EVIDENCE AND POSSIBLE MECHANISM FOR THE PERMANENT DECLINE IN TUBEROINFUNDIBULAR DOPAMINERGIC NEURONAL ACTIVITY AFTER CHRONIC ESTRADIOL ADMINISTRATION IN FISCHER 344 RATS

By

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ABSTRACT

EVIDENCE AND POSSIBLE MECHANISM FOR THE PERMANENT DECLINE IN TUBEROINFUNDIBULAR DOPAMINERGIC NEURONAL ACTIVITY AFTER CHRONIC ESTRADIOL ADMINISTRATION IN FISCHER 344 RATS

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The objective of these studies was to determine if the decline in tuberoinfundibular dopaminergic (TIDA) neuronal function observed during chronic estradiol-17-β (E_2) administration persisted after E_2 was removed. Ovariectomized (OVX) Fischer 344 rats were implanted with an E_2-containing Silastic capsule for 4 weeks. Other OVX rats received an empty or E_2-containing capsule for 4 weeks, after which the capsules were removed, and experiments were performed 4 or 26 weeks later. Anterior pituitary (AP) weight and serum prolactin was greatly increased at the end of the E_2 treatment,that persisted 4 and 26 weeks after E_2 was withdrawn. At the end of E_2 treatment and 4 weeks after E_2 was withdrawn, TIDA function, as evaluated by electrical stimulation of median eminence tissue in vitro after allowing for uptake of ^{3}H-DA, was decreased compared to OVX controls. Also, E_2-treated rats showed a reduced serum prolactin response to drugs that act on central dopaminergic neurons, e.g., morphine, haloperidol, and nomifensine. Therefore, the decline in TIDA neuronal function observed at the end of long-term E_2 treatment persists up to 26 weeks after E_2 removal.

In an attempt to elucidate the mechanism by which E_2 results in a "permanent" decline in TIDA function, F344 rats were given daily bromo-

cryptine injections in addition to a 30-day E_2 treatment. Bromocryptine, a dopaminergic agonist, prevented the E_2-induced increases in serum prolactin and AP DNA content. TIDA neuronal release was reduced in both E_2 and E_2 and bromocryptine treated groups. However, by 30 days after discontinuing treatment only rats given E_2 alone showed a persistent decline in TIDA function. Since animals given bromocryptine and E_2 had small APs and showed recovered TIDA activity after the withdrawal period, the enlarged AP in E_2-treated rats compressed the hypothalamus and may be responsible for the permanent damage to TIDA neurons.

Since permanent damage to hypothalamic neurons by an enlarged AP was speculated to be the result of E_2 treatment, neurons which regulate other AP hormones may also be damaged. To evaluate this possibility, pulsatile release of prolactin, growth hormone (GH) and luteinizing hormone (LH) was evaluated in OVX control rats, chronically E_2-treated rats, and rats 120 days after chronic E_2 treatment. Only the frequency of prolactin pulses, but not the frequency of GH and LH pulses, was reduced in rats 120 days after E_2 treatment. This suggests selectivity in the hypothalamic damage produced by the enlarged AP.

DEDICATION

This thesis is dedicated to my parents William and Viola Gottschall for their enduring love, support and encouragement. Also, to the numerous people who have, possibly unknowingly, touched my life and renewed my spirit of hope in the face of adversity.

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LIST OF ABBREVIATIONS

Word	Abbreviation
anterior pituitary	AP
apomorphine hydrochloride	APO
catechol-o-methyltransferase	COMT
deoxyribonucleic acid	DNA
l-dihydroxyphenylalanine	l-DOPA
dopamine	DA
dorsomedial and ventromedial hypothalamic nuclei	DMN-VMN
endogenous opiate	EOP
estradiol-17-β	E_2
Fischer 344	F344
follicle stimulating hormone	FSH
gamma amino butyric acid	GABA
gonadotropin releasing hormone	GnRH
growth hormone	GH
haloperidol	HALO
5-hydroxytryptophan	5-HTP
luteinizing hormone	LH
medial basal hypothalamus	MBH
median eminence	ME
medial pre-optic area	MPOA
morphine sulfate	MOR
messenger ribonucleic acid	mRNA
nomifensine maleate	NOM
norepinephrine	NE
ovariectomized	OVX
parachlorophenylalanine	PCPA
prolactin (in figures and tables)	PRL
prolactin inhibiting factor	PIF
prolactin releasing factor	PRF
radioimmunoassay	RIA
serotonin	5-HT
thyroid-stimulating hormone	TSH
thyrotropin releasing hormone	TRH
tuberoinfundibular dopaminergic neuron	TIDA
vasoactive intestinal peptide	VIP

INTRODUCTION

Anterior pituitary (AP) hormone release and synthesis are mainly regulated by chemicals (peptides) secreted from hypothalamic neurons which terminate on a portal vascular system that carries these substances directly to the AP. Prolactin secretion from the AP is controlled by a variety of stimulatory and inhibitory factors present in the hypothalamus, but the net influence on prolactin secretion is inhibitory. The net inhibition of prolactin release exerted by the hypothalamus is unique among AP hormones. Pituitary stalk section, electrical lesion of the median eminence which destroys the terminals for hypothalamic hormone release, or transplantation of the AP away from hypothalamic influence, all enhance the release of prolactin but greatly reduce the secretion of all other AP hormones (Meites et al., 1972). Dopamine (DA), a catecholamine, has been shown to be the major substance in the hypothalamus which inhibits the tonic secretion of prolactin (Leong et al., 1983), although DA alone does not account for the total hypothalamic prolactin inhibiting activity.

Estrogen may be the most important peripheral factor involved in regulating prolactin release. Prolactin levels are higher in females than males, decline in females after ovariectomy, and estrogen replacement returns prolactin to non-ovariectomized control levels (Meites et al., 1972). Estrogen appears to affect the release of prolactin not only by a direct action on the lactotrophs, but also by influencing hypothalamic neurotransmitter mechanisms, particularly DA

(Meites, 1974).

Estrogen and DA affect not only the release of prolactin, but can alter the mitotic rate of the lactotrophs as well. Estrogen administration for prolonged periods in the rodent initially results in hypertrophy and hyperplasia of the lactotrophs, and later after one or two passages of the pituitary tissue, can form a neoplasia capable of growth in the absence of elevated estradiol levels (Furth and Clifton, 1966). The estrogen-induced AP hyperplasia can be prevented by administration of a dopaminergic agonist (Lloyd et al., 1975). In fact, even when given alone, dopaminergic agonists can reduce, and dopaminergic antagonists stimulate AP mitotic activity (Jacobi and Lloyd, 1981; Kalberman et al., 1980). Therefore, manipulations that chronically increase prolactin release can augment lactotroph mitoses, and those that decrease prolactin release reduce lactotroph mitotic activity (Pawlikowski, 1982). Intracellular mechanisms which are essential for the release of prolactin may also influence the regulation of mitosis in the lactotrophs; alterations in second messengers, e.g., Ca^{++}, the cyclic nucleotides, products of the cyclooxygenase or lipoxygenase pathways of arachadonic acid have been shown to influence the release of prolactin (Dannies and Tam, 1982). These substances, or products activated by these substances, could also activate a particular gene; the transcription-translation product may turn on DNA synthesis directly, or deactivate a mitotic regulatory repressor gene. The outcome would be the same in either case--augmented DNA synthesis. Both estrogen and DA have been shown to directly (in the case of estrogen) or indirectly (in the case of DA) alter the synthesis of prolactin mRNA levels (Mauer, 1982). Certainly, if estrogen and DA can affect gene products that regulate

prolactin synthesis, then mitotic regulatory activity could also be influenced.

What are the relative roles of estrogen and DA in stimulating and inhibiting mitotic activity of the lactotrophs? Estrogen can induce pituitary prolactinomas in APs that are transplanted under a kidney capsule suggesting at least to one laboratory (Clifton and Meyer, 1956) that the hypothalamus plays a minor role in the development and growth of these tumors. However, administration of a dopaminergic antagonist concomittantly with estrogen, can significantly augment the increase in AP weight and DNA content (almost double), compared to animals treated wth estrogen alone (Kalberman et al., 1980; Gottschall and Meites, unpublished). This indicates an important role for endogenous hypothalamic DA in the growth and possibly the development of prolactinomas. Dopaminergic agonists can also prevent estrogen-induced increases in AP weight and DNA synthesis (Lloyd et al., 1975). It is clear, then, that exogenous stimulation or blockade of DA receptors can dramatically influence the growth of estrogen-induced prolactinomas.

What effect does chronic administration of estrogen have on endogenous hypothalamic dopaminergic mechanisms which control prolactin release? It is now well established that chronic treatment with estradiol decreases the activity of tuberoinfundibular dopaminergic neurons (Sarkar et al., 1983a). This decline in TIDA neuronal function after chronic estrogen treatment has been suggested to be caused by estradiol-induced degeneration of these neurons, as evidenced by (a) a decline in ^{3}H-DA uptake into TIDA neuron terminals (Sarkar et al., 1984a), (b) increased glial reactivity in the arcuate nucleus (Brawer and Sonnenschein, 1976), and (c) distorted fluorescent fibers and the

presence of autofluorescent material in the arcuate nucleus after fluorescent visualization of arcuate nucleus catecholaminergic neurons (Sarkar et al., 1982). If chronic estrogen treatment indeed causes degeneration of TIDA neurons, then reduced activity of these neurons should be sustained even when estradiol is removed. This thesis will present evidence that chronic estradiol treatment can produce a permanent reduction in TIDA neuronal activity in the Fischer 344 (F344) rat, and will attempt to elucidate the mechanism(s) by which estradiol produces this effect. Also, some evidence will be presented as to the specificity of the estradiol-induced decline in neuron function, i.e., are TIDA neurons alone permanently affected by chronic estradiol treatment?

LITERATURE REVIEW

I. Prolactin Secretion in Different Physiological States

The initial experiments which demonstrated neural and/or hypophyseal action on reproductive and other endocrine functions were carried out by Long and Evans (1922, Evans and Long, 1922) who observed that mating of a female rat with a vasectomized male, mechanical stimulation of the cervix, or chronic injection of bovine AP tissue resulted in a prolonged period of vaginal diestrus characterized by the presence of functional corpora lutea in the ovary. That this phenomena involved AP secretion was confirmed by Smith and Engle (1927) who demonstrated that transplantation of AP tissue away from the in situ site also resulted in a "pseudopregnant" state. Astwood (1941) was the first to propose the functional existence of a third "gonadotrophin" which was able to maintain luteal function in the rat, and Evans et al. (1941) demonstrated that prolactin mediates the maintenance of the corpora lutea during pseudopregnancy. Anterior pituitary involvement in milk secretion was demonstrated by Stricker and Grüter (1928), and prolactin, along with adrenocorticotrophic hormones were shown to be the essential requirement to initiate and maintain lactation by the classical experiments of Turner (1939) and Lyons (Lyons et al., 1958).

