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# Maternal Behavior in the Rat: Neuroendocrine and Neuroanatomical Substrates

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# MATERNAL BEHAVIOR IN THE RAT: NEUROENDOCRINE AND NEUROANATOMICAL SUBSTRATES

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But speedily an earnest longing rose
To brace myself to some determined aim,
Reading or thinking; either to lay up
New stores, or rescue from decay the old
By timely interference: and therewith
Came hopes still higher, that with outward life
I might endue some airy phantasies
That had been floating loose about for years,
And to such beings temperately deal forth
The many feelings that oppressed my heart.

Wm. Wordsworth
The Prelude
Bk. 1, Lines 114-123

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Finally, two people who made an indirect but important contribution to my thesis research deserve acknowledgement. When Bob Meisel came as a postdoc to the lab, he made it a much more fun place to be (as well as completing my conversion to a Mets fan by being as willing to talk baseball as to discuss the brain). My husband, Walter Robinson, has shared my goals and offered loving support and patience when needed over the past five years, and it is to him that this thesis is dedicated.

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ABSTRACT

MATERNAL BEHAVIOR IN THE RAT: NEUROENDOCRINE AND NEUROANATOMICAL SUBSTRATES

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A pathway connecting the preoptic region and the ventral midbrain regulates the performance of maternal behavior by female rats. There is also evidence that the facilitatory action of estrogen on maternal behavior is mediated via estrogen target cells in the medial preoptic region. Neither the anatomical elements that constitute this functional pathway nor the neuroendocrine events triggered by the estrogenic signal have been defined.

The first set of studies described in this thesis (Chapter 2) employed a method combining steroid hormone autoradiography and fluorescent dye retrograde tracing to demonstrate that numerous estradiol-concentrating neurons in the medial preoptic region of the rat brain (especially those in the lateral portion of the medial preoptic nucleus, in the medial preoptic area, and in the ventral and caudal bed nucleus of the stria terminalis) have projections to the ventral tegmental area and the midbrain central grey. These anatomical results are described in detail, and it is proposed that the facilitatory effect of estrogen on maternal behavior may involve alterations in function of preoptic estradiol-concentrating neurons that send their axons directly to the midbrain.

A second set of experiments investigated the possibility that estrogen's action on maternal behavior may involve interactions with brain oxytocin systems. It is demonstrated (Chapter 3) that 1. raised

central levels of oxytocin can induce short-latency maternal behavior in ovariectomized, estrogen-primed virgin rats 2. that this effect is estrogen-dependent 3. that the effect is sensitive to duration of test cage habituation 4. that treatment of 16-day pregnant, ovariectomized and hysterectomized estrogen-primed rats with antisera to oxytocin or an analog antagonist of oxytocin delays the short-latency onset of maternal behavior and 5. that injection of oxytocin directly into the ventral tegmental area produces short-latency maternal behavior similar to that elicited in preceding experiments by intracerebroventricular injection. The final chapter (Chapter 4) considers the role of the ventral tegmental area in the regulation of maternal behavior.

Preface

This thesis comprises two sets of studies. The topic of estrogenic facilitation of maternal behavior has been approached both anatomically (Chapter 2: studies of the connectivity of the estradiol-concentrating neurons of the medial preoptic area of the rat brain) and with behavioral techniques (Chapter 3: studies of the estrogen-dependent oxytocin facilitation of responsivity to pups). Taken together, the results from these two lines of research suggest that the facilitatory action of estrogen on maternal behavior involves 1. alterations in the function of preoptic area estradiol-concentrating neurons that send their axons directly to the midbrain and 2. interactions with the brain oxytocin systems. The final chapter (Chapter 4) will consider the possible significance of the ventral tegmental area, demonstrated in this thesis to be both a target of estrogen-concentrating preoptic area neurons and a site of action for oxytocin, in the regulation of goal-directed behaviors, including maternal behavior.

# Chapter 1 Introduction

## I. Estrogen Facilitation of Maternal Behavior in the Rat

### A. Background

In the rat, the onset of maternal behavior is correlated with the birth of the pups (slight prepartum increases can be seen in the frequency of performance of some behaviors, e.g., nest building, but not in others such as placentophagia: See Rosenblatt, 1965; Slotnick et al., 1973; Kristal, 1980). Major endocrine changes occur at the end of pregnancy, particularly in circulating levels of the steroid hormones progesterone and estradiol, and also in plasma titers of the anterior pituitary product, prolactin (Shaikh, 1971; Amenomori et al., 1970; Morishige et al., 1973). The final days of gestation in the rat are marked by a decline from the elevated progesterone levels characteristic of pregnancy, accompanied by a gradual increase in the level of circulating estrogens beginning around day 15 of pregnancy. This results in a sharp increase in the estrogen/progesterone ratio around the time of parturition (Soloff et al., 1979). A sharp rise in the level of circulating prolactin begins on day 21 (the rat gestation period is approximately 22-23 days). These dramatic endocrine changes are followed by the appearance of a novel source of sensory stimulation -- the pups themselves -- in the rat's environment.

A role for hormones in maternal behavior is suggested by the observation that only a small percentage of female rats not recently pregnant will behave maternally at first pup exposure (Wiesner and Sheard, 1933) In fact, nulliparous rats are typically unwilling even

to be in the same part of a cage as a rat pup (Fleming and Luebke, 1981). The difference between these naive rats and mother rats is not that the mothers have had the experience of giving birth. Rats whose pups are delivered by caesarean section at term exhibit normal onset of maternal behavior (Moltz et al., 1966; Mayer and Rosenblatt, 1980).

Two important strategies have been used to explore the relationship between altered hormone levels and facilitated responsiveness to young: they have led to the general conclusion that a rise in estrogen levels is strongly associated with a significant reduction in the latency to show maternal behavior. First, this conclusion is derived from many studies in which hormone regimens mimicking the endocrine changes of pregnancy are administered to virgin rats (for a review see Rosenblatt et al., 1979, or Fahrbach and Pfaff, 1982). The latency of such animals to respond to foster pups with maternal care is then compared with that of animals not receiving hormone treatment. All rats will eventually display maternal behavior if co-housed with pups: the average latency of this response is 5 - 7 days (Rosenblatt, 1967). Well-chosen steroid hormone treatments can reduce this latency to 1 - 3 days (Moltz et al., 1970; Zarrow et al., 1971). Estrogen is a necessary part of the successful regimens. A sequence of progesterone followed by its withdrawal concurrent with a rise in estradiol is the most effective treatment (Bridges, 1984), but progesterone withdrawal alone cannot stimulate the onset of maternal behavior (Rosenblatt et al., 1979). With high (supraphysiological) doses of estrogen, a short period of estrogen priming (24-48 hours) is sufficient, and progesterone priming is not needed. This is particularly true for hysterectomized rats (Siegel and Rosenblatt,

1975a, b).

A second source of evidence for the critical role of estrogen comes from experiments using a pregnancy-terminated model in which the endocrine changes occurring normally at the end of gestation are reproduced (reviewed by Rosenblatt et al., 1979) In these studies, rats are hysterectomized and ovariectomized during the final third of pregnancy. If a single injection of estradiol benzoate, a slowly-absorbed and hence long-acting form of estrogen, is given at the time of surgery, and pups are first presented 48 hours later, the response latency is reduced to approximately one day. As mentioned above, it has also been shown that shown that hysterectomy and ovariectomy combined with a single injection of estradiol benzoate can facilitate the performance of maternal behavior in virgin rats, although there are considerable differences in minimal effective estrogen doses between the two preparations (Siegel et al., 1978).

B. A Possible Brain Site of Estrogen Action, the Medial Preoptic Area

The pregnancy-terminated preparation has also been used in an attempt to localize the brain site of action of estrogen with regard to maternal behavior. Evidence supporting a critical role for the estrogen receptors in the medial preoptic area in regulating the onset of maternal behavior comes from studies involving the implantation of small amounts of crystalline estradiol benzoate directly into the brain (Numan et al., 1977). In ovariectomized and hysterectomized pregnant rats so treated, females receiving unilateral, undiluted estradiol benzoate implants into the medial preoptic area had shorter latencies to respond to pups than did animals with identical estrogen implants

into the hypothalamic ventromedial nucleus, the mammillary bodies, or under the skin of the neck. Animals with cholesterol implants into the medial preoptic area showed no such facilitation as was seen in the estrogen implant group. As will be discussed below, the medial preoptic area contains a high density of cells that exhibit nuclear binding of [$H^3$]-estradiol (Pfaff and Keiner, 1973). These findings suggest that the medial preoptic area is a major site of action in the estrogen facilitation of maternal behavior, although not necessarily the only site. This conclusion is qualified only by the fact that intracranial implants made using undiluted hormone are likely to produce considerable spread of the hormone away from the intended target site (Palka et al., 1966). No other studies testing this finding have been reported. Also, there are no parallel studies in which localized intracranial estrogen treatments have been shown to shorten the latency to respond to pups of nulliparous females.

On the other hand, an extremely strong case can be made that an intact medial preoptic area is essential to the performance of maternal behavior in the rat. Numan (1974) has shown that lesions of this region made five days postpartum severely disrupt maternal behavior. The lesioned mothers, whose preoperative behavior was completely normal, no longer built nests, retrieved, or crouched over pups in the nursing posture. Although such females do not show an aversion to pups -- they will occasionally approach, sniff, and lick young pups -- they give them no care. The performance of these lesioned mothers does not improve significantly with continued exposure to pups.

Medial preoptic area lesions also prevent the eventual onset of maternal behavior in virgin rats housed with foster pups (Numan et al.,

1977). Such lesions also abolish the appearance of maternal behavior in ovariectomized and hysterectomized pregnancy-terminated rats injected with adequate doses of estradiol (Numan and Callahan, 1980).

Control studies have shown that knife cuts of the stria terminalis or the medial corticohypothalamic tract, the prominent fibers of passage of this region, do not reproduce the medial preoptic area deficits (Numan, 1974). However, the severe disruption caused by medial preoptic area lesions is mimicked by bilateral knife cuts severing the connections between the medial preoptic area and the lateral preoptic area (and the rostral part of the anterior hypothalamic area from the lateral hypothalamus; Numan, 1974; Terkel et al., 1979; Numan and Callahan, 1980; Miceli et al., 1983). These findings suggest that the behavioral effects of medial preoptic area lesions are primarily the result of interruption of the lateral connections of the medial preoptic area, presumably of the connections with the medial forebrain bundle.

Studies using the tritiated amino acid autoradiographic tracing technique have shown that fibers from the medial preoptic area descend in the medial forebrain bundle (Conrad and Pfaff, 1976b; Swanson, 1976). Also, studies by Avar and Monos (1967, 1969) have shown that lesions in the region of the medial forebrain bundle (rostral lateral hypothalamic area) produce behavioral deficits similar to those seen after medial preoptic area lesions. Numan, Morrell, and Pfaff (in press), using a more refined HRP-labeling knife cut technique, have replicated this finding and provided important new information on the location of the fibers of importance and their cells of origin (see below). Taken together, these data suggest that further studies of the

brain-endocrine interactions regulating maternal behavior might reasonably choose the medial preoptic area as a starting point.

C. The Medial Preoptic Area--Background

Study of the medial preoptic area offers an excellent opportunity to investigate how a small brain region coordinates diverse functions. In addition to its role in the regulation of maternal behavior, an intact medial preoptic area is also essential for the timing of ovulation (Clemens et al., 1976), the performance of male sexual behavior (Malsbury and Pfaff, 1974), and thermoregulation (Hammel, 1968). This tissue is also importantly involved in the maintenance of water balance (Johnson and Buggy, 1978), autonomic regulation (reviewed by Mancia and Zanchetti, 1979), and in estrogenic effects on motor activity in female rats (Meisel and Fahrbach, unpublished data). The functional complexity of the preoptic area is matched by an anatomical complexity for which there has been only recently full appreciation.

The preoptic area can be recognized in all vertebrate brains, although the medial preoptic region as opposed to the periventricular preoptic region is only poorly developed in such forms as the cyclostomes, the teleost fish, and the amphibians (reviewed by Crosby and Showers, 1969). In all vertebrate species, the preoptic area lies at the level beginning immediately rostral to and then overlapping with the rostral end of the optic chiasm. It extends dorsally to the anterior commissure. Caudally, the preoptic area merges into the hypothalamus: the boundary between the two regions, medial and lateral, is notably indistinct, offering an anatomical representation of the high degree of functional interrelatedness between them.

The preoptic area of mammals has been frequently described, and the pattern seen in the rat (Gurdjian, 1927; Krieg, 1932; Swanson, 1976; Bleier et al., 1979) appears to have considerable generality. Like the hypothalamus, the mammalian preoptic area is divided (by both classical and contemporary authors) into three longitudinal cytoarchitectonic divisions: the lateral, medial, and periventricular zones. The periventricular zone consists of small cells found in close proximity to the optic recess/third ventricle, which often appear to be present in orderly rows parallel to the ependymal layer (Crosby and Woodburne, 1940; Bleier et al., 1979). The medial zone is densely cellular with no prominent longitudinal fiber systems. The lateral zone is dominated by the fiber systems of the medial forebrain bundle, but also contains many neuronal cell bodies. These cells are scattered, making "area" an accurate designation for the lateral zone ("lateral preoptic area"). By contrast, the so-called medial preoptic area actually consists of distinct nuclei embedded in an overall cell-dense "area". These nuclei have been described in detailed atlases based on Nissl-stained material (Gurdjian, 1927; Bleier et al., 1979), but by and large have been disregarded in experimental tract-tracing studies and ignored in conventional atlases (de Groot, 1959; König and Klippel, 1963; Paxinos and Watson, 1982).

D. Medial Preoptic Region Nomenclature

The entire region of the rat brain bounded rostrally by the lamina terminalis, caudally by the anterior hypothalamus, laterally by the medial forebrain bundle-associated lateral preoptic area, dorsally by the anterior commissure, and ventrally by the optic chiasm is

referred to as the "medial preoptic area," even though it contains several distinct nuclear groups. Most atlases only go so far as to distinguish a separate periventricular zone (e.g. Konig and Klippel, 1963; Swanson, 1976; Bleier et al., 1979; Paxinos and Watson, 1982). A recent examination of the subdivisions of the medial preoptic area has provided a useful framework for future anatomical study of this region, and with few exceptions the nomenclature of Simerly et al. (1984) will be employed here. The large, oval medial preoptic nucleus, which extends the length of the preoptic region, will be considered to be embedded in a less cellular medial preoptic area, which is bounded laterally by the lateral preoptic area and dorsally by the bed nucleus of the stria terminalis and the anterior commissure. The medial preoptic nucleus contains a small, tightly-packed cluster of neurons, which is larger in male rats than in female rats and has been previously labeled the "sexually dimorphic nucleus of the preoptic area" (Gorski et al., 1978, 1980). Here, in accordance with Simerly et al. (1984), this nucleus will be referred to as the central part of the medial preoptic nucleus. The medial preoptic nucleus can be further subdivided into a cell-dense medial part and a less cell-dense lateral part. As discussed by Simerly et al. (1984), the volume of all three subdivisions of medial preoptic nucleus is sexually dimorphic. When an inclusive term for the medial preoptic nucleus, medial preoptic area, median preoptic nucleus, and periventricular preoptic area is required, the term medial preoptic region will be used.

The other nomenclature to be used here is more or less standard, with Paxinos and Watson (1982) being used as the primary reference atlas. An issue unresolved in the literature, however, is the

appropriate subdivision of the rostral preoptic region around the optic recess and rostral third ventricle. This periventricular area, bounded dorsally by the nucleus of the diagonal band, is labeled by Kŏnig and Klippel (1963) "nucleus preopticus, pars suprachiasmatica." It contains numerous [$H^3$]-estradiol-concentrating neurons (Pfaff and Keiner, 1973; Stumpf et al., 1975) whose distribution extends across the nuclear boundaries provided by other investigators (e.g. Swanson, 1976; Bleier et al., 1982; Simerly et al. 1984). Since it is the capacity to concentrate gonadal steroids that is the feature of interest here, I will retain the term preoptic suprachiasmatic area as used by Pfaff and Keiner (1973), while recognizing that finer, architectonically-based distinctions are possible. This area appears to be equivalent to the "medial preoptic nucleus" in the nomenclature of Bleier et al. (1982).

In the preoptic region in general, the distribution of estrogen target neurons does not map readily onto accepted (and easily-discriminated) nuclear boundaries. For instance, the estradiol-concentrating cells of the medial preoptic nucleus and the medial preoptic area actually form a continuous group with those of the ventral bed nucleus of the stria terminalis, and estradiol-concentrating cells are often found scattered in the medial portion of the lateral preoptic area. This observation suggests that functional categories of neurons are as likely to be scattered within and across nuclei as they are to be concentrated in a compact group. Connectivity is another way of defining sets of neurons sharing a common function. The intersection of patterns of connectivity with the steroid-hormone concentrating capacity of preoptic region

neurons will be investigated in the anatomical studies described in Chapter 2 of this thesis.

E. Connectivity of the Medial Preoptic Region

Afferents. Information about afferents to the medial preoptic region has been obtained from anatomical and electrophysiological studies. Anatomical approaches have included degeneration-based techniques (at the light microscopic level: reviewed by Nauta and Haymaker, 1969; at the electron microscopic level: reviewed by Palkovits and Záborszky, 1979); Golgi methods (e.g., Millhouse, 1969); and autoradiographic methods using tritiated amino acids as anterograde tracers (e.g., Conrad and Pfaff, 1976b). Conclusions drawn from these studies have been summarized in detail by Palkovits and Záborszky (1979). More recently, the horseradish peroxidase retrograde tracing method has been used to locate neurons sending axons to the preoptic region (Day et al., 1980; Berk and Finkelstein, 1981; Kita and Oomura, 1982). These horseradish peroxidase studies have confirmed previous descriptions of afferent input to the medial preoptic region while adding considerable new information, particularly in regard to the source of ascending projections from midbrain and brainstem.

The most extensive published horseradish peroxidase study of preoptic region afferents is that of Berk and Finkelstein (1981). Although the authors refer to the retrogradely-labeled cells obtained after iontophoretic deposit of tracer as "medial preoptic area" afferents, it is clear from their charts that the size and placement of their injection sites do not permit distinctions among afferents to the medial preoptic nucleus, medial preoptic area, periventricular preoptic

area, median preoptic nucleus, and the preoptic suprachiasmatic nucleus. The term "medial preoptic region" is therefore appropriate here. Neurons projecting to the medial preoptic region were present in the lateral septum, the medial and central nuclei of the amygdala, and in the amygdalo-hippocampal area and ventral subiculum as well as in the dorsal and median raphe nuclei, the midbrain central grey, the peripeduncular nucleus, the lateral parabrachial nucleus, the locus coeruleus, the area adjacent to the medial longitudinal fasciculus, the nucleus of the solitary tract, and the lateral reticular nucleus. A study by Day et al. (1980) combining a histofluorescent technique with horseradish peroxidase retrograde tracing has further demonstrated that the noradrenergic innervation of the medial preoptic region is probably derived mainly from the A1 and A2 medullary cell groups.

These data emphasize the input to the medial preoptic region from the septum, the limbic system, and from midbrain and medullary serotoninergic and catecholaminergic cell groups. A similar distribution of horseradish peroxidase-labeled neurons was reported by Kita and Oomura (1982) after tracer deposit into a region comprising the medial preoptic cell groups and possibly including the ventral portion of the bed nucleus of the stria terminalis. Although the extensive spread of horseradish peroxidase around its application sites makes it difficult to determine local inputs with certainty (Fahrbach et al., 1984), these authors also present information on afferents from cell groups near the medial preoptic region. Both the nucleus accumbens and the bed nucleus of the stria terminalis appeared to project heavily to the medial preoptic region. The existence of such connections finds support in the anterograde tracing studies of Conrad

and Pfaff (1976a,b). Another set of local connections which cannot be confirmed solely by retrograde tracing studies is that between the medial and lateral parts of the preoptic region. There is, however, considerable evidence for such a connection, summarized by Palkovits and Záborszky (1979). Now that retrograde tracers more sensitive than horseradish peroxidase have become available (e.g., wheat germ agglutinin, fluorescent dyes), it is possible that further sets of afferents will be discovered, and that better estimates of the relative strengths of these connections will become available.

Electrophysiological studies have confirmed the existence of many of the direct pathways to the preoptic region revealed in neuroanatomical tracing studies (e.g. Hori et al., 1972; Carrer et al., 1978; reviewed by Renaud, 1979). Such studies have proved especially useful in the description of short hypothalamic-preoptic connections: for example, another important set of afferents to the medial preoptic area and the medial preoptic nucleus comes from the hypothalamic ventromedial nucleus, a brain region rich in estradiol-concentrating neurons (Renaud and Martin, 1975; Anschel et al., 1982).

Electrophysiological studies of the medial preoptic region have also added new information concerning multisynaptic pathways of functional importance. One such pathway appears to be that projecting from the midbrain central grey to the medial preoptic region (MacLeod and Mayer, 1980). Such diffusely-organized, multisynaptic pathways, almost certainly relaying signals from the reticular formation (Morrell and Pfaff, 1983b), presumably account for the medial preoptic responsivity to a variety of sensory cues, including thermal (i.e. skin

temperature: Wit and Wang, 1968; Knox et al., 1973; Boulant and Hardy, 1974) and auditory (Gordon and Heath, 1981) stimulation.

Another "sensory" projection, that from the medial amygdala, provides relatively direct transmission of olfactory information (Scalia and Winans, 1975) The median preoptic nucleus receives a direct neuronal projection from the pontine taste area (Norgren, 1976); it also seems likely that gustatory information is relayed to the preoptic region via the bed nucleus of the stria terminalis.

The medial preoptic region is also characterized by a collection of specialized, nonneural afferents. Autoradiographic studies have repeatedly identified neurons exhibiting nuclear binding of [$^{3}$H]-estradiol in the medial preoptic nucleus, medial preoptic area, and the adjacent periventricular zone (e.g., Pfaff and Keiner, 1973; Stumpf et al., 1975). Biochemical studies of the regional distribution of estrogen binding based upon gross dissection of the rat brain have shown that the highest concentration of estradiol receptors is found in the preoptic area and hypothalamus (McEwen et al., 1975). A recent radioligand binding study in which the Palkovits punch technique was used to determine the estrogen receptor content of individual rat brain nuclei provides more direct, quantitative confirmation of the autoradiographic studies (Rainbow et al., 1982a). The cytoplasmic estradiol receptor content (expressed in terms of fmol/mg protein) is higher in the medial preoptic nucleus and periventricular preoptic area than in any other brain region sampled. It has been further demonstrated that changes in preoptic region nuclear estrogen receptor levels parallel the changes occurring in plasma estradiol over the course of the estrous cycle, with maximal

levels detected at proestrus (McGinnis et al., 1981). Finally, although the highest levels of cytoplasmic estrogen receptors in either male or female rats are found in the periventricular preoptic area and the medial preoptic area (and the overall pattern of brain distribution of gonadal steroid receptors is qualitatively similar in both sexes), the medial preoptic region of male rats has been found to have fewer than half the levels of estrogen receptors found in the preoptic area of female rats (Rainbow et al., 1982b). Sex differences in regional estrogen receptor populations may have important functional correlates, although the relationships may be subtly determined: for example, it has been shown that the preoptic region of the male rat brain retains less [$^3$H]-estradiol sixty minutes after an intravenous infusion than does similar tissue from a female, but that after only thirty minutes the preoptic region of the male actually shows increased estrogen binding relative to the female (Nordeen and Yahr, 1983).

Autoradiographic studies have revealed extensive labeling of neurons in the medial preoptic region and periventricular preoptic area after injection of [$^3$H]-testosterone (Sar and Stumpf, 1973a). Since the rat brain contains both aromatase and 5$\alpha$-reductase capable of metabolizing testosterone to either estradiol or dihydrotestosterone (Naftolin et al., 1975; Selmanoff et al., 1977), the character of this labeling is unclear. Lieberburg and McEwen (1977) have shown that, two hours after administration of [$^3$H]-testosterone, estradiol is the major form of nuclear-associated radioactivity present in the preoptic area, but [$^3$H]-testosterone and [$^3$H]-dihydrotestosterone are also present. After direct infusion of [$^3$H]-dihydrotestosterone, the preoptic area has the greatest brain retention of radioactivity

(Lieberburg et al., 1977); in autoradiographic studies in which [$^3$H]-dihydrotestosterone is administered, many labeled neurons are present in the medial preoptic region (Sar and Stumpf, 1977). These findings support the existence of a preoptic population of androgen receptors (binding dihydrotestosterone and possibly its metabolites, androstenediol and androstenedione) distinct from the population of estrogen receptors.

Whether or not a population of testosterone receptors independent of these primarily dihydrotestosterone receptors also exists in the brain is uncertain. Preoptic labeling is not present in autoradiograms prepared from tissue of animals infused with testosterone which is tritium-labeled in the C-1$\alpha$ and C-2$\alpha$ positions, sites which would be cleaved by the action of estrogen-forming aromatase (Sheridan, 1979). This finding suggests that if such primarily testosterone receptors do exist in the central nervous system, they are not found in the region of interest here.

The existence of brain receptors specific for progestins has proved difficult to demonstrate autoradiographically using [$^3$H]-progesterone as the radioligand. An early study using estrogen-primed guinea pig brain failed to reveal the existence of receptors for progestins in the preoptic region outside of the preoptic suprachiasmatic area (Sar and Stumpf, 1973b). The synthetic progestin R-5020 has a higher affinity for the progestin receptor than the native hormone, and [$^3$H]-R-5020 has been used to demonstrate autoradiographically progestin receptors in the periventricular and medial preoptic areas in rats pretreated with estradiol (Warembourg, 1978). Binding studies have revealed that, while the non-estrogen

stimulated preoptic area contains a low basal level of binding sites, this brain region contains a greater concentration of estrogen-inducible cytoplasmic progestin receptors than does any other brain region sampled (MacLusky and McEwen, 1978; MacLusky and McEwen, 1980; Parsons et al., 1982).

Autoradiographic studies using [$^{3}$H]-corticosterone (Gerlach and McEwen, 1972; Warembourg, 1975a) and the synthetic gluococorticoid [$^{3}$H]-dexamethasone (Warembourg, 1975b) have not revealed the existence of any receptors specific for this class of steroid in the preoptic region. Biochemical studies of regional glucocorticoid binding in the rat brain have reached the same conclusion (reviewed by McEwen et al., 1979).

Many of the neurons of the preoptic region have an intrinsic thermosensitivity (Guieu and Hardy, 1971; Baldino and Geller, 1982). By virtue of its close anatomical relationship and shared vascular connections (Ambach and Palkovits, 1979) with the organum vasculosum of the lamina terminalis, the medial preoptic region is in an excellent position to sample not only circulating hormones but also the contents of the cerebrospinal fluid, since the highly vascularized organum vasculosum is characterized by physiologically permeable fenestrated capillaries (Weindl, 1983). The preoptic region also contains receptors for angiotensin II, a potent dipsogenic peptide (Sirett et al., 1977), and receptors for endogenous opioid peptides (Hammer, 1983).

Catecholamine varicosities are present throughout the preoptic region in "low to medium amount" (Parent, 1979, p. 533). The distribution of serotonin fiber immunoreactivity in the medial preoptic

region has been studied in detail by Simerly et al. (1984), who report that this monoaminergic input is sexually dimorphic.

Many peptidergic fibers are present in the medial preoptic region. Neurotensin, cholecystokinin, and substance P fibers densely innervate the medial preoptic nucleus and surrounding medial preoptic regions (Watson et al., 1983; Loren et al., 1978; Aronin et al., 1983). LHRH, VIP, somatostatin, vasopressin, oxytocin, bombesin-like, TRH, ACTH, and neuropeptide Y fibers are also present (Watson et al., 1983, 1984; Sar et al., 1978; Lechan and Jackson, 1982; Moody and Pert, 1979; Allen et al., 1984). Preoptic peptidergic neurons (identified by immunohistochemical procedures) in the rat contain neurotensin (Kahn et al., 1980; Hara et al., 1982; Watson et al., 1983), substance P (Aronin et al., 1983; Watson et al., 1983), cholecystokinin (Loren et al., 1979; Vanderhaeghen et al., 1980), LHRH (Witkin et al., 1982; Merchenthaler et al., 1984), and TRH (Lechan and Jackson, 1982).

Current knowledge of the preoptic region receptor and peptide populations is certainly incomplete: such a list serves as a reminder that the preoptic region is a brain area where sensory information, integrative limbic system input, and endocrine signals converge. In comparison with other brain sites, the potential for interaction of gonadal steroids with afferent neural signals is striking.

Efferents. Earlier reports that neurons of the medial preoptic region did not have significant extranuclear axonal projections (e.g., Valverde, 1965) were supplanted in the 1970's by autoradiographic studies of preoptic efferents based on anterograde transport of tritiated amino acid tracers (Conrad and Pfaff, 1976b; Swanson, 1976). This technique revealed the existence of ascending and descending long

to be no anterograde tracing studies which describe the specific connections of the preoptic suprachiasmatic area.

Available retrograde tracing studies using horseradish peroxidase confirm the preceding description (Philippson, 1979c; Ottersen, 1980; Morrell et al., 1981). Again, local connections have proved among the most difficult to describe accurately with neuroanatomical tracing techniques based on injected substances, but efferents to many hypothalamic nuclei, including the ventromedial nucleus, and short connections with the lateral preoptic area almost certainly exist (Palkovits and Záborszky, 1979). Unlike the long descending axonal projections which have a weak contralateral component, these local connections tend to be ipsilateral.

Electrophysiological evidence can be adduced to support the concept of a descending pathway from the medial preoptic region to the hypothalamic ventromedial nucleus (Renaud, 1979). Electrical recording methods have also demonstrated a direct influence of the medial preoptic region on neuronal activity in the midbrain central grey and the ventral tegmental area (MacLeod and Mayer, 1980; Maeda and Mogenson, 1980). Possible functions of these preoptic region-to-medial midbrain connections will be discussed below. Chapter 2 of this thesis describes studies in which the contribution of the estradiol-concentrating neurons of the medial preoptic region to this pathway is investigated.

## F. Functional Role for Preoptic-Midbrain Connections in Motivated Behavior: An Hypothesis

One explanation of why medial preoptic region lesions are

effective in disrupting maternal behavior is the idea that the cells in this region are the origin of an important descending pathway which relays integrated information concerning endocrine status and sensory stimuli experienced to brain regions more directly involved in the regulation of the muscle movements that result in changes in behavior. There is direct evidence that a neural pathway connecting the preoptic region and the ventral midbrain is important for maternal behavior. Bilateral substantia nigra lesions cause a severe but temporary disruption of maternal behavior in postpartum rats (Numan and Nagle, 1983). A knife cut lateral to the medial preoptic region paired with a contralateral substantia nigra lesion produced an equivalent disruption of maternal behavior. This suggests that both interventions were acting on a single system that must be damaged bilaterally in order to produce deficient responses to pups.

In contrast to the short-term effects of substantia nigra lesions, bilateral electrolytic lesions of the ventral tegmental area produced severe and lasting deficits in maternal behavior (Numan and Smith, 1984). Maternal behavior was also impaired in lactating rats receiving a unilateral medial preoptic region lateral knife cut and a contralateral ventral tegmental area lesion. Thus, the ventral tegmental area lesions reproduced the effect of medial preoptic lateral knife cuts or medial preoptic lesions on maternal behavior. The size of the lesion appeared to determine the degree of impairment: large ventral tegmental area lesions completely eliminated maternal behavior, while small lesions were especially detrimental to pup retrieval (carrying) and nest building.

That damage inflicted at the level of the ventral midbrain might

reproduce the effects of damage to the medial preoptic region is consistent with the known distribution of medial preoptic efferents (Conrad and Pfaff, 1976b; see preceding section). The relatively great importance of the medial forebrain bundle afferent pathway is indicated first by the finding that bilateral ventral tegmental area lesions produced a behavioral deficit comparable to that produced by bilateral medial preoptic lesions or lateral knife cuts. Second, it has been shown that the effects of cutting other preoptic region efferent pathways either does not interfere with maternal behavior performance (e.g., stria terminalis; Numan, 1974) or produces so many abnormalities (e.g., dorsal medial preoptic region connections; Numan and Callahan, 1981) that a disruption of maternal behavior is possibly a secondary effect.

These data, therefore, imply an especially critical role for the ventral tegmental area of the midbrain in the regulation of maternal behavior. This conclusion is only qualified by the lack of comparable studies assessing the impact on maternal behavior of lesions of other midbrain medial preoptic region targets. In particular, the contribution of the midbrain central grey and the tegmental region just dorsal to the medial lemniscus (see Conrad and Pfaff, 1976b) may be comparable to that of the ventral tegmental area.

The concept that preoptic area efferents to the ventral midbrain are important for maternal behavior receives functional as well as anatomical validation. Evidence has been adduced that the ventral tegmental area provides a "functional interface between the limbic system and the motor system" (Mogenson et al., 1980; p. 69). The fundamental problem of transforming motivation (produced by both

internal and external stimulus changes) into behavior requires that such "interfaces" exist in the brain since very few forebrain neurons are anatomically placed in such a way that they can influence motoneurons directly. In the case of steroid-hormone induced changes in maternal behavior, it is difficult to imagine an anatomical arrangement which would bypass such an interface, since extremely small numbers of spinal motoneurons contain autoradiographically-localizable estrogen receptors (Morrell et al., 1982), and preoptic region estrogen implants are themselves sufficient to promote maternal responsivity (Numan et al., 1977), even though preoptic region neurons do not project directly to the spinal cord (Schwanzel-Fukuda et al., 1983).

The efferent pathways from the ventral tegmental area (including the A10 dopaminergic cell group) ascend to terminate in regions related to somatomotor responses, particularly the nucleus accumbens, which in turn sends a massive input to globus pallidus and through this multisynaptic pathway eventually influences somatomotor cortical function.

An essential feature of the limbic/motor interface concept is that "motivational" signals (endocrine and/or sensory) received elsewhere in the brain are relayed to the ventral tegmental area, which in and of itself has no inherent goal-oriented properties. In the case of hormone-dependent maternal behavior, the relevant conjunction of endocrine and sensory motivational cues appears to occur in the medial preoptic region. The most direct means of transmitting the endocrine part of this signal to the midbrain would be for the preoptic region estradiol-concentrating neurons to send their axons to terminate in the ventral tegmental area. Our knowledge of the connectivity of these

brain regions suggests that this is a plausible account. However, alternate pathways, perhaps involving a relatively local synapse in the lateral preoptic/lateral hypothalamic area could just as easily account for the results described above, until there is actual proof that the preoptic region estradiol-concentrating neurons have extranuclear projections.

Determination of whether or not a direct pathway of this nature exists is of more than just hodological interest. It is likely that alteration in protein synthesis is a mechanism through which steroid hormones alter brain function and behavior. If any of these cellular products are neuroactive secretory proteins, demonstration of their site of action as well as their regulation by estrogen will be necessary.

Anatomical data can in turn provide new insight into functional relationships. If, for example, we could establish that only a restricted subset of the preoptic region targets were addressed by the estradiol-concentrating neurons, we could conclude that the preoptic-to-midbrain medial forebrain bundle pathway contained within it distinct channels for the estrogenic signal. Targets <u>not</u> receiving that particular signal directly might be predicted to be less involved in the estrogen-regulated medial preoptic region functions.

A technique that allows simultaneous demonstration of the steroid hormone-binding capacity and the axonal projections of individual neurons will be used to test the hypothesis that the anatomical substrate of the preoptic-ventral tegmental area interaction with regard to maternal behavior consists, at least in part, of axons from medial preoptic region estrogen target neurons. Since adjacent

brain regions (bed nucleus of the stria terminalis, periventricular preoptic area, lateral preoptic area) are also estrogen target tissues, I have also examined the nature of the descending pathway from these regions. Preoptic region connections to the ventral tegmental area will be compared with connections to a more dorsal midbrain region, the central ("periaqueductal") grey, another target of medial preoptic area efferents.

## II. Estrogen-Oxytocin Interactions in the Regulation of Maternal Behavior

### A. Background

The anatomical approach described above is a first step towards investigating the cellular mechanisms which mediate gonadal steroid regulation of maternal behavior. Another means of understanding the neural events following nuclear binding of steroid hormones is to take as a starting point endocrine or pharmacological treatments which affect the performance of the behavior of interest and, in effect, to use experiments involving hormone manipulations and behavioral observations to infer the nature of estrogen-regulated cellular products. In the brain, obvious candidates for steroid-hormone regulated gene products are neurotransmitters and neuropeptides, their receptors, and the enzyme systems related to their synthesis and degradation.

Until recently, research on the neuroendocrine regulation of the onset of maternal behavior dealt exclusively with the roles played by estrogen, progesterone, and prolactin. As stated above, however,

experimental manipulations of steroid hormone levels produce medium latency (1 - 3 days) maternal behavior and not a rapid onset of behavior like that characteristic of the parturient rat. Exposure to raised levels of estrogen can significantly reduce the latency to respond to cues provided by pups, but considerable sensory priming (contact with pups) is still required.

In 1979, Pedersen and Prange reported that intracerebroventricular (icv) infusions of oxytocin produced maternal behavior in virgin rats with a latency of less than an hour. The quick onset of this nurturing response indicated that raised central nervous system levels of oxytocin could substitute effectively for days of exposure to pups.

B. Oxytocin: Known Functions and Distribution in the Central Nervous System

In mammals, the major recognized functions of oxytocin released into the peripheral circulation from the posterior pituitary are reproductive: stimulation of uterine contractions at parturition (reviewed by Fuchs, 1983) and of milk ejection during suckling (reviewed by Bissett, 1974). The oxytocin secreted from the neurohypophysis is synthesized by neurons located in the paraventricular nucleus, supraoptic nucleus, and the accessory magnocellular groups of the hypothalamus. It is subsequently transported to its terminal release sites in the pituitary. That oxytocin-containing fibers are widely-distributed throughout the central nervous system, however, only became clear with the application of specific immunocytochemical techniques for localization of oxytocin and its associated neurophysin, although these results were anticipated

by Barry's (1954) earlier studies of Gomori-stained material.

The location of immunoreactive oxytocin nerve cell bodies and fibers has been extensively described (Sofroniew, 1980; Rhodes et al., 1981b; Buijs, 1982) and will be reviewed here only briefly. Oxytocinergic projections are found in the septum, amygdala, and olfactory bulb. Descending projections are seen to the dorsal and ventral midbrain, the dorsal parabrachial nucleus, the nucleus of the solitary tract, the dorsal motor nucleus of the vagus, and the lateral magnocellular reticular nucleus. Oxytocin fibers also innervate the spinal cord, particularly the intermediolateral columns (Swanson and McKellar, 1979), and the cerebellum (Nilaver et al., 1984) The cerebral cortex is notably free of oxytocin-containing fibers.

It is generally accepted that neurons located in the hypothalamic paraventricular nucleus are the cells of origin for these pathways: lesions of this region eliminate the immunoreactive fibers seen in the midbrain, brainstem, and spinal cord (Lang et al., 1983). However, with the exception of studies by Sawchenko and Swanson (1982) on the source of the spinal cord and brainstem innervation, there is no direct evidence from studies combining immunocytochemical and retrograde tracing techniques as to which paraventricular neurons send their axons to any particular location.

Oxytocin is also present in the cerebrospinal fluid (Dogterom et al., 1977). In this case, the source is unknown. Electrical stimulation of the paraventricular nucleus results in raised levels of cerebrospinal fluid oxytocin, suggesting that this nucleus is responsible for its release (Jones et al., 1983). Cerebrospinal fluid levels of oxytocin appear to be unrelated to plasma levels

(Robinson and Jones, 1982); the implication is that the release of peptide into the two physiological compartments is separately regulated.

It has been argued that this neuropeptide is a likely candidate for neurotransmitter status. It is present in synaptic vesicles in terminal varicosities (Buijs and Swaab, 1979); it is released by calcium-dependent secretory mechanisms (Buijs and van Heerikhuize, 1982); and some cells in the central nervous system have been demonstrated to exhibit reproducible changes in electrophysiological parameters in response to local application of oxytocin (Morris et al., 1980; Dreifuss et al., 1982).

The functions of oxytocin in the brain are not known. The distribution of oxytocin fibers in the brain stem and spinal cord suggests a role in autonomic regulation (Swanson and Sawchenko, 1980, 1983); behavioral studies have implicated oxytocin in certain memory processes (reviewed by de Wied, 1980); oxytocin and related neurohypophysial peptides stimulate grooming in mice and rats (Meisenberg, 1981; 1982). The results of Pedersen and colleagues suggested that oxytocin might have a role in reproduction by facilitating the performance of short-latency maternal behavior.

In the first studies of Pedersen and Prange (1979), both intact and ovariectomized, estrogen-primed virgin females were treated with intracerebroventricular (icv) oxytocin. To be rated as "fully maternal" animals had to perform all of the behaviors characteristic of the postpartum rat. Full maternal behavior within an hour of oxytocin treament was seen in 11/13 rats ovariectomized and injected subcutaneously with 100 µg estradiol benzoate/kg body weight 48 hours

before icv infusion and pup presentation. No animals injected with the oil vehicle and then given oxytocin exhibited maternal behavior during a two hour observation period; only 2/11 estrogen-primed animals given normal saline icv responded to pups with short latency. A less striking oxytocin effect was seen in intact females, but there was a relationship between oxytocin response and estrous state, with diestrus (low estrogen) animals being least likely to exhibit maternal behavior under these test conditions. Arginine-vasopressin was not effective in facilitating maternal behavior performance in any intact animals.

A subsequent report from Pedersen et al. (1982) used the ovariectomized, estrogen-primed virgin preparation to establish 1. a linear dose-response relationship over the range of 100-400 nanograms of oxytocin and 2. the specificity of the oxytocin facilitation. Out of 18 substances tested only oxytocin and tocinoic acid (the ring structure of the oxytocin molecule) were effective in stimulating short-latency maternal behavior. Arginine-vasopressin produced a significant incidence of maternal behavior, but with a longer latency (2 - 3 hours). The side chain structure of Pro-Leu-Gly-$NH_2$ was not effective at any time.

C. Interaction of Estrogen and Brain Oxytocin: Possible Role in Estrogen-Stimulated, Short-Latency Maternal Behavior

These reports are the first in which endocrine manipulations alone have been shown to turn virgin rats into short-latency pup responders. Induction studies cannot in themselves constitute evidence of a physiological role for a hormone in a particular behavior, but in light of the known peripheral actions of oxytocin (uterine contraction and

milk ejection), the idea of this peptide having a role in the rapid onset of maternal behavior in rats at parturition is attractive. I was particularly intrigued by the estrogen-dependence of the reported effect, since experimental examination of the nature of this dependence might provide insight into the cellular and biochemical mechanisms by which estrogen promotes maternal responsiveness.

Anatomical and functional linkages between estrogen and oxytocin systems have previously been described. High estrogen levels coupled with declining progesterone induce the synthesis of oxytocin receptors in the uterus and mammary glands (Soloff et al., 1979) This suggests that oxytocin target cells could be the site of estrogen action. Alternatively, cells producing, transporting, and releasing oxytocin could be one of the estrogen-sensitive points of this behavior system. Some estradiol-concentrating neurons in the rat hypothalamus also contain oxytocin: these neurons are located in a subdivision of the paraventricular nucleus known to have ample non-neural lobe projections (Rhodes et al., 1982). Other oxytocin cells which do not exhibit nuclear binding of estradiol have been demonstrated to alter their peptide content (as estimated by optical density measurements of immunocytochemical reaction product) in response to ovariectomy and estrogen treatment (Rhodes et al., 1981a). This sequential effect of estrogen and oxytocin on maternal behavior may reflect one of these patterns of overlap or may represent a yet undescribed interaction.

The experiments detailed in Chapter 3 will describe 1. results of investigations into the effects of oxytocin on the maternal responsivity of ovariectomized, estradiol benzoate-treated virgin rats 2. studies of the estrogen-dependence of this oxytocin effect 3. data

on the effect of varying the test cage habituation period on the response to oxytocin 4. experiments in which anti-oxytocic agents are used to delay the onset of maternal behavior in estrogen-primed rats and 5. studies attempting to define a tissue site of oxytocin action. Observations on other effects of intracerebroventricular infusions of oxytocin in rats (activity, grooming, body temperature) will also be presented. These studies represent an attempt to build a case for an estrogen-dependent role of brain oxytocin in the short-latency onset of maternal behavior that occurs at parturition.

# Chapter 2 Anatomical Studies

## I. Introduction

The broad goal of these studies is the demonstration of the existence of populations of estradiol-concentrating neurons that send their axons to specific distant targets. In many instances, distributions of steroid hormone-concentrating cells and the distribution of neurons afferent to a particular brain area have been shown to overlap (Morrell et al., 1984). However, the heterogeneous structure of brain tissue requires nothing less than a combined method with cell-by-cell spatial resolution to prove that steroid hormone-concentrating neurons and not only their near neighbors have the long projections of concern. The point of such research is not to uncover new connections -- although such studies will certainly confirm previous data and may add valuable quantitative information -- but is instead the discovery of the type(s) of neurons participating in a known functional pathway.

The evidence assembled above has led to the hypothesis that the pathway connecting the medial preoptic area to the ventral tegmental area (and more generally, to the midbrain) consists in part of axons from estradiol-concentrating neurons. Such a hypothesis does not exclude other targets for estradiol-concentrating neurons in the preoptic area, nor does it exclude the existence of other pathways which may transmit the same information (i.e. the presence of an estrogenic signal) over more synapses. However, specification of direct synaptic targets of estradiol-concentrating neurons should

facilitate the search for the mechanisms of estradiol action on neurons, whether it be reflected in new protein products, changed patterns of electrical activity, or morphological restructuring.

## II. Methods Background

### A. Studying Neural Circuitry with Retrogradely-Transported Fluorescent Dyes

The demonstration that macromolecules are retrogradely transported in axons (Lasek, 1967; Kristensson, et al., 1968; reviewed by Kristensson, 1978) stimulated the development of sensitive neuroanatomical tracing methods that have replaced classical techniques based on visualization of degeneration subsequent to injury. To identify cells of origin of a pathway, these methods employ exogenous chemical markers which are deposited in the extracellular space, taken up by axons and axon terminals, and retrogradely transported by the living neuron to the cell body. This allows precise localization of neurons afferent to the tracer application site. Tissue processing then consists of procedures which preserve the <u>in vivo</u> distribution of the marker during subsequent tissue handling and ultimately enable its visualization at the light or electron microscopic level. The principles and methodological niceties of such studies have been extensively reviewed (e.g. LaVail, 1978; Blackstad et al., 1981).

The enzymatic marker horseradish peroxidase (HRP) has been the most widely used substance for retrograde tracing of neuronal connections. The pinocytotic uptake, vesicular packaging, transport, and intracellular fate of HRP have been described (Mesulam, 1982), and

visualization techniques have been repeatedly modified in order to improve the sensitivity of HRP tracing (Warr et al., 1981). HRP histochemistry has proved compatible with a variety of other techniques used in the study of neural tissue (reviewed by Steward, 1981), but certain demands of the tissue processing (e.g., aldehyde fixation, pH of reaction solutions) render its combination with procedures which have different critical demands of their own less satisfactory.

Thus, other classes of tracers have been developed as alternatives to HRP. Desired features of new tracers have included greater sensitivity than HRP and greater ease of combination of retrograde tracing with other techniques. Kuypers and colleagues have identified a number of fluorescent substances which are retrogradely transported in axons (Kuypers et al., 1977; Bentivoglio et al., 1979; Bentivoglio et al., 1980; Kuypers, 1981). In the past few years these substances have been used in neuroanatomical tracing studies with increasing frequency. Advantages of using fluorescent dyes as retrograde tracers include their extreme sensitivity in revealing afferent connections (more sensitive than HRP: Sawchenko and Swanson, 1981) and the simplicity of their visualization, which facilitates multiple labeling of individual neurons (van der Kooy et al., 1978; Kuypers et al., 1980) and combination of retrograde tracing with other histochemical procedures (e.g. immunohistochemistry). Disadvantages of using these substances as tracers include their uptake by axons as well as axon terminals (as convincingly demonstrated by Sawchenko and Swanson, 1981, these small, lipophilic dye molecules can diffuse across neuronal membranes); their lack of permanence as a marker, as they fade upon exposure to light; and the lack of information on their intracellular

fate subsequent to internalization. Many of the fluorescent dyes are water soluble, and there is a possibility of their diffusion from retrogradely labeled cells during subsequent processing of tissue sections in aqueous solutions (Kuypers, 1981).

The basic technique is straightforward. Deposition of dye in the target area is followed by an empirically determined survival time appropriate for the particular dye being used and the system being studied. The tissue is fixed by transcardiac perfusion with a buffered paraformaldehyde fixative, cryoprotected by soaking in a sucrose solution, and frozen sections are cut, mounted, and examined with appropriate illumination using the excitation wavelength specific for that dye. The most popular dyes (True blue, DAPI, propidium iodide, primuline, Evans blue) have been used successfully in a variety of vertebrate nervous systems, and fluorescent dye tracing can be considered an established neuroanatomical technique.

B. Use of Steroid Hormone Autoradiography to Localize Target Cells in the Nervous System

Vertebrate brains are a target tissue for gonadal steroids, but not all neurons contain receptors for estrogens and androgens. Autoradiographic procedures can be used to locate the neurons that concentrate steroid hormones (Pfaff and Keiner, 1973; Stumpf and Sar, 1975; Morrell and Pfaff, 1981). When tritium-labeled steroids are used to label receptors, this method permits identification of individual hormone-concentrating cells, a degree of resolution essential in the study of a heterogeneous structure such as the nervous system. In general outline the procedure for steroid hormone autoradiography is

the same regardless of tissue origin or the substance being localized. Basically, exposure of the tissue to radiolabeled steroid hormone for a fixed time is followed by freezing of the tissue after any necessary tissue fixation and dissection. Cryostat sections are thaw-mounted in the dark directly onto emulsion-coated slides. The $\beta$-particles emitted from the tritiated hormone bound to its receptor oxidize the silver halide crystals of the emulsion; these are subsequently reduced during photodeveloping to render them visible. After adequate exposure under low-humidity, light-tight conditions and photodeveloping, the slides are prepared for analysis with the light microscope. It is assumed that the accumulated radioactivity visualized in the light microscope as a collection of reduced silver grains over the cell nucleus results from binding of the radiolabeled steroid hormone to an intracellular receptor protein. Unvarying requirements of this procedure are 1. prevention of diffusion of the bound, radiolabeled substance during tissue processing, which could result in unphysiological redistribution of the hormone under study 2. standardization of the method of tissue apposition to the emulsion in order to obtain reproducible and quantifiable patterns of grain distribution and 3. maintenance of adequate histological detail in order to take advantage of the cell-by-cell resolution of binding attainable with the use of tritium-labeled ligands.

The distribution of estradiol-concentrating neurons in the rat brain has been extensively described using autoradiographic techniques (Pfaff and Keiner, 1973; Morrell, Krieger, and Pfaff, 1985). These autoradiographic results have been repeatedly confirmed by biochemical studies of regional estrogen-binding in the rat brain (reviewed by

McEwen et al., 1982). In the rat, as in all other vertebrate species examined (Morrell and Pfaff, 1978), the vast majority of estrogen-concentrating cells are found in the medial preoptic area, tuberal hypothalamus (hypothalamic arcuate and ventromedial nuclei), limbic structures such as the septum and medial amygdala, and in the midbrain central grey deep to the tectum. As has been emphasized by Morrell and Pfaff (1978), other techniques have shown that all of the brain regions containing large numbers of estrogen-concentrating cells are involved in either neuroendocrine regulation of gonadotropin secretion or in the regulation of reproductive behavior. This overlap strongly implies that the steroid hormone receptors localized autoradiographically are indeed the relevant functional receptors.

C. Studying Axonal Projections of Estradiol-Concentrating Neurons using Fluorescent Dye Retrograde Tracing Combined with Steroid Hormone Autoradiography

The successful combination of fluorescent dye retrograde tracing with steroid hormone autoradiography was first used by Arnold (1980) to demonstrate patterns of connectivity of steroid hormone concentrating neurons in unfixed zebra finch brains. Using the fluorescent dye primuline and tritiated dihydrotestosterone (DHT), Arnold was able to demonstrate that, for example, some DHT-accumulating hypoglossal motoneurons project to the syringeal muscles, and that some DHT-concentrating neurons in the magnocellular nucleus of the anterior neostriatum send axons to the nucleus robustus of the caudal archistriatum. No quantitative data were presented.

Morrell and Pfaff (1982) subsequently adapted this technique for use in the rat central nervous system, using a variety of dyes in formalin-fixed tissue. Their demonstration that some estrogen-concentrating neurons in the ventrolateral subdivision of the ventromedial hypothalamic nucleus send axons to the dorsal midbrain indicated the feasibility of the studies to be described below as well as providing new information on the connectivity of the mediobasal hypothalamus. In fact, their article constitutes the only other published report of this particular combination technique.

## III. Methods

### A. Studying Medial Preoptic Region Projections to the Midbrain using Fluorescent Dye Retrograde Tracing Combined with Steroid Hormone Autoradiography

Experimental Animals. Virgin female Sprague-Dawley rats (Crl:CD(SD)BR) obtained from Charles River Breeding Laboratories were used in these studies. These animals were housed singly in our colony room at 22 $^{o}$C on a 12:12 light:dark cycle with lights off at 1200 hr. All animals were adapted to our facility for at least one week prior to surgery. Animals were approximately 60 days of age at the time of tracer injection (body weight range 150-200 grams). Young adult rats were used in order to minimize any potential confusion of fluorescing endogenous lipofuscins with the retrogradely-transported fluorescent dyes, since lipofuscins accumulate with age in the rat brain (Reichel et al., 1968). Rats to be given radiolabeled estradiol for steroid hormone autoradiography were obtained from Charles River

ovariectomized and adrenalectomized (3 weeks prior to isotope infusion and sacrifice) in order to eliminate competition for receptor sites by endogenous hormones. Those rats (N = 16) were supplied with a 0.9% saline, 2% sucrose solution to drink in addition to ad libitum laboratory chow and water. The completeness of the ovariectomy was verified at the time of sacrifice.

Additional rats (N = 30) of the same strain, age, and weight but not ovariectomized or adrenalectomized were used 1. to develop stereotaxic coordinates for tracer injection 2. to test varying tracer concentrations and survival times and 3. to obtain additional anatomical information. All animals were prepared for tracer injection surgery with i.p. injection of atropine sulfate (0.12 mg) to reduce bronchial secretion (Waynforth, 1980). General anesthesia was induced by i.p. injection of sodium pentobarbital (Nembutal; approximately 40 mg/kg body weight), with Xylocaine applied topically around the wound to ensure local anesthesia. At the completion of surgery, most animals were given a s.c. injection of a 10% solution of pentylenetetrazol (Metrazol; 0.3 cc, Sigma P-6500) in order to speed their recovery from barbiturate anesthesia. Shortly before sacrifice by transcardial perfusion with fixative, each animal was given an i.p. injection of an overdose of Nembutal (approximately 75 mg/kg body weight). Animals receiving very large ventral midbrain fluorescent dye injections sometimes appeared to be a little somnolent, but were otherwise in good health and able to eat, to drink, and to groom themselves.

