning and the late afternoon were prepared as described above and treated in one of two ways.

A) Approximately 20 slices of tissue were placed in a petri dish with 30 ml of ice cold DPBS and subjected to two 90 minute washes while being shaken at 60 Hz. This procedure was carried out on ice. Following the 3 hours of washing, \(^{3}H\)-naloxone binding to the brain slices was assessed as described above.
B) Approximately 20 slices of mediobasal hypothalamus were preincubated in 20 ml of 50mM glycine in normal saline (pH 2.5) for 15 minutes. The tissue was then processed as above, and the amount of $[^3H]$-naloxone specifically bound was determined.

**ONTGENY OF DIURNAL VARIATION IN $[^3H]$-NALOXONE BINDING TO HYPOTHALAMIC TISSUE IN PREPUBERTAL RATS**

Male and female rats aged 9, 15, 23, 26, and 30 days were sacrificed at 0700, 1000, 1300 and 1600 hrs. Mediobasal hypothalamus and cerebral cortex were incubated with a single concentration (10 nM) of $[^3H]$-naloxone, and the amount specifically bound was determined.

**ATTEMPTED MODIFICATION OF DIURNAL RHYTHMS IN $[^3H]$-NALOXONE BINDING TO BRAIN TISSUE**

**NEONATAL GONADECTOMY AND DIURNAL OPIOID BINDING RHYTHMS**

Neonatal animals of both sexes arrived at the university animal care facility at 1 or 2 days of age, and were gonadectomized on day 9 of life, as described earlier, or sham operated. A sham operation consisted of inducing light ether anesthesia, opening
the skin, and closing the incision with stainless steel wound clips. The animals were allowed to recover and placed with their mothers until they were weaned on day 21 of life. On day 30 of life, the animals were sacrificed at 0700 and 1700, slices of mediobasal hypothalamus were prepared, and $^3$H-naloxone binding studies were performed.

**NEONATAL ANDROGENIZATION WITH EV AND DIURNAL OPIOID BINDING RHYTHMS**

Litters of male and female rats arrived at the University Animal Care facility on either day 0 or day 1 of life. The animals were anesthetized by cooling, and injected with 100 g of estradiol valerate in 0.1 ml of sesame oil, or with oil alone. The injection sites were painted with flexible collodion, and the animals were allowed to warm under incandescent lights or in a 37°C incubator. After a suitable time period (30 minutes to 1 hour) the animals were replaced in their home cages with their mothers. Animals were weaned on day 21 of life. On day 30 of life, the animals were sacrificed at 0700 and at 1700, brain slices were prepared, and $^3$H-naloxone binding was determined.
INDUCTION OF DIURNAL RHYTHMS IN OPIOID BINDING WITH PMSG:

PMSG was used to treat immature animals in an attempt to induce diurnal variations in opioid binding to slices of mediobasal hypothalamus. On day 18 of life, female rats were injected with 7.5 I.U. of PMSG in 0.1 ml of saline, or with saline alone, at 1000 hrs. On day 20 of life, the animals were sacrificed at 0700 hrs and at 1700 hrs, and $[^3H]$-naloxone binding was determined.

EFFECT OF TREATMENT WITH BARBITURATE ON DIURNAL RHYTHM IN OPIOID BINDING:

The effect of a barbiturate on the expression of the diurnal difference in opioid binding sites was studied by injecting a group of 30 day old female rats with sodium pentobarbital (31 mg/kg, i.p.) or with saline (0.1 ml, i.p.) at 1000 hrs. The animals were sacrificed at 1700 hrs, and the binding of a single concentration of $[^3H]$-naloxone to slices of mediobasal hypothalamus was assessed. This binding was compared to that in identical (but untreated) groups of animals sacrificed at 0700 hrs and 1700 hrs that same day.
EFFECT OF CHRONIC TREATMENT WITH BARBITURATE ON DEVELOPMENT OF SPONTANEOUS PUBERTY

Female rats were injected daily with sodium pentobarbital (31 mg/kg, i.p.) or with saline (0.1 ml, i.p.) at 1100 hrs., from day 23 of life to day 30 of life. The animals were weighed daily and inspected for VO. On the day of VO, the animals were sacrificed, and the ovaries and uteri were removed, cleaned, and weighed, and the presence of corpora lutea or ova was determined.

EFFECT OF CONSTANT DARKNESS ON DIURNAL RHYTHM IN OPIOID BINDING

Female rats were placed into a room at 21 days of age, and kept in constant darkness (DD) until day 30 of life. All manipulations of the animals (changing of water bottles, cleaning of cages, etc) were performed under a dim red light. On day 30 of life, the animals were sacrificed in the dark (under dim red illumination) at 0700 hrs. and 1700 hrs. The brains were placed into ice cold buffer and transported to the laboratory, where mediobasal hypothalamic slices were prepared and $^3$H-naloxone saturation curves were constructed. Binding was assessed in slices of mediobasal hypothalamus from
control animals housed under normal (LD 14:10) lighting conditions at the same time.

EFFECT OF CONSTANT LIGHT ON DIURNAL RHYTHM IN OPIOID BINDING:

21 day old female rats were placed into conditions of constant light (LL) until day 30 of life. On day 30 of life, the animals were sacrificed at 0700 hrs and 1700 hrs. Medial basal hypothalamic slices were prepared from these animals as well as control animals housed under normal lighting conditions (LD 14:10) and $^3H$-naloxone binding was determined.
EXPERIMENTS WITH ESTRADIOL VALERATE

THE EFFECT OF ESTRADIOL VALERATE ON

$^3$H-NALOXONE BINDING TO MOUSE HYPOTHALAMUS

Sexually mature female Swiss webster mice (25-30 g), or C57BL/6J mice were injected with either 200 g of estradiol valerate (EV) in 0.1 ml of sesame oil, or with the vehicle alone. Beginning two weeks following injection, vaginal smears were examined daily for the presence of persistent estrus.

Two to four months post-injection, the animals were sacrificed by decapitation. The brains were quickly removed and placed into ice cold buffer. Saturation curves were constructed for the binding of $^3$H-naloxone to slices of mediobasal hypothalamus. Ovaries, uteri, thymus glands and anterior pituitary glands were collected and weighed. In some experiments, the pituitaries were placed into organ culture and LH release was studied as described below.
Anterior pituitary glands were bisected in situ, and placed into organ culture as described by Wilkinson and Moger (1981). Briefly, the hemi-glands were placed upon stainless steel grids in the central well of a tissue culture dish containing 500 μl of Medium 199 modified to contain HEPES buffer (0.01 M; Sigma Chemicals), glucose (5g/L), sodium pyruvate (0.001 M) and penicillin and streptomycin (50 IU/ml and 50 g/ml respectively). Cultures were maintained in a 37°C, water saturated atmosphere of 95% air/5% CO₂. Two 60 minute preincubations were carried out to allow spontaneous secretion to stabilize. A sample of incubation medium was obtained after 120 minutes for the determination of basal release of LH, following which the pituitary glands were placed into fresh medium containing $3.4 \times 10^{-9}$ M GnRH. A sample of medium was taken for the determination of GnRH-stimulated LH release after an additional two hour incubation.
DSP4 EXPERIMENTS

PREPARATION OF DSP4

DSP4 was dissolved in physiological saline immediately before use. In the event that the dissolved drug was not used promptly, it was kept on ice. For those experiments in which it was desirable to examine the effects of the cyclized (i.e. aziridinium ion) form of the drug, it was dissolved in 0.05 M phosphate buffered saline (pH 7.4) and allowed to stand at a temperature of 37° C for 45 minutes. Such treatment has been shown to favor production of the aziridinium ion (Zieher and Jaim-Etcheverry, 1980).

EFFECTS OF DSP4 ON BRAIN CATECHOLAMINE CONTENT

Blocks of mediobasal hypothalamus from animals which were treated with DSP4 (80 mg/kg, i.p. in 0.1 ml saline), or saline (0.1 ml, i.p.) and sacrificed at various time intervals after treatment, or from animals used in selected experiments detailed below, were removed and immediately frozen in liquid nitrogen (-210° C). The samples were stored at -80° C until assayed for catecholamine content by Dr. C.J. Gibson, Department of Pathology, University of
Western Ontario, London, Ontario. Noradrenaline and dopamine were measured by high performance liquid chromatography (HPLC) with electrochemical detection (ED) after purification on mini-alumina columns (Felice et al., 1978). Hypothalami were sonicated in 0.1N perchloric acid (with EDTA and sodium metabisulfite added as an antioxidant). Dihydroxybenzyamine (Sigma Chemical Co., St. Louis, Mo.) was added as internal standard. Sonicates were centrifuged at 12,000 x g for 15 minutes. The acid supernatants were purified on 50 mg mini-alumina columns, and the catecholamines eluted in 250 l of 0.5N acetic acid. A 100 l aliquot was injected on the chromatograph. The LC system consisted of a dual piston pump (model 510, Waters Assoc., Mississauga, Ont.); Rheodyne manual injector (model 7125; Mandel Scientific, Rockwood, Ont.); a Whatman precolumn (ODS-Partisil reverse phase C18 particles, 30 ) and Altex Ultrasphere I.I column (5 reverse phase C18 particles; 4.6 mm x 15 cm; Beckman-Altex, Toronto, Ont.). The amines were detected with an LC-4B amperometric controller and glassy carbon electrode (TL-5) (Bioanalytical Systems, West Lafayette, In.) set at a potential of +80 V versus a Ag/AgCl electrode. The mobile phase, pumped at a flowrate of
1.5 ml/minute, was 94% 0.05 M sodium acetate, 0.1 M EDTA and 0.32 mM sodium octyl sulfate (Eastman Kodak, Rochester, N.Y.) and 6% methanol.

DSP4 AND LH SECRETION

EFFECT OF DSP4 ON LH RESPONSE TO NALOXONE

Groups of animals were injected with 50 mg/kg of DSP4 dissolved in 0.1 ml saline, with 50 mg/kg of cyclized DSP4 dissolved in 0.05 M phosphate buffer, pH 7.4, with 200 mg/kg of U14624 (a norepinephrine synthesis inhibitor which blocks dopamine-β-hydroxylase) dissolved in 0.1 ml of dimethyl sulfoxide (DMSO), or with 0.1 ml of saline at 1200 hours. All injections were given intraperitoneally. Each of these treatment groups was further divided into two subgroups. At 1400 hours one of these subgroups was injected with saline (0.1 ml, s.c.), and the other was injected with naloxone HCl (2.5 mg/kg in 0.1 ml saline, s.c.). Fifteen minutes following this injection, the animals were sacrificed by decapitation. Trunk blood was collected and processed for determination of LH concentration.
TIME COURSE OF DSP4 EFFECT ON LH RESPONSE TO NALOXONE

DSP4 (50 mg/kg i.p.) or saline was administered to groups of rats as described above. 2 hours, 1 day, 2 days and 7 days after the administration of DSP4, groups of animals were sacrificed and the LH response to naloxone was determined, as described above.

EFFECT OF DSP4 ON LH LEVELS

Twenty-four day old female rats weighing 40-45 grams were ovariectomized and allowed to recover for 48 hours. At 1400 hours on day 26 of life, animals were injected with saline (0.1 ml, i.p.) or with DSP4 (50 mg/kg, i.p., 0.1 ml). The control animals and half of the DSP4 animals were sacrificed 15 minutes after the administration of drug or vehicle. The second half of the DSP4 group was sacrificed 45 minutes after the administration of the drug. Trunk blood was collected from all groups, at sacrifice, and LH concentration was determined. This experiment was also repeated with xylamine HCl (50 mg/kg, i.p.), and the sampling times were 10, 20, and 40 minutes after administration of the drug.
DOSE RESPONSE CURVE FOR EFFECT OF DSP4 ON LH LEVELS

Twenty three day old female rats were ovariectomized and allowed to recover for 48 hours. On day 25 of life, the animals were injected with saline (0.1 ml), or DSP4 (1, 5, 10, 25, or 50 mg/kg; i.p.; 0.1 ml). The animals were sacrificed 45 minutes after treatment, and the concentration of LH in trunk blood was determined. This experiment was repeated using xylamine HCl (1, 2.5, 5, 10, 25 or 50 mg/kg) in place of DSP4.

EFFECT OF DSP4 ON PROLACTIN LEVELS

Twenty four day old female rats weighing 40-45 grams were ovariectomized and allowed to recover for 48 hours. At 1400 hours on day 26 of life, animals were injected with saline (0.1 ml, i.p.) or DSP4 (50 mg/kg, i.p., 0.1 ml). The control animals and half of the DSP4 animals were sacrificed 15 minutes after the administration of drug or vehicle. The second half of the DSP4 group was sacrificed 45 minutes after the administration of the drug. Trunk blood was collected from all groups, at sacrifice, and prolactin concentration was determined.
DSP4 and RECEPTOR BINDING

EFFECT OF DSP4 IN VITRO ON $^3$H-NALOXONE BINDING

Cerebral cortical slices (400) were incubated with a free concentration of $^3$H-naloxone estimated to be close to the $K_d$ (2nM), and $^3$H-haloxone binding to these slices was determined in the standard manner. The binding assays were carried out in the presence (10$^{-5}$ M) or absence of DSP4.

EFFECT OF DSP4 IN VITRO ON $^3$H-NALOXONE BINDING SATURATION CURVE

Slices of mediobasal hypothalamus (400) were prepared in the standard fashion. Saturation curves were constructed for the specific binding of $^3$H-naloxone to these slices incubated in the presence and in the absence of 10$^{-5}$ M DSP4.

ABILITY OF DSP4 TO COMPETE FOR $^3$H-NALOXONE BINDING SITES

Slices of cerebral cortex and of mediobasal hypothalamus were incubated with a single concentration of $^3$H-naloxone (2nM) in the presence of increasing concentrations of DSP4 (10$^3$-10$^{-4}$ M). The amount of $^3$H-naloxone bound to the slices was expressed as % of the binding in the absence of DSP4.
EFFECT OF DSP4 IN VIVO ON $^3$H-NALOXONE BINDING
- SATURATION CURVE

Animals were injected with DSP4 (50 mg/kg, i.p., in 0.1 ml saline) or saline (0.1 ml, i.p.) and 400 slices of mediobasal hypothalamus were prepared at various time intervals following administration of drug or vehicle. The brain slices were processed in the standard manner, and saturation curves were constructed for the amount of $^3$H-naloxone bound.

EFFECT OF IN VITRO NORADRENALINE UPTAKE INHIBITORS ON IN VITRO DSP4 ACTION ON OPIOID BINDING

Slices of mediobasal hypothalamus were prepared in the standard fashion, and incubated under 1 of four conditions. Group 1 (BUF/BUF) was preincubated for 15 minutes in buffer, and then incubated normally with a single concentration (2 nM) of $^3$H-naloxone. Group 2 (DES/DSP4) was preincubated for 15 minutes in buffer containing $10^{-5}$ M desipramine hydrochloride, and then incubated normally with a single concentration (2 nM) of $^3$H-naloxone, in the presence of $10^{-5}$ M DSP4. Group 3 (BUF/DSP4) was preincubated for 15 minutes in buffer, and then incubated normally with a single concentration (2 nM) of $^3$H-naloxone, in the presence of $10^{-5}$ M DSP4. Group 4 (DES/BUF) was
preincubated for 15 minutes in buffer containing 10^{-5} M desipramine hydrochloride, and then incubated normally with a single concentration (2 nM) of ^{3}H-naloxone. Following the incubations, the amount of ^{3}H-naloxone specifically bound to the mediobasal hypothalamic slices was determined.

In a second set of experiments, cortical slices were preincubated for 60 minutes with DSP4 (10^{-6} M) with and without added desipramine or cocaine (10^{-5} M). [^{3}H]-naloxone was then added and incubations continued in the presence (or absence) of the uptake inhibitors for 3 hours.

**EFFECT OF IN VIVO DESIPRAMINE ON IN VIVO DSP4 ACTION ON OPIOID BINDING**

Animals were placed into 1 of 4 treatment groups. Group 1 (SAL/SAL) received an injection of saline (0.1 ml, i.p.) which was followed at 15 minutes by a second saline injection. Group 2 (SAL/DSP4) received an injection of saline (0.1 ml, i.p.) which was followed in 15 minutes by an injection of DSP4 (50 mg/kg, i.p., in 0.1 ml of saline). Group 3 (DES/SAL) received an injection of desipramine hydrochloride (10 mg/kg, i.p., in 0.1 ml of saline) which was followed in 15 minutes by an injection of saline (0.1
ml, i.p.). Group 4 (DES/DSP4) received an injection of desipramine hydrochloride (10 mg/kg, i.p., in 0.1 ml of saline) which was followed in 15 minutes by an injection of DSP4 (50 mg/kg, i.p., in 0.1 ml of saline). Two hours after the second injection, the animals were sacrificed, slices of mediobasal hypothalamus were prepared and incubated with a single concentration of $^{3}$H-naloxone (2 nM), and specific binding was assessed.

**EFFECT OF DSP4 ON $^{3}$H-FLUNITRAZEPAM BINDING**

**EFFECT OF IN VITRO DSP4 ON $^{3}$H-FLUNITRAZEPAM BINDING**

Slices of mediobasal hypothalamus (230) were prepared in the standard manner. The slices were incubated in the presence or absence of DSP4 (10^{-5} M), and the binding of $[^3]$H-flunitrazepam at two concentrations (2 nM, 8 nM) assessed. The assay for the determination of the specific binding of $[^3]$H-flunitrazepam to brain slices has been described by Whitaker et al., (1984). It is similar to the assay for the specific binding of $[^3]$H-naloxone to brain slices. The slices were incubated in a volume of 500 l of 50 mM TRIS buffer. The $[^3]$H-flunitrazepam was present in a volume of 125 l. Non-specific
binding was determined in the presence of \(10^5\) M clonazepam, delivered in a volume of 5 l. Incubations were carried out for 3 hours, on ice, and the assay wells were protected from light throughout the incubation period. The incubation was terminated, and the slices were washed (on ice, 2 x 1 ml, 2 x 5 minutes) with ice cold buffer and treated as in the \([^{3}\text{H}]\)-naloxone assay.

**EFFECT OF IN VIVO DSP4 ON \(^{3}\text{H}\)-FLUNITRAZepam BINDING**

Animals were injected with DSP4 (50 mg/kg, i.p. in 0.1 ml saline) or with saline alone (0.1 ml, i.p.). Two hours following the administration of drug or vehicle, the animals were sacrificed, and slices (230) of mediobasal hypothalamus, cerebral cortex, and cerebellum were prepared. The slices were incubated with a single concentration (4 nM) of \(^{3}\text{H}\)-flunitrazepam, as described above, and the amount of specific binding was determined.
DSP4 AND PUBERTY

EFFECT OF ADMINISTRATION OF DSP4 ON SPONTANEOUS SEXUAL MATURATION

Prepubertal female rats were divided into control and experimental groups. Control groups received a single injection of saline (0.1 ml, i.p.) and experimental groups received a single injection of DSP4 (50 mg/kg, i.p., in 0.1 ml saline). The injections were administered on day 23 or day 29 of life. One group received two injections of DSP4, on day 24 and on 26 of life. Controls for this group received matching saline injections. A group of 5 day old female animals was also treated, but in this case, the injections (both saline and DSP4) were administered s.c. Animals were weighed daily and observed for vaginal opening (VO). On the day that VO was observed, the animal was sacrificed. The ovaries, uteri, and in some cases adrenal glands were removed, trimmed of excess fat, and weighed. In some experiments, the ovaries were examined for evidence of ovulation, and the number of corpora lutea (CL) and/or ova noted.
EFFECT OF DSP4 ON THE DEVELOPMENT OF INDUCED LH SURGES IN IMMATURE RATS

EFFECT OF DSP4 ON THE INDUCTION OF AN LH SURGE BY GONADAL STEROIDS

Gonadotropin surges were elicited in intact immature female rats by estradiol-progesterone treatment (Caligaris et al., 1968). Twenty-four day old rats were randomly assigned to 4 groups of seven. At noon they received a subcutaneous injection of 10 g of estradiol benzoate (EB) in sesame oil. On day 27 of life, the animals were further treated as follows:

Group 1 - i.p. injection of 50 mg/kg DSP4 in saline at 1000 hrs and s.c. injection of 1 mg progesterone (P) in oil at 1200 hrs.

Group 2 - s.c. injection of 1 mg of P in oil plus i.p. injection of 50 mg/kg DSP4 in saline at 1200 hrs.

Group 3 - s.c. injection of 1 mg of P in oil at 1200 hrs.

Group 4 - no further treatment.
All animals were sacrificed by decapitation at 1700 hrs. Trunk blood was collected, allowed to clot and stored at 4°C overnight. The sera were stored at -70°C until assayed for their LH content. At the time of sacrifice, all animals were examined for VO, and for signs of uterine stimulation.

A second series of experiments was performed to determine if naloxone was capable of interfering with the action of DSP4 on the gonadal steroid-induced generation of an LH surge. As above, twenty-four day old rats were randomly assigned to 4 groups of seven. At 1000 hrs they received a subcutaneous injection of 10 g of estradiol benzoate (EB) in sesame oil. On day 27 of life, the animals were further treated as follows:

**Group 1** - (EP) 1 mg of progesterone in oil (s.c.) at 1200 hrs.

**Group 2** - (ED) 50 mg/kg (i.p.) of DSP4 at 1000 hrs. 1 mg of progesterone in oil (s.c.) at 1200 hrs.

**Group 3** - (END) 2.5 mg/kg of naloxone (s.c.) at 1000 hrs, then 50 mg/kg of DSP4
(i.m.) at 1010 hrs. 1 mg of progesterone in oil (s.c.) at 1200 hrs.

Group 4 - (EN) 2.5 mg/kg of naloxone (s.c.) at 1000 hrs. 1 mg of progesterone in oil (s.c.) at 1200 hrs.

All animals were sacrificed by decapitation at 1700 hrs. Trunk blood was collected, allowed to clot and stored at 4°C overnight. The sera were stored at -70°C until assayed for their LH content. At the time of sacrifice, all animals were examined for VO, and for signs of uterine stimulation.

**EFFECT OF DSP4 ON PMSG-INDUCED OVULATION IN IMMATURE RATS**

Twenty-four day old female rats were randomly assigned to groups of 10. All animals received subcutaneous injections of 5 I.U. of pregnant mare serum gonadotropin (PMSG) in saline at 1000 hours. Forty-eight hours later, experimental animals were injected with DSP4 (50 mg/kg, i.p.) and control animals received a second injection of saline (0.1 ml). Twenty-four hours following this, the animals were examined for VO, sacrificed by decapitation, and the ovaries and uteri removed. The ovaries were examined microscopically for the presence of corpora
lutea, and the presence or absence of ova in the oviduct was noted. In one experiment, the protocol was varied so that the DSP4 was administered simultaneously with the PMSG (i.e. 72 hours before sacrifice).

Further experiments were performed to see if an effect of DSP4 on PMSG-induced ovulation could be influenced by pretreatment with naloxone hydrochloride. Twenty four day old female SD rats were randomly assigned to one of four groups of 8 animals. At 1000 hrs they received a subcutaneous injection of 7.5 I.U of NIH PMSG. On day 26 of life (i.e., the day of induced "proestrus"), the animals were further treated as follows:

**Group 1** - (Control) No further treatment.

**Group 2** - (N) Naloxone 2.5 mg/kg s.c. at 1000 hrs

**Group 3** - (D) DSP4 50 mg/kg i.p. at 1000 hrs.

**Group 4** - (N+D) Naloxone 2.5 mg/kg s.c. at 1000 hrs. Followed by DSP4 50 mg/kg i.p. at 1010 hrs.
Twenty-four hours following this, the animals were examined for VO, sacrificed by decapitation, and the ovaries and uteri removed. The ovaries were examined microscopically for the presence of corpora lutea, and the presence or absence of ova in the oviduct was noted.
RESULTS

BRAIN SLICE ASSAY

TIME COURSE OF $[^{3}\text{H}]$-NALOXONE BINDING TO HYPOTHALAMIC BRAIN SLICES

The time course of the binding of $[^{3}\text{H}]$-naloxone (2nM) to slices of mediobasal hypothalamus is illustrated in Fig. 2. The binding was near maximal at 120 minutes at 30° C, and was half maximal at 30 minutes. At this temperature, the binding was stable for at least 4 hours. Unless otherwise stated, assays were incubated at 30° C for 180 minutes to ensure equilibrium binding. Non-specific binding, i.e. binding determined in the presence of 10$^5$ M naloxone hydrochloride, reached maximal values in 30 minutes, and represented 25%-35% of total binding at this concentration of ligand. Fig. 3 illustrates the time course of binding at 4° C. Saturation occurs before 12 hours, and the binding is stable for up to 30 hours.
Fig. 2 Time course of specific binding of [3H]-Naloxone (2 nM) to 400 µ-slices of MBH from adult male SW mice. Incubation carried out at 30°C.
Fig. 3. Time course of binding of [3H]Naloxone (2 nM) to forebrain slices of adult male SJL mice. Incubation carried out at 4°C.

SPECIFIC BINDING (fm/mg)

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<th>Time (hours)</th>
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10 20 30
REVERSIBILITY OF [3H]-NALOXONE BINDING TO HYPOTHALAMIC BRAIN SLICES

Slices of mediobasal hypothalamus were incubated with [3H]-naloxone (2 nM) at 30°C for 180 minutes. Displacement of the bound [3H]-naloxone was achieved by the addition of a large excess of unlabelled naloxone hydrochloride (10^{-5} M). Fig. 4 illustrates that displacement of the label was very rapid. 50% of the binding was lost in two minutes, and greater than 80% by 40 minutes. When the tissue was incubated at 4°C (Fig 5), the bound ligand was also rapidly dissociated by the addition of a large excess of unlabelled naloxone hydrochloride.

DEMONSTRATION OF PENETRATION OF LIGAND

Figure 6 presents pseudocolor generated autoradiograms of slices of cerebral cortex which have been incubated in the normal manner. The top print, Fig. 6A, was prepared from a superficial section of the brain slice, while Fig. 6B was prepared from a central section of the same slice. Computer assisted densitometry shows that the amount of ligand bound to the tissue is not significantly different in a given slice from the periphery to the centre, and that the amount of ligand bound to the
Fig. 4 Reversibility of binding of [3H]-Naloxone (2 nM) to 400μm slices of MBH from adult male SW mice. Incubation carried out at 30°C. 10 μM unlabelled naloxone HCl was added after 3 hours of incubation.
**Fig. 5** Reversibility of binding of [3H]-Naloxone (2 nM) to 400 μ slices of MBH from adult male SW mice. Incubation carried out at 4°C. 10 μM naloxone HCl was added after 3 hours of incubation.
Fig. 6 Pseudocolor autoradiograph of [3H]-naloxone binding to the surface (A) and interior (B) of a slice of cerebral
surface of the slice does not differ from the amount bound in the centre of the slice. Thus, penetration of ligand does not appear to be a problem in this assay.

**DISPLACEMENT OF [³H]-NALOXONE BINDING TO BRAIN SLICES**

The ability of various agents to compete with [³H]-naloxone for its binding site was studied. Figures 7 and 8 illustrate that several compounds which act through opioid receptors are able to displace [³H]-naloxone binding. The displacement can be described by a sigmoidal dose response curve, in which increasing amounts of competitor displace increasing amounts of radioligand. The concentration of competitor necessary to displace 50% of the bound radioligand (2 nM [³H]-naloxone) is referred to as the IC₅₀. The IC₅₀ for naloxone, levorphanol, dextorphine, morphine sulphate, and DADLE are: 4 × 10⁻⁹ M, 2 × 10⁻⁹ M, 10⁻⁶ M, 8 × 10⁻⁸ M and 5 × 10⁻⁷ M respectively.
Fig. 7 Displacement of [3H]-naloxone (2 nM) from 400μ slices of MBH obtained from adult male SJW mice. Slices were incubated in the presence of increasing concentrations of naloxone HCl (NAL), levorphanol (LEV) or dextrophan (DEX) for 2 hours. Results are expressed as the % of control binding, i.e. binding carried out in the absence of any competitor. IC50 values are given in the text.
Fig. 8 Displacement of [3H]-Naloxone (2 nM) from 400μ slices of MBH obtained from adult male SW mice. Slices were incubated in the presence of increasing concentrations of morphine sulphate (MORPHINE) or of D-ala2-d-Leu5-enkephalin (DADLE) for 2 hours. Results are expressed as the % of control binding, i.e. the binding in the absence of any competitor. IC50 values are given in the text.
STEREOSPECIFICITY OF $^{3}\text{H}$-NALOXONE BINDING TO BRAIN SLICES

Figure 7 also reveals that the binding of $^{3}\text{H}$-naloxone to brain slices is stereospecific. The biologically active opiate agonist levorphanol is able to potently displace $^{3}\text{H}$-naloxone from slices of mediobasal hypothalamus ($IC_{50}$: $2 \times 10^{-9}$ M). However, its biologically inactive stereoisomer dextrorphan is relatively ineffective in displacing $^{3}\text{H}$-naloxone, with an $IC_{50}$ which is 1000 fold greater ($10^{-6}$ M).

SATURABILITY OF $^{3}\text{H}$-NALOXONE BINDING

The specific binding of $^{3}\text{H}$-naloxone to slices of cerebral cortex and mediobasal hypothalamus are illustrated in Figures 9 and 10 respectively. In both tissues, increasing the concentration of $^{3}\text{H}$-naloxone resulted in increasing amounts of the ligand being specifically bound, until saturation was achieved. Non-specific binding represented 30% to 35% and 24% to 27% of total binding in cerebral cortex and mediobasal hypothalamus respectively. Analysis of the binding data shows that the $B_{max}$, a measure of receptor density, is $6.95 \pm 0.45$ fm/mg of tissue weight in adult male mouse mediobasal hypothalamus.
Fig. 9 Saturation curve of [3H]-naloxone to 400μ slices of cerebral cortex obtained from adult male SW mice. Specific binding was determined in the presence of 10 μM naloxone HCl, and incubations were carried out for 2 hours. SDergd = 0.045. Bmax = 6.47±0.47 fm/mg. Kd = 7.45±0.93 nM.
Fig. 10. Saturation curve of [3H]-naloxone binding to 400 μg slices of MIII obtained from adult male SW mice. Specific binding was determined in the presence of 10 μM naloxone HCl, and incubations were carried out for 24 hours. Saturad = 10. Bmax = 6.95±0.45 fm/mg. Kd = 1.49±0.22 nM.
and 6.47±0.47 fm/mg of tissue weight in cerebral cortex obtained from the same animals. The binding affinity is described by the $K_i$, defined as the concentration of radioligand at which 50% of maximal binding is attained. The $K_i$ for mouse hypothalamus is 1.49±0.22 nM, while that for cerebral cortex is 7.45±0.45 nM. Binding described by $K_i$ values in the nanomolar range is of high affinity.

**REGIONAL DISTRIBUTION OF SPECIFIC [3H]-NALOXONE BINDING**

The ability of [3H]-naloxone to bind specifically to a given tissue depends on the presence of specific receptors for the ligand in that tissue. As seen in Figure 11, specific binding of [3H]-naloxone (2 nM) was readily demonstrable in both cerebral cortex and mediotbasal hypothalamus. However, specific binding of [3H]-naloxone was not detectable in brain slices prepared from cerebellum.
Fig. 11 Binding of [3H]-naloxone (2 nM) to 400μm slices of tissue obtained from different parts of the brain of adult male SW mice. The tissue was incubated for 2 hours, and non-specific binding was determined in the presence of 10 μM naloxone HCl. Results are expressed as the mean ± s.e.m. of 8 (total binding) and 5 (non-specific binding) samples per point.
EFFECT OF TISSUE CONCENTRATION ON $[^{3}\text{H}]$-NALOXONE BINDING

The absence of binding artifacts, such as receptor or ligand degradation, can often be confirmed through construction of a tissue quantity versus binding plot (Burt, '1985). Figure 12 illustrates the result of incubating 1, 2, 3 or 4 slices of mediobasal hypothalamus with a single concentration of $[^{3}\text{H}]$-naloxone (2 nM). The increase in specific binding with the increase in tissue was linear, with a correlation coefficient (obtained through linear regression analysis) of 0.98. Although not shown in the figure, both total and non-specific binding increased linearly, each with a correlation coefficient of 0.99.