Following the separation of growth stimulating activity from lactogenic activity in an AP extract, a relatively pure "prolactin" was identified (Riddle, et al., 1933) and a pigeon crop assay was developed

for this preparation. A major breakthrough in "prolactinology" was the development of radioimmunoassays, initially for insulin by Yalow and Bernson (1959), and later the rat prolactin radioimmunoassay was reported by Niswender et al. (1969) which for the first time allowed for quantitative measurement of physiological levels of prolactin in the serum. Prolactin was subsequently shown to be secreted in surge-like patterns in the rat on the afternoon of proestrous day during the 4-day estrous cycle, and during the first half of pregnancy (Smith et al., 1975). During the 1960's and 1970's a wealth of evidence had substantiated the presence of hypothalamic neurotransmitters, particularly DA and serotonin (5-HT), as major modulators of prolactin secretion (Meites, 1977). Li (1972) fully characterized the amino acid sequence of rat, ovine, and human prolactin. Recently, a cloned DNA complementary to rat prolactin messenger RNA was sequenced which includes the codon for the precursor signal peptide, the amino acid sequence was determined from the complementary DNA (Cooke et al., 1980).

A. Estrous and Menstrual Cycles

Intact adult female rats are spontaneous ovulators, but do not exhibit a true luteal phase. Estrous cycles are usually 4 or 5 days in length with ovulation occurring 10 or 11 hrs after a late afternoon surge of luteinizing hormone (LH), on the proestrous day of the cycle, (Everett, 1964). Contiguous with the surge of LH is an afternoon rise in prolactin, which usually begins earlier and is maintained longer than that of LH, and is dependent on ovarian estrogen secretion (Everett, 1964). Early experiments demonstrated that AP prolactin content was higher (Reece and Leonard, 1939) and that AP prolactin release in vitro

was greater, in animals killed on the day of proestrus or estrus as compared to animals killed on diestrus (Sar and Meites, 1967). Studies performed later showed a surge of prolactin in the serum, as measured by radioimmunoassay, beginning early on the afternoon of proestrus. This surge reaches a peak as the lights go off (with a 12 hr on, 12 hr off light cycle) and decreases to near basal values by the morning of estrus (Niswender et al., 1969; Butcher et al., 1974, Smith et al., 1975).

The surge of prolactin is dependent on a slowly rising rate of follicular estrogen secretion during diestrus II and proestrus, since injection of an antiserum to estradiol on diestrus II prevents the proestrus surge of prolactin (Neill et al., 1971). The action of estrogen on the surge of prolactin seems to be mediated through hypothalamic mechanisms since a rostral hypothalamic cut placed immediately behind the optic chiasm blocks the proestrus rise in prolactin secretion (Neill, 1972).

Studies performed in primates, including women, have usually failed to demonstrate any consistent change in the pattern of prolactin secretion throughout the menstrual cycle (Reyes et al., 1975; McNeilly and Chard, 1974; Lenton et al., 1982). However, these studies in women involved collecting only one blood sample per day. Reports that collected blood more frequently showed a midcycle increase in circulating prolactin which was coincident with the LH surge (Robyn et al., 1976), and 2-fold greater amount (Djahanbakhch et al., 1984) than prolactin levels measured the night before the LH surge. Multiple blood sampling is especially important since prolactin has been shown to be secreted in a pulsatile fashion not only in rats (Saunders et al., 1976), but also in primates during the mid-cycle surge of

gonadotrophins. Many of these prolactin pulses are coincident with LH pulses (Backstrom et al., 1982; Belchetz et al., 1978). This is important because the C-terminal fragment of the precursor to gonadotrophin-releasing hormone (GnRH) has recently been isolated and shown to have potent prolactin-inhibiting activity (Nikolics et al., 1985). It is interesting to speculate that if these two peptides, GnRH and the prolactin inhibiting C-terminal fragment, are released simultaneously from the same neurons then each pulse peak of LH should correspond to a pulse nadir of prolactin. Although this does not appear to occur during the episodic release of LH and prolactin pulses, there are a number of physiological situations when prolactin levels are high and LH levels are low and vice versa. The function of the preovulatory surge of prolactin during the menstrual cycle is unknown.

During the estrous cycle of the rat, prolactin may have a role in luteolysis of corpora lutea formed during the previous estrous cycle. Regression of the corpus luteum is primarily dependent on prostaglandin production but stimuli that can alter the rate of prostaglandin synthesis can decrease the time to luteolysis (Rothchild, 1981). Injection of ergot drugs which decrease prolactin levels in the serum significantly increased numbers of corpora lutea compared to saline injected controls. When exogenous prolactin was injected along with the ergot drug, luteolysis occurred similar to saline injected animals (Meites et al., 1972). Therefore, prolactin does play a role in corpora lutea regression during the estrous cycle of the rat, although other AP factors (eg. LH) may also be important.

B. Pregnancy and pseudopregnancy

During the estrous cycle of the rat, progesterone is secreted from corpora lutea for about 2 days before regressing. However, mating or a variety of other cervical stimuli results in maintenance of progesterone secretion until late in gestation in the pregnant rat and for about 11-13 days in the pseudopregnant rat. There is a wealth of evidence which has identified prolactin as the stimulus which converts the corpora lutea of the estrous cycle to actively secreting corpora lutea of pregnancy (Butcher et al., 1972; Smith et al., 1975; Smith et al., 1976). Prolactin, during the first half of pregnancy or during all of pseudopregnancy, is secreted in twice daily surges termed nocturnal and diurnal; the former peaks as the lights turn on, the latter as the lights go off (Smith et al., 1975; Butcher et al., 1972). Blockade of the prolactin surges on day 3 or later, but not on day 1 or day 2 of pseudopregnancy by administration of bromocryptine causes regression of corpora lutea. Regression can be reversed if prolactin is injected simultaneously with bromocryptine (Smith et al., 1976). Ovariectomy the day after mating reduced the magnitude of the nocturnal and diurnal surges on day 2 of pseudopregnancy and the surges of prolactin lasted only for about 6 days (Freeman et al., 1974; Freeman and Sterman, 1978). Steroid replacement can prolong the surges to about day 10. Prolactin surges can be induced after cervical stimulation in the long-term ovariectomized rat which demonstrates that the surges are not absolutely dependent on ovarian secretion (Smith and Neill, 1976a), but maintenance of normal surges requires progesterone (Smith et al., 1975). In the pseudopregnant state, the last prolactin nocturnal surge occurs on day 11, and on day 12 a normal proestrus surge of prolactin occurs

signifying a return to estrous cycles (Smith and Neill, 1976b). Termination of the diurnal and nocturnal surges of prolactin during pregnancy, which happens on day 8 and 10 of pregnancy respectively, appears to be mediated through a rise in the secretion of rat placental lactogen by the developing conceptus which occurs about ths time (Tonkowicz and Voogt, 1983). The rat placental lactogen may feedback on hypothalamic mechanisms which regulate the surges, to inhibit further prolactin secretion. Between days 9 and 12 of pregnancy, corpora lutea appear to be both LH and prolactin dependent, after which functional corpora lutea are maintained by placental hormones (Rothchild, 1981). Serum prolactin is maintained at low levels until the day before parturition, at which time there is a large surge (Linkie and Niswender, 1972).

The most convincing evidence that these surges are neurally mediated endocrine reflexes, in rats is that after pelvic neurectomy, cervical stimulation does not result in pseudopregnancy and there are no prolactin pulses (Kollar, 1953). However, pelvic neurectomy does not seem to interfere with other normal hypothalamic-pituitary ovarian functions. It is this reflex which initiates the prolactin surges. The neural mechanisms which maintain the twice daily surges of prolactin during early pregnancy or pseudopregnancy are not well understood. Lesion of the medial pre-optic area (MPOA) resulted in a prolonged pseudopregnant state and nocturnal surges but no diurnal surges. This demonstrates that the MPOA tonically inhibits the nocturnal surge of prolactin and the development of pseudopregnancy (Clemens et al., 1976; Arita and Kawakami, 1981). Also, in rats which were cervically stimulated, electrical stimulation of the MPOA completely prevented both

the diurnal and noctural surge (Gunnet and Freeman, 1984). Contrary to this, is the observation that stimulation of the MPOA in pentobarbitol anesthetized female rats can induce a diurnal surge of prolactin (Gunnet and Freeman, 1984). It was suggested that these two contradictory functions of the MPOA involve two different neuronal pathways.

A second important hypothalamic area involved in the regulation of the prolactin surges of pseudopregnancy is the dorso medial and ventro medial nuclei (DMN-VMN). Electrical stimulation of these areas results in both prolactin surges and a pseudopregnant state (Beach et al., 1978; Freeman and Banks, 1980). However, like the dual action of the MPOA, lesions of the DMN-VMN in cervically stimulated females selectively abolished the diurnal surge, but the nocturnal surge remained. Therefore, both the MPOA and the DVM-VMN apparently regulate both prolactin surges, but involve independent pathways for each surge. Studies recently carried out (Gunnett and Freeman, 1985) to investigate the interaction of these two areas suggest that the inhibitory action of the MPOA on the nocturnal surge can continue to operate in a DVM-VMN lesioned animal. However, the stimulatory role of the MPOA in the diurnal surge, requires an intact DVM-VMN.

C. Lactation

The role of prolactin in the production and secretion of milk in female mammals is probably its most well known and ubiquitous function (Meites et al., 1972; Cowie et al., 1980). Prolactin levels increase about the time of parturition and are maintained high as long as lactation is continued by the suckling young. Prolactin is released into the circulation as a result of suckling by a typical neuroendocrine

reflex. However, the suckling-induced reflex release of prolactin has many characteristics which differ from the proestrus or cervical-stimulation induced prolactin surges discussed previously. Following return of young to their mothers after a period of separation, prolactin rises within 5 minutes after the initiation of suckling, reaching a peak at 30 minutes and remains at this level for at least 90 minutes (Grosvenor and Whitworth, 1974). Prolactin continues to be secreted until AP stores have been exhausted (Grosvenor et al., 1979). In contrast to the proestrus and cervical-stimulated surges of prolactin, suckling induced prolactin release is not surge-like and does not exhibit a circadian pattern, but is tightly coupled to the stimulus of suckling and responds to a greater degree with a more intense period of suckling. Also, suckling-induced prolactin release strictly requires application of the stimulus for a prolactin response to occur (Neill, 1980). At the spinal level, the central nervous system pathways involved in the suckling-induced release of prolactin appear to be common to the pathway involved in oxytocin release for milk ejection. Just central to the medial geniculate body, the prolactin pathway diverges and passes between the third ventricle and the mammilothalamic tract, to the lateral hypothalamus and up to the MPOA. At the MPOA the pathway joins with a neocortical path which descends from the orbitofrontal region. These two pathways, then pass caudally to the anterior hypothalamic area, where, at least as far as activating prolactin release by electrical stimulation is concerned, it appears to terminate. However, this may be due to changes in neurons or neurotransmitters (Tindal, 1978). The specific neurotransmitters involved in this release of prolactin, particularly between the anterior hypothalamus area and the

median eminence are unknown but both DA and serotonin have been implicated (Leong et al., 1983).