Fluorescent Dyes. Preliminary retrograde tracing studies were

performed using many fluorescent dyes: Evans blue, DAPI, primuline, propidium iodide, bisbenzimide, True blue, Granular blue, and SITS. Selection of the dyes to be used in the combination studies was based on 1. sensitivity (relative number of retrogradely-labeled cells produced by similar size injections) 2. dye brightness in thin (12 µm and 6 µm) cryostat sections 3. lack of diffusion of retrogradely transported dye out of neurons into the surrounding neuropil and 4. mainly cytoplasmic labeling, in order to provide maximum contrast with the nuclear labeling produced by the autoradiographic procedure. All dyes but SITS were retrogradely transported from the midbrain to the preoptic area, but True blue, DAPI, and a combination of DAPI and primuline were judged to be relatively more sensitive and brighter than the others, with DAPI showing a greater resistance to fading than either True blue or primuline. These findings are by and large in agreement with the published literature.

True blue [trans-1,2-Bis(5-amido-2-benzofuranyl)ethylene-dihydrochloride] was obtained from the following source:

Dr. Illing KG
Warthweg 14-18
Postfach 1150
D-6114 Grosz
Umstadt, Germany.

A 10% w/v solution was used. The dye was diluted in distilled, deionized water. It appeared to form a suspension rather than a solution. Diluted True blue was stored under foil wrap at 4 $^{o}$C until immediately before use.

DAPI (4',6-Diaminino-2-phenylindole, supplied in the dihydrochloride hydrate form) was obtained from the Aldrich Chemical Co. (Milwaukee, WI.; Catalog No. 21,708-5) and was used diluted in distilled, deionized water in concentrations (w/v) of either 10% alone or 2.5% in combination with 10% primuline (ICN Pharmaceuticals). These mixtures were also stored under light-tight conditions at 4 $^{o}$C until immediately before use.

Radiolabeled Estradiol. [2,4,6,7,16,27-$^{3}$H(N)]-estradiol was obtained from New England Nuclear (specific activity, 151 Ci/mmol; radiochemical purity initially greater than 98%). This was supplied in aliquots of 1.0 mCi (0.0018 mg) in 1.0 ml of absolute ethanol and stored dark at 4 $^{o}$C until use. Animals received either a nuclear-saturating dose of 0.8 µg estradiol/250 g body weight (Morrell et al., 1982) or a lower dose of 100 µCi/100 g body weight. As no significant differences have been found between autoradiographic results obtained from rats receiving tritiated estradiol by intravenous infusion and rats receiving the same hormone dose injected intraperitoneally (Morrell et al., 1982), all animals in this study received this steroid via i.p. injection. In order to deliver the radiolabeled estradiol via this route with minimal irritation to the animal, the hormone-in-ethanol solution was brought up to twice the initial volume with sterile normal saline. This mixing was done in the 1.0 ml syringe used for the i.p. injection.

Stereotaxic Surgery. Fluorescent dyes were injected using standard stereotaxic procedures by means of a 1.0 µl Hamilton syringe. Coordinates were determined either from the rat brain atlas of deGroot (1959) or König and Klippel (1963). For the ventral tegmental area, from interaural zero the coordinates were A-P +2.4, D-V -2.7, and M-L ± 0.5; for the midbrain central grey, A-P +2.4, D-V -1.0, M-L ± 0.5. ventral tegmental area coordinates used with the head level were measured from bregma: A-P -5.3, D-V -8.4, M-L ± 0.7. An angled approach to the midbrain was also sometimes used to avoid the midbrain central grey. From interaural zero, these coordinates were A-P +2.2, D-V -7.7, and M-L ± 3.2, with the syringe holder fixed at a 15° angle lateral from a line perpendicular to interaural zero.

In order better to adapt these coordinate systems for the small animals used in these studies, a correction factor was employed in the A-P plane. The distance between bregma and interaural zero was determined. If the distance fell in the range of 5.8 - 6.2 mm, the correction factor was zero. Otherwise, the distance was divided by 3, and this number was subtracted from the initial A-P coordinate to determine the actual drill site.

The Hamilton syringe needle was lowered 10 minutes before the start of the dye injection; the injection was made slowly, over 5 minutes; 10 more minutes were allowed to elapse before the needle was raised. Injection volumes ranged from 0.1 µl to 0.5 µl. Both unilateral and bilateral placements were used. Post-infusion survival times were either 3 or 4 days.

Isotope Administration and Perfusion. After allowing 3 or 4 days

for retrograde dye transport, the animals were given half the total hormone dose; after 0.5 hr, the remaining half was injected and perfusion begun 2 hours later, known from previous studies to be a time when specific nuclear binding is high and nonspecific-low affinity binding is low (McEwen et al., 1975). After intracardiac injection of 0.2 ml of heparin, the animals were perfused with 10 mM phosphate-buffered saline (PBS; pH = 7.2) for 4 minutes, followed by 10 minutes of 4% paraformaldehyde in 10 mM PBS. All solutions were at room temperature. The brain was then immediately removed from the skull, soaked for 4 hours in the paraformaldehyde fixative at 4 $^{o}$C and then transferred for storage overnight (approximately 18 hours) into 15% sucrose in 10 mM PBS. The following day the brain was blocked into a rostral block containing the preoptic area and hypothalamus and a caudal block containing the midbrain injection site. The blocks were then frozen onto cryostat chucks in liquid freon stirred over liquid nitrogen and finally transferred into liquid nitrogen for storage until cryostat-sectioning. The brains of dye-injected animals which did not receive radiolabeled estradiol were prepared for sectioning using an identical perfusion and freezing procedure.

Preparation of Autoradiograms. The autoradiographic procedure for steroid hormone localization in brain tissue has been described (Pfaff and Keiner, 1973; Morrell and Pfaff, 1981, 1983a). Briefly, 12 and 6 μm sections were cut on a Bright cryostat (Hacker Instuments) at -28 $^{o}$C and thaw-mounted under safelight conditions onto slides which had been previously coated with Kodak NTB-3 emulsion and dried. A

tissue sampling strategy was used in which each section thaw-mounted onto an emulsion-coated slide was separated from the next section on that slide (3 sections/slide) by either 30, 24, or 18 μm. (The intervening tissue was either discarded or collected as described below for counterstaining.) This separation was chosen to eliminate the possibility of double-counting the same neuron, even if all sections on a slide were analyzed. The autoradiograms were stored in the dark under lead shielding at 4 °C in light-tight slide boxes containing dessicant. Exposure times ranged from 90 days to 1 year. After exposure, the autoradiograms were developed in Kodak D-19 Developer (2 minutes at 16 °C), rinsed in Kodak Liquid Hardener (1 minute at 18-21 °C), fixed in Kodak Fixer (2 rinses 10 minutes each at 18-21 °C), and rinsed in distilled water. The sections were then dehydrated briefly in a series of ethyl alcohols (2 minutes each in 70%, 95%, 100%, 100%, followed by 2 minutes in xylene) and coverslipped with Entellan (Merck). To minimize fading of the fluorescent dyes, developed autoradiograms were stored in the dark at 4 °C except when being viewed. During cryostat sectioning, sections adjacent to those mounted onto emulsion-coated slides were picked up on chrom alum-coated slides for subsequent counterstaining with cresyl violet or neutral red in order to facilitate anatomical localization of retrogradely labeled cells. Tissue obtained from animals treated with fluorescent dye only and not with tritiated estradiol was cryostat-sectioned (6, 12, or 24 μm) and thaw-mounted onto chrom alum-coated slides, cleared briefly in ethanol, and coverslipped with Entellan.

Controls. The brains of three animals receiving neither fluorescent dye nor isotope were processed in an identical fashion to that of animals receiving the combination treatment. Autoradiograms produced from this control tissue were developed with every batch of experimental slides and examined for evidence of positive or negative chemography as well as for endogenous fluorescence. Sections from several injection sites were also processed as autoradiograms in order to ascertain that the fluorescent dyes themselves had no chemographic effects. In this experiment, neither chemography nor endogenous fluorescence complicated interpretation of results. The distribution of estradiol-concentrating neurons was compared with that obtained from earlier studies in our laboratory using rapidly frozen, unfixed tissue. No differences were seen in the pattern of estradiol-concentrating cells, confirming earlier observations (Morrell and Pfaff, 1983a; Shivers et al., 1983a) that at least some perfusion-fixation procedures are compatible with steroid hormone autoradiography of rat brain tissue.

Data Collection and Analysis. The autoradiograms were examined through a Zeiss microscope with standard bright field light (12 V, 100W halogen tungsten lamp source) for the presence of silver grains and with ultraviolet light (HBO 50W mercury bulb light source, Zeiss UG1 excitation filter, 360 nm, standard epifluorescence system with Neofluar objectives) for the presence of retrogradely-transported fluorescent dyes. Criterion for an estradiol-labeled cell was the presence of reduced silver grains over the nucleus in numbers equal to or greater than five times the number of silver grains over an adjacent

cell-sized area of neuropil. This has previously been demonstrated to be a conservative criterion which should effectively eliminate the possibility of counting false positives (Morrell and Pfaff, 1983a). In actual practice, the use of sufficiently long exposure times (longer than four months in almost all cases) and radiolabeled estradiol of high specific activity rendered grain-counting almost unnecessary, as the autoradiographic signal-to-noise ratio was extremely high.

Criterion for dye labeling with True blue was the presence in the cytoplasm of a brilliant blue color; for DAPI, the presence in the cytoplasm and nucleolus of a soft blue color against a background of bright whitish-blue granules; and for the DAPI-primuline combination, soft blue color in the cytoplasm in addition to brilliant whitish-yellow spherical granules. In addition to labeling somal cytoplasm, these dyes often produced labeling of proximal neuronal processes. It was possible to illuminate a section simultaneously with both white light and ultraviolet light: with appropriate adjustment of the intensity of the white light, neurons with silver grains over the nucleus and dye in the somal cytoplasm (hereafter referred to as "double-labeled neurons") were easily identified with all three of the dyes used in this study. Neurons which only concentrated estradiol and neurons which only contained retrogradely-transported dye were also easily distinguished. Both the dye label and the autoradiographic signal were easily detected in 6 and 12 μm thick sections, although the dye appeared brighter in the thicker sections.

After initial viewing, sections were further analyzed by charting the location of double-labeled neurons onto anatomically accurate camera lucida drawings which indicated the position of all

estradiol-concentrating cells. All of this charting was done at a magnification of X1250. The camera lucida drawings were then mapped onto the counterstained adjacent sections in order to produce the final charts of the location of double-labeled neurons. Cell counts were performed on many sections across many brains in order to estimate the percentage of double-labeled neurons in a particular brain region. The tissue sampling strategy described above avoided the possible error of "double-counting" parts of the same neuron in adjacent sections.

In addition to charting, many of the sections were also photographed. Using either 200 or 400 ASA Kodak Ektachrome daylight film, color photomicrographs were made using double exposures in which the section was exposed in turn to the white light and ultraviolet light sources. This double exposure procedure permits optimal focussing on both features of interest, the silver grains and the cytoplasmic fluorescent dye; it is necessary because the silver grains are in the emulsion, below the plane of focus of cell. Black and white photomicrographs were made using Ilford XP1 400, a fine-grained, variable ASA film which was processed with Kodak C41 chemicals. Since the long exposure times necessary for photomicrography resulted in considerable fading of the fluorescent label, individual sections were typically either photographed or charted, but not both. Many sections through the fluorescent dye injection site were examined, and the location of the dye still visible in the midbrain was drawn onto standard templates prepared from the König and Klippel rat brain atlas.

Limits on Interpretation. The steroid hormone binding was studied with well-established autoradiographic technique which is believed to

maximize the number of labeled nuclei detected. The only factor which could reduce the number of steroid-concentrating nuclei (false negatives) seen in these sections is the thickness of the sections. Since the signal-producing $\beta$-particle from tritium travels on average only 2 $\mu$m (Rogers, 1979), sections thicker than 2 $\mu$m will not permit registration of all $\beta$-particles emitted by radiolabeled steroid bound within the tissue. The 6 $\mu$m section represents a compromise between the demands of dye visualization (brighter in thicker sections) and the demands of autoradiographic signal detection. Thus, the number of estradiol-concentrating neurons may be very slightly underestimated in these sections.

It also appears likely that any error in the number of dye-labeled cells counted is in the direction of underestimation. Although protective measures were taken at all stages of preparation of the section for viewing and during storage, dye fading could have occurred during tissue processing or especially during viewing, due to photobleaching (Giloh and Sedat, 1982). It should be noted that very little fading occurred during the autoradiographic exposure period (extremely dry and dark conditions), as sections exposed for 12 months were not discernibly less bright than sections exposed for 2 months, nor were there any differences in approximate numbers of fluorescent cells counted.

Two more factors which may affect the interpretation of these data are first, the variability inherent in the tracer application procedure and second, the uptake of these fluorescent dyes by fibers of passage as well as by axon terminals. While the pattern of the steroid hormone signal may be regarded as fixed across brains, the amount and

distribution of the retrogradely-transported dye label naturally varies with the precise placement of the injection site, the size of the injection site, and the tracer used. The factors regulating tracer uptake are not well understood, and an exact definition of the region of uptake may be impossible. Large dye injection volumes increase the number of retrogradely-labeled cells, and it is not always apparent if the cause of this is the larger area of brain tissue in contact with the dye or the greater amount of dye available for uptake and transport. Conversely, small injection volumes facilitate localization of the injection site, but greatly diminish the number of retrogradely-labeled cells seen. The strategy in this study has been to attempt to compare results obtained from cases featuring large sites with results from cases characterized by small, better-localized sites. The large sites presumably give some idea of the total numbers of cells which may be involved in a particular projection. Detection of a common pattern across cases in which similarly placed tracer injections of different sizes and different dyes will be considered strong experimental evidence for the existence of a demonstrated connection. However, the numbers obtained from the cell counts should be regarded only as imperfect <u>estimates</u> of the strength of the connection. Taking the second caution expressed above into account (uptake of dye by intact axons), it is evident that statements based solely upon these data must be limited to the format, "these neurons send their axons to <u>or</u> through a particular target region." Data from studies using other techniques (e.g., anterograde tracers, electrophysiological recording) may aid in the definition of terminal fields.

Nomenclature. The results will be presented in the format of labeled neurons identified in cell groups defined according to the nomenclature discussed in chapter 1. Most of the data presented here will be based on uncounterstained 6 and 12 µm sections. In such material many of the finer distinctions which can be made in thicker stained sections would be difficult if not impossible to discern. Data will be presented at a level of detail appropriate for the material at hand, with interpretation in light of the most up-to-date parcellation of the medial preoptic region reserved for the discussion section.

Procedure. The autoradiograms in this study were prepared in two batches. The first batch (N = 8) was prepared in October of 1982. These autoradiograms were developed and analyzed throughout 1983. These cases featured mainly relatively large volume (0.25 µl - 0.5 µl) dye injections into the ventral midbrain and employed True blue and DAPI combined with primuline as tracers. A second batch (N = 8) using True blue and DAPI as tracers was prepared in June 1983 and developed and analyzed during the first 9 months of 1984. These cases included smaller volume injection sites (0.1 - 0.2 µl) into dorsal and ventral midbrain, as well as some control placements including the substantia nigra and the region lateral to the midbrain central grey. Table 2 - 1 summarizes the cases analyzed in this study.

TABLE 2 - 1

| BRAIN | DYE | VOLUME[a] | DESCRIPTION OF INJECTION SITE | [$^3$H]-ESTRADIOL DOSE | AUTORADIOGRAPHIC EXPOSURE TIMES |
|---|---|---|---|---|---|
| 1-1 | 10% TB | 0.2 µl | unilateral, confined to VTA | 100 µCi/100 g b.w. | 115-320 days |
| 1-3 | 10% Pr 2.5% DAPI | 0.5 µl | unilateral, ventral and medial midbrain. Spread across midline | nuclear-saturating dose[b] | 112-315 days |
| 1-4 | 10% Pr 2.5% DAPI | 0.2 µl | unilateral VTA, through base of brain | 100 µCi/100 g b.w. | 170-285 days |
| 1-8 | 10% TB | 0.2 µl | bilateral, involving VTA and IPN | nuclear-saturating dose | 68-304 days |
| 1-10 | 10% Pr 2.5% DAPI | 0.2 µl | bilateral VTA | nuclear-saturating dose | 66-305 days |
| 1-11 | 10% Pr 2.5% DAPI | 0.5 µl | unilateral MCG and VTA | nuclear-saturating dose | 66-319 days |
| 2-1 | 10% DAPI | 0.25 µl | unilateral, large dorsal and ventral midbrain injection | nuclear-saturating dose | 101-304 days |
| 2-3 | 10% DAPI | 0.1 µl | bilateral, angled ventral midbrain injection | nuclear-saturating dose | 138-343 days |
| 2-4 | 10% DAPI | 0.1 µl | unilateral ventral midbrain and MCG. Spread to base of brain | nuclear-saturating dose | 132-337 days |
| 2-5 | 10% TB | 0.5 µl | unilateral MCG injection | nuclear-saturating dose | 137-342 days |
| 2-6 | 10% TB | 0.25 µl | bilateral VTA | nuclear-saturating dose | 104-342 days |
| 2-7 | 10% TB | 0.1 µl | bilateral, angled injection lateral to MCG | 100 µCi/100 g b.w. | 96-319 days |

a all fluorescent dyes diluted with distilled, deionized water
b 0.8 µg [$^3$H]-estradiol/250 g body weight

ABBREVIATIONS b.w., body weight; IPN, interpeduncular nucleus; MCG, midbrain central grey; Pr, primuline; TB, True blue; VTA, ventral tegmental area

# IV. Results

## A. Summary

Analysis of autoradiograms prepared from female rats receiving fluorescent dye injections into the ventral tegmental area, the midbrain central grey, or both revealed the existence of numerous estradiol-concentrating neurons in the preoptic area that have midbrain projections. After dye injection into the ventral midbrain, the highest numbers of double-labeled cells were identified in the medial preoptic nucleus and in the surrounding medial preoptic area. Fewer, but still substantial, numbers of double-labeled neurons were found in the ventral part of the bed nucleus of the stria terminalis (preoptic continuation). Small numbers of double-labeled neurons were identified in the dorsomedial portion of the lateral preoptic area. In fact, the region where the ventral bed nucleus of the stria terminalis, the dorsolateral medial preoptic area, and the dorsomedial lateral preoptic area meet in the caudal preoptic region was characterized in several cases by large numbers of dye-labeled, estradiol-concentrating neurons. Almost no estradiol-concentrating neurons in the periventricular preoptic area had midbrain connections, and relatively fewer anterior hypothalamic area than medial preoptic area estradiol-concentrating neurons were double-labeled. In most cases, only a very low percentage of estradiol-concentrating neurons in the preoptic suprachiasmatic area sent axons to the ventral midbrain, although such cells were consistently identified. This general pattern was also seen after midbrain central grey and combined ventral tegmental area/midbrain

central grey injections. In all cases, many dye-filled cells which did not concentrate estradiol and many estradiol-concentrating cells which were not retrogradely labeled with fluorescent dye were found. In fact, the majority of identified neurons in all regions examined were labeled by one method alone.

B. Anatomical Results - Individual Cases

The details of the distribution of double-labeled neurons in the preoptic region will be illustrated by the following representative cases.

Brain 1-1. Unilateral True Blue Injection into the Ventral Tegmental Area

Tracer Injection. This animal received a 0.2 µl unilateral injection of 10 % True Blue into the ventral tegmental area and an isotope injection of 100 µCi [$^{3}$H]-estradiol per 100 grams body weight. Although the latter constitutes slightly less than a nuclear-saturating dose of [$^{3}$H]-estradiol, the pattern and approximate number of estradiol-concentrating cells identified in this brain after adequate autoradiographic exposure time was identical to that seen in other cases in which the nuclear-saturating dose was given (see Table 2 - 1). It was also identical to that seen in autoradiograms prepared from unfixed, fresh-frozen tissue (e.g., Pfaff and Keiner, 1973; Morrell et al., 1985). Figure 2 - 1 represents the apparent center of the dye injection site (for a more extensive representation of this and other fluorescent dye injection sites, refer

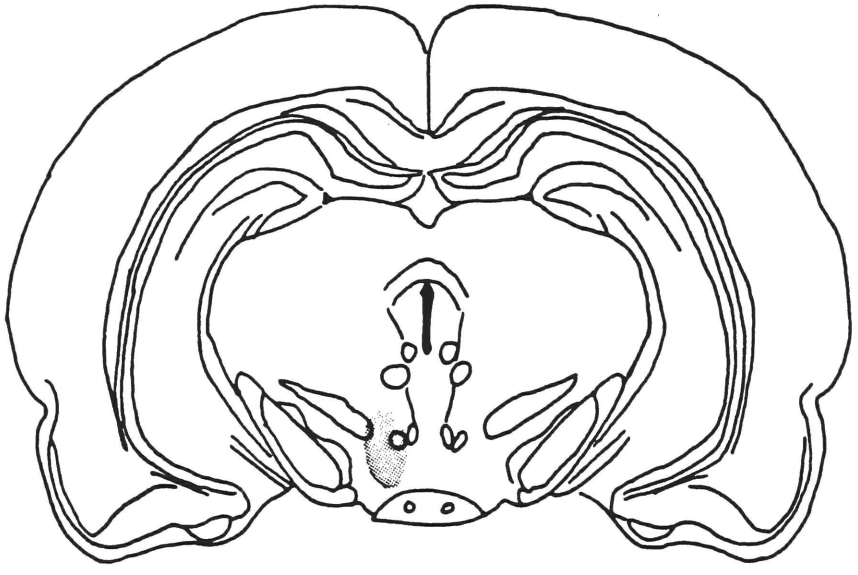


Figure 2 - 1. Center of True blue deposit site for Brain 1 - 1. Shaded area indicates dye location. 0.2 µl of 10% True blue was injected unilaterally into the rostral ventral tegmental area (level A 2420 µ; König and Klippel, 1963). Survival time after tracer injection, 3 days. For complete rostral-caudal extent of dye spread in the ventral midbrain, see Appendix 1.

to Appendix 1). This small site included the rostral ventral tegmental area (at the level of the posterior medial mammillary nucleus) and extended to the level of the rostral interpeduncular nucleus. It appeared to be lateral to the interfascicular nucleus, while including the paranigral subdivision of the ventral tegmental area. There was no dye spread into substantia nigra. Other fiber tracts and nuclei involved in the dye site were the fasciculus retroflexus, the mammillotegmental tract, the interstitial nucleus of Cajal, and the supramammillary region. With the exception of the strong retrograde labeling seen in the medial and lateral habenulae, undoubtedly a result of the position of the fasciulus retroflexus within the tracer injection site, it seems unlikely that incidental dye spread into these regions could account for the pattern of retrograde labeling seen in the preoptic region of this brain.

Fluorescent dye-labeled neurons. In order to establish the pattern of retrograde labeling produced by this injection site, the 24 available 12 μM thick autoradiogram sections each separated by 72 μM were carefully examined. This sampled tissue from the level of the diagonal bands through the anterior hypothalamic area. Approximate numbers of dye-labeled only neurons were estimated, while estradiol-concentrating cells and double-labeled neurons were later actually counted and charted for a sample of sections. The vast majority of the dye-labeled cells seen were ipsilateral to the injection site, although scattered contralateral dye-labeled neurons were present in a weak reflection of the ipsilateral pattern of labeling.

Moderate numbers of True blue-labeled neurons were found in the ventral subdivision of the vertical limb of the diagonal band, very near the base of the brain. In these unstained, thin sections it was difficult to distinguish the medial septum from the dorsal subdivision of the vertical limb of the diagonal band, but small numbers of brightly-labeled neurons were persistently present along the midline. At rostral septal levels, retrogradely-labeled neurons were seen in a distinct cluster in the dorsal septum, but very few dye-labeled neurons were seen throughout the entire extent of the lateral septum. Very few (approximately 10, total, for all sections examined) labeled cells were present in the caudal nucleus accumbens. At the level of the optic recess and more caudally, moderate numbers (approximately 25/section) of retrogradely-filled True blue neurons were seen in the horizontal limb of the diagonal band. Dorsal to this region, only a very few scattered dye-labeled cells were seen in the ventral pallidum.

Scattered, small True blue-labeled neurons were always present in the preoptic suprachiasmatic area. Almost no dye-labeled cells were present in the bed nucleus of the stria terminalis dorsal to the anterior commissure at rostral preoptic levels, but their numbers increased gradually in the caudal direction, with the greatest number being present in the most ventral portion of the preoptic continuation of the bed nucleus, stria terminalis.

Many retrogradely-labeled neurons (several hundred/section) were present in the lateral preoptic area and in the medial region, including both the medial preoptic nucleus and the surrounding medial preoptic area. Caudal to this, similar numbers of dye-labeled cells were present in the lateral hypothalamus, but the number of True

blue-filled cells present medially diminished caudal to the level of the suprachiasmatic nucleus. The only exception to this was seen in the ventral anterior hypothalamic region, where large numbers of dye-labeled cells were present in the crescent-shaped lateral anterior nucleus, just dorsal to the optic tract. The periventricular region appeared dark and devoid of fluorescent dye label throughout.

Double-Labeled Neurons. True-blue labeled, estradiol-concentrating neurons were present in the preoptic suprachiasmatic area, the medial preoptic nucleus, the medial preoptic area, the rostral anterior hypothalamic area, the dorsal lateral preoptic area, and the bed nucleus of the stria terminalis. No True Blue labeled, estradiol-concentrating neurons were seen in the diagonal bands, the periventricular preoptic area, or in the dorsal subdivisions of the medial preoptic region, including the anterodorsal preoptic nucleus and the parastrial nucleus. The percentages of estradiol-concentrating neurons containing fluorescent dye label are shown for each region in Table 2 - 2, which also shows the number of sections examined and the total number of estradiol-concentrating neurons counted in areas of interest ipsilateral to the tracer injection site. Examples of double-labeled neurons are shown in Figure 2 - 2 (a - e).

The highest percentage of double-labeled neurons (in a fully counted region; relatively few of the estradiol-concentrating neurons of the lateral preoptic area/lateral hypothalamus were identified and counted) was seen in the medial preoptic area (8.2%) as opposed to the medial preoptic nucleus (3.9%). This reflects a tendency for greater numbers of True blue-labeled neurons to be found away from the third

Figure 2 - 2. Photomicrographs of estradiol-concentrating, True blue-labeled neurons in the preoptic region of the rat brain. All photomicrographs were taken with a X100 oil Neofluar objective. Initial magnification on film: 250X. Final magnification with photographic enlargement: approximately 1000X. Fields (a)-(e) were photographed with both U.V. and standard white light. Fluorescent dye-labeling indicates that a neuron sends an axon to the medial midbrain.

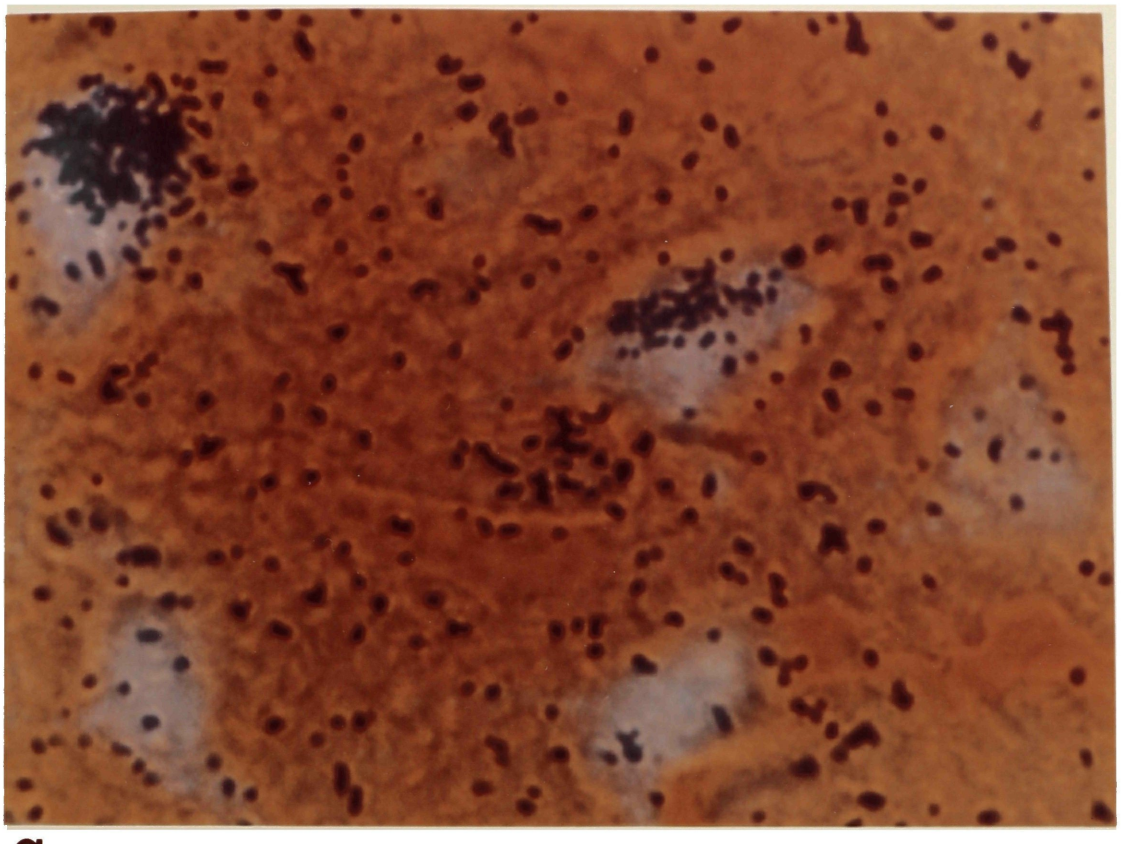
(a) Estradiol-concentrating, True blue-labeled neurons in the dorsal medial preoptic area adjacent to the ventral bed nucleus of the stria terminalis. Neurons only labeled with fluorescent dye and a neuron which bound [$^3$H]-estradiol but does not contain dye can also be seen. Brain 1-1.

(b) True blue-labeled neuron in the lateral medial preoptic area adjacent to cells that contain no label in either cytoplasm or nuclei and near neurons which only concentrate estradiol. Brain 2-6.

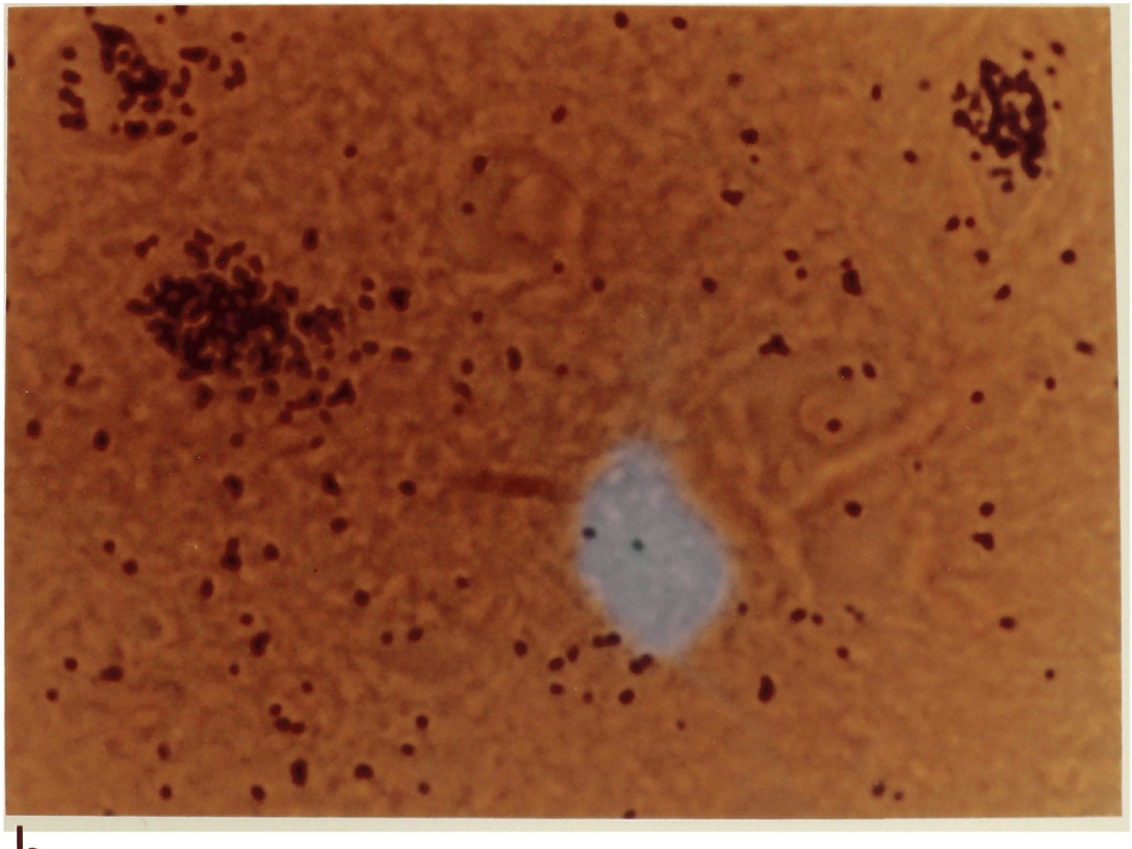
(c) Estradiol-concentrating, True blue-labeled neuron in the medial preoptic area. Arrow points to dye-filled process, a typical characteristic of retrogradely-labeled neurons. Brain 2-6.

(d) Estradiol-concentrating, True blue-labeled neuron in the medial preoptic nucleus. Adjacent to a neuron that exhibits nuclear binding of [$^3$H]-estradiol. Brain 2-6.

(e) Estradiol-concentrating, True blue labeled neuron in the rostral anterior hypothalamic area. Brain 2-6.



a



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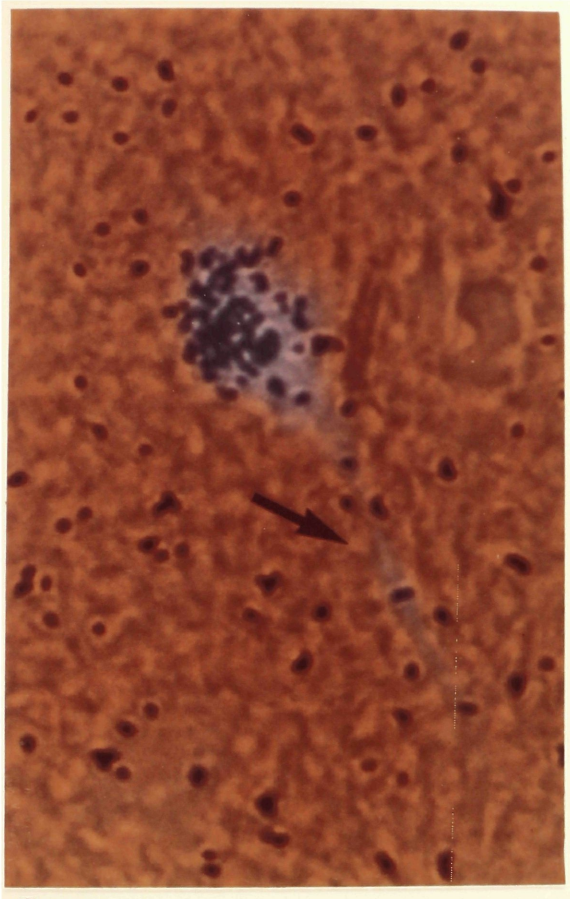
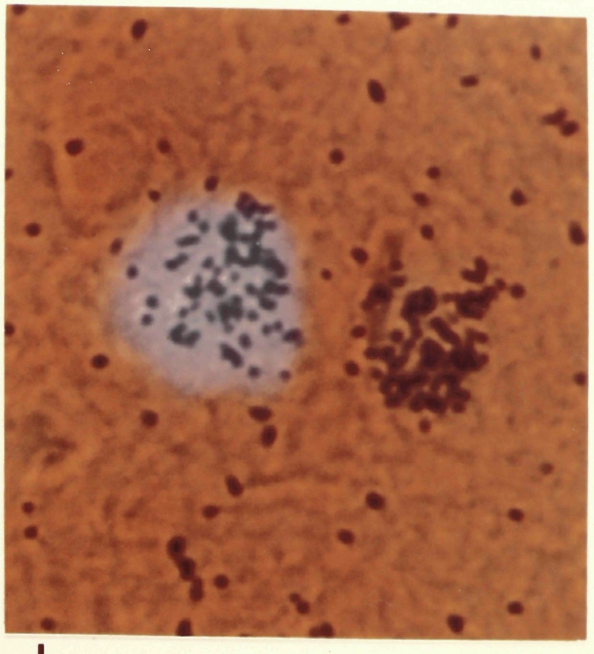
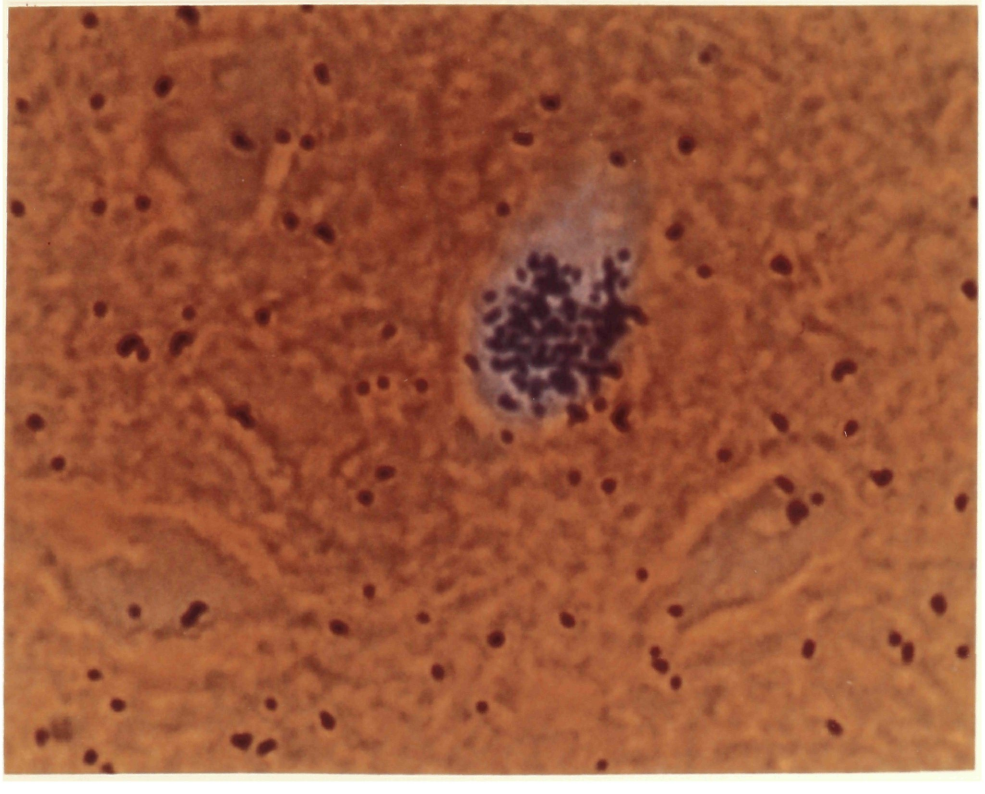


c



d



e

ventricle, while numerous estradiol-concentrating neurons are found in close proximity to the ventricle, especially in the more rostral sections examined. Many double-labeled neurons were found in the regions just lateral to, and just dorsal to, the medial preoptic nucleus. Smaller percentages of double-labeled neurons were found in the preoptic suprachiasmatic area (2.6%) and the bed nucleus of the stria terminalis (3.4%). Double-labeled neurons were scattered throughout the preoptic suprachiasmatic area, while such neurons had a strong tendency to be ventral and caudal in the bed nucleus of the stria terminalis, with the highest numbers present in the preoptic continuation of this bed nucleus. Figure 2 - 3 (a - f) shows the anatomical distribution of the double-labeled neurons, charted from representative single sections. These figures emphasize two important points which hold generally true for this material. First, when regions containing large numbers of estradiol-concentrating neurons are compared (such as the medial preoptic nucleus, medial preoptic area, and bed nucleus of the stria terminalis), the regional percentages of double-labeled neurons reflect the regional distribution of dye-labeled neurons: that is, areas with the greatest numbers of dye-labeled neurons can be correctly predicted to be the areas containing the greatest number of double-labeled neurons. Second, although it is possible for the purposes of data analysis to assign the double-labeled neurons present to individual nuclei and areas, in reality they exhibit a distribution which little respects boundaries assigned on the basis of patterns of Nissl-staining (see especially Figure 2 - 3, section d).

For Brain 1-1, counts of dye-labeled only neurons were available

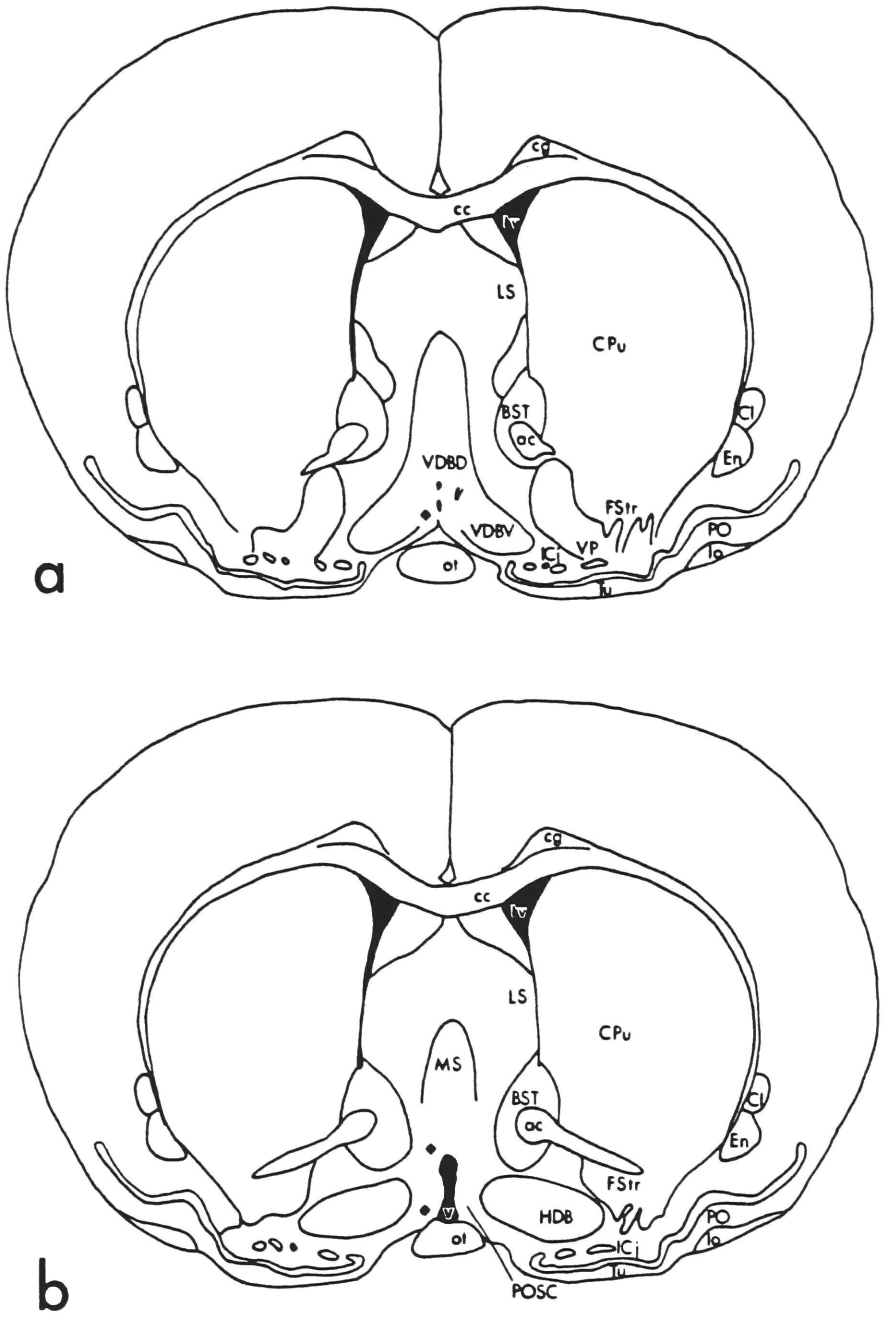
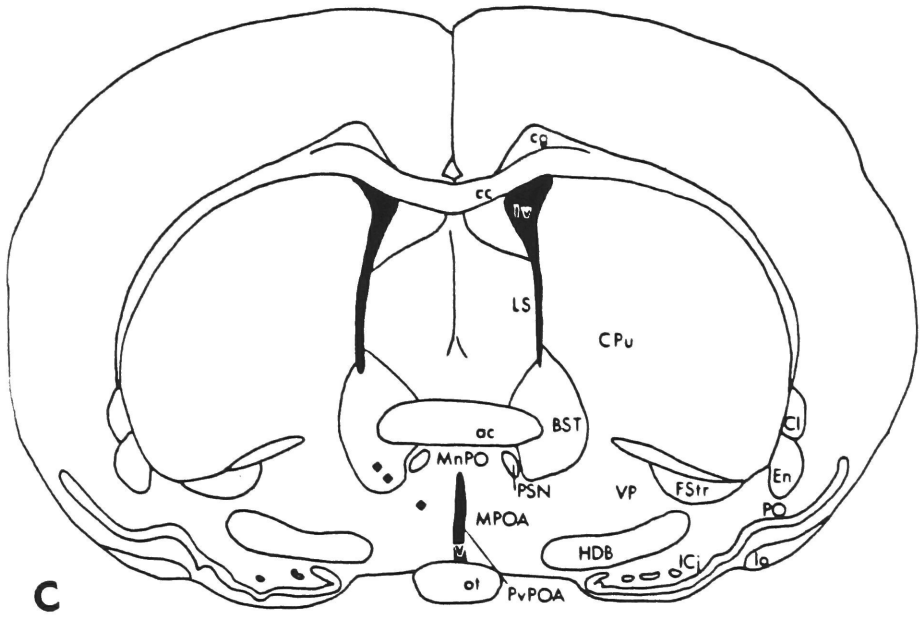



Figure 2 - 3. These charts (a - f) show the location of estradiol-concentrating, retrogradely-labeled neurons identified in the preoptic region of a rat receiving a pressure injection of 0.2 µl of True blue into the ventral midbrain. (See Figure 2 - 1 and Appendix 1 for tracer injection site.) These drawings are of actual, representative 6 and 12 µm sections. All double-labeled cells in a section are shown. One diamond (◆) equals one dye-labeled, estradiol-concentrating neuron. Brain 1 - 1.
(Abbreviations for this and subsequent figures are given at the end of Chapter 2.)



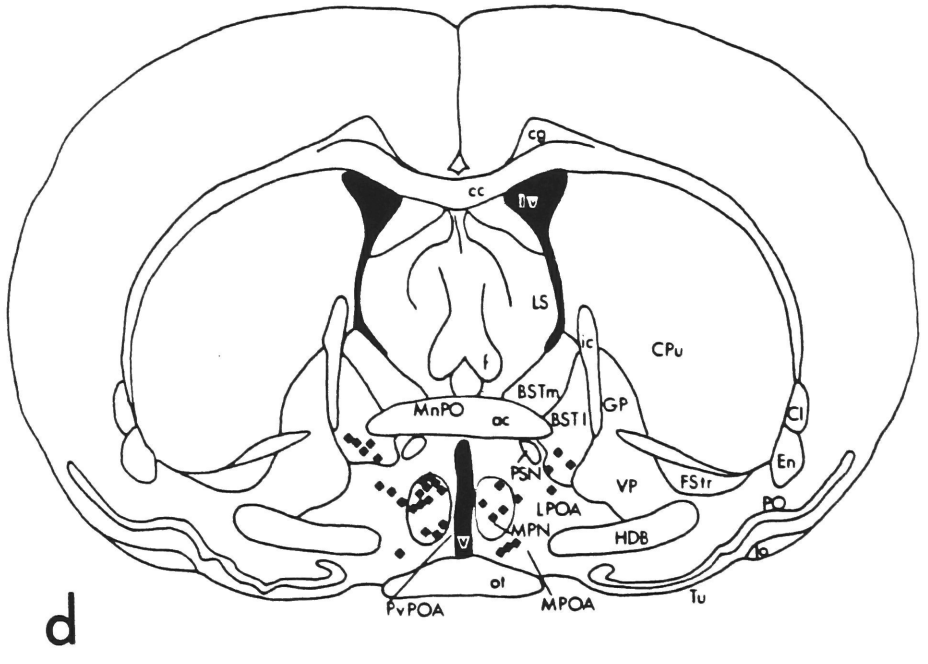
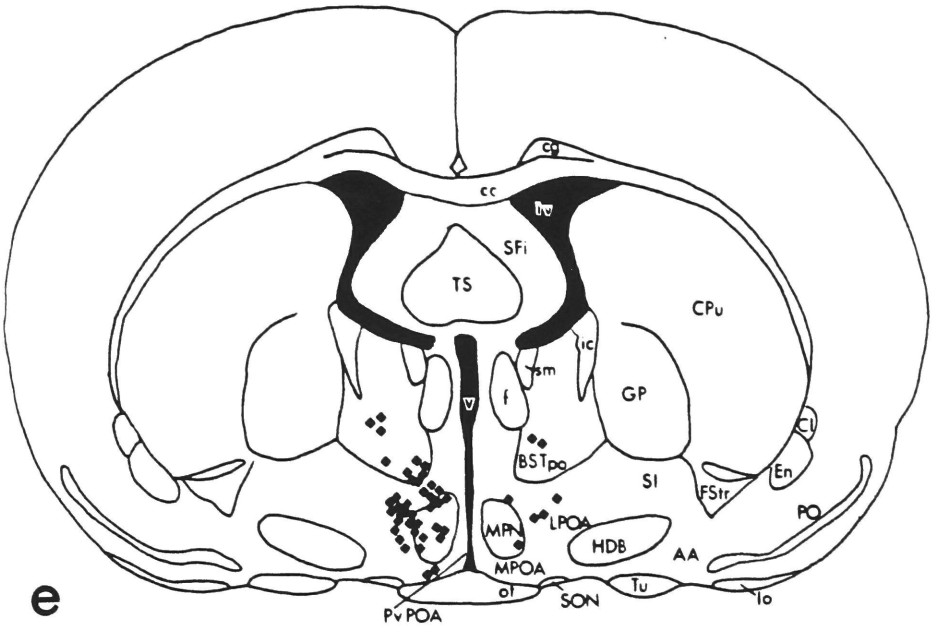




Figure 2 - 3. Continued. Brain 1 - 1.



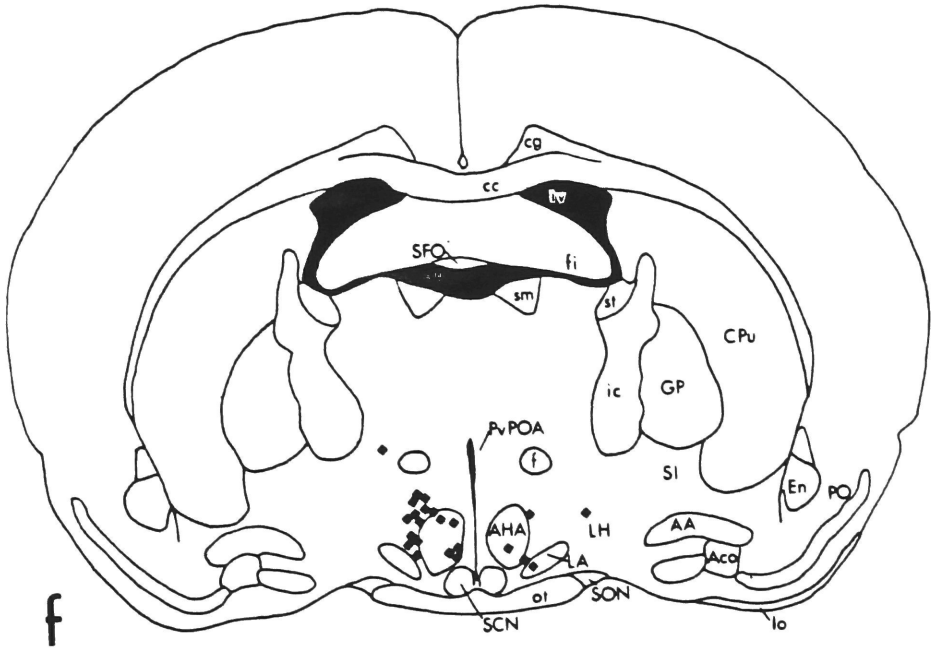




Figure 2 - 3. Continued. Brain 1 - 1.

TABLE 2 - 2 (BRAIN 1 - 1)

UNILATERAL 0.2 µl TRUE BLUE INJECTION INTO VTA

| AREA[a] | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: $E_2$ NEURONS (PER SECTION) | RANGE:DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL $E_2$ NEURONS | TOTAL DOUBLE-LABELED NEURONS | %$E_2$ NEURONS WITH DYE LABEL |
|---|---|---|---|---|---|---|---|
| POSC[b] | 10 | 90 µm | 25 - 102 | 0 - 9 | 609 | 16 | 2.6% |
| MPN | 6 | 60 µm | 168 - 280 | 5 - 12 | 1283 | 50 | 3.9% |
| MPOA | 7 | 72 µm | 27 - 168 | 1 - 20 | 674 | 55 | 8.2% |
| BNST | 7 | 72 µm | 48 - 198 | 2 - 9 | 936 | 32 | 3.4% |
| PvPOA[c] | 6 | 60 µm | 17 - 46 | 0 | 187 | 0 | 0 |
| LPOA/LH[d] | 5 | 54 µm | 0 - 22 | 0 - 6 | 60[e] | 10 | 16.7% |
| AHA[f] | 1 | 12 µm | - | - | 95 | 9 | 9.5% |

a ipsilateral to tracer injection site
b includes midline region
c caudal to the POSC
d boundary vague between LPOA and LH
e incomplete count due to scattered nature of $E_2$-concentrating neurons in this region
f includes lateral anterior nucleus. Rostral section may not be representative of more caudal sections through this region.