EFFECT OF NUMBER OF WASHES ON SPECIFIC BINDING OF $[^{3}\text{H}]$-NALOXONE

The effect of an increasing number of washes on the specific binding of $[^{3}\text{H}]$-naloxone to brain slices was studied (Fig. 13). It was found that specific binding, after falling significantly with one and two washes (each of 5 minutes), remained constant for 2 to 5 washes, (each of 1 ml). Non-specific binding decreased to 10% of total binding after 5 washes. In subsequent experiments, all slices were washed twice.
Fig. 12. Effect of increasing the number of slices of MBH (400 μm, obtained from adult male 89 mice) on the specific binding of [3H]-naloxone (2 nM). Incubations were carried out for 2 hours at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. The correlation coefficient for the linear regression of the data is 0.98.
Fig. 13 Effect of the number of washes on the binding of [3H]-naloxone (2 nM) to slices of MBH (400μ) obtained from adult male SW mice. Incubations were carried out at 30° C for 2 hours. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Washes were done with 1.0 ml of buffer for 5 minutes.
upon termination of the incubation. It was later determined that a wash volume of 0.5 ml was just as effective as 1.0 ml, and the wash volume was therefore decreased (results not shown).

**THERMOLABILITY OF $[^{3}H]$-NALOXONE BINDING TO BRAIN SLICES**

Receptor binding is normally unstable at high temperatures. Figure 14 illustrates that preincubation of hypothalamic slices at 70°C for 45 minutes completely eliminated specific $[^{3}H]$-naloxone binding. Preincubation of the slices at this temperature for 20 minutes resulted in a greater than 90% decrease in specific binding.

**EFFECTS OF METABOLIC INHIBITORS ON $[^{3}H]$-NALOXONE BINDING TO BRAIN SLICES**

In order to demonstrate that the radioactivity detected in the brain slices was due to specific binding, and not to a tissue uptake or internalization process, brain slices were incubated with either ouabain, or sodium azide, both metabolic inhibitors capable of disrupting uptake, an energy requiring process. As seen in Figure 15, neither ouabain nor sodium azide had any effect on the specific binding of $[^{3}H]$-naloxone to slices of
Fig. 14. Thermal stability of [3H]-naloxone binding. Slices of MBII (400 μm) obtained from adult male SW mice were treated as described in the text and specific binding of [3H]-naloxone (2 nM) was determined in the presence of 10 μM naloxone HCl. Incubations were carried out at 30°C for 2 hours. Results are expressed as mean ± s.e.m of 8 (total binding) and 4 (non-specific binding) samples per condition.
Fig. 15 Effect of metabolic inhibitors on [3H]-nafoxone binding. 400μ thick slices of MBH and cerebral cortex were coincubated with either quinidine (1 mM) or sodium azide (10 mM) and [3H]-nafoxone HCl (2 nM) as described in the text. The incubations were carried out at 30°C for 2 hours. Results are expressed as % control binding, i.e. binding in the absence of any competing or interfering agent.
mediobasal hypothalamus. These experiments were performed in TRIS buffer, which is sodium free. When the assay was carried out in the presence of sodium (DPBS), ouabain was also without effect on the specific binding of $[^3H]$-naloxone (8.6 nM: $[^3H]$-naloxone: control SB: 64.9 ± 5.6 vs ouabain 60.6 ± 5.9 fm/mg, P > 0.05; 1.4 nM: $[^3H]$-naloxone: control 34.1 ± 2.6 vs ouabain 30.2 ± 1.6 fm/mg, P > 0.05; data not illustrated).

**EFFECT OF VARIOUS DRUGS ON $[^3H]$-NALOXONE BINDING TO BRAIN SLICES**

A number of drugs are known to interact with opioid receptors. The effects of some of these agents are illustrated in Figure 16. Coincubation with phenoxybenzamine significantly reduced $[^3H]$-naloxone binding to slices of both cerebral cortex and mediobasal hypothalamus. Phentolamine reduced the binding of $[^3H]$-naloxone to both mediobasal hypothalamus and cortex, but the reduction did not achieve statistical significance in the latter case. Neither yohimbine nor clonidine significantly affected cortical or hypothalamic $[^3H]$-naloxone binding, while isoproterenol and clonazepam were without effect on the binding in mediobasal hypothalamus, but their effect on cortical binding
Fig. 16 Effect of various drugs (in vitro) on [3H]-naloxone binding. 400µ slices of MBH and cerebral cortex obtained from adult male SW mice were co-incubated with 10µM of various drugs as detailed in the text, and [3H]-naloxone (2 nM). The incubations were carried out at 30°C for 2 hours. Results are expressed as % control binding, i.e. binding in the absence of any competing or interfering agent. * P < .05 ** P < .025 *** P < .005 vs control.
was not determined. Ascorbic acid also significantly decreased the amount of $[^3H]$-naloxone bound to mediobasal hypothalamus FIG 17. This reduction in specific binding was due exclusively to an action on the total binding of $[^3H]$-naloxone, without any effect on the non-specific binding.

**EFFECT OF CHRONIC EXPOSURE TO NALOXONE ON $[^3H]$-NALOXONE BINDING TO BRAIN SLICES**

The effect of chronic exposure to naloxone, via a subcutaneous silastic implant, on the binding of $[^3H]$-naloxone to slices of MBH is shown in Figure 18. Control mice, exposed to lactose-containing implants, had a $B_{max}$ of $13.66 \pm 2.36$ fm/mg and a $K_d$ of $2.88 \pm 1.54$ nM. Treatment for seven days with a naloxone-containing implant resulted in a large increase in the $B_{max}$ to $34.28 \pm 1.51$ fm/mg. There was a concomitant increase in the $K_d$ to $5.55 \pm 0.48$ nM.

Removing the naloxone-containing implant and allowing the animals to recover for 24 hours before performing the binding assay eliminated the increase in $[^3H]$-naloxone binding seen with the pellet in situ (Fig. 19). Since this experiment was only performed at a single concentration of radioligand (10nM), the
Fig. 17. Effect of ascorbate on [3H]-naloxone binding. 400µ slices of MBH obtained from adult male SW mice were incubated with 1 mM sodium ascorbate and [3H]-naloxone (2 nM) for 2 hours at 30°C. Specific binding was determined in the presence of 10 µM naloxone HCl. The sodium ascorbate was made up in assay buffer containing 50mM-HEPES to maintain buffer pH at 7.4. Results are expressed as mean ± s.e.m. of 6 (total binding) and 4 (non-specific binding) determinations per point.
Fig. 18 Effect of chronic exposure to naloxone HCl on [3H]-naloxone binding. 400 µm slices of MBH were prepared from adult male SW mice which had been implanted with silastic implants containing either 30 mg of naloxone HCl or lactose 7 days earlier. Non-specific binding was determined in the presence of 10 µM naloxone HCl. Incubations were carried out at 30°C for 2 hours.
Fig. 19 Effect of removal of naloxone pellet on [3H]-naloxone binding. Animals were treated as in figure 15. One group had the pellet removed 24 hours before 400μ slices of MEH were prepared and the specific binding of [3H]-naloxone (10 nM) was determined. Specific binding was determined in the presence of 10 μM naloxone hydrochloride. Incubations were carried out at 30°C for 2 hours. Results are the mean ± s.e.m. of 7 (total binding) and 4 (non-specific binding) determinations.
effect on the binding parameters cannot be elucidated.
CHARACTERIZATION OF OPIOID BINDING TO RETINAL FRAGMENTS

TIME COURSE OF \(^3\)H-NALOXONE BINDING TO RETINAL FRAGMENTS

The time course of the binding of \(^3\)H-naloxone to retinal fragments is illustrated in Fig 20. Total and specific binding were rapid, reaching half maximal values in less than 10 minutes. Saturation was reached by 45 minutes, and the binding was stable when the incubation was extended to four hours. The non-specific binding did not increase during the intervals examined. Non-specific binding was equal to approximately 25%-30% of total binding at saturation conditions.

REVERSIBILITY OF BINDING OF \(^3\)H-NALOXONE TO RETINAL FRAGMENTS

The time course of \(^3\)H-naloxone binding to retinal fragments is reprised in Figure 21, here expressed as percent of maximal binding. It can be seen that the addition of a 1000 fold excess of unlabelled naloxone hydrochloride (10^5 M) is able to rapidly displace the labeled naloxone from its binding site. The reversibility of binding was half
Fig. 20 Time course of binding of [3H]-naloxone (1 nM) to retinal fragments from adult male SD rats. Incubation carried out at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone hydrochloride. Each point represents the mean of 5 (total binding) or 3 (non-specific binding) retinal quadrants.
Fig. 21  Reversibility of [3H]-naloxone binding to retinal fragments. Retinal fragments were prepared from adult male SD rats, and incubated with [3H]-naloxone (1 nM) at 30°C. After 3 hours of incubation, 10 μM naloxone HCl was added. The results are expressed as % of maximal total binding.
maximal in approximately 5 minutes, and was complete by 30 minutes.

**DISPLACEMENT OF \( ^{3}H \)-NALOXONE FROM RETINAL FRAGMENTS**

The ability of various agents to compete with \( ^{3}H \)-naloxone for its binding site in retinal fragments was studied, and is illustrated in Figure 22. The displacement can be described by a sigmoidal dose response curve, in which increasing amounts of competitor displace increasing amounts of radioligand. The \( IC_{50} \) values for naloxone, levorphanol, and dextrorphan, are \( 3.3 \times 10^{-9} \) M, \( 1.1 \times 10^{-8} \) M, and \( 8.7 \times 10^{-6} \) M respectively.

**DEMONSTRATION OF STEREOSPECIFICITY OF BINDING OF \( ^{3}H \)-NALOXONE TO RETINAL FRAGMENTS**

Figure 22 also reveals that the binding of \( ^{3}H \)-naloxone to brain slices is stereospecific. The biologically active opiate agonist levorphanol is able to potently displace \( ^{3}H \)-naloxone from slices of medio-basal hypothalamus (\( IC_{50} \): \( 1.1 \times 10^{-8} \) M). However, its biologically inactive stereoisomer dextrorphan is ineffective in displacing \( ^{3}H \)-naloxone, with an \( IC_{50} \) which is almost 1000 fold greater (\( 8.7 \times 10^{-6} \) M).
Fig. 22 Displacement of [3H]-naloxone (1 nM) from retinal fragments obtained from adult male SD rats. Fragments were incubated in the presence of increasing concentrations of naloxone HCl, levorphanol or dextorphpan for 2 hours. Results are expressed as the % of control binding, i.e., the binding in the absence of any competitor. IC50 values are given in the text.
Saturability of $^3$H-naloxone binding to retinal fragments

The specific binding of $^3$H-naloxone to fragments of retina from an adult male SD rat is illustrated in Figure 23. Increasing the concentration of $^3$H-naloxone resulted in increasing amounts of the ligand being specifically bound, until saturation was achieved. Analysis of the binding data revealed a $B_{\text{max}}$ of $12.93 \pm 0.89 \text{ fm/mg}$ of retinal tissue, and an $K_d$ of $3.41 \pm 0.53 \text{ nM}$. This binding may be characterized as high affinity binding, by virtue of the fact that the $K_d$ is in the nanomolar range, and that saturation binding occurs in the nanomolar range.

Effect of tissue concentration on $^3$H-naloxone binding to retinal fragments

Construction of a tissue concentration versus binding plot, illustrated in Figure 24, shows that there is a linear relationship between the number of slices incubated, and the amount of ligand specifically bound. The correlation coefficient for the regression of this data is 0.99. Both total and non-specific binding also increased linearly, each with a correlation coefficient of 0.99.
Fig. 23 - Retinal binding of [3H]-naloxone. Retinal fragments were prepared from adult male SD rats and incubated in the presence of [3H]-naloxone for 2 hours at 30°C. Non-specific binding was determined in the presence of 10μM naloxone HCl, and varied from 25-35% of total binding over the concentration range examined.
Fig. 24. Effect of increasing the number of retinal fragments obtained from adult male SD rats on the specific binding of [3H]-naloxone (1 nM). Incubations were carried out at 30°C for 2 hours. Non-specific binding was determined in the presence of 10µM naloxone HCl. The correlation coefficient for the linear regression of the data is 0.99.
SEX DIFFERENCES IN RETINAL BINDING OF $^3$H-NALOXONE

Retinal fragments obtained from female rats bound more $^3$H-naloxone than did those obtained from male rats. Figure 25 illustrates the differences seen between adult male and female SD rats. The affinity of the binding site is similar in both sexes ($K_d$: $1.28 \pm 0.2$ nM vs $1.55 \pm 0.27$ nM, male vs female). However females have almost 80% more receptors than do the males ($\text{P}_{\text{max}}$: $4.32 \pm 0.28$ fm/mg vs $7.72 \pm 0.59$ fm/mg, male vs female). Figure 26 illustrates that these differences are maintained in LE rats, where the binding parameters are as follows: $\text{P}_{\text{max}}$: $13.64 \pm 2.45$ fm/mg vs $30.89 \pm 3.23$ fm/mg, male vs female; $K_d$: $4.95 \pm 1.61$ nM vs $2.39 \pm 0.52$ nM, male vs female.

STRAIN DIFFERENCES IN RETINAL BINDING OF $^3$H-NALOXONE

As indicated above, retinas obtained from animals with pigmented eyes bound more $^3$H-naloxone than did those obtained from albino animals. In Figures 27 and 28, the data are replotted to highlight the interstrain differences in $^3$H-naloxone binding seen in the pigmented LE and the albino SD strains of rat. Note that the male LE rat had both a higher $\text{P}_{\text{max}}$ and a higher $K_d$ than did its albino
Fig. 25  Sex differences in retinal opioid binding. Retinal fragments were prepared from adult male and female SD rats. The fragments were incubated with [3H]-naloxone for 2 hours at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Binding parameters are given in text.
Fig. 26 Sex differences in retinal opioid binding. Retinal fragments were prepared from adult male and female LE rats. The fragments were incubated with [3H]-naloxone for 2 hours at 30° C. Non-specific binding was determined in the presence of 10 μM naloxone-HCl. Binding parameters are given in text.
Fig 27 Strain differences in retinal opioid binding. Retinal fragments were obtained from adult female LE and SD rats. Data are replotted from figs. 47 and 48. Binding parameters are given in text.
Fig 28 Strain differences in retinal opioid binding. Retinal fragments were obtained from adult male LE and SD rats. Data are replotted from figs. 25 and 26. Binding parameters are given in text.
counterpart ($R_{\text{max}}: 13.64 \pm 2.45$ fm/mg vs $4.32 \pm 0.28$ fm/mg; $K_d: 4.95 \pm 1.61$ nM vs $1.28 \pm 0.2$ nM—LE vs SD).

The females, on the other hand differed only in their receptor density, and not in affinity ($R_{\text{max}}: 0.89 \pm 3.23$ fm/mg vs $7.72 \pm 0.59$ fm/mg; $K: 2.39 \pm 0.52$ nM vs $1.55 \pm 0.27$ nM; LE vs SD).

**Retinal Binding of $^3$H-Naloxone in Different Strains of Hamster**

Preliminary experiments with adult female GS hamsters and the CHF 147 cardiomyopathic and CHF 148 noncardiomyopathic control hamsters, shown in Figure 29 have revealed that at a single point on the binding curve (2 nM $^3$H-naloxone), there are marked differences in the amount of $^3$H-naloxone specifically bound. The binding in retina from GS hamsters ($4.12 \pm 1.09$ fm/mg) is significantly higher than that in retinal fragments from the CHF147 hamsters ($1.00 \pm 0.13$ fm/mg). The amount of $^3$H-naloxone bound by retinal fragments from CHF 148 hamsters ($3.50 \pm 0.40$ fm/mg), while not significantly different from the GS controls, was significantly higher than that seen in the CHF 147 animals. When this experiment was repeated with 60 day old male hamsters, a similar pattern was seen (Fig. 30), i.e. at a single point on the binding curve (2 nM), the specific binding
Fig. 29 Strain differences in opioid binding to hamster retina. Retinal fragments were prepared from adult female Golden Syrian, CHF 147, and CHF 148 hamsters. The fragments were incubated with [3H]-naloxone (2 nM) for 3 hours at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Results are presented as the mean±s.e.m. of a total of 15 (total binding) and 9 (non-specific binding) retinal quadrants per group, and are the pooled results of 2 experiments. * P < 0.001 vs 6S. ** P < 0.005 vs 148.
Fig. 30 Strain differences in opioid binding to retina from male hamsters. Retinal fragments were prepared from 60 day old Golden Syrian, CHF 147, and CHF 148 hamsters. The fragments were incubated with [3H]-naloxone (2 nM) for 3 hours at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Results are presented as the mean±s.e.m. of 12 (total binding) and 5 (non-specific binding) retinal quadrants per group. * P < 0.01 vs 65 and 148.
Fig. 31 Saturation analysis of strain differences in opioid binding to hamster retina. Retinal quadrants were prepared from 60 day old female Golden Syrian, CHF 147, and CHF 148 hamsters. The fragments were incubated with [3H]-naloxone for 3 hours at 30°C. Non-specific binding was determined in the presence of 10 μM Naloxone HCl. Results are presented as mean of 5 (total binding) and 3 (non-specific binding) retinal quadrants per group, and are the pooled results from 2 separate experiments.
(in fm/mg) was GS: 3.49 ± 0.20; 147: 2.40 ± 0.20; 148: 3.38 ± 0.52. Saturation binding curve (Fig 31) analysis revealed that the affinity of the binding site was not different in 60 day old female hamsters (Kd: GS: 1.01 ± 0.35; 147: 1.17 ± 0.09; 148: 1.75 ± 0.91 nM). The density (Bmax) of the retinal 3H-naloxone binding site was the same in both GS (8.18 ± 1.37 fm/mg) and 148 (11.24 ± 3.52 fm/mg) hamsters. These values were both significantly greater than the Bmax of 3.41 ± 0.14 fm/mg as determined in retina obtained from 147 hamsters.

EFFECT OF LIGHT ON RETINAL BINDING OF 3H-NALOXONE

The effect of obtaining and incubating the retinae in darkness is shown in Fig. 32. No difference was seen in the density of 3H-naloxone binding sites in retinae obtained in the dark (Bmax: 376 ± 49 fm/mg), or under normal room illumination (Bmax: 466 ± 54 fm/mg). There was, however, a marked increase in the affinity of the binding site for 3H-naloxone when retinal quadrants were obtained and incubated in the dark (Kd: dark: 0.40 ± 0.2 nM; light: 1.73 ± 0.38 nM). Retinal quadrants which were obtained and incubated in the dark had a purple tinge upon termination of the incubation. This
Fig. 32 Effect of light on retinal [3H]-naloxone binding. 30 day old female rats were sacrificed at 0500 hrs under dim red illumination, and the retinae were dissected under red illumination. Control animals were sacrificed under normal room illumination within 30 minutes. Retinal quadrants (5 for TB and 3 for NSB) were incubated with increasing concentrations of [3H]-naloxone at 30°C for 3 hours. Non-specific binding was determined in the presence of 10μM naloxone HCl.
(dis)coloration was never seen in retiae obtained in the light and incubated in the normal fashion.
DIURNAL RHYTHMS IN THE RAT

ONTGENY OF DIURNAL VARIATIONS IN THE ABILITY OF NALOXONE HCl TO RELEASE LH IN PREPUBERTAL RATS.

The development of the ability of naloxone to differentially release LH in the morning and the afternoon is illustrated in Figures 33 (males) and 34 (females). In general, naloxone did not significantly release LH when administered to prepubertal male rats, either in the morning or the afternoon. The situation was quite different in female rats. Significant responses to naloxone were observed at both 0800 and 1700 hrs in all ages examined except 2 days old. Careful examination of the data, plotted as % increase over basal levels (Figures 35) reveals that beginning at day 26, and continuing through day 32, there was a marked decrease in the ability of naloxone to release LH in the female rat in the late afternoon, as compared to the early morning.
Fig. 33 Ontogeny of the diurnal response of LH to naloxone in male SD rats. Animals were given 0.1 ml of saline (s.c.) or 2.5 mg/kg naloxone HCl at 0800 (top panel) or 1700 hrs (bottom panel). Animals were sacrificed and blood collected 15 minutes after administration of drug or vehicle. (See text for details). Results are presented as mean±s.e.m. N=7-14 animals per group.
Fig. 34 Ontogeny of the diurnal response of LH to naloxone in female SD rats. Animals were given 0.1 ml of saline (s.c.) or 2.5 mg/kg naloxone HCl at 0800 (top panel) or 1700 hrs (bottom panel). Animals were sacrificed and blood collected 15 minutes after administration of drug or vehicle. (See text for details). Results are presented as mean±s.e.m. N=7-14 animals per group.
Fig. 35 Diurnal variation in naloxone-induced increase in serum LH in male (top panel) and female (bottom panel) SD rats. Animals were given saline (0.1 ml, s.c.) or naloxone HCl (2.5 mg/kg) at 0700 (AM) or 1700 (PM) hrs. Blood was sampled 15 minutes after administration of drug. Results are expressed as naloxone-induced % increase over basal levels of LH, and are presented as mean±s.e.m. N=7-14 animals per group.
24 HOUR PATTERN OF 3H-NALOXONE BINDING TO HYPOTHALAMIC SLICES FROM PREPUBERTAL FEMALE RATS

A bimodal pattern in the binding of $[^{3}H]$-naloxone (10 nM) to slices of mediobasal hypothalamus was observed in 30 day old female rats (Fig. 36). Binding was maximal in the morning, shortly after the light phase had begun, and declined steadily over the course of the day, reaching a nadir (60% of the AM value) in the late afternoon. A transient increase in binding was observed with the onset of the dark phase.

SATURATION ANALYSIS OF DIURNAL CHANGES IN $^{3}H$-NALOXONE BINDING TO BRAIN TISSUE FROM PREPUBERTAL RATS

Eadie-Hofstee analysis of saturation curves constructed in the early morning and the late afternoon (Fig. 37) reveals that the decrease in binding seen in the late afternoon in 30 day old females is due to a decrease in the density of binding sites ($B_{\text{max}}$: $30.18\pm2.44$ fM/mg vs $19.32\pm1.19$ fM/mg; am vs pm) and not to a change in the affinity of the binding site ($K_{d}$: $3.09\pm0.40$ nM vs. $2.59\pm0.31$ nM; am vs pm). The SDevad values for the am and pm curves were 0.101 and 0.089 respectively. Non-
Fig. 36 24 hour pattern of [3H]-naloxone binding to hypothalamus. Slices of MBH were prepared from 30 day old female SD rats every 4 hours for a 24 hour period. The specific binding of [3H]-naloxone (10 nM) was determined after 3 hours of incubation at 30° C. Non-specific binding was determined in the presence of 10µM naloxone. Results are presented as mean±s.e.m. of 15 (total binding) and 10 (non-specific binding) slices per time point.
Fig. 37 Saturation analysis of diurnal changes in [3H]-naloxone binding to hypothalamus. 300μm slices of MBH were obtained from 26 day old female SD rats, and the specific binding of [3H]-naloxone was assessed. Incubations were run for 3 hours at 30° C. Non-specific binding was determined in the presence of 10 μM naloxone HCl.
specific binding did not differ significantly between the morning and the afternoon assays, and represented 25%-30% of total binding.

**EFFECT OF DISSOCIATION OF ENDOGENOUS LIGAND FROM BRAIN TISSUE ON THE DIURNAL PATTERN OF $^3$H-NALOXONE BINDING TO BRAIN TISSUE**

Figure 38 indicates that prolonged washing of hypothalamic slices obtained in the late afternoon did not have any effect on the amount of $[{}^3$H]-naloxone bound (control tissue: $R_{\text{max}} = 26.8\pm 1.4$ fM/mg, $K_d = 1.79\pm 0.19$ nM; washed tissue: $R_{\text{max}} = 22.6\pm 1.5$ fM/mg, $K_d = 1.65\pm 0.24$ nM). Similarly, washing of hypothalamic tissue obtained in the early morning did not affect binding at a single concentration of ligand (8 nM $[{}^3$H]-naloxone; control tissue: 13.0±2.5 fM/mg bound; washed tissue: 12.3±0.8 fM/mg bound). Preincubation of hypothalamic tissue obtained in the late afternoon in an acidic glycine buffer was also without effect on the amount of $[{}^3$H]-naloxone bound (10 nM $[{}^3$H]-naloxone; control tissue: 26.0±1.6 fM/mg bound; glycine treated tissue: 25.1±1.9 fM/mg bound).
Fig. 38 Effect of dilution washing on the binding of [3H]-Naloxone to hypothalamus in the late afternoon. 300 μ slices of MBH obtained from 30 day old female SD rats and treated as described in the text. Incubations were carried out at 30°C for 3 hours. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Results are presented as the mean ± s.e.m. of 5 (total binding) and 3 (non-specific binding) slices per point.
ONTGENY OF DIURNAL VARIATION IN $^3$H-NALOXONE BINDING TO HYPOTHALAMIC TISSUE IN PREPUBERTAL RATS

The patterns of binding of $[3^H]$-naloxone to slices of both cerebral cortex and mediobasal hypothalamus are illustrated in Figures 39-41. Binding of this ligand to cortex did not change significantly over the course of the day, in either sex, at any age examined, with the exception of 23 day old females, where a slight, but statistically significant decrease in binding was found over the course of the day. Male rats exhibited statistically significant decreases in the amount of $[3^H]$-naloxone bound to slices of mediobasal hypothalamus over the course of the day at all ages examined except day 26. The magnitude of these changes varied from 65% (day 9) to 34% (day 23). The binding patterns observed in female animals were quite distinct from those seen in the males. Hypothalamic binding was constant over the day in 9 and 15 day old females, and an irregular pattern was noted in 23 day old females. The diurnal pattern of hypothalamic $[3^H]$-naloxone binding noted above (Figure 36) was well in place by day 26 of life.
Per point and/regional/2 experiments per group

All data are given in text. Results are presented as mean ± SEM at 8 h, 14 h, and 17 h. Averages of all

days were

not shown. The data is shown in Figure 1. The standard concentrations of the

ligands are as follows: 10 ng/ml for the control and 100 ng/ml for the experimental groups.

The results are shown in Table 1.
Results are presented as mean ± SEM of 8 (18) and 5 (NSB) slices per point, and represent 2 experiments.

Fig. 4. Ontogeny of diurnal variations in [3H]-halothane (2 nM) binding to brain. Details are given in text.
Fig. 41 Ontogeny of diurnal variations in [3H]-naloxone (2 nM) binding to brain. Details are given in text. Results are presented as mean±s.e.m. of 8 (TB) and 5 (NSB) slices per point, and represent 2 expts per group.
ATTEMPTED MODIFICATION OF DIURNAL RHYTHMS IN 
$^3$H-NALOXONE BINDING TO BRAIN TISSUE

NEONATAL GONADECTOMY AND DIURNAL OPIOID 
BINDING RHYTHMS

Technical problems prevented the construction of 
complete saturation curves on brain tissue obtained 
from male animals castrated at day 9 of life. 
However, binding was performed (at 30 days of age) at 
one concentration of $^3$H-naloxone (10 nM), and the 
results are presented in Figure 42. Hypothalamic 
tissue from control male rats bound significantly 
less $^3$H-naloxone in the afternoon, than did tissue 
obtained in the early morning. This diurnal 
difference in binding was not seen in gonadectomized 
animals. There was no apparent difference in $^3$H- 
naloxone binding to cortical tissue obtained either 
in the morning or in the afternoon. In agreement with 
earlier findings, and as shown in Figure 43, control 
female rats showed a decrease in the density of 
hypothalamic opioid binding sites in the afternoon, 
compared to the morning ($B_{\text{max}}$: AM: 32.5±2.8 fm/mg vs 
PM: 16.5±1.6 fm/mg). The affinity of the binding site 
did not change ($K_d$: AM: 1.27±0.36 nM vs PM: 1.76±0.54 
nM). Gonadectomy at the age of 9 days had the effect 
of eliminating the AM/PM diurnal differences in $B_{\text{max}}$.
Fig. 42 Effect of infantile gonadectomy on diurnal variation in opioid binding in male rats. Male SD rats were orchidectomized on day 9 of life. On day 30 of life, 300μ slices of cerebral cortex and MBH were prepared and incubated with [3H]-naloxone (10 nM) for 24 hours at 4°C. Non-specific binding was determined in the presence of 10μM naloxone HCl. Results are presented as the mean ± s.e.m of 5 (total binding) and 3 (non-specific binding) slices per group.
Fig. 46 Effect of infantile ovariectomy on diurnal variations in opioid binding in females. Female SD rats were ovariectomized on day 9 of life. 300 µ slices of MBH were prepared on day 30 of life, and the specific binding of [3H]-naloxone was determined. Incubations were carried out at 4°C for 24 hours. Non-specific binding was determined in the presence of 10 µM naloxone HCl.
(AM: 23.9±2.2 fm/mg vs PM: 25.3±4.8 fm/mg). However, a sharp decrease in the affinity of the binding site was seen in the late afternoon ($K_d$: AM: 1.40±0.43 vs PM: 7.22±2.54 nM).

NEONATAL ANDROGENIZATION WITH ESTRADIOL VALERATE AND DIURNAL OPIOID BINDING RHYTHMS

Neither the male control animals, nor the male animals injected with EV on day 1 of life, showed a diurnal rhythm in $^3$H-naloxone binding to slices of mediobasal hypothalamus tissue on day 30 of life (Fig 44). No change was seen in $B_{max}$ between the morning and the afternoon test points ($B_{max}$: Control: AM: 19.9±3.3 fm/mg vs PM: 22.8±2.1 fm/mg; EV: AM: 22.9±2.7 vs PM: 20.3±1.7 fm/mg), but there was a tendency for the affinity of the binding site to decrease in the afternoon in both groups ($K_d$: Control: AM: 1.1±0.6 nM vs PM: 3.1±0.5 fm/mg; EV: AM: 1.6±0.5 vs PM: 2.6±0.4 fm/mg).

The response of female animals treated with EV at day 9 of life differed from that of the males (Fig 45). A diurnal variation in $^3$H-naloxone binding to slices of mediobasal hypothalamus was apparent on day 30 of life. As has been seen before, the variation was due to a decrease in the density of
Fig. 44 Effect of neonatal androgenization on diurnal pattern of opioid binding in male rats. Male SD rats were treated with EU (100 µg in sesame oil) on either day 0 or day 1 of life. Controls received vehicle alone. Slices of MBH were prepared on day 30 of life. Slices were incubated with [3H]-naloxone for 24 hours at 4° C. Non-specific binding was determined in the presence of 10 µM naloxone HCl.
Fig. 45 Effect of neonatal androgenization on diurnal pattern of opioid binding in female rats. Female SD rats were treated with EU (100µg in sesame oil) on either day 0 or day 1 of life (bottom panel). Controls received vehicle alone (top panel). 300µ slices of MBH were prepared on day 30 of life. Slices were incubated with [3H]-naloxone for 24 hours at 4° C. Non-specific binding was determined in the presence of 10µM naloxone HCl. SDerad for the top curves are less than 0.12, while those for the bottom curves are between 0.15 and 0.17.
binding sites in the afternoon, while the receptor affinity remained unchanged ($B_{\text{max}}$: AM: 16.3±1.0 fm/mg vs PM: 10.7±0.7 fm/mg; $K_d$: AM: 1.8±0.2 nM vs PM: 1.2±0.2 nM). This diurnal variation was not seen in the animals that had been subject to the neonatal E\textsubscript{V} treatment ($B_{\text{max}}$: AM: 14.7±1.3 fm/mg vs PM: 15.9±1.5 fm/mg; $K_d$: AM: 1.8±3 nM vs PM: 2.4±0.5 nM).