After parturition, a period of lactational anestrus occurs, the length of which appears to be related to the intensity and frequency of suckling by the young (Van der Shoot, et al., 1978). However, since suckling induced release of prolactin is also related to the intensity of the suckling stimulus, at least in rats, hyperprolactinemia could be involved in the lactational anestrus (Meites et al., 1978; McNeilly, 1984). A number of mechanisms have been suggested to explain the anestrus during lactation, and many of these involve the hyperprolactinemia which occurs during this time. The mechanism which has the best support is the effect of suckling and/or prolactin on the GnRH control of gonadotrophin secretion. Immediately before parturition, there is a massive reduction in circulating steroids. When suckling begins postpartum, concentrations of follicle stimulating hormone (FSH) return to normal within a few days, but LH remains below normal diestrus levels even when estradiol is low (Taya and Greenwald, 1982). The degree of pulsatile LH secretion, which is taken to be a reflection of pulsatile GnRH secretion, is decreased in lactating rats. The greater the degree of the suckling stimulus the greater the decrease in pulsatile LH release (Fox and Smith, 1984). The ability of lactating mothers to release LH after injection of GnRH was reduced in lactating mothers compared to non-lactating controls both in vivo (Lu et al., 1976a) and from AP tissue incubated in vitro (Lu et al., 1976b; Smith, 1985). This effect is probably due to a decrease in AP GnRH receptors which were observed to be reduced by 50% in lactating mothers compared to diestrus females (Smith, 1984). All of these results suggest a decrease in GnRH

secretion and demonstrate diminished ability of GnRH to release LH in lactating rats. Evidence supporting prolactin as the agent which reduces GnRH or GnRH action on the AP is that treatment of lactating mothers with ergocornine, which reduces PRL secretion, produced increases in LH release even in the presence of suckling pups (Lu et al., 1976b). Furthermore, increasing circulating prolactin by AP transplants, transplantation of a prolactin-secreting AP tumor, or injections of prolactin significantly reduced LH levels (Meites et al., 1978). In contrast to these results, Smith (1978) has shown that prolactin can decrease LH secretion in ovariectomized lactating rats only in the presence of suckling pups. Therefore, although prolactin may be a significant factor responsible for the inhibition of gonadotrophin release during postpartum lactation, it is not the sole factor, and a more direct effect of the suckling stimulus may also inhibit GnRH release.

In addition to the potential hypothalamic effects of prolactin on GnRH during lactation, there is evidence for direct inhibition of ovarian function by prolactin (McNeilly, 1984). In vitro, addition of prolactin (Van der Shoot et al., 1982) appears to inhibit the secretion of estradiol by suppressing the levels of FSH-induced aromatase within granulosa cells. A similar effect was observed in granulosa cells from lactating rats (Taya and Greenwald, 1982). However, administration of GnRH or LH can induce follicle growth and ovulation in lactating rats (Taya and Greenwald, 1982), sows (Hausler et al., 1980), or cows (Riley et al., 1981). Therefore, the relative contribution of the direct action of prolactin on the ovary in producing lactational anestrus remains obscure.

D. Stress

The first indirect evidence that acute stress resulted in prolactin release from the AP was by Nicoll et al. (1960) who observed that various stressors such as restraint, injection of formaldehyde, cold or heat could induce lactation in estrogen-primed rats. This was confirmed when stress was shown to deplete AP prolactin content (Grosvenor et al., 1965). With the development of the prolactin radioimmunoassay, it was observed that ether stress increased serum prolactin levels in female rats at all times during the estrous cycle except on the afternoon of proestrus when prolactin levels were already high (Neill, 1970). Ether and Nembutal anesthesia also caused release of prolactin (Ajika et al., 1972). Krulich et al. (1974) demonstrated that even mild stressors, e.g., handling of animals or transfer from room to room, could increase serum prolactin levels. In addition to stress-induced decreases in growth hormone (GH) secretion, prolactin appeared to be more susceptible to these mild stresses than other AP hormones. The release of prolactin in response to acute stress is a hypothalamic-mediated event. The effect of chronic stress on AP prolactin secretion is not as clear. Restraint stress for 2 hours a day for 20 days increased serum prolactin on day 9 and 19 when the samples were taken prior to the stress. However, there was no effect of stress on prolactin levels on day 10 and 20 when the samples were taken following the stress period (Riegle and Meites, 1976). Moreover, in another study, chronic (42 days) cold exposure, forced exercise, or daily immobilization tended to decrease serum prolactin levels in single samples taken throughout the treatment period (Tache et al., 1978). The functional role of increased circulating prolactin during acute stress is not known.

E. Puberty and old age

Although particularly recognized for its role as a luteotropin and in mammary gland function in the female rat, prolactin appears to be important in events occurring during reproductive development. Prolactin levels gradually increase during the pre-pubertal period in the rat (Voogt et al., 1970; Ojeda et al., 1976), and during this time a circadian diurnal pattern is established; levels which slowly increase in the late afternoon and evening (Kimura and Kawakami, 1980). Hyperprolactinemia, induced by administration of the dopaminergic antagonist, sulpiride or by injecting exogenous prolactin, results in precocious puberty (Clemens and Meites, 1977; Advis and Ojeda, 1978; Advis et al., 1981a). Prolactin appears to advance puberty in females by facilitating the steroidogenic response of the ovary to gonadotropins which are at low levels during the juvenile period; injected prolactin has been shown to elevate ovarian LH receptors (Advis et al., 1981b). As might be expected, suppression of prolactin by ergot drugs delays the onset of puberty and suppresses the steroidogenic ability of the ovary (Advis et al., 1981a). No effect was observed on LH or FSH levels in these studies, suggesting a direct action of prolactin on the ovary (McNeilly, 1984). A contrary, but interesting observation is that implantation of prolactin into the hypothalamus of pre-pubertal female rats advanced the onset of puberty, suggestive of a central action of prolactin (Clemens et al., 1969). As can be seen, prolactin plays an important role in reproductive development, although whether its most important action is at the hypothalamic and/or ovarian level is not clear.

Towards the opposite end of reproductive development, i.e., old age, prolactin secretion is radically altered as compared to the young adult

rat. Regularly cycling middle-aged animals (10-12 mos) exhibit an altered proestrus surge shown to be increased in Sprague-Dawley rats (Wise, 1982) but decreased in mice (Brawer and Finch, 1983) or Long-Evans rats (Gottschall and Davis, 1980). Following cessation of cycles, due to hypothalamic defects (Meites, 1982), estradiol levels are moderately elevated (11-25 mos) and as a consequence, prolactin levels are also increased (Lu et al., 1979). Ovariectomy at a young age prevents this age-related hyperprolactinemia (Lu et al., 1979). Aging in female rats is also associated with a high incidence of prolactin-secreting pituitary microadenomas (Takahashi and Kawashima, 1983) and frank prolactin-secreting pituitary tumors (Huang et al., 1976), which may be related to the earlier recurrent estrous cycles and to the acyclic period of moderately elevated estradiol levels (Finch et al., 1984). This phenomenon will be discussed further in section VIc.

F. Male and Female

The male rat has lower circulating levels of prolactin than the female rat. This, for the most part, is due to the action of estradiol which increases prolactin release in the female. Ovariectomy decreased circulating prolactin to approximately male levels (MacLeod and Fontham, 1970), and this is surprising, in part because adult female APs contain about three times as many lactotrophs as males (Takahashi and Kawashima, 1982). Estradiol replacement in ovariectomized females returns prolactin to pre-castrate levels. The secretion of prolactin in the male rat is not surge-like at any time of the animal's life, in contrast to the female. However, neonatally castrated males can exhibit afternoon prolactin surges when given estrogen. It is thought that androgens,

during neonatal life, prevent the organization of a rostral hypothalamic "surge center." In fact, the hypothalamic MPOA of the rat (Gorski et al., 1978), as well as the human is sexually dimorphic (Swaab and Fliers, 1985). With increasing age, male rats become hyperprolactinemic but not in as great numbers or to the same extent as in females, possibly owing to the fact that female APs have a greater number of lactotrophs (Takahashi and Kawashima, 1982).

II. Agents that Promote Prolactin Secretion

Neuroendocrine substances which affect AP hormone secretion can generally be divided into three categories: 1) neurotransmitters/ neuromodulators which do not act on the AP, but whose activity either directly or indirectly regulates the activity of releasing hormone neurons; 2) hypothalamic hypophysial releasing or release-inhibiting hormones are the chemicals present in neurons whose terminals end on capillaries in the median eminence. The portal vasculture carries the hormones to the AP where they act directly on specific binding sites to affect AP hormone synthesis and release; and 3) substances, usually present peripherally, which can act on neurotransmitter/neuromodulator neurons, releasing/release-inhibiting neurons or directly on the AP to influence hormone release. Of the substances discussed in Sec II and III, serotonin and the endogenous opiates belong to category #1, prolactin releasing factors and prolactin-inhibiting factors belong to category #2 and estrogen, testosterone, progesterone, and corticosterone belong to category #3. Present evidence suggests that gamma-amino butyric acid (GABA) and acetylcholine may belong to category #1 or

category #2.