ABBREVIATIONS AHA, anterior hypothalamic area; BNST, bed nucleus of the stria terminalis; $E_2$, estradiol; LH, lateral hypothalamus; LPOA, lateral preoptic area; MPN, medial preoptic nucleus; MPOA, medial preoptic area; POSC, preoptic suprachiasmatic area; PvPOA, periventricular preoptic area; VTA, ventral tegmental area

TABLE 2 - 3 (BRAIN 1 - 1)

UNILATERAL 0.2 µl TRUE BLUE INJECTION INTO VTA

| AREA[a] | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: TRUE BLUE NEURONS (PER SECTION) | RANGE:DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL TRUE BLUE NEURONS | TOTAL DOUBLE-LABELED NEURONS | %DYE NEURONS CONCENTRATING ESTRADIOL |
|---|---|---|---|---|---|---|---|
| POSC[b] | 10 | 90 µm | 1 - 65 | 0 - 9 | 276 | 16 | 5.8% |
| MPN | 4 | 42 µm | 9 - 26 | 5 - 12 | 114 | 36 | 31.6% |
| MPOA | 5 | 54 µm | 9 - 87 | 1 - 20 | 342 | 48 | 14.0% |
| BNST | 5 | 54 µm | 4 - 31 | 2 - 9 | 108 | 23 | 21.3% |
| PvPOA[c] | 4 | 42 µm | 0 - 1 | 0 | 1 | 0 | 0 |

a ipsilateral to tracer injection site
b includes midline region
c caudal to the POSC

ABBREVIATIONS BNST, bed nucleus of the stria terminalis; MPN, medial preoptic nucleus; MPOA, medial preoptic area; POSC, preoptic suprachiasmatic area;PvPOA, periventricular preoptic area; VTA, ventral tegmental area

for a subset of the total sections counted. This information is shown in Table 2 - 3. The relatively high percentage of dye-labeled neurons which also concentrate estradiol can be compared to the relatively low percentage of estradiol-concentrating neurons that are also dye-labeled. This suggests the interpretation that, while most of the estradiol-concentrating cells in the preoptic region are not directly involved in the functional pathway to the ventral tegmental area, a significant number of the preoptic area neurons which send axons to the ventral midbrain can respond to estrogenic signals. This conclusion must, of course, be qualified by the lack of surety that all or even a majority of the preoptic region neurons with this connection are revealed in these retrograde tracing studies.

Brain 1-3. Unilateral DAPI/Pr Injection into the Ventral Tegmental Area and the Midbrain Central Grey

Tracer Injection. This animal received a 0.5 ul unilateral injection of 10% primuline/2.5% DAPI into the ventral midbrain and a nuclear-saturating dose of [$^{3}$H]-estradiol. The midbrain injection site is large and involves both the ventral tegmental area and ventral periaqueductal grey (see Figure 2 - 4), as well as the medial edge of the ipsilateral substantia nigra. Slight damage caused by the tip of the injection needle was localized just medial to the medial lemniscus, at the most rostral extent of the interpeduncular nucleus. Additional nuclei included in the injection zone (to a greater or lesser extent: see detailed drawings of the injection site in Appendix 1) are the interstitial nucleus of Cajal, the nucleus of

Darkschewitsch, the red nucleus, the interpeduncular nucleus, the rostral linear nucleus, and the central linear nucleus. Fiber tracts in the region of dye spread are the fasciculus retroflexus, the mammillotegmental tract, the medial longitudinal fasciculus, and the superior cerebellar peduncle. The pattern of retrograde labeling seen after this combined midbrain central grey/ventral tegmental area injection will be described in detail for comparison with the pattern seen after a more restricted injection into the ventral tegmental area (Brain 1-1, above). Autoradiographic localization of estradiol-concentrating cells in this brain was identical to that reported previously (Pfaff and Keiner, 1973), and to that seen in all other autoradiographic preparations in this study.

Fluorescent Dye-Labeled Neurons. This large midbrain injection site produced extensive retrograde labeling of preoptic area neurons. As described for Brain 1-1, a series of 24 12 μm sections were examined for the presence of somal fluorescent dye from the preoptic suprachiasmatic region to the rostral anterior hypothalamic area.

The pattern of DAPI-labeled cells seen in Brain 1-3 was in general outline quite similar to the pattern of True blue labeling observed in Brain 1-1, although the total number of retrogradely-labeled cells was much higher in Brain 1-3, and labeling was present in several additional regions. Many labeled cells were seen both ipsilateral and contralateral to the actual injection site, possibly reflecting the spread of tracer across the midline as well as actual bilateral projections.

As in Brain 1-1, many scattered, small, dye-labeled neurons were present in the preoptic suprachiasmatic area, from the level of the

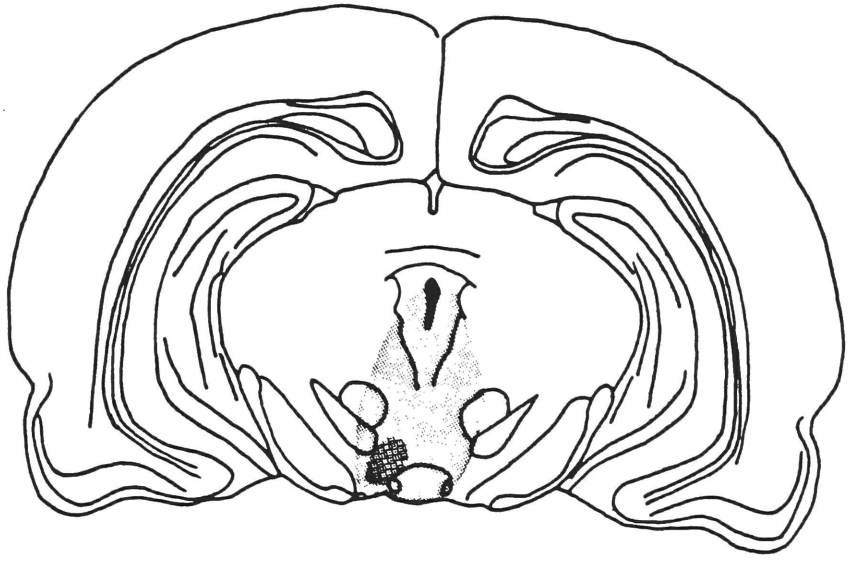


Figure 2 - 4. Center of DAPI/Primuline deposit site for Brain 1 - 3. Shaded area indicates dye location; double-hatched area shows position of tip of injection needle. 0.5 µl of 2.5% DAPI/10% Primuline was injected unilaterally into the medial midbrain (level A 1950 µ; König and Klippel, 1963). Survival time after tracer injection, 3 days. For complete rostral-caudal extent of dye spread in the medial midbrain, see Appendix 1.

optic recess to the level of the rostral medial preoptic area. Small numbers of dye-labeled cells were also present in the lateral septum, both limbs of the diagonal bands, and in the rostral bed nucleus of the stria terminalis (both dorsal and ventral to the anterior commissure). Scattered labeled cells were also present in the ventral pallidum, although the exact boundaries of this region were difficult to discern in this unstained tissue.

Massive numbers (hundreds/section) of retrogradely-labeled neurons were present in both the medial and lateral preoptic areas, with the lateral region exhibiting relatively greater numbers of fluorescing neurons. Large numbers of DAPI-labeled neurons were present at the caudal levels of the lateral preoptic area, and this lateral pattern of strong retrograde labeling continued into the lateral hypothalamus. Hundreds of dye-labeled neurons were seen in the medial preoptic nucleus and the surrounding medial preoptic area. More caudally, in the anterior hypothalamic area, fewer dye-labeled cells were present, although the lateral anterior nucleus was again extremely well-labeled. Dye-labeled neurons were extremely rare in the periventricular preoptic region, although small numbers (approximately 10/section) were consistently seen in the median preoptic nucleus, dorsal to the third ventricle.

Relative to the numbers of dye-labeled neurons in the rostral subdivisions of the bed nucleus of the stria terminalis, numbers of labeled cells were increased in the preoptic continuation of this bed nucleus. A few scattered dye-labeled cells were present in the septal triangular nucleus.

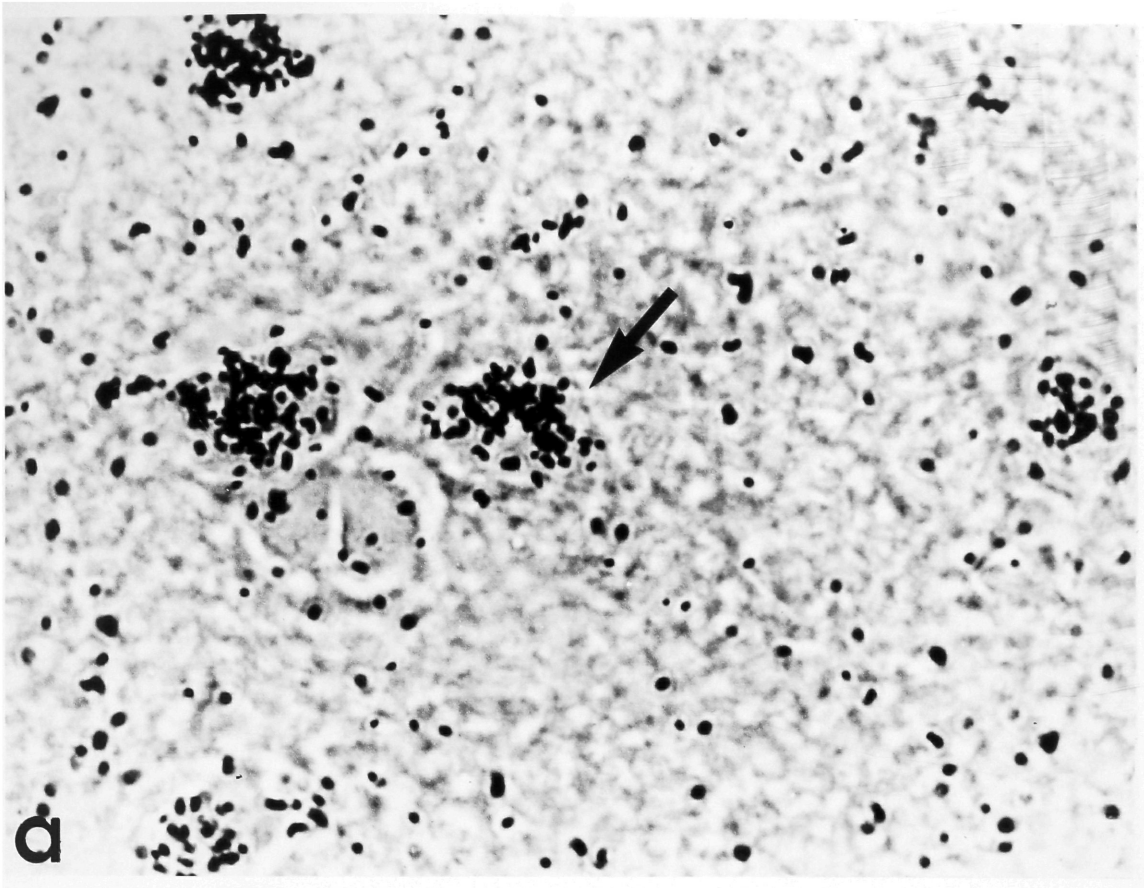
The pattern of labeling described here is characteristic of that

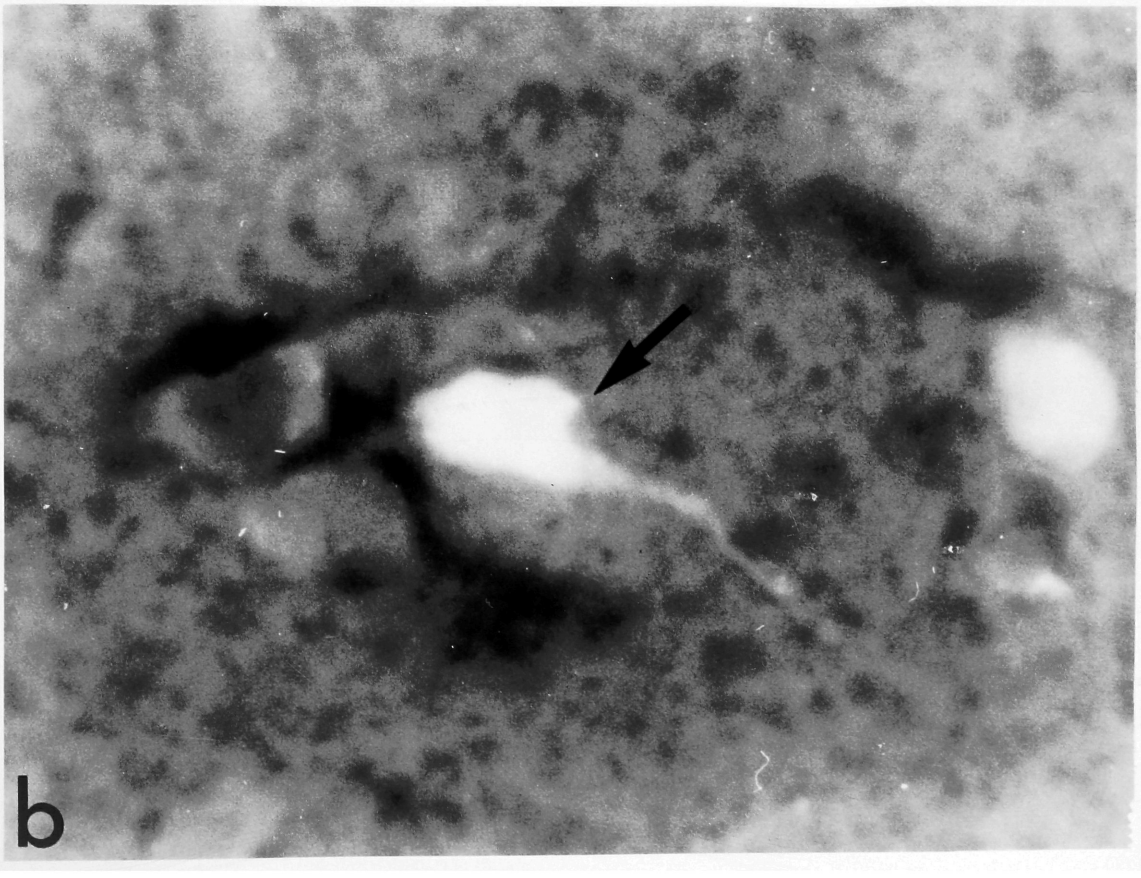
seen after medial midbrain injections of retrograde tracers. In addition, in Brain 1-3 a consistent pattern of dye labeling was present in the caudate nucleus. These DAPI-labeled neurons were restricted to the medial caudate nucleus, to a narrow band of tissue just lateral to the ipsilateral lateral ventricle. As will be discussed below, this retrograde labeling is almost certainly the result of spread of the fluorescent dye from the ventral tegmental area injection site into the medial edge of the rostral pole of the substantia nigra.

Double-Labeled Neurons. In confirmation of the pattern observed in Brain 1-1, DAPI-labeled, estradiol-concentrating neurons were present in the preoptic suprachiasmatic area, the medial preoptic nucleus, the medial preoptic area, the bed nucleus of the stria terminalis, the lateral preoptic area, and the anterior hypothalamic area. Almost no DAPI-labeled, estradiol-concentrating neurons were present in the periventricular preoptic area. Photomicrographs of double-labeled neurons are shown in Figure 2 - 5.

Figure 2 - 6 presents in detail the actual distribution of double-labeled neurons in a representative section through the preoptic suprachiasmatic region at the level of the optic recess, as well as the locations of neurons which only concentrate estradiol and of neurons which only contain retrogradely-transported DAPI. It is apparent that DAPI-labeled, estradiol-concentrating neurons are found scattered throughout this region in significant numbers, with a tendency to be concentrated near and around the midline. An important point illustrated in this figure is that there is no segregation of these cells by type: that is, cells which both concentrate estradiol

Figure 2-5. Photomicrographs of estradiol-concentrating DAPI-labeled neurons in the preoptic area of the rat brain. Photomicrographs were taken with a X100 oil Neofluar objective. Initial magnification on film: 250X. Final magnification with photographic enlargement: approximately 1000X. Fields (a) and (b) are the same. Field (a) was photographed with standard white light to show accumulations of silver grains representing [$^3$H]-estradiol concentrated in the nuclei of cells in the dorsolateral medial preoptic area. Field (b) was photographed with U.V. illumination to reveal location of fluorescent dye. Arrow points to same cell in (a) and (b), which is an estradiol-concentrating cell with a projection to the medial midbrain. At the far right of the field, a second double-labeled neuron is also visible. To the left of the cell identified by the arrow two neurons which concentrated [$^3$H]-estradiol but do not contain dye can be seen.


a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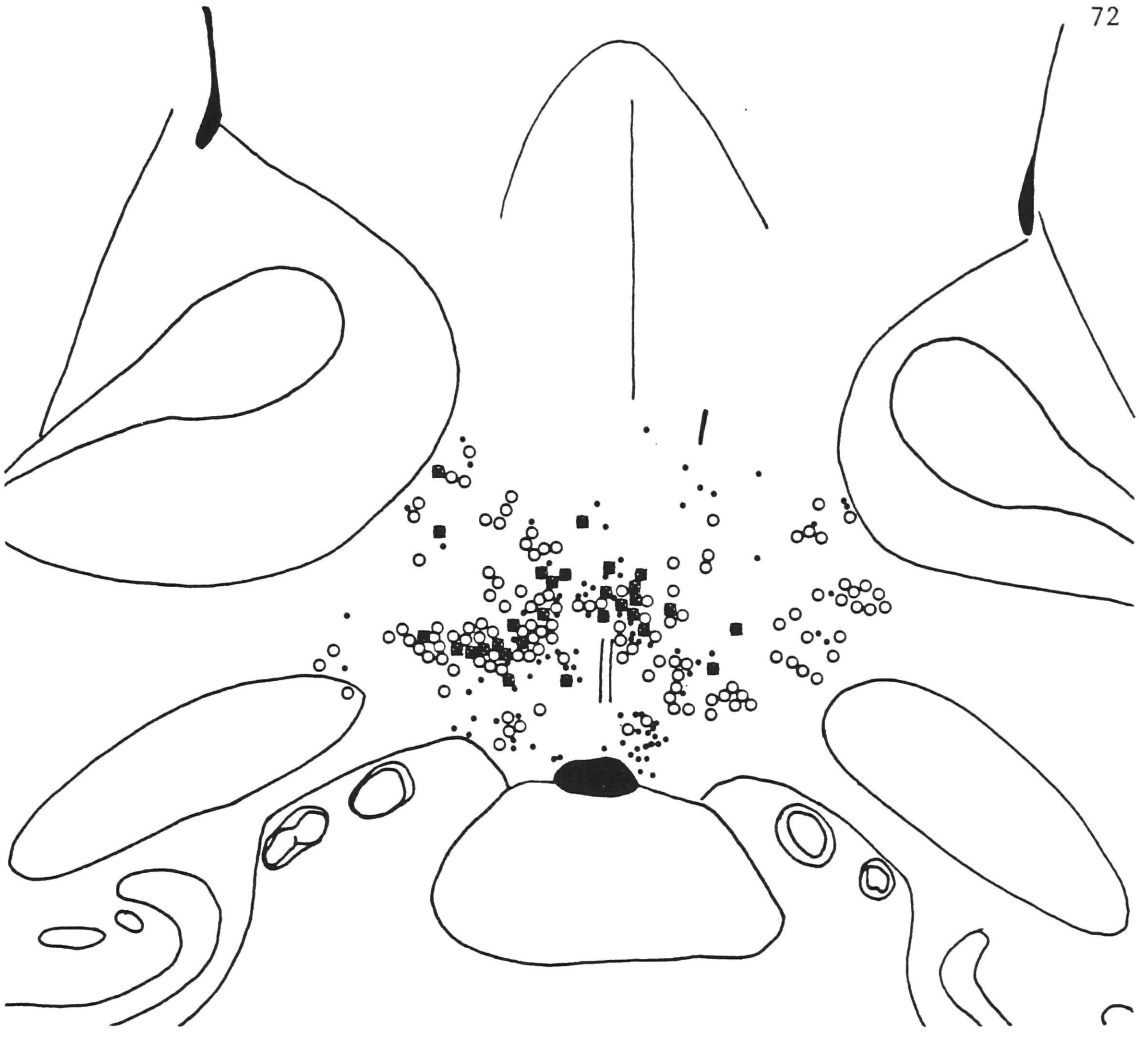


Figure 2 - 6. This section through the preoptic suprachiasmatic area (level of the optic recess) shows the location of three types of neurons: estradiol-concentrating neurons ( • ), dye-labeled neurons ( O ), and neurons which both concentrate estradiol and are retrogradely-filled with dye transported from the midbrain ( ⊠ ). This rat (Brain 1 - 3) received a pressure injection of 0.5 µl DAPI/Primuline into the medial midbrain. This drawing is of an actual representative 6 µm section. Although tissue features outside of the preoptic suprachiasmatic area are indicated as an aid to anatomical localization, dye-labeled and estradiol-concentrating cells in those regions are not shown. Note the heterogeneous nature of the cell population of the preoptic suprachiasmatic area.

and project to the midbrain can be found immediately adjacent to cells which have only one or neither of these characteristics. This "local heterogeneity" was a feature of every brain region which contained double-labeled neurons.

Figure 2 - 7 (a - f) presents diagramatically on transverse sections the distribution of DAPI-labeled, estradiol-concentrating neurons through the rostral-caudal extent of the preoptic region. Because of the extremely large numbers of cells involved, double-labeled neurons only are displayed on these charts.

The major difference between these results and the pattern of double-labeling seen in Brain 1-1 is the absolute number of double-labeled cells revealed by the larger tracer injection. Otherwise, the distribution is similar. DAPI-labeled, estradiol-concentrating neurons were found throughout the rostral-caudal extent of the medial preoptic nucleus and the medial preoptic area, with relatively greater numbers present in the caudal half of the medial preoptic area. Double-labeled neurons were much less likely to be present in the medial one-third of the medial preoptic nucleus than in the lateral two-thirds, reflecting the consistent tendency for dye-labeled neurons to be present in highest numbers away from the periventricular zone.

As in Brain 1-1, double-labeled neurons in the bed nucleus of the stria terminalis were concentrated in the caudal and ventral portions of that nucleus. The region of intersection in the caudal preoptic region between the ventral portion of this bed nucleus, dorsal and lateral medial preoptic area and the dorsal and medial lateral preoptic area was characterized by large numbers of DAPI-containing

neurons exhibiting nuclear estradiol binding, distributed in apparent disregard for the boundaries defining these cell groups.

Cell counts derived from camera lucida drawings for a large number of sections from Brain 1-3 are presented in Table 2 - 4. Assuming that estradiol binding is stable across brains, in comparison with Brain 1-1, it can be seen that the larger injection site has revealed that a significant proportion of estradiol-concentrating neurons in the preoptic suprachiasmatic nucleus, medial preoptic nucleus, and the medial preoptic area (13%, 17%, and 22%, respectively) send their axons to the midbrain, with smaller contributions from the bed nucleus of the stria terminalis, the lateral preoptic area, and the rostral anterior hypothalamic area. The increased retrograde dye-labeling present in Brain 1-3 (which, in effect, "reveals" the nature of the estradiol-concentrating neurons) could be due both to the larger amount of dye available for retrograde transport from the ventral midbrain and to the addition of cells afferent to the midbrain central grey. As will be discussed below, there is evidence for a significant contribution from the latter. Whether or not the entire preoptic region projection to the midbrain is revealed by even such a large injection of tracer cannot be determined.

Brain 2-6. Bilateral True Blue Injection into the Ventral Tegmental Area and Interpeduncular Nucleus

Tracer Injection. As shown in Figure 2 - 8, this animal received a bilateral injection of 10% True blue (0.25 µl per side) into the

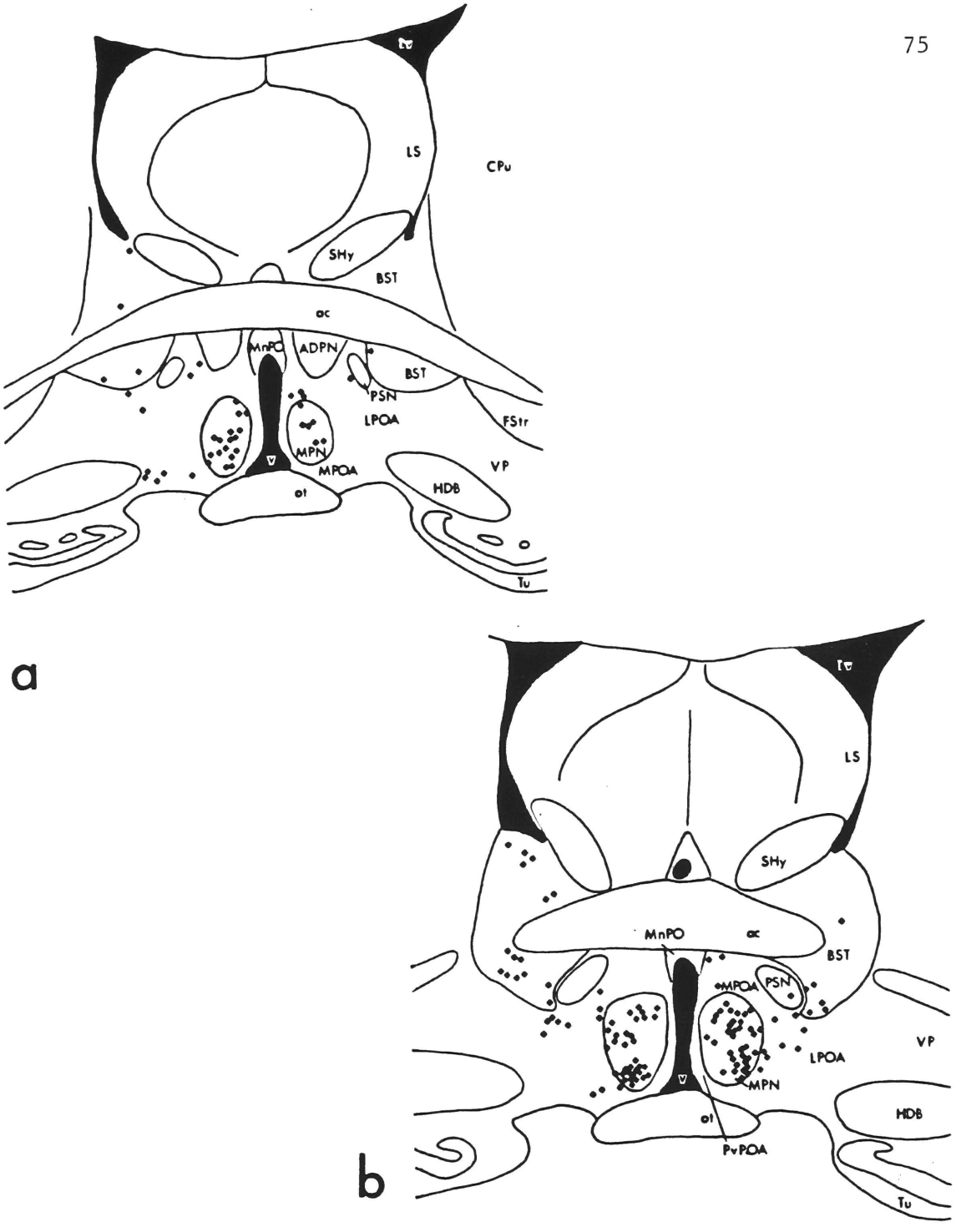



Figure 2 - 7. These charts (a - f) show the location of estradiol-concentrating, retrogradely-labeled neurons identified in the preoptic region of a rat receiving a pressure injection of 0.5 µl DAPI/Primuline into the medial midbrain. (See Figure 2 - 4 and Appendix 1 for tracer injection site.) These drawings are of actual, representative 6 and 12 µm sections. All double-labeled cells in a section are shown. One diamond (◆) equals one dye-labeled, estradiol-concentrating neuron. Brain 1 - 3.

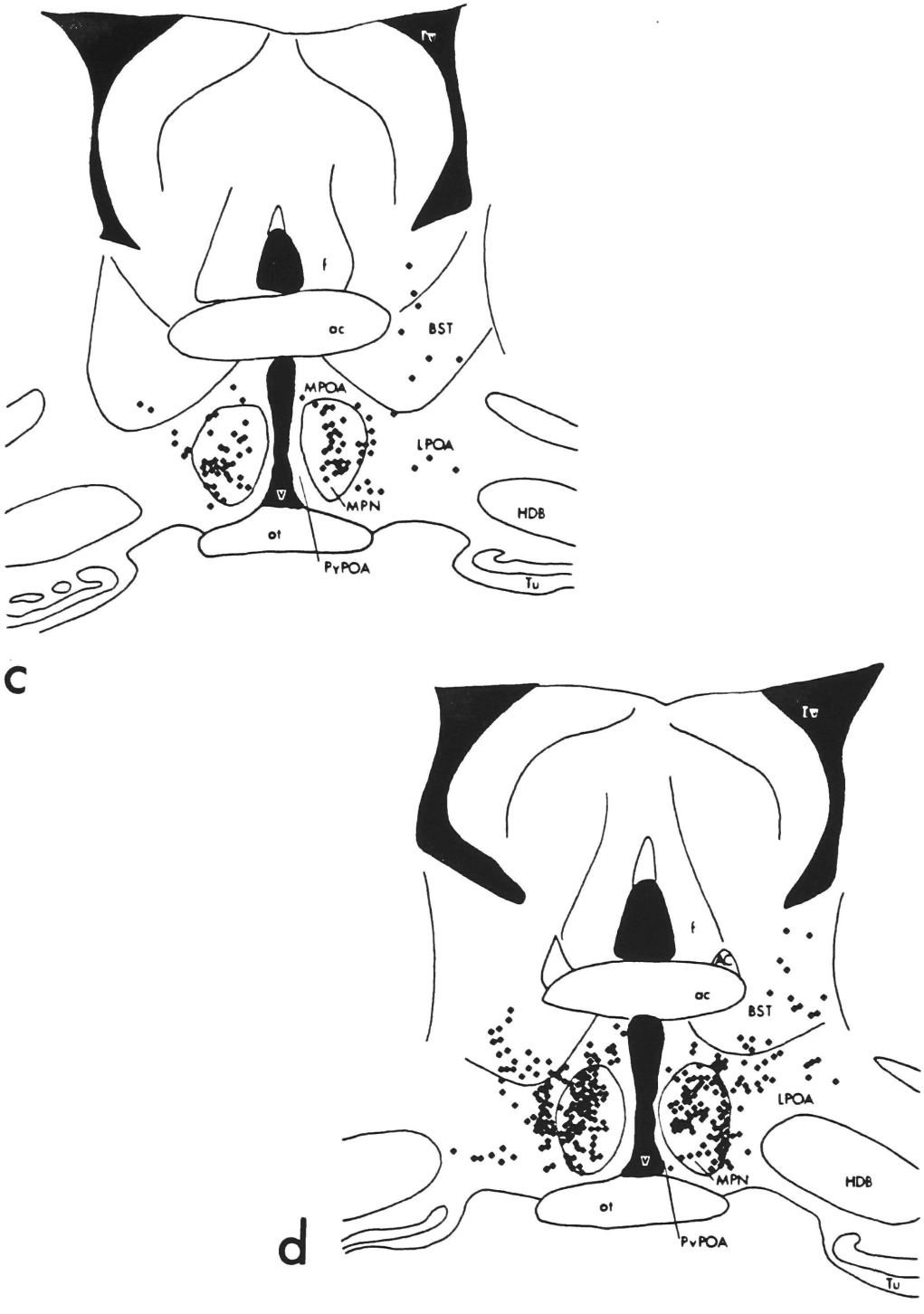



Figure 2 - 7. Continued. Brain 1 - 3.

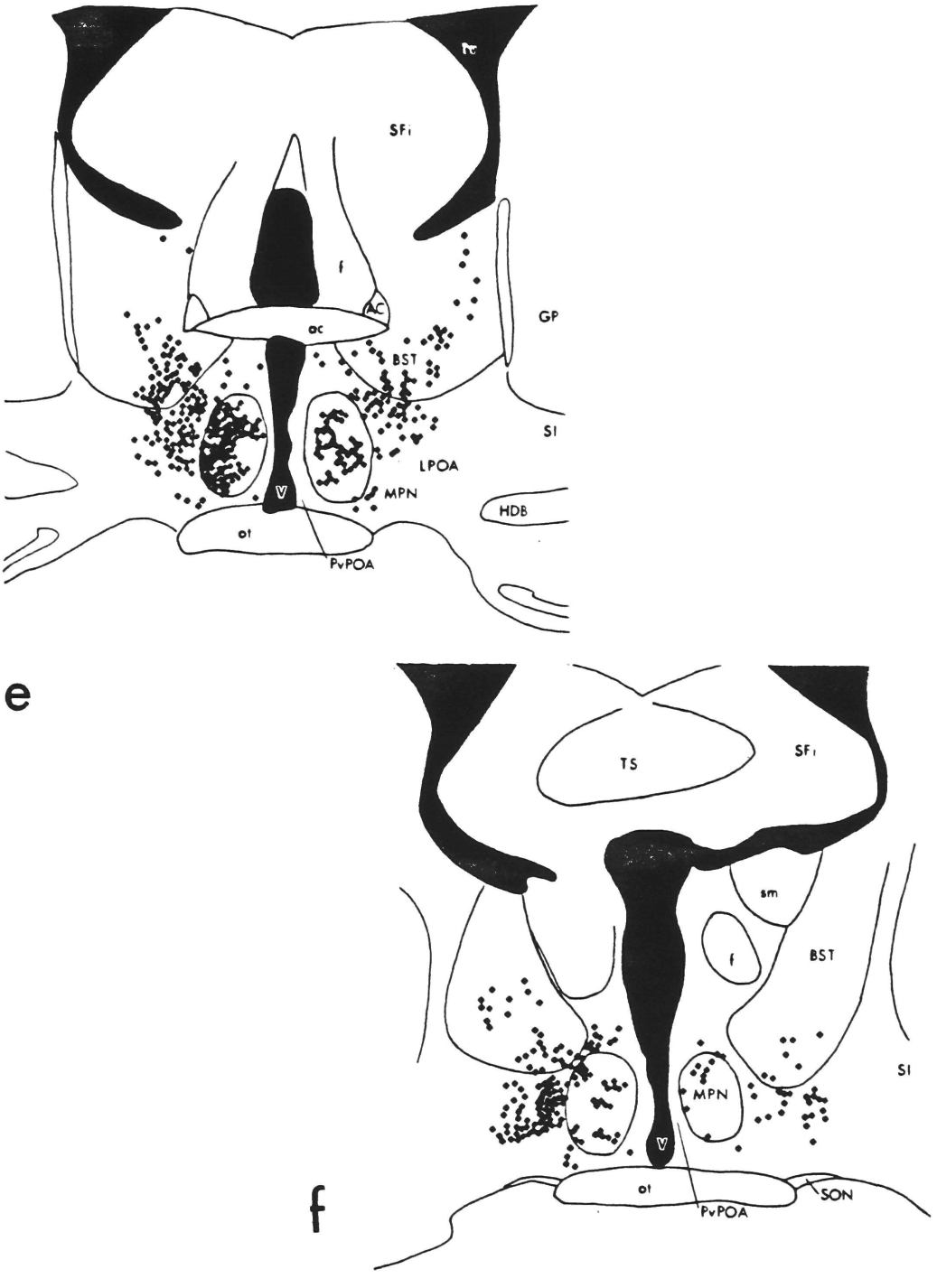



Figure 2 - 7. Continued. Brain 1 - 3.

TABLE 2 - 4 (BRAIN 1 - 3)

UNILATERAL 0.5 µl DAPI/PR INJECTION INTO VTA AND ADJACENT AREAS

| AREA[a] | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: $E_2$ NEURONS (PER SECTION) | RANGE: DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL $E_2$ NEURONS | TOTAL DOUBLE-LABELED NEURONS | %$E_2$ NEURONS WITH DYE LABEL |
|---|---|---|---|---|---|---|---|
| POSC[b] | 6 | 48 µm | 81 - 212 | 6 - 32 | 961 | 125 | 13.0% |
| MPN | 22 | 174 µm | 142 - 309 | 8 - 132 | 5864 | 1020 | 17.4% |
| MPOA | 29 | 228 µm | 17 - 187 | 2 - 74 | 3170 | 700 | 22.1% |
| BNST | 39 | 306 µm | 15 - 487 | 0 - 55 | 7202 | 434 | 6.0% |
| PvPOA[c] | 41 | 330 µm | 15 - 64 | 0 - 2 | 1114 | 7 | 0.6% |
| LPOA/LH[d] | 16 | 126 µm | 14 - 82 | 0 - 20 | 553[e] | 98 | 17.7% |
| AHA[f] | 18 | 144 µm | 18 - 444 | 1 - 33 | 2792 | 191 | 6.8% |

a ipsilateral to tracer injection site
b includes midline region
c caudal to the POSC
d boundary vague between LPOA and LH
e incomplete count due to scattered nature of $E_2$-concentrating neurons in this region
f includes lateral anterior nucleus. Rostral sections may not be representative of more caudal sections through this region.

ABBREVIATIONS AHA, anterior hypothalamic area; BNST, bed nucleus of the stria terminalis; $E_2$, estradiol; LH, lateral hypothalamus; LPOA, lateral preoptic area; MPN, medial preoptic nucleus; MPOA, medial preoptic area; POSC, preoptic suprachiasmatic area; PR, primuline; PvPOA, periventricular preoptic area; VTA, ventral tegmental area

caudal and ventral portions of the ventral tegmental area and into the lateral edges of the interpeduncular nucleus. There was no indication of dye spread into the midbrain central grey, and hence it was of great interest to compare the pattern of dye labeling seen in this brain to that observed in Brains 1-1 and 1-3. Also, in Brain 2-6 it was possible to sample tissue caudally to the level of the ventromedial hypothalamic nucleus. This permits direct comparison of the present results with those of Morrell and Pfaff (1982). The injection sites were symmetric.

Fluorescent Dye-Labeled Neurons. To a great extent, the distribution of True blue labeled neurons in Brain 2-6 replicates that described above for Brains 1-1 and 1-3. Only the differences and the caudal pattern will be noted here. Again, there was minimal septal labeling (some medial septal True blue-labeled cells were present), but labeled neurons were present in small numbers in both the septal triangular nucleus and in the septofimbrial nucleus.

Occasionally, well-labeled magnocellular preoptic area neurons were observed.

As examination of the more limited material available for Brains 1-1 and 1-3 indicated, initial heavy retrograde dye labeling in the rostral anterior hypothalamic area diminishes significantly as one examines more caudal sections, while the lateral regions continue to contain many dye-filled cells. A similar pattern was also seen in the material from animals that received ventral tegmental area dye injections but no [$^{3}$H]-estradiol. A striking difference in Brain 2-6 in the anterior hypothalamic region was the absence of large numbers of retrogradely labeled cells in the lateral anterior nucleus.

Examination of sections caudal to the preoptic/anterior hypothalamic region revealed several additional groups of True blue-labeled neurons. Although sections through the hypothalamic paraventricular nucleus have not yet been charted in detail, preliminary examination of this material revealed scattered (but consistently present) dye-labeled neurons in the medial parvocellular subdivision of the paraventricular nucleus. Occasionally, dye-labeled cells retrogradely filled from the ventral midbrain were also seen in the lateral parvocellular subdivision. Rarely, a dye-labeled magnocellular neuron was seen in the posterior magnocellular subdivision. No dye-labeled cells were present in the anterior magnocellular subdivision (the "anterior commissural nucleus") of the paraventricular nucleus. A perifornical cluster of brightly-labeled cells was present from anterior hypothalamic levels through the medial basal hypothalamus. The zona incerta and lateral hypothalamus were characterized by numerous dye-labeled neurons (hundreds/section), while scattered dye cells were seen in the central nucleus of the amygdala and in the rostral medial amygdala. Fluorescent cells were rarely present in the arcuate nucleus, although some were present in the retrochiasmatic tuberal region ventral to the third ventricle (2 - 10 per section). Scattered True blue-labeled neurons were present in the dorsomedial hypothalamic nucleus: the ventromedial hypothalamic nucleus was conspicuously dark (fewer than 20 dye-labeled cells per section in the entire nucleus), although many dye-labeled neurons were present in the lateral hypothalamus at the same level. True blue neurons were extremely rare in the periventricular hypothalamic region.

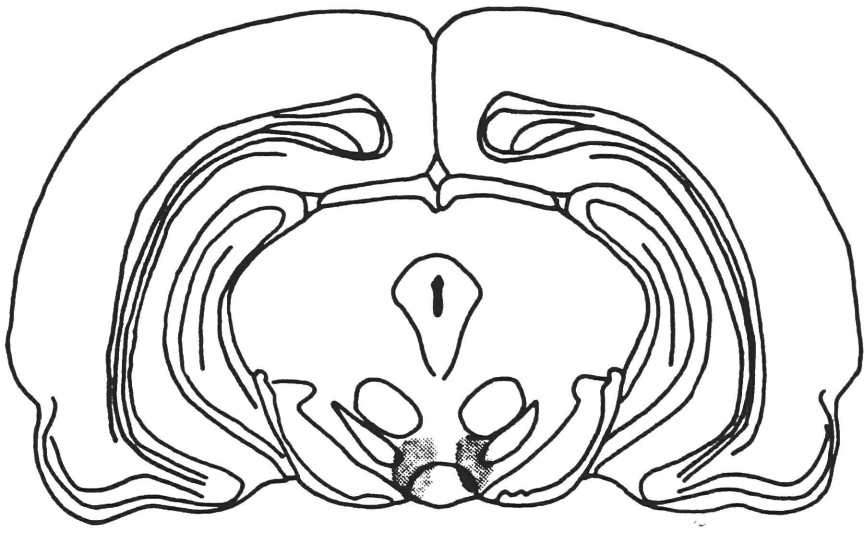


Figure 2 - 8. Center of True blue deposit site for Brain 2 - 6. Shaded area indicates dye location. 0.25 µl of 10% True blue was injected bilaterally into the ventral tegmental area at the level of the interpeduncular nucleus (level A 1760 µ; König and Klippel, 1963). Survival time after tracer injection, 4 days. For complete rostral-caudal extent of dye spread in the ventral midbrain, see Appendix 1.

TABLE 2 - 5 (BRAIN 2 - 6)

BILATERAL 0.25 µl TRUE BLUE INJECTION INTO VTA

| AREA[a] | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: $E_2$ NEURONS (PER SECTION) | RANGE: DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL $E_2$ NEURONS | TOTAL DOUBLE-LABELED NEURONS | %$E_2$ NEURONS WITH DYE LABEL |
|---|---|---|---|---|---|---|---|
| POSC[b] | 2 | 12 µm | 239 - 324 | 4 - 15 | 582 | 19 | 3.3% |
| MPN | 9 | 72 µm | R:156 - 408<br>L:171 - 406 | R:3 - 43<br>L:6 - 49 | R:2863<br>L:3058 | R:156<br>L:201 | R:5.5%<br>L:6.6% |
| MPOA | 12 | 96 µm | R: 49 - 165<br>L: 51 - 175 | R:0 - 17<br>L:3 - 12 | R:1372<br>L:1305 | R: 80<br>L: 88 | R:5.8%<br>L:6.7% |
| BNST[c] | R:14<br>L:13 | R:108 µm<br>L:102 µm | R: 88 - 297<br>L: 57 - 363 | R:3 - 20<br>L:1 - 16 | R:2891<br>L:3213 | R:161<br>L:113 | R:5.6%<br>L:3.5% |
| PvPOA[d] | 13 | 102 µm | R: 30 - 97<br>L: 27 - 90 | R:0<br>L:0 - 1 | R: 770<br>L: 785 | R: 0<br>L: 1 | R: 0<br>L:0.1% |
| AHA[e] | 1 | 6 µm | - | - | R: 539<br>L: 339 | R: 21<br>L: 12 | R:3.9%<br>L:3.5% |

a symmetric, bilateral tracer injection. The preoptic-to-midbrain projection has a contralateral component; both midbrain injection sites must be considered a potential source for retrograde-labeling observed in the preoptic region.

b right and left totals for this area only

c one left section missing due to tissue damage

d caudal to the POSC. Does not include "preventricular periventricular area" (Bleier et al., 1982) or median preoptic nucleus

e Rostral section may not be representative of more caudal sections through this region.

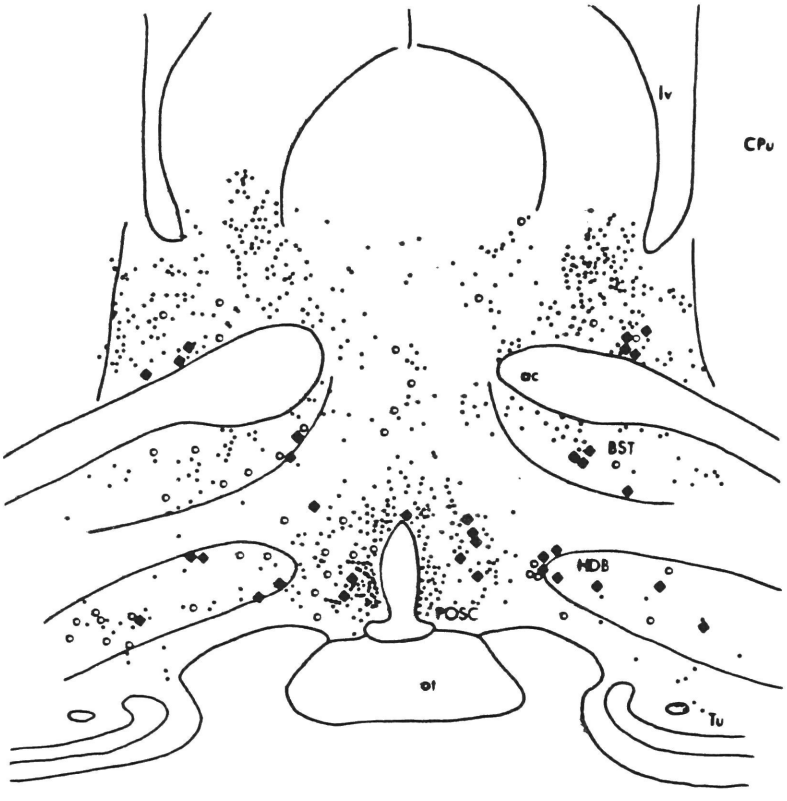
ABBREVIATIONS AHA, anterior hypothalamic area; BNST, bed nucleus of the stria terminalis, $E_2$, estradiol; L, left side of the brain; MPN, medial preoptic nucleus; MPOA, medial preoptic area; POSC, preoptic suprachiasmatic area; PvPOA, periventricular preoptic area; R, right side of the brain; VTA, ventral tegmental area

Double-labeled Neurons. Double-labeled neurons were numerous in the medial preoptic nucleus, the medial preoptic area, and in the bed nucleus of the stria terminalis: they were also present in the preoptic suprachiasmatic area and in the rostral anterior hypothalamic area. Table 2 - 5 shows the percentages of estradiol-concentrating neurons found in these regions that were also labeled with dye retrogradely-transported from the ventral midbrain. Double-labeled cells were also occasionally identified in the paraventricular nucleus (medial parvocellular subdivision). Estradiol-concentrating and double-labeled neurons were counted from charts made from seven sections through the arcuate nucleus and the ventrolateral subdivision of the hypothalamic ventromedial nucleus (total tissue: 60 μm). On both the right and left sides of the third ventricle, fewer than 1% of the estradiol-concentrating neurons of the arcuate nucleus were found also to project to the ventral midbrain. A slightly higher percentage (right side: 3.0%, 21/725; left side: 1.6%, 14/888) of estradiol-concentrating neurons in the ventromedial nucleus were also dye-labeled.

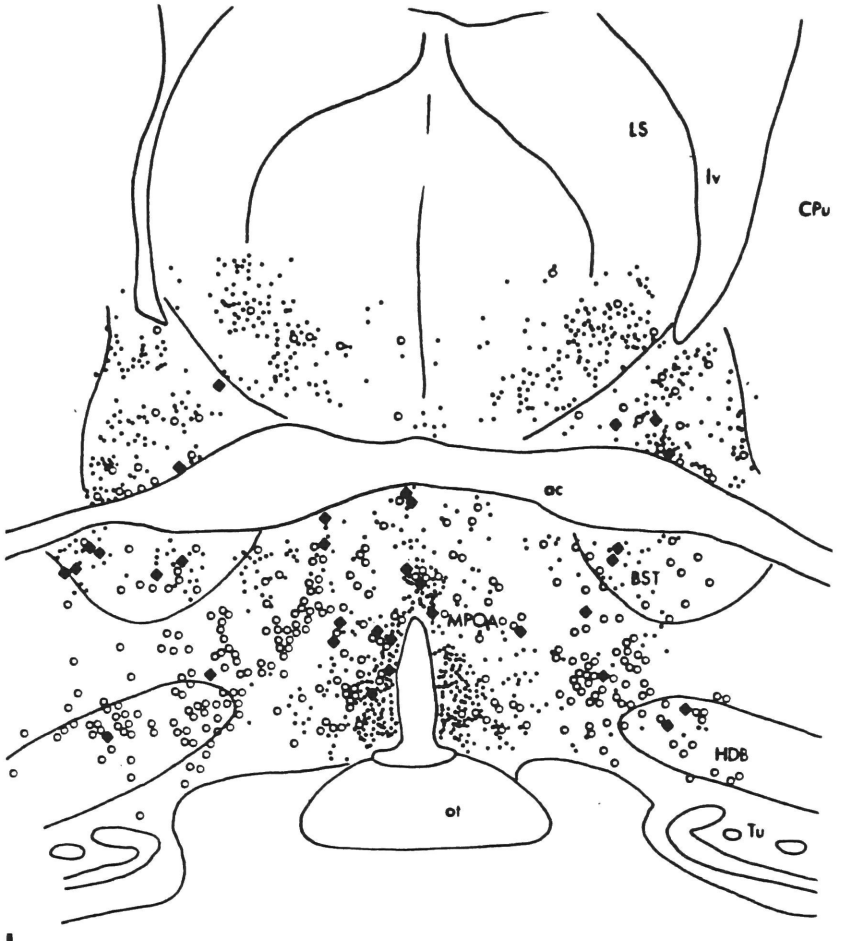
Figure 2 - 9 (a - e) illustrates in detail the distribution of estradiol-concentrating and double-labeled neurons in the preoptic region of Brain 2-6, while also showing the approximate location of dye-only labeled neurons. These drawings are of representative single sections and each symbol represents one counted, identified neuron. This set of charts makes clear the pattern of partial overlap between the distribution of dye-labeled (lateral-tending) and estradiol-concentrating (medial-tending) neurons. The location of double-labeled neurons in sections from Brain 2-6 can be seen to be

Figure 2 - 9.