**INDUCTION OF DIURNAL RHYTHM IN OPIOID BINDING WITH PMSG**

21 day old female rats did not show any difference in the amount of $^3$H-naloxone bound (6, 7, or 8 nM) to slices of mediobasal hypothalamus obtained in the morning or in the afternoon. However, treatment with 7.5 I.U. of PMSG on day 18 of life resulted in the appearance of a significant decrease in the amount of $^3$H-naloxone (7 nM) specifically bound to hypothalamus in the afternoon on day 21. At 6 nM $^3$H-naloxone, a tendency toward decreased binding was seen, but the difference did not achieve significance (Fig 46).
Fig. 46 Induction of diurnal variation in [3H]-naloxone binding to hypothalamus in immature rats with PMSG. 18 day old female SD rats were treated with 7.5 I.U. of PMSG. Controls received saline. 300μ slices of MBH were prepared on day 20 of life and the specific binding of [3H]-naloxone was determined. Slices were incubated at 30°C for 3 hours. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Slices were obtained at 0700 (AM) and at 1700 (PM) hours. Results are expressed as mean ± s.e.m.
EFFECT OF TREATMENT WITH BARBITURATE ON DIURNAL RHYTHM IN OPIOID BINDING

Treatment with sodium pentobarbital at 1000 hrs prevented the appearance of the late afternoon decrease in the amount of $[^3H]$-naloxone bound to hypothalamic tissue from 30 day old female rats (Fig. 47). This inhibition of the pm decrease in $[^3H]$-naloxone binding was observed at both a low (2 nM) and a high (10 nM) concentration of radioligand.

EFFECT OF CHRONIC TREATMENT WITH BARBITURATE ON DEVELOPMENT OF SPONTANEOUS PUBERTY

Chronic treatment with sodium pentobarbital was essentially without effect on the spontaneous development of puberty (Table 1); there being no difference in the day of VO, ovarian and uterine weight. While the barbiturate treated group was slightly heavier than the control group, and maintained this minimal weight difference throughout the experiment, the rates of growth were identical for both groups of animals (Fig. 48). Furthermore, normalizing the ovarian and the uterine weight for body weight, did not influence the lack of difference between these two groups.
Fig. 47 Barbiturate blockade of diurnal variation in [3H]-naloxone binding. 30 day old female SD rats were injected with saline or sodium pentobarbital (31 mg/kg, i.p.) at 1000 hrs and sacrificed at 1700 hrs. 300μ slices of MBH were incubated with [3H]-naloxone (2 nM, 10 nM) for 3 hours at 30° C. Binding was also determined in slices of MBH obtained from untreated animals at 0700 hours. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Results are given as mean ± s.e.m.
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Number of animals is given in parentheses

* P < 0.025 vs. control
Fig. 48 Effect of chronic barbiturate on body weight. Female SD rats were injected with sodium pentobarbital (31 mg/kg, i.p.) or with saline (0.1 ml i.p.) at 1100 hours from day 23 to day 30 of life. Results are presented as mean±s.e.m. N=10 animals per group.
EFFECT OF CONSTANT LIGHT ON DIURNAL RHYTHM IN OPIOID BINDING

Preliminary experiments, performed at 2 concentrations of $^3$H-naloxone (7.5 nM and 8.5 nM), showed that exposure to conditions of constant light for a period of 9 days appeared to eliminate the late pm decrease in specific binding of $^3$H-naloxone to slices of mediobasal hypothalamus seen in control animals (Fig. 49). These initial experiments were repeated, and saturation binding curves were constructed. As seen in Figure 50, there was no difference in either $B_{\text{max}}$ or $K_d$ in mediobasal hypothalamic tissue obtained in the early morning or in the late afternoon from 30 day old rats maintained under LL for 9 days. This contrasts with control rats, housed under a LD of 14:10, where the late afternoon decrease in binding was seen.

EFFECT OF CONSTANT DARKNESS ON DIURNAL RHYTHM IN OPIOID BINDING

When prepubertal female rats were housed under conditions of constant darkness, the effects on diurnal $^3$H-naloxone binding rhythms were remarkably similar to those seen with exposure to constant light. As illustrated in Fig 51, exposure to 9 days of LL completely eliminated the diurnal difference in $^3$H-
Fig. 49 Effect of constant light on mediobasal hypothalamic [3H]-naloXone binding. Female SD rats were housed in constant light from 21 to 30 days of age. Animals were sacrificed on day 30 as described in the text, and specific binding of [3H]-naloXone to 300μ slices of MBH was determined. Incubations were carried out at 4°C for 24 hours. Non-specific binding was determined in the presence of 10μM naloXone HCl.
Fig. 50 Saturation analysis of effect of constant light on diurnal variation in [3H]-naloxone binding. Rats were housed under constant light from day 21 to day 30 of life. Saturation curves were constructed for [3H]-naloxone binding to 300μ slices of MBH on day 30 of life. Assays were run at 30°C for 3 hours. Non-specific binding was determined in the presence of 10μM naloxone HCl. Slices were obtained at 0700 (AM) and 1700 (PM) hrs.
Fig. 51 Effect of constant darkness on diurnal variation in [3H]-naloxone binding. Female SD rats were housed in constant darkness from 21 to 30 days of age (bottom panel). Controls were housed under 14:10 LD (top panel). Animals were sacrificed on day 30 as described in the text, and specific binding of [3H]-naloxone to 300μl slices of MBH was determined. Incubations were carried out at 4°C for 24 hours. Non-specific binding was determined in the presence of 10μM naloxone HCl.
naloxone binding to mediobasal hypothalamus seen in control animals housed under an LD of 14:10. The experimental curves are virtually superimposable, and no change in either $B_{\text{max}}$ or $K_d$ was detected.
Estradiol Valerate Experiments

Effect of EV on Organ Weights in SW Mice

The effect of EV treatment on organ weights is shown in Figure 52. The weights of the ovaries, the uteri and the thymus glands, as well as the body weight were not affected by treatment with estradiol valerate. There was a significant increase in the weight of the pituitary glands in the EV treated group (1.01±0.07 mg vs 1.39±0.12 mg, control vs EV; p<0.01).

Effect of EV on Basal and GnRH-Stimulated LH Release from Pituitary Glands in EV Mice

Exposure to GnRH in vitro resulted in a significant increase in LH output from pituitary glands obtained from both control and EV-treated Swiss Webster mice. As shown in Figure 53, anterior pituitary glands from the EV-treated group had both a greater basal release (229.2±58.8 ng/ml/mg tissue wt) and a higher GnRH-stimulated release of LH (1624.7±385.7 ng/ml/mg tissue wt) than did the control glands (120.7±36.8 and 1175±242.6 ng/ml/mg tissue respectively), although these differences did not achieve statistical significance.
Fig. 52 Effect of estradiol valerate on body and organ weights in mice. Female SW mice (8 animals / group) were treated with EV (200 μg in oil) or vehicle 3 months earlier. Results are presented as mean±s.e.m. * P < 0.01 vs control.
Fig. 53 Basal and GnRH-stimulated release of LH from pituitary glands in vitro. Female SW mice were treated with EU as described in the text, 3 months prior to experiment. Results are presented as mean±s.e.m. of 8 control and 7 EU incubations. * P < 0.001 vs basal.
EFFECT OF EV ON \(^{3}\text{H}-\text{NALOXONE BINDING TO SLICES OF MEDIOBASAL HYPOTHALAMUS IN SW MICE}\)

Results of the opiate binding studies are shown in Fig. 54. Eadie-Hofstee analysis of the binding data reveal that the EV treatment had no effect on the affinity of the opiate receptors (K\(_d\): 2.78±0.56 vs. 2.32±0.61 nM; control vs. EV). However, there was a significant increase in the density of hypothalamic opiate receptors seen in the EV-treated animals (B\(_{\text{max}}\): 14.68±2.08 vs. 26.91±2.90 fmole/mg tissue; C vs EV).

EFFECT OF EV ON ORGAN WEIGHTS IN C57BL/6J MICE

The effect of EV treatment on organ weights in C57BL/6J mice is shown in Figure 55. Neither ovarian weight nor body weight were affected four months after the administration of 200 g of EV. However, there was a significant increase in the weight of both the pituitary glands (2.09±0.8 mg vs 3.16±0.14 mg, control vs EV; p< 0.0005), and the uteri (78.59±6.5 mg vs 159.84±12.2 mg, control vs EV; p< 0.0005) in the EV treated group.
Fig. 54 Hypothalamic binding of [3H]-naloxone 3 months after treatment with 200 μg of EU in female SW mice. Assay was performed on 400μm slices of MBH. Incubation was at 30°C for 2 hours. Non-specific binding was in the presence of 10 μM naloxone HCl. Binding parameters are presented in the text.
Fig. 55 Effect of estradiol valerate on body and organ weights in mice. Female C57BL/6J mice were treated with EV (200 mg) in oil or vehicle 4 months earlier. Results are presented as mean±s.e.m. N=16 control and 13 EV animals. * P < 0.0005 EV vs control.
EFFECT OF EV ON $^3$H-NALOXONE BINDING TO SLICES OF MEDIOBASAL HYPOTHALAMUS IN C57BL/6J MICE

Results of the opiate binding studies are shown in Fig. 56. As was the case with the SW mice, Eadie-Hofstee analysis of the binding data reveal that the EV treatment had no effect on the affinity of the opiate receptors ($K_d$: 1.75±0.19 vs. 1.68±0.29 nM; control vs. EV). The previously seen EV-induced increase in the density of hypothalamic $^3$H-naloxone binding sites was also replicated in this experiment ($B_{max}$: 21.50±1.15 vs. 32.30±2.66 fmole/mg tissue; C vs EV).
Fig. 56 Hypothalamic binding of [3H]-naloxone 4 months after treatment with 200 μg of EV in female C57BL/6J mice. Assay was performed on 300μ thick slices of MBH. Incubation was at 30° C for 3 hours. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Binding parameters are presented in the text.
DSP4 EXPERIMENTS

EFFECT OF DSP4 ON LH RESPONSE TO NALOXONE

Figure 57 illustrates that in prepubertal female rats (age 26 days), the normal LH response to naloxone was completely eliminated 2 hours following the administration of DSP4 (50 mg/kg, i.p. in 0.1 ml saline). Administration of the norepinephrine synthesis inhibitor U-14624 also eliminated the normal LH response to naloxone after 2 hours (200 mg/kg, i.p. in 0.1 ml DMSO).

TIME COURSE OF DSP4 EFFECT ON LH RESPONSE TO NALOXONE

As seen in Figure 58, in adult male rats, which normally demonstrate a much more sluggish response to naloxone than do prepubertal females, the LH response was significantly inhibited when compared to control animals 2 hours following exposure to DSP4. This inhibition persisted 2 days following DSP4 exposure. However, seven days after DSP4 exposure, the response appeared to be increased compared to controls, although this did not reach significance.
Fig. 57 Effect of DSP4 and U14624 on LH response to naloxone. 26 day old female SD rats were treated with saline, DSP4 (50 mg/kg, i.p. in saline) or U-14624 (200 mg/kg in 0.1 ml DMSO) at 1200 hrs. At 1400 hrs, animals from each group were given either saline or naloxone HCl (2.5 mg/kg, s.c.). Blood was collected 15 minutes later. Results are presented as the mean±s.e.m. N=7 animals per group.
Fig. 58 Time course of DSP4 effect on LH response to naloxone. Adult male SD rats were treated with DSP4 (50 mg/kg, i.p.) or saline. The animals were challenged with naloxone HCl (2.5 mg/kg) at various intervals after the DSP4. 15 minutes following naloxone, the animals were sacrificed and blood was collected. Results are presented as mean + s.e.m. N=7 rats per group.
EFFECT OF DSP4 ON LH LEVELS IN OVARIECTOMIZED RATS

DSP4 had the effect of rapidly reducing the circulating levels of LH in the short term (48 hours) ovariectomized rat. LH was maximally inhibited 15 minutes after treatment with DSP4 (control 210±50 ng/ml vs DSP4 55±4 ng/ml; p<0.01). Forty five minutes after the administration of DSP4, the LH level was still depressed at 30±10 ng/ml. Figure 59.

DOSE RESPONSE OF LH TO DSP4 IN OVARIECTOMIZED RATS

Figure 60 illustrates the dose-response relationship between DSP4 and serum LH in the short-term (48 hour) ovariectomized rat. Blood was sampled 15 minutes after the administration of DSP4. A multiphasic response was observed, with serum LH levels depressed to approximately 60% of control values by a dose of either 1 or 5 mg/kg. Increasing the dose to 10 or 25 mg/kg further depressed serum LH to approximately 40% of control levels. Finally, a dose of 50 mg/kg resulted in a depression of serum LH to approximately 10% of the control value.
Fig. 59 Effect of DSP4 on LH levels. Female SD rats were ovariectomized on day 24 of life. On day 26 they were given DSP4 (50 mg/kg, i.p.). Animals were sacrificed and blood was sampled immediately prior to, 15 and 45 minutes after the administration of the drug. Results are presented as the mean±s.e.m. N=7 rats per group. * P < 0.01 vs control by ANOVA.
Fig. 60 Dose response curve of effect of DSP4 on LH levels in ovariectomized rats. 26 day old female rats which had been ovariectomized 48 hours earlier were injected with DSP4 as indicated. Animals were sacrificed and blood was obtained 45 minutes after administration of drug. Results are given as the mean±s.e.m. N=7 animals per point. *P < 0.05 vs control by ANOVA.
EFFECT OF DSP4 ON PROLACTIN LEVELS IN OVARIECTOMIZED RATS

The response of serum prolactin to DSP4 was very similar to that of serum LH, and is illustrated in Figure 61. DSP4 (50 mg/kg, i.p. in 0.1 ml saline) had the effect of rapidly reducing the circulating levels of prolactin in the short term (48 hours) ovariectomized rat. Prolactin was maximally inhibited 15 minutes after treatment with DSP4 (control 28.9±11.3 ng/ml vs DSP4 7.2±6.0 ng/ml; p<0.01). Forty five minutes after the administration of DSP4, the prolactin level was still markedly depressed at 8.7±3.0 ng/ml.
Fig 61 Effect of DSP4 on prolactin levels. Female SD rats were ovariectomized on day 24 of life. On day 26 they were given DSP4 (50 mg/kg, i.p.). Animals were sacrificed and blood was sampled immediately prior to, 15 and 45 minutes after the administration of the drug. Results are expressed as the mean±s.e.m. * P < 0.05 ** P < 0.01 vs control values by ANOVA. N=6-7 animals per point.
DSP4 AND OPIOID BINDING STUDIES

EFFECT OF DSP4 IN VITRO ON $^3$H-NALOXONE BINDING

DSP4 reduced the specific binding of $^3$H-naloxone to slices of cerebral cortex, as determined at a single concentration (2 nM) by more than 80%. Inspection of Figure 62 reveals that this decrement was due solely to a decrease of total binding, while non-specific binding was unchanged.

EFFECT OF DSP4 IN VITRO ON $^3$H-NALOXONE SATURATION BINDING

Incubation of slices of mediobasal hypothalamus with increasing concentrations of $^3$H-naloxone in the presence of DSP4 (Fig 63) resulted in the elimination of any specific binding at low ligand concentrations, and in an overall decrease of approximately 75% in the $B_{max}$ (control: 18.92±2.14 fm/mg, vs DSP4: 5.29±0.85 fm/mg; p<0.001) and 50% in the $K_d$ (control: 4.21±0.89 nM, vs DSP4: 2.07±1.72 nM; p<0.001).
Fig. 62 Effect of DSP4 in vitro on [3H]-naloxone binding. 400μ slices of cerebral cortex were prepared from adult male SW mice and incubated with [3H]-naloxone (2 nM) for 3 hours at 30° C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Results are expressed as mean ± s.e.m of 8 (total binding) or 5 (non-specific binding) determinations.
Fig. 63  In vitro DSP4 and [3H]-naloxone binding. 400μ slices of MBH were obtained from 26 day old female SD rats, and incubated with [3H]-naloxone for 3 hours at 30° C. Non-specific binding was determined in the presence of 10 μM naloxone HCl.
ABILITY OF DSP4 TO COMPETE FOR $^{3}$H-NALOXONE BINDING SITES

DSP4 was able to displace $^{3}$H-naloxone from its binding site in both mediobasal hypothalamus and cerebral cortex. Figure 64 shows the displacement of the radioligand from slices of cerebral cortex obtained from adult male SW mice, while Figure 65 shows displacement from hypothalamic slices obtained from 26 day old female SD rats. The $IC_{50}$ values for DSP4 in these experiments were $9.6 \times 10^{-8}$ M and $7.8 \times 10^{-8}$ M respectively.

EFFECT OF IN VIVO DSP4 ON $^{3}$H-NALOXONE BINDING TO BRAIN SLICES

Two hours following the administration of DSP4 to 26 day old female SD rats, the $R_{\text{max}}$ of $^{3}$H-naloxone binding to slices of mediobasal hypothalamus was decreased by 44\% (control: $16.02\pm0.28$ fm/mg vs DSP4: $8.32\pm0.02$ fm/mg), while the $K_{d}$ was unchanged (control: $1.13\pm0.10$ nM vs DSP4: $1.15\pm0.01$ nM) (Fig 66). In adult male rats, a response in the opposite direction was observed, and hypothalamic slices had an increase of 33\% in the density of $^{3}$H-naloxone binding sites 2 hours following DSP4 administration (control: $12.49\pm1.30$ fm/mg vs DSP4: $16.71\pm0.89$ fm/mg). Again, there was no change in the $K_{d}$ (control: $3.16\pm0.77$ nM
Fig. 64 Ability of DSP4 to compete for cortical [3H]-naloxone binding sites. 400μ slices of cerebral cortex were prepared from adult male SW mice. The slices were incubated with [3H]-naloxone (2 nM) in the presence of increasing concentrations of DSP4. Results are expressed as the % of control binding, i.e. binding performed in the absence of any competitor. The IC50 value is given in the text.
**Fig. 65** Ability of DSP4 to compete for hypothalamic [3H]-naloxone binding sites. 400μ slices of MBH were prepared from 26 day old female SD rats. The slices were incubated with [3H]-naloxone (2 nM) in the presence of increasing concentrations of DSP4. Results are expressed as the % of control binding, i.e. binding performed in the absence of any competitor. The IC50 value is given in the text.
Fig. 66 Effect of DSP4 in vivo on hypothalamic [3H]-naloxone binding in females. 26 day old female SD rats were treated with saline (0.1 ml, s.c.) or DSP4 (50 mg/kg, i.p.). 400 μ slices of MBH were prepared 2 hours after the administration of drug, and were incubated with [3H]-naloxone for 2 hours at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Binding parameters are given in the text.
vs DSP4: 2.8±0.37 nM) (Fig 67). Seven days following the administration of DSP4 to adult male rats, the density of $^3$H-naloxone binding sites in slices of mediobasal hypothalamus was indistinguishable from that in untreated control animals ($B_{max}$: control: 12.49±1.30 fm/mg vs DSP4: 15.32±2.33 fm/mg), although the affinity of the binding site was slightly (although not significantly) increased ($K_i$: control: 3.16±0.77 nM vs DSP4: 6.63±1.77 nM) (Fig 68).

**EFFECT OF IN VITRO DESIPRAMINE ON IN VITRO DSP4 ACTION ON OPIOID BINDING**

Figure 69 illustrates the effects of pretreatment with desipramine on the DSP4-induced decrease in $^3$H-naloxone binding. Inclusion of DSP4 in the incubation medium resulted in a 90% decrease in the amount of $^3$H-naloxone bound to slices of mediobasal hypothalamus at a single concentration (2 nM) of radioligand. Preincubation of the tissue with desipramine alone did not affect the amount of $^3$H-naloxone bound. Such treatment, however, was unable to eliminate the DSP4-induced decrease in $^3$H-naloxone binding. There was, however, a slight protective effect of the desipramine preincubation, in that DSP4 was unable to decrease opioid binding to the same
Fig. 6.7 Effect of DSP4 in vivo on hypothalamic [3H]-naloxone binding in males. Adult male SD rats were treated with saline (0.1 ml, s.c.) or DSP4 (50 mg/kg, i.p.). 400μ slices of MBH were prepared 2 hours after the administration of drug, and were incubated with [3H]-naloxone for 2 hours at 30° C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Binding parameters are given in the text.
Fig. 68 Effect of DSP4 in vivo on hypothalamic [3H]-naloxone binding in males. Adult male SD rats were treated with saline (0.1 ml, s.c.) or DSP4 (50 mg/kg, i.p.). 400μ slices of MBH were prepared 7 days after the administration of drug, and were incubated with [3H]-naloxone for 2 hours at 30°C. Non-specific binding was determined in the presence of 10 μM naloxone HCl. Binding parameters are given in the text.
Fig. 69 Effect of in vitro treatment with desipramine and DSP4 on opioid binding. 400μ slices were prepared from adult female SW mice. The slices were preincubated on ice for 15 minutes with combinations of desipramine and DSP4 as detailed in the text. Following the preincubation, the slices were incubated with [3H]-naloxone (2 nM) for 3 hours at 30°C. Non-specific binding was determined in the presence of 10μM naloxone HCl. Results are presented as mean±s.e.m. * P<0.005 **P<0.01 vs SAL/SAL.
extent in the desipramine-treated tissue as in the control tissue.

Table 2 indicates that coincubation with the noradrenaline uptake inhibitors cocaine or desipramine was unable to prevent the DSP4 induced decrease in $^3$H-naloxone binding to cortical brain slices.

**EFFECT OF IN VIVO DESIPRAMINE ON IN VIVO DSP4 ACTION ON OPIOID BINDING**

As shown in Figure 70, an injection of desipramine did not have any effect on the binding of $^3$H-naloxone to slices of mediobasal hypothalamus obtained from the treated animals 2 hours later. However, pretreatment with desipramine was able to completely block the 40% decrease in binding seen 2 hours following administration of DSP4 alone.

**EFFECT OF IN VITRO DSP4 ON $^3$H-FLUNITRAZEPAM BINDING**

The specific binding of $^3$H-flunitrazepam to slices of mediobasal hypothalamus obtained from male SW mice was not affected by the presence of $10^{-5}$ M DSP4, at either a low (2 nM) or a high (8 nM) concentration of radioligand Fig. 71.
TABLE 2

EFFECT OF NORADRENALINE UPTAKE INHIBITORS ON DSP4-INDUCED REDUCTION IN [3H]-NALOXONE BINDING

<table>
<thead>
<tr>
<th>UPTAKE INHIBITOR</th>
<th>CONTROL</th>
<th>DSP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>4.81±0.18</td>
<td>2.98±0.19</td>
</tr>
<tr>
<td>Desipramine</td>
<td>-</td>
<td>2.40±0.17</td>
</tr>
<tr>
<td>Cocaine</td>
<td>-</td>
<td>2.58±0.12</td>
</tr>
</tbody>
</table>

Cortical slices (n=6 per group) were preincubated for 60 minutes with DSP4 (10⁻⁶ M) with and without desipramine or cocaine (10⁻⁵ M). [³H]-Naloxone (1 nM) was then added, and incubation was continued for 3 hours at 30°C. All DSP4 treated groups are significantly lower than the control group (p<0.01). Values are in fm/mg ± s.e.m. Experiment performed twice.
Fig. 70 Effects of in vivo desipramine and DSP4 on opioid binding. Adult female SW mice were treated with desipramine and/or DSP4 as detailed in the text. 2 hours after the drug treatment, the animals were sacrificed and 400μ slices of MBH were prepared. The slices were incubated with [3H]-naloxone (2 nM) for 3 hours at 30°C. Non-specific binding was determined in the presence of 10μM naloxone HCl. The results are presented as mean±s.e.m. *P<0.01 vs all other groups by ANOVA.
Fig. 71 Effect of DSP4 in vitro on benzodiazepine binding. Slices of MBH (230μ) were prepared from adult male SW mice. The slices were incubated with or without DSP4 (10μM) and the binding of [3H]-flunitrazepam (2 nM, 8 nM) was determined. Incubations were carried out at 0° C for 3 hours. Non-specific binding was determined in the presence of 1 μM clonazepam. Results are given as mean ± s.e.m.
EFFECT OF IN VIVO DSP4 ON [3H]-FLUNITRAZEPAM BINDING

Two hours following treatment with DSP4, the specific binding of [3H]-flunitrazepam (4 nM) was unchanged in either cerebral cortex or cerebellum obtained from adult male SW mice. However, there was a significant decrease in the amount of [3H]-flunitrazepam bound to hypothalamic slices (Fig. 72). If the interval between administration of DSP4 and the determination of [3H]-flunitrazepam binding was extended to 4 days, a different picture emerged. Hypothalamic slices from the DSP4 treated group bound significantly more [3H]-flunitrazepam (8 nM) than did those from the control group (DSP4: 68.9±5.7 fm/mg vs control: 28.3±3.3 fm/mg, p< 0.001). In cerebellum, [3H]-flunitrazepam binding was also increased (B_max: Control: 29.8±5.5 fm/mg vs DSP4 51.4±10.5 fm/mg; K_d: Control: 1.5±0.9 nM vs DSP4: 2.4±1.7 nM; Fig 73). In cerebral cortex, the binding curves were superimposable (B_max: Control: 138.4±28.5 fm/mg vs DSP4 156.4±21.1 fm/mg; K_d: Control: 5.5±2.1 nM vs DSP4: 7.5±1.7 nM; Fig 74).
Fig. 72: Effect of in vivo DSP4 on benzodiazepine binding. Adult male SW mice were treated with DSP4 (50 mg/kg, i.p.) or saline (0.1 ml). 2 hours after drug treatment, the animals were sacrificed and 230 μ slices of MBH, cerebral cortex and cerebellum were prepared. The slices were incubated with [3H]-flunitrazepam (4 nM) for 3 hours at 0°C. Non-specific binding was determined in the presence of 1 μM clonazepam. Results are presented as mean±s.e.m. *P<0.025, DSP4 vs control.
Fig. 73 Effect of in vivo DSP4 on benzodiazepine binding. Adult male SW mice were treated with DSP4 (50 mg/kg) or saline. 230μm slices of cerebellum were prepared 4 days later, and specific binding of [3H]-flunitrazepam was measured. Incubations were carried out at 0°C for 3 hours. Non-specific binding was determined in the presence of 1 μM clonazepam. Binding parameters are given in the text.
Fig. 74 Effect of in vivo DSP4 on benzodiazepine binding. Adult male SW mice were treated with DSP4 (50 mg/kg) or saline. 230μ slices of cerebral cortex were prepared 4 days later, and specific binding of [3H]-flunitrazepam was measured. Incubations were carried out at 0°C for 3 hours. Non-specific binding was determined in the presence of 1 μM clonazepam. Binding parameters are given in the text.
EFFECT OF DSP4 ON SEXUAL MATURATION

Preliminary observations (Fig. 75) showed that the administration of DSP4 to 5 day old female rats resulted in a delay in VO (34.2±0.3 days (saline) vs 36.1±0.3 days (DSP4), p<0.01), but no change in body weight at VO (114.3±3.4 grams (control) vs 112.2±3.1 grams (DSP4)). It was decided to investigate the results of administering DSP4 later in life on the normal progression to puberty. An unexpected observation was that DSP4 resulted in a brief loss of weight on the first night following the injection. Subsequently, the animals gained weight at the same rate as the saline injected controls. Growth curves for the animals administered DSP4 are presented in Figures 76-78. In those groups which received DSP4 on day 5 and day 23 of life, VO was delayed, though in all cases VO occurred at the same body weight (Table 3). Exposure to DSP4 did not affect the weight of the ovaries, uterus or adrenal glands, or the number of corpora lutea or ova present on the day of VO, with the exception of uterine weight in the rats injected on day 29 (Control: 202.1±14.5 mg vs DSP4: 159±17.5 mg; p<0.05) (Table 3). DSP4 was treated in such a manner as to favor production of the aziridinium ion, and administered to 29 day old females. These animals
Fig. 75 Effect of perinatal DSP4 on sexual maturation. 5-day-old female SD rats were treated with DSP4 (50 mg/kg, i.p.) or saline. The age and body weight on the day of vaginal opening are presented as mean ± s.e.m. *P < 0.01 vs control.
Fig. 76 Growth curve of animals exposed to DSP4. Female SD rats were treated with DSP4 (50 mg/kg) or saline on day 23 of life, and weighed daily until vaginal opening occurred. Results are presented as the mean±s.e.m.
Fig. 77 Growth curve of animals exposed to DSP4. Female SD rats were treated with DSP4 (50 mg/kg), cyclized DSP4 (DSP4 incubated in 0.05M PBS for 45 minutes @ 37°C) or saline on day 29 of life, and weighed daily until vaginal opening occurred. Results are presented as the mean ± SEM.
Fig. 78 Growth curve of animals exposed to DSP4. Female SD rats were treated with DSP4 (50 mg/kg) or saline on days 24 and 26 of life, and weighed daily until vaginal opening occurred. Results are presented as the mean±SEM.
### TABLE 3

**EFFECT OF DSP4 ON PUBERTY**

<table>
<thead>
<tr>
<th>AGE AT ADMINISTRATION</th>
<th>DAY 23</th>
<th>DAY 29</th>
<th>DAY 24 + DAY 26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (11)</td>
<td>DSP4 (10)</td>
<td>Control (10)</td>
</tr>
<tr>
<td>Age at Y.O. (days)</td>
<td>33.5±0.8</td>
<td>36.0±0.4**</td>
<td>36.6±0.7</td>
</tr>
<tr>
<td>Body Wt at Y.O. (grams)</td>
<td>111.4±4.6</td>
<td>112.4±2.4</td>
<td>116.5±3.5</td>
</tr>
<tr>
<td>Ovarian Wt at Y.O. (mg/pr)</td>
<td>30.8±1.8</td>
<td>28.3±2.0</td>
<td>29.3±2.5</td>
</tr>
<tr>
<td>Adrenal Wt at Y.O. (mg/pr)</td>
<td>31.5±2.2</td>
<td>33.0±1.5</td>
<td>42.4±2.5</td>
</tr>
<tr>
<td>Uterine Wt at Y.O. (mg)</td>
<td>162.4±15.5</td>
<td>149.2±13.4</td>
<td>202.1±14.5</td>
</tr>
<tr>
<td># of C.L. at Y.O.</td>
<td>7.4±2.3</td>
<td>6.2±1.3</td>
<td>6.6±2.3</td>
</tr>
</tbody>
</table>

Number of rats per group in parentheses. Values are means ± s.e.m. * P < 0.05 ** P < 0.01 vs. control
did not grow in a manner different from their littermates which received the uncyclized DSP4 (Fig 77).

**EFFECT OF DSP4 ON INDUCTION OF GONADOTROPIN SURGES IN IMMATURE RATS**

**EFFECT OF DSP4 ON INDUCTION OF AN LH SURGE BY GONADAL STEROIDS**

Treatment of EB-primed immature rats with P elicited an LH surge (Fig. 79). If the animals received DSP4 at the same time as the P, the LH surge was inhibited, and LH levels were not significantly different from those animals treated with EB alone. In contrast, when DSP4 was administered 2 hours prior to the progesterone, the LH surge was not inhibited, and the LH levels attained were not significantly different from those in steroid-treated animals not exposed to DSP4.

Figure 80 illustrates that pretreatment with naloxone, before the administration of progesterone, had no effect on the induction of the LH surge. Pretreatment with DSP4, as shown above, blocked the generation of the surge. The administration of naloxone before the DSP4 was able to partially block
Fig. 79 Effect of DSP4 on gonadal steroid-induced LH surge. Female SD rats received 10 μg of EB at 1000 hrs on day 24 of life. On day 27 of life the animals were further treated as follows: group 1: no further treatment. Group 2: 1 mg P at 1200 hrs. Group 3: 50 mg/kg of DSP4 at 1000 hrs and 1 mg P at 1200 hrs. Group 4: 50 mg/kg at 1200 hrs and 1 mg P at 1200 hrs. Animals were sacrificed and blood collected at 1700 hrs on day 27 of life. Results are presented as the mean ± s.e.m. * P < 0.05 vs group 2 ** P < 0.05 vs group 3.
Fig. 80 Effect of naloxone on the influence of DSP4 on gonadal-steroid induced generation of LH surges. 24 day old female SD rats were treated with 10 μg of EB at 1000 hours. On day 27 of life, the animals were further treated as follows: Group 1: (EP) 1 mg of P at 1200 hrs. Group 2: (E+N) 2.5 mg/kg of naloxone HCl at 1000 hrs and 1 mg of P at 1200 hrs. Group 3: (E+D) 50 mg/kg of DSP4 at 1000 hrs and 1 mg of P at 1200 hrs. Group 4: (E+N+D) 2.5 mg/kg of naloxone HCl at 1000 hrs, then 50 mg/kg of DSP4 at 1010 hrs, 1 mg of P at 1200 hrs. Animals were sacrificed at 1700 hrs on day 27 of life, and blood was collected. * P < 0.004 vs groups 1 and 2. ** P < 0.05 vs group 4 *** P < .01 vs groups 1 and 2.
the inhibitory effect of DSP4, but a normal surge was not generated.