A. Serotonin (5-hydroxytryptamine; 5-HT)

Convincing evidence demonstrates that 5-HT stimulates the release of prolactin. Meites (1963) first reported that 5-HT initiated mammary secretion in rats, and Kamberi et al. (1971) first reported that 5-HT administered intracerebroventricularly (5-HT does not cross the blood brain barrier) increased serum prolactin levels. Systemic injection of tryptophan or 5-hydroxytryptophan (5-HTP), synthesis precursors of 5-HT, increased brain 5-HT turnover and serum prolactin concentrations (Meites and Clemens, 1972; Lu and Meites, 1973; Mueller et al. 1976). Synthesis precursors of 5-HT may exert non-specific actions on other monoaminergic neurons, although the non-specific effects of tryptophan and 5-HTP appear not to involve the hypothalamic catecholaminergic neurons since catecholamine levels in the hypothalamus were not altered after 5-HT precursor administration. Sub effective doses of 5-HTP, when injected together with the specific 5-HT reuptake inhibitor, fluoxetine, resulted in a significant rise in prolactin levels (Clemens et al., 1977). In male rats, blockade of 5-HT synthesis by injection of parachlorophenylalanine (PCPA) or depletion of 5-HT by the specific serotonergic neurotoxin, 5,7 dihydroxytryptamine, significantly reduced brain 5-HT and serum prolactin levels, while having no effects on brain norepinephrine or DA content (Gil-Ad et al., 1976). 5-HT does not cause release of prolactin when added to AP tissue in vitro (Birge et al., 1970; Meites and Clemens, 1972). Injection of PCPA blocked the estrogen-induced (Caligaris and Taleisnik, 1974) or suckling-induced (Kordon et al., 1973/74) surges of prolactin secretion. Metergoline or

cinanserin, both 5-HT receptor antagonists, blocked the stress-induced increase in prolactin (Demarest et al., 1985a). Increases in prolactin after 5-HTP administration were demonstrated after cutting all afferent input to the medial basal hypothalamus, and no effect was observed after extra hypothalamic lesions (Ohgo et al., 1976), suggesting the importance of intrahypothalamic 5-HT. However,another study showed that lesion or electrical stimulation of the mesencephalic raphe nucleus resulted in decreased and increased prolactin secretion, respectively (Advis et al., 1979). These results taken together demonstrate that 5-HT is involved in a neuronal circuit(s) mediating prolactin release. Most evidence suggests that 5-HT acts through a putative prolactin-releasing factor to cause the secretion of prolactin (Clemens et al., 1978).

B. Opiates

The ability of morphine to cause the release of prolactin was first suggested by Meites et al. (1962) who demonstrated mammary gland activation after injection of morphine in estrogen-primed rats. Isolation of the endogenous opiates (EOP; Hughes et al., 1975; Hughes et al., 1977) from brain tissue, and their relative high concentration in the hypothalamus, suggested that EOPs may have neuroendocrine functions. Indeed, intracerebroventricular or systemic administration of the EOP's have been shown to influence the release of a number of AP hormones, including prolactin (Dupont et al., 1977; Bruni et al., 1977; Van Vugt and Meites, 1980). The neuroendocrine actions of EOPs are similar to morphine, and can be blocked with the opiate receptor antagonist, naloxone. Beta-endorphin was shown to be 500 to 2000 times more potent than the enkephalins in raising serum prolactin levels in the rat (Cusan

et al., 1977). It is generally agreed that morphine and the EOPs do not alter AP hormone secretion by acting directly on the AP. No effect of the EOPs on prolactin secretion was observed in AP cell culture (Rivier et al., 1977), or on whole AP tissue (Shaar et al., 1977), using high concentrations of met-enkephalin. However, the EOPs, particularly beta-endorphin, have been shown to be in high concentration in the hypothalamic-hypophysial portal blood in the rat (Sarkar and Yen, 1985) and in monkeys (Wardlaw et al., 1980). The reason for the presence of beta-endorphin in the portal blood is not clear, and may represent an overflow from hypothalamic or pituitary activity.

The EOPs are involved in a number of physiological stimuli which alter prolactin secretion. Naloxone in a dose-related fashion, can block the suckling-induced (Miki et al., 1981) and stress-induced (Van Vugt et al., 1979) release of prolactin. A single injection of naloxone can block the proestrus surge of prolactin (Ieiri et al., 1980), and also the diurnal surges of prolactin after cervical stimulation (Sirinathsinghji and Andsley, 1985), although the involvement of EOPs in the proestrus surge of prolactin are controversial (Piva et al., 1985). The neuronal circuits through which the EOPs act to effect prolactin release are not certain, although both dopaminergic and serotonergic systems appear to be involved (Van Vugt et al., 1979; Arita and Porter, 1984; Koenig et al., 1979)

Administration of morphine or EOPs to rats which results in a marked rise in serum prolactin also decreases the turnover of DA in the median eminence (Deyo et al., 1979; Van Vugt et al., 1979), and diminishes DA levels in hypophysial portal blood (Gudelsky and Porter, 1979). These results are consistent with the belief that the EOPs increase prolactin

by inhibiting the activity of tuberoinfundibular dopaminergic (TIDA) neurons. However, the decrease of DA in portal blood after morphine administration may not be solely responsible for the rise in prolactin (Arita and Porter, 1984). It appears that a stimulatory factor, possibly via a serotonergic-prolactin-factor-releasing system, also takes part in the opiate-induced increase in prolactin release (Koenig et al., 1979).

C. Prolactin-Releasing Factor(s) (PRF)

It has been a number of years since the postulation of a putative PRF from the prolactin-releasing activity of hypothalamic extracts (Meites et al., 1960). It may be that physiological increases in prolactin secretion, produced during suckling, proestrus, pregnancy etc., are at least partly due to PRF(s) secretion from the hypothalamus which act on the AP to release prolactin. Unfortunately, numerous peptides have been shown to release prolactin from AP cells in vitro. Some of these include thyrotrophin-releasing hormone (TRH; Tashjian et al., 1971), substance P (Kato et al., 1976), vasoactive intestinal peptide (VIP; Kato et al., 1978), epidermal growth factor (Johnson et al., 1980), fibrobast growth factor (Schonbrunn et al., 1980), cholecystokinin (Malarkey et al., 1981), angiotensin II (Steele et al., 1981), neurotensin (Enjalbert et al., 1982), bombesin (Westendorf and Schonbrunn, 1982), and vasopressin (Shin, 1982). Some of these substances are present in the hypothalamus (Palkovits, 1984), and are in high concentrations in the portal blood. However, probably the most important criteria for the identification of a substance as a hypothalamic-hypophysial hormone (see Sec IV below) is the ability of the particular neuronal system hormone to alter its secretion into the

portal blood at times during physiological changes in secretion of the hypophysial hormone. To date, evidence has been presented that only two of the above peptides (TRH and VIP) show alterations in release during physiological changes in prolactin release.

Initially, TRH was isolated as a peptide that regulates thyroid stimulating hormone (TSH) release from the AP (Bowers et al., 1970), but TRH was soon shown to also markedly increase prolactin secretion (Tashjian et al., 1971). The most convincing evidence that TRH is a physiologically significant factor regulating prolactin release is that the rise in prolactin during suckling causes the release of both prolactin and TSH in the rat (Blake, 1974). TRH levels are increased in hypophysial portal blood during suckling (Fink et al., 1981) or after mammary nerve stimulation (de Greef and Visser, 1981). Also, the amount of TRH required to release prolactin in vitro (10^{-9} M; Woolf and Letourneau, 1979) is in the same range of concentrations found in the portal blood (Eskay et al., 1975). Studies have shown that suckling augments the PRL responsiveness to TRH (Leong and Neill, 1982). However, suckling does not induce a rise in circulating TSH levels in women and in many physiological states, prolactin and TSH release do not occur together, e.g., during stress, the afternoon of proestrus, cold temperature.

The isolation of VIP was originally from porcine intestine, but it has been found to be widely distributed in the mammalian central nervous system, with high concentations in the hypothalamic suprachiasmatic nucleus but only moderate levels in the median eminence (Palkovits, 1984). VIP is present in rat portal blood (Said and Porter, 1979; Shimatsu et al., 1981) and can bind specifically to sites on AP

membranes (Bataille et al., 1979). Like TRH, VIP releases prolactin *in vitro* at concentrations similar to those found in portal blood (Enjalbert et al., 1980). Passive immunization with antiserum to VIP can partially block the suckling-induced rise in prolactin, and completely prevent the stress-induced rise in prolactin (Abe et al., 1985). Therefore, VIP may be involved in the stress-induced increase in prolactin. In summary, VIP appears to be a PRF involved in the release of PRL in certain physiological states although more work is required to confirm this. The status of TRH as a physiological releasor of prolactin remains in question.

D. Estrogen

The ability of chronic estrogen administration to stimulate prolactin secretion will be discussed in detail in Section V. Here, the potential for acute estrogen action to modulate the release of prolactin will be briefly covered. Estradiol was first shown to cause prolactin secretion *in vivo* by Turner (1939) and *in vitro* by Nicoll and Meites (1962). Estradiol benzoate induced prolactin release in a dose-related fashion (Chen and Meites, 1970). Acute estradiol can act directly on the AP to antagonize the inhibitory effect of DA on prolactin secretion (Lu et al., 1971; Raymond et al., 1978). Also, estradiol has been shown to greatly enhance prolactin responsiveness to TRH, possibly by increasing TRH binding sites on the lactotrophs (DeLean et al., 1977), even in the presence of inhibitory amounts of DA (Raymond et al., 1978; Labrie et al., 1980). The effect of acute estradiol administration on VIP and other agents influencing prolactin secretion has not been investigated. Estradiol given acutely *in vivo* may act via the hypothalamus as well as

directly on the pituitary to increase prolactin secretion.

One of the most studied effects of estrogen is its direct action on the AP to increase prolactin synthesis by augmenting prolactin gene transcription (Mauer, 1982). The estradiol-induced increase in prolactin mRNA occurs via a biphasic mechanism, the first phase is dependent on the conversion of the cytosolic estrogen receptor to the activated nuclear form, and is protein synthesis independent. The second phase is possibly mediated by the ability of estrogen to alter the responsiveness of the AP to another regulator of prolactin gene transcription, e.g., DA. This phase can be blocked by a protein synthesis inhibitor (Shull amd Gorski, 1985).

III. Agents that Inhibit Prolactin Secretion

A. Prolactin Inhibiting Factor (PIFs)

The control of prolactin secretion by the hypothalamus is predominantly inhibitory, which is unique among AP hormones. The inhibitory influence of the hypothalamus on prolactin secretion was first suggested when AP transplants underneath the kidney capsule in short-term hypophysectomized female rats resulted in maintenance of active corpora lutea and/or development of the mammary gland. This indicates a continuous secretion of prolactin in the blood in the absence of hypothalamic influence. Secretion of all other AP hormones was diminished, as indicated by atrophy of target glands (Everett, 1966). Prolactin was assumed to be released from the AP after elimination of hypothalamic influence, and the inhibitory factor was demonstrated to be contained within the hypothalamus. This was performed

by testing acid extracts of hypothalamus in vitro on AP hormone secretion, and it was found that prolactin secretion was inhibited (Talwalker et al., 1963). The putative prolactin inhibitory substance was named prolactin inhibitory factor or PIF. Subsequently, much of the PIF activity of the hypothalamus was attributed to dopamine (DA) which will be discussed in detail in Sec. IV. However, DA-free extracts concentrated from the mediobasal hypothalamus and the organum vasculosum of the lamina terminalis have been shown to contain PIF activity (Enjalbert et al., 1977). Recently, the C-terminal fragment of human pre-pro GnRH has been shown to have potent PIF activity (Nikolics et al., 1985; discussed in Sec. I). This 56 amino acid peptide may, along with DA, make up the total hypothalamic prolactin inhibition.