These charts show the location of estradiol-concentrating, retrogradely-labeled neurons (◆) identified in the preoptic region of a rat which received a bilateral pressure injection of 0.25 ul of True blue into the ventral tegmental area. (See Figure 2 - 8 and Appendix 1 for tracer injection site.) Also shown are the location of neurons which project to the ventral midbrain but do not concentrate estradiol (○), and the location of estradiol-concentrating cells (•) which are not dye-labeled. These drawings are of actual, representative 6 and 12 μm sections. Each symbol represents one neuron. Note that estradiol-concentrating neurons tend to be medial, while neurons retrogradely-labeled from the midbrain are predominant more laterally.
Brain 2 - 6.




a

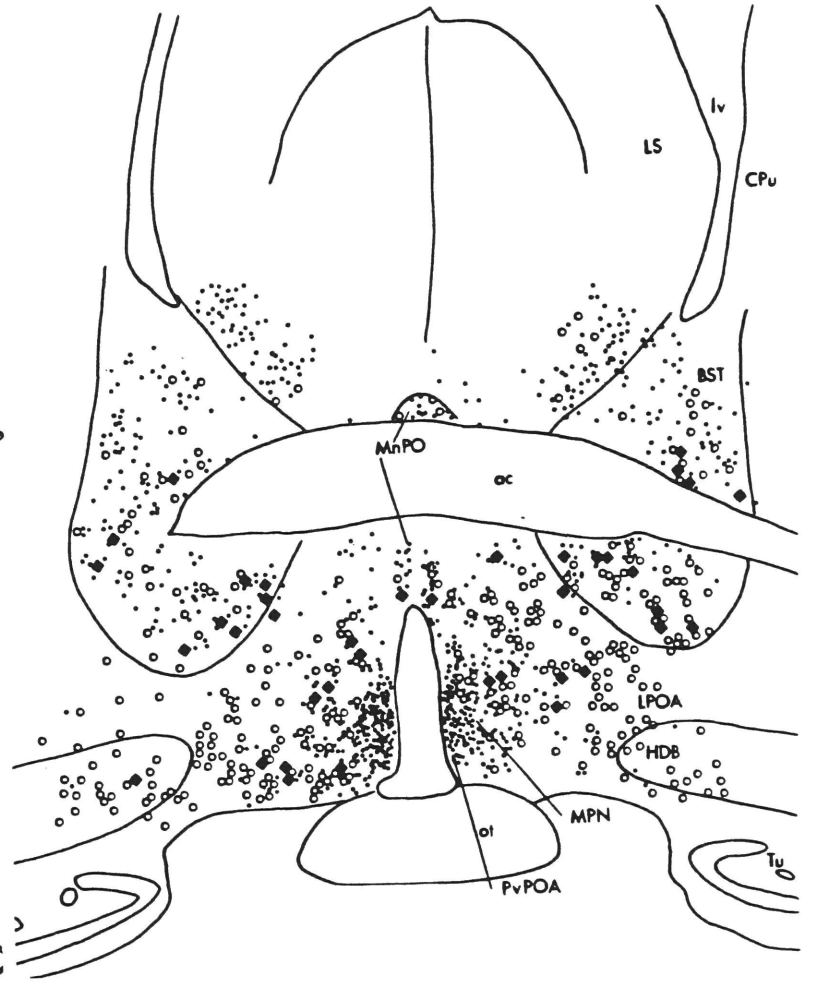



b

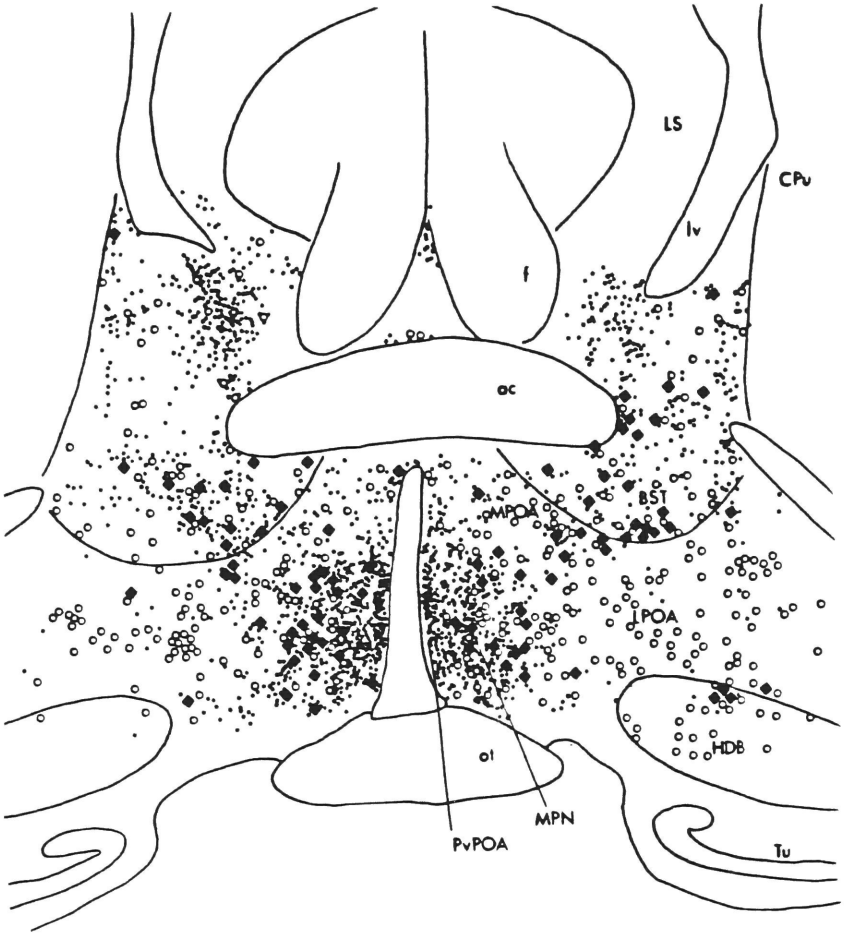
(•) estradiol-concentrating neuron

(○) dye-labeled neuron

(◆) estradiol-concentrating, dye-labeled neuron




c

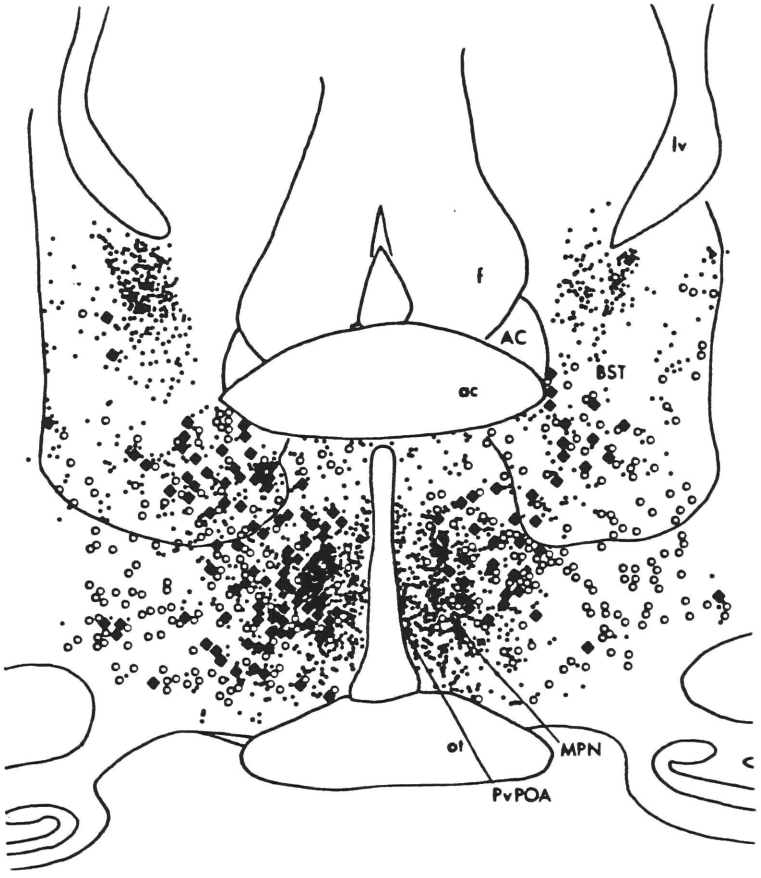



d

( • ) estradiol-concentrating neuron

( ○ ) dye-labeled neuron

( ◆ ) estradiol-concentrating, dye-labeled neuron




e

TABLE 2 - 6 (BRAIN 2 - 6)

BILATERAL 0.25 µl TRUE BLUE INJECTION INTO VTA

| AREA[a] | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: TRUE BLUE NEURONS (PER SECTION) | RANGE:DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL TRUE BLUE NEURONS | TOTAL DOUBLE-LABELED NEURONS | %DYE NEURONS CONCENTRATING ESTRADIOL |
|---|---|---|---|---|---|---|---|
| MPN | 4 | 24 µm | R: 39-64<br>L: 41-101 | R: 3-18<br>L: 6-58 | R: 2[illegible]<br>L: 268 | R: 46<br>L: 117 | R: 20.2%<br>L: 43.7% |
| MPOA | 5 | 30 µm | R: 38-77<br>L: 52-99 | R: 2-18<br>L: 6-26 | R: 318<br>L: 367 | R: 35<br>L: 54 | R: 11.0%<br>L: 14.7% |
| BNST | 5 | 30 µm | R: 36-59<br>L: 32-43 | R: 6-20<br>L: 6-21 | R: 244<br>L: 200 | R: 70<br>L: 55 | R: 28.7%<br>L: 27.5% |
| PvPOA[b] | 5 | 30 µm | R: 0-1<br>L: 0-1 | R: 0-1<br>L: 0-1 | R: 1<br>L: 3 | R: 0<br>L: 2 | —[c] |

a symmetric, bilateral tracer injection. The preoptic-to-midbrain projection has a contralateral component; both midbrain injection sites must be considered a possible source for retrograde-labeling observed in the preoptic region.

b caudal to the POSC. Does not include "preventricular preoptic area" (Bleier et al., 1982) or median preoptic nucleus.

c numbers too small to present as meaningful percentages

ABBREVIATIONS BNST, bed nucleus of the stria terminalis; L, left side of the brain; MPN, medial preoptic nucleus; MPOA, medial preoptic area; POSC, preoptic suprachiasmatic area; PvPOA, periventricular preoptic area; R, right side of the brain; VTA, ventral tegmental area

quite similar to that found in Brain 1-1 (see Figure 2 - 3) and Brain 1-3 (see Figure 2 - 7).

Finally, Table 2 -6 contains data on the percentage of dye-labeled neurons identified in the preoptic region which also exhibited nuclear binding of [$^{3}$H]-estradiol. Axons from such cells are a substantial component of the pathway connecting the preoptic region with the ventral tegmental area: in the medial preoptic nucleus and the bed nucleus of the stria terminalis, for example, more than 25% of the True blue-labeled neurons were also estradiol target cells.

Brain 2-5. Unilateral True Blue Injection into the Midbrain Central Grey

Tracer Injection. This animal received a unilateral injection of 0.5 ul of 10% True blue into the midbrain central grey. There appeared to be only minimal ventral spread of tracer into the dorsal part of the ventral tegmental area (see Figure 2 - 10). There was also no spread of dye into or around the cerebral aqueduct, thus ensuring no spurious labeling could occur via a ventricular route. The ipsilateral nucleus of Darkschewitsch, the interstitial nucleus of Cajal, and the mammillotegmental tract were in the region of the injected tracer, but it seems unlikely that connections of these brain structures contributed to the pattern of dye-label observed in the diencephalon. The injection site was centered on the rostral midbrain central grey. Sections were examined from the diagonal bands caudal through the medial basal region of the hypothalamus.

Fluorescent Dye-Labeled Neurons. Again, retrogradely-labeled cells were present in the medial septum, the diagonal bands (both vertical and horizontal limbs), and the preoptic suprachiasmatic area. Numerous True blue-labeled neurons were present in the medial and lateral preoptic areas. More labeled cells were counted in the medial (including the medial preoptic nucleus) area than in the lateral preoptic area. The periventricular area contained no discernible dye-labeled neurons, although a few small labeled cells were present in the median preoptic nucleus. The pattern of dye labeling was reversed in the anterior hypothalamic level, with True blue-labeled cells more numerous in the lateral hypothalamus and very few such cells seen in the anterior hypothalamus outside of the lateral anterior nucleus, which was prominently dye-labeled. Dye-labeled neurons were very infrequently found in the medial parvocellular subdivision of the hypothalamic paraventricular nucleus. Caudally, True blue-labeled cells were present in the zona incerta, the arcuate nucleus, the medial amygdala, and especially in the ventromedial hypothalamic nucleus, in both the medial and ventrolateral subdivisions. These projections were mainly ipsilateral.

Double-Labeled Neurons. Table 2 - 7 presents the results from cell counts made from a sample of sections from Brain 2-5, in terms of percentages of estradiol-concentrating neurons which are also dye-labeled. As is typical for this material, the region containing the highest percentage of double-labeled neurons was the medial preoptic region (ipsilateral side, with regard to tracer injection: 8.1%). Again, double-labeled neurons were also found in the preoptic suprachiasmatic area (not shown in Table 2 - 7 due to the small number

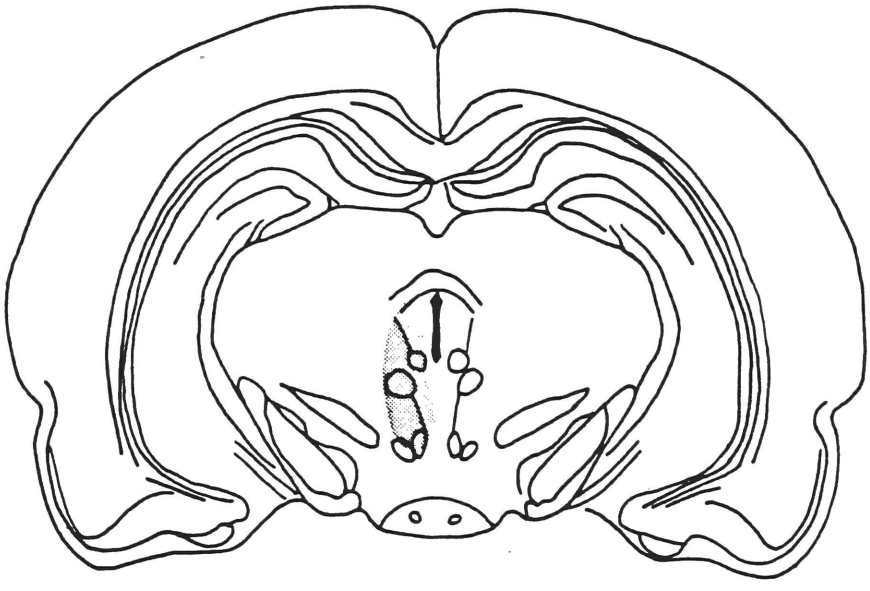


Figure 2 - 10. Center of True blue deposit site for Brain 2 - 5. Shaded area indicates dye location. 0.5 µl of 10% True blue was injected unilaterally into the rostral midbrain central grey (level A 2420 µ; König and Klippel, 1963). Survival time after tracer injection, 4 days. For complete rostral-caudal extent of dye spread in the midbrain, see Appendix 1.

TABLE 2 - 7 (BRAIN 2 - 5)

UNILATERAL 0.5 µl TRUE BLUE INJECTION INTO MCG

| AREA | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: $E_2$ NEURONS (PER SECTION) | RANGE: DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL $E_2$ NEURONS | TOTAL DOUBLE-LABELED NEURONS | %$E_2$ NEURONS WITH DYE LABEL |
|---|---|---|---|---|---|---|---|
| MPN | 5 | 54 µm | Ipsi:295-443<br>Contra:318-525 | Ipsi:10-21<br>Contra: 1-9 | Ipsi:2121<br>Contra:2194 | Ipsi:73<br>Contra:23 | Ipsi:3.4%<br>Contra:1.0% |
| MPOA | 5 | 54 µm | Ipsi: 84-254<br>Contra:206-297 | Ipsi:13-17<br>Contra: 3-14 | Ipsi: 928<br>Contra:1218 | Ipsi:75<br>Contra:46 | Ipsi:8.1%<br>Contra:3.8% |
| BNST | 7 | 78 µm | Ipsi:285-655<br>Contra: 53-571 | Ipsi: 4-23<br>Contra: 1-8 | Ipsi:2707<br>Contra:2413 | Ipsi:70<br>Contra:27 | Ipsi:2.6%<br>Contra:1.0% |
| PvPOA | 7 | 78 µm | Ipsi: 19-106<br>Contra: 25-74 | Ipsi: 0<br>Contra: 0 | Ipsi: 432<br>Contra: 408 | Ipsi: 0<br>Contra: 0 | Ipsi: 0<br>Contra: 0 |
| AHA[a] | 2 | 24 µm | Ipsi:271,283<br>Contra:225,301 | Ipsi: 5,6<br>Contra: 4,5 | Ipsi: 554<br>Contra: 526 | Ipsi:11<br>Contra: 9 | Ipsi:2.0%<br>Contra:1.7% |
| MePN[b] | 3 | 30 µm | 16 - 73 | 0 - 1 | 134 | 2 | 1.5% |
| ARC | 2 | 12 µm | Ipsi: 85,106<br>Contra: 92,135 | Ipsi: 1,3<br>Contra: 1,1 | Ipsi: 191<br>Contra: 229 | Ipsi: 4<br>Contra: 2 | Ipsi:2.1%<br>Contra:0.9% |
| VL-VM[c] | 2 | 12 µm | Ipsi: 85,112<br>Contra: 96,182 | Ipsi:16,20<br>Contra:5,9 | Ipsi: 197<br>Contra: 278 | Ipsi:36<br>Contra:14 | Ipsi:18.3%<br>Contra: 5.0% |

a caudal to the POSC
b midline structure: right and left totals summed for this area only
c rostral portion of VL-VM

ABBREVIATIONS AHA, anterior hypothalamic area; ARC, arcuate nucleus; BNST, bed nucleus of the stria terminalis; Contra, contralateral to the tracer injection site; $E_2$, estradiol; Ipsi, ipsilateral to the tracer injection site; MCG, midbrain central grey; MePN, median preoptic nucleus; MPOA, medial preoptic area; POSC, preoptic suprachiasmatic area; PvPOA, periventricular preoptic area; VL-VM, ventrolateral subdivision of the hypothalamic ventromedial nucleus

of rostral sections available for counting), the medial preoptic nucleus, the bed nucleus of the stria terminalis, and the rostral anterior hypothalamic area. The anatomical distribution of these double-labeled neurons was not different from that shown in Figures 2 - 7 and 2 - 9, and hence no charts are presented. Two notable features of the labeling found in Brain 2 - 5 are indicated in Table 2 - 7. First, the median preoptic nucleus was carefully examined throughout its full rostral-caudal extent and found to include several double-labeled neurons (2/134). Second, although the arcuate nucleus contained very few midbrain-projecting neurons, the ipsilateral ventrolateral subdivision of the hypothalamic ventromedial nucleus contained the highest percentage of double-labeled neurons counted in this brain (18.3%). These results are in extremely good agreement with the more extensive autoradiographic/fluorescent dye retrograde tracing studies of Morrell and Pfaff (1982).

Brain 2-7. Bilateral True Blue Injections into the Lateral Midbrain

Tracer Injection. As illustrated in Figure 2 - 11, this case features bilateral injections of 10% True blue (0.1 μl per side) into the lateral midbrain (mesencephalic reticular formation) without incursion into either the midbrain central grey or the ventral tegmental area. The symmetric midbrain injection sites produced a bilaterally symmetric pattern of retrograde labeling in the preoptic area and hypothalamus.

Fluorescent Dye-Labeled Neurons. The pattern of fluorescent dye labeling seen in this brain differs markedly from that produced by

tracer injections into the midbrain central grey and the ventral tegmental area, although there is some overlap, particularly in lateral structures.

Scattered True blue cells were seen in the horizontal limb of the nucleus of the diagonal band, in the magnocellular preoptic area, and in the lateral preoptic area. Along the midline, scattered cells were present in the medial septum and in the preoptic suprachiasmatic area. A few dye-labeled cells were counted in the median preoptic nucleus, while the medial preoptic region was dark, containing only 1 - 3 True blue labeled cells per section. Small numbers of retrogradely-filled neurons were present in the lateral hypothalamus and in the zona incerta, in addition to a few in the rostral ventromedial hypothalamic nucleus.

Overall, these injection sites produced sparse retrograde labeling in the diencephalon, with a notable absence of fluorescent label in the medial preoptic region and in the anterior hypothalamus. The only exception to this pattern was seen bilaterally in a very circumscribed portion of the bed nucleus of the stria terminalis adjacent to the dorsal and lateral medial preoptic area. 20 - 30 dye-labeled neurons were clustered together in this region in sections from the level of the lateral subdivision of the bed nucleus of the strai terminalis through the preoptic continuation of that nucleus.

Double-labeled Neurons. Given the low numbers of retrogradely-labeled cells present in the preoptic region of Brain 2-7, it is not surprising that very few double-labeled neurons were identified in the medial preoptic nucleus, the medial preoptic area, and the anterior hypothalamic area. However, double-labeled neurons

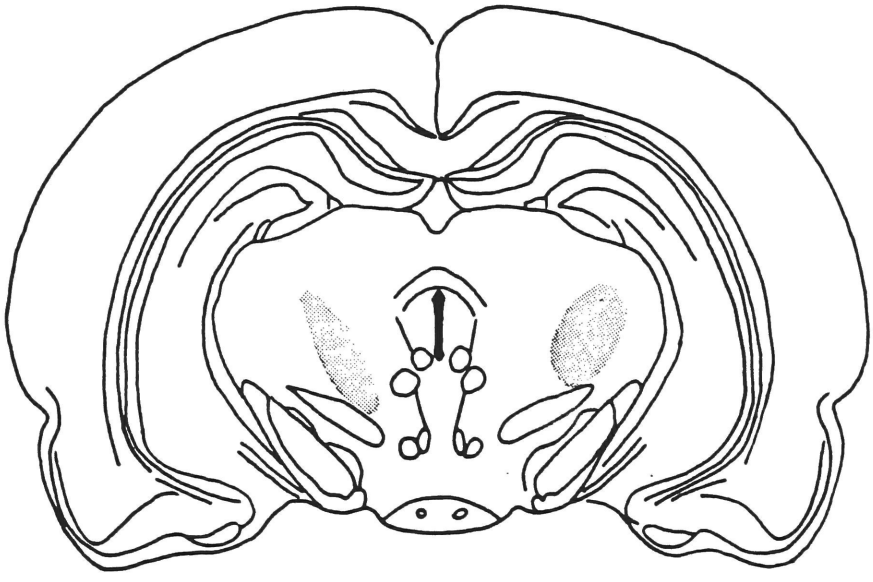


Figure 2 - 11. Center of True blue deposit site for Brain 2 - 7. Shaded area indicates dye location. 0.1 µl of 10% True blue was injected bilaterally into the lateral midbrain, dorsal to the medial lemniscus (level A 2420 µ; König and Klippel, 1963). Survival time after tracer injection, 4 days. For complete rostral-caudal extent of dye spread in the midbrain, see Appendix 1.

were consistently identified in that ventral portion of the bed nucleus of the stria terminalis in which retrogradely-filled True blue neurons were found, in most cases between 5 and 10/side/section. Double-labeled neurons were, of course, identified in a similar region in the other cases discussed here: the exceptional feature of Brain 2-7 is the absence of accompanying dye- and double-labeling in the medial preoptic area and the medial preoptic nucleus.

The only other region consistently found to contain double-labeled neurons in this brain was the ventrolateral subdivision of the hypothalamic ventromedial nucleus. Thus, a lateral midbrain placement of tracer as compared with the previously discussed medial midbrain dye injection sites resulted in a very different pattern of retrograde and hence double-labeling in the preoptic region.

Other Cases.

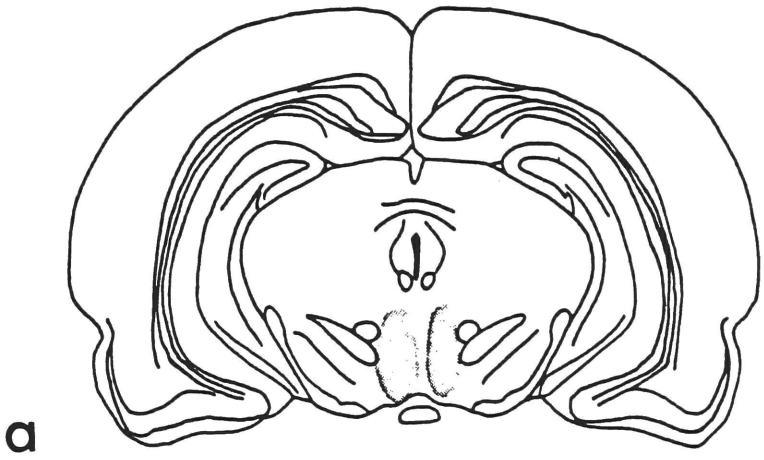
Three other brains were judged to be of anatomical interest and were examined in detail. The injection sites are illustrated in Figure 2 - 12. In Brain 1-8, True blue was deposited bilaterally in the ventral tegmental area and the ventral midbrain central grey, with considerable spread into the interpeduncular nucleus. In 1-10, bilateral injections of DAPI/Pr were successfully confined to the ventral tegmental area and the ventral portion of the central grey, with no spread into the interpeduncular nucleus. Finally, in Brain 1-11, considerable spread of DAPI/Pr up the needle track produced a large "medial midbrain" injection site, with fluorescent dye present in the ventral tegmental area, the midbrain central grey, and the dorsal midbrain at the level of the superior colliculus.

Figure 2 - 12. Centers of tracer deposit sites for Brains 1 - 8, 1 - 10, and 1 - 11. Shaded area indicates dye location.

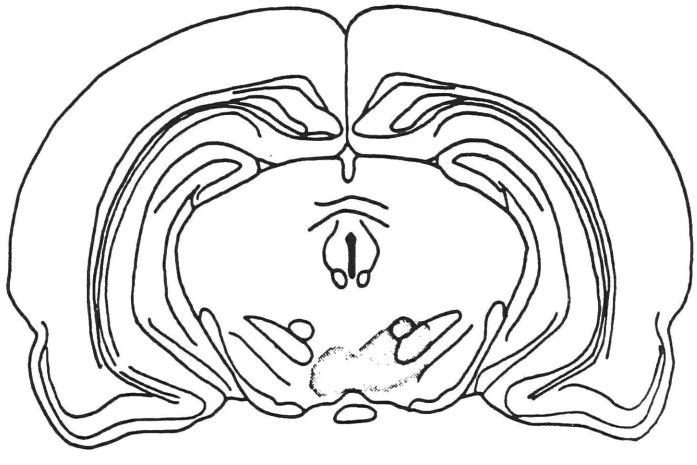
(a) Brain 1 - 8. Bilateral injection of 0.2 µl of 10% True blue into the ventral tegmental area. A 2180 µ.
(b) Brain 1 - 10. Bilateral injection of 0.2 µl of 10% Primuline/2.5% DAPI into the ventral tegmental area. A 2180 µ.

(c) Brain 1 - 11. Unilateral injection of 0.5 µl of 10% Primuline/2.5% DAPI into the medial midbrain. A 2180 µ.

Anatomical levels based upon atlas plates in König and Klippel, 1963. Survival time after tracer injections, 3 days. For complete rostral-caudal extent of dye spread in the ventral midbrain, see Appendix 1.



a



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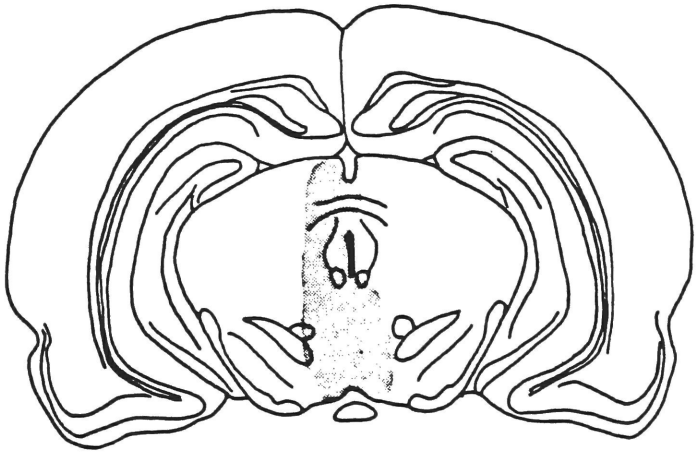


c

TABLE 2 - 8 (BRAIN 1 - 11)

UNILATERAL 0.5 µl DAPI/PR INJECTION INTO "MEDIAL MIDBRAIN"

| AREA[a] | TOTAL SECTIONS COUNTED | TOTAL TISSUE SAMPLED | RANGE: $E_2$ NEURONS (PER SECTION) | RANGE:DOUBLE-LABELED NEURONS (PER SECTION) | TOTAL $E_2$ NEURONS | TOTAL DOUBLE-LABELED NEURONS | %$E_2$ NEURONS WITH DYE LABEL |
|---|---|---|---|---|---|---|---|
| MPN | 9 | 78 µm | 68 - 175 | 1 - 21 | 1087 | 80 | 7.4% |
| MPOA | 18 | 150 µm | 10 - 73 | 0 - 13 | 548 | 86 | 15.7% |
| BNST | 20 | 162 µm | 24 - 74 | 0 - 10 | 1031 | 55 | 5.3% |
| PvPOA | 20 | 150 µm | 7 - 37 | 0 - 1 | 441 | 3 | 0.7% |

a ipsilateral to tracer injection site

ABBREVIATIONS BNST, bed nucleus of the stria terminalis; $E_2$, estradiol; MPN, medial preoptic nucleus; MPOA, medial preoptic area; PR, primuline; PvPOA, periventricular preoptic area

In both distribution of retrogradely-labeled cells and incidence of double-labeling, Brains 1-8 and 1-10 confirm the results described in detail above, adding weight to the conclusion that many preoptic region neurons which concentrate estradiol also send axons to the dorsal and ventral midbrain. The pattern of retrogradely-labeled cells seen in Brain 1-11 is congruent with that described above, although, as in 1-3, the very extensive injection site produced extremely large numbers of dye-labeled neurons in the preoptic region. This case was unusual in that a relatively large number of dye-labeled neurons were present in the lateral septum. Table 2 - 8 contains the results of counts made from camera lucida drawings of dye-labeled, estradiol-concentrating neurons. Again, the highest percentages of double-labeled neurons (ipsilateral to the injection site) were in the medial preoptic area (15.7%) and the medial preoptic nucleus (7.4%): too few anterior hypothalamic area and preoptic suprachiasmatic area sections were available to count. The distribution of double-labeled neurons did not differ from that illustrated above for Brains 1-1, 1-3, and 2-6 (Figures 2 - 3, 2 - 7, and 2 - 9).

## V. Discussion

### A. Main Findings

The main finding of these combined autoradiographic-fluorescent dye retrograde tracing studies is that considerable numbers of estrogen-concentrating cells in the preoptic region of the rat brain are neurons that have long projections to the midbrain. These neurons thus have the capacity to transmit steroid hormone-regulated signals

directly to their midbrain targets.

Many estradiol-concentrating neurons which send axons to the midbrain were found in the medial preoptic nucleus and the surrounding medial preoptic area; in the ventral portion of the preoptic continuation of the bed nucleus of the stria terminalis; in the rostral anterior hypothalamic area; and in the lateral preoptic area/lateral hypothalamus continuum. Scattered dye-labeled, estradiol-concentrating cells were found in the preoptic suprachiasmatic region; such cells were extremely rare in the periventricular preoptic area, in the lateral septum, and in the diagonal band nuclei. A previous report of such projections from the ventromedial hypothalamic nucleus to the midbrain (Morrell and Pfaff, 1982) was confirmed in these studies.

In all cases these so-called "double-labeled neurons" were found adjacent to neurons which only concentrate estradiol and neurons which only project to the midbrain.

Double-labeled neurons were present in the preoptic suprachiasmatic area, the medial preoptic nucleus, the medial preoptic area, the bed nucleus of the stria terminalis, the anterior hypothalamic area, and the lateral preoptic area after injections of fluorescent retrograde tracers into the ventral tegmental area alone, the midbrain central grey alone, or after combined large injections which included both structures. Double-labeled neurons were seen in the ventromedial hypothalamic nucleus only after midbrain central grey dye injections and <u>not</u> after tracer injections successfully confined to the ventral midbrain (ventral tegmental area and interpeduncular nucleus). Injection of retrograde tracer into the lateral midbrain

(mesencephalic reticular formation) resulted in significant numbers of double-labeled neurons only in a small region of the caudal and ventral bed nucleus of the stria terminalis and in the ventrolateral subdivision of the ventromedial hypothalamic nucleus.

B. Diencephalic Neurons Afferent to the Ventral Tegmental Area and the Midbrain Central Grey.

Descending afferents to the medial structures of the midbrain have been previously studied in retrograde tracing experiments using horseradish peroxidase (Phillipson, 1979c; Morrell et al., 1981). Projections to the ventral tegmental area and the midbrain central grey have also been identified in numerous autoradiographic studies in which anterograde transport of [$^{3}$H]-amino acids was used to label efferent pathways from the preoptic area and the hypothalamus (Conrad and Pfaff, 1976a, 1976b; Swanson, 1976; Krieger et al., 1979; Saper et al. 1977; Swanson et al. 1978). However, with the exception of the brief report from Morrell and Pfaff (1982), the sensitive fluorescent dyes have not previously been used in retrograde tracing studies to locate neurons afferent to the dorsal and ventral midbrain. The retrograde tracing results obtained in this study by and large confirm earlier accounts, with the distinction that in every case the total number of afferent neurons identified is greater than has previously been described.

C. Estradiol-Concentrating Regions Afferent to the Midbrain

Medial Preoptic Region: Medial Preoptic Nucleus and Medial Preoptic Area. Despite the early recognition of the medial preoptic

nucleus in the rat (Gurdjian, 1927), almost all published experimental neuroanatomical studies of the medial preoptic region have made no distinction between the cell-dense "nucleus" and the surrounding "area". To some extent this simplification has been justified, as it was only recently that significant subdivisional differences in efferent or afferent pathways and structure have been identified (see discussion of medial preoptic region nomenclature in Chapter 1). For the purpose of clarity in comparison of these results with those extant in the neuroanatomical literature, the phrase "medial preoptic region" will be used instead of the traditional "medial preoptic area" to mean the entire region consisting of the medial preoptic nucleus and the surrounding area bordered ventrally by the optic chiasm, dorsally by the anterior commissure and the bed nucleus of the stria terminalis, laterally by the lateral preoptic area, and medially by the periventricular preoptic area. The terms "medial preoptic nucleus" and "medial preoptic area" will be used exclusively as defined in the preceding chapter.

In all cases in which fluorescent retrograde tracers were successfully deposited in the ventral tegmental area, the midbrain central grey, or both, hundreds of retrogradely-labeled neurons were identified in the medial preoptic neuron and the medial preoptic area, throughout the entire rostral-caudal extent of this region. The labeling was mainly ipsilateral, although a weaker contralateral component was always present even when there was no significant spread of injected dye across the midline. In transverse section, the distribution of dye-labeled neurons appeared to be continuous across the medial preoptic region, lateral preoptic area, and the ventral

portion of the preoptic continuation of the bed nucleus of the stria terminalis. Moving caudally, many retrogradely-labeled neurons were also present in the rostral anterior hypothalamic area (at the level of the suprachiasmatic nucleus): their numbers greatly diminished caudal to this region.

This pattern of a strong medial preoptic region projection to certain medial midbrain structures is in agreement with studies by Conrad and Pfaff (1976b) and by Swanson et al. (1978) in which preoptic region efferents were investigated autoradiographically using tritiated amino acids as anterograde tracers [an earlier study from Swanson (1976) assigned the medial preoptic region a more limited set of projections: this report can now be seen to be accurate, but incomplete]. Similar medial preoptic region projections have also been described for the guinea pig (Anderson and Shen, 1980).

The present data corroborate and extend the results of retrograde tracing studies by Morrell et al. (1981) and Phillipson (1979c), in which horseradish peroxidase injections were made respectively into the midbrain central grey and the ventral tegmental area. The tendency, reported by Conrad and Pfaff (1976b), for the more lateral portions of the medial region to have the heaviest long descending projections was confirmed. In the present material, the medial preoptic area and approximately the lateral two-thirds of the medial preoptic nucleus contained greater numbers of retrogradely-labeled neurons after fluorescent dye injections into the midbrain central grey and the ventral tegmental area than did the most medial one-third of the medial preoptic nucleus. This difference may correlate to some degrees with the subdivision of the medial preoptic nucleus into

medial and lateral parts by Simerly et al. (1984), although the exact boundary between these subnuclei is difficult to delineate in thin, uncounterstained material.

As mentioned in the Results section above, this tendency for retrogradely-labeled neurons to be found lateral to the most medial preoptic region means that the region in which the density of estradiol-concentrating neurons is highest (very medial, as described by Pfaff and Keiner, 1973) does not overlap with the region of densest retrograde dye-labeling from the midbrain. The connections of this medial region rich in estradiol-concentrating neurons were not revealed in these studies. However, the number of estradiol-concentrating neurons, though comparatively lower, is still extremely high throughout the entire medial preoptic nucleus and medial preoptic area relative to other brain regions. There is ample overlap in the distributions of these two tyes of label to permit the possibility of substantial double-labeling.

As the results above indicate, considerable double-labeling was indeed found after tracer injections into the midbrain central grey and ventral tegmental area. No notable differences in the distribution of double-labeled neurons through this region were detected when ventral tegmental area - only and midbrain central grey - only cases were compared.

Figures 2 - 3, 2 - 7, and 2 - 9 clearly illustrate the general pattern of double-labeling observed in the medial preoptic region. To summarize, dye-labeled, estradiol-concentrating neurons were found mainly in the lateral portion of the medial preoptic nucleus, although scattered double-labeled neurons could occasionally be found more

medially. The majority of the double-labeled neurons present in the surrounding medial preoptic area were found just lateral and dorsal to the medial preoptic nucleus, while the region immediately dorsal to the medial preoptic nucleus contained few estradiol-concentrating neurons, few retrogradely-labeled neurons, and few double-labeled neurons.

The massive retrograde labeling found in the medial preoptic region after medial midbrain dye injections contrasts with the low number of fluorescing cells found after an injection of a similar quantity of dye in a lateral midbrain region dorsal to the medial lemniscus and lateral to the central grey, in what may be regarded as the rostral portion of the mesencephalic reticular formation. Evidence for a very weak medial preoptic region projection to the lateral midbrain may be found in the reports of Conrad and Pfaff (1976b), Morrell et al. (1981), and Shammah-Lagnado et al. (1983). Only rare medial preoptic region dye-labeled neurons (1 - 3/section) were identified in this case, and double-labeled neurons were correspondingly rare, although it is possible that more might have been revealed if a larger or more caudal injection of tracer had been made.

Bed nucleus of the stria terminalis (septal divisions and preoptic continuation). Injection of retrograde tracer into both the ventral tegmental area and the midbrain central grey yielded scattered dye-labeled neurons in the rostral (septal) subdivisions of the bed nucleus of the stria terminalis and numerous fluorescing cells in the ventral portion of the preoptic continuation of that nucleus. The distribution of these retrogradely-labeled neurons is identical to

that described by Morrell et al. (1981) after a large horseradish peroxidase deposit was made into the ventral portion of the midbrain central grey and the adjacent reticular formation. At all levels, the majority of the labeled neurons occupied the ventral part of the bed nucleus of the stria terminalis. In transverse sections, dye-labeled neurons appeared to be distributed in a continuous pattern from the dorsolateral preoptic area and anterior hypothalamic area into the bed nucleus of the stria terminalis. These anatomical findings are also in agreement with those of Phillipson (1979c), who reported finding retrogradely-labeled neurons in the bed nucleus of the stria terminalis subsequent to a restricted iontophoretic deposit of horseradish peroxidase into the ventral tegmental area. In anterograde tracer studies using [$^{3}$H]-amino acids, several reports have further confirmed the existence of bed nucleus of the stria terminalis afferents to medial regions of the midbrain. Labeled fibers were present in both the ventral tegmental area and the midbrain central grey after a small tracer injection into the rostral bed nucleus of the stria terminalis at the level at which the anterior commissure can be seen as a long, continuous band in transverse section (Swanson and Cowan, 1979). The same midbrain targets also received a labeled projection after [$^{3}$H]-amino acid injection into the ventral portion of the preoptic continuation of this bed nucleus (Conrad and Pfaff, 1976b; Swanson, 1976). Conrad and Pfaff (1976b), on the basis of their anterograde studies, emphasized the close similarity of the axonal projections of the bed nucleus of the stria terminalis and the medial preoptic region. A comparable impression is gained by examination of the present material. Further evidence of

this close relationship is the finding that many estradiol-concentrating neurons in the ventral portions of the bed nucleus of the stria terminalis share with many estradiol-concentrating neurons in the medial preoptic region the property of long axonal projections to the ventral tegmental area and the midbrain central grey. This projection was mainly ipsilateral, with only a weak contralateral component. In all of the medial injection site cases examined, it appeared that relatively fewer cells in the bed nucleus of the stria terminalis were retrogradely labeled in comparison to the medial preoptic region and the lateral preoptic area. This was reflected in a lower percentage of double-labeled neurons in this bed nucleus.

There is no suggestion in the literature that one of the differences between bed nucleus, stria terminalis and medial preoptic region projections is that the bed nucleus axons have a wider distribution in the mesencephalic reticular formation. The lateral midbrain injection site case in this study (Brain 2 - 7), however, produced significantly more retrogradely labeled cells in the strial bed nucleus than in the medial preoptic region. In particular, the discrete ventral group of dye-labeled cells seen in sections from Brain 2 - 7 was not present in any other case examined in this study.

Anterior hypothalamic area (including the lateral anterior nucleus). As in the medial preoptic region, large numbers of neurons in the anterior hypothalamus concentrate radiolabeled estradiol (Pfaff and Keiner, 1973). Many retrogradely-labeled cells are found in the rostral portion of this area after tracer injection into the midbrain central grey and the ventral tegmental area. The existence of this

set of midbrain afferents has been previously demonstrated by a variety of neuroanatomical tracing techniques (e.g., Conrad and Pfaff, 1976c; Phillipson, 1979c). Caudal to the level of the suprachiasmatic nucleus, the numbers of dye-labeled cells in this area diminish greatly, with a single striking exception in cases in which the fluorescent dye injection site included the midbrain central grey. In these cases, a distinct cell group in the ventrolateral portion of the anterior hypothalamus, the lateral anterior group of Bleier et al. (1979) consistently contained large numbers of brightly fluorescing retrogradely-labeled neurons. This dye-labeled cluster was extremely prominent in several cases, in part due to the lack of retrograde label in the immediately adjacent hypothalamic region.

It appears that this cell group with strong projections to the midbrain central grey coincides with the ventrolateral anterior hypothalamic area group (VL-AHA) described by Morrell et al. (1981). In that report, this cell group was found to contain large numbers of horseradish peroxidase-filled neurons after enzyme deposition in the dorsal midbrain.

Tracer injection sites which involved some portion of the midbrain central grey or the brain region just lateral to the central grey always resulted in retrogradely-labeled neurons in the hypothalamic lateral anterior nucleus (see Figure 2 - 13 for an example of the distribution of these cells). In Brain 1-1, in which the dye True blue appeared to be largely confined to the ventral tegmental area, True blue-labeled neurons were also identified in the ventral anterior hypothalamic area. In Brain 2-6, however, extremely few dye-labeled neurons were found in the lateral anterior nucleus

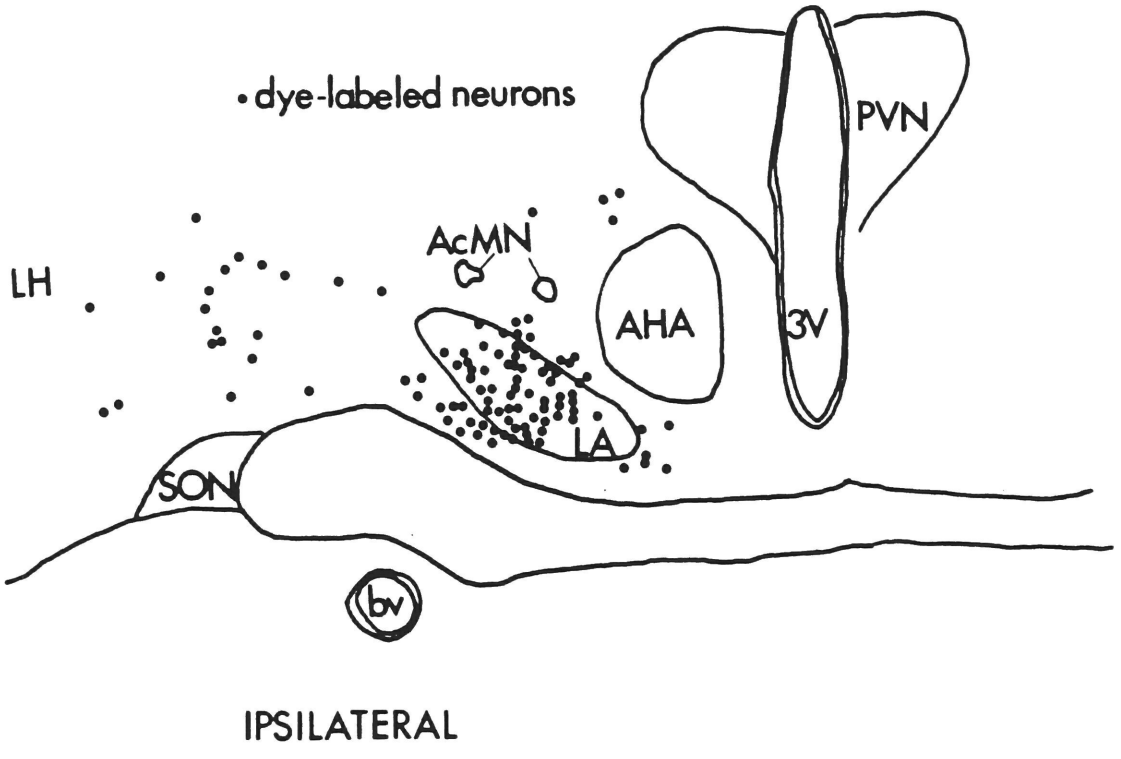



Figure 2 - 13. This figure shows the distribution of retrogradely-labeled neurons in the caudal anterior hypothalamus at the level of the hypothalamic paraventricular nucleus. This representative section was prepared from a camera lucida drawing of a section from Brain 1-3 (DAPI injection into the ventral tegmental area and midbrain central grey. (Each • represents one dye-labeled neuron.) This is approximately the most caudal extent of the lateral anterior hypothalamic cell group. Dye-labeled neurons are numerous in this region throughout its extent.

ABBREVIATIONS AcMN, accessory magnocellular neurons; AHA anterior hypothalamic area; bv, blood vessel; LA, lateral anterior nucleus of the anterior hypothalamus; LH, lateral hypothalamus; PVN, paraventricular nucleus; SON, supraoptic nucleus; 3V, third ventricle.

after bilateral tracer injection in the caudal ventral tegmental area. Examination of the complete injection sites (see Appendix 1) suggests that Brain 1-1 is characterized by a more rostral ventral tegmental area dye placement and a slighty more dorsal spread of tracer in comparison to Brain 2-6, in which the dye is well-confined to the caudal portion of the ventral tegmental area. Thus it is possible that the lateral anterior nucleus projects strongly only to the midbrain central grey and not to or through the ventral midbrain. The question cannot be resolved on the basis of the present material. Beyond the previous description of a strong midbrain central grey projection, little is known about this anterior hypothalamic nucleus. Its separate existence has not been taken into account in most neuroanatomical tracing studies of this region. It is not identified in the standard rat brain reference atlases of either Konig and Klippel (1963) or Paxinos and Watson (1982). Bleier et al. (1979) describe the lateral anterior nucleus as a "broad curved band of medium-sized cells lying laterally in the caudal part of the anterior area", with "its ventral and lateral edge"... "sharply demarcated by a prominent fiber bundle" (ibid, p. 156). Thus, it is particularly easy to identify in sections stained for fiber tracts (e.g. by Luxol fast blue) as well as by a Nissl stain (e.g. cresyl violet). Some scattered estradiol-concentrating neurons were present in this region, and a small subset of these were also retrogradely-labeled after dye injection into the midbrain central grey. Nevertheless, the majority of the double-labeled neurons found in the anterior hypothalamic region were located in that part of the anterior hypothalamic area immedately caudal to (and in actuality continuous with) the medial

preoptic nucleus/medial preoptic area complex and immediately ventromedial to the preoptic continuation of the bed nucleus of the stria terminalis.

Preoptic suprachiasmatic region (including the anteroventral periventricular nucleus). The rostral-most extension of the medial preoptic region, the so-called preoptic suprachiasmatic region, is an ill-defined area containing several distinct cell groups present in the periventricular zone around the optic recess and the nascent third ventricle. Its caudal border is formed by the medial preoptic nucleus, and a dorsal boundary is formed by the nucleus of the diagonal band. This region has recently been variously subdivided into several distinct nuclear groups, including the anteroventral periventricular nucleus (AVPv) of Simerly et al. (1984). Examination of steroid hormone autoradiograms, however, reveals that estradiol-concentrating cells are present in high density throughout this region (as defined by König and Klippel, 1963, and Pfaff and Keiner, 1973). For the purposes of this study this entire region will be considered to be the "preoptic suprachiasmatic area". As described above, many estradiol-concentrating neurons in this area were retrogradely labeled with fluorescent dye after midbrain injections into either the ventral tegmental area, the midbrain central grey, or both.

Little is known about the connectivity of this region. Morrell et al. (1981) reported that horseradish peroxidase-filled neurons were present in the region around the optic recess after large dorsal midbrain injections of this retrograde tracer, particularly in cases in which the injection was placed in the rostral midbrain. Cells in

this region have also been demonstrated to project to the median eminence (Lechan et al., 1982). The present study suggests that, in addition, the preoptic suprachiasmatic region may share with the adjacent medial preoptic region a projection to the medial portion of the ventral midbrain. A subset of these cells concentrate estradiol.

Luteinizing hormone-releasing hormone (LHRH) immunoreactive cell bodies have been localized to this region (e.g, Silverman and Krey, 1978; Witkin et al., 1982; Merchenthaler et al., 1984). In female rats circulating levels of estrogens appear to regulate the LHRH content of these neurons (e.g., Shivers et al., 1983b). This is apparently not the result of direct action of estradiol on LHRH-containing cells, as combined immunocytochemical-steroid hormone autoradiographic studies have revealed that LHRH neurons do not concentrate radioactive estradiol (Shivers et al., 1983a). In the guinea pig (Silverman and Krey, 1978), the hamster (Jennes and Stumpf, 1980), and in the rat (Witkin et al., 1982; Merchenthaler et al., 1984) LHRH-positive fibers have been detected in the region of the supramammillary commissure: this tract continues caudally in the ventral tegmentum to an apparent terminal field in the interpeduncular nucleus. LHRH fibers have also been described as a component of the fasciculus retroflexus, and immunocytochemically-identified LHRH fibers are also prominent in the midbrain central grey around the third ventricle and the cerebral aqueduct. Thus, it is possible that some of the non-estradiol-concentrating neurons retrogradely-labeled in this study in the rostral medial preoptic region and preoptic suprachiasmatic region represent LHRH-containing cell bodies.

Some of the questions regarding the nature of efferents from the

preoptic suprachiasmatic area would be resolved by anterograde tracing studies of this region using tritiated amino acid autoradiography. Such data are not to my knowledge available.

The lateral preoptic area and the lateral hypothalamus. Although the lateral preoptic and hypothalamic regions of the rat brain are not typically thought of as steroid hormone target tissue, scattered cells in this region are consistently found that exhibit nuclear binding of radioactive estradiol (Pfaff and Keiner, 1973). Many of these cells are located just ventral to the preoptic continuation of the bed nucleus of the stria terminalis and just lateral to caudal medial preoptic area. Many of the cells in this region are retrogradely labeled with fluorescent dye after tracer injections into the dorsal midbrain or the ventral tegmental area. Some of these neurons are double-labeled. However, an accurate estimate of the percentage of cells in this region which do so is difficult to obtain, as the scattered nature of the estradiol-binding cells makes precise charting via camera lucida drawings difficult.

The projection from the lateral preoptic area/lateral hypothalamic continuum to the midbrain has been extensively described both by anterograde (Swanson, 1976; Saper et al., 1979; Berk and Finkelstein, 1982) and retrograde tracing methods (Phillipson, 1979c; Morrell et al., 1981). The impression produced by examination of the present material that fewer cells project to the midbrain from the anterior lateral preoptic area than from the lateral preoptic area/lateral hypothalamic transition region and more caudal lateral hypothalamus sections is in agreement with the reports of Swanson (1976) and Saper et al. (1979). The reverse pattern is seen in the

medial preoptic and anterior hypothalamic region (see discussion above).

The periventricular preoptic area. It was a very infrequent occurrence for dye-labeled cells to be present in the periventricular area after midbrain retrograde tracer injections. Thus, despite the considerable number of estradiol-concentrating neurons counted in this region, double-labeled neurons were found only rarely.

The projections from this region have proved resistant subjects for experimental study, as tracer injections are difficult to confine to this region. The limited autoradiographic data of Swanson (1976) suggest projections to the periventricular grey, the median eminence, lateral hypothalamus, hypothalamic ventromedial nucleus, and the external fiber lamina of the mammillary complex. The projection to the median eminence has been confirmed in a retrograde tracing study using wheat germ agglutinin by Lechan et al. (1982). Further connections of the periventricular preoptic area were not revealed in this study. A recent brief report from this laboratory (Morrell and Pfaff, 1984) indicated a projection from the periventricular preoptic area (and the periventricular hypothalamus) to the medial amygdala. Using the combined fluorescent dye retrograde tracing-steroid hormone autoradiographic technique, these investigators determined that some of the cells retrogradely-labeled in this study also concentrate [$^{3}$H]-estradiol. The possibility of a minor periventricular region-medial amygdala connection was previously indicated by Ottersen's (1982) horseradish peroxidase study of the afferent connections of the amygdaloid complex.

Septal nuclei. The autoradiographically-revealed pattern of

cells concentrating radioactive estradiol described by Pfaff and Keiner (1973) was identical to that observed in this study. After fluorescent tracer injections into the midbrain, scattered dye-labeled neurons were present in the lateral septum, medial septum, and (after ventral tegmental area injections which involved the interpeduncular nucleus and the fasciculus retroflexus) the septal triangular nucleus. Only insignificant numbers of double-labeled neurons were identified in these septal regions. (Some double-labeled neurons may have been missed in the lateral septal area rostral to the level at which tissue sampling began). These descending projections appear to constitute a minor component of the septal efferents. The projections to the ventral tegmental area have previously been described by Meibach and Siegel (1977) and by Swanson and Cowan (1979). Morrell et al. (1981) reported only sparse horseradish peroxidase labeling in the septal nuclei after dorsal midbrain deposits of retrograde tracer. No septal afferents to the ventral tegmental area were revealed in the horseradish peroxidase studies reported by Phillipson (1979c).

The median preoptic nucleus. This small nucleus positioned between the dorsal tip of the third ventricle and the anterior commissure occasionally contained estradiol-concentrating neurons. A few retrogradely-labeled neurons were often present in this nucleus after medial midbrain dye injections (none were seen in the case featuring a lateral mesencephalic reticular formation injection site). Double-labeling was sometimes observed in this region, but the numbers of neurons involved are so small that percentage of overlap was not estimated in most cases.

Although no efferents from the median preoptic nucleus were

revealed in the 1976 autoradiographic tracing study by Swanson, a circumscribed projection to the supraoptic nucleus has been subsequently described (Miselis et al., 1979). The only additional published descriptions of median preoptic nucleus efferents are incidental observations of anterograde transport of horseradish peroxidase conjugated with wheat germ agglutinin in a study of median preoptic nucleus afferents by Saper and Levisohn (1983). These investigators reported that a few labeled fibers could be traced caudally to both the ventral tegmental area and the midbrain central grey. The present report confirms these observations.

Diagonal band nuclei (including the magnocellular preoptic region). Estradiol-concentrating neurons are scattered throughout the diagonal band area (Pfaff and Keiner, 1973). Retrogradely-labeled neurons were consistently found in the diagonal band nuclei after fluorescent dye injection into the dorsal and ventral midbrain, but estradiol-concentrating, dye-labeled neurons were identified only rarely. Again, it is possible that some regions of overlap were missed in this study in the rostral portions of the diagonal band nuclei as tissue sampling typically began at a level just rostral to the optic recess.

The nomenclature and nuclear boundaries that will be used in the following discussion will be that employed in the rat brain atlas of Paxinos and Watson (1982), with the qualification that the magnocellular preoptic neurons are to be considered as a distinct cellular group within the lateral portion of the nucleus of the horizontal limb of the diagonal band. In the rostral ventral portion of the vertical limb of the diagonal band, many retrogradely-labeled

neurons were present near the midline and near the base of the brain, with scattered dye-filled neurons stretching out laterally in lower density from this ventral cluster. Labeling in this region was seen only after ventral midbrain dye injections, as would be predicted from the anterograde tracing studies of Conrad and Pfaff (1976b). Phillipson (1979c) also noted significant retrograde labeling in the nucleus of the vertical limb of the diagonal band after small horseradish peroxidase injections into the ventral tegmental area.

The dorsal portion of the vertical limb of the diagonal band is not easily differentiated in unstained material from the medial septum, but it appeared that retrogradely-labeled neurons were present near the midline in this region after dye injection into both dorsal and ventral midbrain.

The nucleus of the horizontal limb of the diagonal band (including the magnocellular preoptic neurons) contained dye-labeled neurons after retrograde tracer injections into either the dorsal midbrain or the ventral tegmental area. Rarely were double-labeled neurons were detected in this area, which contains relatively few estradiol-concentrating neurons. The pattern of labeling in this lateral region was similar to that illustrated by Morrell et al. (1981). Retrogradely-labeled magnocellular preoptic neurons were seen mainly in one case, Brain 2-6, in which the injection site was confined to the ventral tegmental area. No labeled neurons were seen in this region after dye injection into the rostral lateral mesencephalic reticular formation.

Ventromedial hypothalamic nucleus and the arcuate nucleus. These medial basal hypothalamic nuclei were not the focus of this study:

fewer sections were sampled from this region and observations on their connectivity will be presented here only briefly.

As previously reported by Morrell and Pfaff (1982), examination of this material revealed that a significant proportion (15 - 30%) of the estradiol-concentrating neurons in the ventrolateral portion of the ventromedial nucleus send axons to the dorsal midbrain (example: Brain 2-5). Very little retrograde dye-labeling and hence almost no double-labeling was observed in cases in which the tracer injection site was confined to the ventral tegmental area (Brain 2-6) or to the midbrain region lateral to the midbrain central grey (Brain 2-7). A sparse projection from the ventromedial hypothalamic nucleus to the ventral midbrain and also to the lateral midbrain has been described in two anterograde tracing experiments (Saper et al., 1977; Krieger et al., 1979): these observations were not substantially expanded in the present study. One possible explanation for the relative lack of ventromedial hypothalamic nucleus labeling is that the case (Brain 2-6) in which fluorescent dye was confined to the ventral tegmental area featured an injection site placed caudally in this region (at the level of the interpeduncular nucleus). Ventromedial hypothalamic nucleus axons seen just dorsal to the supramammillary complex may turn in a lateral direction in the rostral ventral tegmental area (Krieger et al., 1979).

Retrogradely-labeled neurons were present in the arcuate nucleus (including in the rostral retrochiasmatic region ventral to the third ventricle) after dye injections including the midbrain central grey. As reported by Morrell and Pfaff (1982) in their more extensive study, very few of these dye-labeled neurons also concentrated radioactive

estradiol (see Brains 2-5 and 2-6).