EFFECT OF DSP4 ON PMSG-INDUCED OVULATION IN IMMATURE RATS

PMSG was administered to sexually immature female rats (24 days old) to induce ovulation. DSP4, administered on day 26 of life, markedly attenuated the stimulatory effects of PMSG on the reproductive tract of immature female rats observed on day 27 of life (Table 4). Both the number of animals exhibiting VO, and the number of animals which ovulated were significantly greater in the control group than in the DSP4 group. In addition, the number of corpora lutea present in those animals which ovulated was significantly decreased in the treated group, as was ovarian weight. Uterine weight was not affected.

Animals receiving DSP4 concurrently with the PMSG showed a lower incidence of VO (DSP4 & PMSG concurrently 67%, DSP4 and PMSG separately, 36%) when the experiment was terminated. Uterine weight was significantly reduced (Table 5). Regardless of the protocol, animals treated with DSP4 were significantly lighter than control animals.
<table>
<thead>
<tr>
<th></th>
<th>CONTROL (10)</th>
<th>DSP4 (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Wt</strong> (grams)</td>
<td>69.8±1.2</td>
<td>61.5±1.3*</td>
</tr>
<tr>
<td><strong>Ovarian Wt</strong> (mg/pr)</td>
<td>29.3±1.6</td>
<td>19.7±1.6*</td>
</tr>
<tr>
<td><strong>Uterine Wt</strong> (mg)</td>
<td>85.3±8.5</td>
<td>84.5±10.4</td>
</tr>
<tr>
<td><strong>Animals with C.L.</strong></td>
<td>9/10</td>
<td>6/10</td>
</tr>
<tr>
<td><strong>C.L./rat</strong></td>
<td>11.4±1.1</td>
<td>7.5±0.9**</td>
</tr>
<tr>
<td><strong>Rats with V.O.</strong></td>
<td>9/10</td>
<td>7/10</td>
</tr>
</tbody>
</table>

Number of rats per group given in parenthesis.

* P < 0.05 vs. control  ** P < 0.01 vs. control
### TABLE 5

**EFFECT OF DSP4 INJECTION EITHER COINCIDENT WITH OR 48 HOURS AFTER PMSG ON PUBERTAL DEVELOPMENT**

<table>
<thead>
<tr>
<th>PMSG followed by DSP4 at 48 hr (6)</th>
<th>Coinjection of DSP4 &amp; PMSG (11)</th>
<th>PMSG Only (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Wt</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(grams)</td>
<td>62.0±2.4*</td>
<td>59.7±1.6**</td>
</tr>
<tr>
<td><strong>Uterine Wt</strong></td>
<td>135.1±8.4</td>
<td>111.7±5.1</td>
</tr>
<tr>
<td>(mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ovarian Wt</strong></td>
<td>22±2.7</td>
<td>21.9±1.6**</td>
</tr>
<tr>
<td>(mg/pr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ova</strong></td>
<td>1.8±1.7**</td>
<td>4.5±1.6*</td>
</tr>
</tbody>
</table>

Number of rats per group is given in parentheses.

* P < 0.01 vs control ** P < 0.05 vs control  P < 0.025 vs control
When naloxone was administered at 1000 hrs on the morning of proestrous (i.e., 48 hours after the PMSG) it was able to block the DSP4-induced weight loss seen in control animals 24 hours later. Furthermore, naloxone prevented an increase in ovarian weight, but was without effect on the decrease in ovarian weight induced by treatment with DSP4 (Fig. 81).

EFFECT OF DSP4 ON HYPOTHALAMIC AMINE CONTENT

The effect on hypothalamic amine content of the administration of DSP4 during the various experimental protocols is given in Figures 82, 83 and 84. In the experiments where pups were exposed to DSP4 at various ages and allowed to proceed to puberty, all of the animals had amine levels which were not significantly different from control animals on the day of sacrifice (day 34), with the exception of the group administered DSP4 at 23 days of age. In this case, hypothalamic noradrenaline was significantly depressed when compared to untreated controls (Figure 82). Hypothalamic dopamine was not affected by DSP4 in this or any of the other experiments. Upon termination of the PMSG experiments, the hypothalamic noradrenaline content
Fig. 81 Influence of naloxone on the effect of DSP4 on PMSG-induced changes in body and organ weights. Animals were treated with combinations of PMSG, DSP4 and naloxone as detailed in the text. Results are given as mean ± s.e.m.
Fig. 82 Effect of DSP4 on hypothalamic noradrenaline content in puberty experiments. Animals were treated with DSP4 on the days indicated, and sacrificed on day 34 of life. Results are presented as the mean ± S.E.M. Determinations were performed on 7–10 hypothalami per group. * P < 0.01 vs control.
FIG. 93 Effect of DSP4 on hypothalamic noradrenaline content in animals treated with PMSG. Hypothalamic noradrenaline content was determined in animals treated with DSP4 and PMSG as follows: Group 1: PMSG with saline. Group 2: PMSG with DSP4. Group 3: PMSG then saline. Group 4: PMSG then DSP4. (Details in text). Results are presented as the mean ± s.e.m. Determinations were performed on 5–8 hypothalami per group. * P < 0.05 vs group 3, † P < 0.05 vs group 1.
Fig. 84 Effect of DSP4 on hypothalamic noradrenaline content in animals treated with gonadal steroids. All animals received 10 μg of EB on day 21 of life. On day 27 of life the animals were further treated as follows: Group 1: no further treatment. Group 2: 1 mg of P at 1200 hrs., Group 3: 50 mg/kg of DSP4 at 1200 hrs and 1 mg of P at 1200 hrs. Group 4: 50 mg/kg of DSP4 at 1000 hrs and 1 mg of P at 1200 hrs. Results are presented as the mean±s.e.m. Determinations were performed on 7-8 hypothalami per group. * P < 0.05 vs group 1.
of the DSP4 treated animals was significantly lower than that of the control groups. This was true, regardless of whether or not the DSP4 and the PMSG were administered together or separately (Figure 83). Animals given DSP4 in an attempt to inhibit the progesterone-induced LH surge following E2 priming also had significantly lower noradrenaline levels than did the control animals. Whether or not the DSP4 and progesterone were co-administered did not significantly affect the amine levels (Figure 84).

The manipulations used in studying the effects of DSP4 on 3H-naloxone binding were also examined for their effect on hypothalamic amine levels, and are summarized in Table 6. In no case was dopamine affected by the administration of DSP4. While the hypothalamic noradrenaline content of adult male rats was not affected 2 hours following the administration of the drug, mature female rats showed a depletion of approximately 50% two hours following exposure to DSP4.
TABLE 6

<table>
<thead>
<tr>
<th>TIME FOLLOWING DSP4</th>
<th>CONTROL</th>
<th>2HR</th>
<th>24 HR</th>
<th>48 HR</th>
<th>7 DAYS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.37±0.18</td>
<td>0.65±0.13*</td>
<td>0.95±0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.55±0.21</td>
<td>1.53±0.27</td>
<td>1.12±0.23</td>
<td>0.8±0.11</td>
<td>0.98±0.16§</td>
</tr>
</tbody>
</table>

Time course of effect of DSP4 on hypothalamic concentration of noradrenaline in adult male rats. Values are expressed as g NA/gram of tissue ± s.e.m. * P< 0.010 vs control; P< 0.025 vs control; § P< 0.05 vs control. N.B. Dopamine content was unchanged.
DISCUSSION

METHODOLOGICAL CONSIDERATIONS

It was the intent of this thesis to examine the general topic of diurnal variations in the complement of opioid receptors in the brain of prepubertal rodents. The development of these rhythms was traced, and a functional correlate of changes in opioidergic tone — viz. changes in the control of LH release was established. Several interventions were examined in an attempt to see if these had any effect on the diurnal rhythm in opioid binding. However, prior to performing the experiments designed to examine the genesis and nature of such rhythms, it was necessary to develop an appropriate tool for these investigations. While numerous investigators have described methods for investigating opioid receptors in nervous tissue (for references see Zukin, 1984), the assays which were available at the outset of these investigations were unsatisfactory for an undertaking of this nature.

For example, autoradiographic techniques are much too cumbersome and time consuming for use in a project of this magnitude. Furthermore, while
autoradiography is an excellent tool, particularly for the regional localization of binding sites for a given ligand in a given tissue (Leslie et al., 1985; Herkenham, 1984), it is a major undertaking in terms of time and effort to characterize the binding and to construct binding curves for saturation analysis, and to determine binding constants. Autoradiography is best suited to investigations dealing with a small number of animals. The very nature of receptor binding studies dictates that they must be cross-sectional measures of individual animals. It is not possible to sample an animal at more than one time point. This in turn determines that the amount of variability in studies of this type is going to be greater than the intra-animal variability would be if an animal could be sampled repeatedly (Wirz-Justice, 1986). Because of the inter-animal variability in receptor binding, we felt that the use of very small numbers of experimental subjects could have skewed or biased the results, and looked for a method which would allow the use of groups of 7-10 animals per experimental group. There are other pitfalls with the method of in vitro (and in vivo) receptor radioautography. Reported differences in ligand binding seen with the autoradiographic technique may
actually be due to changes in the quenching of tritium \( \beta \)-emission by myelin (Rainbow et al., 1984). This potentially serious problem has been addressed recently, and strategies developed to deal with it (Herkenham and Sokoloff, 1984; Kuhar and Unmerstal, 1985; Geary and Wooten, 1985; Geary et al., 1985).

Opioid, as well as other neurotransmitter, hormone or drug, binding sites have traditionally been examined in homogenate preparations (Zukin, 1984). A tremendous amount of information has been developed studying neurotransmitter and drug receptors in brain homogenate preparations, (Marangos et al., 1984). However, it has become increasingly apparent that using a thoroughly disrupted tissue preparation in a radioligand binding assay presents a range of problems which must be addressed. In the first case, preparation of a tissue homogenate, crude membrane preparation or synaptosomal fraction isolates the receptor from its natural environment. Since receptors are coupled to an effector mechanism, it would be useful to examine these entities in intact tissue, where this mechanism may be in place. Significant differences have been found, for instance, in the binding of benzodiazepines to intact
and to broken cells cultured from cerebral cortex (Sher et al., 1982). Mechanical disruption of brain tissue has similarly been shown to affect the properties of histaminic (Tuong et al., 1980), α₁-adrenergic (Sladeczek et al., 1983) and muscarinic (Gilbert et al., 1979) receptors in brain and other tissues. Receptor-linked effectors are also affected by cellular disruption. Porzig (1982) has reviewed the evidence and affirmed that membrane preparations and cultured cells from the same tissue exhibit marked differences in their adenylate cyclase systems.

Performing binding assays in vivo would, of course, eliminate many of the problems associated with a disrupted tissue preparation. However, in vivo assays are not without their own particular problems. These assays require large amounts of radioligand, and the resultant expense precludes their use in many laboratories (Kuhar et al., 1986), including our own. There are also other practical problems to deal with. One must be concerned with the existence of the blood-brain barrier. The entry of certain ligands, neuropeptides among them, into the central nervous system following peripheral administration, would be severely restricted (Meisenberg and Simmons, 1983;
Pardridge, 1983). Metabolism or degradation of the ligand after systemic administration is also a potential problem (Gorenstein and Snyder, 1980; Pardridge and Meitus, 1981; Herkenham, 1984). Finally, the presence of residual unbound or non-specifically bound ligand in the tissue may result in unacceptably high levels of background noise (Herkenham, 1984).

It was felt that a preparation which maintained some degree of cellular integrity was desirable in our investigations of opioid receptor regulation. Autoradiographic studies are performed on dead tissue which has been frozen and thawed, and while this technique is quite adequate for detecting binding sites, it is not well suited to a study of receptor regulation, particularly under physiological conditions. In addition, a preparation which would allow quantification of receptors which were available to the endogenous ligand was also needed. Homogenate and membrane preparations allow free access of the radioligand to all unoccupied receptors, including those which have been internalized. However, only those receptors located on the surface of the cell membrane at a given moment are available for interaction with endogenous ligand.
Thus, a preparation which would permit the recognition only of membrane-bound receptors was needed. Furthermore, an assay system which required minimal tissue preparation, was rapid, and would permit studies to be performed on groups of 7-10 animals at several times over the course of a day was needed. Opioid receptors may (Abood et al., 1980; Wilkinson et al., 1981) or may not (Pert and Snyder, 1973a) be sensitive to freezing. To avoid such uncertainties, it was also necessary that the binding assay could be performed on fresh tissue.

The brain slice suggested itself as a suitable candidate for such a study. These relatively thick slices (100-700) are easy to prepare in quantity from a variety of animal species. They display structural integrity (Bennett et al., 1983; Bak et al., 1980), exhibit appropriate electrical activity (Dingledine, 1984), continue to respire and metabolize, although at a somewhat reduced rate than in situ (Lipton and Whittingham, 1984), and can, with appropriate care, be maintained for several hours (Hatton, 1984; Bak et al., 1980). Brain slices have traditionally been used to study electrophysiology and pharmacology in the central nervous system.

Other investigators have made sporadic attempts to use brain slices in radioreceptor assays. Davis et al. (1975) were the first group to report the use of brain slices (brainstem) in a study of opioid binding sites. They found that $[^3H]$-morphine binding was higher in the slices than it was in brainstem homogenates. Slices from morphine tolerant animals also bound less morphine than slices obtained from naive animals, apparently because of a morphine-induced increase in $K_d$. This difference was not apparent in homogenates. However, while the change in binding represented a decrease in the $B_{max}$ of the binding site, a much larger change in the $K_d$ was observed. The 250% increase in $K_d$ seen in the slices from morphine tolerant animals, may well be artifactual, resulting from the presence of a large amount of residual morphine in the slice. This brings out a problem which will be repeated over and over in the studies to be cited below. None of the studies using brain slices in radioligand binding assays properly characterized the binding to these slices. Extrapolations were made from other published studies which had used the homogenate preparations. It must
be emphasized that it is essential that all new methods of binding must be rigourously characterized with respect to time course of binding, stereospecificity, degree of non-specific binding, saturability, tissue linearity, etc (Burt, 1985; Marangos et al., 1984). For instance, in the study by Davis et al. (1975), the binding was allowed to proceed for 16 minutes at 37°C. We have shown that in our relatively thin slices (300-400 μ), the binding (at 30°C) is not at equilibrium until 60-90 minutes have elapsed. The problem of changes induced in opioid binding by chronic exposure to morphine was addressed again by this same group, using the ligand \([^3\text{H}]-\text{d-al}a^2-\text{met-enkephalinamide} \) (DALA) in brain slices (Davis et al., 1978). Once again, a decrease in the \(B_{\text{max}}\) was found in conjunction with an increase in \(K_d\). Obviously the retention of morphine in the slices (which were not washed following incubation) is a major confounding variable. Again, while reference is made to some preliminary characterization of the assay, the details are not presented. Of some interest, are the reports emanating from this same laboratory which demonstrate that \(^3\text{H}-\text{naloxone} \) and \(^3\text{H}-\text{morphine} \) (but not \(^3\text{H}-\text{DALA}\) binding in brain slices demonstrates some degree of
positive cooperativity, while none is seen in brain homogenates (Davis et al., 1978; Davis et al., 1977).

The brain slice, then, clearly has some advantages over homogenates, in that it allows for the study of site-site interactions not apparent in disrupted cell preparations.

Others have used the brain slice to pursue the binding of opioid compounds in the central nervous system. Huang and Takemori, showed, in preliminary studies, that slices of cerebral cortex and corpus striatum accumulated low concentrations of etorphine (< 5 nM) in a manner compatible with receptor binding, i.e. uptake was saturable, stereospecific, and did not require metabolic energy (Huang and Takemori, 1976). With hindsight, some would consider that the experimental conditions were not ideal since the presence of sodium ions in the incubation buffer would certainly have greatly reduced specific binding of the agonist etorphine (Pert and Snyder, 1974). However, Werling et al. (1985) have pointed out recently the importance that "experiments in which possible binding site interactions are examined be conducted under conditions which maximize the likelihood that physiologically relevant interactions be observed. Such conditions would include the use of
physiological temperatures and the presence of receptor regulating cations". Takemori and his co-workers continued to study opiate (\textsuperscript{3}H-morphine) binding sites in this type of incubation system. Quantitative binding assays, as we now understand them, were never performed. Instead, the ability of naloxone to displace pre-bound ligand was carefully examined (Kitano and Takemori, 1977; Kitano and Takemori, 1979). Thus, binding of morphine (10nM) was stereospecific and extremely sensitive to displacement with naloxone; the effect was maximal at concentrations of less than $10^{-10}$ M and was readily seen at $10^{-12}$ M. Striatal slices from morphine-dependent mice showed an enhanced sensitivity (affinity) to naloxone, whereas slices from cortex and brainstem did not. This result is quite different from that described by Davis et al. (1975, 1977, 1978) who observed a marked decrease in affinity of morphine binding to slices of brainstem obtained from tolerant rats. Strict comparisons between these experiments are difficult to make, since the assay methods, buffers and animals were dissimilar. However, Oishi and Takemori (1982) draw attention to the problem of residual morphine contained within the slices from morphine-dependent animals. Their efforts
to wash out the drug were not completely successful, but they showed that mouse brain tissue, 6 hours after morphine withdrawal, did not contain detectable quantities of morphine. Slices of corpus striatum from these acutely withdrawn mice still showed the affinity shift previously attributed to morphine dependence (Oishi and Takemori, 1982). Thus, in the presence of sodium ions, the antagonists naloxone and naltrexone can discriminate between brain tissue from naive or morphine-tolerant mice. This discrimination is not consistently seen in brain tissue homogenates (Brunello et al., 1984).

A further report from the laboratory of Takemori describes the influence of enkephalin pretreatment, in vitro and in vivo, on the morphine-releasing effect of naloxone (Vaught et al., 1981). Injection of leucine enkephalin (leu-enk) but not met-enkephalin (met-enk) 15 minutes before sacrifice, or preincubation of striatal slices with leu-enk (0.1 pM to 1.0 nM) markedly enhanced the ability of naloxone to release $[^3]H$-morphine. Again, binding curves were not examined but the evidence suggests a receptor-mediated process which might readily be studied in slice binding assays. Also, the shift in apparent affinity of the opiate binding site is consistent
with the in vivo data, i.e., prior administration of leu-enk to mice markedly enhanced morphine-induced analgesia (Vaught and Takemori, 1979). These experiments, on the rapid induction of opiate receptor modifications, appear to complement the studies reported by Pert and Snyder (Pert and Snyder, 1976) and by Lewis et al. (1984). These papers describe the enhancement of opiate binding in rat and mouse brain which followed the injection of opiate agonists or antagonists.

Attempts have been made to characterize the binding, in brain slices, of radioligands to a number of other classes of neurotransmitter receptors, including (but not limited to) dopaminergic (Martres et al., 1984), muscarinic cholinergic (Gilbert et al., 1979), benzodiazepine (Whitaker et al., 1984) and adrenergic (Wilkinson and Wilkinson, 1985) sites. Recently, this technique has also been extended to the use of muscle slices (Watson-Wright and Wilkinson, 1986). For a review on the use of tissue slices in radioligand binding assays, including a more complete discussion on the advantages and problems of this method, as well as a discussion of the use of intact cells (e.g. cell cultures,
dispersed tissue preparations, etc.) see Appendix I and references therein.

The brain slice appears, then, to fulfill the requirements for a binding system which this investigation demands. It is rapid, simple, inexpensive, involves minimal tissue preparation, maintains some degree of tissue integrity, and it appears that it is possible to maintain the receptors in an environment which has some relationship to the in situ situation. It cannot be emphasized too strongly, that in order to provide useful information, this binding system, as well as all others, must be well characterized (Burt, 1985). To that end, the following series of experiments were performed.
CHARACTERIZATION OF [3H]-NALOXONE BINDING TO BRAIN SLICES:

A radioreceptor assay has been developed and characterized for the binding of [3H]-naloxone to thick slices of brain tissue (mediobasal hypothalamus and cerebral cortex). While it is true that the first step in the action of a drug, hormone, or neurotransmitter on its target tissue, is often the binding of that substance to a specific receptor, not all binding sites are, in fact, true receptors. A rigid set of criteria must be satisfied to demonstrate that a binding site is a physiologically relevant receptor. These criteria have been reviewed by Burt (1985), and are discussed here with respect to the characterization of the slice binding assay with brain tissue. In order for information derived from binding experiments to be meaningful, the binding must be in a "steady-state", i.e. a dynamic equilibrium between receptor, ligand and receptor-ligand complex, must exist. For our purposes, the time to equilibrium may be defined as that amount of time necessary for the specific binding to reach a maximum. This time period is a function of the association and dissociation rate constants for the receptor binding reaction (Enna, 1980). The time
course of $[^3H]$-naloxone binding to slices of brain tissue at $30^\circ$ C showed that saturation was not achieved until approximately 2 hours of interaction between the tissue slice and the radioligand had occurred. This result clearly differs from the time course seen in tissue homogenates, where saturation occurs in 5-10 minutes (Pert and Snyder, 1973a; Snyder et al., 1975). This may well represent time necessary for the ligand to diffuse into the tissue slice. That the ligand does fully, and evenly, penetrate the slice is demonstrated unequivocally in Figure 6. In this pseudo-color autoradiograph, through the middle of a slice of cerebral cortex, no difference in ligand binding is discernable between the periphery and the centre of the slice. It has also been demonstrated that specific binding to brain slices will saturate when the binding is carried out at $4^\circ$ C. Under these circumstances, saturation is reached before 12 hours, and is stable for up to 30 hours. In order to be certain that equilibrium had been reached, all incubations were carried out for 2-3 hours ($30^\circ$ C) or 24 hours ($4^\circ$ C). An interesting phenomenon which was observed, is that at low concentrations (1 nM), cooling of mediobasal hypothalamus, and to a lesser extent cerebral cortex,
from rat, mouse and hamster, results in an increase in specific binding. A closer examination of the binding in rat hypothalamus and cortex provided evidence that cooling of the tissue induced a high affinity state of the $[^3H]$-naloxone binding site. $B_{\text{max}}$ values were not significantly affected although there was a tendency to lower values in hypothalamus (Wilkinson et al., 1986). Artificially induced increases in membrane viscosity have been shown to increase $[^3H]$-naloxone binding (Heron et al., 1981). It has also been shown that there is an inverse relationship between membrane lipid fluidity and the ability of serotonin to bind to a crude membrane preparation (Heron et al., 1980), and treatment known to change membrane viscosity has been reported to affect $\beta$-adrenergic receptors in rat lung membranes (Scarpace et al., 1985) but not in brain slices (Wilkinson et al., 1986). Viscosity decreases exponentially with increased temperature (Fung, 1981), and the increased binding which we have seen with cooling of brain tissue may be explained by changes in membrane viscosity.

Saturability is a sine qua non for the demonstration of a true receptor binding system. Opioid binding to slices of both cerebral cortex and
mediobasal hypothalamus demonstrates saturability, and kinetic analysis of the binding curves shows the binding to be of high affinity, i.e. $K_d$ in the nanomolar range. Saturation occurs with nanomolar concentrations of ligand, in agreement with previously published studies using homogenate assays (Law and Loh, 1978; Pert and Snyder 1973b).

The addition of a large excess of unlabelled naloxone was able to rapidly displace the bound radiolabelled ligand. Rapid reversibility of the binding of $[^{3}\text{H}]$-naloxone in this assay is one of several pieces of evidence which indicate that the bound radioactivity represents true binding, and is not the result of a tissue uptake process. $[^{3}\text{H}]$-naloxone which had been taken up or internalized by the cell would not be so readily displaced by the excess naloxone. Also consistent with the argument that what is being measured is binding, and not tissue accumulation, are the observations that saturable binding continues at 4°C, and that the binding is not affected by the metabolic inhibitors sodium azide or ouabain. Furthermore, the initial assays were carried out in the absence of sodium (i.e. in TRIS buffer). Tissue uptake is an energy-requiring process which can be blocked by the
appropriate metabolic inhibitors, is sodium dependent (Iversen, 1975), and is virtually eliminated at low temperature (Silverstein et al., 1977; Pastan and Willingham, 1981; Wileman et al., 1985). Even in the presence of sodium, ouabain was without effect on the specific binding of \[^{3}H\]-naloxone to brain slices. This is further evidence that true specific binding and not tissue uptake is being measured in this assay system.

A prerequisite in demonstrating the presence of a specific receptor is that there must be good correlation between the affinity of a particular substance occupying the binding site and its pharmacological profile; i.e., the studies presented here demonstrate that substances which act through opioid receptors are able to displace \[^{3}H\]-naloxone from its binding site in the slice assay, whereas non- opioids do not. Non-specific binding of naloxone represented approximately 25% of the total binding over the concentration range of \[^{3}H\]-naloxone used in these experiments. Further studies revealed that specific binding reached a stable value after the tissue was washed twice. This degree of non-specific binding compares well with that seen by other investigators using homogenate preparations, and as a
result, all subsequent assays included 2 vashes upon termination of the incubation phase. Furthermore, the IC50 values of the compounds examined parallel the biological activity of these compounds (Snyder and Pert, 1975a). While not conclusive in and of itself, this is strong supporting evidence that we are measuring a functional receptor, and not merely a non-specific binding site or "acceptor" site with this assay (Burt, 1985). Of equal significance is the observation that the binding site displays stereospecificity. The biologically active opiate levorphanol is a potent displacer of 3H-naloxone, with an IC50 of 2 nM. The inactive enantiomer dextrorphan, which is essentially devoid of biological activity, is unable to displace 3H-naloxone binding from brain slices, except at high concentrations and even then only minimally. This is in keeping with the stereospecificity of the opioid receptor in brain tissue as demonstrated with an homogenate assay (Pert and Snyder, 1973b), and fulfills another of the basic criteria for identification of a true receptor (Burt, 1985).

The specific binding of [3H]-naloxone to slices of both cerebral cortex and mediobasal hypothalamus has been shown to be saturable, and is of high
affinity, i.e. saturation occurs when the tissue is incubated with nanomolar concentrations of radioligand. It must, however, be pointed out that while saturation is necessary to demonstrate the presence of a specific receptor, it is hardly sufficient. Indeed, saturable and stereospecific binding to inanimate objects such as glass fibre filters (Snyder and Pert, 1975a) and talc (Cuatrecasas and Hollenberg, 1975) has been described.

The distribution of opioid receptors throughout the brain has been well characterized in a number of species, including human (Pert and Snyder, 1973a; Kuhar et al., 1973; Snyder and Pert, 1975b; Chang et al., 1979). The characterization of the slice assay with $^3$H-naloxone confirmed the findings of these earlier investigations. High binding was found in the mediobasal hypothalamus and an intermediate level of $^3$H-naloxone binding was seen in the cerebral cortex. Specific binding was not detectable in slices of rat and mouse cerebellum, a tissue which is virtually devoid of opioid receptors.

A linear relationship was found between the amount of $^3$H-naloxone specifically bound and the
number of brain slices present. Construction of a tissue concentration vs specific binding curve is a good way of detecting the presence of binding artifacts. Degradation of the ligand or of the receptor itself by tissue derived enzymes or other factors would manifest itself by a deviation from linearity. The excellent correlation coefficients determined on the linear regression of the data from these experiments, indicate that such degradation is not a problem with this assay.

Because of the protein nature of receptors, it is to be expected that they would be adversely affected by heat. The complex tertiary structure of proteins is disrupted by such treatment, and ultimately, the protein will be heat-denatured (Burt, 1985). Specific binding of \(^3\text{H}\)-naloxone to slices of mediobasal hypothalamus was completely eliminated by heating the tissue at 70° C for 45 minutes, and was reduced by 90% when the heat was applied for only 20 minutes.

The effects of coincubating slices of either cerebral cortex or mediobasal hypothalamus with \(^3\text{H}\)-naloxone and various drugs, some known to affect \(^3\text{H}\)-naloxone binding to nervous tissue were
examined. There was no effect of clonazepam (a benzodiazepine agonist) or of isoproterenol (an adrenergic agonist) on the specific binding of $[^3H]$-naloxone to mediobasal hypothalamus. The effect of these drugs in cerebral cortex was not determined. Neither of these drugs has previously been shown to affect opiate binding in vitro.

In this system, preincubation of mediobasal hypothalamus with 1 mM ascorbate resulted in a decrease of approximately 60% in the specific binding of $[^3H]$-naloxone. This decrease was attributable to a reduction in the amount of total binding; non-specific binding was not affected by treatment with ascorbate. These findings are in agreement with those of other investigators. Dunlap et al. (1979) first reported the destruction of stereospecific opiate binding sites in guinea pig brain homogenates (70%-80% inhibition of specific binding sites for $[^3H]$-etorphine, 70-100% reduction for $[^3H]$-naloxone). They have subsequently shown that $^3H$-clonidine (a-adrenergic agonist) binding sites are also quite sensitive to ascorbate (78% reduction). The binding of several other ligands was also affected by exposure to ascorbate, but these binding sites were not nearly as sensitive ($[^3H]$-QNB (muscarinic
cholinergic antagonist): 21% reduction; \([^3\text{H}]-\text{DHE}\) (a-
adrenergic antagonist): 21% reduction) (Leslie et al., 1980). In all cases examined, the reduction in specific binding was revealed by Scatchard analysis to be due to a decrease in receptor density, and not to a change in the affinity of the receptor. Hadjiconstantinou and Neff (1983) have summarized the evidence showing a similar action of ascorbate on dopamine receptors. It is not possible to confirm that the ascorbate-induced decrease in specific \[^3\text{H}]-\text{naloxone}\) binding seen in brain slices is due to a loss of receptors, since the binding at only one concentration of \[^3\text{H}]-\text{naloxone}\) was determined. Nevertheless, brain tissue appears to respond to the presence of ascorbate in this assay in the same manner as it does in an homogenate assay.

The \(\alpha\)-adrenergic antagonist phenoxybenzamine (PBZ) reduced the specific binding of \[^3\text{H}]-\text{naloxone}\) to slices of cerebral cortex and mediobasal hypothalamus by 67% and 54% respectively, when present in a concentration of 10 \(\mu\text{M}\). Cicero et al. (1974) first reported that PBZ inhibited the binding of \[^3\text{H}]-\text{naloxone}\) to whole rat brain homogenate, with an IC\(_{50}\) of \(9.2 \times 10^{-7}\) \(\text{M}\). Further studies by Spiehler et al. (1978) showed that PBZ acted as an
irreversible inhibitor of $[^3H]$-naloxone binding to whole forebrain homogenates prepared from male SW mice. They determined that the decrease in binding was due to a loss of receptors (i.e. a decrease in $B_{max}$) and not to a PBZ-induced change in receptor affinity for $[^3H]$-naloxone. Again, while the experiments with brain slices were performed at only a single concentration of naloxone, the loss of specific $[^3H]$-naloxone binding is clearly compatible with that seen in the homogenate preparation.