B. Acetylcholine

There is relatively minimal information regarding the action of acetylcholine on prolactin release, much of which is conflicting. Early work (Libertun and McCann, 1973, McLean and Nikitovitch-Winer, 1975) suggested a stimulatory role for acetylcholine on prolactin release since very large doses of central or systemically administered atropine inhibited prolactin secretion in male and female rats. However, other reports showed that intracerebroventricular injection of acetylcholine, or the cholinergic drugs pilocarpine or physostigmine could decrease serum prolactin--an effect which could be blocked by atropine (Grandison et al., 1974). Cholinergic agonists were also shown to block the afternoon surge of prolactin in OVX estrogen-primed rats, inhibit the rise of prolactin during restraint stress and partially block nocturnal surges of prolactin in the pseudopregnant rat (Grandison et al., 1974,

Grandison and Meites, 1976, Subramanian and Gala, 1976a, Subramanian and Gala, 1976b). It was suggested that acetylcholine was affecting prolactin release through central catecholaminergic mechanisms (Grandison and Meites, 1976). More recently, cholinergic drugs have been shown to inhibit prolactin by a direct AP action (Mükherjee et al., 1980; de Galarreta et al., 1981). Cholinergic receptors are present on the AP (Schaeffer and Hsueh, 1980). To date, though, the mechanism and physiological relevance of the ability of acetylcholine to affect prolactin release is unclear.

C. Gamma-amino Butyric Acid (GABA)

Much evidence has been accumulating in recent years indicating a role for GABA in the regulation of AP function, particularly prolactin secretion. The synthetic enzyme glutamic acid decarboxylase is present in hypothalamic tissue (Tappaz et al., 1977) and autoradiography has localized ^{3}H-GABA in different regions of the hypothalamus, including the ME (Makara et al., 1975). Binding sites have been demonstrated for GABA on the AP (Grandison, 1981) and GABA was recently shown to be in greater concentrations in hypophysial portal compared to peripheral blood. Also, electrical stimulation of the ME results in almost an 8-fold elevation of GABA in the portal circulation (Mitchell et al., 1983). Although early experiments suggested that GABA was effective in suppressing prolactin release when administered centrally (Mioduszewski et al., 1976), most recently work demonstrates that GABA acts directly on the AP to inhibit prolactin secretion. Incubation of GABA with AP preparations in vitro resulted in inhibition of prolactin secretion (Enjalbert et al., 1979; Grossman et al., 1981), although large

concentrations are required relative to dopamine (Arimura and Schally, 1977). Muscimol, a GABA agonist, can reduce prolactin levels in hypophysectomized rats bearing an AP transplant under the kidney capsule, and can inhibit the rise in prolactin after administration of the DA synthesis blocker, alpha-methyl para tyrosine (Muller et al., 1979). Interestingly, the GABA-containing dipeptide, homocarnosine, is also present in portal blood under basal prolactin conditions (Mitchell et al., 1983) and can inhibit prolactin when incubated with AP tissue in vitro, albeit at relatively high concentrations of 6 μM (Schally et al., 1977). Even with respect to the evidence that GABA can suppress prolactin release in vitro, this does not rule out the possibility of a central action of GABA to inhibit prolactin.

D. Corticosterone, progesterone and testosterone

Adrenalectomy in the rat produces an increase in plasma prolactin (Ben David et al., 1971), which can be prevented by administration of glucocorticoids (Chen et al., 1976). Corticosterone or a glucocorticoid agonist can inhibit the ether or restraint stress-induced increase in prolactin in adrenalectomized (Harms et al., 1975) or in intact male rats (Euker et al., 1975). It appears that the influence of corticosterone on prolactin secretion is exerted at both the AP and hypothalamic levels. Incubation of AP tissue in media containing 10 μg/ml corticosterone decreases prolactin release but does not affect other AP hormones. After adrenalectomy, medial basal hypothalamic DA turnover was not altered but a higher concentration of 5-HT was found in the anterior hypothalamus (Leung et al., 1980). Doses of fluoxetine, a 5-HT agonist, and crypoheptadine, a 5-HT antagonist, were ineffective in

altering prolactin release in intact rats, but fluoxetine increased and crypoheptadine suppressed serum levels of prolactin in adrenalectomized rats (Leung et al., 1980). Therefore, the influence of the glucocorticoids, specifically corticosterone, on AP prolactin secretion is complex, with actions at both the hypothalamic and AP level.

Even less is known regarding the effect of testosterone and progesterone on prolactin secretion. Progesterone is essential for maintenance of normal diurnal and nocturnal surges of prolactin which occur during pregnancy in the rat (Neill, 1980). The estrogen-induced increase in prolactin release is partially antagonized by concurrent administration of progesterone (Chen and Meites, 1970). Castration of male rats or testosterone when administered alone does not alter or slightly lowers circulating prolactin levels (Meites, 1977). However, testosterone or the reduced metabolite, 5 -dihydrotestosterone can completely inhibit the estradiol induced increase in prolactin, under chronic treatment conditions (Brawer et al., 1983).

IV. Evidence that Dopamine is the Principal PIF of the Hypothalamus

In the last 15 years an enormous amount of evidence has substantiated DA as a PIF which regulates prolactin release. In this section, the evidence which has allowed DA to meet the criteria for a hypothalamic-hypophysiotropic hormone will be presented.

A. Criteria for Hypothalamic-Hypophysiotropic Hormones

Certain conditions must be verified for a substance to be considered as a hypophysiotrophic hormone. Many of these criteria were established based upon the work of Geoffrey Harris (1955), who was the first to propose and substantiate a central control of AP function and Andrew Schally (Schally, 1978) and Roger Guillemin (Guillemin, 1978) were the first to isolate and characterize hypophysiotrophic hormones from the hypothalamus. These criteria are important because they aid in distinguishing true hypophysiotrophic action from a pharmacological effect of a substance on AP hormone release. For a substance to be considered as a hypothalamic-hypophysiotrophic hormone, some of the criteria which must be verified are: 1) the factor should exist in the median eminence and be present in portal blood at higher concentrations than in the systemic circulation; 2) the purified factor should be effective in altering AP hormone release in vitro at a concentration similar to that found in the portal blood; 3) the synthetic factor should act identically with the purified hypothalamic factor, and agonists and antagonists should result in appropriate hormone changes; 4) presence of binding sites for the factor should be located specifically on (in) the appropriate AP cell population; and 5) changes in portal blood levels of the factor should correspond to physiological variations in hormone secretion.

In considering DA as a prolactin-inhibiting hormone, all of the above criteria have been realized.

B. Dopamine in the Median Eminence and Hypothalamic-Hypophyseal Portal Blood

Although several nuclei containing DA have been located in the hypothalamus, it appears that only one, originating in the arcuate and periventricular nuclei is involved in the regulation of AP function. This group of perikarya is termed A_{12} by Fuxe et al. (1979). The A_{12} neurons can be divided into two groups: DA-containing neurons terminating in the pars intermedia and pars nervosa have perikarya which originate in the rostral and caudal arcuate nucleus, respectively, and are termed tuberohypophysial dopaminergic neurons; DA-containing neurons terminating in the external layer of the median eminence have perikarya diffusely distributed throughout the arcuate and periventricular nuclei, and are termed tuberoinfundibular dopaminergic (TIDA) neurons (Björklund et al., 1973). The TIDA neurons appear to be intimately involved in the control of prolactin secretion. DA has not only been visualized by fluorescent anatomical methods, but also quantitated by chemical means. There are relatively high concentrations of DA in the median eminence, about 100 ng/mg protein (Rinne and Sonninen, 1968). Even though there was good evidence in the early 1970's that DA played a crucial role in regulating prolactin secretion , it wasn't until a sensitive assay was developed to measure plasma DA (Ben-Jonathan et al., 1977), that DA was quantitated in the portal blood. In female rats under pentobarbital anesthesia and using a radioenzymatic assay for measurement, DA was found to be in concentrations of 1-3 ng/ml in the portal blood (Ben-Jonathan et al., 1977). Others using urethane anesthesia and a liquid chromatographic-electrico-chemical detection system observed portal blood DA levels in the 5 ng/ml range (Plotsky et al., 1978),

compared to peripheral levels of 0.1-0.3 ng/ml. This portal blood concentration of DA was sufficient to inhibit prolactin secretion when infused *in vivo* after blockade of *in situ* of TIDA synthesis (Gibbs and Neill, 1978). Therefore, DA meets the criteria of being in high concentrations in the median eminence and hypophysial portal blood (greater than peripheral levels), and can inhibit prolactin release at concentrations similar to those found in the portal plasma.

C. Action of Dopamine and Dopaminergic Agonists and Antagonists

Pasteels (1961) and Talwalker et al. (1961) first showed independently that acid extracts of rat hypothalamus inhibit the *in vitro* secretion of prolactin from AP tissue. Subsequently, many reports indicated *in vivo* that dopaminergic drugs could decrease prolactin release and anti-dopaminergic drugs increase prolactin. At the time, the belief was that these dopaminergic drugs changed hypothalamic PIF activity, and therefore altered the amount of PIF released into portal blood (Ratner et al., 1965; Nagasawa and Meites, 1970; Lu and Meites, 1972; Meites et al., 1972). However, it was observed that TIDA neuron terminals in the median eminence are adjacent to capillaries which drain directly into the portal blood (Fuxe et al., 1974), and that hypothalamic extracts lose their PIF activity if first incubated with a monoamine oxidase (Shaar and Clemens, 1973). This suggests that DA itself could be PIF. The most convincing evidence for DA's direct action on the lactotroph is work performed *in vitro*. Consistent reports showed that DA can reduce prolactin by a direct effect on the AP *in vitro* at concentrations of 5×10^{-7} M (Koch et al., 1970; MacLeod and Lehmeyer, 1974), and one report observed a decrease in prolactin at 10^{-9} M DA

(Shaar and Clemens, 1974). The decrease in prolactin by DA observed in vitro can be prevented by DA receptor blockers (MacLeod and Lehmeyer, 1974).