Other Regions with Descending Afferents to the Midbrain. Ventral midbrain injection sites which included the ventral tegmental area resulted in scattered retrogradely-labeled neurons in the nucleus accumbens, in accord with the anterograde tracing studies of Conrad and Pfaff (1976) and Nauta et al. (1978). The ventral pallidum (included by some authors within the substantia innominata) also contained scattered dye-labeled cells in these cases. A similar finding based on horseradish peroxidase studies was reported by Phillipson (1979). In the one case in which the injection site clearly included the rostromedial tip of the substantia nigra (Brain 1-3), a narrow longitudinal band of dye-labeled neurons was seen adjacent to the ipsilateral lateral ventricle. These cells appeared to be on the lateral edge of what Kemp and Powell (1971) described as a "narrow zone, relatively free of neurons, beneath the ependyma of the lateral ventricle in which there is a large number of glial cells" (ibid, p. 386). Although such a projection was not demonstrated in the horseradish peroxidase retrograde tracing study in the rat of Bunney and Aghajanian (1976), these authors do not describe their injection sites in detail, and it is not clear if they have included an extremely rostromedial tracer placement. A topographically organized medial caudate-to-medial substantia nigra relationship was observed by Domesick (1977) in an autoradiographic anterograde tracing study, and the older literature based mainly on degeneration studies in cats fully concurs with the localization of the striato-nigral projection observed in this study (Voneida, 1960; Niimi et al., 1970; Grofova, 1975).

In some cases the brain was prepared for cryostat-sectioning by blocking the edges in such a way that little dorsal or lateral cortex remained. In a few cases, however, the brain was not blocked and intact sections were mounted onto the emulsion-coated slides. In these cases it was not unusual for retrogradely-labeled neurons to be identified in the cerebral cortex. These neurons were found mainly in the cortical region just dorsal to the rhinal sulcus: their appearance and location suggested that they were layer 5 pyramidal cells. Occasionally, dye-labeled cells were also present in the frontal pole of the cerebral cortex. (This was an ipsilateral projection.) A similar population of retrogradely-labeled cortical neurons was identified in the horseradish peroxidase study of Morrell et al. (1981). The existence of direct corticomesencephalic projections has also been demonstrated in the rat in studies using anterograde tracing methods (e.g. Beckstead, 1979).

D. Estimating the Strength of Preoptic-Mesencephalic Connections

The agreement of the present neuroanatomical results with those obtained in previous studies using different tracing techniques is evidence that the neuronal labeling observed in this study is solely the result of axonal and terminal uptake of tracer from the injection site, and subsequent retrograde axonal transport of this material. This rules out the possibility of spurious labeling produced by spread of tracer away from the injection site (e.g. dye transport via a ventricular route). Given the finding that cells free

of fluorescent label were invariably found adjacent to labeled cells, it also seems unlikely that significant (if any) apparently "retrograde" label was produced by leakage of transported dye out of labeled soma and its subsequent uptake by neighboring soma. Such a possibility was further minimized by the short post-dye injection survival times employed and by restricting data analysis to only those cases in which the neuropil, glial cell populations, and ependymal cells lining the ventricular walls were entirely free from the characteristic blue fluorescence of the dyes used as tracers in this study.

Despite the conventionality of the pattern of retrograde-labeling seen in this material after midbrain tracer injection, it is striking that previous studies have not indicated the strength of these preoptic region-midbrain connections. For example, retrograde tracing studies employing horseradish peroxidase as the tracer molecule (e.g. Phillipson, 1979c; Morrell et al., 1981) have not revealed the large numbers of retrogradely-labeled cells seen in the medial preoptic nucleus and the surrounding medial preoptic area (sometimes, several hundred in a 6 or 12 μm section) in this study. One factor very likely to have contributed to the extensive retrograde labeling seen here is, as was mentioned in the methods section above, the extremely great sensitivity of certain of the fluorescent dye neuroanatomical tracers. The physicochemical events occurring at the neuronal membrane which account for the high levels of uptake and retrograde transport of these substances have not been determined. It is also possible that the limitations of the other techniques (in terms of numbers of retrogradely-labeled cells seen) are related to the histochemical

visualization procedures necessary for the detection of axonally-transported materials.

Another factor which may have contributed to the large number of preoptic region cells whose midbrain connectivity was revealed in this study is that the dye injections, which were designed to maximize the retrograde labeling obtained, often combined two important preoptic targets, the ventral tegmental area and the midbrain central grey. In previous studies, confinement of tracer to a very restricted target area rather than optimization of labeling obtained was often the primary goal (e.g. Phillipson, 1979c).

Two remaining and perhaps more important factors must be taken into consideration in the interpretation of these results. The first is the contribution of dye uptake by fibers of passage to the pattern of labeling detected; the second issue is the possible contribution of afferents to midbrain structures other than the midbrain central grey or the ventral tegmental area inadvertently or unavoidably included in the dye injection sites analyzed here.

Recently, evidence has accumulated in favor of earlier concepts (e.g. Beattie et al., 1930) that forebrain neurons may project to pontine, medullary, and spinal levels. Various modern neuroanatomical tracing techniques have been used to demonstrate the existence of cells in hypothalamic and preoptic regions which have such long, descending axons (Swanson and Kuypers, 1980; Hosoya, 1980; Sofroniew and Schrell, 1980; Watkins et al., 1981; Schwanzel-Fukuda et al., 1984). Anterograde tracing studies using [$^{3}$H] amino acids indicate that many of these axons travel medially through the midbrain (e.g. Conrad and Pfaff, 1976a, b) and would be well-placed to take up fluorescent

dyes from our midbrain central grey and ventral tegmental area injection sites. Thus, it is possible that many of the estradiol-concentrating neurons which were identified in this study as having midbrain connections may not actually have the capacity to influence midbrain function directly.

Several types of evidence argue that many preoptic region neurons may terminate in synaptic contacts in the ventral tegmental area and the midbrain central grey, particularly neurons located in the medial preoptic nucleus and the medial preoptic area. First, in anterograde tracing studies in which [$^{3}$H] amino acids are placed in the preoptic region, the number of labeled fibers observed diminishes substantially caudal to the ventral tegmental area and the periaqueductal grey (Conrad and Pfaff, 1976b; Swanson et al., 1978). In addition, the scattered silver grain patterns often characteristic of terminal fields have been observed in these regions. Second, retrogradely-labeled neurons have been identified in the preoptic region after horseradish peroxidase applications to the midbrain (Phillipson, 1979c; Morrell et al., 1981) which did not apparently produce damage at the injection site. It is well-established that horseradish peroxidase is rarely taken up by intact axons (Mesulam, 1982). Third, electrophysiological studies have indicated short-latency effects on midbrain neural activity resulting from preoptic region stimulation (Maeda and Mogenson, 1980). Finally, an extensive quantitative study by Schwanzel-Fukuda et al. (1984) used immunocytochemical detection of retrogradely-transported wheat germ agglutinin to localize forebrain neurons projecting directly to the medullary region and spinal cord of the rat. The tracer delivery

method (implantation of a slow-release polyacrylamide gel pellet) was chosen to maximize the number of retrogradely-labeled cells detected. The results of this study demonstrated that, while significant numbers of wheat germ agglutinin-filled neurons were present in the lateral hypothalamus, the paraventricular nucleus, the bed nucleus of the stria terminalis, and the medial basal hypothalamus after gel pellet implants into the medulla and spinal cord (the lateral hypothalamus was particularly well-labeled), extremely few labeled cells were seen in the medial preoptic nucleus, the medial preoptic area, the lateral preoptic area, and the anterior hypothalamic area. These findings strongly imply that the majority of estradiol-concentrating neurons in the medial preoptic nucleus, the medial preoptic area, the lateral preoptic area, and the anterior hypothalamic area counted in our study as having dorsal and ventral midbrain projections are correctly identified. A midbrain site of termination for the dye-labeled, estradiol-concentrating neurons of the bed nucleus of the stria terminalis is also possible, but not proved.

It seems unlikely that the midbrain structures inadvertently included in the fluorescent dye injection sites contributed in any significant way to the observed preoptic region pattern of retrograde-labeling. Many of these structures (e.g. the substantia nigra, the accessory occulomotor nuclei) have distinct patterns of afferents.

The only previously described preoptic region projections to the interpeduncular nucleus, which often contained fluorescent dye after ventral midbrain injections, arises in the nucleus of the diagonal band (Contestabile and Flumerfelt, 1981), and the existence of even a

sparse projection is a matter of dispute (Marchand et al., 1980).

Caudally, the midline raphe nuclei have been demonstrated to receive input from the medial preoptic region, the lateral preoptic area, the diagonal band nuclei, and the bed nucleus of the stria terminalis (Aghajanian and Wang, 1977). Large dye injections may have identified afferents to these nuclei as well as to the ventral tegmental area and the midbrain central grey.

Another probable additional source of preoptic region retrograde label in this study is the mesencephalic reticular formation laterally adjacent to the medial target areas. Several studies have demonstrated the existence of sparse but direct projections from the medial and lateral preoptic region (Morrell et al., 1981; Shammah-Lagnado et al., 1983). Overall, the mesencephalic reticular formation appears to share weakly in the afferents to the medial midbrain structures. Anterograde tracing studies, however, while confirming the existence of these lateral projections, support the conclusion that the majority of preoptic region retrograde labeling observed in this material, particuarly that found in the medial preoptic region, represents afferents to (and possibly through) the midbrain central grey and ventral tegmental area.

E. Functional Implications

This study has identified a set of neurons which share two characteristics: they exhibit nuclear binding of estradiol and they send their axons to the medial midbrain. Although the neurons which are members of this set can be described as being located within particular nuclear groups, in reality they are distributed across the

preoptic region with little regard for conventional cell group boundaries.

These experiments were motivated by a desire to define the nature of the anatomical elements composing the functional pathway connecting the preoptic region to the midbrain. The existence of such a functional pathway was inferred from numerous lesion and knife cut studies in which bilateral damage to this pathway was shown to impair performance of maternal behavior (Avar and Monos, 1967, 1969; Numan, 1974; Numan et al., 1977; Terkel et al., 1979; Numan and Callahan, 1980; Jacobson et al., 1980; Miceli et al., 1983; Numan and Nagle, 1983; Numan and Smith, 1984; Gray and Brooks, 1984). [Similar functional pathways have been defined for other motivated or regulatory behaviors such as male sexual behavior and drinking associated with the maintenance of body fluid balance (Malsbury and Pfaff, 1974; Swanson and Mogenson, 1981). These topics will not be considered here although it should be borne in mind that the anatomical substrate attributed to the functional pathway for maternal behavior may be shared by these other functions.]

An extended period of care for the young is an intrinsically interesting biological adaptation of many avian and mammalian species, but the maternal behavior of rats has been selected for study here because achieving an understanding of its regulation will provide a partial means to the end of answering the question of how changes in circulating levels of steroid hormones alter behavior. To reach this long-term goal two classes of questions are posed as research problems. The first category consists of signal questions. The answers to such questions involve identifying signals and where (by what groups of

cells) they are received in the nervous system. For example, estrogen is a signal which increases the tendency of rats to respond to young pups with nurturing behavior instead of indifference or aggression, and one of the places this modulating signal is received appears to be the medial preoptic region of the brain. Future research will involve determining the intracellular signals by which estrogen regulates cell function and the signals used by the estrogen target cells to communicate with other neurons. The other category of questions consists of organizational questions, which ask on what principles the system is constructed. For example, maternal behavior in the rat consists by and large of responses which are not unique to the maternal state but which will be displayed many other times in the life of the animal (e.g., carrying objects, manipulating nest material). But rats will not direct these actions towards pups with short latency unless exposed to raised levels of estrogen, and hence one of the organizational features of maternal behavior is that its latency and the nature of the objects at which actions are directed but not the form of its motor components are regulated by the action of a steroid hormone on the brain.

A purely anatomical study such as that described in this chapter goes a very short way towards answering either type of question. However, our full appreciation of functional relationships depends on our knowledge of the elements--in this case the nerve cells--which make up the system. The explanatory power of functional studies lies in their interpretation in light of accurate anatomical information.

In the large vertebrate brain it is no easy matter to identify a set of cells involved in a particular function. Convergence of many

different types of evidence is required before such a set of cells can be confidently identified, especially when the endpoint is a complicated behavior extended in time over several weeks. Considerable caution must be exercised in assigning function to an anatomical pathway. Thus, the claims to be made in the following discussion are restricted to correlation with previous studies of the functional pathway relevant for maternal behavior and to outlining suggestions for future functional studies based on the present anatomical findings.

A role for the medial preoptic region in the regulation of maternal behavior is well-established, but poorly understood. This brain region appears to have a dual role, serving as the primary site for estrogen's actions on maternal behavior latency (Numan et al., 1977), but the medial preoptic region must also be intact for normal performance of maternal behavior in postpartum animals (Numan, 1974; Jacobson et al., 1980), and for the induction of maternal behavior to occur in non-estrogen-primed virgin rats continuously housed with foster pups (Numan et al., 1977; Miceli et al., 1983). The action of estrogen on the brain does not appear to be an essential component of the maintenance phase, although tests have been devised which reliably sort out estrogen-supported maternal behavior from that performed in the absence of this hormone (Leroy and Krehbiel, 1978; Stern and MacKinnon, 1976; Mayer and Rosenblatt, 1979). Thus, with regard to maternal behavior, the preoptic region must be more than simply a target site for estrogen, as the results of damage to this area are much more severe than the effects of ovariectomy. The other reasons for its importance to this behavior have not yet been determined. One suggestion is that it serves as a site where sensory information,

particularly olfactory information, is integrated with endocrine signals.

Although the importance of the medial preoptic region itself (and its lateral projections) has been clearly established for both the onset and maintenance of maternal behavior, whether or not the dual role attributed to the medial preoptic region is shared by other portions of the preoptic-to-midbrain functional pathway is not known as fully. Most tests of the effects of damage to this pathway have been carried out in postpartum animals (e.g., Numan and Nagle, 1983; Numan and Smith, 1984). In the one study, however, in which ventral tegmental area lesions were made prior to parturition (Gaffori and Le Moal, 1979), severe disruptive effects on maternal behavior were also reported. Thus, it is not unreasonable to assume, although not yet extensively tested, that the same pathway mediates both the preoptic area's influence on timing the onset of maternal behavior and its role in the maintenance phase. It is interesting to speculate whether or not the estrogen target cells which facilitate the onset of maternal behavior also support the hormone-independent forms of the behavior. Estrogen might then be considered only one of several relevant afferents, or it may have a primacy in that it might influence the capacity of cells to respond to other inputs.

The brain lesion studies of Numan (1974) which gave the first indication of the importance of this brain region for maternal behavior involved damage approximately localized to the ventral two-thirds of the medial preoptic region and the lateral three-fourths of this region. Examination of the lesion placements shows that the medial preoptic nucleus, the medial preoptic area, possibly the most medial

part of the lateral preoptic area, and the most rostral part of the anterior hypothalamic area would have been damaged by these lesions. In most cases even the ventral portion of the bed nucleus of the stria terminalis appears to be completely spared. These lesions completely eliminated retrieval and nest building, and only 1/10 of the lesioned females was ever observed to nurse her young.

Knife cuts in the parasagittal plane which sever connections between the medial preoptic/anterior hypothalamic region and the lateral preoptic area/lateral hypothalamus continuum, however, would have transected efferents from the bed nucleus of the stria terminalis as well as from the medial preoptic nucleus and the medial preoptic area; they also may have directly damaged neurons located in the region where the medial preoptic area, the bed nucleus of the stria terminalis, and the lateral preoptic area intersect (Numan, 1974; Numan and Callahan, 1980; Miceli et al., 1983). This is, of course, the area in which a high number of estradiol-concentrating neurons with projections to the midbrain was consistently detected in the present study. Such knife cuts reproduce the behavioral effects of medial preoptic region lesions.

Examination of lesions effective in preventing the onset of pup-induced maternal behavior in virgin rats suggests that damage restricted to the medial preoptic nucleus and the medial preoptic area is sufficient to impair responsiveness to pups.

The convergence of the lesion and knife cut data is interpreted to mean that the two manipulations are disrupting the same system. Close comparison of the nature of the behavioral deficits produced by medial preoptic region lesions and lateral knife cuts suggests that this is

indeed true, with nursing being the least affected component and retrieving and nest building being the most-affected component in both types of studies (Numan and Callahan, 1980; Jacobson et al., 1980; Numan and Smith, 1984). Interpretations of such studies is not always straightforward because even damage restricted to the medial preoptic region disrupts a number of ascending and descending neural systems. In two studies a relationship was proposed between damage in the dorsal medial preoptic region (Jacobson et al., 1980) or transection of a particular set of dorsal and caudal efferents from the medial preoptic region (Terkel et al., 1979), but the evidence for such a correlation is weak and indirect, with a confounding factor always being the fact that larger lesions or longer knife cuts are typically the most effective disruptors of normal behavior.

Only one experiment to date has provided direct correlational evidence between exact cells damaged and maternal behavior performance, and it is relevant to compare the present results with those findings (Numan et al., submitted). It had been previously demonstrated that damage to the lateral hypothalamus/medial forebrain bundle reproduces the effects of medial preoptic damage or knife cuts lateral to the medial preoptic area on maternal behavior (Avar and Monos, 1967, 1969). This is easily understood in light of knowledge that projections to and from this region travel largely in the medial forebrain bundle (Conrad and Pfaff, 1976b). There is a spatial segregation of efferents within the medial forebrain bundle, and this makes it possible to damage some pathways without harming others. In this particular study, however, the location of the cell bodies of origin of the cut fibers was not assumed but rather was directly demonstrated by means of a technique in

which the knife cut was produced by a horseradish peroxidase-coated wire knife. It was predicted that deficits in maternal behavior performance would be produced by knife cuts which would yield a large number of horseradish peroxidase-filled neurons in that part of the preoptic region in which the effective lesions had been located--that is, that they would be found primarily in the medial preoptic nucleus and the ventral portion of the medial preoptic area. Since autoradiographic tracing studies have demonstrated that the axons from this region travel in the ventral part of the medial forebrain bundle, it was expected that ventral lateral hypothalamic knife cuts would produce the most extreme deficits in maternal behavior and the highest numbers of retrogradely-labeled neurons in the medial preoptic nucleus and the immediately adjacent area. Dorsal cuts were expected to produce less of a maternal behavior impairment and greater numbers of horseradish peroxidase-labeled cells in other brain regions such as the lateral preoptic area and the bed nucleus of the stria terminalis. The anatomical predictions were entirely confirmed in this study, but the behavioral tests revealed the opposite of what had been expected: the dorsal lateral hypothalamic cuts produced the most extreme maternal behavior deficits.

Examination of the charts of the location of horseradish peroxidase-filled cell bodies reveals that the dorsal knife cuts resulted in strong retrograde labeling in the region where the dorsal and lateral medial preoptic area, ventral bed nucleus of the stria terminalis, and the lateral preoptic area share a common border as well as considerable numbers of peroxidase-labeled neurons in the ventral portion of the bed nucleus of the stria terminalis.

One interpretation of these findings, which indicate that maternal behavior can proceed even when many of the direct medial preoptic nucleus-to-midbrain connections are severed is that it is the short connections of the medial preoptic nucleus that are critical, and that the relevant anatomical pathway consists of short projections from the medial subdivisions of the preoptic region to the lateral preoptic region and the lateral hypothalamus, and then to the midbrain via a relay in these regions (Numan et al., submitted). The second link of this chain would be damaged by the dorsal lateral hypothalamic knife cuts. Another explanation would focus on the relatively small number of medial preoptic nucleus neurons labeled in the dorsal knife cut studies and suggest that the axons from a critical subset of medial preoptic nucleus neurons differ from other medial preoptic nucleus in that their axons travel in the dorsal portion of the medial forebrain bundle. A third interpretation is that the neurons critical for this behavior are those which were identified in both the present study and the Numan et al. (submitted) study, the neurons (many of them estradiol-concentrating) in the more dorsal and lateral portions of the medial preoptic region (including the ventral bed nucleus of the stria terminalis).

Both of these studies produced somewhat unexpected results and have shifted the experimental focus from the medial preoptic nucleus itself to the more lateral parts of this region. Numan et al., (submitted) have proposed that estrogen action enters into this functional pathway by means of binding to target cells in the medial preoptic nucleus which then send their axons to the lateral preoptic area/lateral hypothalamus; the axons of these lateral neurons then

descend to the ventral midbrain to influence systems controlling the motor aspects of behavior. The present results imply that the first link in this chain is not a prerequisite, as a substantial number of the elements which project directly to the midbrain and which must be intact if the behavior is to be performed are themselves estradiol target cells.

The major problem with such an interpretation is the results of the lesion studies which demonstrate that damage to the medial preoptic nucleus and the adjacent medial preoptic area alone is sufficient to render the animal incapable of nurturing pups. Perhaps descending efferents from both the medial and lateral cells of the medial preoptic region have a role in regulating maternal behavior performance in rats. It is possible that both sets of efferents must be damaged before the behavior is severely disrupted. Additional deficits were observed in the dorsal cut animals (loss of weight, hypoactivity) which raise the possibility that it was partial damage to the maternal behavior pathway plus the burden of the additional deficits that resulted in the poorer maternal behavior of the dorsal cut group.

Several types of studies are needed to resolve this issue. First, a better localization of the critical site of estrogen action within the medial preoptic region is needed. Experiments in which the behavioral effects of small implants of dilute estradiol into the medial and ventral preoptic region, the dorsal and lateral preoptic region, and the ventral bed nucleus of the stria terminalis would be particularly useful. Discrete lesions of the region identified in both studies as containing relatively large numbers of retrogradely-labeled cells (the dorsal and caudal part of the medial preoptic region) should

be examined for their effect on the hormonally-mediated onset and maintenance of maternal behavior. The present study also suggests that the midbrain central grey is as likely as the ventral tegmental area to receive projections from both estradiol-concentrating and non-steroid target cells located in the medial preoptic region. The effect of lesions of this region on maternal behavior should also be examined in experiments that parallel the studies demonstrating a role for the ventral tegmental area. From an anatomical point of view, characterization of the axonal projections of the many estradiol-concentrating neurons of the medial preoptic region not revealed in this study will add important information to our understanding of both the anatomical and functional pathways underlying this behavior.

A slightly different strategy of combining knife cuts lateral to the preoptic region with horseradish peroxidase or other retrograde tracing methods might also prove extremely useful. In this approach deposits of retrograde tracer would be made in the midbrain after behaviorally disruptive or ineffective (by design) knife cuts had been produced. Comparison of the pattern of retrograde labeling produced in the medial preoptic region after both types of knife cuts might reveal the location of the functionally relevant cells in the medial preoptic region by the absence of labeling in the absence of maternal behavior.

The evidence summarized above that the ventral tegmental area of the midbrain is one of the targets of the estrogen-regulated output of the medial preoptic region. It is of interest to note that the effective dorsal lateral hypothalamic knife cuts produced in the study

by Numan et al. (submitted) also resulted in large numbers of labeled neurons in the ventral tegmental area, suggesting that some of the deficits produced by the knife cuts are a result of damage to an ascending pathway from the ventral midbrain whose function can be regulated by the preoptic region. The function of the ventral tegmental area efferents in relationship to maternal behavior--that is, proposals for the next stage along the pathway from the preoptic region to the midbrain to maternal behavior will be discussed in Chapter 4.

Anatomical Abbreviations

| | |
|---|---|
| AA | anterior amygdala |
| ac | anterior commissure |
| AC | nucleus of the anterior commissure |
| ACo | cortical amygdaloid nucleus |
| ADPN | anterior dorsal preoptic nucleus |
| AHA | anterior hypothalamic area |
| BST | bed nucleus of the stria terminalis |
| BSTl | bed nucleus of the stria terminalis, lateral division |
| BSTm | bed nucleus of the stria terminalis, medial division |
| BSTpo | bed nucleus of the stria terminalis, preoptic continuation |
| cc | corpus callosum |
| cg | cingulum |
| Cl | claustrum |
| CPu | caudate putamen |
| f | fornix |
| fi | fimbria |
| FStr | fundus striati |
| GP | globus pallidus |
| HDB | horizontal limb of the nucleus of the diagonal band |
| ic | internal capsule |
| ICj | islands of Calleja |
| LA | lateral anterior hypothalamic nucleus |
| LPOA | lateral preoptic area |
| lo | lateral olfactory tract |
| LS | lateral septum |
| lv | lateral ventricle |
| MnPO | median preoptic nucleus |
| MPN | medial preoptic nucleus |
| MPOA | medial preoptic area |
| MS | medial septum |
| PO | primary olfactory cortex |
| POSC | preoptic suprachiasmatic area |
| PvPOA | periventricular preoptic area |
| ot | optic tract |
| SCN | suprachiasmatic nucleus |
| SFi | septofimbrial nucleus |
| SHy | septohypothalamic nucleus |
| SI | substantia innominata |
| sm | stria medullaris |
| SON | supraoptic nucleus |
| st | stria terminalis |
| TS | triangular septal nucleus |
| Tu | olfactory tubercle |
| V | third ventricle |
| VDBD | vertical limb of the nucleus of the diagonal band, ventral division |
| VDBV | vertical limb of the nucleus of the diagonal band, dorsal division |
| VP | ventral pallidum |

# Chapter 3 Behavioral Studies: Estrogen Dependent Oxytocin Effects on Maternal Behavior

## I. Introduction

In contrast to the reports of Pedersen and Prange (1979) and Pedersen et al. (1982; summarized in Chapter 1 of this thesis), Rubin et al., (1983) found no effect of central infusions of oxytocin on maternal behavior latency using similar (but not identical) test conditions. My first studies were therefore designed to replicate the findings of Pedersen et al. (1982) and to provide a fuller description of the behavioral effects of raised central levels of oxytocin in rats (Experiment 1). In Experiment 2, I examined the effects of intracerebroventricular oxytocin infusion in ovariectomized virgin rats not primed with estrogen. The effect of testing maternal behavior in familiar and novel environments is presented in Experiment 3, in which the ovariectomized, estrogen-primed preparation was again used. In Experiment 4, an attempt was made to assess the physiological importance of oxytocin facilitation of maternal behavior by using anti-oxytocin antiserum and an analog antagonist of oxytocin to reduce the effective action of endogenous oxytocin before and during first pup contact in hysterectomized and ovariectomized 16 day pregnant rats treated with estrogen. Experiment 5 consists of studies using microinjections of oxytocin directly into brain tissue rather than into the lateral ventricle with the objective of defining the oxytocin target sites.

## II. Experiment 1

In Experiment 1, the effects of intracerebroventricular (icv) injections of 400 nanograms of oxytocin (n = 20) vs. normal saline (n = 16) or no icv treatment (n = 12) on short-latency maternal behavior were studied in estrogen-primed virgins. The effects of icv infusion of oxytocin on body temperature were studied in an additional group of animals. Hyperthermia causes lactating rats to shorten nursing bouts (Leon et al., 1978; Woodside et al., 1980). While such a strong relationship has not been demonstrated between thermoregulation and other aspects of maternal behavior, the possibility of other interactions exists. In light of previous reports on effects of neurohypophysial hormones on body temperature (for a review, see Meisenberg and Simmons, 1983), I conducted this experiment to learn if oxytocin-induced changes in body temperature might be mediating the observed facilitation of responsivity to pups.

### A. Materials and Methods

Subjects and housing. Sprague-Dawley rats (ZM/SD) obtained from Zivic-Miller Laboratories, Inc. (Allison Park, PA) were used in all experiments. Virgin rats weighing 200-225 grams were singly housed in our facility one week before ventricular cannulation. Timed-pregnant females purchased from Zivic-Miller Laboratories at 12 or 14 days gestation served as pup donors. Test subjects were housed in plastic cages (20 cm x 20 cm x 40 cm) with food and water available ad libitum. Lights were on from 0600-1800 hr; room temperature was 20-22 $^{o}$C. Virgin test subjects were always housed in separate

rooms from pregnant and lactating rats to prevent incidental transmission of auditory or olfactory pup-related cues.

Surgery and estrogen treatment. Stainless steel guide cannulae (22 gauge; Plastic Products, Roanoke, VA) 4.1 mm long were stereotaxically implanted into the left lateral ventricle of test animals. Atropine sulfate (0.12 mg) was injected intraperitoneally (i.p.) ten minutes prior to anesthetization with i.p. sodium pentobarbital (Nembutal). After cannulation, animals were weighed daily, and, in order to accustom them to the handling necessary for icv injection, their implants were regularly inspected and cleaned. Eight days after cannulation, the animals were ovariectomized under Metofane anesthesia (Pittman-Moore) and given a subcutaneous (s.c.) injection of estradiol benzoate (EB) in corn oil vehicle (100 μg EB/kg body weight, 10 μg/0.1 ml corn oil). Various control groups (see Experiment 2 below) received the corn oil vehicle alone. Some animals were ovariectomized but not cannulated. These animals were weighed and handled daily according to the same procedure used for test animals.

Oxytocin infusion and maternal behavior tests. Oxytocin (gift of Dr. V. Hruby, University of Arizona School of Medicine, Tucson, AZ) was dissolved in sterile normal saline, pH = 5.4, a few minutes before use to yield a solution of 400 ng oxytocin/10 μl. In a rat uterine strip bioassay this oxytocin exhibited approximately 500 units activity/mg (Dr. W. Sawyer, Columbia University, NYC, NY; personal communication, December 1982). Two hours before oxytocin infusion animals were removed from their home cages and placed in larger test cages (36 cm x 47 cm x 20 cm). The test cage floor was fitted with a

Plexiglas divider 5 cm high and 0.6 cm thick which divided the cage into quadrants. An adult test animal could easily cross the Plexiglas dividers, but they proved an effective barrier to pup movement. The cage contained a 2.5 cm layer of wood shavings and approximately 30 strips of paper towel (1 cm x 26.5 cm). At the end of the 2 hour habituation period (48 hours after the EB injection), the animal was removed from the test cage and handheld while being injected with either oxytocin or the saline vehicle. The injection was made with a Hamilton 10 ul syringe fitted with a 31 gauge injection needle 0.5 mm longer than the guide cannula. The 10 µl injection volume was delivered over a period of 1 - 2 minutes. While the test animal was being injected, the presence or absence of a nest, its nature (flat or high-sided) and its location in the test cage were noted. In all cases new paper towel strips were added and the paper spread evenly over all quadrants. Three rat pups 12-24 hours old were then placed in the test cage, one in each quadrant which had not previously contained a nest. Behavioral observation began immediately at the conclusion of the injection, after the animal was replaced in the center of the cage. All testing took place in the light part of the cycle between 1000 and 1600 hr. Food and water were available ad libitum.

During the first hour with pups the test animal was observed continuously. A minute-by-minute record was kept in which all instances of the following behaviors were noted: sniffing a pup, licking a pup, carrying a pup, grouping two or three pups, crouching over pups, nest building, eating, drinking, cannibalizing, and grooming. In addition, the number of cage quadrants entered and the

amount of time spent grooming were also recorded once every minute. At the end of the first hour each animal was given a maternal behavior score (see below) and left undisturbed.

Two and four hours later the position of the test animal, pups, and nest were recorded, and the nest quality was assessed. Any animal found with three grouped pups at two or four hours post-infusion was given a retrieval test in which two pups were removed from the group and placed in other quadrants. The test animal was then observed for 15 minutes to see if retrieval of the scattered pups and regrouping occurred (each animal was given only one retrieval test). Any animal retrieving and grouping the three pups was assigned a latency of 0 days.

The maternal behavior of the test animals towards pups was assessed daily for up to ten days following the initial pup presentation. Between 0900 and 1000 hr the position of the test animal, pups, and nest were recorded and the nest quality was assessed. Next, the pups were removed and three freshly-fed pups (2 - 8 days old) were introduced, one being placed in each of the three quadrants not containing the nest. The animal was observed continuously for 15 minutes. Pup retrieval, grouping of pups in the nest, and crouching over pups were recorded.

Behavioral criteria and scoring. Continuous observation post-icv infusion permitted assessment of the intensity and latency of the individual responses which, taken together, constitute maternal behavior in the rat. Two summary measures were also used to compare performance: a maternal behavior composite score and latency of pup response. The five category maternal behavior score used to summarize

the first hour behavior observations was similar to that used by Pedersen and Prange (1979). One point was given for each category in which criterion was met. Licking was recorded whenever a bout of three or more seconds was observed. To receive a point for licking during the first hour, the rat must have been observed licking pups in at least 10/60 observation intervals. Carrying required the female to pick up a pup in her mouth and to carry it to another quadrant. Two or three pups had to be carried within the first hour to score a point. The destination of carried pups was recorded, and a point was awarded for grouping if all three pups were gathered into one quadrant so that they touched one another. Crouching required the female to adopt the nursing posture over at least two pups, with the back arched upward and the hindlegs spread for at least ten consecutive seconds during the initial hour of pup contact. Maternal nest building, for which a point was scored, was distinguished from nonmaternal nest building. Any manipulation of paper was scored as nonmaternal nest building; maternal nest building required at least half of the available paper to be gathered around a pup (or pups). Nest quality was rated separately on a five point scale: 0 = no nest, 1 = flat sleeping nest using some of the available paper, 2 = small maternal nest, 3 = maternal nest with high sides, 4 = excellent maternal nest. Thus, a maternal behavior score of 5 and a high nest rating would indicate performance on the part of the virgin comparable to that of a parturient female.

Latency to respond maternally was the first day on which retrieval and grouping of pups in a maternal nest was observed. Two consecutive successful daily retrieval tests were required before

assignment of a latency. The first day of pup contact was designated day 0. The maximum latency score that could be achieved in these experiments was 10.

Body temperature. The possible contribution of an oxytocin-induced change in body temperature to changes in behavior was assessed in estrogen-primed animals with the same paradigm used to test responsiveness to pups, with the exceptions that ovariectomy preceded oxytocin administration by five days instead of 48 hours and no pups were presented. Forty-six hours before the ovariectomized female rat was moved to the test cage, 100 μg EB/kg body weight (in corn oil) was injected s.c. At 48 hours post-EB injection the animal received an icv injection of either 400 ng oxytocin dissolved in 10 μl sterile normal saline or the vehicle alone. Rectal temperatures were measured using a YSI telethermometer (Model 43TG) with a No. 402 rectal probe. Temperatures were recorded three times per hour one hour before and two hours after the oxytocin or saline treatment.

Verification of cannula placement. At the end of behavioral testing all females were given an overdose of Nembutal and perfused intracardially with normal saline followed by 10% formalin. The brain was removed from the skull and 100 μm frozen sections were cut to determine the location of the guide cannula. All animals for which data are presented here had cannulae placed in the left lateral ventricle at the level of the preoptic area with no accompanying tissue damage. Figure 3 - 1 illustrates a typical lateral ventricle cannula placement.

Data analysis. When using nonparametric statistics, the performance of the groups was compared with the Mann-Whitney U-test

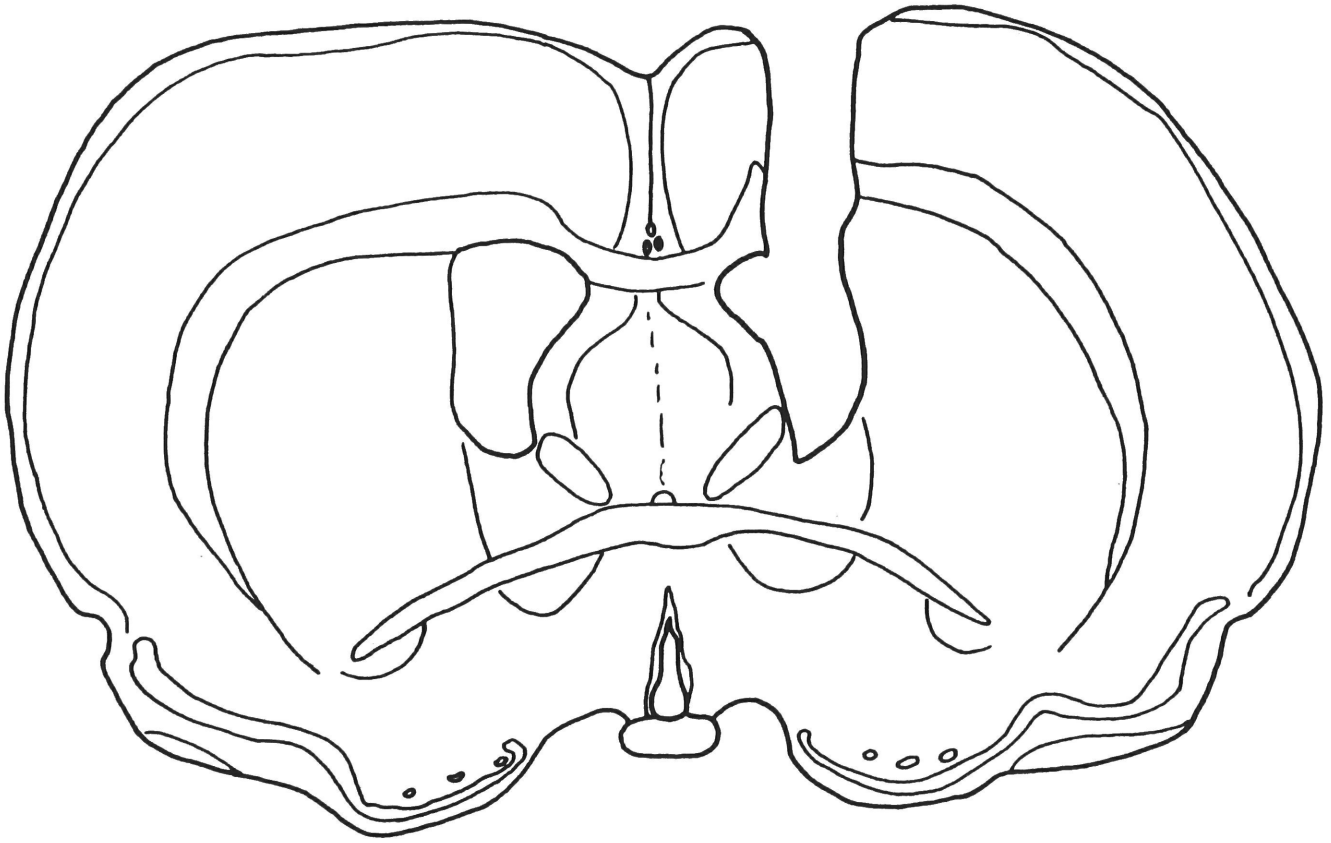


Figure 3 - 1. This figure, produced by microprojection of a 100 μm cresyl violet-stained section, shows a representative lateral ventricle cannula placement. This animal responded with short-latency maternal behavior to infusion of 400 nanograms oxytocin dissolved in 10 μl normal saline in Experiment 1. The anatomical level is approximately A 7190 μ (Konig and Klippel, 1963).

or, in some cases, with a one-tailed test for the significance of a difference of a proportion (Bruning and Kintz, 1977). For interval scale data the t-test was used to determine the existence of significant differences between the groups. Other statistical tests are described as used. Stats Plus (Human Systems Dynamics, 1982), a general statistics package for the Apple IIe Personal Computer, was used to carry out these statistical tests.

B. Results

Maternal behavior scores. The distribution of maternal behavior scores for the first hour of pup contact is shown in Figure 3 - 2. [Data obtained from the group injected with normal saline and data from the uncannulated animals were on every measure indistinguishable, and will henceforth be presented as a combined EB-ONLY group (n = 28).] The proportion of animals in the oxytocin group displaying full maternal behavior (maternal behavior score = 5) during this time was double that in the group receiving only normal saline or no icv injection (50% vs. 25%), with 65% in the oxytocin group showing substantial pup interest (scores of 4 or more). The median scores for the oxytocin and saline groups were 4 and 0, respectively, and the Mann-Whitney U-test (one-tailed, corrected for ties) indicated that there was a significant difference in the maternal behavior scores achieved by the two groups ($p < .01$). In both groups there was a strong tendency for animals to show either full maternal behavior or none at all, as can be seen by the low proportion of animals obtaining scores of 1, 2, or 3 (see Figure 3 - 2).

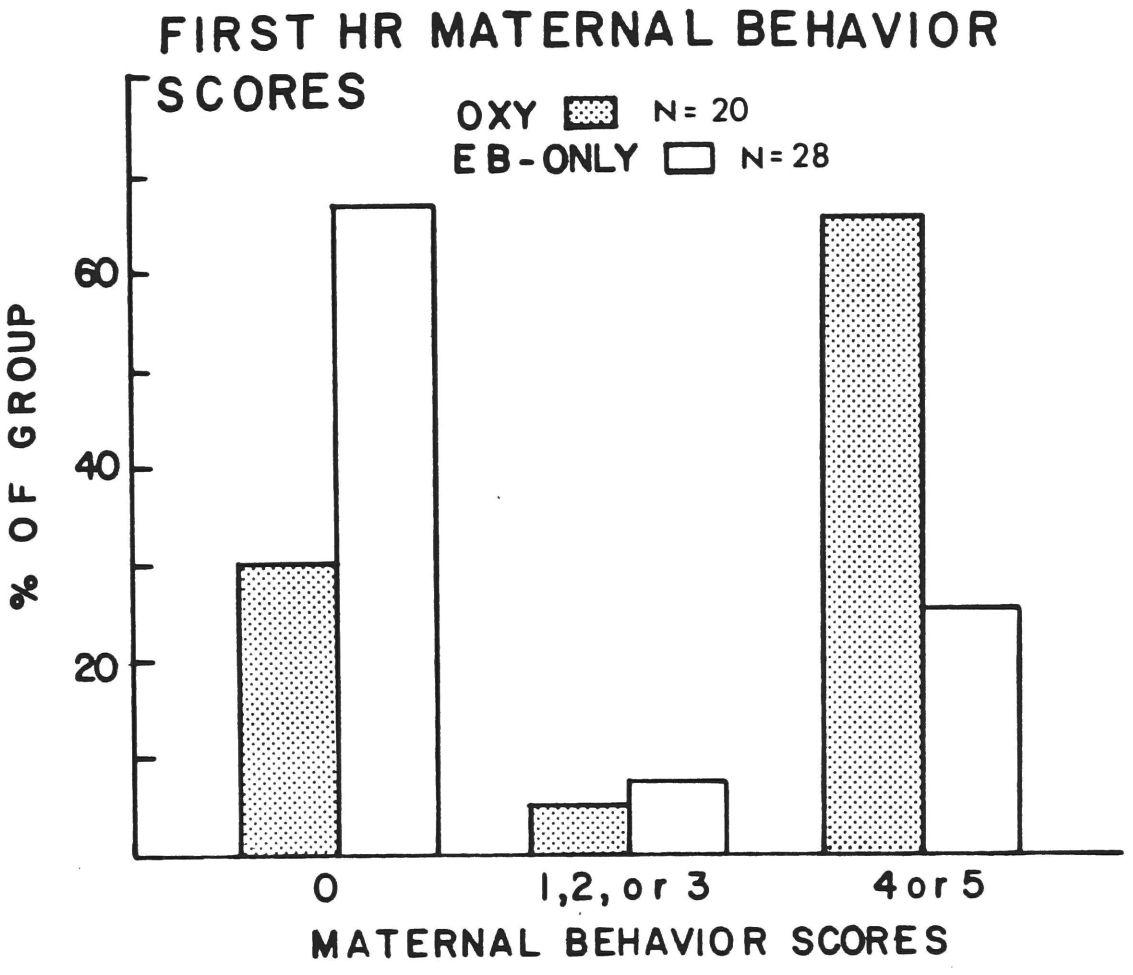



Figure 3 - 2. First hour maternal behavior scores (scoring method given in text). The oxytocin (OXY) group (n=20) received 100 µg estradiol benzoate (EB)/kg body weight s.c. and 400 nanograms oxytocin icv. The EB-only group (n=28) received 100 µg/kg EB and either normal saline (n=16) or no icv infusion (n=12). Both groups were ovariectomized and estrogen-treated 48 hours before first pup contact.

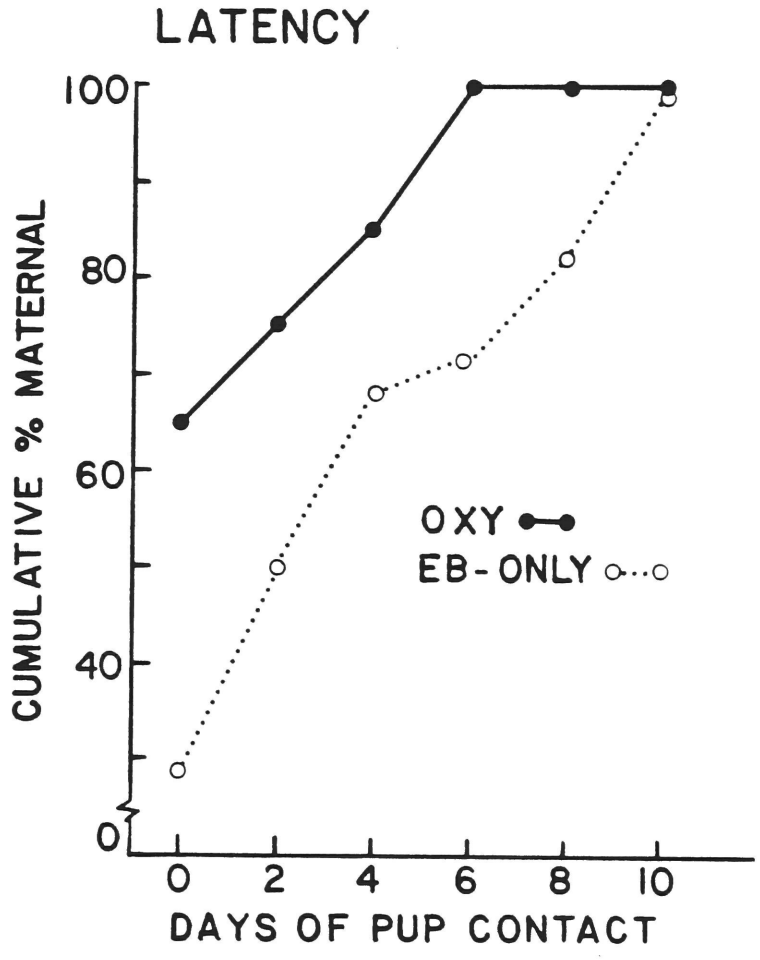



Figure 3 - 3. Latency to respond maternally, expressed in terms of days of pup contact. The cumulative percentage maternal is shown for the oxytocin (OXY) (n=20) and EB-ONLY (n=28) groups. The first day an animal retrieved and grouped three pups was designated the latency.

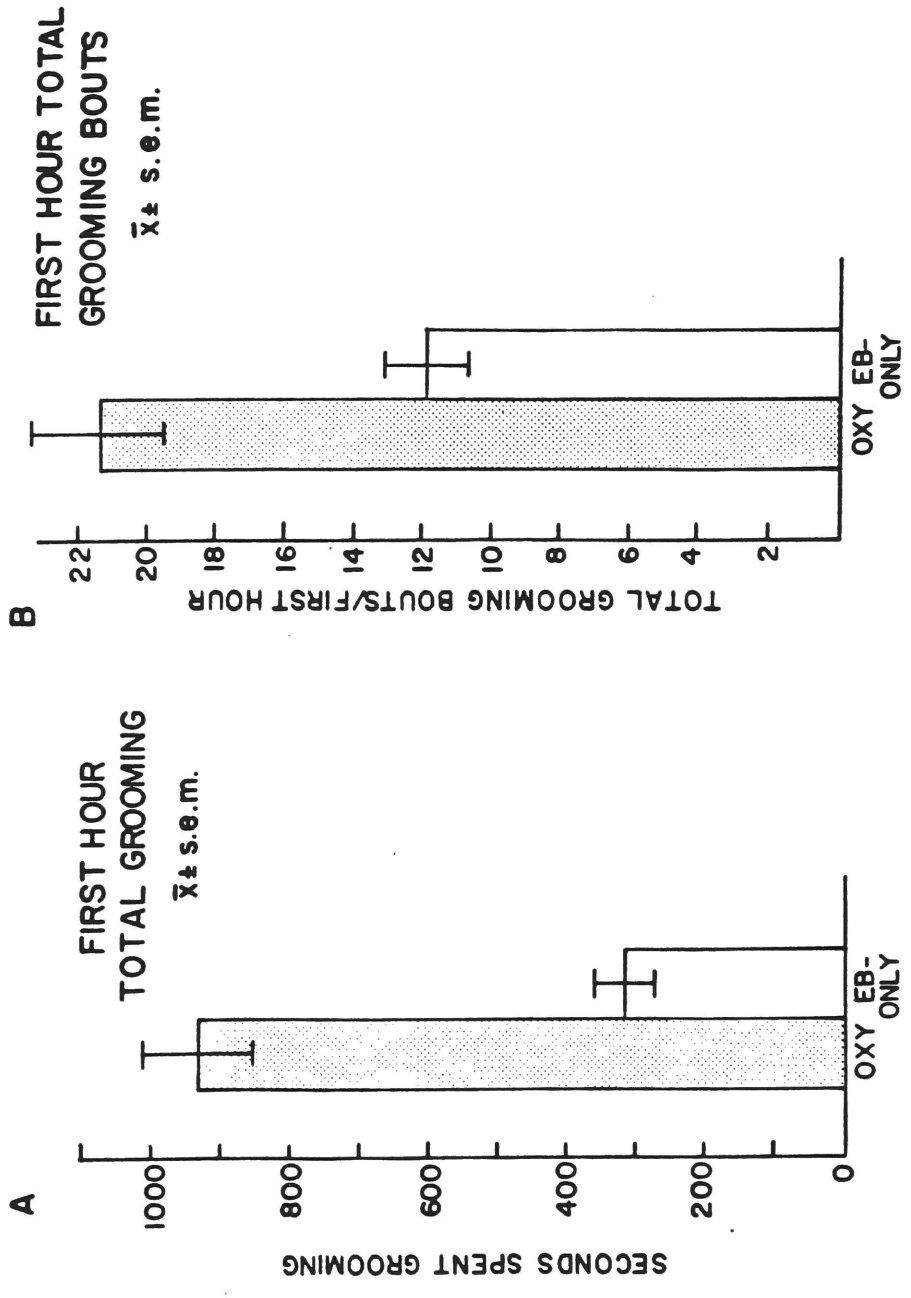
Latency. As can be seen in Figure 3 - 3, administration of 400 ng oxytocin icv signficantly altered the distribution of latency scores as measured in days of pup exposure required to elicit consistent pup retrieval (one-tailed Mann-Whitney U-test, corrected for ties, $p<.01$). The median score for the oxytocin group was 0 days (mean $\pm$ s.e.m. = 1.40 $\pm$ 0.51); the median score for the EB-Only group was 2 days (mean $\pm$ s.e.m. = 3.7 $\pm$ 0.70). Once an animal retrieved three pups, it always retrieved pups on subsequent days. The response latencies of animals in the oxytocin group that did not respond on day 0 did not differ from the response latencies of the control females (one-tailed Mann-Whitney U-test, n.s.).

Grooming. Figure 3 - 4 shows group means for total number of seconds spent grooming during the observation hour and also the total number of grooming bouts three seconds or longer in duration. Estrogen-primed rats given 400 ng of oxytocin spent approximately 3 times as many seconds grooming as did saline-treated or noncannulated controls (mean score $\pm$ s.e.m. of 932.7 $\pm$ 80.3 seconds vs. 313.1 $\pm$ 47.8 seconds; median of 941 seconds vs. 237 seconds; $p<.001$, one-tailed Mann-Whitney U-test). The number of individual bouts was also increased in the oxytocin-treated animals (see Figure 3 - 4), although by slightly less than a factor of two, indicating that length of individual grooming bouts must also have increased. The animals appeared to be grooming normally, with no undue attention given to any specific body surface. Notes taken during the observations suggest that the face and the head area (especially around the cannula in cannulated animals) were the most frequently groomed areas. There was a difference between the groups in the latency of the first grooming

Figure 3 - 4. First hour grooming measures.

(A) First hour total grooming ($\bar{x} \pm$ s.e.m.) expressed in seconds. All grooming and scratching were recorded and totaled. Median scores: oxytocin (OXY), 941 seconds; EB-only, 237 seconds. OXY EB-only, $p < .001$, one-tailed Mann-Whitney U-test.

(B) First hour total number of grooming bouts 3 seconds or longer in duration ($\bar{x} \pm$ s.e.m.). Median scores: OXY, 18; EB-Only, 10. OXY EB-Only, $p < .001$, one-tailed Mann-Whitney U-test.


A
FIRST HOUR
TOTAL GROOMING
x̄ ± s.e.m.
SECONDS SPENT GROOMING
0
200
400
600
800
1000
OXY
EB-ONLY
B
FIRST HOUR TOTAL
GROOMING BOUTS
x̄ ± s.e.m.
TOTAL GROOMING BOUTS/FIRST HOUR
2
4
6
8
10
12
14
16
18
20
22
OXY
EB-ONLY

bout longer than 3 seconds in duration (oxytocin group: 3.7 ± 1.05 minutes, median = 2; EB-ONLY group: 5.4 ± 0.66 minutes, median = 5; one-tailed Mann-Whitney U-test, $p < .01$). A comparison between the amount of grooming performed by animals in the oxytocin group responding to pups during the first hour (n = 13) and nonmaternal oxytocin-treated animals (n = 7) indicated that there was no difference between the two groups (maternal: 884.2 ± 80.75 seconds; nonmaternal: 1022.71 ± 179.1 seconds; two-tailed Mann-Whitney U-test, n.s.).

Activity. The total number of cage quadrants entered during the first hour after pup presentation did not differ significantly between the oxytocin and the EB-ONLY groups (two-tailed Mann-Whitney U-test, n.s.). The group means ( ± s.e.m.) were 39.4 ± 3.8 for the oxytocin group (median: 38) and 37.1 ± 7.2 for the control group (median: 25). Within the oxytocin-treated group there was again no difference between pup responders (n = 13) and nonmaternal animals (n = 7): the respective group means for quadrants entered were 39.2 ± 5.04 and 39.6 ± 6.1.

Other responses. Some authors have reported that icv injections of neurohypophysial hormones can result in short-latency abnormal motor responses including convulsions and occasionally barrel rotation (Kruse et al., 1977; Rubin et al., 1983) Although we have observed these responses in animals treated with high doses of oxytocin obtained from commercial sources, none of the animals in the experiments reported here were so affected. Food and water were available during the observation period: the proportion of animals drinking during the hour did not differ (oxytocin, 5/20; EB-ONLY,

9/28) but animals treated with oxytocin were significantly less likely to eat chow during this time than were animals treated with vehicle (3/20 vs. 15/28, respectively; $p<.01$, one-tailed test for significance of a difference of a proportion). In the course of a series of maternal behavior experiments using ZM/SD rats we have occasionally observed cannibalism of pups. In this experiment, one saline-treated animal was excluded from subsequent data analysis for this reason.

Individual maternal responses. Table 3 - 1 summarizes the percentage of animals in each group exhibiting the individual maternal responses described above. Table 3 - 2 compares the performance of short-latency pup responsive animals from the oxytocin and EB-ONLY groups on some selected behavioral measures.