Phentolamine, another $\alpha$-adrenergic antagonist, was also able to significantly inhibit $[^3H]$-naloxone binding to slices of cerebral cortex. Mediobasal hypothalamus exposed to this drug bound less $[^3H]$-naloxone than did control tissue, but the difference did not reach statistical significance. Cicero et al (1974) found that phentolamine was also able to inhibit $[^3H]$-naloxone binding to brain homogenates, but it was only one sixth as potent as phenoxybenzamine. Both clonidine and yohimbine, an $\alpha_2$-adrenergic agonist and antagonist respectively, were without significant effect on the binding of $[^3H]$-naloxone to either cerebral cortex or mediobasal hypothalamus.
It has been demonstrated by several groups that chronic exposure to an opiate antagonist results in an increase in the number of opioid binding sites (see Zukin and Temple, 1986 for review). Tang and Collins (1978) reported that animals chronically exposed to naloxone developed a supersensitive response to the analgesic actions of morphine. Chronic administration of naloxone for 4 weeks resulted, one week after discontinuation of the naloxone in a 40% increase in the number of $[^3H]$-naloxone binding sites in whole rat brain homogenate, without any change in the affinity of the binding site for the ligand (Lahti and Collins, 1978). Hahn (1984), using SHR and WKY rats, has shown that sustained exposure to naloxone via a subcutaneous implant, beginning prenatally, and maintained through nursing and into adulthood results in a 63% increase in the number of $[^3H]$-naltrexone binding sites in homogenates prepared from whole forebrain, without any change in the $K_d$ of the receptor. Zukin et al. (1982) demonstrated that chronic exposure to the long acting opiate antagonist naltrexone via a subcutaneous pellet (5 weeks) or an osmotic minipump (8 days) resulted in a large increase in the number of specific binding sites for $[^3H]$-dihydromorphine
(DHM), [\(^3\)H]-naloxone, [\(^3\)H]-DADLE and [\(^3\)H]-etorphine in whole brain homogenates without any effect on receptor affinity. Regional analysis showed that the largest increases in [\(^3\)H]-etorphine binding induced by chronic exposure to the opiate antagonist were in the mesolimbic system (+130%) and the hypothalamus (+54%). In an attempt to validate the slice assay, adult male mice were implanted with silastic capsules containing 30 mg of naloxone HCl each. The pellets were left in situ for 7 days. This form of treatment resulted in an increase in \(E_{\text{max}}\) of [\(^3\)H]-naloxone binding sites in mediobasal hypothalamus of 150%, as well as a somewhat reduced receptor affinity for the ligand. Removal of the naloxone pellet 24 hours prior to the assay resulted in the elimination of the naloxone-induced increase in specific binding (as determined at a single concentration on the binding curve). Residual ligand in the tissue slice would manifest itself as a decrease in the affinity of the receptor (i.e., an increase in \(K_d\)). Our results, in general, are in close agreement with the work of Zukin et al., and others. Thus the slice assay appears to be able to detect pharmacologically-induced changes in the opioid receptor complement in brain tissue.
The brain slice radioligand binding assay which has been developed has thus been shown to be a useful tool for the study of [3H]-naloxone binding sites in the rodent brain. These binding sites fulfill the criteria of a true receptor as described by Burt (1985). They are saturable, of high affinity, thermolabile and the binding is readily reversible. Appropriate pharmacological and stereospecificity has been demonstrated, and the affinity of the binding site for various opioid drugs parallels the biological potency of these compounds. The binding is not diminished by metabolic inhibitors, occurs in the absence of sodium, and proceeds (although at a reduced rate) to saturation at low temperature, ruling out the possibility that an uptake site is being detected. Finally, the assay is able to detect drug-induced changes in opiate binding which have been shown to occur in the homogenate assay, and is also able to detect the upregulation of opioid binding sites induced by chronic exposure to an opiate receptor antagonist. The slice assay, then, is an appropriate tool to use in an investigation of opiate receptors in rodent brain tissue.

In considering the results of the binding studies presented in this Thesis, it must be
remembered that $[^3H]$-naloxone labels opioid receptors in a non-specific manner. Martin's laboratory first proposed the existence of multiple forms of the opioid receptor (Gilbert and Martin, 1976; Martin et al., 1976). The topic of multiple opioid receptors has been well reviewed in recent years (Chang et al., 1980; Chang and Cuatrecasas, 1981; Paterson et al., 1983; Goldstein and James, 1984). Goldstein and James (1984) have summarized six lines of evidence to support the notion of multiple opioid receptors:

1) Different pharmacological properties are exhibited by different families of opiate alkaloids.

2) The rank order potency of individual opioids differs in different tissues.

3) The $K_d$ of naloxone differs in a given tissue, depending on which agonist is being blocked.

4) When constructing displacement curves, the rank order potency of competing ligands is dependent on which radioligand is being displaced.
5) Tolerance to a specific opioid compound can be developed in an animal, while sensitivity to a second opioid is unchanged.

6) Selective alkylation can abolish or diminish the response to one opioid ligand, while not affecting the response to another, indicating that multiple opioid receptor types are not interconvertible, but are physically distinct entities.

The best evidence available indicates that LH release is under the influence of \( \mu \) opioid receptors. Schulz et al. (1981) have shown that injection of an antiserum to \( \beta \)-endorphin into the mediobasal hypothalamus was effective in increasing the amount of LH released into the general circulation in prepubertal female rats, while an antiserum to met-enkephalin was ineffective. Anti-dynorphin\(_{1-13}\) had some LH-releasing ability, but was less potent than anti-\( \beta \)-endorphin. Similarly, Forman et al. (1983) demonstrated that systemic administration of an antiserum to \( \beta \)-endorphin to adult male rats was able to induce a prompt increase in the amount of circulating LH. \( \beta \)-endorphin interacts primarily with the \( \mu \)-opioid receptor, although there is some
interaction with the d-site (Holaday and Tortella, 1984; Herz, 1984). This peptide is also found in high concentration in the hypothalamus, and of the endogenous opioids, is the most potent in inhibiting LH release (Forman et al., 1983). Using specific opioid receptor antagonists, Panerai et al. (1985) determined that both u and k receptors were involved in the control of LH release, but that the u receptor was approximately 10 times as sensitive as the k receptor.

Efforts should be made toward establishing specific assays for e.g. u receptors, in order to determine specifically which opioid receptor subtypes are involved in the diurnal rhythm in [³H]-naloxone binding sites. Preliminary experiments (Wilkinson et al., in preparation) show clearly that the opioid agonist peptide ligand [³H]-DAGO satisfies all of the criteria for binding to a u-receptor in brain and is unaffected by ouabain, indicating that it is being bound to the tissue, and not actively taken up. Furthermore, its behavior in a sodium containing incubation medium (i.e. decreased binding) is appropriate for an opioid agonist (see Werling et al., 1985). Additional examination of the binding of [³H]-DAGO and other opioid agonists in physiological
medium (i.e. isotonic, containing cations, etc.) should do much to further our understanding of the role of the endogenous opioids in vivo.
CHARACTERIZATION OF $^3$H-NALOXONE BINDING TO RETINAL FRAGMENTS

It is well recognized that rhythmic phenomena may be modified by light. That some rhythms are entrained to patterns of illumination is without doubt (Rusak and Zucker, 1979). Furthermore, several studies have clearly shown that reproductive function can be manipulated with light (for review, see Schwartz, 1982). The retinal-hypothalamic tract, in rodents, is a monosynaptic neural connection between the ganglion cells of the retina, and the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Conrad and Stumpf, 1975; Rusak and Zucker, 1979;). Evidence also exists of a similar direct connection between the retina and the SCN in man (Sadun et al., 1984). The importance of light in the control of rhythmic phenomena, the observation that hypothalamic opioid receptors varied in a diurnal fashion, and finally, the recent discovery of endogenous opioid peptides in the retina (Brecha et al., 1979; Altschuler et al., 1982; Hoffman, 1983; Su and Lam, 1983), led to an attempt to study opioid binding in relatively intact fragments of retinal tissue. It was felt that if the brain slice technique could be adapted to retina, binding studies might
possibly offer some insight on the effects of light on hypothalamic receptor rhythms.

The retina, a direct extension of the central nervous system, (Conrad and Stumpf, 1975) derives from neuroectoderm, the precursor of the brain, and it has been described as the "prototypical brain slice" by McIlwain (1984). Because of its common embryological origin with forebrain, retina has all of the characteristics of brain tissue (Hadjiconstantinou and Neff, 1984). It was reasoned, thus, that retina should behave in a binding assay in a manner similar to slices of mediobasal hypothalamus and cerebral cortex. Endogenous opiates are believed to play an important modulatory role in the retina. Enkephalin immunoreactivity has been detected in the avian (Brecha and Karten; 1979; Brecha et al., 1979) and guinea pig (Altschuler et al., 1982) retina. Application of opioid peptides directly to retinal tissue affected the release of GABA from GABAergic amacrine cells, as well as the firing pattern of ganglion cells in goldfish (Djamgoz et al., 1981). Iontophoresis of D-alá2-met5-enkephalinamide, an opioid peptide, onto ganglion cells of the amphibians Necturus maculosus (mudpuppy) and Ambystoma tigrum (tiger salamander) resulted in an inhibition.
of light evoked spike activity in all types of ganglion cells (Dick and Miller, 1981). Opioid binding sites have been found in autoradiographic and homogenate preparations of retinalae obtained from rabbits (Slaughter et al, 1985), rats (Medzihradsky, 1976; Howells et al., 1980; Wamsley et al, 1981), chickens (Slaughter et al., 1985), toads (Howells et al.), cows (Borbe, 1985; Osborne and Herz, 1985), goldfish (Slaughter et al, 1985) and man (Wamsley et al., 1981). One report of the detection of opioid binding in relatively intact pieces of chicken retina has appeared in abstract form (Su, 1984).

We reasoned that the retina, - a prototypical brain slice -, would be a suitable candidate for the slice radioligand binding assay, and could potentially provide new data on the effects of light on hypothalamic receptor rhythms. To that end, a series of experiments designed to rigorously characterize the binding of $[^3H]$-naloxone to quadrants of fresh rodent retina was undertaken. The same criteria for the definition of a functional receptor site were used in our investigation of retinal opioid binding as were used in our characterization of the brain slice assay (Burt, 1985).
CHARACTERIZATION OF $^{3}$H-NALOXONE BINDING TO RETINAL QUADRANTS

Autoradiographic studies have localized retinal opioid receptors to the inner plexiform layer and to the layer of the ganglion cells (Wamsley et al., 1981), but no attention has been paid to the radial distribution of the binding sites. To avoid potential problems due to asymmetrical radial distribution of these receptors, it was decided to use retinal quadrants in these studies. However, further anatomical investigation is required to ascertain the precise geographic distribution of retinal opioid receptors. Please note that in this Thesis, the terms fragment and quadrant are used interchangeably. $^{3}$H-naloxone bound to fragments of retina with a time course similar to but somewhat faster than that for brain slices. Equilibrium was reached in approximately 45 minutes, and the binding was stable for up to 4 hours. As with the brain slices, the binding was rapidly reversible, being nearly complete 30 minutes after the addition of a large excess of unlabelled naloxone. Investigators using retinal homogenate preparations for the investigation of opioid binding have used incubations of 15 minutes at 37° C (Howells et al., 1980; Medzihradsky, 1976), and
1 hour at 24°C (Slaughter et al., 1985). In no case was the time course of binding to this particular tissue characterized and reported. Thus it is uncertain whether or not the binding was at equilibrium, although this time course appears to be appropriate for homogenate preparations of brain tissue (Pert and Snyder, 1973a; Snyder et al., 1975). As already emphasized, it is essential to rigorously characterize all aspects of the binding of a given ligand to a novel tissue. Our incubations were carried out for 2-3 hours to ensure that equilibrium was reached. Again, as was the case with the brain slice assay, the rapid displacement of bound $[^3H]$-naloxone from retinal quadrants argues in favor of a specific binding mechanism as opposed to an uptake process as being the operative phenomenon here. The binding was shown to be stereospecific, with dextrorphan being almost 1000 times less potent in displacing $[^3H]$-naloxone from fresh retinal tissue than its biologically active enantiomer levorphanol. Although only 3 compounds which interact with opioid receptors were examined (naloxone, levorphanol, and dextrorphan), the ability of these substances to displace $[^3H]$-naloxone binding from retina paralleled their biological potency (Snyder and Pert, 1975a).
Non-specific binding of [³H]-naloxone to retinal fragments comprised approximately 25%-35% of the total binding over the ligand concentration range studied. This compares favorably to the degree of NSB found in a homogenate prepared from rat retinae (Howells et al., 1980). Specific binding in retinal quadrants was saturable, and of high affinity (Kₐ in the nanomolar range). It is unlikely that either tissue or ligand degradation play an important role in this binding assay, as construction of a tissue concentration versus binding curve indicated good linearity. As explained earlier, deviation from linearity could indicate the presence of a degrading or destabilizing factor released from the intact tissue.

Obtaining, preparing and incubating the retinae under darkness had the apparent effect of increasing the affinity of the retinal binding site for [³H]-naloxone. The significance of this observation is unclear. Such an increase in affinity may represent a change in the content of endogenous ligand in the retina. A difference of this nature would, in all probability, not be apparent with a homogenate preparation, since the initial preparation of the retinal homogenate and the washing that this entails
would tend to remove endogenous ligand. A second possibility is that the change in receptor affinity is the result of a light-induced conformational change in the receptor. The modification by environmental lighting of retinal opioid receptors suggests that these receptors may play a role in the local circuit processing of visual information. Similar changes have been found for both benzodiazepine and GABA receptors in retina (Biggio et al., 1981). Furthermore, Dubocovich et al., 1985 have reported a dark-induced increase in the specific binding of \(^{3}\text{H}\)-spiperone to dopamine receptors in rabbit retina. Unfortunately, these experiments were performed at only a single point on the binding curves, and it is not possible to attribute the binding differences to changes in receptor density or affinity. In addition, the studies of Dubocovich et al., (1985), involved prolonged (1 week) exposure to constant darkness, rather than an acute challenge within the normal photoperiod.

In summary, the \(^{3}\text{H}\)-naloxone binding assay to retinal quadrants satisfies Burt's (1985) criteria, and appears to be labelling a true functional receptor and not merely an "acceptor" site. The binding is reversible, stereospecific, of high
affinity, saturable, and demonstrates appropriate pharmacological specificity. This assay is an appropriate investigative tool for the study of opioid binding in fresh rodent retinal tissue, and its physiological regulation.

**SEX DIFFERENCES IN OPIOID BINDING TO RETINAL QUADRANTS.**

The density of binding sites was consistently greater in female rats than in males. Albino (SD) female rats had 80% more retinal opioid binding sites than did male rats, and pigmented (LE) female rats 125% more binding sites. Relatively few studies have examined opioid receptors in the retina (Medzihradsky, 1976; Howells et al., 1980; Wamsley et al., 1981; Su, 1984; Borbe, 1985; Slaughter et al., 1985; Osborne and Herz, 1985), and this is the first report of such a sex difference. Sex steroids are well known to affect opioid receptors in brain tissue. In brief, several studies have found higher opioid binding in female brain tissue than in male tissue (see for example, Hammer, 1984; Bicknell, 1986). However, this is a gross oversimplification of an exceedingly complex relationship, and the reader is referred to APPENDIX II for further details. As detailed in APPENDIX II, gonadal steroids
also influence CNS levels of endogenous opioid peptides. While it is conceivable that a similar situation exists in retina, the lack of a sex difference in the affinity of the retinal opioid binding site would argue against a difference in the level of these peptides being responsible for the differences in binding. Because the retinal tissue is relatively intact and unwashed, differences in the amount of endogenous ligand are likely to be preserved. These, in turn, are likely to affect the $K_d$ in this binding system. Studies examining the effect of gonadectomy and steroid replacement on retinal opioid binding might contribute to an understanding of the physiological role of these differences, particularly with respect to their role in determining hypothalamic opioid binding rhythms.

**STRAIN DIFFERENCES IN OPIOID BINDING TO RETINAL QUADRANTS**

A further interesting and unexpected observation was that retinal obtained from pigmented (LE) animals bound more $[^3H]$-naloxone than did those obtained from albino (SD) animals. The difference in male animals could be explained by a greater density of binding sites in the presence of a somewhat lowered affinity of the binding site. Females, on the other hand, did
not differ in the affinity of the binding site. The increase in binding seen in female pigmented rats was due solely to an increase in the $B_{\text{max}}$. Also of interest was the observation that sex differences are maintained across strains of animals. Female rats of both the LE and the SD strains had a greater density of retinal opioid binding sites than did the males of the respective strains. This is the first report of such strain differences in the binding of opioid receptor ligands to retinal tissue.

It has, however, been reported that $^{45}\text{Ca}$ binding is higher in pigmented than in unpigmented retina (Drager, 1985). This difference is attributed to the binding of calcium to melanin. In addition, it has been shown that MPP$^+$, a toxic metabolite of the neurotoxin MPTP binds to neuromelanin (D'Amato et al., 1986) and MPTP is actively taken up by pigmented retina, while accumulation in nonpigmented retina is considerably lower (Lyden et al., 1985). These studies indicate that melanin may affect the binding of a particular substance in a given tissue. It is thus possible that increased amounts of opioid binding in pigmented retinæ may be due to a modification of opioid binding by this pigment. There is, in fact, significant binding of $[^{3}\text{H}]$-
diprenorphine to the substantia nigra from human (Pfeiffer et al., 1982) and monkey (LaMotte et al., 1978) brain, although the binding is less than in many areas of the brain which do not contain significant neuromelanin. Another explanation for the greater number of opioid-binding sites in pigmented retina is that increased local calcium concentration (due to the attraction of melanin for calcium noted above) might modulate opioid binding. The question of calcium effects on opioid receptors has been reviewed by Chapman and Way (1985). Calcium has been shown to have either an inhibitory (Pert and Snyder 1973b) or no (Pert and Snyder, 1974) effect on $[^3H]$-naloxone binding. This explanation does not therefore seem tenable in the light of these earlier findings. Note, however, that these earlier studies were all performed on tissue homogenates. The calcium ion may exert entirely different effects in an intact tissue environment. A recent report by Pfeiffer (1985) has shown that calcium may exert different effects at different classes of opioid receptors, although he too was unable to demonstrate an effect on $[^3H]$-naloxone binding (in homogenates). Thus, studies performed on the ability of calcium or neuromelanin to influence opioid agonist and antagonist binding
would be of value in resolving the questions that the present observations have raised.

Preliminary experiments have extended the observations of strain differences in retinal opioid binding from rats to hamsters. Single point binding studies showed that retinae obtained from both GS hamsters and CHF 148 hamsters bound more $[^3H]$-naloxone than did those obtained from CHF 147 hamsters. Similar results were obtained when young male hamsters were used, but the differences were not as exaggerated. This may be due to either an age effect, or a sex difference. The GS hamster has a deeply pigmented retina. The CHF 148 hamster retina is unpigmented, while the CHF 147 hamster retina is lightly pigmented. At first glance these results appear to be discrepant with the findings in rats. Extrapolating from the rat data, one might reasonably expect the GS animals to have considerably higher levels of retinal opioid binding than both the CHF 147 or CHF 148 animals, based on the present of pigment (melanin). While such is the case for the CHF 147 hamsters, it is not for the CHF 148 animals. Furthermore, the difference in pigment content of the 147 and 148 retinae is not very large.
The primary difference between the CHF 147 and the CHF 148 hamsters is that the former are both dystrophic and cardiomyopathic, and the latter are the "normal" controls. While the muscular dystrophies have traditionally been thought of as being degenerative diseases of muscular tissue, it is well recognized that abnormalities of the endocrine and central nervous systems are an important component of these disorders (Dubowitz, 1979; Yoshioka et al., 1980). Evidence of a derangement of neuroendocrine function is found in the observation of abnormal gonadotropin secretion from pituitaries of dystrophic mice and hamsters (Wilkinson, 1984). Homogenates prepared from whole brain or cerebellum obtained from dystrophic mice (129/ReJ-dy (M) and 129B6F-1/J-dy (V)) had the same B_max and K_d for [^3^H]-flunitrazepam (benzodiazepine) binding sites as did tissues obtained from control littermates (Wilkinson and Khan, 1982). However, in these same animals, [^3^H]-DHA (β-adrenergic) binding sites were reduced by approximately 30% when compared to controls. This reduction in binding sites was due entirely to a decrease in B_max and not to a difference in K_d. This reduction in [^3^H]-DHA binding sites was seen in homogenates prepared from whole brain, cortex and
cerebellum. The diminished binding of $^3$H-naloxone to retinal quadrants obtained from dystrophic hamsters (CHF 147) may thus be a reflection of more generalized differences in the central nervous system, and not be due to an influence of melanin on the binding system.
It is well known that the endogenous opioid peptides play an important role in the regulation of reproductive functioning and sexual maturation in the rat (Bhanot and Wilkinson, 1983b). For example, administration of the opioid antagonist naloxone has been shown to result in a rapid increase in circulating levels of LH in immature female rats (Blank et al., 1979; Ieri et al., 1979) indicating the presence of a tonic opioidergic inhibition of gonadotrophin release. This opioidergic inhibition is not seen in immature males however, (Maric et al., 1982; Schulz et al., 1982). Furthermore, this opioid mediation of reproductive function changes as the animal matures. Schulz et al. (1982) have shown a progressive increase in the naloxone-induced release of LH in females up to the age of 16 days, after which the response decreases to adult levels. This contrasts with the results of Cicero et al. (1986) who have shown that female rats 10 or 25 days of age responded to naloxone with prompt increases in circulating LH, while 15 and 20 day old animals did not respond significantly. Both Cicero et al. (1986) and Schulz et al. (1982) performed their experiments
in the morning. When the results of our AM experiments are examined, they are more in keeping with the findings of Schulz et al. (1982), in that we have found significant naloxone-induced increases in circulating LH in female rats of all ages examined except for day 2. Furthermore, the response was maximal at day 15, and thereafter gradually decreased to adult levels. Immature male rats did not respond significantly to naloxone with changes in the circulating levels of LH.

Our interest in the study of diurnal variations in opioid receptors was stimulated by reports of the establishment of a diurnal pattern of LH release with the approach of sexual maturity. Andrews and Ojeda (1981) first reported a changing pattern of LH release in the female rat as puberty approached. They found an increase in the amplitude of LH pulses in the afternoons of the peripubertal period. Meijs-Roelofs et al. (1983), by using a very large series of animals, were able to conclusively demonstrate the existence of higher circulating levels of LH in the afternoon than in the morning, beginning approximately 5 days before first ovulation, in addition to higher mean levels of LH as puberty approached. Using very frequent sampling of
individual animals with a continuous blood collection and replacement technique, Urbanski and Ojeda (1985) have demonstrated an increase in LH pulse amplitude and mean LH levels in the afternoon, as well as the presence of afternoon "mini-surges" as female rats approach puberty. These findings all point to the development of a diurnal pattern of LH secretion in the prepubertal female rat.

The ability of the opiate antagonist naloxone to induce the release of LH has been shown to vary over the course of the day. Blank and Mann (1981) have shown that immature female rats release more LH in response to the administration of naloxone in the morning than they do in the afternoon. These results, taken in conjunction with the findings of diurnal changes in the pattern of LH release which are seen as puberty nears, are suggestive of a diurnal variation in the tonic opioidergic inhibition of gonadotropin release at this stage of development. The present investigations (this Thesis) have confirmed and extended the findings of Blank and Mann (1981). Immature male rats did not respond to naloxone either in the early morning or in the late afternoon. Female rats, however, did show a marked diurnal response to naloxone, releasing significantly
more LH in the early morning than in the late afternoon on day 15, and from day 26 on. In our colony, vaginal opening normally occurs near day 34 of life. This rhythm, while very reproducible, is easily disrupted by changes in photoperiod. Indeed, while the rhythm was readily observable with an L:D of 14:10, it completely disappeared when the photoperiod was changed to 12:12 (data not shown).

The existence of a diurnal rhythm in the binding of the opioid antagonist [3H]-naloxone to mediobasal hypothalamic tissue obtained from female rats nearing puberty has been demonstrated. While several others have reported a circadian or diurnal variation in opiate binding to nervous tissue (Naber et al., 1981; Kafka et al., 1982; Lee et al., 1984), this is the first study to examine such rhythms in discrete brain regions, and to attempt to correlate such variations with changes in physiological function. The existence of such rhythms, however, does not come as a complete surprise. Recent speculation in the literature has proposed that changes in opioid-mediating LH release may be accounted for by changes in opioid receptors (Cicero et al., 1986; Gabriel et al., 1986).
Initial experiments with 30 day old female rats revealed a bimodal pattern of $[^3H]$-naloxone binding to hypothalamus. Maximal binding occurred early in the light phase and again early in the dark phase. A steady decline over the course of the day in the amount of $[^3H]$-naloxone bound was found. The amplitude of this rhythm (defined as % difference between peak and nadir values) was approximately 60%. The form of our rhythm appears, at first, to deviate markedly from that reported by Naber et al. (1981), yet some similarities are apparent. In both cases, shifts from the light phase into the dark phase, and vice versa are accompanied by increases in the amount of $[^3H]$-naloxone bound. The major difference in the two rhythms, however, is that we found a steady decrease in the binding of opioid receptor antagonist over the course of the light phase of the day, while Naber et al. (1981) did not. There are several plausible explanations for these differences. Our investigations were performed on immature female animals, and not adult males. Furthermore, we restricted our investigations in this case to the mediobasal hypothalamus, while Naber et al. (1981) examined forebrain (i.e. whole brain less cerebellum). In addition, we have employed the
technique of radioligand binding to intact brain slices (Bhanot and Wilkinson, 1983; Jacobson and Wilkinson, 1984; see discussion above), whereas the other group utilized an homogenate preparation. The use of intact slices permits the characterization of opioid binding sites located on the cell membrane, whereas a binding assay utilizing a brain homogenate preparation permits the characterization of all binding sites available in the cell. Thus, differences in the processing of receptors throughout the day can account for the differences seen with these two techniques. Finally, our animals were maintained under an LD cycle of 14 hours of light followed by 10 hours of darkness, while those used by Naber et al. (1981) were maintained under a 12:12 lighting regimen. As will be discussed below, such small variations in LD can have serious consequences on diurnal or circadian rhythms.

The initial experiments were performed at a single concentration of radioligand. In order to properly characterize the AM/PM differences, saturation binding curves were constructed using mediobasal hypothalamus from 30 day old female rats. Inspection of these curves reveals that the AM/PM differences are due to a decrease in the density
(B$_{\text{max}}$) of binding sites in the late afternoon, as compared to the early morning. That there was no difference in the apparent Kd argues against a change in the affinity of the binding site being responsible for the decreased amount of ligand bound in the afternoon.

However, the possibility still exists that changes in the number of occupied receptors may account for the observed differences, i.e. differences in hypothalamic content of endogenous opioids might modify $[^3\text{H}]$-naloxone binding. An attempt was made to assess this question by dissociating endogenous ligand from the receptors at both the zenith (early AM) and the nadir (late afternoon) of the rhythm. Two methods were used to accomplish this. In the first case, the tissue was treated with the dilution wash technique. This method involves prolonged washing of the tissue for an extended period of time prior to performing the binding assay. Such treatment has been used to dissociate endogenously bound ligand from peptide receptors (Clayton, 1982; Smith and Ojeda, 1985). Dilution washing resulted in no change in either the B$_{\text{max}}$ or the Kd of the $[^3\text{H}]$-naloxone binding site in the mediobasal hypothalamus. Furthermore, dilution
washing of tissue obtained in the early morning had no effect on the amount of ligand bound. This indicates that the decrease in binding seen in the late afternoon is probably not due to an increase in the number of occupied receptors, but is rather due to a true decrease, or downregulation of the number of receptors present in the tissue. Further confirmation of this is found in the observation that a short preincubation in an acidic glycine buffer was also without effect on the amount of [3H]-naloxone bound to mediobasal hypothalamus. This treatment has been shown to dissociate ligand-receptor complexes (Ascoli, 1984). Our results contrast with those of Mash et al. (1985) who reported that the apparent circadian variation of muscarinic receptors in rat forebrain and brain stem could be abolished by treatments known to dissociate endogenous ligand from these receptors. They conclude that the rhythm was actually due to changes in the number of occupied receptors, while the actual number of receptors present was constant over the course of the day. In contrast, the diurnal variation seen in opioid receptors in hypothalamus appears to be due to a true change in the number of receptors present in the tissue, and possibly within the cell membrane.
It is unclear exactly how these diurnal changes in receptor number are accomplished. However, a change in the rate of receptor synthesis or degradation (i.e. receptor turnover) is one possibility. Furthermore, it must be remembered at all times that the cell membrane is a very dynamic structure. Recycling of internalized receptors is another means in which the number of receptors available for ligand occupancy could be modulated. This latter possibility is amenable to being tested by a comparison of the slice binding method with the homogenate preparation; this would measure both internalized and surface (i.e. total) receptors. The regulation of the coupling between adrenergic receptors and the activation of the adenylate cyclase system has been well studied (Harden, 1983; Lefkowitz et al., 1984). In this system, agonist-induced downregulation of the receptor system occurs in a matter of minutes. It has also been shown that the brain slice radioligand binding assay can detect such rapid changes (Wilkinson et al., 1984; Wilkinson and Wilkinson, 1985).

We are, at present, unable to explain what determines the timing of these particular rhythms. Neuronal activity may well play a key role in
determining the circadian pattern of opioid receptor binding in the hypothalamus. To investigate this possibility, an investigation into the effects of barbiturate anesthesia on these rhythms was undertaken. Barbiturates act on the central nervous system in such a manner as to inhibit excitatory synaptic transmission (Richards, 1972; Willow and Johnston, 1983). There is a long history of the use of barbiturate anesthesia in reproductive physiology to "block" neural activity, and thus determine its role in the genesis of various phenomena. The seminal studies of Everett and Sawyer which first showed that barbiturates can block ovulation in the rat are well reviewed by Everett (1977) in his Dale Lecture. It has also been shown that appropriately administered barbiturates can prevent the LH surge (Naftolin et al., 1972), by inhibiting the pituitary response to hypothalamic GnRH (Carter and Dyer, 1979), as well as the hypothalamic release of GnRH (Sarkar et al., 1976). Furthermore, Donham et al. (1986) have recently shown that the administration of barbiturates can block the generation of a diurnal rhythm, viz. the daily neural-clock-timed surge of LH which normally appears around day 1/8 in the prepubertal female hamster (see Introduction).
In exploratory experiments, 30 day old female rats were treated with 31 mg/kg of sodium pentobarbital, a dose known to block the estradiol-induced LH surge in immature female rats (Smith and Ojeda, 1985). Treatment with sodium pentobarbital at 1000 hrs prevented the appearance of the late afternoon decrease in the amount of $^3$H]-naloxone bound to hypothalamic tissue at both a low (2 nM) and a high (10 nM) ligand concentration. These findings need to be reproduced, but are consistent with the hypothesis that the diurnal variation in opioid binding to hypothalamus in immature female rats is of central origin, and may be mediated by neuronal activity. In future work, it would be instructive to examine the effects of the non-barbiturate anesthetic Alphathesin (alphaxolone plus alphadolone), which is unable to block the release of GnRH, (Sarkar et al., 1976), but does prevent the estrogen stimulated LH surge in ovariectomized female rats (Dyer and Mansfield, 1984).

There is a striking similarity between the binding rhythm and the pattern of circulating estradiol in prepubertal female rats, i.e. estradiol levels fall significantly between 0800 and 1600 hours (Ramaley, 1982). Studies from this laboratory have
previously drawn attention to the association between low levels of estradiol and the inability of naloxone to release LH (Wilkinson and Bhanot, 1985). The present studies therefore suggest a relationship between low estradiol levels and a low level of hypothalamic opiate binding. The influence of gonadal steroids on opiate receptor rhythms is a potentially fruitful area of investigation, and is readily testable.

The ontogeny of the diurnal variation in \([^{3}H]\)-naloxone binding sites in mediobasal hypothalamus has also been examined. In neither male nor female animals did the number of cortical opioid binding sites vary during the time intervals studied, with the exception of 23 day old females, where a small but statistically significant decrease in the number of binding sites was seen over the course of the day. The significance of this decrease is not known. Female rats aged 9 and 15 days did not show any change in mediobasal hypothalamic opioid binding sites. The irregular pattern of binding seen in 23 day old females may represent a transition to the peripubertal rhythm seen in 26 and 30 day old females. This rhythm, in which binding was high in the early morning, and then dropped in the afternoon,
is correlated with the decreased opioidergic tone seen in the naloxone response experiments reported originally by Blank and Mann (1981) and confirmed in this Thesis. Male animals did display a generalized decrease in the amount of $[^{3}H]$-naloxone bound to mediobasal hypothalamus at most ages studied. However, these changes could not be correlated with a naloxone-induced release of LH. Since the release of LH in the immature male does not appear to be under opioidergic control, as demonstrated above, it is conceivable that the diurnal changes in opioid binding sites seen in male animals are involved in other physiological processes. Endogenous opioids are, however, intimately involved with LH release in prepubertal females. In neither sex was there any diurnal rhythm in the amount of $[^{3}H]$-naloxone bound to cerebral cortex, a tissue not commonly thought to be involved in the control of LH release (Kalra and Kalra, 1983). When considered in light of the evidence demonstrating the peripubertal emergence of a diurnal pattern of LH release, the existence of large AM/PM differences in the number of $[^{3}H]$-naloxone binding sites in an area known to be important for the control of the release of LH (Kalra and Kalra, 1983), as well as the temporal coupling of
these diurnal variations with similar changes in the inhibitory opiatergic tone exerted on the LH release, would seem to confirm the importance of rhythmic phenomena in the initiation of puberty in the female rat. One must consider the possibility, however, that the changes in opioid receptors are not the sole determinant of the diurnal variations in LH response to naloxone. Since the endogenous opioids exert their influence on LH release through the intermediacy of GnRH (Blank et al., 1979; Cicero et al., 1979), it is possible that diurnal changes in the sensitivity of the pituitary gland to GnRH are, at least in part, responsible for the changes in opioid mediation of LH release. Blank and Mann (1981) demonstrated a near parallelism between the LH response to naloxone and to GnRH over the course of the day. A major point of diversion from this parallelism occurred in the late afternoon, when sensitivity to GnRH returned to morning levels (i.e. high) while sensitivity to naloxone was low. A search for diurnal variations in pituitary GnRH receptors, similar to that reported here for opioid receptors, would be an appropriate means of further investigating this question.