The identification of stereospecific binding sites for DA on AP membranes (Brown et al., 1976; Caron et al., 1976; Calabro and MacLeod, 1978; Cronin et al., 1978) further supports the view that DA acts directly on the lactotroph to inhibit prolactin release. These binding sites have not only been identified on AP membrane preparations, but specifically on lactotrophs (Goldsmith et al., 1979). The competition curves of dopaminergic agonists for ^{3}H-dihydroergocryptine (a specific DA receptor agonist) binding sites parallels the agonists' ability to inhibit PRL release in vitro (Cronin et al., 1978). The AP DA receptors are a pure population of the D_2 type (binding does not increase cAMP levels) and appear to exist in an interconvertable high and low affinity form (Sibley et al., 1982). The K_i for DA agonists binding to the receptor in the high affinity form correlate perfectly with the IC_{50} of the agonists' ability to inhibit prolactin secretion in vitro (George et al., 1985). Also, the K_i for DA of 7 nM, which displaces ^{3}H-dihydroergocryptine from high affinity form receptors, is well within the range of DA concentrations which have been reported in portal blood. Hence, DA binding sites located on the lactotrophs and the ability of DA agonists to inhibit prolactin secretion strengthens the hypothesis that DA is a true hypophysiotropic hormone.

D. Variations in TIDA Neuronal Activity, Hypophyseal Portal Blood Dopamine and Physiological Changes in Prolactin Secretion

Although a number of studies have measured portal blood DA during physiological changes in prolactin secretion, e.g., proestrus, most experiments have used biochemical methods to evaluate TIDA neuron activity. This can indicate changes in potential portal blood DA (Reymond and Porter, 1982), without the use of anesthesia. The most common method of evaluating TIDA neuron activty is based on the fact that since DA content does not appear to change with increasing or decreasing activity of the neurons (under most conditions), synthesis of DA must keep pace with release. Therefore, synthesis of DA should be a reflection of neuronal activity of dopaminergic neurons (Moore and Demarest, 1982). The two available methods to measure TIDA activity are 1) determining the rate of decline of DA after administration of the tyrosine hydroxylase inhibitor alpha-methyl-para tyrosine and is termed DA turnover, or 2) measuring accumulation of l-DOPA after inhibition of l-aromatic amino acid decarboxylase by 3-hydroxybenzylhydrazene (NSD 1015), which is an index of DA synthesis (Moore and Demarest, 1982).

During the estrous cycle in the rat, DA levels in the hypophysial portal blood were lower on the afternoon of proestrus as compared to estrus (Ben-Jonathan et al., 1977), and is at least partially responsible for the afternoon proestrus surge of prolactin. This proestrus fall in portal blood DA may be due to the action of estrogen since adrenalectomized, ovariectomized rats treated with estradiol 24 hours earlier showed decreased DA in the portal blood (Cramer et al., 1979). A slower turnover of DA, and decreased DA synthesis in the median eminence on the afternoon of proestrus as compared to estrus (Demarest

et al., 1981) has also been observed which agrees with the results of DA in portal blood. No changes were observed in DA turnover during the estrous cycle by Homma and Wuttke (1980). Whether the change in TIDA neuronal activity, and therefore portal blood DA during proestrus is alone sufficient to account for the prolactin surge is not known.

The diurnal and nocturnal surges of prolactin which take place during early pregnancy or after cervical stimulation in the rat appear to occur out of phase with changes in DA synthesis in the median eminence (Voogt and Carr, 1981; McKay et al., 1982) and with the levels of DA in hypophysial portal blood (DeGreef and Neill, 1979), i.e., during the prolactin surges portal blood DA is low, in between the prolactin surges portal blood DA is high. This circadian pattern of TIDA neuronal activity appears to be independent of circulating prolactin levels (Demarest et al., 1983), because the neuronal pattern persists even after the prolactin pattern is abolished by administered haloperidol, a DA receptor antagonist. The importance of DA in the development of these prolactin surges is questionable, however, because peripheral infusion of DA, to mimic the DA changes in portal blood which normally occur after cervical stimulation, did not result in nearly as large of a rise in prolactin compared to a normal cervical stimulation-induced prolactin surge (DeGreef and Neil, 1979). Therefore, although changes in hypophysial portal blood DA and TIDA synthesis are inversely correlated with the circadian prolactin surges of pseudopregnancy, DA is not solely responsible for these prolactin surges. Similar findings have been reported regarding hypophysial portal blood DA and the suckling-induced rise in prolactin in rats. In the rat, a decrease in portal DA by itself is not sufficient to account for the suckling-induced rise

in prolactin (Neill et al., 1982).

Thus, the above results imply that during physiological increases in prolactin secretion in the rat changes in DA in the portal blood, although occurring in the appropriate direction, are not of sufficient magnitude to account for the total prolactin response. This has led to the belief that even though DA seems indisputably to be PIF, its role is more important during the tonic or basal conditions of prolactin release than in physiologically stimulated prolactin release. It is interesting that in the estradiol-treated, stalk-transected monkey, DA infusion can account for most if not all of the inhibitory effect of the hypothalamus on prolactin release. Existence of other PIFs other than DA do not need to be postulated for the tonic suppression of prolactin release by the hypothalamus (Neill et al., 1982).

An important physiological mechanism demonstrating DA's role in the basal secretion of prolactin is that DA is involved in prolactin short-loop feedback. Systemic or intracerebroventricular injection of prolactin increases DA turnover (Hökfelt and Fuxe, 1972; Gudelsky et al., 1976), DA synthesis (Johnston et al., 1980), tyrosine hydroxylase activity (Nicholson et al., 1980) and DA in the portal blood (Gudelsky and Porter, 1980). Anti-dopaminergic drugs, e.g., haloperidol, can also increase DA in the portal blood, an effect which appears to be mediated by the haloperidol-induced increase in circulating prolactin, since pre-treatment with prolactin antiserum greatly attenuates the DA response (Gudelsky and Porter, 1980). It appears, therefore, that prolactin may feed back on TIDA neurons in a positive fashion. Since DA inhibits prolactin at the AP level, this may be one mechanism by which prolactin regulates its own secretion.

There is good evidence that the basal secretion of prolactin progressively increases during aging in both male and female rats (Meites et al., 1984). Circulating levels of prolactin are highest in the oldest female rats which often exhibit prolactin-secreting pituitary adenomas. It might be expected, because of the prolactin-TIDA neuronal short-loop feedback mechanism, that DA in the portal blood would be increased with age. However, the major stimulus for the age-related hyperprolactinemia is believed to be a decrease (not an increase) in TIDA neuronal activity in old rats. Both old males and females show reduced median eminence DA content (Gudelsky, 1981; Wilkes et al., 1979), decreased DA turnover and synthesis (Simpkins et al., 1977; Demarest et al., 1982) and decreased DA in the portal blood (Gudelsky, 1981; Sarkar et al., 1984a), as compared to young rats. It follows logically that prolactin-TIDA neuron short-loop feedback is impaired in old rats, as has recently been observed (Sarkar et al., 1983c). Therefore, the failure of TIDA neurons to respond to prolactin during aging may be of significance with regard to the development of hyperprolactinemia and prolactin-secreting tumors.

E. Regulation of Synthesis, Release and Inactivation of Dopamine

The catecholamines, including dopamine are synthesized both in the central nervous system and the periphery from the amino acid precursor tyrosine, by a similar enzymatic sequence. Centrally, tyrosine is actively taken up in the neuron and converted to l-DOPA by tyrosine hydroxylase (Levitt et al., 1965). Tyrosine hydroxylase catalyzes the rate-limiting step in the sequence, and requires molecular oxygen and a reduced pteridine as cofactors for its activity. l-DOPA is converted to

DA by the relatively non-specific ubiquitous enzyme aromatic l-amino acid decarboxylase (Carlsson et al., 1972). The synthesis of DA takes place in the terminals of neurons as the uptake mechanism for tyrosine and both synthetic enzymes are present in synaptosomal fractions (Cooper et al., 1982).

The steady-state concentration of DA in TIDA neurons is maintained relatively constant under most conditions. This is due, at least in part, to DA end-product feedback inhibition on tyrosine hydroxylase. When neuron activity increases, DA is released from the terminals and DA intraneuronal concentration temporarily falls. Along with the increase in neuronal activity, tyrosine hydroxylase appears to increase its affinity for tyrosine and the pteridine co-factor and decrease its affinity for DA (Udenfriend et al., 1965; Costa and Neff, 1966). All of these changes in tyrosine hydroxylase increase its activity to replace the released DA. With decreased neuronal activity, DA binds to an allosteric site on tyrosine hydroxylase to decrease its activity (Cooper et al., 1982).

Catecholamines are stored within vescicles at the neuron teminals. The vescicles are synthesized in the perikarya and transported to the terminal via axonal flow (Dahlstrom et al., 1973). The existence of two storage pools for DA has been proposed (Axelrod, 1974). There is a readily releasable pool which is closely associated with the internal synaptic membrane and is believed to consist of newly synthesized catecholamine. The other, a larger storage pool may be more distant from the synaptic membrane and its function is unknown. Neuron impulses result in Ca^{++}-dependent (DeRobertes and Vas Ferreira, 1957) release of newly synthesized DA (Kopin et al., 1968).

Mesotelenecephalic DA neurons appear to have autoreceptors, which are activated by DA upon release of DA into the synaptic cleft. Activated autoreceptors can inhibit synthesis and possibly further release of DA from the neuron (Moore and Demarest, 1982). The regulation of the release from TIDA neurons is somewhat controversial. Evidence has been reported which suggests the lack of autoreceptors (Demarest and Moore, 1979a) or the presence of autoreceptors on TIDA neurons (Sarkar et al., 1983b). Autoreceptors on TIDA neurons were suggested as evidenced by the ability of DA and its receptor agonists to decrease the release of ^{3}H-DA from median eminence (ME) tissue in vitro. Whether this action occurred via a receptor or by end-product feedback inhibition by the agonist after uptake into the neuron is in question. Nevertheless, ^{3}H-DA agonist binding was demonstrated in homogenates of bovine ME tissue (Cronin et al, 1978). Release of DA from TI neurons can be agumented by prolactin, a mechanism by which prolactin can regulate its release (Sec IV-D) (Perkins and Westfall, 1978; Demarest et al., 1985b).

Inactivation of DA, and the catecholamines in general, is thought to occur by at least two mechanisms: 1) reuptake back into the neuronal terminal, and 2) enzymatic degradation. The uptake of DA into TIDA neurons is also controversial. Sarkar et al. (1983c) showed that the uptake of ^{3}H-DA into whole median eminence tissue was linear over time and temperature and was sodium dependent. It could be effectively blocked by the catecholamine reuptake inhibitor, nomifensine, and by non-radioactive DA. However, others have shown that ^{3}H-DA uptake in neuronal homogenates is lower in median eminence as compared to the striatum (Demarest and Moore, 1979b). In favor of a reuptake mechanism in TIDA neurons is that nomifensine injected in vivo can increase DA

release into hypophysial portal blood (Sarkar et al., 1983b) and decrease prolactin secretion (Cocchi et al., 1979).