Licking. A significantly greater proportion of the oxytocin-treated animals exhibited licking to criterion, i.e., licked pups during ten or more minutes of the first hour. On average, the oxytocin-treated animals licked pups during twice as many intervals as did controls (medians: 18 vs. 2, $p<.001$, one-tailed Mann-Whitney U-test, corrected for ties). All animals in the oxytocin group licked a pup at least once during the hour, as did all but nine of the EB-ONLY animals, but persistence and repetition of licking clearly distinguished responders from non-responders. Table 3 - 2 shows the latencies and frequencies of pup licking for maternal animals. It can be seen that the intensity of the licking behavior, as measured by the number of minutes during which licking was observed, was the same for oxytocin-induced and spontaneously maternal animals. In both groups the nonmaternal animals were observed licking much less frequently.

TABLE 3 - 1

Maternal Responses of Ovariectomized, Estrogen-Primed Virgins

during First Hour of Pup Contact

% Animals Showing Response[a]

| Group | Licks Pups | Carries Pups | Groups Pups | Crouches Over Pups | Builds a Maternal Nest | Builds Any Nest |
|---|---|---|---|---|---|---|
| EB + 400 ng oxytocin n = 20 | 65 | 70 | 65 | 60 | 65 | 95 |
| EB-ONLY n = 28 | 29 | 32 | 25 | 25 | 29 | 68 |
| | $p < 0.01$[b] | $p < 0.01$[b] | $p < 0.01$[b] | $p < 0.01$[b] | $p < 0.01$[b] | $p < 0.02$[b] |

[a] see text for criteria for performance of the individual responses

[b] one-tailed test for significance of a difference between two proportions (Bruning & Kintz, 1977)

TABLE 3 - 2

Comparison of Responses of Maternal[a] Animals from the OXY and EB-ONLY Groups

Median Scores

| Group | Latency to Lick Pups | Total # Intervals Observed Licking | Latency to Retrieve First Pup | First Hr Nest Rating | Latency of First Grooming Bout |
|---|---|---|---|---|---|
| EB + 400 ng oxytocin n = 13 | 8 min. | 24 | 17 min. | 3 | 2 min. |
| EB-ONLY n = 7) | 3 min. | 22 | 4 min. | 3 | 6 min. |
| | n.s.[b] | n.s.[b] | $p < 0.03$[b] | n.s.[b] | $p < 0.01$[b] |

[a] data presented here from animals receiving maternal behavior scores of either 4 or 5. See text for criteria for performance of the individual responses.

[b] two-tailed Mann-Whitney U-test

There is a tendency for the spontaneously maternal (EB-ONLY group) animals to exhibit licking earlier in the hour than their oxytocin-treated counterparts, although this difference was not significant.

Carrying. Table 3 - 1 shows the percentages of oxytocin and EB-ONLY animals which carried pups from one cage quadrant to another (typically, to the nest) during the observation hour. Approximately double the proportion of oxytocin-treated animals exhibited any carrying (oxytocin: 15/20, 75%; EB-ONLY: 12/28, 43%) or carrying to criterion (14/20, 70% vs. 9/28, 32%). All animals, regardless of treatment group, that attained a maternal behavior score of 4 or 5, carried to criterion during the first hour of observation. Some nonresponding animals in both groups (oxytocin: n = 2; EB-ONLY: n = 3) exhibited carrying of one or two pups in the absence of the full array of caregiving behaviors.

Grouping. The maternal rat efficiently cleans, feeds, and keeps her young warm by gathering them together into a restricted nest area. Under the above test conditions, 65% (13/20) of the oxytocin-treated animals vs. 25% (7/28) of the EB-ONLY animals responded to the presentation of pups scattered over three cage quadrants by grouping them. All animals, regardless of treatment, that grouped three pups during the first hour fell into the category that received total maternal behavior scores of 4 or 5.

Crouching. Even though virgin rats do not lactate, such animals will crouch over grouped pups in the nursing posture when rendered maternal either by hormone treatment or prolonged exposure to pups. In Experiment 1, 60% (12/20) of the oxytocin-treated animals vs. 25%

(7/28) of the EB-ONLY animals crouched over grouped pups ($p < .01$, one-tailed test for significance of a difference between two proportions).

Nest building. Table 3 - 1 shows the proportion of animals in both groups that built nests out of shredded paper towels and wood shavings during the first observation hour. All but one of the oxytocin-treated animals manipulated and carried paper during the first hour, while approximately two-thirds of the control animals showed interest in the available paper. "Maternal nests" were distinguished fron "nonmaternal nests" on the basis of appearance (high-sided vs. flat pallet) and whether or not pups were inside. The percentage of oxytocin-treated animals building maternal nests was approximately double that of EB-ONLY animals ($p < .01$, one-tailed test for significance of a difference between two proportions). Table 3 - 1 shows further that the tendency to build a nest was enhanced in almost all animals in the oxytocin group, regardless of whether or not a maternal nest was built. By contrast, there was no difference in the tendencies of the two groups to build nests during the two hour period of habituation to the test cage. When maternal animals from both the oxytocin and EB-ONLY groups are compared (see (Table 3 - 2), no difference in quality of the maternal nest is found after the first hour of pup contact.

Maintenance of induced maternal behavior. Once an oxytocin-treated animal had initiated maternal behavior during the first observation hour, that behavior was maintained or improved over the next four hours and over the succeeding days, as gauged by the first retrieval test and the morning observations. The same was true

of the spontaneously maternal animals in the saline group.

Oxytocin-induced changes in body temperature. The group mean body temperatures measured after icv infusion of oxytocin (n = 7) or sterile normal saline (n = 7) are shown in Figure 3 - 5a. The mean changes in body temperature from the post-infusion baseline are shown in Figure 3 - 5b. A pattern was observed only in the oxytocin group of a slight, short-duration increase in rectal temperature (compared across the three time points using the Friedman ANOVA, $p<.01$). This effect was consistent (one-tailed t-test comparing differences between pre- and post-infusion means, $p<.05$).

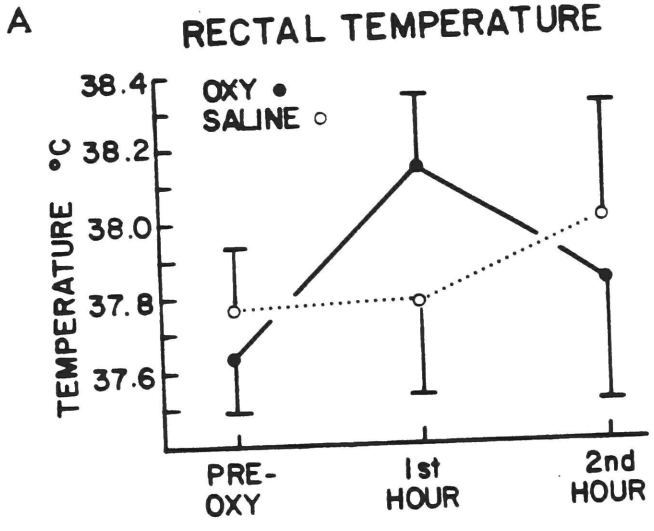
C. Discussion

A significantly higher proportion of estrogen-primed virgin ZM/SD rats given an icv infusion of 400 ng oxytocin exhibited short-latency maternal behavior than did estrogen-primed rats infused with normal saline only or given no icv injection. Within an hour of treatment, approximately two-thirds of the oxytocin-treated animals repeatedly licked, carried, and grouped foster pups: they crouched over them and built high-sided nests around them. Only 25% of the vehicle-treated animals responded to pups. These results replicate the findings of Pedersen et al. (1982), who reported that 72% of estrogen-primed ZM/SD virgins injected with 400 ng oxytocin showed short-latency maternal behavior compared with only 18% of saline-infused controls. As described by these investigators (and others: see Fleming and Rosenblatt, 1974), the behaviors characterizing the maternal state in the rat had a strong tendency to appear as a unit. Further, the continuous observation employed in the present study demonstrates that

Figure 3 - 5. Oxytocin-induced changes in body temperature.

(A) Group mean rectal temperature ($^{o}C$) for oxytocin (OXY) (n=7) and saline (n=7) groups ($\bar{x} \pm$ s.e.m.). Both groups were ovariectomized 5 days before s.c. estrogen injection (100 µg EB/kg body weight). The OXY group received 400 nanograms oxytocin icv while the saline group received only the vehicle. The OXY group means varied significantly across the three time points (Friedman ANOVA by ranks, $p < .01$) while the saline group showed no change.

(B) Group mean rectal temperature change in $^{o}C$ ($\bar{x} \pm$ s.e.m.), with reference to the first hour preinfusion baseline. OXY saline only during the first hour after icv infusion, one-tailed t-test, $p < .05$.


A
RECTAL TEMPERATURE
OXY •
SALINE ○
TEMPERATURE °C
38.4
38.2
38.0
37.8
37.6
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OXY
1st
HOUR
2nd
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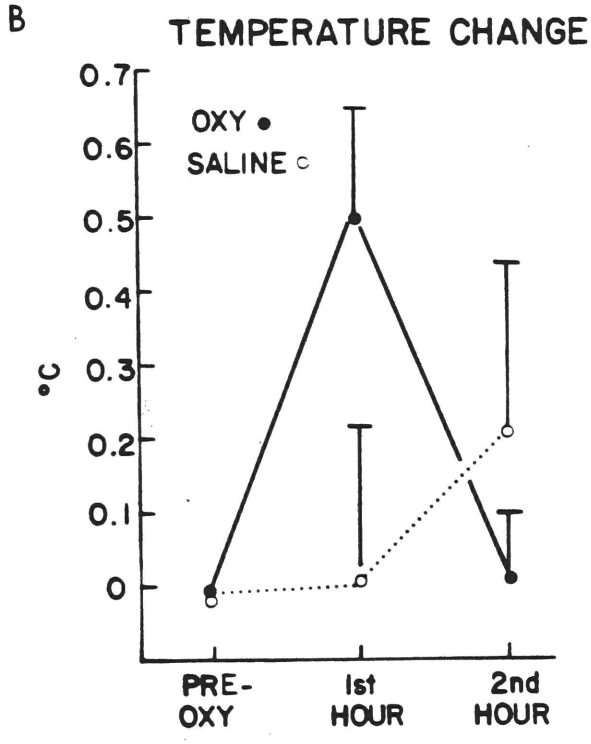

B
TEMPERATURE CHANGE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
°C
OXY ●
SALINE ○
PRE-
OXY
1st
HOUR
2nd
HOUR

the effect of oxytocin is not to increase the intensity or to change the quality of the behavior of individual maternally-responsive rats (compared with spontaneously maternal rats; see Table 2), but rather to increase the proportion of animals responding with short latency.

The slight increase in body temperature over pretreatment levels observed after icv administration of oxytocin is consistent with a previous report of the effects of centrally-administered oxytocin on body temperature in male rabbits (Lipton and Glyn, 1980). The effect observed here in rats was of shorter duration, although comparison is difficult because of dosage and procedural differences. This small effect seems unlikely to have influenced the performance of maternal behavior in this paradigm, especially as hyperthermia might be hypothesized to disrupt rather than to facilitate the expression of some aspects of rodent maternal behavior.

These results contrast with those obtained by Rubin et al. (1983), and this disparity raises the issue of the physiological importance of the "oxytocin effect." It is not yet clear which features of our experimental procedure (which was designed to be as similar as possible to that described by Pedersen and Prange, 1979) were critical in stimulating the rapid onset of maternal responsiveness. Five differences in experimental procedure, which can be tested systematically in future studies, should be noted. First, test animals were obtained from a different supplier and therefore from a different breeding stock of the Sprague-Dawley strain. While there is no a priori reason to think that ZM/SD rats differ from other Sprague-Dawley rats in responsivity to oxytocin, estradiol, or pup stimulation, strain- and supplier-related differences in behavior have

been shown to exist between ZM/SDs and other albino strains under a variety of test conditions (Ray and Barrett, 1975). Ascher et al., (1982) have reported that a longer period of estrogen-priming (10 days) and a higher dose of oxytocin (800 ng) significantly increased the proportion of Charles River Sprague-Dawley rats responding to foster pups with short-latency maternal behavior. The optimal combination of steroid hormone and oxytocin treatments has not yet been defined for the rapid induction of maternal behavior in any strain.

A second factor which may have contributed to variability of effect is a different source of peptide. Like Ascher et al. (1982), I found in pilot experiments with both ZM/SD rats (n = 12) and Charles River Long-Evans rats (n = 11) that Bachem oxytocin, Lot No. R2622, used by Rubin et al. (1983) in their experiments 1A and 2A, was much less effective in maternal behavior induction that oxytocin obtained from Dr. V. Hruby (University of Arizona Medical School, Tucson, AZ) or Peninsula Laboratories (San Carlos, CA) despite their having equivalent activity in a rat uterine strip bioassay (Dr. W. Sawyer, Department of Pharmacology, Columbia University, NYC, NY; personal communication, December 1982). The reason for these peptides' differing activities in two different bioassays is unclear. A discussion of sources of impurities in peptide preparations can be found in Margolis and Longenbach (1980).

In pilot studies (data not reported here), 5 of 9 ovariectomized ZM/SD virgin rats treated 48 hours prior to icv infusion of 400 ng of Peninsula oxytocin (Lot No. 003249) with 100 µg EB/kg body weight exhibited full maternal behavior (and increased time spent grooming)

with a latency of less than one hour. Thus, the inability of oxytocin obtained from Peninsula Laboratories to stimulate rapid onset of maternal behavior in the paradigm used by Rubin et al. (1983; Experiments 1B and 2B) suggests that the differences in our results are not wholly dependent on source of peptide.

Third, unlike Rubin et al., (1983), I routinely used 12 - 24 hour old pups as stimuli in the initial tests. It has been previously demonstrated (Stern and MacKinnon, 1978) that one and two day old pups are more effective stimuli for inducing maternal behavior in virgins than are older pups. Next, Rubin et al. (1983) introduced pups into the virgins' home cages, while in our procedure (identical to that of Pedersen and Prange, 1979) subjects were switched to test cages only two hours prior to oxytocin infusion and introduction of pups. It is possible that some aspect of the rats' responses to the novel environment interacted with the hormone treatment to facilitate responsivity to pups (see Experiment 3, this chapter). Finally, the smaller injection volume used in the Rubin et al. (1983) study (4 $\mu$l vs. our 10 $\mu$l) might have resulted in a less widespread brain distribution of oxytocin.

Performance of maternal behavior can be reliably induced in nonpregnant female rats in the absence of hormonal manipulations, but the latency measured from the beginning of pup contact is typically 5 - 7 days (Rosenblatt, 1967). Hormonal treatments have been described which reduce this latency (these typically mimic the endocrine changes known to occur during pregnancy in the rat: Moltz et al, 1970; Zarrow et al., 1971; Siegel and Rosenblatt, 1975a, b. See Chapter 1 above for fuller discussion), although at least a day of pup stimulation is

still needed for a majority of females to show this behavior. The almost immediate response to oxytocin suggests that very little, if any, pup stimulation was required to activate the maternal behavior of the oxytocin responders. In this respect, the estrogen- and oxytocin-treated virgin is like the parturient female, whose response to pups is immediate: both apear somehow "prepared" to respond to young pups. These results are most similar to those presented by Mayer and Rosenblatt (1980), who showed that the maternal response latency of hysterectomized, ovariectomized, EB-treated virgins can be reduced to times on the order of one hour, comparable to those achieved with oxytocin administration. This reduction could be obtained either by 1. olfactory-vomeronasal deafferentation of the test subjects or by 2. changes in the mode of presentation of the pups. The relationship of the present findings to these studies will be considered in the general discussion below.

The effects of a single icv injection of oxytocin appeared to be transient, so that if an animal did not respond immediately to the combination of oxytocin and the presence of pups, its subsequent latency to respond maternally was no different than if it had been saline-treated.

Compared to saline-treated animals, rats receiving oxytocin appeared alert but not abnormally reactive to environmental stimuli in a way difficult to quantify. As measured by number of cage quadrants entered during the first hour, general activity was not affected by oxytocin. This suggests that the oxytocin-treated animals were not more responsive to pups simply because they encountered them more frequently than did less mobile controls. However, amount of time

spent grooming was significantly increased in the oxytocin group, apparently independently of the tendency to behave maternally. Previous studies have reported slight or no effects (Delanoy et al., 1978; Meisenberg, 1982) of icv oxytocin on grooming in rats. This stands in contrast to the striking effects of icv injection of neurohypophysial hormones on murine scratching and grooming (Meisenberg, 1981). In a study using rats by Meisenberg (1982), larger doses of oxytocin (1 and 10 µg) infused icv in a larger vehicle volume (50 µl) into male Sprague-Dawley rats etherized during the injection significantly increased cumulative seconds of grooming in the five minute period 6 - 11 minutes post-injection to levels 4-5 times the saline baseline. A similar effect was obsrved during this time interval in our estrogen-treated females, despite the many differences in protocol and the presence of pups. During this period, approximately 5 times as much grooming was seen in the oxytocin group compared to the EB-ONLY group.

Like grooming, nest building was also increased in both maternal and nonmaternal rats treated with icv oxytocin. Neurohypophysial hormones have been shown to increase the amount of time mice spend burrowing through bedding materials or "foraging" (Delanoy et al., 1978), but it is not clear if these activities are related to the organized building of nests observed in this experiment. After icv injection of doses of arginine vasopressin ranging from 1.5 ng to 5 µg, Kruse et al. (1977) mention incidental observations of increased nest building activity in rats. Although nest building is an important component of rodent maternal behavior directly related to the survival of the young, it is unlike the other maternal behaviors

evaluated here in that it involves a response to variably available materials and not to the relatively invariant cues produced by neonatal pups. It is context-appropriate on other occasions (e.g., lowered ambient temperatures), and it is performed by males as well as females. The high proportion of nest building in the EB-ONLY group (in contrast to the lower rate of maternal responsiveness), the apparent dissociation of the nest building response to infused oxytocin from the maternal behvior response, and the lesser ability of other neurohypophysial hormones (Pedersen et al., 1982) to stimulate short-latency maternal behavior in this preparation (in contrast to the reported vasopressin stimulation of nest building) suggest that nest building may be under separate neuroendocrine conrol that the cluster of pup-related maternal responses. (Note, however, that experiments involving preoptic region or ventral midbrain damage tend to have a relatively larger disruptive effect on retrieving and nest building, with nursing behavior typically being the least disturbed component of maternal behavior in such studies [discussed by Numan, 1983; Numan and Smith, 1984]. These findings suggest that at least one pup-directed response, retrieving, shares some of the neural circuitry regulating nest building. Interestingly, medial preoptic/anterior hypothalamic-lesioned male rats can still exhibit some nest building in response to lowered ambient temperature [van Zoeren and Stricker, 1977], although the nests built in these circumstances were much smaller than those built by unlesioned control rats.)

## III. Experiment 2

Pedersen and Prange (1979) reported that oxytocin facilitation of short-latency maternal behavior appeared to be dependent on prior estrogen priming. In order to extend these observations, we studied the grooming, nest building, and responsivity to pups of ovariectomized, oil-treated ZM/SD virgins receiving icv injections either of 400 nanograms of oxytocin (n = 9) or normal saline (n = 9). I wished to examine 1. the estrogen dependence of oxytocin-induced maternal behavior 2. possible estrogen modulation of oxytocin-induced grooming and 3. possible oxytocin stimulation of nest building independent of estrogen priming or the performance of maternal behavior. The method was the same as that described for Experiment 1, with the exception that the oxytocin solution for icv injection was diluted in glass vials coated with an organosilane concentrate surface-treating agent (Prosil-28; Specialty Chemicals, Gainesville, Fl). This procedure (recommended by Stewart, 1983) was added to reduce adsorption of the peptide to the surface of the glass in order to maintain full potency of the solution. With regard to the data analysis, since 3/9 of the oxytocin-treated animals and 1/9 of the saline group killed pups, some of the measures (e.g., latency of maternal behavior) will necessarily exclude these animals. Inclusion or exclusion of cannibals will be indicated for each result reported.

### A. Results

Maternal behavior scores. During the first post-infusion hour only 1/9 oxytocin-infused ovariectomized animals exhibited any

any maternal behavior (maternal behavior score of 4: did not lick pups to criterion). No saline-injected, oil-treated subjects responded to pups during this observation period, although one saline animal became fully maternal between the two and four hour post-infusion assessments. Further, there was no difference between the groups in latency to become maternal as measured by days of pup contact required to elicit retrieval (median scores, oxytocin: 5, Oil-Only: 5; mean $\pm$ s.e.m., oxytocin: 5.0 $\pm$ 1.5, Oil-Only 4.2 $\pm$ 0.72, Mann-Whitney U-test, n.s. Cannibals were excluded from these measures, so oxytocin n = 6, Oil-Only n = 8). There was no difference between the groups in incidence of cannibalism: Fisher exact probability test, p = .247, n.s.

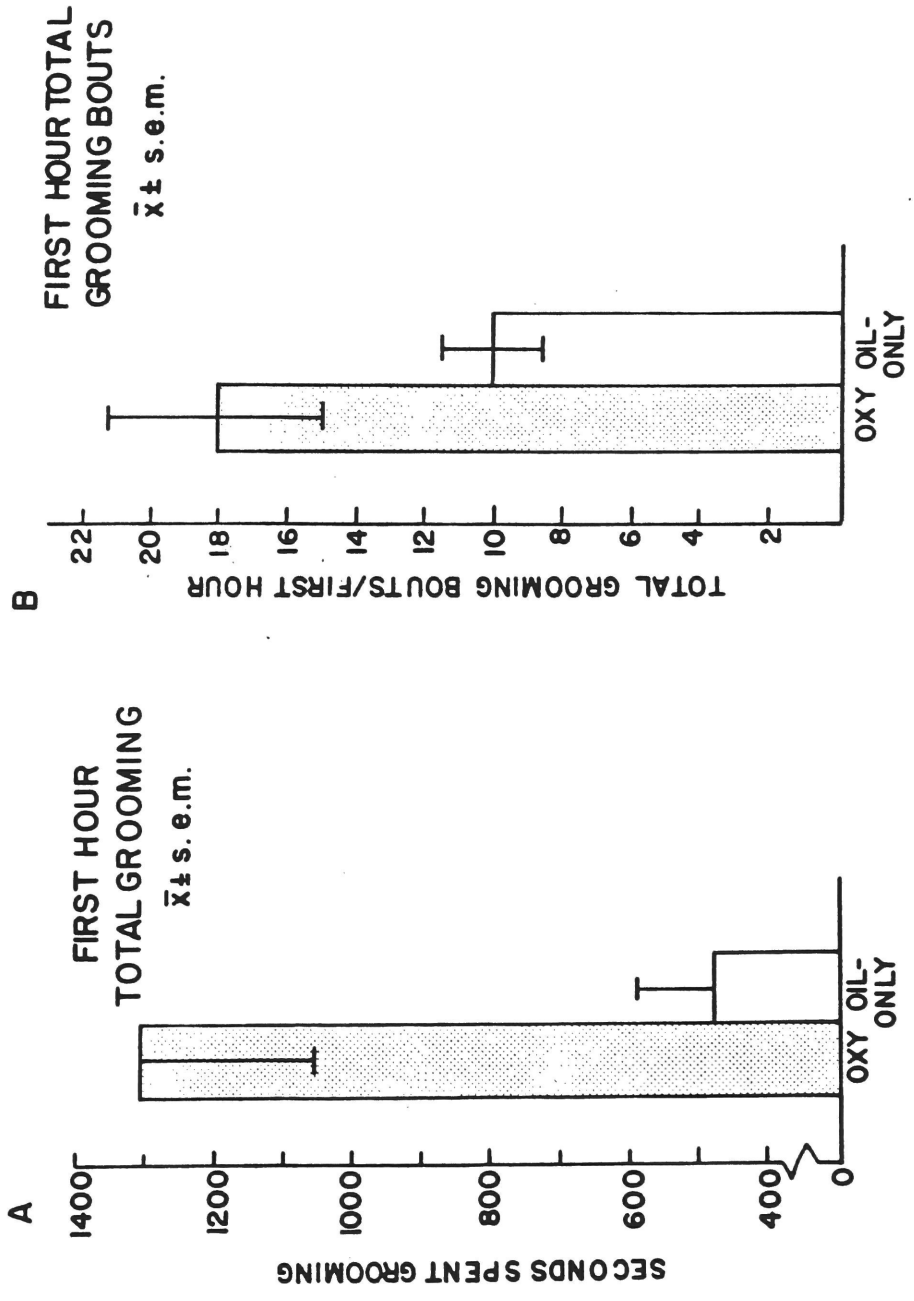
Grooming. Figure 3 - 6a shows the number of seconds the ovariectomized, oil-treated animals spent grooming during the first post-infusion hour. The oxytocin-treated animals spent significantly more time grooming than their saline-treated counterparts. As in Experiment 1, both the frequency and length of individual grooming bouts were increased (see Figure 3 - 6b for total grooming bouts/first hour). When animals that killed pups were included in this analysis, this did not affect the conclusions drawn. The grooming total, with cannibals included, was: oxytocin (n = 9), Oil-Only (n = 9); median, oxytocin: 1242 seconds, Oil-Only: 421 seconds; mean $\pm$ s.e.m., oxytocin: 1194 $\pm$ 157.8 seconds, Oil-Only: 453.6 $\pm$ 86.6 seconds. With cannibals excluded, oxytocin (n = 6), Oil-Only (n = 8); median, oxytocin: 1181.5 seconds, Oil-Only: 455 seconds; mean $\pm$ s.e.m., oxytocin: 1207.8 $\pm$ 493.1 seconds, Oil-Only: 489.5 $\pm$ 89.3 seconds.

Activity. There was no difference between the groups in activity

Figure 3 - 6. First hour grooming measures.

(A) First hour total grooming ($\bar{x} \pm$ s.e.m.) expressed in seconds. All grooming and scratching were recorded and totaled. Both the oxytocin (OXY) (n=9) and Oil-Only (n=9) groups were ovariectomized and given a s.c. injection of corn oil 48 hours before icv infusion with either 400 nanograms oxytocin or normal saline. OXY $>$ Oil-Only, $p < .01$, one-tailed Mann-Whitney U-test.

(B) First hour total grooming bouts 3 seconds or longer in duration ($\bar{x} \pm$ s.e.m.). OXY $>$ Oil, only, $p < .01$, one-tailed Mann-Whitney U-test.


A
FIRST HOUR
TOTAL GROOMING
x̄ ± s.e.m.
SECONDS SPENT GROOMING
1400
1200
1000
800
600
400
0
OXY
OIL-ONLY
B
FIRST HOUR TOTAL
GROOMING BOUTS
x̄ ± s.e.m.
TOTAL GROOMING BOUTS/FIRST HOUR
22
20
18
16
14
12
10
8
6
4
2
OXY
OIL-ONLY

measured by number of quadrants visited in the home cage. Including the animals that killed pups in this analysis did not alter this conclusion. For all animals, oxytocin (n = 9), Oil-Only (n = 9); median, oxytocin: 40, Oil-Only, 22; mean $\pm$ s.e.m., oxytocin: 38.1 $\pm$ 11.6, Oil-Only: 25.1 $\pm$ 5.9 (two-tailed Mann-Whitney U-test, n.s.).

Nest building. Some nest building was performed during the first post infusion hour by 6/9 of the oxytocin-treated animals, but only by 2/9 of the saline-injected subjects. When the presence or absence of any kind of nest at all was assessed at the end of the first post-infusion hour for all animals, there was not significant difference in the percentage of animals building nests (Fisher exact probability test, $p = .069$). However, when the cannibals, which typically spent very little time in nest building, are omitted from this analysis, 5/6 oxytocin vs. 2/8 Oil-Only animals had nests (Fisher exact probability test, one-tailed, $p = .049$), suggesting a stimulatory effect of oxytocin on nest building. This impression was confirmed by comparison across groups of the total number of 60 second intervals during the first hour in which animals were observed manipulating nest material. With cannibals excluded, the oxytocin group was observed nest building significantly more often than the saline-injected animals (two-tailed Mann-Whitney U-test, corrected for ties, $p < .05$).

B. Discussion

As previously suggested (Pedersen and Prange, 1979), the facilitative effect of oxytocin on maternal behavior is dependent upon prior estrogen priming. This was not true of the induction of

grooming described in Experiment 1, as oxytocin-treated animals again spent much more time grooming than their saline counterparts. There was again no effect of oxytocin on activity in the test cage. Whether or not oxytocin alone increases the tendency to build a nest is ambiguous: although a higher proportion of the oxytocin-treated animals than of the saline animals built nests in Experiment 2, the difference was not significant unless the animals that cannibalized pups were omitted from the analysis. It seems likely that pup killing may be incompatible with nest building, as these animals usually remained active and awake during the observation hour, while most other nonmaternal animals groomed, built a nest, and eventually appeared to sleep. These results demonstrate that oxytocin effects on maternal behavior and grooming can be dissociated, with the induction of grooming being independent of estrogen treatment or the lack thereof.

## IV. Experiment 3

The test procedure used by Pedersen and colleagues (Pedersen et al. , 1982) and in Experiments 1 and 2 incorporated a transfer of the animal from its home cage to a larger, unfamiliar test cage 1 - 2 hours before the icv infusion and start of behavioral testing. In an attempt to specify more fully the critical features of my test paradigm, the effect of varying the test cage habituation period on the response to oxytocin was examined.

A. Method

Three groups of ovariectomized, estrogen-primed nulliparous ZM/SD rats were tested using the procedure detailed in Experiment 1. The first group received the typical two hour habituation to the test cage; a second group were tested in their "home cage" (same size as the test cage, one week habituation, no change of bedding during that time); the third group received no habituation to the test cage and were transferred from their home cage to the test cage immediately after the icv infusion. Approximately half of the animals in each group received 400 ng of oxytocin dissolved in 10 µl normal saline (OXY); the other half received 10 µl of normal saline (NS). No habituation: OXY, n = 7, NS, n = 6; 2 hour habituation: OXY, n = 8, NS, n = 7; 1 week habituation: OXY, n = 8, NS, n = 7). This design permitted comparison of the effects of duration of cage habituation on the baseline response as well as on oxytocin-facilitated maternal behavior.

In order to facilitate simultaneous testing of animals, minute-by-minute behavior checklists were not kept. Instead, the animals' behavior was assessed at 1, 2, 3, and 4 hours post-infusion, with a 15 minute retrieval test at 4 hours. The icv infusion was given at 1300 hr, so that the entire test was carried out during the light part of the cycle (lights off at 1800 hr). The maternal behavior of the animals was assessed the next morning (0900-1000 hr) and a 15 minute retrieval test was given using freshly-nourished pups. Animals were then anesthetized and sacrificed by perfusion with 10% formalin in order to examine the cannula placement. No animals were excluded from the data analysis because the cannula did not enter the

lateral ventricle. No animals in this experiment killed pups. As in Experiment 2, the glass vials used for the oxytocin solution were pretreated with Prosil-28 to minimize adsorption of the peptide onto the glass.

B. Results

The results are presented in terms of the proportion of females behaving maternally (receiving a maternal behavior score of either 4 or 5) at a particular behavior assessment. Retrieval was inferred from the presence of grouped pups in a nest if not actually observed during the preceding hour. Data are also presented on the proportion of each group successfully completing a retrieval test within 15 minutes of being presented with three scattered pups.

Figure 3 - 7 indicates the percentage maternal after one hour of pup contact for each of the six groups. For each of the three different durations of cage habituation, the performances of the oxytocin- and saline-treated animals were compared using the Fisher exact probability test (one-tailed probability, $\alpha = .05$). There was a significant difference in the proportion of animals responding maternally only in the case of the animals transferred to the test cage two hours before the icv infusion and the introduction of pups ($p = .034$). The performance of this oxytocin-treated group replicates the short-latency oxytocin induction of maternal behavior in ovariectomized, estrogen-primed ZM/SD rats that was observed in Experiment 1. A short-latency induction of coordinated responsivity to pups was absent both in the groups given one week of pre-test cage habituation or no prior cage habituation at all. The three oxytocin

groups were compared by means of the $\chi^2$ statistic and found to have significantly different distributions of responders and nonresponders ($\chi^2_{(2)} = 6.61$, $p = .036$) at one hour post-infusion. The $\chi^2$ statistic for the comparable saline groups did not achieve statistical significance ($\chi^2_{(2)} = .506$, $p = .687$), suggesting that the two hour cage habituation period was by itself ineffectual in promoting the performance of short-latency maternal behavior.

Figure 3 - 8 indicates the proportion of animals in each group that carried three scattered pups to the nest during a 15 minute retrieval test conducted 4 hours after the introduction of foster pups into the test cage. None of the oxytocin groups differed significantly from its counterpart saline group on this measure. The largest difference in performance was again seen between the oxytocin- and saline-treated groups habituated to the test cages two hours before testing began: 6/8 (75%) of the oxytocin animals retrieved three pups compared to only 2/7 (28%) of the saline group. The $\chi^2$ statistic for the three oxytocin groups ($\chi^2_{(2)} = 9.51$, $p = .009$) supports the claim that the tendency of an oxytocin-treated animal to perform this active component of maternal behavior was <u>not</u> independent of duration of pre-test cage habituation.

Figures 3 - 9 and 3 - 10 illustrate the proportions of the groups found with three warm pups grouped in a nest the next morning and the proportions of animals retrieving three pups within 15 minutes at the end of the next morning's maternal behavior assessment. All of the animals, regardless of habituation duration or icv treatment, were equally likely to be found with pups in a nest the next morning. There also appeared to be no statistically significant relationship

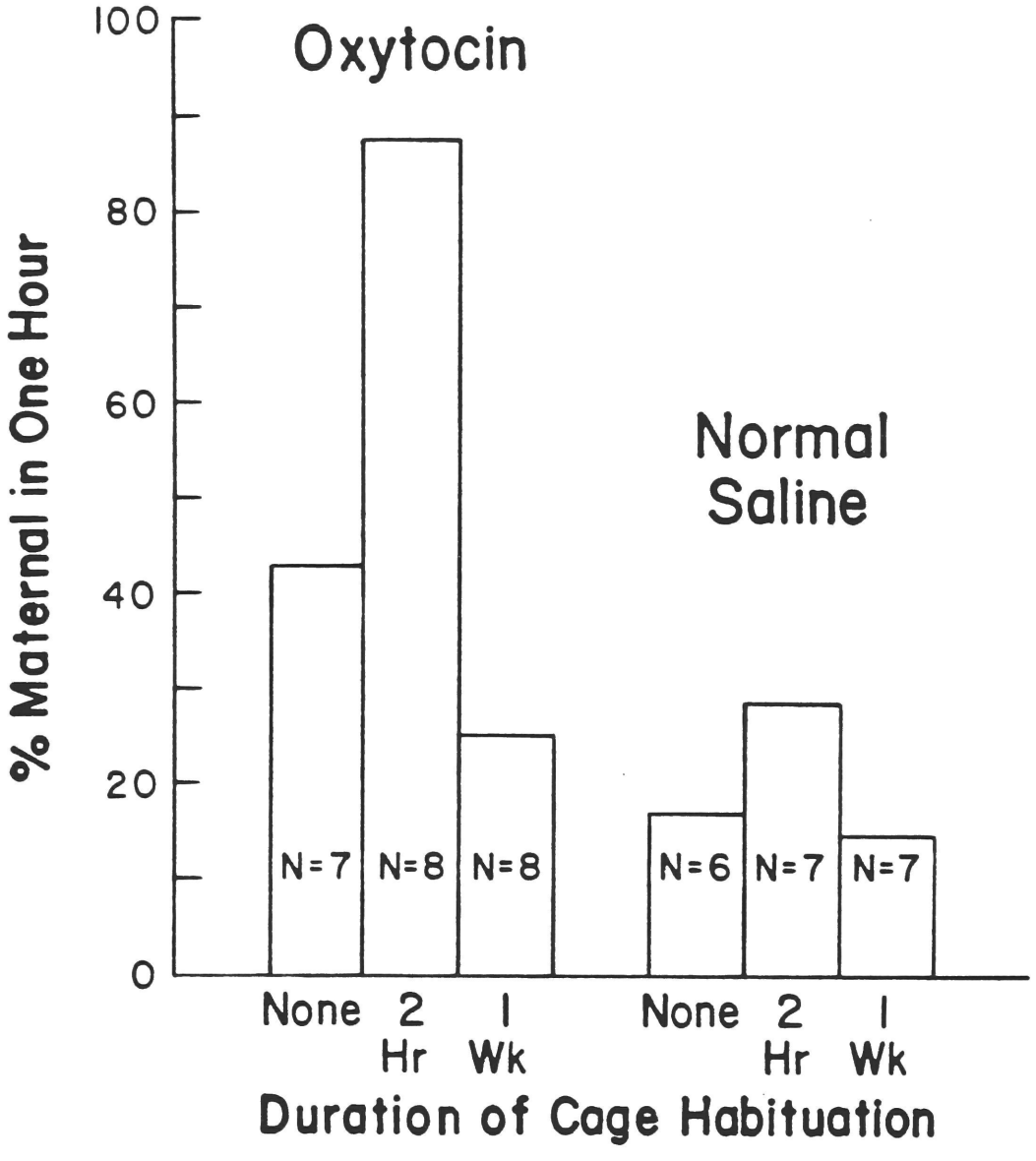



Figure 3 - 7. Effect of duration of cage habituation on oxytocin induction of short-latency maternal behavior in estrogen-primed virgins. Oxytocin groups: $\chi^2_{(2)} = 6.6$, $p = .036$. Saline groups: $\chi^2_{(2)} = .506$, n.s.

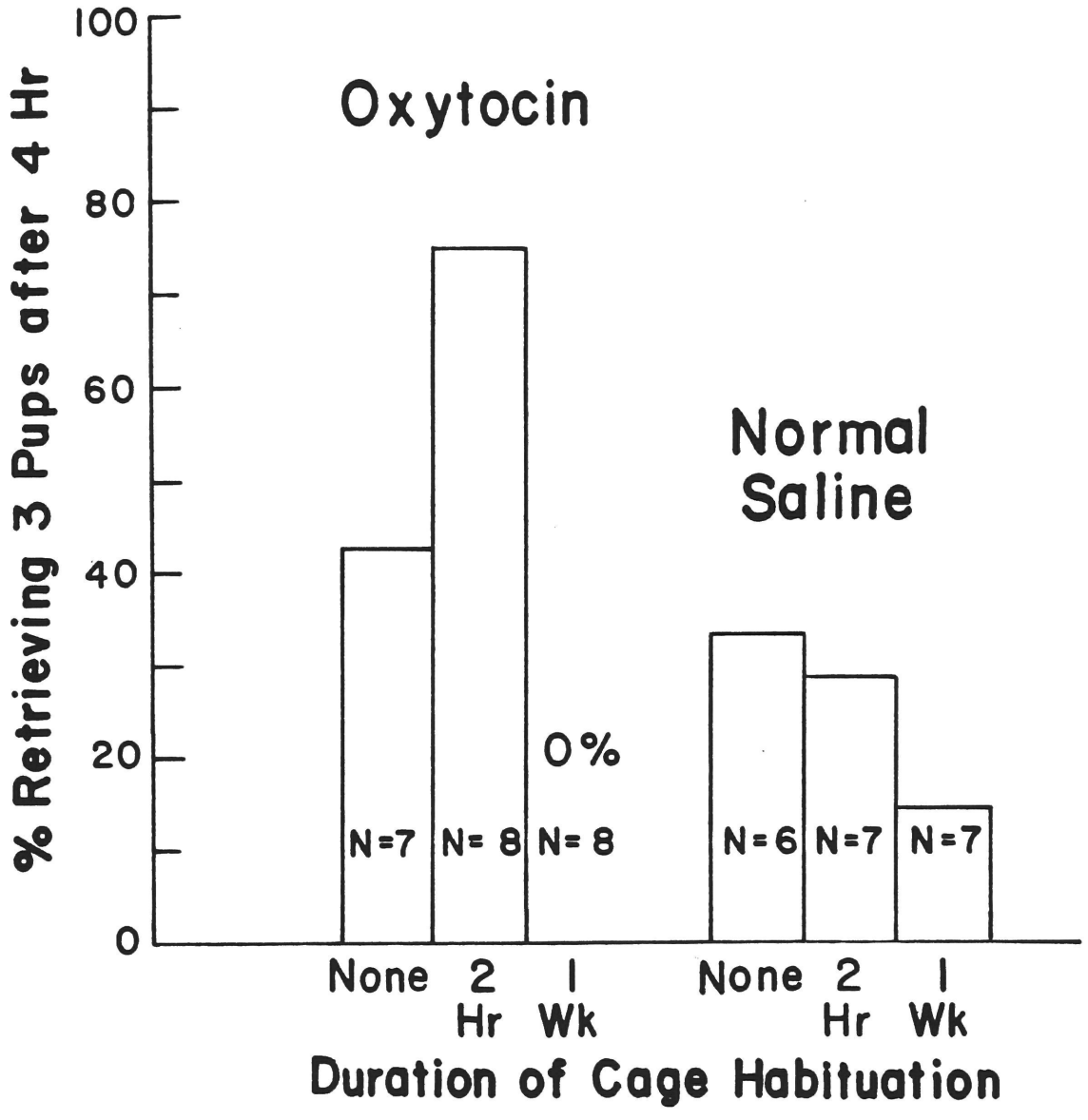



Figure 3 - 8. Effect of duration of cage habituation on retrieval of 3 scattered pups during a 15 minute retrieval test conducted 4 hours after icv infusion and introduction of pups into test cage. Oxytocin groups: $\chi^2_{(2)} = 9.51$, $p = .009$. Saline groups: $\chi^2_{(2)} = .698$, $p = .674$, n.s

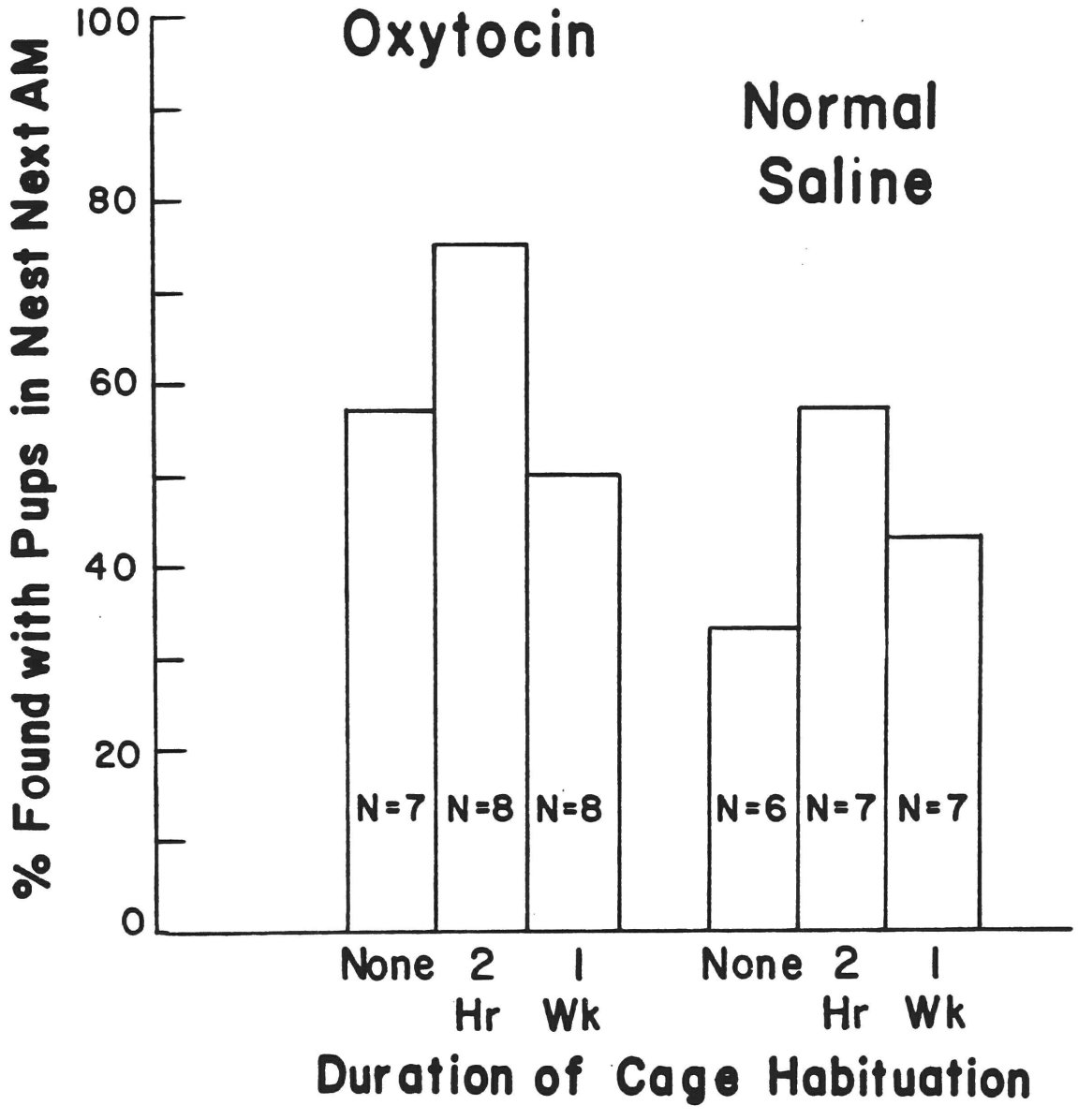



Figure 3 - 9. Effect of duration of cage habituation on proportion of groups found with 3 warm pups grouped in a nest the next morning. Oxytocin groups: $\chi^2_{(2)} = 1.12$, $p = .577$, n.s. Saline groups: $\chi^2_{(2)} = .760$, $p = .663$, n.s.

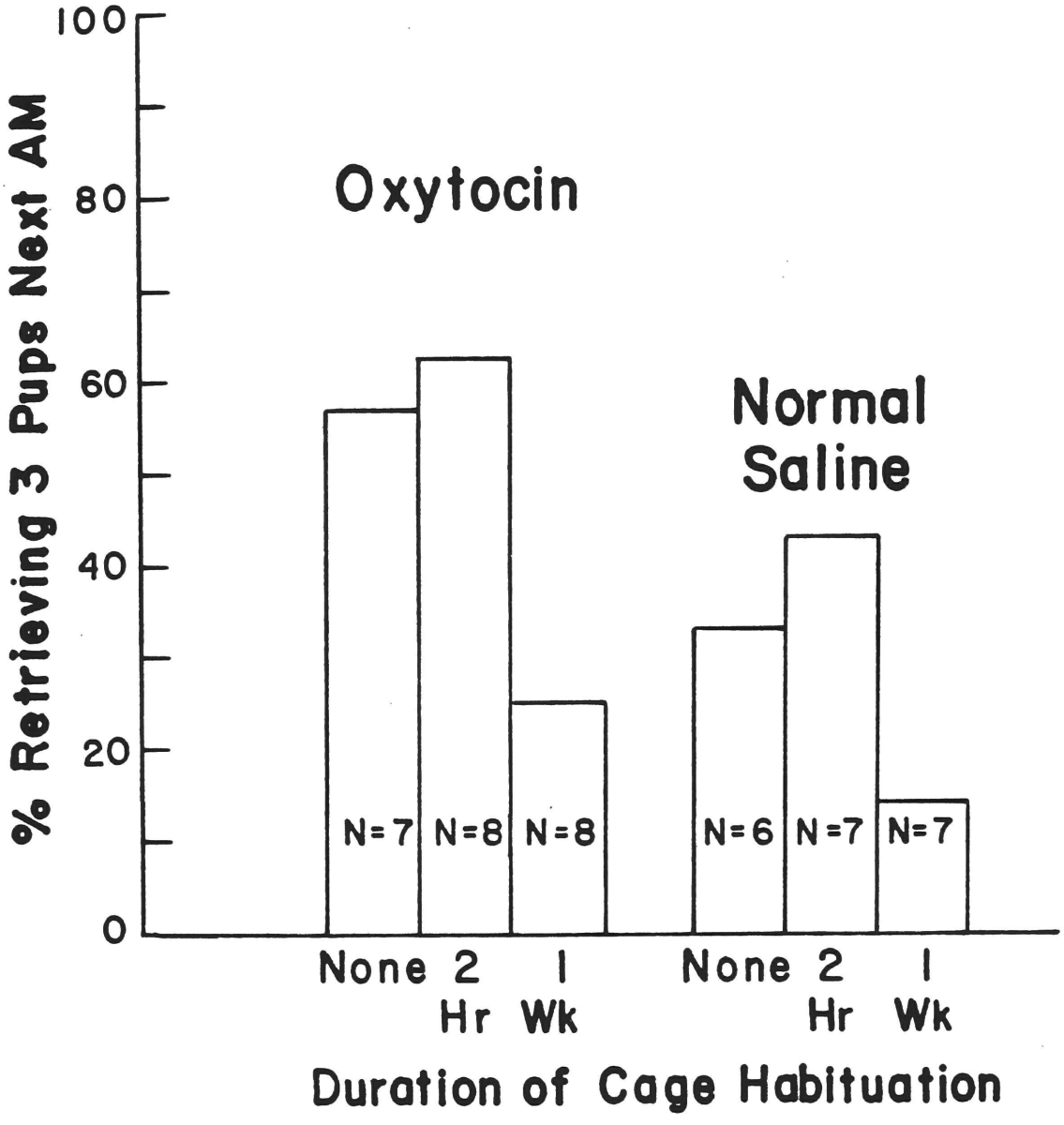



Figure 3 - 10. Effect of duration of cage habituation on proportion of groups retrieving 3 scattered pups during a 15 minute retrieval test conducted the next morning. Oxytocin groups: $\chi^2_{(2)} = 2.60$, $p = .272$, n.s. Saline groups: $\chi^2_{(2)} = 1.41$, $p = .500$, n.s.

between treatment and proportion of animals retrieving three pups the next morning, although Figure 3 - 10 suggests that the animals given the long term (one week) habituation to the test cage were least likely to carry scattered freshly-fed pups to the nest regardless of whether they had received icv oxytocin or normal saline.

C. Discussion

The expected pattern of response (predicted by the results of Experiment 1) was observed in the estrogen-primed animals infused with either oxytocin or normal saline and tested in cages that they had been housed in for only two hours: after only one hour of contact with foster pups, 87.5% of the oxytocin group vs. 28.5% of the normal saline animals exhibited full maternal behavior. However, when animals were given the same treatment and behavior tests in their home cages, there was no difference between the performances of the saline- and oxytocin-treated animals (25.0% vs. 14.3%). There was also no statistically significant difference between the oxytocin- and saline-treated groups which were placed in their test cages with the foster pups immediately after receiving the icv infusion. This same pattern of results--significant responsivity to pups seen only in the oxytocin-treated animals of the short-term habituation group--was also evident in the results of the retrieval test given after four hours of pup contact. However, by the next morning (approximately 20 hours after the icv infusion), the differences among the groups were no longer as pronounced. Surprisingly, transfer to the test cage immediately after the oxytocin infusion (the no habituation condition), does not appear to inhibit the oxytocin effect as much as

does the one week habituation (home cage test).

How might the familiarity of the test environment influence the display of maternal behavior? There was no difference in the latency to respond across the saline-treated groups, suggesting that it is the combination of the presence of raised levels of oxytocin in the central nervous system and short-term habituation to a novel environment which facilitates the performance of maternal behavior (in this experiment and in experiment 1 above) in estrogen-primed virgins. In most maternal behavior experiments, animals are tested in their home cages. It has been suggested that testing female hamsters (cycling virgins) in a neutral test environment rather than in their home cages enhances the probability of nurturing behavior and depresses the incidence of cannibalism, but the evidence for this view is indirect (Marques and Valenstein, 1976). Introduction into a new cage is known to stimulate neuroendocrine changes presumed to be stress-related (e.g., Bassett et al., 1973; Jolles et al., 1979; Green et al., 1979). This raises the possibility that some aspect of the rats' endocrine/neurochemical responses to the novel environment intracted with the oxytocin treatment to facilitate responsivity to pups. When this undefined factor is absent because the animals are tested in a very familiar environment--their home cage--the combination of two days of estrogen priming and 400 ng of oxytocin cannot elicit short-latency maternal behavior in ovariectomized virgin rats.

The balance of endogenous factors which synergize with exogenous oxytocin in an estrogen-primed animal appears to be rather delicate. Extreme novelty (the no habituation condition) did not further enhance

the likelihood of inducing short-latency maternal behavior with oxytocin. This suggests that too much of the novelty-induced factor may be inhibitory; alternatively, time (several hours) may be required for this factor to "develop". Or perhaps it may be wrong to characterize the novelty-induced state as "potentiating" the central effects of oxytocin: instead, the oxytocin may be allowing the animal to overcome a novelty-induced inhibition of maternal behavior, and the dose used too small to overcome the effects of acute novelty as compared to two hours' old novelty. This explanation is the least appealing of the three since, although it might be argued that the lack of an independent effect of duration of cage habituation on responsivity to pups in the saline group reflects only an inability to lower further an already extremely low level of responsivity. According to such a hypothesis the animals tested in their home cages with oxytocin should also exhibit short-latency maternal behavior. This experiment demonstrates that they do not.

Since the action of even 400 ng of oxytocin is likely to be transient (half-clearance time for oxytocin in rat cerebrospinal fluid is estimated to be approximately 20 minutes: Mens et al., 1983), the two hour cage habituation period may have provided a serendipitious conjunction of raised central levels of oxytocin and the endogenous effects of novelty. Note that a major difference between the test procedure used successfully by Pedersen et al. (1982) and in the present experiments and the method employed by Rubin et al. (1983) is that the latter investigators tested the animals in their home cages. This apparently minor procedural change may partially explain the inability of Rubin et al. (1983) to elicit short latency maternal

behavior with icv-administered oxytocin. (A relatively long--24 hour--pre-test cage habituation period may have also inhibited the oxytocin induction of maternal behavior in the experiments of Bolwerk and Swanson [1984]. However, since the procedure used by these authors is not comparable to the method described here in other features such as dose of oxytocin, nature of the steroid hormone priming regimen, and strain of rat used, it is difficult to interpret this study in relation to the present results).

The apparent dependence of the oxytocin induction of short-latency maternal behavior on non-pup related environmental cues suggests that oxytocin activity is but one factor taken into account by the estrogen-primed brain regulating the behavioral response of the virgin rat to initial pup presentation. Identification of the relevant cues and the changes in neural and neuroendocrine function they evoke should provide a clearer understanding of the mechanism by which oxytocin elicits this complex behavioral sequence.

Such research might also elucidate behaviorally critical neuroendocrine changes that occur in the parturient rat. While it is highly speculative to suggest, for example, that the stress of being placed in a novel environment could mimic the stress of the first parturition, a reasonable strategy for exploring the relationship of the endocrine manifestations of stress to maternal behavior might begin with the use of adrenalectomized and adrenal-product replaced (steroids, amines) animals in the standard test procedure described above.