It must be noted, that the present efforts concentrate exclusively on the role of opioid
peptides and opioid peptide receptors in controlling LH release. It is, however, well known, that many other neurotransmitters are intimately involved in the control of GnRH and gonadotropin release, including catecholamines (Barraclough and Wise, 1982; Ramirez et al., 1984), serotonin (Arendash and Gallo, 1978; Chen et al., 1981; Horn and Fink, 1985), GABA (Mansky et al., 1982; Lamberts et al., 1983), acetylcholine (Everett et al., 1949; Eneroth et al., 1977a, 1977b; Kamberi, 1972; Libertun and McCann, 1974), and other peptides (see for example, Kalra, 1985). (For review, see Wilson 1979). It is recognized that opioid peptides and receptors are not the sole determinants of LH release, but rather a single component in a very complex regulatory system. A more complete understanding of the processes involved in the transition from sexual immaturity to competent adult reproductive functioning must await an investigation of receptors for these other neurotransmitters, and the possibility that rhythmic changes in these receptors may be responsible for determining diurnal or circadian patterns of gonadotropin release.

To summarize, then, in immature female rats, a diurnal variation in the number of [3H]-naloxone
binding sites in slices of mediobasal hypothalamus, but not cerebral cortex emerges as the animal approaches puberty. The emergence of this rhythm is paralleled by an afternoon decrease in the ability of naloxone to release LH. The decrease in the amount of $[^3H]^{-}$naloxone bound is due to a true decrease in the number of binding sites, and not to an increase in receptor occupancy, or to a change in the affinity of the receptor. The decrease in receptor numbers could be responsible for the emergence of a diurnal pattern of LH release as the animal nears puberty, and may well be involved in the initiation of the preovulatory LH surge necessary for the expression of reproductive maturity. The next section of this thesis deals with attempts to manipulate the diurnal rhythm in opioid binding seen in slices of mediobasal hypothalamus.
Several neurotransmitter receptor systems are known to be affected by gonadal steroids. Estrogen has been shown to exert a modulatory influence on serotonergic (Biegon and McEwen, 1982), cholinergic (Rainbow et al., 1983, Morley et al., 1983), benzodiazepine (Wilkinson et al., 1983a), adrenergic (Wilkinson and Herdon, 1982, Vacas and Cardinali, 1980) and dopaminergic (Hruska et al., 1980) receptors in the central nervous system. In addition, cyclic changes through the estrous cycle, and sex differences have been noted for a variety of neurotransmitter systems (for examples, see Fischette et al., 1983; Shephard et al., 1982 and Avissar et al., 1981).

As noted above, the endogenous opioid peptides play an important role in the control of reproductive function. LH release is under a tonic opioidergic inhibition (Blank et al.; 1979; Schulz et al., 1982; Bicknell, 1985; Millan and Herz, 1985; Howlett and Rees, 1986), and ovulation can be blocked by the administration of opioid peptides.
(Koves et al., 1981). These phenomena are readily reversible with the opiate receptor antagonist naloxone. Recent studies in the rat have demonstrated that either one-time exposure to high doses of estradiol valerate or chronic exposure to more physiological levels of estradiol through subcutaneous silastic implants result in an increase in the number of \([^3H]\)-naloxone binding sites in the hypothalamus (Wilkinson et al., 1983b). The estradiol-induced increase in opiate binding occurs in the anterior hypothalamus (Wilkinson et al., 1985), an area important in the control of reproductive cyclicity, and essential for the midcycle LH surge (Kalra and Kalra, 1983).

[Note that these experiments ignored the possible influence of rhythmic changes in receptor binding.]

In additional studies (this Thesis) both SW and C57BL/6J mice responded to a single large injection of EV in the same manner. The finding of an estrogen-induced increase in \(^3H\)-naloxone binding to murine hypothalamic tissue confirms work previously reported in the rat (Wilkinson et al., 1983). In agreement with these experiments, it has been shown that the increase in \(^3H\)-naloxone binding is due to an actual increase in the density
of opiate binding sites, and not to a change in the affinity of the binding site. In these investigations, a single injection of EV resulted in a 90% increase in the density of opiate binding sites in SW mice. This compares favorably with the 97% increase seen in Wistar rats (Wilkinson et al., 1983) following a single large injection of EV. The density of the binding sites in the C57BL/6J mice increased somewhat less than the SW mice, but the 50% increase induced by EV was still highly significant.

Cultured pituitary glands obtained from EV-treated SW mice responded appropriately to exogenously administered GnRH, with a prompt release of LH, which while slightly higher, was not significantly different from control animals. Finch's group did not perform in vitro studies on GnRH-induced LH release (Mobbs et al., 1981). However, they reported that the control and EV-treated animals did not respond differently to an injection of GnRH. It must be noted that the dose of releasing factor used (60 ng, i.p.) is massive, and this may not represent the normal in vivo response to GnRH.
Brawer (1983) has suggested that exposure to physiological levels of endogenous estradiol over a lifetime may account for aging of the hypothalamus (see also Finch et al., 1984). In the female rat, aging is associated with an interruption of cyclic reproductive functioning. The acyclic state is characterized by the presence of polyfollicular ovaries and constant vaginal estrus (Finch, 1978; Meites, 1982; Ascheim, 1983). Such changes are remarkably similar to those seen in rats following the administration of a large dose of EV (Brawer et al., 1978). An EV-induced acyclic state with persistent vaginal estrous has also been identified in mice, and put forth as a model for the aging of reproductive function (Mobbs et al., 1981). It has also been proposed that the anovulatory polycystic ovarian state induced by a single injection of EV in young cycling female rats is a good model of the polycystic ovarian syndrome (PCO) in humans (Brawer et al., 1978; Schulster et al., 1984).

Is it possible that estrogen-induced anovulation is due to a disruption in the diurnal signal for GnRH release? Recently, it has been shown that the circadian pattern of LH release is abnormal in teenage girls with PCO (Zumoff et al.;
While pulsatile LH release was coupled to sleep in a population of control girls of the same age, 5 out of 6 PCO subjects showed a phase shift with a dissociation of the pulsatile secretion from the sleep period, and one subject did not show pulsatile secretion. Circadian rhythms in circulating LH have also been detected in older women with PCO, but were not well described in relation to the pattern seen in normal adult females (Kazer and Laughlin, 1986). This constellation of observations, i.e. EV affects opioid receptors, EV can induce a PCO-like syndrome, and PCO has been associated with abnormalities in the circadian pattern of LH release, led to the present investigation of whether or not EV treatment has any influence on binding rhythms.

It has been recognized for some time, that neonatal androgenization causes sexual differentiation of the hypothalamus (Barraclough, 1968; Gorski, 1971; Macluskey and Naftolin, 1981; Thomas and Ani, 1982; Gorski, 1985). A masculine (acyclic) pattern of gonadotrophin release develops if the brain is exposed to androgens in the critical prenatal or perinatal period. If neonatal
female rats are exposed to aromatizable androgen or to estrogen itself, a pattern of anovulatory persistent estrous develops in adulthood, and the normal female cyclic pattern of gonadotropin release is not realized (see refs above as well as Harris, 1964; Barraclough, 1966; Flerko, 1968; Arnold and Gorski, 1984). Non-aromatizable androgens, such as dihydrotestosterone (DHT), when administered during the critical period, have no effect on the subsequent development of cyclic activity in female rats (Luttge and Whalen, 1970; Brown-Grant et al., 1971). Furthermore, estradiol is more potent than testosterone in blocking the development of typically feminine patterns of gonadotropin secretion in the adult, when administered neonatally (Gorski and Wagner, 1965; Sutherland and Gorski, 1972). These facts, in conjunction with the observation that antiestrogens are able to block the androgenizing effects of testosterone in the neonate (Doughty and Mcdonald, 1974), lead to the conclusion that it is through its conversion to estrogen that testosterone is able to affect the sexual differentiation of the hypothalamus. It is generally believed that the high levels of estrogen present in the circulation
of infantile male and female rats (Weisz and Gunsalus, 1973; Ojeda et al., 1975), do not enter the brain because the hormone is tightly bound to a-fetoprotein (Raynaud et al., 1971; Flapinger et al., 1973). This prevents the spontaneous masculinization of all female rats during the critical period. Testosterone of testicular origin, however, can pass into the brain, and (upon aromatization) there exert its action in the normal male (Lieberburg et al., 1977; Goy and McEwen, 1980; Arnold and Gorski, 1984).

The treatment of neonatal rats with a single large dose of estradiol valerate (200 μg) would certainly induce androgenization of the female hypothalamus. A diurnal rhythm in [³H]-naloxone binding to slices of mediobasal hypothalamus was seen in control female animals examined on day 30 of life. Females that were androgenized with EV did not show any difference in hypothalamic [³H]-naloxone binding between 0700 and 1700 hrs. Neither the control males nor the EV-treated, males displayed any diurnal variation in hypothalamic opioid binding in this particular experiment. While a diurnal rhythm in hypothalamic [³H]-naloxone binding had been seen earlier in normal male rats
on day 30, this was not a constant finding. Neonatal EV appears to have eliminated the diurnal variation in $[^3H]$-naloxone binding to slices of mediobasal hypothalamus in female rats examined on day 30 of life. Caution must be exercised in making this conclusion, however, as only 2 time points were examined. While it can be said with confidence, that the normal AM/PM differences were eliminated, the rhythm in opioid binding may simply have been phase-shifted, and it may be manifest at another point in time. Interventions have been reported which do not eliminate, but rather phase shift, circadian or diurnal rhythms. (Wirz-Justice et al., 1982; Naber et al., 1982). Clearly, however, this observation that neonatal androgenization with EV appears to affect the normally present late afternoon decrease in hypothalamic opioid binding in peripubertal female rats may represent a partial explanation for the anomalous circadian pattern of LH release reported in teenage girls with PCO. These studies must be extended by examining the hypothalamic binding of $[^3H]$-naloxone to androgenized female rats at more time points over the course of the day. The effect
of. EV treatment in the adult should be examined similarly.
EFFECT OF INFANTILE GONADECTOMY ON DIURNAL VARIATIONS IN HYPOTHALAMIC OPIOID BINDING

Neonatal or infantile ovariectomy is a means of preventing or minimizing exposure of the developing central nervous system to sex steroids prior to the onset of puberty. The peripubertal development of diurnal variations in the hypothalamic binding of \[^{3}\text{H} \] -naloxone may be under steroidal influence. It has been demonstrated (this Thesis) that neonatal androgenization with EV appears to eliminate or shift this rhythm in the 30 day old female rat. Furthermore, as noted above, the opioid receptor rhythms seem to follow the diurnal variation in circulating estradiol levels in peripubertal rats (Ramaley, 1982). Urbanski and Ojeda (1986) have shown that the development of prepubertal afternoon "minisurges" of LH is ovary dependent, and will not appear if female rats are ovariectomized neonatally. To examine the influence of gonadal steroids on the development of the opioid binding rhythm, infantile rats (both male and female) were gonadectomized on day 9 of life. Binding studies were performed on hypothalamic tissue obtained from these animals on day 30 of life.
The patterns of circulating steroids have been well described in immature rats (Kamberi et al., 1980; Dohler and Wuttke, 1975; Ramaley, 1979; Weisz and Gunsalus, 1973). Estradiol levels in both males and females are high at birth and decline thereafter, only to rise again beginning near day 9 of life. Peak estradiol levels are reached on approximately day 15, following which the levels begin to decrease (Kamberi et al., 1980). This is a time of rapid induction of estradiol receptors in the hypothalamus (McEwen et al., 1976). Male rats have relatively high levels of circulating testosterone at birth, and these levels are maintained until approximately day 19 of life, while the levels in female rats are uniformly low (Dohler and Wuttke, 1975). By removing the gonads on day 9, the process of sexual differentiation has been allowed to proceed, as the animals were exposed to endogenous gonadal steroids during the critical period. However, the prepubertal influence of gonadal steroids was interrupted by the gonadectomy.

Infantile ovariectomy resulted in an abolition of the normal diurnal variation in hypothalamic binding of \([^3H]\)-naloxone in 30 day old rats. The
appearance of a diurnal decrease in the affinity of the binding site was an unexpected observation and may be a consequence of differences in the amount of endogenous ligand present in the hypothalamus. β-endorphin is known to vary in a circadian manner in the pituitary gland as well as in various brain regions, including the hypothalamus (Kerdelhue et al., 1983). It is not clear what effect infantile ovariectomy has on such circadian variations in the content of endogenous opioid peptides in the brain. Opioid levels are sensitive to steroids (Wardlaw et al., 1982b). For example, chronic treatment with estrogen induces a significant decrease (45%-50%) in hypothalamic immunoreactive β-endorphin (Forman et al., 1985). Lim and Funder (1984) reported a doubling of the amount of immunoreactive β-endorphin both in the circulation and in the neurointermediate lobe of the pituitary gland following short-term ovariectomy. This increase was reversible by estradiol in a dose-dependent manner. Ovariectomized adult rats treated with estradiol (by subcutaneous implant) for three weeks showed a 30% decrease in hypothalamic β-endorphin (Wardlaw et al., 1985). Others have reported that estrogen can increase striatal levels of immunoreactive met-
enkephalin (Dupont et al., 1980). Ferin's group has extensively studied steroid-induced changes in immunoreactive β-endorphin in the portal blood of primates. They feel that portal blood reflects β-endorphin activity in the hypothalamus, while systemic blood levels are a reflection of pituitary activity (Ferin et al., 1984). Ovariectomy was accompanied by a disappearance of detectable β-endorphin (Wehrenberg et al., 1982) which was reversible by the administration of gonadal steroids (Wardlaw et al., 1982a). Their studies of the menstrual cycle showed that during menstruation, when gonadal steroids were at their nadir, β-endorphin was undetectable in portal blood. As the steroid levels increased, i.e. in the late follicular and luteal phases, increasing amounts of β-endorphin were found in the portal circulation. While the highest concentrations were detected in the presence of progesterone, the administration of this steroid alone to ovariectomized monkeys had no effect on β-endorphin, indicating a synergistic role for this steroid with estrogen (Wardlaw et al., 1982a; Wehrenberg et al., 1982). Changes in the hypothalamic content of β-endorphin have also been
detected during the estrous cycle of the rat (Barden et al., 1981). However, in spite of the well recognized effects of gonadal steroids on endogenous opioid levels, an explanation for an ovariectomized-induced diurnal difference in the amount of endogenous hypothalamic opioid peptides, and as a consequence the affinity of the binding site, is not readily apparent.

It is unclear, at present, whether steroids affect the amount of β-endorphin released from the hypothalamus, or affect the hypothalamic synthesis or processing of the peptide. Based on studies made with pregnant rats, Wardlaw and Franz (1983) propose that estradiol alone stimulates hypothalamic release of β-endorphin to a larger extent than it does synthesis. However, in the presence of progesterone, they believe that hypothalamic synthesis is stimulated to a greater degree than is release. Their observations are supported to a large extent by Petraglia et al. (1985). Studies to determine the effect of infantile ovariectomy on diurnal patterns of hypothalamic β-endorphin content as well as turnover would assist in explaining the observations presented here.
While complete saturation binding curves were not prepared from the orchidectomized male rats or their controls, a late afternoon decrease in the amount of $[^{3}H]$-naloxone bound to hypothalamus was noted in the control group. This difference was not apparent in the castrate group, indicating that exposure to gonadal steroids may well be necessary in males for the development of the diurnal rhythms in hypothalamic opioid binding sites. Again, the physiological significance of diurnal variations in hypothalamic opioid binding sites in male rats is unclear at this point.
INDUCTION OF DIURNAL RHYTHMS IN OPIOID BINDING BY PMSG

One way to better understand the genesis and the function of the patterns of hypothalamic opioid receptor changes over the course of the day is to attempt to modify these rhythms by various interventions. Such is the case with the androgenization or the infantile gonadectomy of animals presented here. Of equal importance in our understanding of these phenomena, might be an attempt to induce such rhythms where they are not normally present. Diurnal variations in the binding of \[^3H\]naloxone to hypothalamic slices were not apparent in female rats until the age of 26 days. Puberty in our rat colony occurs around day 34 of life. It was reasoned that since the females began to display a diurnal variation in opioid binding as puberty approached, it might be possible to artificially induce a diurnal rhythm, if "puberty" could be imposed on the animals. Treatment with PMSG has been shown to reproducibly induce ovulation in prepubertal female rats via an increase in circulating gonadotropins and steroids (Zarrow and Quinn, 1963; Lunn and Bell, 1968; Wilson et al., 1974; Parker et al., 1976; Kostyk et
This model has been well characterized and much used as a model of puberty in female rats (see Ramaley, 1974), as well as to study the role of catecholaminergic systems in the generation of the GnRH surge which precedes ovulation (Wilkinson et al., 1979; Sarkar et al, 1981; Sarkar and Fink, 1981).

Administration of PMSG to immature (18 day old) female rats was able to induce a diurnal variation in the binding of $[^3H]$-naloxone to slices of mediobasal hypothalamus. Hypothalamic tissue obtained from control animals treated with saline bound the same amount of $[^3H]$-naloxone in the early morning as in the late afternoon. The endocrine consequences of a single injection of PMSG to an immature female rat are multiple, and closely mimic the pattern of hormonal secretion found just prior to puberty in the normal rat (Walker and Mahesh, 1976; Ojeda et al., 1980). A marked increase in the amount of circulating estradiol follows the administration of PMSG by 20-24 hours (Wilson et al., 1974; Shasida and Johnson, 1976). This initial increase in circulating estrogen levels is followed by an increase in the concentration of hypothalamic GnRH (Sorrentino and Sundberg, 1975; Sarkar and
Fink, 1979). This in turn results in a generalized increase in the pituitary secretion of gonadotropins. Circulating concentrations of both LH and FSH increase, as does that of prolactin (Johnson et al., 1975; Sasomoto and Johke, 1975). The gonadotropins induce the ovaries and the adrenal glands to secrete a number of steroids, among which are included progesterone, and testosterone, approximately 52-56 hours after the PMSG was given (Wilson et al., 1974; Parker et al., 1976). Approximately 12 hours after the release of the gonadotropins, ovulation takes place. (Wilson et al., 1983). It is not clear which of the PMSG-induced alterations in hormonal environment is responsible for the induction of the diurnal rhythm in hypothalamic opioid binding. Possibly some combination of hormones is needed to induce the rhythm. However, these experiments point the way for a series of experiments to be performed, in which manipulation of the various hormonal variables, singly and in combination, would be examined for effects on the development of the receptor binding rhythm.
EFFECT OF MANIPULATING THE LIGHT-DARK CYCLE ON DIURNAL VARIATIONS IN HYPOTHALAMIC OPIOID BINDING

Light, be it natural or artificial, is a ubiquitous environmental constituent which affects many biological processes (Wurtman, 1975; Wurtman et al., 1985). Cycling female rats placed into an environment of constant darkness (DD) continue to cycle normally, and to ovulate, for long periods of time (Hoffman, 1973). Animals raised in DD have been reported to exhibit precocious (Relkin, 1968a, Relkin, 1968b) or delayed puberty (Fiske, 1941, Wurtman, 1975). Schwartz (1982) has recently reviewed the effects of constant bright illumination on reproductive processes in the female rat. Female rats housed in constant light (LL) display a syndrome characterized by a cessation of estrous cycles (Wise et al., 1975). The ovaries, which become polyfollicular, do not release ova. High levels of estradiol released by the polyfollicular ovaries result in a persistent vaginal cornification (Fiske, 1941), enlarged stimulated uteri (Daane and Parlow, 1971) and continuous behavioral estrus or sexual receptivity (Hoffman, 1967).
Abnormalities in the timing of the LH surge and release of ova mark the onset of constant illumination-induced persistent estrus (Lawton and Schwartz, 1967; McCormack and Sridaran, 1978). This is followed by vaginal cornification with occasional breakthrough ovulation, and finally by a state of persistent vaginal cornification, and the total cessation of ovulation (Schwartz, 1982). Many variables interact to determine the timing and degree of the animal's response to constant illumination. Strain of animal (Hoffman, 1970), age at which beginning of exposure to LL begins (Takahashi and Suzuki, 1969; Hoffman, 1973; Takeo et al., 1975; Shirama, 1978), intensity and spectrum of light (Wurtman and Wiesel, 1969; McCormack and Sridaran, 1978; Piacsek and Hautzinger, 1978; Albers et al., 1980), and the nutritional status of the animal (Piacsek and Meites, 1967; Walker and Bethea, 1977) all have been shown to play a role in the response to constant illumination. A more detailed discussion of the spectral composition and intensity of light is given by Wurtman (1975).

It is beyond the scope of this thesis to examine the many circadian or diurnal rhythms which
manifest themselves in nature. However, light is not without effect on reproductive rhythms in man. Puberty occurs earlier in blind girls than it does in normal sighted females (Hollowich, 1979). Oligomenorrhea, in the face of normal libido and fecundity, is a frequent finding in blind women. Light has also been proposed as a therapeutic intervention in the treatment of menstrual dysfunction. For instance, it has been shown that menstrual cycles can be regularized by night time illumination at midcycle (Greenleaf, 1981).

There is good reason to believe that changes in illumination may effect changes in the hypothalamus, if such hypothalamic changes are involved with rhythmic phenomena. The retinal-hypothalamic tract, in rodents, is a monosynaptic neural connection between the ganglion cells of the retina, and the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Conrad and Stumpf, 1975; Rusak and Zucker, 1979). Evidence also exists that a similar direct connection between the retina and the SCN in man (Sadun et al., 1984). The SCN are intimately involved with the generation and control of rhythmic phenomena (Ramaley, 1980; Rusak and Zucker, 1979). There exists, thus, a direct route.
for external environmental information (i.e., light) to influence hypothalamic function, and ultimately hypothalamic rhythms.

Housing of prepubertal female rats in LL or in DD for a period of 9 days (i.e. until day 30 of life) had the same effect on hypothalamic opioid receptors. In both cases, no differences were observed in the binding at 0700 and 1700 hours. It is unclear whether or not modification of the LD cycle eliminated or shifted the diurnal variation in hypothalamic opioid binding sites. However, the functional consequences of small alterations in photoperiod may be significant. While a diurnal difference in the response of LH to naloxone HCl in 30 day old female rat was easily demonstrated when the animals were housed under an LD of 14:10, the diurnal difference completely disappeared when the animals were housed under an LD of 12:12 (data not shown). It is unknown, at the present, whether this change in photoperiod also eliminated the diurnal difference in hypothalamic opioid binding.

It has also been demonstrated that keeping animals in LL or DD is able to affect pubertal development. Placement into LL at birth results in
an advancement of VO (Fiske, 1941, Piacsek and Hautzinger, 1974). Controversy exists as to the results of placing female animals into DD at birth, with both advances (Relkin, 1968a; 1968b) and delays (Fiske, 1941; Wurtman, 1975) in VO reported. However, the effect on VO of placing prepubertal animals into DD or LL at the age of 21 days has not yet been examined. It would be extremely instructive to extend the experiments reported here in two different directions. In the first instance, a more rigorous study of the diurnal patterns of hypothalamic opioid receptors is indicated, with more time points being examined. Secondly, placing animals into DD or LL at this rather late stage of development and allowing the animals to progress to puberty, would provide useful information on the functional consequences of photoperiod-induced changes in the diurnal difference in hypothalamic opioid binding.

Of interest is the fact that while endogenous rhythms are in fact present in prepubertal rats, it is only near day 25 or 26 of life that the animal is able to respond to signals from the environment and set these rhythms (Ramaley, 1979; Ramaley, 1980) This is also a key time period for synaptic
connections to the SCN (Campbell and Ramaley; 1974; Ramaley, 1980). This is the age at which the diurnal difference in hypothalamic opioid binding is first apparent in female rats, and in the present experiments, the animals were subjected to DD or LL over this critical period. It is interesting to speculate that these unusual photoperiods somehow influenced the development of the SCN, and ultimately affected the generation of the diurnal difference in hypothalamic opioid binding. Autoradiographic examination of opioid binding sites in the SCN presents an interesting means of quantifying the role of this nucleus in this phenomenon. Further, as described elsewhere, it would be instructive to correlate possible changes in retinal opioid binding with those seen in the hypothalamus.
EXPERIMENTS WITH DSP4

The next section of this Thesis deals with the use of the putative noradrenergic neurotoxin DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride). As will be documented below, biogenic amines are implicated in the control of puberty. The initial intent of these investigations was to demonstrate that interference with aminergic neurotransmission would have a deleterious effect on puberty in female rats, and that such an effect was, at least in part, mediated through opioid mechanisms, perhaps through an alteration in the hypothalamic diurnal rhythm of $^3$H-haloxone binding sites. DSP4 has been referred to as both a selective (Hall et al., 1984; Dooley et al., 1983; Bickford et al., 1984) and a specific (Jaim-Etcheverry and Zieher, 1980, 1983) adrenergic neurotoxin, i.e. DSP4 appears to be without effect on levels of dopamine, serotonin, adrenaline, acetylcholine, GABA or glutamic acid (Jaim-Etcheverry and Zieher, 1983). In view of this specificity, DSP4 seemed to be an ideal probe with which to study the opiateergic control of LH secretion in the rat; an adrenergic component of the opiateergic system appears to be crucial in the regulation of LH
release (Kalra and Kalra, 1984). The neurotoxin was administered to female rats at several ages, and was found to be without effect on the development of sexual maturity. These experiments were extended to examine the influence of DSP4 on the artificial induction of LH surges in immature rats, and to examine the effects of DSP4 on LH secretion itself. While these experiments have not so far been successful in their original intent, they have provided new insight into the mode of action of the neurotoxin DSP4, and have indicated that this tertiary haloalkylamine interacts directly with opioid receptors. Although an effect of DSP4 on opioid receptor rhythms has not yet been observed, the results of these experiments are of sufficient interest to merit discussion here. This discussion is divided into two parts. Immediately below will be found a consideration of the effects of DSP4 on sexual maturity, the induction of gonadotropin surges, and LH release. This is followed by a discussion of the effects of DSP4 on opioid receptors in brain tissue.
EFFECT OF DSP4 ON SPONTANEOUS PUBERTY

Puberty, the attainment of sexual maturity, is marked by the first ovulation and subsequent cyclic reproductive functioning in the female rat. While the basic mechanisms controlling sexual development are complex and controversial, it is well established that the central nervous system is an essential participant in the process (Reiter and Grumbach, 1982; Ojeda et al., 1980). First ovulation is preceded by a proestrous-like LH surge (Kamberi et al., 1980) which in turn is dependent on a sudden increase in the release of GnRH from the hypothalamus (Sarkar and Fink, 1979). A large body of evidence implicates noradrenergic and dopaminergic systems in the control of GnRH release (Sarkar and Fink, 1981; Lofstrom et al., 1977a; Lofstrom et al., 1977b; Barraclough and Wise, 1982), and in the onset of puberty (Advis, Simpkins, Chen and Meites, 1978; Wuttke et al., 1980). In an attempt to further delineate the role of the noradrenergic system in the control of puberty in the female rat, the effects of DSP4 on both spontaneous and induced sexual maturation in the female rat have been studied.
DSP4 was first reported to have a long lasting inhibitory effect on the uptake of noreadrenaline (NA) more than 10 years ago (Ross et al., 1973). Since that time, several reports have appeared in the literature detailing the actions of this drug on both peripheral and central noradrenergic systems. In addition to the inhibition of NA uptake (Ross and Renyi, 1976; Jonsson et al., 1982), DSP4 has been reported to deplete the NA content of brain and peripheral tissues (Jonsson et al., 1981), to decrease the activity of the enzyme dopamine β-hydroxylase (Ross, 1976), to influence significantly the postnatal development of central noradrenergic neurons (Jonsson et al., 1982), and to alter both α- and β-adrenergic receptors in the central nervous system (Dooley et al., 1983). DSP4 is the prototypical member of a class of compounds which are tertiary haloalkylamines. These compounds are known to undergo cyclization to form an aziridinium ion, a highly reactive species thought to be responsible for the uptake blocking activity (Jaim-Etcheverry and Zieher, 1983; Ransom et al., 1982).

However, in spite of this well-documented deleterious effect on noradrenergic neurons, it has not been possible to interrupt the normal pubertal
progression of the immature female rat with a single neonatal exposure to DSP4. In all instances during which exposure to DSP4 resulted in a delay in vaginal opening, there was a concomitant delay in weight gain, and both the control and the treated animals showed VO at the same body weight. The close association between the onset of puberty and somatic growth is well described (Kennedy and Mitra, 1963; Frisch, 1980), and the present results are in accord with this.

Previous attempts to study the effects of a disruption of catecholaminergic systems on the spontaneous progression to puberty have relied on the use of the neurotoxin 6-hydroxydopamine (6-OHDA). Ruf and Holmes (1974) found that intraventricular administration of 6-OHDA to 23 day old female rats resulted in an 8 day delay in vaginal opening. This treatment was also accompanied by a slight but transient decrease in body growth, but control animals opened at a significantly lighter body weight. Administration of a second dose of 6-OHDA on day 24 had the effect of more severely retarding both body growth and vaginal opening. This result is of particular interest in that during the current study, administration of DSP4 on both day 23 and day 24
resulted in a significant delay in vaginal opening with no significant change in body weight. Chronic administration of 6-OHDA to neonatal female rats, in a regime known to deplete central catecholamines (Clark et al., 1972) has been found to delay vaginal opening, but this delay was accompanied by a significant decrease in body weight on the day of VO (Sarkar et al., 1981). Other investigators have found that the administration of reserpine early in life results in a significant delay in VO (Carraro et al., 1965; Hyppa and Rinne, 1971).

Neither 6-OHDA nor reserpine are specific in their action. 6-OHDA is not a selective neurotoxin, and there is evidence that it can damage not only neural cells, but meningeal, glial, and other extraneuronal tissue as well (Sievers et al., 1983). Reserpine is known to deplete both noradrenaline and dopamine as well as serotonin (Shore, 1972). The non-specificity of action of 6-OHDA and reserpine is avoided by the use of DSP4. This tertiary haloalkylamine has been shown to have actions compatible with a specific neurotoxic effect on central as well as peripheral noradrenergic neurons (Jaim-Etchéverry and Zieher, 1980). It is believed that the compound, which is able to cross the blood-
brain barrier, acts by forming a cyclic aziridinium ion under physiological conditions, which binds to the noradrenaline uptake site and results in an irreversible inhibition of the membrane uptake mechanism (Jaim-Etcheverry and Zieher, 1983). The inability of an acute insult to noradrenergic fibre systems to adversely affect the onset of puberty demonstrates both the resilience of these systems, and the strength of the normal drive to puberty. Previous work has shown that by seven days after electrolytic (Katzman et al., 1971) or chemical (Smith et al., 1982) lesioning, active regeneration of catecholaminergic neurons has begun.

In view of the remarkable capacity of the central noradrenergic system for regeneration, it is possible that in the current study, sufficient recovery had occurred following early exposure to DSP4 to allow a normal progression to puberty. Indeed, on day 30 of life (i.e. from 6 to 25 days after DSP4) none of the groups studied had hypothalamic noradrenaline levels significantly lower than the control group. Jonsson et al. (1982) have previously shown that the administration of DSP4 to rats at various developmental stages had the effect of decreasing the noradrenaline concentration of
various regions, both 1 day and 2–3 months following the DSP4. They did not examine the effect of DSP4 given to prepubertal females on hypothalamic amines during the same time course we have used here, although the drug is known to affect hypothalamic amine levels.