The three major catabolic metabolites of DA in the central nervous system are dihyroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and to a much lesser extent methoxytyramine (MTA). Deamination occurs by the action of monoamine oxidase, which exists as a mitochondrial membrane bound enzyme (Nukada et al., 1963), and requires reuptake of DA into the neuron terminal. The aldehyde is then further oxidized to the acid by aldehyde dehydrogenase. Extraneuronally, possibly of glial origin, DA can be O-methylated by catechol-O-methyltransferase (COMT) (Alberici et al., 1955; Axelrod, 1974).

V. Relation of Estrogen to AP Hormone Secretion and Development of Pituitary Prolactinomas

In many instances, the secretory function and cellular proliferation in endocrine glands are closely correlated. A chronic increase in hormone secretion is usually accompanied by enhanced mitotic activity of that secretory cell type. With regard to prolactin secretion, there is an increase in cellular proliferation of the lactotrophs in many cases of increased prolactin release; e.g., blockage of DA receptors (Jacobi and Lloyd, 1981; Kalberman et al., 1980) or lesion of the MBH (Cronin et al., 1982) which destroys TIDA neurons both of which enhance prolactin secretion and increase mitotic activity of the lactotrophs. Estrogen is a potent physiological stimulator of prolactin release (Meites, 1974) and results not only in lactotroph proliferation but also in the

development of prolactinomas (Furth and Clifton, 1966). In this section, the relation of physiological and pharmacological doses of estrogen to secretion and mitosis of prolactin-secreting cells will be discussed.

A. Estrogen Actions on the Anterior Pituitary

Estrogen administration was first shown to enhance the pituitary prolactin content in rats and guinea pigs by Reece and Turner (1936). Since mammary gland stimulation was also observed, prolactin was assumed to be increased in the serum as well. Also, estrogen injected *in vivo* can enhance AP prolactin release *in vitro* (Ratner et al., 1963), suggesting estrogen can act directly on the AP to increase prolactin. After the development of radioimmunoassay, daily injections of estradiol benzoate were shown to increase serum prolactin concentrations in a dose-related fashion (Chen and Meites, 1970) although the largest pharmacological doses of estradiol did not elevate prolactin to the same extent of the lower optimal dose. Chronic estrogen treatment (at relatively high doses) can inhibit the secretion of LH, FSH, and thyrotropin TSH release from the AP, and these same doses can increase the release of prolactin and possibly GH (Meites, 1974).

1. *In vitro* stimulation of prolactin

Estradiol, but not testosterone, progesterone, cortisol, or corticosterone, added the incubation media of AP tissue cultures increases the release of prolactin into the media (Nicoll and Meites, 1962). Estrogen also causes prolactin release in primary cultures of AP cells (Labrie et al., 1980; West and Dannies, 1980). However, estrogen's ability to promote prolactin secretion *in vitro* is rather modest

compared to the large response observed *in vivo*. Two findings appear to explain this discrepancy: 1) estradiol can almost completely reverse the inhibition of prolactin release by DA or DA agonists, and 2) estradiol can also sensitize the lactotrophs to the prolactin-releasing action of TRH. The DA agonist bromocryptine maximally inhibited (70%) prolactin secretion in primary AP cell cultures (Labrie et al., 1983). However, maximal inhibition of prolactin by bromocryptine observed after preincubation with estradiol was only 20%. It has been suggested that estrogen can exert an "anti-dopaminergic"-like action. However, the potent anti-dopaminergic effect of estrogen cannot be explained by an estradiol-induced decreased number or affinity of DA receptors (Labrie et al., 1983), suggesting this is a post-receptor phenomenon. Furthermore, estrogens can increase the number of TRH binding sites on AP cells in culture (Gershengorn, 1979) and this action temporarily parallels estrogen's ability to sensitize the prolactin response to TRH (DeLean et al., 1977). Although estradiol can cause a specific release of prolactin, the mechanism of release is not well understood. It is thought that under basal conditions of release *in vitro*, the most recent synthesized prolactin is preferentially released (Walker and Farquhar, 1980). Estradiol is known to increase the synthesis of prolacin (Mauer and Gorski, 1977; Stone et al., 1977) at least in part by increasing transcription of prolactin messenger RNA (Mauer, 1982; Schull and Gorski, 1985).

2. *In vivo* stimulation of prolactin

Painting of the skin of mice at twice weekly intervals with estrin preparations called "alpha-folliculin" or keto-hydroxy estrin and the

observation of mammary and pituitary tumor development was the first report to establish that the carcinogenic effect of any substance was restricted to tissue remote from the site of application of the cancer-causing agent (Cramer and Horning, 1936). In this report, hypopituitarism and strain differences in the susceptibility to estrogen was also observed (Cramer and Horning, 1936; McEwen et al., 1936). Later, it was found that the F344 rat is particularly susceptible to estrogen at the pituitary level and stilbesterol-induced pituitary adenomas up to 500 mg in weight have been observed (Dunning et al., 1947). As is true with most experimental neoplasms induced by hormone imbalances, the estrogen-induced pituitary adenoma is initially only capable of growth in animals with the same hormone imbalance required for tumor induction (Furth et al., 1960). However, ultimately these tumors will produce autonomous variants capable of growth in a usual endocrine environment. Normally, primary estrogen-induced tumors are dependent on elevated estradiol levels for their growth, but can become autonomous after one or two passages of the pituitary tissue (Furth and Clifton, 1966). Whether the change in estrogen sensitivity of these tumors with time is an exclusive characteristic of the autonomous cells or is just an adaptive phenomenon acquired gradually is not known (Clifton and Furth, 1961; Furth, 1969). Estrogen-induced pituitary adenomas secrete copious amounts of prolactin (Meyer and Clifton, 1956) and contain hyperplastic and hypertrophic lactotrophs (Gersten and Baker, 1970). Somatotrophin release is also moderately increased with chronic estradiol treatment (Lloyd et al., 1972; Gottschall et al., 1986). However, after estradiol is removed, GH, but not prolactin, returned to control levels (Gottschall et al., 1985). Chronic estrogen

can decrease circulating levels of TSH, thyroxin and triiodothyronine, LH, and FSH (Nakagawa et al., 1980).

In vivo, chronic estradiol dramatically increases prolactin synthesis (Wiklund et al., 1981) and this appears to be due to an increase in prolactin mRNA synthesis (Mauer, 1982) and turnover (De Nicola et al., 1978) and increased DNA synthesis (De Nicola et al., 1978). Therefore, estradiol administration appears to specifically augment prolactin release and synthesis in addition to increasing DNA synthesis and mitotic activity of the lactotrophs.

3. Interaction of estrogen and dopamine on lactotrophic mitotic activity and prolactin secretion

As mentioned earlier, the proliferation of endocrine cells appears to be related to the cell's secretory response. During various states of chronic prolactin hypersecretion, there is an increase in mitotic activity of AP cells. Lactating rabbits which maintain high levels of prolactin for extended periods, exhibit increased AP mitoses (Allanson et al., 1969). Administration of estrogens (Hunt, 1947) in a single dose (Lloyd et al., 1972; Lloyd et al., 1973) or maintaining high estradiol levels by implanting an estrogen-containing pellet (Kalberman et al., 1979) increased AP mitotic activity and DNA synthesis. In each of these studies, prolactin concentrations in the serum were markedly enhanced. Furthermore, estrogen can increase DNA polymerase (Mastro and Hymer, 1973) and thymidine kinase activity (Valotaire et al., 1975) in the AP, both indicative of enhanced DNA synthesis. Dopaminergic receptor agonists such as bromocryptine inhibit estrogen-induced prolactin secretion (Lu et al., 1971) from normal and tumorous APs (Quadri et al.,

1972). By inhibiting the release of prolactin, bromocryptine can also decrease the stimulation of DNA synthesis and mitotic activity as a result of estrogen treatment (Lloyd et al., 1975; Kalberman et al., 1980). The DA antagonist sulpiride injected daily during estrogen treatment was able to stimulate larger APs, prolactin secretion, and DNA synthesis when compared to rats treated only with estrogen (Jahn et al., 1982). This action of sulpiride was interesting in that acute injection of sulpiride was only effective in raising prolactin during early estrogen treatment (7 and 21 days) and was completely ineffective in increasing prolactin secretion at 45 days of estrogen treatment. Moreover, antagonists injected in vivo increased AP DNA synthesis and decreased AP prolactin content even in the absence of estrogen (Jacobi and Lloyd, 1981). Therefore, the mitotic activity of the lactotrophs can be augmented by substances which increase prolactin secretion, i.e., estrogen and DA antagonists, and decreased DNA synthesis is observed when prolactin secretion is low, i.e., DA agonists. This close coupling of secretion and mitosis of the lactotrophs may be important when hypothalamic mechanisms controlling prolactin release do not function normally.

Other factors may contribute in altering prolactin secretion and mitosis of the lactotrophs after chronic estradiol treatment. In F344 rats, estrogen-induced prolactinomas were shown to develop a direct arterial blood supply to the tumor which may dilute hypothalamic factors reaching the AP via the portal circulation, and thereby reduce their influence on prolactin secretion (Elias and Weiner, 1984). After stilbestrol treatment in F344 rats, the lactotrophs appear in two distinct populations based upon gradient sedimentation which may vary in

their sensitivity to estrogen or hypothalamic factors (Phelps and Hymer, 1983). Also, when an AP is transplanted under the kidney capsule preceding estrogen treatment, the in situ AP exhibited greater growth and tumor development than the transplant (Welsch et al., 1971). This would suggest a positive hypothalamic influence on lactotroph proliferation as a result of estrogen and indeed, increased prolactin-releasing activity of hypothalamic extracts was observed after chronic estrogen treatment (Ratner and Meites, 1964; Nakagawa et al., 1980).

Chronic administration of GnRH agonists (Lamberts et al., 1981) or GnRH antagonists (De Quijada et al., 1983) can inhibit growth of estrogen-induced transplantable prolactin secreting tumors. Tumor inhibition by GnRH analogs appears to be mediated by chemical castration as evidenced by lowered estradiol levels and atrophy of the ovaries and the uterus. Ovariectomy or administration of the anti-estrogen tamoxifen can also inhibit pituitary tumor growth (Lamberts, 1984).