A critical role for adrenal products in the endocrine regulation of the onset of maternal behavior has been largely discounted because

adrenalectomzed rats can exhibit maternal behavior even though lactation is disrupted (Thoman and Levine, 1970), and the performance of 16-day pregnant rats hysterectomized, ovariectomized, and treated with estradiol benzoate is not altered if adrenalectomy is carried out with the ovariectomy (Siegel and Rosenblatt, 1978). There are some hints that early postpartum maternal behavior might be disrupted in adrenalectomized rats (Hennessy et al., 1977), although the reported retrieval deficits are minor.

Stress-induced effects need not necessarily be adrenal-mediated. The close anatomical relationship of oxytocinergic fibers to brainstem and spinal cord structures regulating autonomic responses as well as the diversity of its afferents has been emphasized in the recent literature (e.g., Swanson and Sawchenko, 1983).

Stressors such as forced swimming or immobilization have been shown to release pituitary oxytocin into the peripheral circulation (Lang et al., 1983). The effect of such treatments on central oxytocin release has not been studied, nor are the effects of such stressors on maternal behavior performance known.

Note finally that the comparison of the effects of novelty--certainly a mild stress, if "stress" indeed it be--and parturition, certainly an extreme life stress, is not entirely disproportionate, as the rat experiences a damping of adrenal responsiveness prior to parturition (Ota et al., 1974), which may serve as one of its functions to protect maternal behavior from any disruptive effects of stress.

In summary, this experiment has demonstrated that length of habituation to the test cage can be a critical factor in determining

an ovariectomized, estrogen-primed virgin rat's response to centrally-administered oxytocin and the presence of foster pups. The feature of the environment and the neuroendocrine mechanisms underlying this effect are not yet known.

## V. Experiment 4

The above results suggested a role for a central nervous system response to endogenous oxytocin in the onset of maternal behavior at parturition, but the behavior induction studies with estrogen-primed virgins cannot speak to this question. To test this hypothesis directly, I have attempted to reduce the effectiveness of action of endogenous oxytocin before and during first pup contact. I have done this by treatments intended to reduce the amount of oxytocin available to act at receptors (by administering antisera to oxytocin icv) or by competitive inhibition of oxytocin's actions at the level of the receptors (by administering an analog antagonist of oxytocin icv). I predicted that these treatments would disrupt the performance of maternal behavior by increasing latency to respond to pups. In order to gain maximal control over the timing of the changes in levels of steroid hormones postulated to be critical in the hormonal induction of maternal behavior, and in order to be sure that our anti-oxytocic treatments would be acting at a time when maternal behavior would otherwise be elicited with high probability, I chose to use the pregnancy-terminated model developed by Siegel and Rosenblatt (1975). In this preparation 16 day pregnant rats are hysterectomized, ovariectomized, and immediately injected s.c. with estradiol benzoate.

With a high dose of estrogen (100 µg/kg body weight), 80% or more of the animals respond maternally to foster pups on the first day of testing (48 hours after surgery and estrogen treatment). Use of this preparation enabled the experiments to be conducted within a defined system which is believed to reproduce the critical endocrine events accompanying the natural termination of pregnancy by the birth of the young (for a review of experiments based on this preparation, see Rosenblatt et al., 1979).

A. Materials and Methods

Animals and housing. Four-day pregnant Sprague-Dawley rats (first pregnancy) were obtained from Zivic-Miller Laboratories, Inc. for use in the experiments described here. Other timed-pregnant rats purchased from Zivic-Miller at 12 or 14 days gestation served as pup donors. Rats were housed singly under a 12:12 light-dark cycle with lights on at 0600 hr. Test subjects were housed in a separate room from other pregnant or lactating females.

Surgical procedures and steroid hormone treatments. On day eight of pregnancy, rats under Nembutal anesthesia were implanted with left lateral ventricle cannulae and given 0.02 ml Terramycin intramuscularly. After cannulation, animals were weighed daily, and implants were inspected and cleaned to accustom the rats to the handling necessary for icv injection. On day 16 of gestation, pregnancy was terminated by ovariectomy and hysterectomy under Metofane anesthesia. Immediately following surgery the animals received a s.c. injection of estradiol benzoate (EB) in corn oil vehicle (20 or 100 µg EB/kg body weight) or the vehicle alone. Some

16-day pregnant animals were ovariectomized, hysterectomized, and EB-treated without prior cannulation.

Experimental groups. The following six experimental groups were formed. Baseline groups received either 20 μg EB/kg body weight (n=7) or 100 μg EB/kg body weight (n=8) or corn oil (n=7). These groups will hereafter be referred to as EB20, EB100, and CO. Three disruption groups with lateral ventricle cannulae received 100 μg EB/kg body weight and either 1) icv infusion of antiserum to OXY (n=8; 2 μl administered 3 times daily at 0800, 1400, and 2000 hr beginning day 15 of pregnancy, with an additional final 5 μl infusion preceding pup presentation by 30 min) 2) icv infusion of 800 ng/10 μl normal saline vehicle of an analog antagonist to OXY, $d(CH_2)_5$-8-ornithine-vasotocin twice, 2 hr and several minutes preceding pup presentation (n=6) or 3) icv infusion of either an antiserum to antidiuretic hormone (ADH; n=3) or undiluted normal rabbit serum (NRS; n=4) according to the anti-OXY schedule given above. (These animals did not differ in their maternal behavior performance and will be treated as an icv control group, combined n=7). These three groups will subsequently be referred to as anti-OXY, OXY-antag., and anti-ADH/NRS, respectively.

Materials. Two antisera to oxytocin were used. Four animals received icv infusions of undiluted anti-oxytocin (rabbit) obtained from Immuno Nuclear Corp. (Stillwater, MN), Lot. #17162. A second group (n=4) received microinfusions of an anti-oxytocin (rabbit) obtained from Dr. T. van Wimersma Greidanus (Rudolf Magnus Institut voor Farmacologie, Utrecht) and used diluted 1:10 in 10 mM phosphate-buffered saline (pH = 7.4). The analog antagonist to

oxytocin, d$(CH_2)_5$-8-ornithine-vasotocin ([1 $\beta$-mercapto-$\beta$, $\beta$-cylopenta methylene propionic acid, 8-ornithine] vasotocin) was a gift from Dr. M. Manning (Medical College of Ohio, Toledo, OH). It has been pharmacologically characterized (Bankowski et al., 1980) as an antagonist to oxytocin in both uterine and mammary gland preparations. The anti-antidiuretic hormone (rabbit) was also obtained from Dr. van Wimersma Greidanus and was used diluted 1:10 in 10 mM phosphate buffered saline.

Antibody characterization. The anti-oxytocin and anti-antidiuretic hormone antisera obtained from Dr. van Wimersma Greidanus have been used extensively in our laboratory for immunohistochemical analyses of magnocellular neurons in rat hypothalamus (e.g., Rhodes et al., 1981b), and we are confident of their specificity. Since I had no prior immunohistochemical experience with Immuno Nuclear anti-oxytocin, I stained Zamboni's- and paraformaldehyde-fixed 75 $\mu$m vibratome tissue sections through rat hypothalamus using this reagent and the ABC method (Vector Laboratories, Inc., Burlingame, CA) with diaminobenzidine for visualization. The cellular pattern of staining revealed in the subdivisions of the magnocellular nuclei of the rat hypothalamus after performing a standard immunocytochemical procedure with Immuno Nuclear anti-OXY antiserum was identical to that reported in previous studies from our laboratory with other anti-OXY antibodies and histochemical procedures.

Behavioral testing. Two hours before first exposure of the test subjects to foster pups (46 hours after surgery and EB treatment), animals were moved into test cages (36 cm x 47 cm x 20 cm) containing

a 2.5 cm layer of wood shavings and approximately 30 strips of paper towel (1 cm x 26.5 cm). At 48 hours after hysterectomy and ovariectomy (at approximately 1300 hr), three foster pups between 12 and 24 hours of age were scattered in the females' cages. These pups remained until the next morning (approximately 0900 hr), when they were replaced by freshly-fed pups in the age range 1-5 days. The first day of pup contact was designated as day 0 of testing.

Animals were tested in groups of 6, with animals from at least 3 of the groups being tested on any given day. Ten 1 minute observations were made of each animal during the first hour of contact with pups. Subsequent observations were made at the end of 2,3,4, and 5 hours of pup contact. A five-category maternal behavior score was used to summarize the observations, as previously described. One point each was given for licking of pups, carrying of pups, grouping of pups, crouching over pups in a nursing posture, and building of a maternal nest. Nest quality was rated on a five-point scale: 0 = no nest, 1 = flat sleeping nest, 2 = small nest containing pups, 3 = maternal nest with high sides around pups, 4 = excellent maternal nest using all available paper. Animals with scores of 4 or 5 and a nest quality rating $>$ 2 were rated maternal. If an animal did not respond maternally during the first 5 hours of observation (latency of 0 days), a latency was assigned which was defined as the first day on which retrieval and grouping of 3 pups in a maternal nest was observed.

Controls for General Disruptive Effects of $d(CH_2)_5$-8-ornithine-vasotocin on Behavior. To ensure that any observed effects were due to interference with mechanisms regulating

the onset of estrogen-facilitated maternal behavior and not to treatment-induced malaise or a nonspecific inattention to external cues that would render the animal incapable of performing maternal behavior, I also studied the effects of icv infusion of $d(CH_2)_5$-8-ornithine-vasotocin on established maternal behavior. Eight 8 day pregnant rats, housed under a 12:12 L:D cycle with lights off at noon, were implanted with lateral ventricle cannulae. The day of birth of the litters was designated day 0. Litters were culled or supplemented so that all contained 8 pups. Starting on day 1 postpartum two daily observation sessions were held, one during the light part of the cycle (1100-1200 hr) and one in the dark (1600-1700 hr). Each animal was observed once every 5 minutes for a total of 12 recorded observations/hour. A checklist was used, adapted from that described by Bertino (1982). Items recorded were: nursing, resting with pups, resting without pups, grooming, pup grooming, object manipulation, locomotion, eating, drinking, and time away from litter. Two hours and a few minutes prior to the Day 5 am observation the animals were given and icv infusion of either 10 µl normal saline or 800 ng $d(CH_2)_5$-8-ornithine-vasotocin dissolved in 10 µl normal saline. The daily observations were then carried out as usual. Day 6 was the final day of testing. In order to determine if the icv infusion of an antagonist to oxytocin would have any effect on milk ejection, the litters were weighed before the start of the test on day 5 and then 2, 5, and 24 hours after the icv treatment.

Verification of cannula placement. At the end of testing (5 or 6 days), the females were given an overdose of Nembutal and perfused

intracardially with normal saline followed by 10% formalin. 100 μm frozen sections were cut to determine the location of the cannula tip. All animals for which data are presented here had cannulae placed in the left lateral ventricle at the level of the preoptic area with no accompanying tissue damage. No animals had to be discarded from this study because of improper cannula placement.

Data analysis. The significance of group differences was analyzed by nonparametric methods, including the Kruskal-Wallis one way analysis of variance and the Mann-Whitney U-test. For between groups comparisons of proportions the Fisher exact probability test was used. A two-factor mixed design analysis of variance for repeated measures on one factor (Bruning and Kintz, 1977) was used to analyze the effects of the OXY antag. on the maternal behavior of lactating rats. A significance level of .05 was applied throughout. Four animals which cannibalized during the first hour of pup contact were not tested further. These animals - one from the baseline group EB20, one from the corn oil group, one from the disruption group anti-OXY, and one from the disruption group receiving anti-ADH - were not included in subsequent data analyses. The weights of the litters of the lactating mothers receiving icv saline or $d(CH_2)_5$-8-ornithine-vasotocin were compared using the Student t-test. The Stats Plus general statistics package for the Apple IIe Personal Computer (Human Systems Dynamics, 1982) was used to carry out these statistical tests.

B. Results

Effects of anti-oxytocin antisera and an oxytocin analog antagonist on maternal behavior latency. Compared with 16-day pregnant, hysterectomized, and ovariectomized rats receiving 100 ug EB/kg body weight and no icv injection, 16-day pregnant animals receiving the same surgery and EB treatment plus either anti-OXY antiserum or $d(CH_2)_5$-8-ornithine-vasotocin exhibited significantly longer latencies to respond to foster pups with maternal behavior. Figure 3-11 shows for all groups cumulative percentage maternal expressed in days of pup contact. The Kruskal-Wallis test (corrected for ties) indicated there was an overall significant difference in latency among the groups, $H(5) = 177.5$, $p < .001$. Subsequent Mann-Whitney U-tests showed anti-OXY or OXY antag. EB controls, and the two groups receiving 100 µg EB/kg body weight plus anti-OXY or $d(CH_2)_5$-8-ornithine-vasotocin could not be distinguished in latency from the CO group (CO vs. anti-OXY: $z = -1.04$, n.s.; CO vs. OXY-antag.: $z = -0.21$, n.s. Also, as expected, the pregnancy-terminated animals receiving no EB had significantly longer response latencies than either of the EB-baseline groups (EB20 vs. CO: $z = -1.67$, $p < .05$, one-tailed test, corrected for ties; EB100 vs. CO: $z = 2.31$, $p < .01$, one-tailed test). Finally, the latency of the CO group was also significantly longer than that of the Anti-ADH/NRS group ($z = 1.98$, $p < .03$, one-tailed test). The group receiving 100 µg EB/kg body weight plus anti-ADH or NRS had the same latency as the two EB-only baseline groups (EB100 vs. anti-ADH/NRS: $z = 0.46$, n.s.; EB20 vs. anti-ADH/NRS: $z = 0.51$, n.s.).

As is evident in Figure 3-11 the differences among the groups

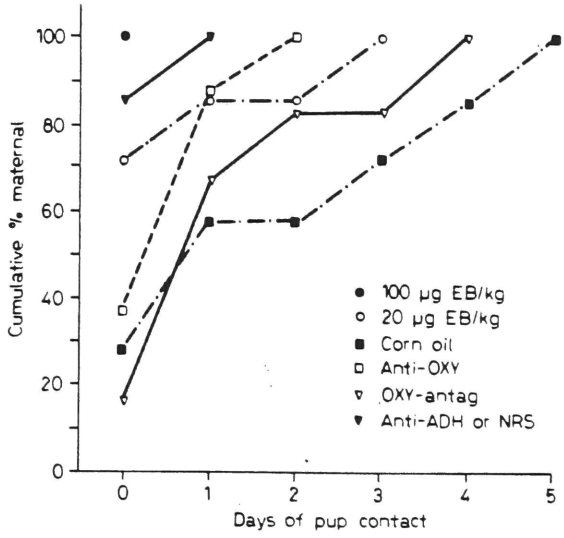



Figure 3 - 11. Delaying effect of icv infusion of anti-oxytocic agents on the onset of maternal behavior. The cumulative percentage of rats ovariectomized and hysterectomzed on day 16 of gestation responding maternally to foster pups is shown for 6 days of pup contact. The anti-OXY, OXY-antag., and anti-ADH/NRS groups all received 100 µg EB/kg body weight at the time of surgery. The groups differ significantly in response latency measured in days of pup contact, $p < .001$ (Kruskal-Wallis test, corrected for ties).

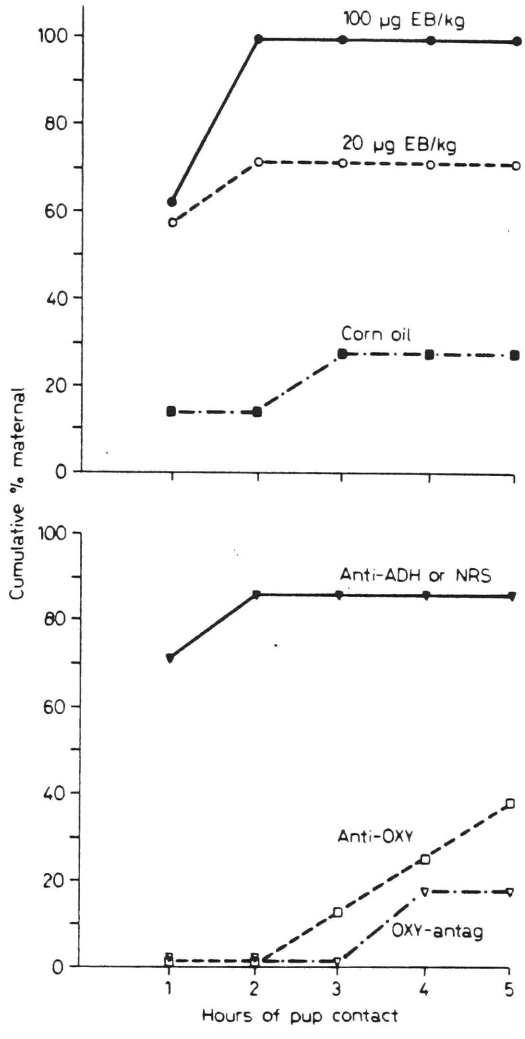



Figure 3 - 12. Cumulative percentage of rats responding maternally to pups during the first five hours of pup contact (same animals whose response latency is summarized by days in Figure 3 - 11). After 1 hour or 5 hours there was no difference in proportion of group responding maternally among the groups receiving only EB and the group receiving EB and NRS or anti-ADH. The anti-OXY and OXY-antag. groups (which also received 100 µg EB/kg body weight) were not different from the group receiving only corn oil (proportions compared with the Fisher exact probability test, one-tailed, $\alpha = .05$).

TABLE 3 - 3

PERCENTAGE OF 16-DAY PREGNANT, HYSTERECTOMIZED AND OVARIECTOMIZED RATS SHOWING MATERNAL BEHAVIOR DURING FIRST HOUR WITH PUPS

| Group | N | Licking Pups | Carrying Pups | Grouping Pups | Crouching over Pups | Nest Building |
|---|---|---|---|---|---|---|
| 100 µg EB/kg b.w. | 8 | 75 | 87.5 | 75 | 62.5 | 75 |
| 20 µg EB/kg b.w. | 7 | 71.4 | 57.1 | 57.1 | 57.1 | 71.4 |
| corn oil | 7 | 42.9 | 28.6 | 14.3 | 14.3 | 14.3 |
| 100 µg EB/kg b.w. + anti-OXY | 8 | 37.5 | 12.5 | 12.5 | 0 | 12.5 |
| 100 µg EB/kg b.w. + OXY antagonist | 6 | 50 | 33.3 | 16.7 | 16.7 | 0 |
| 100 µg EB/kg b.w. + anti-ADH or NRS | 7 | 71.4 | 71.4 | 71.4 | 71.4 | 71.4 |

Abbreviations ADH, anti-diuretic hormone; b.w., body weight; EB, estradiol benzoate; NRS, normal rabbit serum; OXY, oxytocin.

were most pronounced on the first day of pup contact (day 0). All animals which were not maternal on day 0 eventually displayed maternal behavior in $\leq 5$ days, as determined by the daily retrieval tests. Since the final icv infusion was made shortly before pup presentation, this could represent either a wearing-off of the anti-oxytocic effects or the combination of a long period of steroid priming (16 days gestation and 2 days EB treatment) with proximity to pups, or both.

Effects of anti-oxytocin antisera and an oxytocin analog antagonist on responsivity to pups during the first hour of pup contact. Figure 3-12 shows the cumulative percentages of maternal animals in the baseline and disruption groups during the first 5 hr of pup contact. At the end of the first hr, the ranking seen at the end of day 0 had already been established: EB100 = EB20 = anti-ADH/NRS > anti-OXY = OXY antag. = CO (proportions compared with the Fisher exact probability test, one-tailed, $\alpha = .05$). Table 3-3 shows the percentages of animals which, during the first hour with pups, performed the individual behaviors that constitute maternal care. Many animals which did not attain maternal behavior scores of 4 or 5 nevertheless showed some pup interest as indicated by frequent observations of pup licking and occasional observations of pup carrying. Table 3-3 also shows that crouching over grouped pups and nest building were the two components of maternal behavior least likely to be elicited within an hour from animals in the CO, anti-OXY, and OXY-antag. groups.

Nest quality ratings after 5 hours of pup contact. The group median nest quality ratings received after the first 5 hours with pups present were EB100, 3; EB20, 3; CO, 1; anti-OXY, 1; OXY antag., 1;

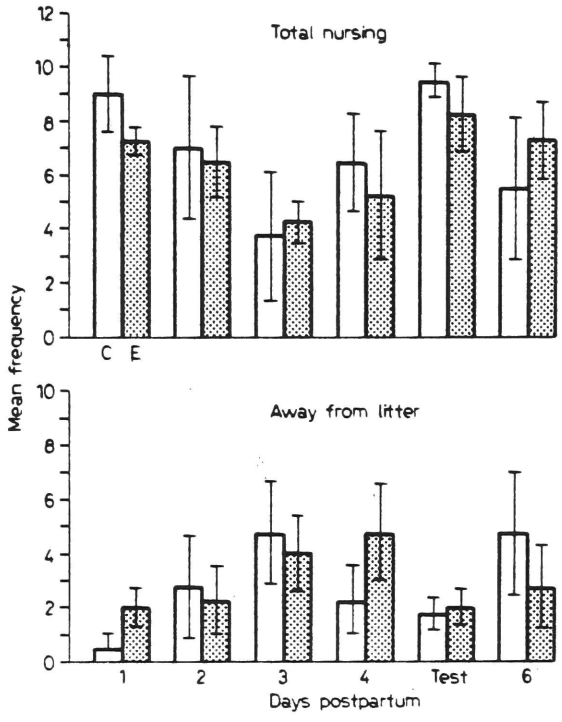



Figure 3 - 13. Lack of effect of $d(CH_2)_5$-8-ornithine vasotocin on behavior of lactating females. During daily observation sessions, rats were observed to be either nursing or away from their litters with the mean frequencies ( $\pm$ s.e.m.) indicated. Two hours and 5 minutes prior to the observation session on day 5 postpartum, the experimental group ⊡ (n=4) received an icv infusion of 800 ng of $d(CH_2)_5$-8-ornithine-vasotocin in 10 µl normal saline, while the control group □ (n=4) received 10 µl normal saline. Groups did not differ in their responses to icv infusion.

TABLE 3 - 4

EFFECT OF $d(CH_2)_5$-OVT AND NORMAL SALINE ON TOTAL NURSING AND TIME AWAY FROM LITTER

Analysis of Variance: Two Factor Mixed Design: Repeated Measures on One Factor (Bruning & Kintz, 1977)

AM OBSERVATIONS

| | Total Nursing | | | | | Time Away from Litter | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Source | SS | df | MS | F | P | SS | df | MS | F | P |
| TOTAL | 58 | 47 | - | - | - | 381.25 | 47 | - | - | - |
| Between Subjects | 66 | 7 | - | - | - | 53.08 | 7 | - | - | - |
| Conditions (NS vs. $d(CH_2)_5$-OVT) | 2 | 1 | 2.0 | 0.19 | n.s. | 0.33 | 1 | 0.33 | 0.04 | n.s. |
| Error (between subjects) | 64 | 6 | 10.7 | - | - | 52.75 | 6 | 8.79 | - | - |
| Within Subjects | 515 | 40 | - | - | - | 328.17 | 40 | - | - | - |
| Trials(days) | 118 | 5 | 23.6 | 1.86 | n.s. | 57.51 | 5 | 11.50 | 1.41 | n.s. |
| Trials X Conditions | 17 | 5 | 3.4 | 0.29 | n.s. | 84.25 | 5 | 16.85 | 2.07 | n.s. |
| Error (within subjects) | 380 | 30 | 12.7 | - | - | 244.25 | 30 | 8.14 | - | - |

TABLE 3 - 4 (CONTINUED)

EFFECT OF $d(CH_2)_5$-OVT AND NORMAL SALINE ON TOTAL NURSING AND TIME AWAY FROM LITTER

Analysis of Variance: Two Factor Mixed Design: Repeated Measures on One Factor (Bruning & Kintz, 1977)

PM OBSERVATIONS

| | Total Nursing | | | | | Time Away from Litter | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Source | SS | df | MS | F | P | SS | df | MS | F | P |
| TOTAL | 758 | 47 | - | - | - | 694 | 47 | - | - | - |
| Between Subjects | 418 | 7 | - | - | - | 315 | 7 | - | - | - |
| Conditions (NS vs. $d(CH_2)_5$-OVT) | 19 | 1 | 19 | 0.28 | n.s. | 17 | 1 | 17 | 0.34 | n.s. |
| Error (between subjects) | 399 | 6 | 66.5 | - | - | 298 | 6 | 50 | - | - |
| Within Subjects | 340 | 40 | - | - | - | 379 | 40 | - | - | - |
| Trials (days) | 60 | 5 | 12 | 1.60 | n.s. | 58 | 5 | 11.6 | 1.33 | n.s. |
| Trials X Conditions | 54 | 5 | 10.8 | 1.44 | n.s. | 58.5 | 5 | 11.7 | 1.34 | n.s. |
| Error (within subjects) | 226 | 30 | 7.5 | - | - | 262.5 | 30 | 8.75 | - | - |

TABLE 3-5

Changes in Litter Weight

after ICV Infusion of $d(CH_2)_5$-8-OVT or Normal Saline

| Hours After IVC Infusion | Group (8 pups/litter) $d(CH_2)_5$-8 OVT | Normal Saline |
|---|---|---|
| 2 hours | 0.8±0.7[a] grams | 1.3±0.8 grams |
| 5 hours | 2.3±1.4 grams | 3.0±0.7 grams |
| 24 hours | 16.8±2.6 grams | 11.6±4.5 grams |

a $\bar{X} \pm$ s.e.m. Groups were compared using the Student t-test. No significant differences were found at any time.

ABBREVIATIONS icv, intracerebroventricular; OVT, ornithine-vasotocin

anti-ADH/NRS, 4. The differences in nest quality produced by no EB (CO group) or reduced OXY effectiveness (anti-OXY or OXY antag. groups) were significant [Kruskal-Wallis test (corrected for ties), $H(5) = 155$, $p < .001$]. Subsequent Mann-Whitney U-tests indicated that, again, EB100 = EB20 = anti-ADH/NRS > anti-OXY = OXY-antag. = CO.

Effect of an oxytocin analog antagonist on established maternal behavior. Lactating rats treated with $d(CH_2)_5$-8-ornithine-vasotocin on day 5 postpartum did not differ from controls infused with normal saline in frequency of performance of any of the maternal behaviors on the checklist. Both groups of rats were observed to be nursing more frequently and away from the pups less during the hour immediately following the second icv infusion, but this effect was not significant (see Figure 3-13). Table 3-4 shows the results of the analysis of variance for the two representative measures, time spent with pups in the nest and incidence of nursing during the hour observation periods. No difference in litter weights was found at 2,5, or 24 hours when litters nursed by mothers receiving central administration of the antagonist were compared with litters of animals receiving normal saline. These data are summarized in Table 3-5.

C. Discussion

The performance of the three groups treated with EB100, EB20, or corn oil replicates in Sprague-Dawley females obtained from Zivic-Miller the finding of Siegel and Rosenblatt (1975) that pregnant rats hysterectomized and ovariectomized on day 16 of gestation and treated with estrogen exhibit significantly faster onset of maternal

behavior compared with control females treated with oil. The longer latency of the CO group indicates the critical role of estrogen in the hormonal stimulation of maternal behavior.

I conclude, from comparison of the performance of the anti-oxytocin and oxytocin-antag. groups with that of the two EB-only groups, that a temporary reduction in oxytocin action in the central nervous system significantly delays the onset of estrogen-dependent maternal behavior. The lack of any difference between the EB-only groups and the anti-ADH/NRS group suggests that the inhibitory effect of anti-oxytocin antisera and $d(CH_2)_5$-8-ornithine-vasotocin on maternal behavior was specific to actions on the brain oxytocin systems, and did not represent a general inhibitory effect of icv injection or rabbit-derived antibodies. The prediction that icv administration of anti-oxytocic agents would disrupt the onset of estrogen-stimulated maternal behavior, even in a preparation as extremely responsive as the 16 day pregnant, hysterectomized, ovariectomized EB-treated rat (100% maternal within 2 hours in this study with 100 µg EB/kg body weight) is confirmed.

Short pretreatment (beginning 1 hour before pup introduction) with $d(CH_2)_5$-8-ornithine-vasotocin was as effective in delaying the onset of the behavior as longer (4 days) pretreatment with anti-OXY antisera. This suggests that lengthy pretreatment was not necessary to produce the effect, and by extension, that oxytocin could act simultaneously with (or as a result of) pup stimulation to facilitate maternal behavior.

A disruptive effect of icv $d(CH_2)_5$-8-ornithine-vasotocin on maternal performance was not seen in lactating rats tested 5 days

postpartum, suggesting that oxytocin is not importantly involved in the maintenance phase of maternal behavior. In order to determine if the presence of an oxytocin antagonist in the brain was compatible with the performance of maternal behavior, we purposely chose to examine its effects using a non-disruptive observational procedure in lactating rats, in which the strength of the behavior is presumably greatest. It is possible that a disruptive effect might have been seen if the pregnancy-terminated preparation had been used or that a more rigorous test involving transfer to a new cage, retrieval of scattered pups, building a new nest, etc. might have revealed differences between the groups' performances. A test in which the animal is moved to a new cage and required to carry and to group pups and to build a new nest would be more similar to that used in the oxytocin induction of behavior in virgins, and would place more emphasis on the active components of maternal behavior. The goal of this experiment was to explore a possible physiological role for oxytocin in the maintenance phase of normal, undisturbed maternal behavior. The delineation of test conditions under which the behavior can be successfully disrupted should be an important strategy in determining how brain oxytocin alters behavior. Future studies on the importance of brain oxytocin in the maintenance phase of maternal behavior should use the specific anti-oxytocin antibody as well as the $d(CH_2)_5$-8-ornithine-vasotocin, and might make use of varied durations of mother-pup separations.

The effectiveness of $d(CH_2)_5$-8-ornithine-vasotocin in delaying the onset of maternal behavior suggests that oxytocin receptors in the brain may share features with the

better-characterized uterine receptor, since this analog antagonist was defined as anti-oxytocic by virtue of its effectiveness in antagonizing the contractile actions of oxytocin on the rat uteus (Bankowski et al., 1980). (This substance is also an antagonist of the milk-ejection response to oxytocin). There have been reports that oxytocin itself can influence the firing rate of hypothalamic magnocellular neurons electrophysiologically identified as oxytocinergic on the basis of their firing patterns (Moss et al., 1972). Central injections of oxytocin have been shown to stimulate the milk-ejection reflex in the rat (Freund-Mercier and Richard, 1981). In this experiment no deleterious effect of central administration of the antagonist, $d(CH_2)_5$-8-ornithine-vasotocin, on milk output was found. The litter weight estimate of milk output, however, is an extremely crude and insensitive measure of the milk ejection reflex: it is likely that a subtle change in the activity of the oxytocinergic magnocellular neuron population would not be detected by this means.

The results of experiment 4, together with the previous demonstration that exogenous oxytocin can facilitate the performance of maternal behavior in ovariectomized, estrogen-treated virgins, suggest a role for oxytocin action in the heightened responsivity to pups displayed by the parturient female. Oxytocin could act in concert with the central nervous system effects of estrogen or could possibly mediate some of the effects of estrogen with regard to maternal behavior. These possibilities will be explored further in the General Discussion at the end of this chapter.

## VI. Experiment 5

As mentioned in Chapter 1, oxytocin is a normal constituent of the cerebrospinal fluid (Dogterom et al., 1977). This radioimmunoassayable oxytocin almost certainly does not represent neurohypophysial material, as it has been shown that cerebrospinal fluid and plasma oxytocin levels can vary independently in response to both natural (suckling) and artificial (electrical stimulation) inducers of oxytocin release (Robinson and Jones, 1982; Jones et al., 1983). It is possible that one link in the "maternal behavior circuit" involves release of oxytocin into the cerebrospinal fluid (perhaps via the oxytocinergic innervation of the choroid plexus and other circumventricular organs: reviewed by Kozlowski et al., 1978), distribution of the peptide by this means throughout the central nervous system, and, ultimately, action at a distance from the release site. However, the well-documented presence of oxytocin in neurons and its neurotransmitter-like characteristics such as its presence in terminal varicosities (Buijs and Swaab, 1979) and release by $Ca^{++}$-dependent mechanisms (Buijs and van Heerikhuize, 1982) strongly suggest that an equally plausible account would involve depolarization-dependent synaptic release of oxytocin at or near its receptors.

Thus, Experiment 5 involves microinjection of small volumes of oxytocin directly into potential target sites, using the same estrogen-primed virgin test procedure outlined in Experiment 1 above. The strategy used to choose the tissue test sites was to compare information from studies of oxytocin distribution in the brain with

what is known about neural circuitry important for maternal behavior. Making the assumption that immunocytochemical descriptions of the location of oxytocin fibers in the brain represent physiological sites of oxytocin release, I explored systematically a number of sites at which the two types of information overlap. I expected that a positive site would contain endogenous oxytocin and would have high sensitivity--that is, that lower levels of oxytocin would be required to elicit estrogen-dependent behavior from this site than from the lateral ventricle.

A. Methods

Potential tissue target sites were screened in the first phase of this experiment. These sites were the ventral tegmental area; the midbrain central grey; the medial amygdala; the medial preoptic area; the lateral septum; and (as a control site) the caudate putamen. The procedure was similar to that used in Experiment 1, with the exception that minute-by-minute records were typically not kept in order to facilitate screening of several animals at once. Virgin female rats obtained from Zivic-Miller were housed in our facility two weeks prior to surgery. The details of the surgical procedure (and drugs used at this time) are given above. In addition, all animals received i.m. injections of .02 ml Bicillin-LA (Wyeth Laboratories) at the time of surgery to reduce the chance of infection associated with the wound. The implanted cannulae were either purchased from Plastic Products (22 gauge) or made in our laboratory from 23 gauge thin wall stainless steel hypodermic tubing obtained from Small Parts, Inc. (Miami, FL). Appropriate coordinates were determined from the atlases of König and

Klippel (1963) and Paxinos and Watson (1982); they were tested in female rats of the same age and weight as those used in these studies (see Table 3-6 for actual coordinates used). One week after implant surgery the rats were ovariectomized under Metofane anesthesia. At this time they received a priming dose of EB, 100 µg/kg body weight. Two days (46 hours) later the rats were transferred from their home cages to the test cages. Two hours later (48 hours after EB treatment), the rats received intracranial infusions of either oxytocin (typically 100 or 200 ng/0.25 µl/side of brain; see Table 3-6) or normal saline. Each rat was given bedding material and three foster pups 12 to 24 hours old. At one, two, three, and four hours post-infusion, each cage was checked for the presence of a nest and indications of responsivity to pups. At four hours all animals were given a retrieval test in which the three pups were scattered (one to each cage quadrant not containing a nest) or, in the case of non-maternal animals, moved to a new location. The animals were then observed to see if retrieval of pups to a nest occurred during the next 15 minutes. The procedure on the next morning and on following days until the latency to respond maternally was determined was identical to that described for Experiment 1, as was the subsequent tissue processing for anatomical localization of cannula placement and method of data analysis. Since it was noted that animals were sometimes found with three warm pups grouped in the nest at the start of morning observations even when they did not successfully retrieve three pups to the nest within 15 minutes, latencies both for grouping pups in a nest overnight and retrieval are presented.

Animals implanted with bilateral medial preoptic area cannulae

TABLE 3 - 6

EFFECTS OF MICROINFUSION OF OXYTOCIN AND NORMAL SALINE ON SHORT-LATENCY MATERNAL BEHAVIOR[a]

| CANNULA PLACEMENT | STEREOTAXIC COORDINATES | INFUSION | MATERNAL<1 Hr ALL PLACEMENTS | MATERNAL<1 Hr "GOOD" PLACEMENTS | FOR ALL PLACEMENTS: MEDIAN 1 HR NEST RATING | MEDIAN LATENCY: PUPS IN NEST | MEDIAN TO RET. ≤15 MIN. |
|---|---|---|---|---|---|---|---|
| Ventral Tegmental Area | Bilateral (head up 5 mm: from interaural zero) AP +2.4 ML ±0.5 DV -2.7 | 200 ng OXY/ 0.25 µl NS (n=17) 0.25 µl NS (n=9) | OXY: 10/17[b] NS: 0/9 | OXY: 7/10[b] NS: 0/7 | OXY: 3[c] NS: 1 | OXY: 0[d] NS: 1.5 | OXY: 0[c] NS: 3 |
| Midbrain Central Grey | Bilateral (head level: 10° perpendicular to interaural line: from bregma) AP -6.8 ML ±1.0 DV -4.5 | 200 ng OXY/ 0.5 µl NS (n=13) 0.5 µl NS (n=7) | OXY: 4/13 NS: 1/7 | OXY: 3/11 NS: 1/4 | OXY: 2 NS: 1 | OXY: 0.5 NS: 0 | OXY: 1 NS: 0.5 |
| Medial Preoptic Area | Bilateral (head level: from bregma) AP 0 ML ±0.8 DV -6.9 | 100 ng OXY/ 0.25 µl NS (n=10) 0.25 µl NS (n=9) | OXY: 4/10 NS: 4/9 | OXY: 4/10 NS: 4/9 | OXY: 2 NS: 2 | OXY: 1 NS: 0 | OXY: 1 NS: 1 |

a additional animals implanted in the substantia nigra, lateral septum, and lateral preoptic area are not included on this chart due to poor cannula placements or small size of group. See also TABLE 3 - 7.

b $p<.01$, Fisher exact probability test, one-tailed

c $p<.01$, Mann-Whitney U-test, one-tailed

d $p<.02$, Mann-Whitney U-test, one-tailed

TABLE 3 - 7

EFFECTS OF MICROINFUSION OF OXYTOCIN ON SHORT-LATENCY MATERNAL BEHAVIOR

| CANNULA PLACEMENT | STEREOTAXIC COORDINATES | INFUSION | MATERNAL<1 Hr ALL PLACEMENTS | MEDIAN 1 HR NEST RATING | MEDIAN LATENCY: PUPS IN NEST | MEDIAN TO RET. ≤ 15 MIN. |
|---|---|---|---|---|---|---|
| Medial Amygdala | Bilateral (head level: from bregma) AP -2.6 ML ±3.0 DV -8.0 | 200 ng OXY/ 0.5 µl NS (n=12) | OXY: 4/12 | OXY: 2 | OXY: 0.5 | OXY: 1 |
| Caudate Nucleus | Unilateral (head level: from bregma) AP -0.5 ML +3.0 DV -4.3 | 400 ng OXY/ 0.5 µl NS (n=7) | OXY: 0/7 | OXY: 2 | OXY: 1 | OXY: 1 |
| Septum | Unilateral (head level: from bregma) AP -0.5 ML +0.5 DV -4.1 | 400 ng OXY/ 0.5 µl NS (n=5) | OXY: 0/5 | OXY: 2 | OXY: 2 | OXY: 2 |
| Lateral Ventricle | Unilateral (head level: from bregma) AP -0.5 ML +1.6 DV -4.3 | 400 ng OXY/ 0.5 µl NS (n=4) | OXY: 2/4 | OXY: 3 | OXY: 0.5 | OXY: 0.5 |

were tested as described under Experiment 1, with grooming and pup-directed behaviors recorded every minute during the first post-infusion hour.

Tables 3-6 and 3-7 provide a summary of this set of experiments.

B. Results

A total of 112 rats were tested with foster pups after intracranial infusions of either oxytocin or normal saline. Of these animals, nine killed a pup; these were not tested further and they have been omitted from the following data analysis. In only one case--that of an animal implanted with bilateral ventral tegmental area cannulae--could the cannibalism be associated with an unusually large amount of tissue damage at the injection site.

When comparisons were made between the responses of animals receiving microinfusions of oxytocin and saline into the ventral tegmental area, the midbrain central grey, and the medial preoptic area, significant differences were present only in the ventral tegmental area group (see Table 3 - 6). Animals receiving bilateral infusions of oxytocin into the ventral tegmental area were more likely to respond to foster pups with full maternal behavior in less than one hour than were their saline counterparts (Fisher exact probability test, one tailed, $p = .004$). They also had significantly higher quality nests at the end of that hour (Mann-Whitney U-test, one-tailed $p < .01$). The latencies (in days) of the oxytcin-treated animals both to be found with three warm, well-grouped pups at the start of a morning observation and to retrieve three scattered, freshly-fed pups within 15 minutes were significantly shorter than those recorded for

the saline group. All of the cannula placements were judged to be adequate in the sense that spread of infusate to the target area would almost certainly have occurred. However, when a subset of cases was considered in which the placements were rated as especially "good" on the basis of their symmetry and appropriate dorsal-ventral position of the tip of the injection needle, the same between-groups differences were still present. Figure 3 - 14 shows the location of the effective ventral tegmental area cannula placements.

No significant differences in maternal behavior were found between the oxytocin- and saline-treated animals in the midbrain central grey and medial preoptic area groups. However, when total grooming during the first post-infusion hour was compared in the two medial preoptic area treatment groups, oxytocin was found to promote short-latency grooming (Median grooming scores: oxytocin group, n=10, 408 seconds; saline group, n=9, 139 seconds. Mann-Whitney U-test, one-tailed, $p < .01$).

No apparent induction of short-latency maternal behavior was seen in several groups given localized infusions of oxytocin. This includes a group of animals given bilateral infusions of oxytocin into the medial amygdala (see Table 3 - 7). Figure 3 - 15 shows the location of the cannula placements in this preliminary study. There appears to be no pattern to the cannula placements of the responding and nonresponding animals that would account for the differences in behavior. Also, it seems unlikely that slight differences in cannula tip location would be of critical significance given that such a large infusion volume (0.5 $\mu$l) was used.

It was of special interest to compare results obtained from

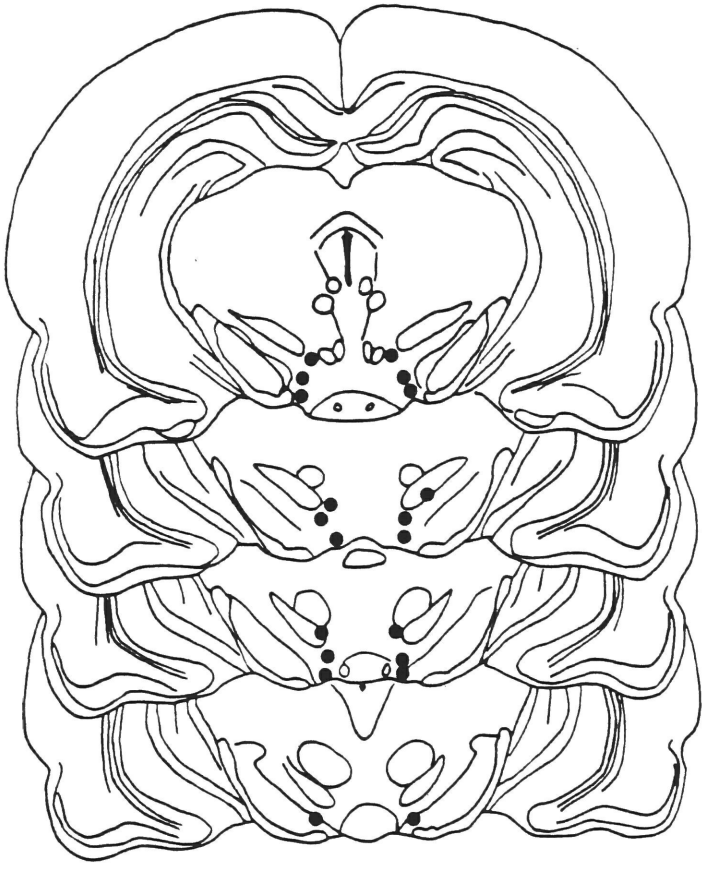


Figure 3 - 14. Ventral midbrain sites of oxytocin activation of short-latency maternal behavior. Ovariectomized, estrogen-primed virgin rats (ZM/SD) received bilateral infusions of oxytocin (200 ng/0.5 µl/ side). ● = tip of cannula track. Cannula placements shown for 10 animals responding maternally to foster pups ≤ 1 hour. Transverse sections through the rat midbrain approximate levels A 2420 µ - A 1760 µ (König and Klippel, 1963).

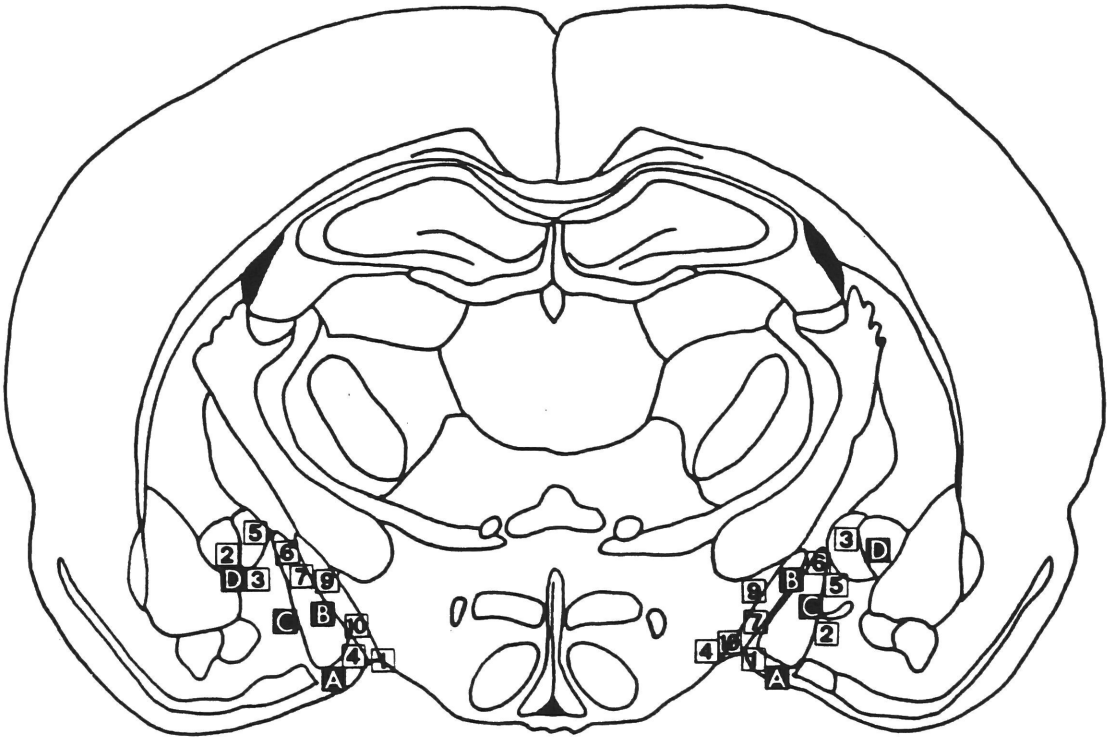



Figure 3 - 15. Amygdala sites of oxytocin microinfusion. Ovariectomized, estrogen-primed virgin rats (ZM/SD) received bilateral infusions of oxytocin (200 ng/0.5 µl/side). Blocks containing numbers or letters indicate the tip of the cannula track: A, B, C, and D show the location of the pairs of placements from animals responding maternally to foster pups $\leq$ 1 hour. Numbers indicate placements from animals <u>not</u> responding to pups with short-latency maternal behavior. Transverse section through the rat diencephalon, approximately level A 4620 µ (König and Klippel, 1963).

from estrogen-primed females receiving unilateral oxytocin infusions into the septum, lateral ventricle, and caudate nucleus (see Table 3 - 7). I reasoned that if infusions into the caudate nucleus (not considered an oxytocin target tissue) elicited the same response as infusions aimed at the lateral septum, then diffusion of the injected substance into the adjacent lateral ventricle could be invoked to account for any effects of septal injections on maternal behavior, because the two injection sites are equidistant from the ventricular wall. No short-latency maternal behavior (see Table 3 - 7) was elicited by oxytocin injection into the caudate nucleus or the septum. The only short-latency responses were found in the lateral ventricle group (2/4 fully maternal in less than one hour). These three groups were compared by means of the Kruskal-Wallis one-way analysis of variance: there were no differences on any behavioral measure. (This comparison is less instructive than inititally planned because of the loss of three animals--one due to pup cannibalism, the other two to respiratory ailments--in the lateral ventricle group before completion of testing.)

C. Discussion

Lesions of the ventral tegmental area abolish maternal behavior (Gaffori and Le Moal, 1979; Numan and Smith, 1984), and the ventral tegmental area receives substantial input from the critical medial preoptic region (Conrad and Pfaff, 1976b; also Chapter 2, this thesis). In the ventral midbrain, scattered neurophysin and oxytocin fibers are seen in the ventral tegmental area (Swanson, 1977; Buijs, 1978; Kozlowski et al., 1983; Fahrbach and Morrell, unpublished

observations). At this level the main body of oxytocin fibers travel caudally and dorsally between the medial lemniscus and the zona compacta of the substantia nigra. (There is no evidence yet whether the immunocytochemical raction product detected in this region represents axon terminals in addition to fibers of passage.) Of the various tissue sites tested in the screening studies described above, only the ventral tegmental area consistently yielded positive responses to oxytocin. The results of the present study clearly indicate that the oxytocin sensitivity of the ventral midbrain merits further examination. Such studies must be expanded considerably before it can be concluded that the ventral tegmental area is a physiological site at which oxytocin acts to facilitate maternal behavior. Lower doses of oxytocin and control cannula placements will have to be included in future studies. Other peptides, especially other neurohypophysial peptides, should be screened for their action at this site on maternal behavior to rule out the possibility that some nonspecific, pharmacological stimulatory action on cells in this region resulted in the facilitation of maternal behavior. Demonstration of oxytocin release sites and oxytocin receptors in this region will also be necessary.

Finally, although the cannulae were aimed at the ventral tegmental area and although the histological results indicated that this goal was by and large achieved, the spread of the 0.5 µl injection volume in the ventral midbrain almost certainly extended to the more lateral portions of this region. As mentioned above, the substantia nigra may also receive an oxytocinergic innervation from the hypothalamic paraventricular nucleus. Lesions of the substantia

nigra produce a transient disruption of maternal behavior in lactating rats (Numan and Nagle, 1983); action of oxytocin at this brain site instead of in the more medial ventral tegmental area cannot be ruled out in the interpretation of the results of Experiment 5.

Spread of injected fluid may also occur dorsally around the chronically-implanted guide cannula. The results of infusion of oxytocin into the midbrain central grey, however, appear to rule out action of the peptide at this site in the experiments involving ventral tegmental area sites. In this experiment, the responses of animals receiving oxytocin injections into the midbrain central grey did not differ from those of animals receiving saline treatment. As reviewed in Chapter 2 of this thesis, the medial preoptic region sends many fibers to the midbrain central grey; like the ventral tegmental area, the midbrain central grey also contains scattered oxytocin fibers (Swanson, 1977; Buijs, 1978; Kozlowski et al., 1983; Fahrbach and Morrell, unpublished observations).

A possible role for the amygdala in the regulation of maternal behavior is suggested by the facts that 1. lesions of the amygdala facilitate the performance of maternal behavior by virgin rats (Fleming et al., 1980) 2. the amygdala has reciprocal connections with the medial preoptic region (Ottersen, 1980; Berk and Finkelstein, 1981) and 3. oxytocin is present in the medial amygdala in fibers and axon terminals (Buijs and Swaab, 1979). Since connections of the medial preoptic region with the medial amygdala may be especially important for the performance of maternal behavior by virgin females (Miceli et al., 1983) and some early (and incomplete) accounts of the brain distribution of oxytocin receptors have indicated that they may

be present in the amygdala (de Kloet et al., 1984), this target site seemed especially likely to yield a positive maternal behavior response. Although the results at present are certainly not complete, they hardly offer encouragement. The same may be said of septal sites.

The medial preoptic area itself contains immunoreactive oxytocin fibers and scattered oxytocin-containing cell bodies (Rhodes et al., 1981b; Fahrbach and Morrell, unpublished observations). To test the possibility that the same neurons what mediate some of the effects of estrogen on maternal behavior might be the site of oxytocin action (implicit in this hypothesis is another that the responsivity of those cells to oxytocin would be directly modulated by estrogen), I compared the maternal behavior of animals receiving preoptic infusions of either oxytocin or normal saline. Although raised levels of preoptic oxytocin did not facilitate the performance of maternal behavior by these estrogen-primed virgins, grooming was significantly increased in these animals.

This finding is particularly interesting in light of a recent report by Ferris and Albers (1984) reporting that intracranial injections of both oxytocin and vasopressin stimulate flank grooming in the golden hamster and that the medial preoptic area was the most sensitive site from which this behavior could be elicited. Flank-grooming in the hamster is part of a behavioral sequence of flank marking. This sequence itself can be elicited by vasopressin (but not oxytocin) injection into the medial preoptic area (Ferris et al., 1984). The relationship of oxytocin-induced grooming in the rat to this species' broader behavioral adaptation is not yet clear, but

these two sets of results suggest that the medial preoptic region may have a special function in the regulation of grooming.

It is tempting at this point to remember that, in addition to being a response to novelty (Bindra and Spinner, 1958), mild stress (Colbern et al., 1981), intracranial infusion of exogenous peptides (e.g., ACTH and prolactin in addition to oxytocin and vasopressin: Jolles et al., 1979; Drago et al., 1980), and unclean fur, grooming is also a thermoregulatory response of heat-stressed rodents. The grooming response to experimentally induced, abnormally high levels of preoptic oxytocin may reflect a regulatory role for this peptide in coordinating thermoregulatory and maternal responses. One problem with this assumption of overlapping function which suggests that the medial preoptic region coordinates a variety of motivated behaviors is that preoptic-lesioned rats still groom in the heat (Roberts and Martin, 1977) or in response to intraventricular infusions of ACTH (Colbern et al., 1977). Thus, it is likely that the neural substrates for grooming are represented at many different levels of the central nervous system. This is in contrast with maternal behavior, which does not survive preoptic lesions, possibly because its representation is so strongly tied to a single specific endocrine input, the estrogenic signal.

The only previous studies linking oxytocin and the ventral midbrain concern oxytocin regulation of catecholamine function in this region (Kovács and Telegdy, 1983). The relevance of such studies to maternal behavior will be considered in Chapter 4.

# VII. General Discussion

Changes in central nervous system levels of oxytocin can both facilitate (increased levels) or delay (decreased levels) the performance of maternal behavior in estrogen-primed rats. In these studies the timing of the behavior onset and not the quality of the behavior was altered. The established maternal behavior of lactating rats was not disrupted by a treatment successful in delaying the onset of estrogen-dependent maternal behavior. This suggests that oxytocin's role may be most powerful in directing the naive animal's first responses to pups.