EFFECT OF DSP4 ON PMSG-INDUCED OVULATION

Treatment with PMSG has been shown to reproducibly induce ovulation in prepubertal female rats (Lunn and Bell, 1968; Wilson et al., 1974; Kostyket et al., 1978). This model has been well characterized and much used to study the role of catecholaminergic systems in the generation of the GnRH surge which precedes ovulation (Sarkar et al., 1981; Sarkar and Fink, 1981). We have been able to demonstrate that DSP4 interferes with the effects of PMSG on ovulation. The ability of PMSG to release GnRH, and ultimately gonadotrophins was clearly inhibited by the administration of this neurotoxin, as evidenced by the decreased ovarian weight and lower incidence of ovulation. Sarkar, Smith and Fink (1981) have previously demonstrated that intraventricular treatment with 6-OHDA inhibits the surges of GnRH and LH induced with PMSG, while
intravenous administration of the drug had no effect. Unlike 6-OHDA, DSP4, a substance which has been shown to readily cross the blood-brain barrier (Zieher and Jaim-Etcheverry, 1980; Jonsson et al., 1981). We have thus avoided the technical difficulties and the stress to the animals which occur with intraventricular drug administration. This result confirms the normal stimulatory influence of the noradrenergic system in the PMSG model, as proposed by Lofström et al. (1977b). The inhibitory effect of DSP4 on the system was present regardless of whether the DSP4 was administered concurrently with, or 48 hours after the PMSG, although the effect was greater in the former case. This is taken to indicate that the noradrenergic component of the response to PMSG is active early in the process of ovulation.

EFFECT OF DSP4 ON GONADAL STEROID-INDUCED LH SURGE

Yet another means of inducing a surge of GnRH and of LH in prepubertal females is that of administering progesterone (P) to estradiol benzoate (EB)-primed animals (Caligaris et al., 1968). The stimulatory influence of P on the release of gonadotrophins in ovariectomized rats is mediated through noradrenaline (Kalra et al., 1972). We have
successfully confirmed the involvement of noradrenaline in this process by the use of DSP4. The LH releasing action of P was completely abolished by treating the primed animals with DSP4 simultaneously. However, when the DSP4 was administered two hours before the P was administered, a normal LH surge was observed.

Animals given DSP4 in an attempt to inhibit the progesterone-induced LH surge following EB priming had significantly lower noradrenaline levels than did the control animals. Whether or not the DSP4 and progesterone were co-administered did not significantly affect the amine levels. Although the noradrenaline levels at the end of the experiment were the same, it is not clear whether differences in the amine levels at the time of P administration may account for the discrepancies in the generation of an LH surge seen with the varied temporal administration of DSP4 and P. On the other hand, such differences may be related to DSP4-induced changes in opioid receptors, rather than to effects on noradrenergic neurons (see below).
EFFECT OF DSP4 ON NALOXONE-INDUCED LH RELEASE

Within 2 hours of the injection of DSP4, the stimulatory effect of the opiate antagonist naloxone on LH release in immature female and mature male rats is abolished. Administration of the norepinephrine synthesis inhibitor U-14624 was also able to eliminate the response of LH to naloxone in prepuberal female rats. This drug acts through an inhibition of the enzyme dopamine β-hydroxylase (Johnson et al., 1970), an action which DSP4 shares (Ross, 1976). The response to naloxone in DSP4 treated animals is restored in 7 days. In male rats, paradoxically, the abolition of the response to naloxone was achieved without any change in hypothalamic NA. This observation led to the speculation that DSP4 may interact directly with hypothalamic opioid receptors so as to prevent the stimulatory effect of naloxone on circulating levels of LH. Further experiments examining the action of DSP4 in vivo on LH release were performed. It has previously been shown that LH levels in the short-term ovariectomized (48 hours post-surgery, age 28 days) immature female are rapidly reduced after opioid injection (Bhanot and Wilkinson, 1983). In the same preparation, DSP4 was able to maximally inhibit
LH output within 15 minutes, and to maintain this inhibition of circulating LH for at least 45 minutes. Dose response studies revealed a bimodal effect of DSP4 on LH levels in this model. There was a trend (non-significant by ANOVA) toward lowered LH levels with doses of 1 and 5 mg/kg, and a significant lowering of LH levels above this dosage. DSP4, thus, appeared to have effects similar to an opioid agonist. These observations gave added credence to the proposition that some of the effects of DSP4 might be mediated through endogenous opioid systems. This novel interaction between DSP4 and opioid receptors is discussed below.

It was further reasoned that if DSP4 were acting through opioid receptors, it might display some additional opioid-like effects on systems other than LH release. Prolactin release is known to be partially under the control of endogenous opioids (Van Vugt and Meites, 1980; Shaar et al., 1980). Administration of the opioid antagonist naloxone to rats induces a prompt decrease in the amount of circulating prolactin (Shaar et al., 1977; Bruni et al., 1977); while exposure to exogenous (Bruni et al., 1977, Clemens and Sawyer, 1974) as well as endogenous (Dupont et al., 1977; Cusan et al., 1977;
opioid agonists induce the release of prolactin into the circulation in both rodents and man. However, administration of DSP4 to ovariectomized prepubertal rats, while causing a decrease in circulating LH, did not cause prolactin to increase. Rather, prolactin levels were decreased in a pattern similar to LH. The action of opioid agonists and antagonists on prolactin release is believed to be mediated through dopamine (Vân Vugt et al., 1979). That acute treatment with DSP4 did not result in increased circulating prolactin does not rule out an interaction between DSP4 and opioid receptors. DSP4 may have removed an opioidergic inhibition of dopamine release, and thus lowered circulating prolactin levels. The evidence for such a direct interaction between DSP4 and opioid receptors is discussed below.

The apparent direct influence of DSP4 on central opioid systems suggested that some experiments (i.e. the effect of DSP4 on PMSG-induced ovulation, and on gonadal steroid-induced LH surges) should be re-examined with a view to delineating opioid or noradrenergic involvement. In and of itself, naloxone had no effect on the induction of an LH surge by appropriate treatment with gonadal steroids. However,
naloxone was able to partially block the inhibitory effect of DSP4 on such a surge, without completely preventing it. This may be interpreted as evidence that at least a part of the action of DSP4 in this system is mediated via opioid receptors. Further studies using selective opioid antagonists might pinpoint the locus of DSP4's inhibitory effect on gonadal steroid-induced LH surges. Similarly, naloxone was able to prevent the DSP4-induced overnight weight loss in DSP4-treated animals in the PMSG experiment. The protective effect of naloxone in this system also extended to the increase in uterine weight. In the light of these observations, the fact that naloxone pretreatment had no effect on the decrease in ovarian weight induced by DSP4 in PMSG-treated animals, might lead one to conclude that there is a dissociation of opioid and non-opioid mechanisms involved in the effects of DSP4 on the action of PMSG in immature female rats. Thus some of the effects of DSP4 may be mediated through an interference with the endogenous opioid systems, and some may act through other means, including the well known effects on the noradrenergic system.
THE EFFECT OF DSP4 ON OPIOID RECEPTORS

The observations which suggested that DSP4 might mediate some of its effects through an interaction with opioid receptors has been reviewed in the Discussion. Briefly, changes in basal and naloxone-induced LH release were seen following treatment with DSP4 which could not be accounted for by an effect of DSP4 on hypothalamic noradrenaline or dopamine concentrations. DSP4 is known to undergo cyclization to form an aziridinium ion. This ion is believed to be responsible for the noradrenaline uptake blocking action of DSP4, via an intrinsic irreversible alkylating activity (Zieher and Jaim-Etcheverry, 1980; Ross et al., 1973). Several other drugs are known which irreversibly block binding sites through the intermediacy of an aziridinium ion; for example phenoxybenzamine and dibenamine bind to α-adrenergic sites (Henkel et al., 1978). However, phenoxybenzamine interacts with brain opiate receptors (Cicero et al., 1975; Spiehler et al., 1978) and potentiates the analgesic effect of morphine (Cicero et al., 1975). The close structural resemblance of phenoxybenzamine (Henkel et al., 1978) to DSP4 and other tertiary haloalkylamines, as well as the observations discussed above led to an
examination of whether DSP4-opioid receptor interactions occur. The data strongly suggest that in addition to a noradrenaline-depleting effect, DSP interacts directly with \( ^3H \)-naloxone binding sites in brain slices.

Binding assays carried out in vitro, both in brain slices (this Thesis) and in homogenates (see Jacobson et al., 1985; Wilkinson et al., 1985b), show clearly that the supposedly specific noradrenergic neurotoxin DSP4 competes with \( ^3H \)-naloxone for an opioid binding site. Studies carried out on hypothalamic slices obtained from rats pretreated with DSP4 revealed that the loss of the LH response to naloxone in immature females was accompanied by a 44% decrease in the density of hypothalamic opioid receptors. Interestingly, in the male rats, the opposite was true, i.e. hypothalamic opioid receptor binding was increased after DSP4 treatment, even though the LH response to naloxone was abolished. Clearly, the interaction of DSP4 with those hypothalamic neurons which control the control of LH release is complex, but obviously is far from specific insofar as the noradrenergic system is concerned.
It seems unlikely that DSP4-induced neuronal destruction has taken place within 2 hours of drug injection, a process which could account for a loss of opioid binding. Of greater likelihood, is an interaction of DSP4 with hypothalamic opioid receptors in vivo. There is now strong evidence that an opioidergic system located within the hypothalamus is responsible for tonic inhibition of LH secretion (Kalra and Kalra, 1984). Since the in vitro data indicates a direct influence of DSP4 on hypothalamic opioid binding sites, it is likely that the lack of effect of naloxone on LH release in DSP4 pre-treated rats is in some way related to this phenomenon.

A direct interaction of a non-opioid with the opioid receptor is not without precedent, as discussed for phenoxybenzamine above. Phentolamine (Cicero et al., 1974) as well as some dopaminergic agents (Cicero et al., 1975) are also able to interact with the opioid receptor. An effect of DSP4 on β-adrenergic binding sites has not been found in our system (M. Wilkinson, personal communication), nor has a direct influence of DSP4 on benzodiazepine receptors been found (data not shown). This suggests that DSP4 has at least some specificity for the
opioid receptor which has not previously been described.

At the relatively high doses of DSP4 (50 mg/kg) which are normally used to inhibit noradrenaline uptake, it is likely that opioid receptors in vivo are being blocked. A similar argument has been put forward by Blank et al. (1983), in their work on monoaminergic agonists such as phentolamine and prazosin. Other studies in our laboratory have shown that other "specific" neurotoxins such as chloroethyl clonidine, a putative irreversible α-adrenergic ligand, may well have a significant opioidergic component to its action when used in high doses (Wilkinson et al., 1985b). The fallacy of automatically attributing the action of these so-called "specific" neurotoxins to an effect on their nominal target neurotransmitter has been recognized by other investigators (Bennett et al., personal communication; Bennett et al., 1985), and caution is mandated in interpreting the results of such experiments.

In vivo administration of DSP4 was without effect on the binding of [3H]-flunitrazepam to slices of either cerebral cortex or cerebellum. There was,
however, a significant depression in the binding of \[^3H\]-flunitrazepam to slices of mediobasal hypothalamus. This effect could not be seen when hypothalamic slices were incubated \textit{in vitro} with DSP4. A direct action of DSP4 on the benzodiazepine receptors would have been seen as a change in binding following \textit{in vitro} exposure. The observation that 4 days following DSP4 administration, \[^3H\]-flunitrazepam binding to slices of mediobasal hypothalamus and cerebellum was \textit{increased}, in addition of the lack of short term \textit{in vitro} effects of DSP4 on benzodiazepine binding supports earlier suggestions of a modulation of benzodiazepine receptors by catecholaminergic mechanisms. The increase in binding seen following DSP4 treatment contrasts with the report of Medina and Novas (1983), who reported a decrease in \[^3H\]-flunitrazepam binding in homogenates following DSP4 treatment, but are consistent with the increase in \[^3H\]-flunitrazepam binding seen by Sabuto et al. (1981) following treatment with the catecholaminergic neurotoxin 6-hydroxydopamine. Further attention on the mechanisms of DSP4 interactions with these, and other receptor types, is merited.
Both DSP4 and desipramine are able to inhibit the uptake of noradrenaline, the former irreversibly, and the latter reversibly. The inhibitory effect of DSP4 on noradrenaline uptake can be antagonized by desipramine (Ross, 1976; Ross and Renyi, 1978). Since it has been demonstrated that DSP4 is able to inhibit opioid binding to hypothalamus, experiments were performed to search for a protective action of desipramine against its inhibitory action of DSP4.

Preincubation of slices of mediobasal hypothalamus with desipramine was unable to eliminate the DSP4-induced decrease in \(^{3}H\)-naloxone binding. A slight protective effect of the desipramine was seen, in that DSP4 could not reduce opioid binding to the same extent in desipramine-treated slices as it could in control slices. However, these data may indicate that the action of DSP4 on the opioid binding site is occurring largely independently of the interaction of DSP4 with the noradrenaline uptake site, where desipramine acts. Further evidence in favor of a site of action of DSP4 independent of the noradrenaline uptake mechanism is that a combination of preincubation and coincubation with either desipramine of cocaine, another reversible noradrenaline uptake inhibitor, was without effect on
the ability of DSP4 to inhibit opioid binding in brain slices.

The situation was somewhat different in vivo. Injection of desipramine was able to completely block the 40% decrease in the binding of $[^{3}H]$-naloxone to slices of mediobasal hypothalamus seen two hours following administration of DSP4 alone. This anomalous result would appear to indicate that the effect of DSP4 on opioid binding sites in vivo is somehow being mediated through an interaction with the noradrenaline uptake site. Of course, the possibility exists that desipramine is able to interact with the opioid receptor. As of the writing of this Thesis, these findings have not been reproduced. Early indications are that the DSP4 may not be working properly, and attempts to settle this matter must await the arrival of fresh supplies of the drug.
SUMMARY

1) An assay which makes use of intact brain slices for the study of opioid receptors has been developed and validated.

2) This assay has also proven to be a reliable tool for the investigation of opioid receptors in intact retinal tissue. Both sex and strain differences in opioid binding to retinal tissue have been described.

3) Naloxone is less able to induce the release of LH in the late afternoon than it is in the morning in prepubertal female rats. The emergence of this diurnal decrease in opioid tone has been examined, and it was found that the rhythm appears between days 23 and 26 of life.

4) The hypothalamic, but not the cortical, complement of opioid receptors decreases in the late afternoon in prepubertal female rats. This diurnal decrease in the number of opioid receptors develops with the same temporal pattern as does the difference in the ability of naloxone to release LH, cited in #3 above. It is proposed that there is a functional link
between these two phenomena, and that the development of a decrease in the opioidergic inhibition of LH release may be crucial to the establishment of a pattern of LH release which is important in the control of sexual maturation in the female rat.

5) The diurnal rhythm of hypothalamic opioid binding is disrupted by neonatal androgenization, and infantile gonadectomy.

6) The diurnal rhythm of hypothalamic opioid binding, which is not normally present until between days 23 and 26 of life, can be induced by treatment of 18 day old female rats with PMSG.

7) The diurnal rhythm of hypothalamic opioid binding is susceptible to interference by changes in photoperiod. Animals housed in conditions of total darkness or continuous light for 9 days did not show the diurnal difference in opioid binding seen when housed under a 14:10 LD photoperiod.

8) Administration of the neurotoxin DSP4 at various ages was without effect on the spontaneous development of puberty in female rats.

9) DSP4 interferes with the action of PMSG in the induction of ovulation in immature female rats.
10) DSP4 interferes with the steroid-induction of an LH surge in immature female rats.

11) LH release is profoundly affected by DSP4 in a number of models which were examined. These include naloxone-stimulated LH release in intact immature female rats as well as basal release in 48 hour ovariectomized rats. These effects may be mediated, in part, through opioidergic systems.

12) DSP4 interacts directly and potently with opioid receptors. Some of the actions attributed to its effects on noradrenergic systems may in fact be due to this newly described interaction.
APPENDIX I

MINI REVIEW

TISSUE SLICES IN RADIOLIGAND BINDING ASSAYS:
STUDIES IN BRAIN, PINEAL AND MUSCLE

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Summary

The use of tissue homogenates in receptor binding assays raises serious questions as to the physiological value of a preparation which examines receptors (binding sites) in disrupted tissue. In order to usefully study the regulatory properties of neurotransmitter receptors under physiological conditions, the necessity for tissue preparations which retain some degree of cellular integrity is clear. We review here the experiments which have utilized intact tissue - largely in the form of thick slices - to perform radioligand binding assays.

There are many reports which note marked differences between studies in intact versus broken cell preparations. For example, significant discrepancies in $K_D$ and $B_{max}$ values are apparent for $[^3H]$ quinuclidinyi benzilate (muscarinic) and $[^3H]$ ouabain (Na+/K+ - ATPase, sodium pump) sites in brain and muscle respectively. A further example is the well-described stimulatory effect of GABA on benzodiazepine binding sites which is not seen in tissue slices. Other examples are highlighted.

For all ligands so far examined, binding to slices is reversible, stereospecific, saturable, displaceable by appropriate drugs and of high affinity (mM). The method developed in our own laboratory is inexpensive, rapid and involves a minimum of tissue preparation. The technique is so simple as to allow many workers to enter this field who would not otherwise have done so. We suggest that metabolically active tissue slices offer the simplest approach to the study of cell-surface receptor regulation in living tissue.

The characterization and quantification of a large variety of membrane-bound receptors, or binding sites, was made possible with the development of the radioligand binding assay (1-3). The past five years or so have witnessed an exponential growth in the number of publications devoted to studies in the nervous system alone (2). To date, hormone, drug and neurotransmitter
receptor sites have probably been examined in all available tissue types, so that much is now known on cellular communication via specific recognition sites (receptors) located within cell membranes. In brief, the assay technique consists of an incubation step in which a thoroughly disrupted tissue homogenate or cell membrane fraction is allowed to interact with a radioactively labelled drug (ligand); when this binding reaction reaches equilibrium, the unbound ligand is rapidly separated from the ligand-receptor complex by filtration through glass fiber filters or by centrifugation (3). Scintillation counting of the bound ligand readily provides quantitative data on the nature of the binding site.

It is probably true to say that this type of binding assay has provided information quite unobtainable by any other means and has certainly led directly to the discovery, for example, of the endogenous opiate peptides. Nevertheless, the use of tissue homogenates raises serious questions as to the value of a preparation which examines receptors free of their natural environment. This is not to imply that the study of isolated receptor proteins (4) is not of value, merely that investigations of receptor-mediated physiological events ought to be examined in intact tissue. In terms of receptor binding data this has been repeatedly stressed. For example, Porzig (5) has drawn attention to marked differences in the β-receptor-adenylate cyclase system of fragmented membranes and living cells. Sher et al. (6) have reported discrepancies in the binding of benzodiazepine ligands to intact versus broken cell preparations. The pharmacological specificity of brain histamine (H2) receptors and the binding of [3H]-prazosin (alpha1) to intact or broken cells exhibit important differences in the nature of the receptors (7,8). In fact, cellularly intact tissues have seldom been used to investigate binding sites. This is clearly an area which needs to be examined more carefully.

An ideal system would be to perform ligand binding in vivo. However, this approach is severely compromised through a combination of cost (the need for large amounts of labelled compounds) and, for the brain, the difficulties of drug penetration of the blood-brain-barrier (though see 9,10). These problems have been overcome to some extent with the autoradiographic technique which localizes binding sites in ultra-thin tissue sections (approximately 10-30 m) (11,12). This elegant technique has been quickly adapted for use in the quantification of receptor binding sites either through direct scintillation counting of the ultra-thin sections (13,14) or by densitometry (15,16,17). The primary advantage of this method remains its ability to localize and visualize receptor sites but for some laboratories it is financially impractical to own a cryostat microtome/microdot densitometer merely to perform binding assays. It is additionally important to remember that the thin sections consist of frozen and thawed dead cells. In order to usefully study the regulatory properties of, for example, neurotransmitter receptors under physiological conditions, the need for tissue preparations which retain some degree of cellular integrity is clear.

The success of the autoradiographic method prompted us to consider the use of tissue slices in binding assays. In particular, the brain slice is commonly regarded as a thin (100-700 m) section of a brain region which is capable of being maintained for many hours in vitro. The use of the brain slice in neurobiological, predominantly electrophysiological, studies has recently received renewed attention (18,19,20). Structural integrity can be largely maintained (21,22), respiratory function appears to be good (20) and electrical activity is comparable to that seen in brain in situ (20). Our
efforts to use tissue slices in radioligand binding assays are described below. Included in this discussion are descriptions of various attempts by other workers to utilize slices in binding assays.

Opiate binding sites in brain slices

Opiate receptors in brain slices have attracted more attention than any other type of binding site. Our own work has attempted to rigorously characterize \( ^{3}H \)-naloxone (NAL) binding, but other laboratories have also reported numerous studies particularly with respect to morphine tolerance.

We have characterized and quantified the binding of NAL to slices (300-400 \textmu m) of rat, mouse and rabbit brain (23-27). Of some importance were our efforts to establish that NAL binding to slices possessed those characteristics previously described for receptors in tissue homogenates (for a critical review see ref. 28). For example, the binding is readily reversible, saturable, stereospecific and thermolabile. Non-specific binding is usually \( \leq 25\% \). Initial studies (23,24) were performed in hypotonic TRIS buffer (50 mM; pH 7.4) in order to more readily compare data obtained from homogenate assays, which are normally run in TRIS. More recent experiments have employed isotonic Dulbecco's PBS (a balanced salt solution) as the incubation medium (26,27; see also section on \- adrenergic binding, below). Thus, NAL binding assays are now run routinely in PBS. The slices can therefore be maintained in a metabolically active form, given oxygen and glucose. We have reported diurnal rhythms of opiate binding in immature rat brain (26) and the ontogenesis of opiate sites in rabbit brain (27).

Recent work by LaBella and Pinsky (29) addresses the critical issue of whether the intact cells within the brain slice are metabolically active and capable of internalizing opiate ligands. Binding of various tritiated ligands (naloxone, met-enkephalin, etorphine and DADL) was examined. Extensive characterization of the receptor sites was not included in this short paper, but the results clearly indicate that opiate peptides, but not drugs such as naloxone, appear to be internalized as expected. For example, the absence of glucose or the presence of ouabain reduced, but did not eliminate, accumulation of \( ^{3}H \)-met-enk and \( ^{3}H \)-DADL at 37°C. An important issue here is whether the labelled drugs were retained in the slice by an uptake mechanism into nerve terminals as has been described by George and Van Loon (29a).

Davis et al., in 1975, appear to be the first group to have reported opiate binding assays with brain slices (30-32). These authors were able to show that brainstem slices were superior to homogenates in their ability to detect an effect of morphine tolerance. Slices from tolerant rats bound significantly less \( ^{3}H \)-morphine (MOR) than did slices from naive rats (30), but this alteration in binding was not observed in homogenates. However, the change in \( B_{\text{max}} \) values was accompanied by a large reduction in \( K_{D} \) (i.e. 6.62 nM, control, versus, 22.6 nM for morphine tolerant rats) which suggests that residual morphine was present in the slices. In a subsequent paper (32) these workers addressed the same problem, but with a different ligand \((\text{d-ala}^{2}\text{-met-enkephalinamide; DALA})\). Again a reduction in \( B_{\text{max}} \) and a shift in \( K_{D} \) was observed. The morphine content of the slices is clearly a confounding factor which needs closer scrutiny (see also Discussion in ref. 37). Further data from this laboratory suggests that tissue slices may be especially valuable in the study of cooperativity (31,32). For example, the binding of NAL and MOR exhibited strong positive cooperativity whereas DALA did not. Cooperative
interactions were not seen in brain homogenate assays. Thus, some important differences appear to exist between intact and broken cells in terms of site-site interactions.

Complementary, and probably contemporary, experiments have been performed by Takemori and co-workers (33, 34-37, 39). In preliminary studies, slices of cerebral cortex and corpus striatum accumulated low concentrations of etorphine (<5 nM) in a manner compatible with receptor binding (33). With hindsight the experimental conditions were not ideal since the presence of sodium ions in the incubation buffer would certainly have greatly reduced specific binding of the agonist etorphine (33a). Quantitative binding assays, as we now understand them, were never performed. Instead the ability of naloxone to displace pre-bound ligand was carefully examined (34, 35). Thus, binding of MOR (10 nM) was stereospecific and extremely sensitive to displacement with naloxone; the effect was maximal at concentrations of less than 10⁻¹² M and was readily seen at 10⁻¹⁰ M. Striatal slices from morphine-dependent mice showed an enhanced sensitivity (affinity?) to naloxone, whereas slices from cortex and brainstem did not. This result is quite different from that described by Davis et al. (30-32; see above), who observed a marked decrease in affinity of MOR binding to slices of brainstem obtained from tolerant rats. Strict comparisons between these experiments are difficult to make since the assay methods, buffers and animals were dissimilar. However, Oishi and Takemori (37) draw attention to the problem of residual morphine contained within the slices from MOR-dependent animals. Their efforts to wash out the drug were not completely successful, but they showed that mouse brain tissue, after MOR withdrawal (6 h), did not contain detectable quantities of MOR. Slices of corpus striatum from these acutely withdrawn mice still showed the affinity shift previously attributed to MOR-dependence (37). Thus, in the presence of sodium ions, the antagonists naloxone and naltrexone can discriminate between brain tissue from naive or MOR-tolerant mice. This discrimination is not consistently seen in brain tissue homogenates (38).

A further report from this laboratory (36) describes the influence of enkephalin pretreatment, in vitro and in vivo, on the morphine-releasing effect of naloxone. Injection of leucine enkephalin (LEU-ENK), but not MET-ENK 15 minutes before sacrifice, or preincubation of striatal slices with LEU-ENK (0.1 pM to 1.0 nM) markedly enhanced the ability of naloxone to release [³H]-MOR (23, 24). Also, the shift in the apparent affinity of the opiate binding sites is consistent with in vivo data i.e. prior administration of LEU-ENK to mice markedly enhanced morphine-induced analgesia (39). These experiments, on the rapid induction of opiate receptor modifications, appear to complement the studies reported by Pert and Snyder (40) and by Lewis et al. (40a). These papers describe the enhancement of opiate binding in rat and mouse brain which followed injection of opiate agonists or antagonists.

Pieces of intact retina (McIlwain's "prototypical tissue slice" (41)) are easily used in opiate binding assays. Su (42) has described binding of [³H]LEU-ENK, [³H]MET-enkephalinamide and [³H]-NAL to pieces of chick retina. Binding affinity was in the nanomolar range. These results have been confirmed in rat retina, which binds [³H]-NAL reversibly, stereospecifically, with high affinity and low non-specific binding (40b).
Beta-adrenergic binding in slices of brain and muscle and intact pineal gland

In early studies we were unsuccessful in labelling \( \beta \)-adrenoreceptors with the widely-used ligand dihydroalprenolol (DHA). This failure was no doubt due to the lipid solubility of the DHA (43,44). The work of Staehelin and co-workers (see ref. 45, for example) led us to employ the water-soluble ligand \(^{3}\text{H}\text{-CGP-12177}.\) This hydrophilic \( \beta \)-adrenergic antagonist is unable to penetrate cell membranes and can be used to quantify cell surface receptors with acceptably low NSB. We have characterized and quantified the binding of \(^{3}\text{H}\text{-CGP to } \beta \text{-adrenoreceptors in slices of cerebral cortex and intact pineal glands (25,46) and in slices of skeletal muscle (47, 48). This assay method allows the demonstration of isoproterenol-induced down-regulation (internalization) of \( \beta \)-adrenoreceptors. Receptor recycling is observed at 37\( ^\circ \text{C} \) in the absence of \( \beta \)-agonist but can be blocked by low temperature (0\( ^\circ \text{C} \)) or by monensin. \( \beta \)-Adrenoreceptors can also be labelled and quantified in intact, single pineal glands of rat, mouse and hamster (46). Rat pineals contain approximately 10 times more binding sites than do hamster or mouse pineals and up to 8 times more sites than found in rat cerebral cortex. Rat pineal \(^{3}\text{H}\text{-CGP binding can be up- and down-regulated but not to the same degree as seen in brain slices (see also homogenate data, 46a).\)"

In mouse skeletal muscle \(^{3}\text{H}\text{-CGP binds to thick (1 mm) slices of fast-twitch (extensor digitorum longus [EDL]) and slow-twitch (soleus) muscle (47). In both muscles the majority of specific binding is to the \( \beta \) receptor subtype. \( B_{\text{max}} \) is approximately twice as high in the soleus as in the EDL, whereas affinity is higher in the fast rather than the slow-twitch muscle. These data agree well with those of Williams et al. (47a) who used muscle homogenates. We have now determined that \( \beta \)-adrenoreceptors are increased in soleus muscle slices from dystrophic (CHF 147) hamsters (48). We have compared the binding of \(^{3}\text{H}\text{-CGP in soleus and extensor digitorum longus (EDL) muscles of male Golden Syrian (GS) and Canadian Hybrid Farms 147 (CHF 147) dystrophic hamsters. The predominant receptor subtype was \( \beta \). \( B_{\text{max}} \) was higher in the soleus than in the EDL while affinities were similar. In young (60-day-old) CHF 147 animals, \( B_{\text{max}} \) for soleus was higher than in GS whereas affinity was decreased. These differences were no longer evident in 300-day-old hamsters. No changes in binding characteristics were seen at either age in EDL. The increased CGP binding in soleus but not EDL and in young but not old dystrophic animals correlates positively with the severity and incidence of necrotic lesions in these muscles (49).\)

Benzodiazepine \((^{3}\text{H}\text{-flunitrazepam; FNZ})\) binding in brain slices

We have characterized and quantified the binding of \(^{3}\text{H}\text{-FNZ to slices of cerebral and cerebellar cortex of the mouse (50). The binding site has many of the characteristics expected of a benzodiazepine receptor; for example, \(^{3}\text{H}\text{-FNZ binding is reversible, stereospecific, saturable and of high affinity. The binding is sensitive to elevated temperature but not to metabolic poisons such as ouabain and sodium azide. These latter drugs have been used to determine whether uptake processes contribute to the observed binding. This assay allowed us to confirm the known reduction of \(^{3}\text{H}\text{-FNZ binding in "emotional" mouse brain. In addition we have confirmed that the neurotoxin DSP4 can modify FNZ binding sites (see 50).\)"

An aspect of the benzodiazepine receptor which has received considerable attention is the ability of GABA to modulate the affinity of the binding; that is, GABA can elevate \(^{3}\text{H}\text{-FNZ binding through a change in } K_{D} \text{ rather than in maximum binding capacity (see 50). We have demonstrated that the }^{3}\text{H}\text{-FNZ binding in brain is modified by GABA (see 50).\)
binding site in intact tissue (slices) is not responsive to GABA. This result raises the interesting possibility that the GABA-induced change in FNZ binding is an artifact which results from the use of broken cells. Alternatively, it is conceivable that an endogenous benzodiazepine ligand, as well as endogenous GABA, remains bound to the receptors within the tissue slice and prevents the stimulatory effect of GABA. In support of this idea Schiller and Farb (51) report that GABA does not potentiate FNZ binding in brain cell cultures grown in the presence of flurazepam. This conflict suggests that GABA-benzodiazepine interactions in the living brain need to be re-examined.

Miscellaneous studies in brain: GABA, cholinergic and dopamine binding sites

Johnston et al. (52) examined uptake and binding of muscimol, a potent GABA agonist, to rat cerebral cortical slices. \( ^{3} \text{H} \)-muscimol was actively transported into (presumed) nerve terminals by a sodium-dependent GABA uptake mechanism. In the absence of sodium, however, and at an incubation temperature of 4°C, GABA-displaceable specific binding was observed. This binding was sensitive to a series of GABA agonists and antagonists but not to GABA-uptake inhibitors. The very low specific activity of the tritiated muscimol (3.5 Ci/mmc) was probably responsible for the 'poor' specific binding. These results do suggest that the study of GABA binding sites in tissue slices is possible, particularly since high specific activity ligands are now available (e.g. bicuculline; approximately 80 Ci/mmc). Also, pertinent commentaries have recently appeared which discuss whether GABA binding might profitably be examined under more physiological conditions (52a-52c).