The estrogen-dependent effects of oxytocin on maternal behavior latency reported here and by others (Pedersen et al., 1982) are interpretable within the preexisting framework of knowledge about the neuroendocrine regulation of maternal behavior. The effect of oxytocin in this paradigm is to elicit with short latency a behavior that all nonparturient, non-hormone-treated rats are capable of performing with a longer latency. Hormone treatments can reduce this latency to one or two days of pup exposure, but in virgin rats only a combination of endocrine treatments involving hysterectomy and specific "naturalistic" strategies of pup presentation or olfactory deafferentation (Mayer and Rosenblatt, 1980) have been shown to reduce this latency to the order of minutes. Taken together, these findings have led to the view that, while estrogen action on the brain facilitates the performance of maternal behavior, all rats but those which have undergone a full pregnancy find pups aversive, and that maternal behavior is not elicited until this aversion (which appears

to have an olfactory basis) is overcome (e.g., see Mayer and Rosenblatt, 1975; Fleming and Luebke, 1981). It is possible that the parturient female lacks this aversion because the mode of stimulus presentation is optimal. However, this seems unlikely to account for the entire difference as full-term caesarean-delivered rats respond maternally with short latency regardless of method of pup presentation (Mayer and Rosenblatt, 1980). The missing coordinating factor in the virgin's performance may be neuroendocrine rather than environmental. It is possible that oxytocin release in the brain (or enhanced responsivity to oxytocin) may be a part of the neuroendocrine events which overcome the pup aversion and act in concert with raised levels of estrogen to produce the short-latency onset of maternal behavior characteristic of the parturient and near-term female. The sum of endogenous oxytocin and exogenous peptide produced by the procedure described above may resemble raised levels presumed, although not yet proved, to be present in the rat central nervous system at parturition.

The estrogen dependence of the oxytocin effect on maternal behavior may represent the summation of two independent facilitatory mechanisms, neither of which alone is sufficient for short-latency onset. It has not yet been tested whether, in the absence of the estrogen pretreatment, a higher dose of oxytocin than that used in these experiments can stimulate rapid onset of maternal behavior. An alternative hypothesis is that estrogen pretreatment may in some way prepare the brain for oxytocin action. This latter possibility is attractive in light of the demonstrated estrogen induction of oxytocin receptors in the uterus and mammary gland (Soloff et al., 1979) as

well as reports that some estradiol-concentrating cells in the rat brain also contain oxytocin (Rhodes et al., 1982). Such findings support the hypothesis that estrogen may regulate the functioning of brain oxytocin through effects on oxytocin biosynthesis or release and/or on oxytocin receptor populations.

There is some preliminary evidence for the existence of oxytocin receptors in the rat brain (e.g., van Leeuwen et al., 1984) but these receptors have not yet been characterized, and the distribution of oxytocin receptors in the brain is not known. Functional studies such as those reported in this chapter provide ample justification for the studies at the cellular level of oxytocin action in the brain. The present results suggest that there may be at least two populations of oxytocin receptors in the central nervous system: one which is susceptible to the regulatory influence of estrogen and which underlies the short-latency induction of maternal behavior in the parturient animal and the estrogen-primed virgin and another which is estrogen-insensitive and mediates the short-latency induction of grooming. Altered brain levels of oxytocin have also been shown to affect the performance of male rats on several tasks which may involve learning and memory (Bohus et al., 1978; Kovács et al., 1979): as these peptide effects have a very different time course from the effects described here, they may represent yet a third oxytocin mechanism involved in behavior.

Even in advance of knowledge of specific anatomical details, several possible functional links between brain estrogen and oxytocin action, differing in important ways, can be distinguished. An obligatory relationship would require that estrogen facilitation of

maternal behavior always occur via changes in the functioning of the brain oxytocin systems. In this case, oxytocin would be said to mediate estrogen's effects on maternal behavior, and any interference with oxytocin action would be predicted to eliminate estrogenic facilitation of maternal behavior. Also, any other means of causing the same change in oxytocin function should be able to substitute for estrogen priming.

In the extreme alternative case, oxytocin would be described as acting in parallel with or in concert with estrogen to facilitate maternal behavior, but not by being part of the estrogen-regulated neural circuitry. That is, cells containing oxytocin or receptors for oxytocin would not necessarily be found to be elements in the minimal neural pathways essential for the performance of short-latency maternal behavior. This would not rule out the possibility of estrogen effects on brain oxytocin, but would relegate these effects to being only a part of the mechanisms by which estrogen regulates maternal behavior onset. The oxytocin effect on behavior need not be restricted to estrogen-dependent behaviors or responsiveness to pups, but could perhaps represent a more general change in stimulus responsivity, perhaps a regulation of responses to novel environmental cues.

I find the latter hypothesis more plausible. I think of the steroid action on the brain as "priming" the animals to respond to young with unlearned, species-specific nurturing behavior, and the oxytocin as acting to permit expression of estrogen priming immediately during the first exposure to pups. In the absence of enhanced central nervous system oxytocin function (either produced by

experimental manipulation or presumed to be present normally at parturition in the parturient rat), this estrogen priming can also be revealed by continued exposure to pups.

This position is supported by several types of evidence. First, oxytocin administration does not alter the quality or intensity of short-latency maternal behavior, only the proportion of estrogen-primed animals expressing it. This finding implies that oxytocin is not directing the behavior, but rather is determining whether or not it will be displayed at a particular time. Second, the results of experiments using various cage habituation periods show that non-pup related environmental cues can alter the display of maternal behavior despite the presence of estrogen and oxytocin. This suggests that oxytocin activity is but one factor taken into account by the estrogen-primed brain regulating the behavioral response of the virgin rat to initial pup presentation. Again, parallels to the work of Mayer and Rosenblatt (1980) are relevant. Like oxytocin injections, the treatments they used (olfactory-vomeronasal deafferentation or staggering the introduction of pups during the early hours of the light part of the cycle rather than presenting three at once in the dark) allow virgin rats to overcome their aversion to rats and hence reveal the maternal behavior priming effects of estrogen.

I have proposed that oxytocin may have a permissive role to play in the expression of maternal behavior, and that estrogen acts independently in other, as yet undetermined ways, to promote responsiveness to pups. Of course, in addition to independent maternal behavior priming actions, estrogen might also act to make

subsequent oxytocin action more effective in the ways discussed above. The widespread distribution of oxytocin pathways in the brain suggests a variety of central nervous system functions for this peptide. Estrogen sensitivity at some points in the pathways and not at others would allow the steroid hormone to amplify selectively one or more aspects of this system, in coordination with the peripheral endocrine events of parturition also regulated by estrogen.

The anatomical findings reported in Chapter 2 of this thesis support the idea that estrogen acts on a medial preoptic → ventral tegmental area circuit important for both the onset and maintenance of maternal behavior. Estrogen may <u>also</u> act on the ventral tegmental area (which lacks its own estrogen receptors) via a paraventricular nucleus (oxytocinergic) → ventral tegmental are connection important only for facilitating the onset of the first performance of maternal behavior. More speculatively, the medial preoptic region projections to the hypothalamic paraventricular nucleus may be the pathway via which the preoptic region regulates the onset of maternal behavior, while the direct medial forebrain bundle projection to the ventral midbrain may be critical for the maintenance of postpartum maternal behavior.

Thus, the rise in estrogen which occurs during the last days of gestation in the rat may have as one of its effects the enhancement of brain oxytocin function. This would in turn promote the short-latency behavioral expression of the long-term priming effects of the steroid hormone levels of pregnancy at the birth of the pups. The oxytocin facilitation of estrogen-dependent maternal behavior may represent one of several neuroendocrine mechanisms ensuring that contact with

offspring is maintained until the behavior is fully established and can be sustained solely by continuing interaction with pups.

# Chapter 4 The Role of the Ventral Tegmental Area in Maternal Behavior

Several different approaches to the neuroendocrine and neuroanatomical substrates of maternal behavior in the rat have led to the ventral tegmental area. In Chapter 2 of this thesis a combined steroid hormone autoradiography/retrograde tracing technique was used to demonstrate that estradiol-concentrating neurons in the preoptic region project to the ventral midbrain. In Experiment 5 of Chapter 3, the ventral tegmental area is identified as a site at which oxytocin can act (at least in pharmacological doses) to induce short-latency maternal behavior in estrogen-primed female rats. Previous studies by Gaffori and Le Moal (1979) and Numan and Smith (1984) have demonstrated the disruptive effect of lesions of the ventral tegmental area on both the onset and maintenance of maternal behavior.

The morphology of the local cell populations and the connectivity of the ventral tegmental area of the rat have been extensively described (Phillipson, 1979a,b,c; Beckstead et al., 1979; Simon et al., 1979; Swanson, 1982; Albanese and Minciacchi, 1983; Poirier et al., 1983). This interest is in large part inspired by the presence of numerous dopamine-containing cells (the A10 cell group) in this region (reviewed by Fuxe et al., 1974). The ventral tegmental area consists of a mixed population of dopaminergic and nondopaminergic cells. Recent studies have shown that the various efferent targets of the ventral tegmental area receive their projections from discrete subsets of ventral tegmental neurons (Swanson, 1982; Albanese and Minciacchi, 1983). These identified projection neurons are by and

large interminged and scattered across the subdivisions of the ventral tegmental area, but some clustering of neurons with shared output can be discerned. Such a cluster is formed by the ventral tegmental neurons which project to the nucleus accumbens: although such cells are found throughout the ventral tegmental area, they are most densely concentrated in the ventral half of the area (Swanson, 1982). This is the part of the ventral midbrain which receives medial preoptic region efferents via the medial forebrain bundle (Conrad and Pfaff, 1976b), as well as input from the bed nucleus of the stria terminalis (Swanson and Cowan, 1979).

The function of the many ascending and descending pathways originating in the ventral tegmental area is not known. Evidence from many different studies suggests that the pathway to the nucleus accumbens (which is predominantly dopaminergic; Swanson, 1982) is involved in the regulation of locomotion. For example, injections of dopamine directly into the nucleus accumbens iniate locomotion in rats (Pijnenburg et al., 1976); electrolytic or 6-hydroxydopamine (dopamine-depleting) lesions of the A10 cell group produce abnormal daily atterns of locomotor activity, including hyperactivity in the dark part of the cycle (Galey et al., 1977); 6-hydroxydopamine lesions of the nucleus accumbens (Kelly et al., 1975) or haloperidol (a dopamine antagonist) injections into the accumbens (Pijnenburg et al., 1975) block amphetamine-induced enhancement of locomotor activity. Transplants of embryonic mesencephalic tissue containing dopamine cells into the nucleus accumbens of rats with 6-hydroxydopamine lesions of the ventral tegmental area to a great extent normalized the responses of the animals to amphetamine and apomorphine (Dunnett et

al., 1984). (Many of these studies also provide evidence for a similar functional role for the anteromedial cortex, which also receives a dopaminergic innervation from the ventral tegmental area.)

Is there a role for a medial preoptic → ventral tegmental area nucleus accumbens multisynaptic pathway in the regulation of maternal behavior? Smith and Holland (1975) reported that prepartum accumbens lesions severely disrupted postpartum maternal behavior, but the extent of the lesions they produced is not clear from their brief report, and the effect was reversed by subsequent lesions of the posterior hypothalamus. Apart from this study, there is no direct evidence for this position. Initiation of forward locomotion, however, is an important component of maternal behavior. It shares this feature with other motivated behaviors such as feeding and aggression. None of the evidence presented to date is inconsistent with the formulation of Mogenson et al. (1980) that projections from hypothalamic and limbic structures via the medial forebrain bundle to the ventral tegmental area can regulate the initiation of motivated behaviors via an ascending ventral midbrain projection to the nucleus accumbens, which in turn projects to the globus pallidus and the substantia nigra and thus provides a "way in" to the motor system. Approaches to the study of maternal behavior which used of manipulations of central dopamine systems (with diminished dopamine function in the forebrain, particulary in the accumbens, predicted to be correlated with disrupted maternal behavior) would provide a strong test case for the general validity of this model.

Locomotion is a component of another behavior, exploration, which is elicited in rats by mild novelty (being placed in an unfamiliar

open field, having a small object placed in the cage). Depletion of dopamine in the rat forebrain (accumbens, olfactory tubercle, lateral septum, and deep layers of the frontal and piriform cortices) produces animals with two specific locomotor deficits: they explore a novel open field less and they explore a novel object less (Fink and Smith, 1979; 1980). This alteration in behavior is seen in the absence of abnormalities in basal activity levels and does not simply represent impaired locomotion as the responses of dopamine-depleted and dopamine-normal animals are equivalent in familiar environments.

Whether or not pups are a novel stimulus for primiparous mothers under natural conditions is not known, but in the laboratory virgin rats are isolated from pups unto the start of maternal behavior testing. Any factors facilitating approach to a novel stimulus should act to reduce the latency of maternal behavior. The work of Kovács and his colleagues has suggested that neurohypophysial hormones, including oxytocin, can affect brain catecholaminergic neurotransmission (Kovács et al., 1979; Kovács and Telegdy, 1983), although the pattern of results described to date is complex and difficult to correlate with function. Dopamine-mediated oxytocin effects on responses to novel objects might then be categorized with other manipulations that reduce the latency of virgin rats to approach pups, such as olfactory/vomeronasal deafferentation (Fleming and Rosenblatt, 1974), presenting pups one at a time (Mayer and Rosenblatt, 1980), and testing animals in very small cages (Terkel and Rosenblatt, 1971).

This is a further elaboration of the idea expressed in Chapter 3 that oxytocin may have a general effect on central neurotransmission

which permits the maternal behavior-specific estrogen priming to be expressed. Although the results of the experiments using anti-oxytocic agents suggest a role for endogenous oxytocin mediation of rapid onset maternal behavior, it is possible that standard experimental tests of responsivity to pups introduce inhibition not present at normal parturition, and that the apparent need for an agent to overcome this inhibition is artifactual. Resolution of this issue will require attempts to interfere with central oxytocin function in parturient animals and study of the effects of altered levels of central oxytocin on responses to "novel" stimuli other than pups.

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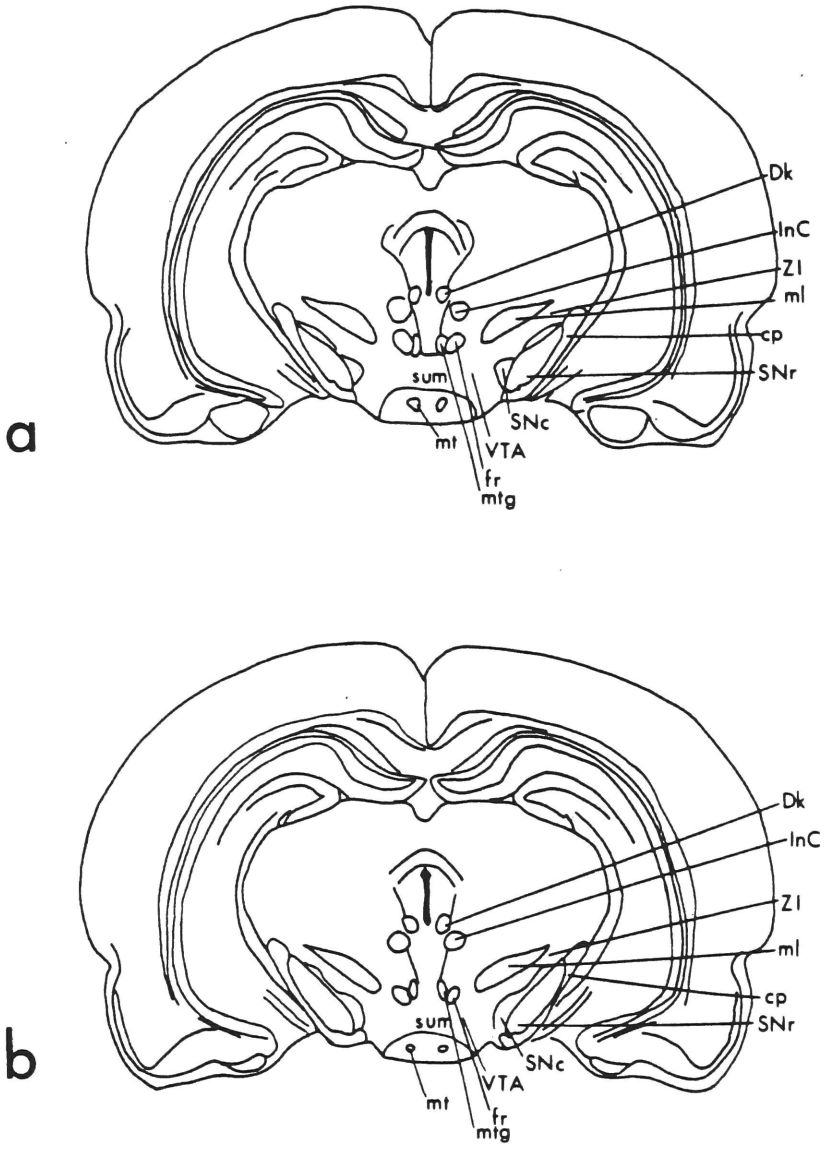
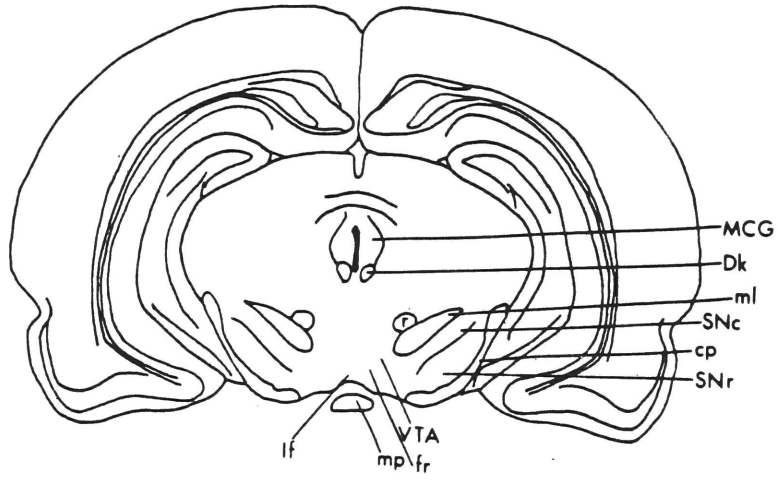



Appendix 1

These sections (a - e) identify the structures in and around the medial midbrain tracer injection sites described in Chapter 2 and illustrated in the subsequent drawings in this appendix. They are adapted from the following atlas plates of Kŏnig and Klippel (1963): A 2580 μ(a), A 2420 μ(b), A 2180 μ(c), A 1760 μ(d), A 1270 μ(e). ABBREVIATIONS CLi, central linear raphe nucleus; cp cerebral peduncle; Dk, nucleus of Darkshewitsch; fr, fasciculus retroflexus; If, interfascicular nucleus; IP, interpeduncular nucleus; MCG, midbrain central grey; ml, medial lemniscus; mp, posterior mammillary nucleus; mt, mammillothalamic tract; mtg, mammillotegmental tract; RF reticular formation; RLi, rostral linear raphe nucleus; SNc, substantia nigra, compact area; Snr, substantia nigra, reticular area; sum, supramammillary decussation; tfp, transverse fibers of the pons; VTA, ventral tegmental area; ZI, zona incerta.

c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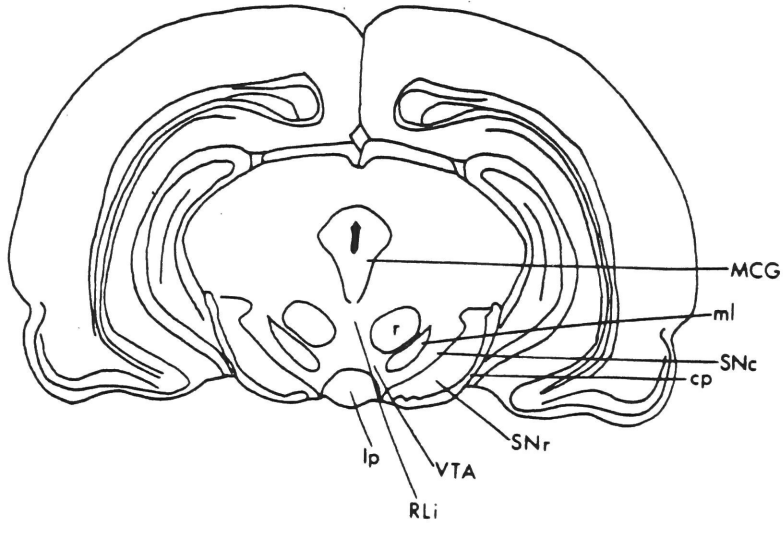
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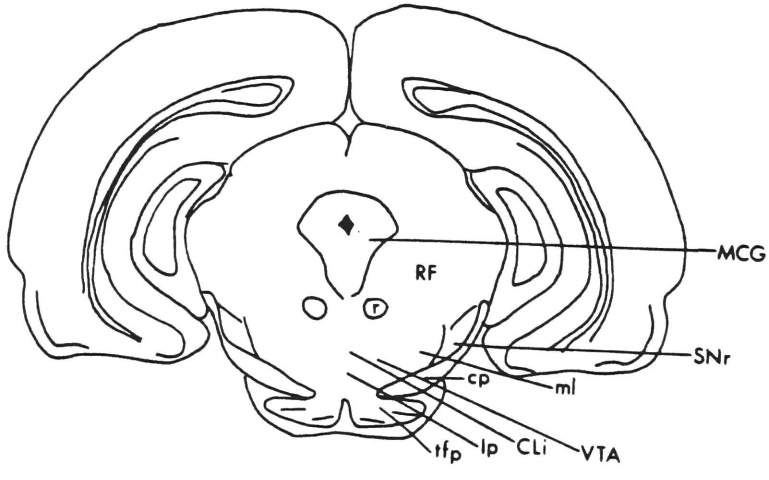
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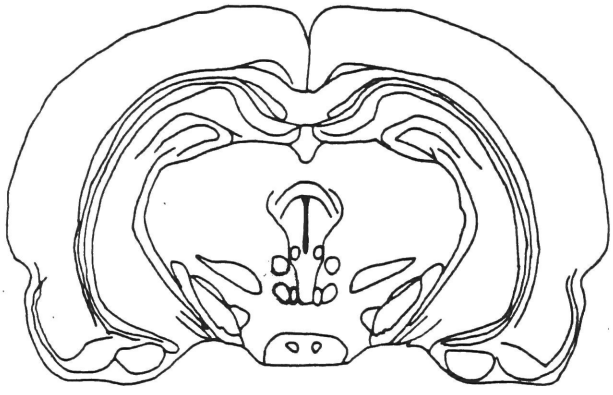
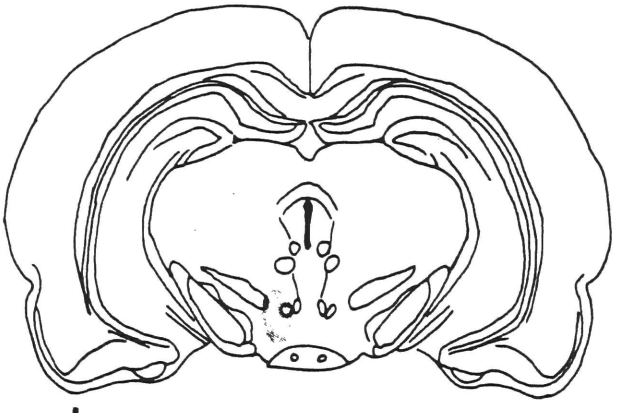


a

Brain 1 - 1. Unilateral 10% True blue injection into the ventral tegmental area. 0.2 µl. Post-injection survival time, 3 days.



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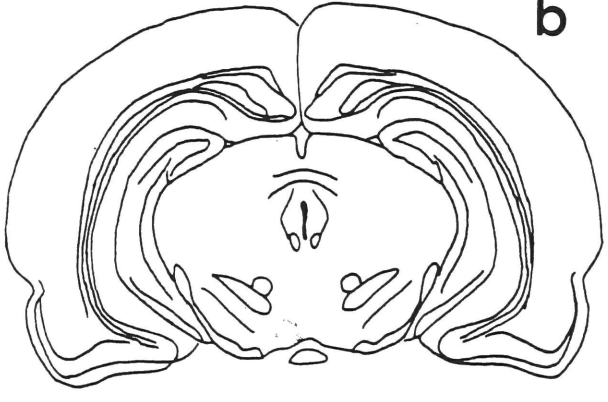

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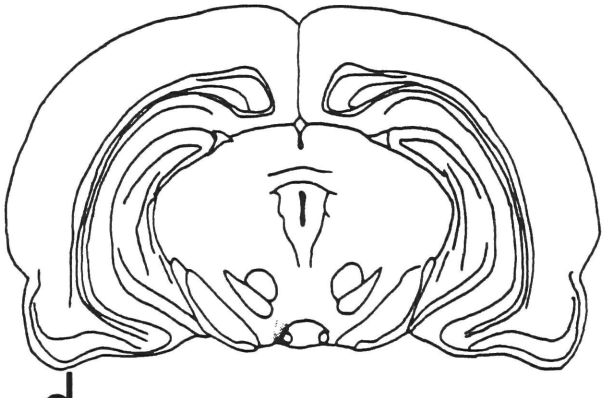

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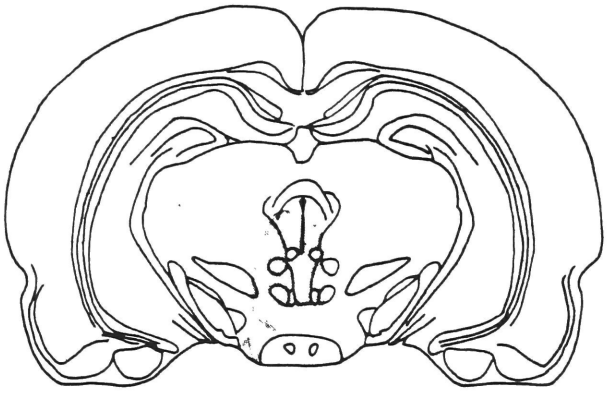
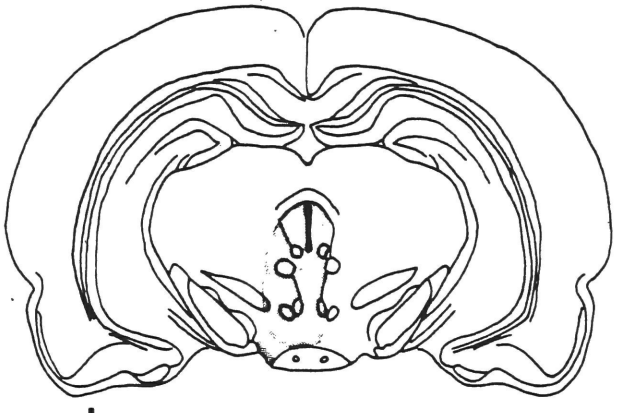


a

Brain 1 - 3. Unilateral 10% Primuline, 2.5% DAPI injection into the medial midbrain. 0.5 µl. Post-injection survival time, 3 days.



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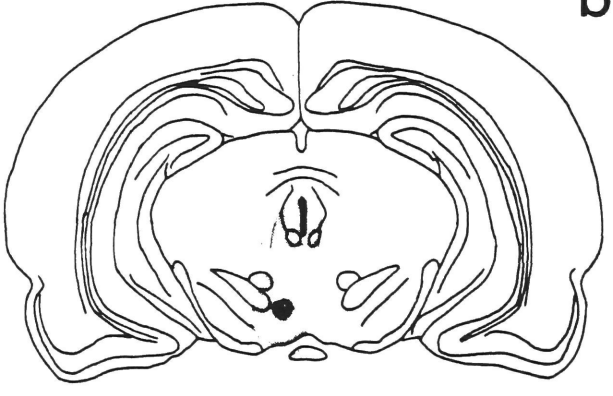

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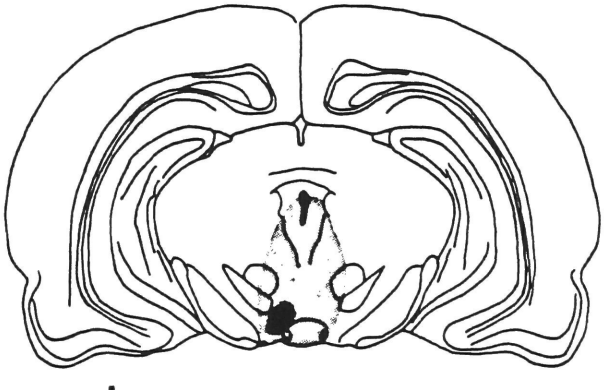

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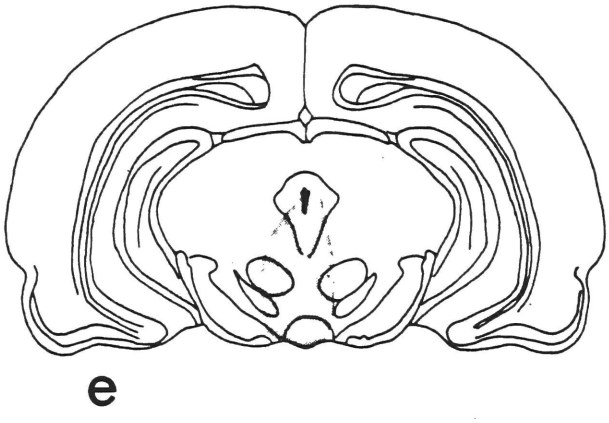

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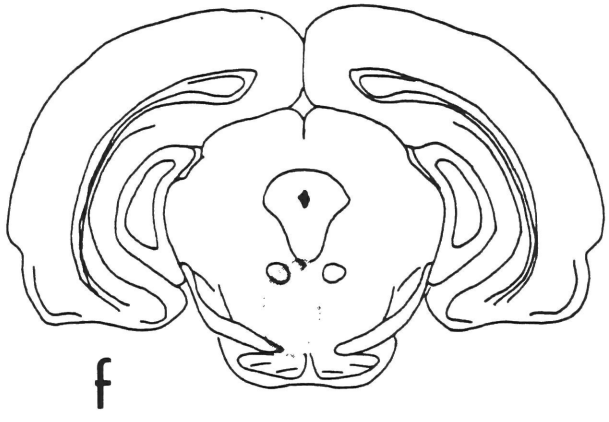

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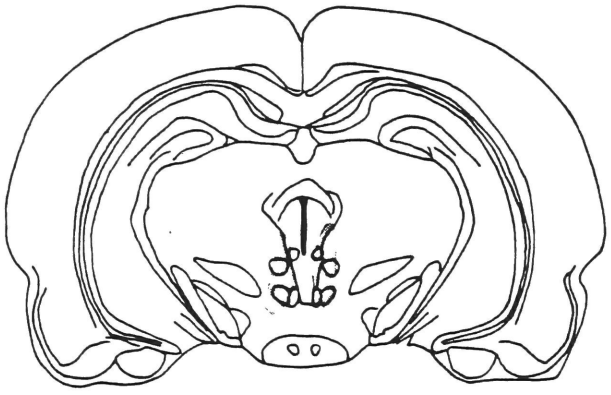
Brain 1 - 3. Continued.



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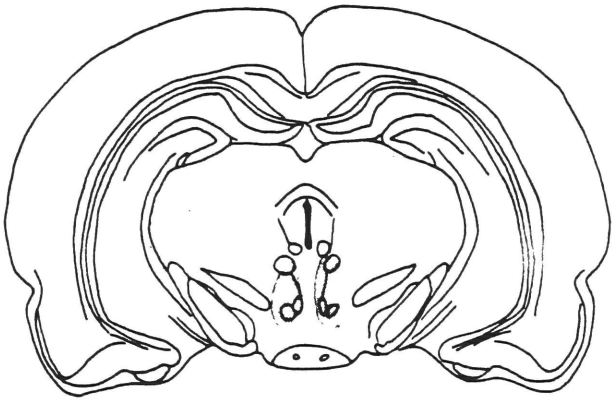
Brain 1 - 8. Bilateral 10% True blue injection into the ventral tegmental area and the interpeduncular nucleus. 0.2 µl per side. Post-injection survival time, 3 days.



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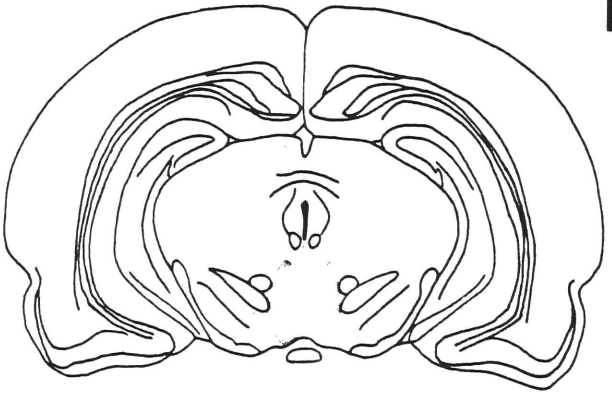

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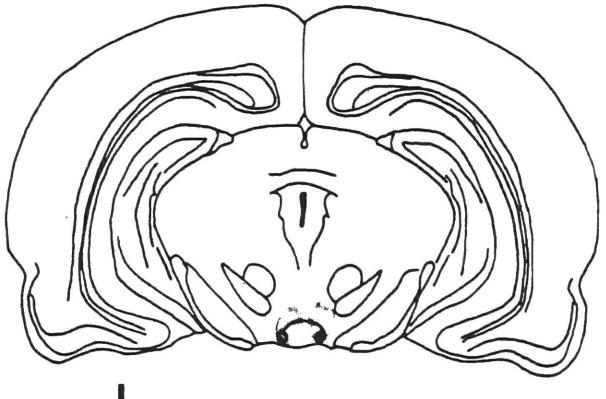

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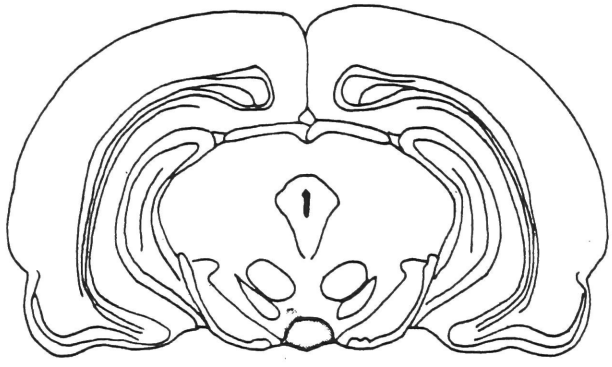

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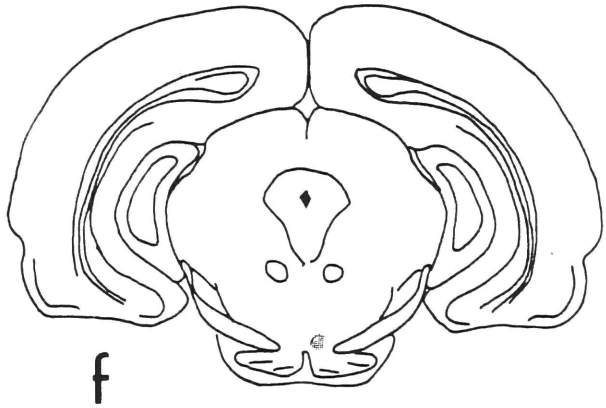

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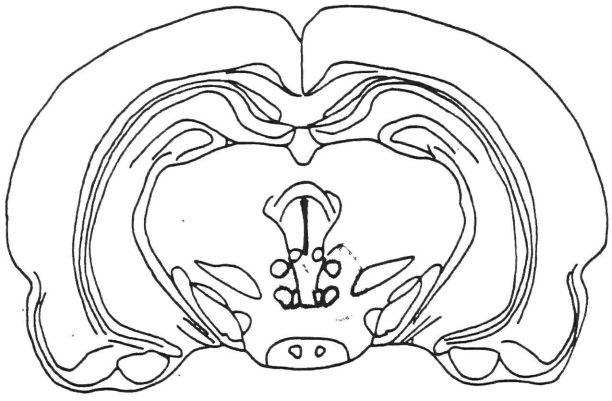
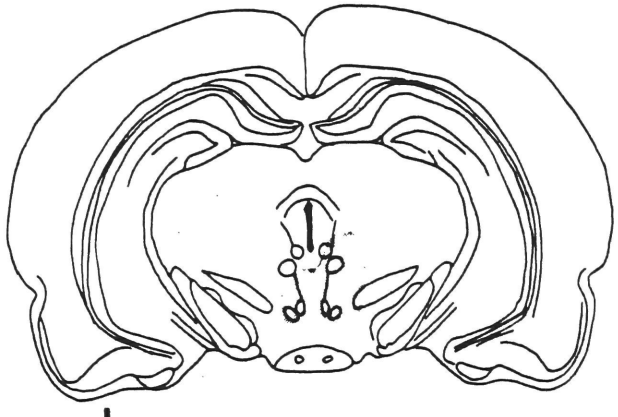


a

Brain 1 - 10. Bilateral 10% Primuline, 2.5% DAPI injection into the ventral tegmental area. 0.2 µl per side. Post-injection survival time, 3 days.



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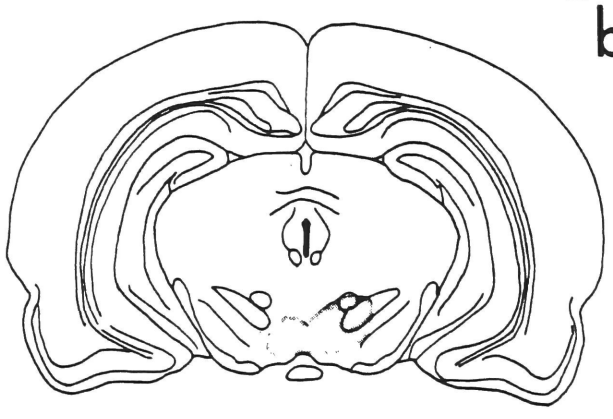

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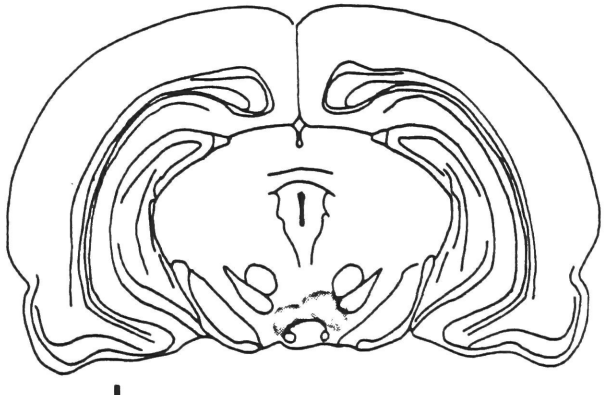

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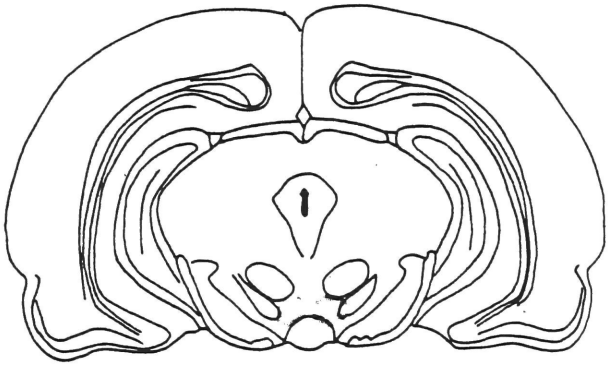

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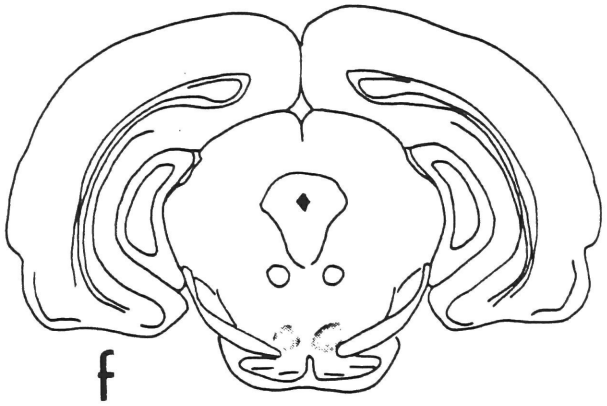

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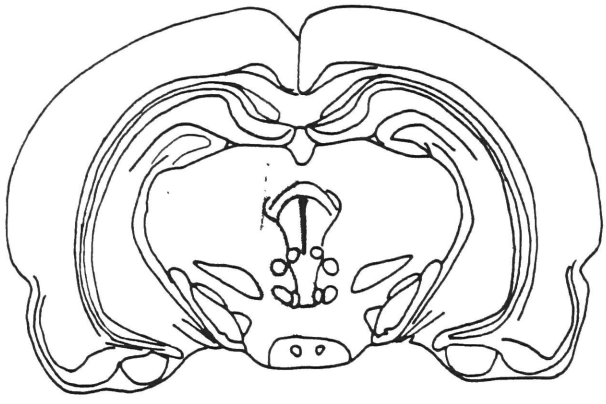

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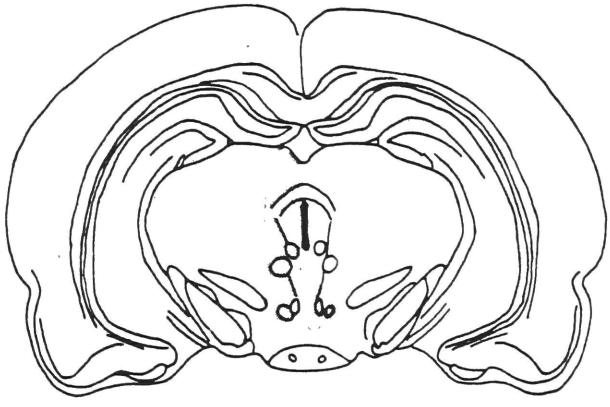
Brain 1 - 11. Unilateral 10% Primuline, 2.5% DAPI injection into the midbrain central grey and ventral tegmental area. 0.5 µl. Post-injection survival time, 3 days.



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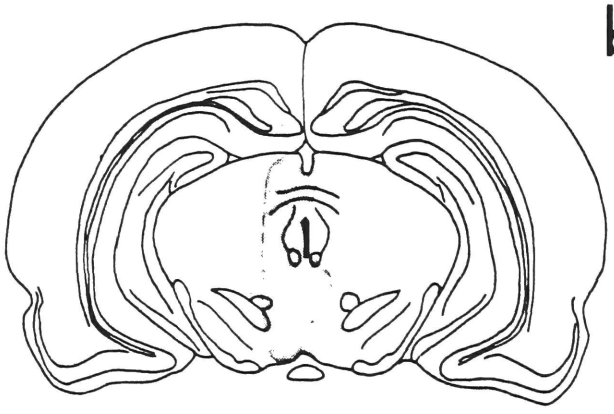

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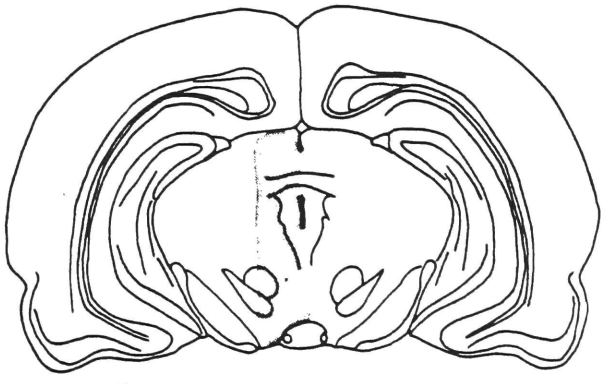

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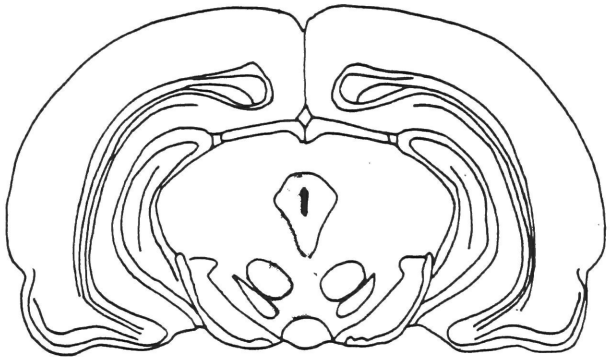

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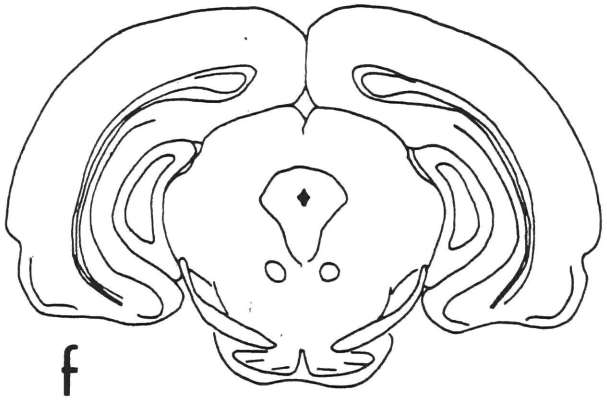

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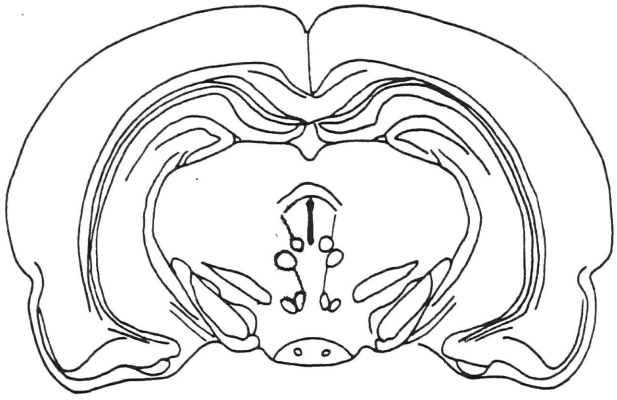

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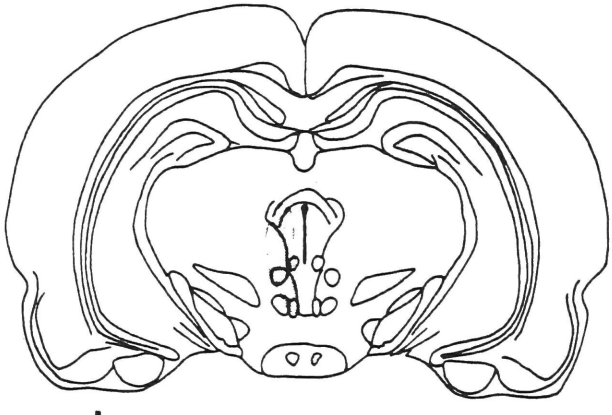
Brain 2 - 5. Unilateral 10% True blue injection into the rostral midbrain central grey. 0.5 µl. Post-injection survival time, 4 days.



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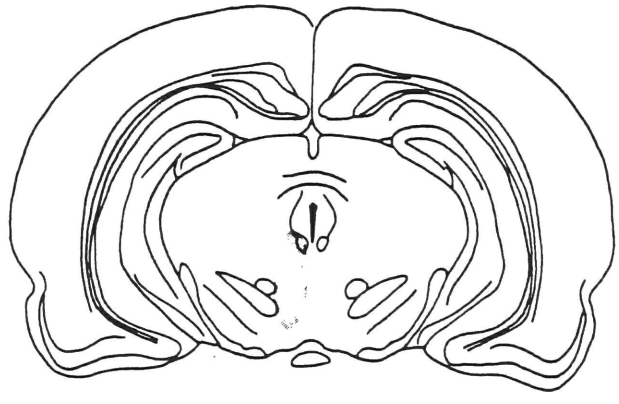

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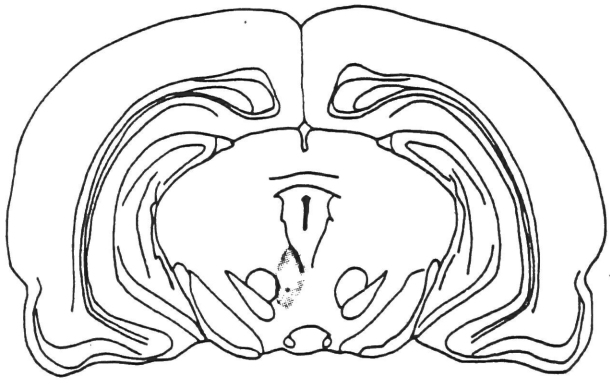

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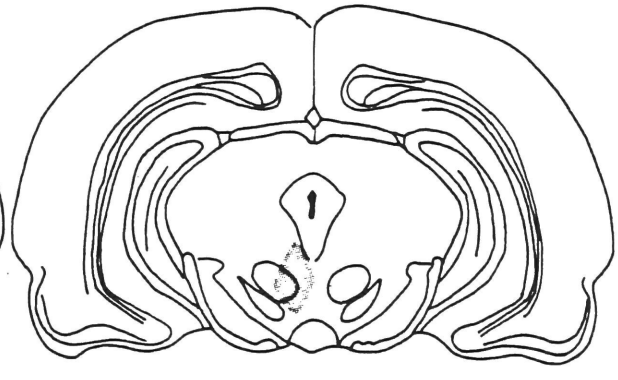

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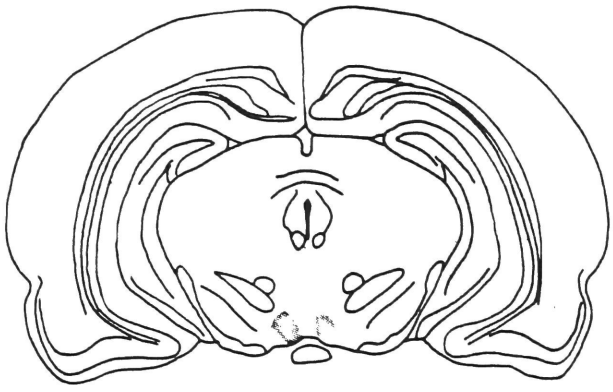
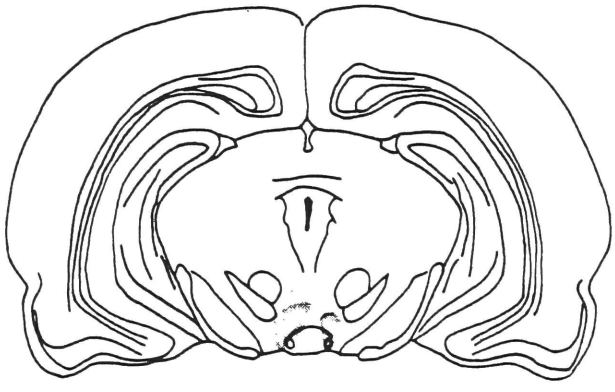
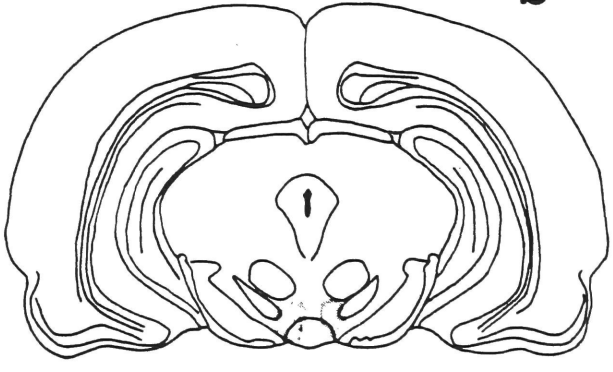


a

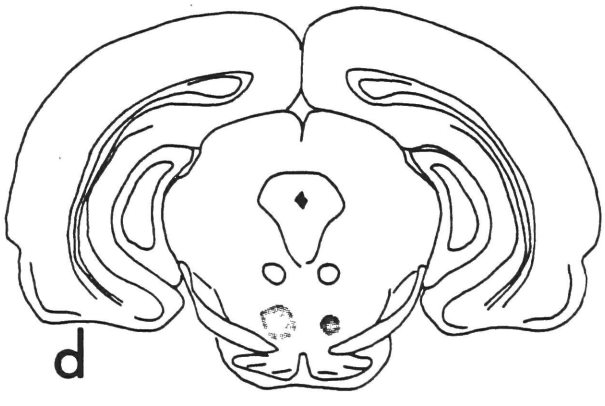
Brain 2 - 6. Bilateral 10% True blue injection into the ventral tegmental area. 0.25 µl per side. Post-injection survival time, 4 days.



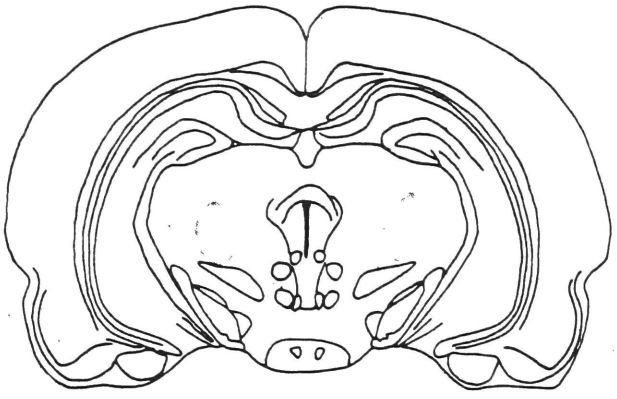
b



c

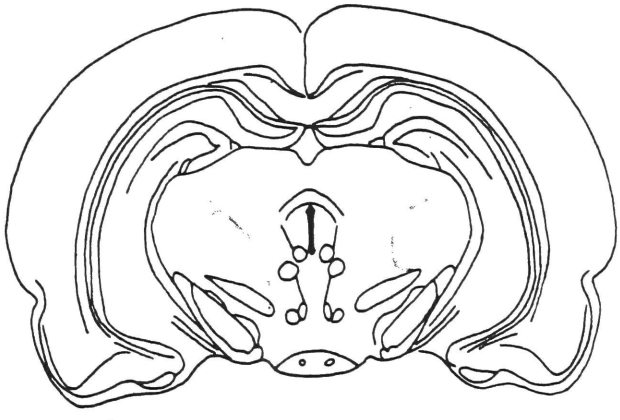


d

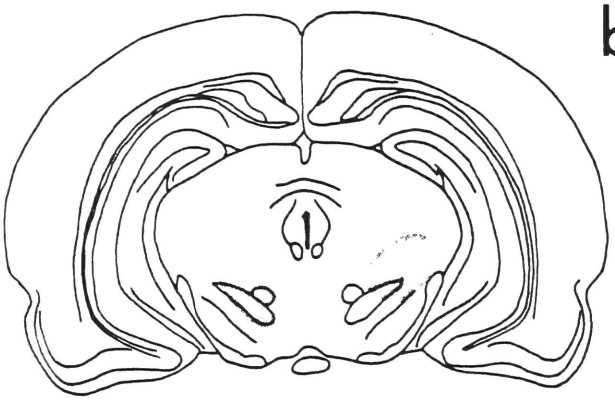


a

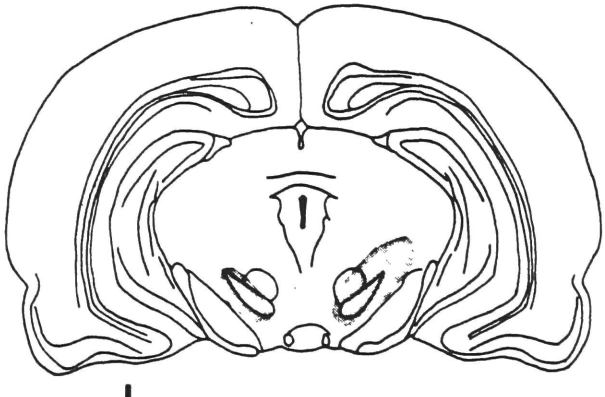
Brain 2 - 7. Bilateral 10% True blue injection into the lateral midbrain. 0.1 µl per side. Post-injection survival time, 4 days.



b



c



d

End