Two studies from Iversen's laboratory (53,54) sought to examine muscarinic receptors in striatal and hippocampal slices. The ligand used was \( ^{3} \text{H} \)-quinuclidinyl benzilate (QNB). Their experiments amply demonstrated the utility of brain slices to compare binding data and cyclic nucleotide responses (53). For example, receptor occupancy appears linearly related to the magnitude of the cGMP response. The binding of \( ^{3} \text{H} \) QNB to brain slices was subsequently examined more rigorously and the results compared to those from homogenate assays. Maximum binding (B_max) of \( ^{3} \text{H} \) QNB was very similar in slices and homogenates of striatum and hippocampus. In contrast, the affinity (K_i) values were quite different. In addition, competition experiments revealed a similar shift in the ability of muscarinic agonists and antagonists to displace \( ^{3} \text{H} \) QNB. The authors conclude that this discrepancy, between slices and homogenates, represents a fundamental difference in the nature of the respective binding sites. A further word of caution should be added with respect to the lipophilic character of the ligand, a property which may alter binding characteristics. Gossuin et al. (55) report on the high non-specific binding of \( ^{3} \text{H} \) QNB to intact cells compared to the use of \( ^{3} \text{H} \)-N-methylscopolamine (NMS), a hydrophilic drug. The suggestion that \( ^{3} \text{H} \)-NMS is a more appropriate ligand with which to quantify cell-surface receptors has been recently made by Lee and El-Fakahany (56), and implies that it could also be used with success in slice assays.

Abood et al (56a,56b) have described experiments in which \( ^{3} \text{H} \)-nicotine appears to bind to cerebral cortical slices (400-800 m; 30-50 mg wet weight) from rat brain. Specific binding was stereospecific and represented 45% of total binding. Scatchard analysis gave a K_d value of 19 nM. Significantly, these authors were not able to detect specific \( ^{3} \text{H} \)-nicotine binding to brain homogenates. Their conclusion was that \( ^{3} \text{H} \)-nicotine binds to a non-
cholinergic site. In view of recent widespread reports that $[^3]H$-nicotine is a useful ligand for autoradiographic localization of cholinergic sites (7,9), the work of Abood et al needs to be re-examined with higher specific activity $[^3]H$-nicotine.

Dopaminergic binding sites have been quantified in rat striatal slices (57) with the ligands $[^3]H$-apomorphine (APO) and $[^3]H$-domperidone (DOM). Incubations were performed in physiological buffer and three distinct classes were identified (D-2, D-3 and D-4). $B_{\text{max}}$ and $K_D$ values were comparable to those obtained from homogenate assays. An important conclusion from this report is that the known receptor subtypes are unlikely to be the artifactual product of cell disruption. Also, the surprising observation that guanylnucleotides reduced D-2 and D-3 sites in the slices suggests that contrary to popular belief this type of regulatory subunit is accessible from outside the cell. These same authors have reported on the disadvantages of $[^3]H$DOM as a ligand because of its lipid solubility (58). It seems likely that a reasonable degree of specific binding of $[^3]H$ DOM to slices can only be achieved by first homogenizing, and then filtering, the slices (as described in ref. 57). Experiments in our laboratory indicate that there is very little displaceable (specific) binding of $[^3]H$DOM to striatal slices when the tissue is washed, intact, according to our published procedure (for example ref. 46). In conclusion, $[^3]H$ DOM and $[^3]H$ APO may also label intracellular sites in slice incubations. The use of a hydrophilic ligand such as $[^3]H$(-)-DO 710 may provide data on cell surface dopamine receptors (58).

Miscellaneous studies in muscle.

The first attempt to investigate muscarinic binding sites was made by Paton and Rang (59) using small intact strips of intestinal smooth muscle (probably 6-8 cells thick) equilibrated with $[^3]H$ atropine. This very early study suggested the presence of a cholinergic binding site in this tissue. Several later studies continued this work (60-63). For example, Burgen et al. (62) used the irreversible muscarinic ligand $[^3]H$ propylbenzilylcholine mustard (PrBCM). However, the authors noted some discrepancy between the low potency of atropine to block PrBCM binding and the much lower concentration of atropine needed to prevent agonist action on contraction. Ward and Young (63) subsequently made a more thorough investigation of PrBCM receptor sites in guinea-pig intestine by carefully measuring IC$_{50}$ values for a variety of agonists and antagonists. In general, the IC$_{50}$ values for inhibition of binding were higher in intact strips than in broken cell preparations. The authors suggest that some type of access-limitation factor probably affects the irreversible binding of the ligand. As alluded to earlier for other ligands, the lipophilic nature of PrBCM probably complicates the issue. The hydrophilic, $[^3]H$ N-methylscopolamine could usefully be applied in this system. Note, however, that Snyder et al. (64) reported an excellent correlation between pharmacological potencies and IC$_{50}$ values for displacement of $[^3]H$ QNB in guinea pig ileum.

Muscarinic binding sites have also been examined in slices of rabbit iris muscle and compared directly with assays in tissue homogenates (65). The results of this work are very similar to those described by Gilbert et al. (54) for $[^3]H$QNB binding in striatum (see earlier). Briefly, $K_D$ and IC$_{50}$ values for muscle slices were numerically higher (i.e. of lower affinity) than those for tissue homogenates, although receptors in slices show typical binding characteristics: saturation, high affinity, reversibility and drug specificity.
Taft et al. (65) also examined alpha-adrenergic (\(^3\)H)WB-411; WB) binding in slices and homogenates of rabbit iris. The results clearly show that slices of iris muscle contain \(\alpha\)-adrenergic binding sites which have all the expected characteristics.

In 1973, Clausen and Hansen (66) began an investigation of \(^3\)H]ouabain binding to the sodium-potassium pump of intact skeletal muscle. These experiments culminated in a method to examine \(^3\)H]ouabain binding in biopsies. ("slices") of rat and human skeletal muscle (67,68). Their initial report on receptors in intact rat soleus muscle strongly suggested that \(^3\)H]ouabain binds to the surface of the plasma membrane by a reversible and saturable process. In subsequent reports this group confirmed that muscle fragments (2-14 mg) also allowed the assay of the number of \(^3\)H]ouabain sites. Close agreement was obtained in a comparison of binding to intact or cut muscle in a variety of preparations including manipulation of thyroid status and potassium deficiency (67,69). Furthermore, values of \(K_D\) for \(^3\)H]ouabain binding are identical whether determined in biopsies, whole muscle or muscle homogenates (67). The usefulness of this ligand is probably due in part to its hydrophilic nature (70).

The method is readily adaptable to human muscle biopsies. In a further careful study Norgaard et al. (68) obtained tissue samples (50-75 mg) with a Bergstrom needle from the vastus lateralis muscle. The number of binding sites did not vary with age (25-80 years) or sex. Samples from other muscles (intercostal; rectus abdominis) yielded similar data. The number of sites was approximately 2-fold higher than previously determined from homogenate assays; the value of the \(K_D\) (1.8x10^{-8} M) represents a 27-fold increase in the affinity of the \(^3\)H]ouabain binding site. This report also demonstrates that \(^3\)H]digoxin and \(^3\)H]ouabain appear to label identical sites. The method clearly allows, within hours, the quantitative determination of the number of \(^3\)H]ouabain binding sites in human muscle biopsy samples, and should prove to be useful in the investigation of muscle disease. For example, the effects of denervation on \(^3\)H]ouabain binding in intact extensor digitorum longus (EDL) and soleus (SOL) muscles of the developing rat have been studied by Ward and Warham (71). They observed an age-related increase in binding (EDL>SOL) which was significantly prevented by denervation in SOL but not in EDL. The values obtained for normal levels of binding agreed well with the earlier data of Clausen and Hansen (66).

Molecular studies of calcium-selective voltage-dependent channels were made possible by the ready availability of a variety of radiolabelled ligands, notably the 1,4-dihydropyridines (72). However, although much effort has been expended on the characterization and quantification of these sites in tissue homogenates, several authors have pointed out discrepancies between the affinity of the binding sites and the pharmacological potency of the blocking drugs (see e.g. 73,74). It is conceivable that cell disruption adversely affects this type of binding site. An attempt to overcome this problem, with mixed results, has been performed in vivo (75). The need for a binding assay for the voltage-sensitive calcium channel in intact, isolated tissue is evident. A recent report by Schwartz et al. (76) describes the quantification of dihydropyridine (\(^3\)H]PN-200/110) receptors in intact frog sartorius muscle. The great difficulty associated with the use of lipid-soluble ligands is again illustrated by these experiments. Most of the observed uptake of \(^3\)H]PN-200/110 is non-saturable into the tissue 'lump'. Nevertheless, estimates of \(K_D\) and \(B_{max}\) were determined, and these compared favourably with values obtained from skeletal muscle homogenate assays.
Discussion

This review has summarized various efforts to utilize non-disrupted, intact tissue - largely in the form of slices - in radioligand binding assays. In total, the evidence suggests that this useful method awaits exploitation on a larger scale. For example, the ease and simplicity of the technique should enable many workers to enter this field who would not otherwise do so. The low cost of tissue slicers compared to that of a high-speed refrigerated centrifuge is merely one of several advantages. The technique is simple, rapid and involves a minimum of tissue preparation. It is now possible to examine receptor internalization and recycling in intact, fresh tissue as has been described for isolated tumor cells or frog erythrocytes. A further advantage of the use of fresh, non-homogenized tissue is the capability to examine the state of receptor occupation at the time of death e.g., the presence of endogenous ligands, which remain bound to the receptors, should modify the equilibrium binding of labelled ligand. This has been attempted with the homogenate assay but this is clearly compromised through homogenization and washing of the tissue (77). A further advantage is that the assay offers a rapid method of drug screening and could be employed as a radioreceptor assay for the quantification of drug levels.

A similar case has recently been made for the use of suspensions of intact brain cells; El-Fakahany and co-workers (56,78) have also expressed concern that receptor data from homogenates may not reflect actual in vivo binding conditions. Their method is obviously a great improvement on cell-free assays. Unlike the slices, dissociated cells allow almost instantaneous access of ligand to the receptors. Although not described in these papers (56,78), this assay can obviously provide valuable data on binding kinetics to fresh, non-neoplastic brain cells. However, the retention of tissue structure - for example in a brain slice - could conceivably be critical for normal neuron function and neuron-glia interaction, including receptor recycling. Nonetheless, a cautionary note on the use of lipophilic ligands is worth re-emphasizing. The ability to examine receptor recycling in intact cells or tissue slices is facilitated by the availability of ligands which can selectively label cell surface sites (e.g., CGP-12177 (ȳ), pindolol (ȳ), N-Me-scopolamine (muscarinic). These compounds are hydrophilic in nature. Lipophilic ligands such as dihydroalprenolol (ȳ), domeridone (dopamine) or PN-200/110 (calcium channel) are extremely difficult to wash out and therefore should be avoided (see discussion in 43,44) if at all possible. If unavoidable, it is possible to achieve low non-specific binding by homogenizing and then filtering the individual slices (see 57). On the other hand, the use of lipophilic ligands, in conjunction with hydrophilic ligands, should permit the assay of total as well as cell surface receptors.

An obvious problem in the use of thick slices is the difficulty of knowing the precise concentration of ligand in the center of the slice. Put another way, is diffusion of ligand into the slice impeded? In our own work we have been careful to establish the time-course of the binding and also whether this is readily reversible. An examination of the time taken to reach equilibrium clearly shows that diffusion is a significant component of the binding reaction. For example, equilibrium binding of ligands to tissue homogenates at 30° is rapid and normally complete by 45-60 mins. Binding to slices reaches a plateau somewhere between 2 and 3 hours. In all cases, however, this is rapidly reversible on addition of an excess of unlabelled ligand. We believe it is a reasonable assumption that the plateau phase
represents penetration of the ligand to all parts of the slice. We have recently confirmed this for \([^3]H\)-naloxone binding by comparing autoradiographs of thin sections taken from the center of slices with those taken from more superficial sites (Jacobson, Leslie and Wilkinson; in preparation).

In conclusion, we suggest that slices offer the simplest approach to the study of receptors in living tissue. The limited number of ligands already tried leads us to suppose that most, if not all, types of binding site could be successfully labelled in tissue slices once suitable hydrophilic labelled ligands become available. A large multifunctional group of compounds already labelled and almost certainly hydrophilic are the peptides. The use of agonist peptide ligands in slice assays will be a challenging exercise. This view is forcefully emphasized by Motulsky et al (80) who have recently outlined their concerns with respect to agonist binding assays which use intact cells. In essence 'their argument suggests that the techniques developed for antagonist binding to membrane fragments are inapplicable. Several suggestions are offered to overcome the problems inherent in agonist binding. Some of them - such as the use of low temperature incubation and metabolic inhibitors - have been used already in slice assays (see 23, 24, 46). An appreciation of the difficulties outlined by Motulsky et al (80) is essential, for further progress in the use of living tissue in binding assays.

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References

INTRODUCTION

Opiates profoundly influence reproductive and endocrine function (Ferin et al., 1984; Millan and Herz, 1985; Bicknell, 1985). From the initial observation by Barraclough and Sawyer in 1955 that the administration of morphine could block ovulation in rats, and the subsequent report that this could be prevented by pretreatment with the opiate antagonist naloxone (Packman and Rothchild, 1976), to the recognition of the high incidence of reproductive dysfunction in both male (Azizi et al., 1973; Cicero et al., 1975) and female (Santen et al., 1975) narcotic users, an action of the opiates on gonadotrophin secretion has long been suspected. It is now well established that there is an opiateergic component to the negative feedback control of gonadotrophin releasing-hormone (GnRH) release by the gonadal steroids. (Blank et al., 1979; Cicero et al.,)

This chapter is excerpted from a review by William Jacobson and Michael Wilkinson entitled "The Effects of Steroids on Neurotransmitter Receptors in the Central Nervous System", and submitted to Progress in Neurobiology. This review was derived from the comprehensive examination of William Jacobson for the degree of Doctor of Philosophy.
Acute blockade of opiate receptors with the narcotic antagonist naloxone causes a prompt elevation of circulating LH levels both in the rat (Bhanot and Wilkinson, 1983; Cicero et al., 1979; Blank et al., 1979) and in man (Ropert et al., 1981). Thus, it appears that there is a tonic opiateergic inhibitory influence on the release of hypothalamic GnRH (Rotsztejn et al., 1978a; 1978b; Wilkes and Yen, 1981; Blank and Roberts, 1982). It was the similarity in the action of the endogenous opioid peptides with the well known central inhibitory action of testosterone on the control of gonadotrophin release (Cicero et al., 1979) which initially led investigators to look for a modulatory role of gonadal steroids on opiate systems.

**STEROIDS AND OPIATE LEVELS**

Although this review is concerned with the action of gonadal steroids on opiate receptors, it is pertinent to describe the work of many investigators who have demonstrated an unequivocal modulation of endogenous opiate systems by gonadal steroids.
Mueller (1980) has shown a sex difference in the levels of peripherally detectable β-endorphin with female rats having lower levels than males. This contrasts with a recent report in humans where females showed higher levels than males, regardless of the phase of the menstrual cycle (Furuhashi et al., 1984). Chronic estrogen treatment of intact cycling rats induces anestrus and significantly depletes central and peripheral levels of β-endorphin (Forman et al., 1985). Mueller et al. (1980) also showed that estradiol benzoate (EB) was able to reduce the basal concentration of β-endorphin both in the circulation and in the neurointermediate lobe of the pituitary gland. Lim and Funder (1984) reported a doubling of the amount of immunoreactive β-endorphin in the neurointermediate lobe of the pituitary gland following short-term ovariectomy. This increase is reversible by estradiol in a dose-dependant manner. Others have reported that estrogen can increase striatal levels of immunoreactive met-enkephalin (Dupont et al., 1980). While variations in the amount of both β-endorphin and met-enkephalin have been reported through the rat estrus cycle in brain and pituitary, (Hong et al., 1982a; Lee et al., 1980), there is one report of no change in hypothalamic β-
endorphin content through the cycle (Wardlaw et al., 1981). Evidence for a modulatory role of steroids on opioid systems was found, however, in the changes in β-endorphin content seen during pregnancy, (see Newnham et al., 1984; Wardlaw and Frantz, 1983) and following the implantation of estradiol bearing capsules into ovariectomized female rats (Wardlaw et al., 1981). Ferin's group has extensively studied steroid-induced changes in immunoreactive β-endorphin in the portal blood of primates. They feel that portal blood reflects β-endorphin activity in the hypothalamus, while systemic blood levels are a reflection of pituitary activity. (Ferin et al., 1984). Ovariectomy was accompanied by a disappearance of detectable β-endorphin in portal blood (Wehrenberg et al., 1982) which was reversible by the administration of gonadal steroids (Wardlaw et al., 1982a). Their studies of the menstrual cycle showed that during menstruation, when gonadal steroid levels were at their nadir, β-endorphin was undetectable in the portal blood. As the steroid levels increased, i.e. in the late follicular and the luteal phases, increasing amounts of β-endorphin were found in the portal circulation. While the highest concentrations were detected in the presence of progesterone, the
administration of this steroid alone to ovariectomized monkeys had no effect on \( \beta \)-endorphin, indicating a synergistic role for this steroid with estrogen (Wardlaw, 1982a; Wehrenberg et al., 1982). Note, however, that Casper and Alapin-Rubilovitz (1985) have reported that progestins alone can increase opioid activity in postmenopausal women. Finally, using the prolactin and LH responses to the narcotic antagonist naloxone as a probe, Shoupe et al. (1985) have shown that the activity of the endogenous opioid system is low in oophrectomized women (see also Casper and Alapin-Rubilovitz, 1985). The administration of conjugated estrogens raises the activity of this system, and it is still further enhanced by the inclusion of progesterone in the treatment regimen. Studies on rodents have also revealed that gonadal steroids are able to influence endogenous opiate systems in the CNS (Wardlaw et al., 1982b; Barden et al., 1981).

Bhanot and Wilkinson (1984) have shown that the inhibitory influence of endogenous opiates is dependant upon the presence of gonadal steroids. Forty-eight hours following ovariectomy, prepubertal rats showed significant increases in circulating levels of LH following administration of naloxone, an
opiate antagonist. However, 1 week after ovariectomy, nalofoxone was no longer able to induce an increase in circulating LH levels. Careful priming of the ovariectomized animals with EB resulted in a prompt reinstatement of the LH response to nalofoxone. The subsequent findings of Petraglia et al. (1984) confirm a role for steroids in the modulation of endogenous opiate systems. Using the adult rat as a model, they observed a time-dependent disappearance of the ability of endogenous opioid peptides to inhibit LH secretion following gonadectomy, in agreement with the findings of Bhanot and Wilkinson (1984) in prepubertal animals. In some cases, Petraglia et al (1984) were able to reinstate the LH response to nalofoxone with careful priming of the long-term animals with EB.

The observation that the presence or absence of gonadal steroids can so broadly affect an action of opioid agonists and antagonists would lead one to suspect that gonadal steroids might influence opiate receptors. The effects of both short term and long term exposure to gonadal steroids, as well as the effects of castration on opiate binding sites have been examined. In addition, some studies have
undertaken the examination of sex differences in opiate binding. These reports will be reviewed here.

**STEROIDS AND OPIATE RECEPTOR CHANGES**

Hahn and Fishman (1979) reported that 3 weeks after orchidectomy, there was an approximate doubling of the density of $[^{3} H]$-naltrexone binding sites in rat forebrain (i.e. whole brain minus cerebellum) membranes. The increase in receptor density was not accompanied by a change in $K_s$ of either the low affinity or the high affinity sites which the authors measured. Furthermore, 7 days of treatment with testosterone propionate was able to return the density of $[^{3} H]$-naltrexone binding sites to control levels.

Several investigators have unsuccessfully attempted to duplicate this work. Wilkinson et al (1981), using $[^{3} H]$-DHM ( ), $[^{3} H]$-DADLE (delta), or $[^{3} H]$-naloxone (NAL, a "universal" opiate receptor ligand), were unable to demonstrate any increase in opiate binding in either forebrain or hypothalamic membranes following castration in the male rat. Furthermore, steroid replacement with either testosterone or estradiol was without effect on $[^{3} H]$-
DHM, [³H]-DADLE, or [³H]-naloxone binding to forebrain membranes. The findings of Wilkinson et al. (1981) were echoed by Diez and Roberts in 1982. Both male and female mice, as well as young and old male rats were used in their experiments, and whole brain (minus cerebellum) and hypothalamus were examined. Again, castration had no effect on the binding of NAL or [³H]-met-enkephalin. Cicero et al. (1983) have undertaken a very careful replication of the original experiments of Hahn and Fishman. Once again, they too were unable to demonstrate any castration-induced change in either the number or the affinity of DHM, DADLE or [³H]-naltrexone binding sites in the male rat, in either whole brain (less cerebellum) or hypothalamus. In addition, careful analysis of their data reveals that castration is not able to induce any shift in the relative proportions of and delta binding sites in whole brain (less cerebellum).

The controversy has been further fueled by two recent reports. Piva et al., (1985) found that eight weeks following castration of adult male rats, there was a significant, and testosterone-reversible decrease in the $K_a$ of the DHM binding site in membranes prepared from whole brain. They were unable to detect any change in the $R_{max}$ of the binding. Hahn
and Fishman (1985) have successfully repeated their earlier observations of a castration-induced increase in the number of [3H]-naltrexone binding sites in membranes prepared from whole forebrain, and have extended these studies to include [3H]-naloxone. It is striking that Hahn and Fishman (1979; 1985) were able to show a castration effect on opiate receptors in whole brain homogenate, where both steroid and opiate receptors are diffusely distributed. Wilkinson et al. (1981), repeating their experiments as exactly as possible were unable to demonstrate such an effect on hypothalamic tissue where both steroid receptors and opiate receptors are concentrated. If the effect reported by Hahn and Fishman (1979; 1985) were not artifactual, it certainly makes sense that it would be present in hypothalamus. There are several factors which may explain the lack of corroboration of the original observations of Hahn and Fishman. Wilkinson et al. (1981) and Cicero et al. (1983) discuss the inherent variability of ligand binding studies, referring to their "notoriously fickle" nature and to unknown "subtle procedural differences" which may occur between laboratories. Indeed, Hahn and Fishman (1985) attribute the inability of other investigators to replicate their results to methodological factors.
They claim that when samples were filtered individually and rapidly, the castration-induced increases in opiate antagonist binding sites were present, whereas they were not apparent if the samples were filtered together in a standard filtration manifold. It is not clear how their samples were filtered in the original report (Hahn and Fishman, 1979). One aspect which at first seems readily apparent is the fact that the original binding assays of Hahn and Fishman were performed on unwashed membranes. The purpose of washing or preincubating membranes is to remove endogenous ligand from the tissue, and to dissociate as much endogenous ligand as possible from the receptors, thus making them available for detection in the binding assay (Burt, 1980). However, Hahn and Fishman (1985) report that washing the membranes was without effect on the castration-induced increase in opiate binding sites.

As described above, gonadal steroids do exert an influence on the amount of endogenous opioid peptides present in the central nervous system. It is not unreasonable, therefore, to expect that these changing amounts of endogenous opiates in and of themselves might cause differences in receptor
occupancy and in binding to the opiate sites. Hahn and Fishman themselves claim that a castration-induced reduction in endogenous ligand may explain the increase in \[^{3}H\]naltrexone binding. Petraglia et al. (1982) have shown, however, that there is no change in hypothalamic \(\beta\)-endorphin content following castration in the male rat. However, a reduction in endogenous ligand would be seen as an apparent increase in the affinity of the binding (i.e. a decrease in \(K_D\)) with no effect on the \(B_{\text{max}}\). Hahn and Fishman report just the opposite. In fact, when the experiments were repeated by Cicero et al. (1983) and Diez and Roberts (1982), there was still no difference between castrate and non-castrate animals regardless of whether the membranes had been preincubated or not. It thus seems unlikely that a castration-induced change in the amount of endogenous ligand can account for the changes seen by Hahn and Fishman, or for the fact that they were not observed by the others. Species and strain differences can similarly be ruled out, because all three investigators attempted to replicate the original experiments as closely as possible, and these factors were standardized. The major difference between the studies of Hahn and Fishman and the others is the
choice of radioligand for the binding studies. The original investigation was performed with $^3$H-naltrexone, a non-specific opiate receptor ligand which is not available commercially. The ligands in the other studies cited above included $^3$H-DHM (receptors), $^3$H-DADLE (delta receptors), $^3$H-met-enkephalin (delta receptors with some activity) and $^3$H-naloxone, (general). In only one case, that of Cicero et al. (1983) was $^3$H-naltrexone used. However, the observations of Hahn and Fishman have not been replicated. In addition, naloxone and naltrexone are thought to bind to the same classes of receptors. Thus, it seems unlikely that receptor specificity explained the observed differences.

Wilkinson et al. (1981) investigated the binding of $^3$H-DHM, and of $^3$H-DADLE to forebrain membranes prepared from animals treated in one of two ways known to induce an LH surge via short term exposure to estrogen: treatment of prepubertal female rats with pregnant mare serum gonadotrophins (PMSG) (Wilkinson et al., 1979a) and treatment of ovariectomized rats with estrogen/progesterone (Wilkinson et al., 1979b). Binding of these opiate receptor ligands to forebrain membranes was not modified by these manipulations. In order to maintain
circulating levels at near constant values, steroids are often administered in subcutaneous silastic capsules. (See for example Chazal et al., 1977). This technique has been exploited in examining the effects of prolonged exposure to the gonadal steroids on opiate receptors. Wilkinson et al. (1981) demonstrated that in rats ovariectomized 21 days prior to implantation, 72 hours of exposure to estradiol capsules was unable to modify opiate (DHM) binding sites in either whole forebrain or hypothalamic membranes. Furthermore, Diez and Roberts (1982) were unable to demonstrate any effect of a series of injections of steroid (100 ug/kg of TP or EB twice daily for 7 days) on NAL binding. In an attempt to maintain more persistent hormone levels, silastic implants containing TP or EB were used in intact male and female rats respectively. Again, no effect on NAL binding sites was apparent 8 days after implantation.

The reason for the inability of several groups to reproduce the data of Hahn and Fishman (1979; 1985) remains unresolved. This conclusion should not obscure the fact that in some circumstances gonadal steroids do affect opiate receptor populations, for example during chronic treatment with estradiol
(Wilkinson et al., 1983, Wilkinson et al., 1985). One week after the removal of an estradiol-containing silastic capsule which had been implanted for 3 months, hypothalamic membranes from ovariectomized rats had a significantly greater density of NAL binding sites than did control tissue (Wilkinson et al., 1983). This difference was not seen in membranes prepared from amygdala or cerebral cortex, and there was no change in the affinity of the binding site. A recent report by Wilkinson et al. (1985) has shown that the estrogen-induced increase in NAL binding sites occurs in (but may not be limited to) the anterior hypothalamus, an area essential for the generation of the midcycle LH surge (Kalra and Kalra, 1983). The effects of a single s.c. injection of 2 mg of estradiol valerate (EV) to intact female rats were also studied by Wilkinson et al. (1983). It was found that at a single point on the binding curve, membranes prepared from the hypothalami of the EV treated animals bound significantly more NAL than did control membranes. This difference was not seen in amygdala. In a similar study of female mice 4 months after an injection of EV (200 g), the density of NAL binding sites was significantly elevated in hypothalamic slices from the treated animals, while
the Kᵢ was unchanged (Jacobson et al., 1984). Chronic estrogen treatment has recently been demonstrated to induce a significant (45-50%) decrease in the hypothalamic content of immunoreactive β-endorphin (Forman et al., 1985). The increase in the number of opiate receptors seen in intact females following chronic treatment with estradiol is similar to the upregulation of receptors seen in other systems following denervation (Davies and Lefkowitz, 1981), and is therefore not incompatible with the proposition of Naftolin and Brawer (1978) that high doses of estrogen can induce a functional chemical deafferentation of the hypothalamus.

A critical direction for future research to take would be an examination of opiate receptor binding in discrete brain (and specifically hypothalamic) nuclei. Preliminary work by Bicknell et al. (1986), in which they have microdissected various regions of the rat hypothalamus, reveals a sex difference in [³H]-etorphine binding. Females possessed higher binding than did males. In addition, examination of their data suggests that in the castrate male, the stalk-median eminence binds more [³H]-etorphine compared to intact controls, a result which is compatible with the data of Hahn and Fishman (1979;
1985). All of this work clearly needs to be re-examined with the technique of quantitative autoradiography in order to specifically localize changes in binding to specific cell groups. Some progress has been made already with respect to sex differences in opiate binding. Iyengar and Rabii (1984), have examined the anatomic distribution of $[^3\text{H}]$-naloxone binding sites in both prepubertal and adult male and female rats. While they did not perform saturation experiments, and thus were unable to attribute differences in binding to changes in either the affinity or the density of the binding sites, their experiments did reveal several discrete differences in the amount of ligand bound. Their results, summarized in Table 2, clearly demonstrate sex differences as well as ontogenetic differences in the regional binding of $[^3\text{H}]$-naloxone in the brain. Hammer (1984) has also found that autoradiographically determined $[^3\text{H}]$-naloxone binding in the medial preoptic area (MPOA) is greater in the adult female (during diestrus) than in the adult male rat, particularly in the sexually dimorphic nucleus (SDN) of the MPOA. Differences in the binding of the opiate antagonist to the SDN throughout the estrous cycle were also apparent. Binding appeared to vary
inversely with the circulating gonadal steroid levels, and was greatest in diestrus, least during proestrus, and intermediate on the day of estrus. Hammer (1984) found no difference in binding to the suprachiasmatic nucleus either between sexes or within the estrous cycle, a result which is quite different to that obtained by Iyengar and Rabii (1984) who reported a 2-fold enrichment in adult female SCN. Note also that these same authors found a reversal of opiate binding of male/female MPOA compared to Hammer (1984).

A subsequent study (Hammer, 1985a), designed to examine the ontogenesis of opiate binding in the rat sexually dimorphic nucleus (SDN), clearly shows the appearance of labelling specifically in the female by day 3 after birth. Binding increased steadily to postnatal day 10. In contrast, male pups showed no such specific labelling of the SDN, nor did the labelling increase with age. By 10 days after birth, labelling was clearly less dense than in the female brain. Sex-depdendant morphological changes in the SDN are probably caused by gonadal steroids; e.g., perinatal treatment of female pups with androgens induces a morphology characteristic of the male brain (Jacobson et al., 1981). Thus, Hammer (1986) was able
to demonstrate that treatment of neonatal female rat pups with testosterone resulted in a male pattern of opiate binding, whereas castration of male pups increased opiate labelling to female levels. This author postulates that there may be some similarity between the low levels of opiate binding in estrogen-dominated (proestrus) adult rats (Hammer, 1984) and binding density in neonatal rats; i.e., aromatized testosterone (males) and ovarian estrogen (females) might serve to reduce opiate binding. This appears unlikely, since the pattern of increase of opiate binding is paralleled by elevations in estrogen secretion in neonatal female rats (Dohler and Wuttke, 1975). It seems more likely that the reversible variations in opiate binding in adult, cycling is due to estrogen-progesterone interactions whereas in the neonatal brain estradiol is stimulatory and testosterone inhibitory to the appearance of opiate binding sites. This is borne out by a preliminary report from Hammer (1985b) which describes blockade of receptor development or by the use of the estrogen-antagonist tamoxifen.

In summary, opiatergic systems are clearly influenced by gonadal steroids. Brain levels, as well as portal and peripheral blood levels of
immunoreactive β-endorphin have been shown to vary with the hormonal environment. The normal tonic inhibition of gonadotrophin release can be eliminated by gonadectomy and reestablished with careful steroid priming. Short term castration does not appear to affect the opiate receptor complement, but this is a contentious issue, and there is some evidence to support both sides of the story. It seems reasonably certain, however, that long term estrogen treatment is accompanied by changes in opiate binding to hypothalamic tissue. The development of autoradiographic techniques, which allow the examination of discrete sub-hypothalamic nuclei should help resolve some of the problems. In addition, this method has uncovered some interesting steroid-mediated changes in neonatal opiate binding.
APPENDIX III

WILLIAM JACOBSON
CURRICULUM VITAE

PUBLICATIONS


ABSTRACTS


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