B. Anatomy and Pathophysiology of Estrogen-Induced Prolactinomas

The percentage of AP cell types in the normal adult female rat consists of about 30% lactotrophs, 30% somatotrophs, 15% gonadotrophs, 15% unidentified (or undifferentiated) chromophobic cells with the remainder consisting of thyrotrophs, corticotrophs and unclassified cells (Takahashi and Kawashima, 1982). Estradiol treatment results in profound changes; most dramatic is a marked increase in the number of lactotrophs. Estradiol implantation into the pars distalis resulted in hypertrophied and hyperplastic lactotrophs (Gersten and Baker, 1970). Ultrastructurally, the lactotrophs contained extensive development of the rough endoplasmic reticulum arranged in concentric whorls (Shiino

and Rennels, 1975), an increased Golgi region volume density and a smaller number and size of secretory granules (Mc Comb et al., 1981). All of these cellular alterations are indicative of increased synthesis and secretion of prolactin and an increased mitotic activity of the lactotrophs. Continued estrogen treatment results in compression of the pituitary sinusoids and thickening of the sinusoid walls due to the increase in lactotroph cell number. Further estrogen administration results in a gland which is nodular in appearance with marked sinusoid compression, and occasionally open sinuses which appear as blood lakes (Clifton and Meyer, 1956). The mean weight of these Wister rat APs was 172 mg after 230 days of estrogen treatment, compared to 10 mg control AP. As mentioned earier, the F344 strain is particularly susceptible to the AP hyperplastic effect of estrogen (Dunning et al., 1947). After only 60 days of treatment with stilbesterol, almost 60% of total AP cells were immunocytochemically identified as lactotrophs (Phelps and Hymer, 1983). This is certainly a low estimate (because many of the lactotrophs may have lacked sufficient granules to stain immunocytochemically) since the total number of cells in the AP increased 1100%. When chronic estrogen treatment is discontinued, the number and size of secretory granules in the lactotrophs increased dramatically, and secondary lysozomes were present (Shiino and Rennels, 1975). The glands, although still hypertrophic, lost the nodular appearance and returned to a more normal shape (Treip, 1983). The question of whether primary estradiol-induced multiplication of lactotrophs is a true neoplasia or just hyperplasia has not been resolved, but the current belief is that it is a hyperplasia. Estrogen treatment produces an immediate and dramatic reduction on somatic

growth. This is more apparent in male than female (which exhibit a growth stasis) rats. The mechanism responsible for the estrogen-induced dimunition of growth appears to be complex. Estradiol can reduce dietary intake (Josimovich et al., 1967) and depress levels of an insulin-like growth factor and carrier protein even in the presence of normal or elevated growth hormone levels (Drazmin et al., 1979). In the male, estradiol may suppress anabolic steroid production by inhibiting gonadotrophin release from the AP (Attardi et al., 1980).

The induction of hyperprolactinemia after high doses of estrogen to male rats is associated with reduced circulating TSH, LH and FSH levels (Smythe et al., 1981), although in female rats GH may be elevated (Gottschall et al., 1986). Hyperprolactinemia also increases adrenal weight and corticosterone levels (Gottschall and Meites, unpublished). Estradiol-induced hyperprolactinemia produces marked mammary gland development (even in the absence of the adrenal), the uteri are usually hypertrophic and the ovaries are smaller than normal. In the rat, hyperprolactinemia results in a puzzling renal proximal tubular degeneration with polyuria and proteinuria (Furth and Clifton, 1966).

VI. Estrogen Influence on the Central Nervous System

The view that steroid hormones can influence the brain and behavior have been held for many years. The experiments of Berthold (1849), on the castration effects on sexual and aggressive behavior in the rooster, and Brown-Sequard and D'Arsonval (1891) work on the effects of testicular extracts on rejuvenation processes in the aged human, demonstrate the long-held belief that the gonads affect behavior.

However, it has not been until the last three decades that information regarding the mechanism(s) and site of action of steroids has been achieved. Brain implants of crystalline steroid were used to localize brain regions where steroids may be acting (Harris et al., 1958; Kent and Liberman, 1949). Technological advances allowed for measurements of the affinity and number of steroid receptors (Jensen and Jacobson, 1962) and the receptors could be specifically localized by autoradiography (McEwen et al., 1972). These tools made possible the identification of specific brain sites where steroids act to modify or even induce particular physiology and/or behavior. Many studies dealing with steroids throughout the sixties and seventies were concerned with steroid modulation of neuroendocrine reproduction function and behavior. This section will focus on the location of the sites and mechanism of action of steroids in the brain, and more importantly, how chronic administration of estradiol can have profound permanent actions on reproductive and other neuroendocrine processes.

A. Distribution of Estrogen Target Sites in the Brain

Much of the work on steroid-concentrating sites in the brain has used the rat as a model. The map of estrogen-concentrating cells in the rat brain obtained by autoradiography after injection of ^{3}H-estradiol has revealed estrophilic perikarya in the hypothalamic hypophysiotropic area and also the corticomedial amygdala (Leiberburg and McEwen, 1977). Fewer and less intensive ^{3}H-estradiol was found in nuclei of cells of the midbrain central gray and hippocampus (Pfaff and Keiner, 1973). There is little sex difference in ^{3}H-estradiol uptake, i.e., short-term castrated males exhibit almost an identical pattern of uptake as do

short-term castrated females (McEwen, 1981). When radioactivity was quantitated after extraction from nuclear fractions of brain tissue, the AP showed the greatest uptake followed by the pre-optic area, the medio-basal hypothalamus, the corticomedial amygdala, and the rest of the hypothalamus (McEwen, 1981). The percent of total perikarya containing estradiol in specific areas of the hypothalamus has also been analyzed by autoradiography (Morrell and Pfaff, 1983). The pre-optic area, ventromedial nucleus, arcuate nucleus, and medial amygdaloid nucleus contain 24, 29, 21 and 40% respectively, estrogen-concentrating cells relative to the total number of perikarya in these areas. This uptake was shown to be saturable and an active selective process in a particular sub population of neurons.

B. Effect of Estrogen on Neurons and Neurotransmitter Systems

Steroids in general, and estradiol specifically, easily traverse the cell membrane because of their lipophilic nature, and classically, were believed to bind to cytosolic receptors in specific target cells. This receptor-estrogen complex is then translocated across the nuclear membrane after "activation" originally described as an increase in affinity of the receptor for polyanions (Higgins et al., 197; Milgrom et al., 1973). The activated estrogen-receptor complex then interacts with the genome, possibly by altering promoter elements which control initiation of transcription, resulting in the synthesis of steroid regulated proteins (Chambon et al., 1984). Although for many years most work has suggested a cytosolic intracellular population of steroid receptors, some recent evidence has postulated that this receptor, whether bound to steroid or not, actually resides in the nucleus

(Gorski, 1985). Recently, steroids have been demonstrated to have non-genomic mediated effects that are of very short latency (Szego, 1984).

1. Genomic, transcription-translation mediated effects

There are only a few instances where steroid hormone action has been shown to result in direct genomic activation in the brain. Estradiol administration can transiently activate hypothalamic cell nuclear RNA polymerase II activity (Kelner et al., 1980). Unlike the uterus, the brain activation lacks the sustained activation of RNA polymerase II and does not alter RNA polymerase I activity. This may be because of the absence of estrogen-induced hypertrophy and hyperplasia in the brain. Indirect demonstrations of genomic involvement after estrogen administration include the ability of the RNA synthesis inhibitor, actinomycin D, to block estrogen action on female sexual behavior (Quadagno et al., 1971; Terkel et al., 1973), and the preovulatory LH surge (Jackson, 1972; Kalra, 1975).

The major end result of genomic activation by estrogens is the production of enzymes and other cell proteins believed to be the substances which manifest hormone action. The importance of these products is shown by their ability to 1) alter neuronal transmission, synthesis or plasticity, 2) produce stabilizing factors like nerve growth factor, 3) result in programmed cell loss, or 4) activate, de-activate or modulate pre- or post-synaptic receptor populations.

There are an abundant number of examples which illustrate estrogen-induced alteration of neuronal function by transcription products. Due to the diversity of estrogen's effects, an important qualification in

estrogen-mediated action is that they are primary, i.e., a direct effect of estrogen on a particular neuron, or secondary, i.e., an effect mediated by the estrogen-induced release of a hormone or neurotransmitter (McEwen, 1981). Examples of the genomic-action of steroids will be given in Sec. VI C and D.

2. Effects which may operate on neural membranes

Some actions of steroids are of short latency, too short to be acting via transcription-translation. In some instances, these short-lateny events have been reproduced on isolated synaptosomes or membranes. The best example of a short-latency action is the alteration in diencephalon neuronal discharge rates within seconds after 17-β-estradiol iontophoresis (Kelly et al., 1977). No effect was observed after 17-α-estradiol administration. The importance of these short-latency effects as a major means of influencing neuroendocrine function has not been determined.

C. Estradiol-induced Adult Anovulatory Syndrome

Elevated estrogens, administered exogenously or endogenous high circulating levels result in a well-characterized anovulatory state in the female rodent that has been postulated as a model for aging (Finch et al., 1984). A single injection of a large dose of estradiol benzoate (Brown-Grant, 1974) or estradiol valerate (Brawer et al., 1978), or exposure to constant light (Brawer et al., 1980) causes the cessation of regular estrous cycles in female rats and a rapid onset of persistent vaginal cornification. The ovary contains many large follicles which produce substantial amounts of estradiol and can maintain estradiol

levels of 30 pg/ml, the upper limit of the physiological range. This estradiol level can be sustained for up to six months or longer.

The potential action of chronic estradiol administration on the hypothalamus was first suggested by observations of hypothalamic neuroanatomy after multiple monthly injections of estradiol valerate (Brawer and Sonnenschein, 1976). Histologically, the arcuate nucleus contained multiple pathological foci characterized by degenerating axons and dendrites. These foci were most apparent in the lateral regions of the nucleus. The neuronal degeneration was associated with reactive microglia containing cellular debris and reactive astrocytes that contained many pools of electron dense material and large bundles of fine filaments. The microglial response is a long-established marker for neuronal degeneration present in diseases where cell death is a well-known phenomenon (Bernheimer et al., 1973). Interestingly, the arcuate nucleus pathology progressed with the same time course, intensity and distribution in rats given monthly injections or a single injection of estradiol valerate (Brawer and Finch, 1983). The lesion appears to be an estrogen-ovarian dependent phenomenon, since OVX prior to a single injection of estradiol or placement in constant light, does not result in the pathology (Brawer et al., 1980). However, chronically elevated estradiol in ovariectomized rats by multiple injections can produce the arcuate nucleus lesion.

D. Estrogen and Hypothalamic Mechanisms which Regulate Prolactin Release

Recently, it has been shown using autoradiography that arcuate

nucleus neurons containing immunoreactive tyrosine hydroxylase are the major neurotransmitter class of perikarya of this nucleus that concentrate ^{3}H-estradiol. These cell bodies are well established to belong to TIDA neurons. This important work shows that estradiol has at least the capability of directly affecting a final common pathway involved in regulating prolactin release; ie. TIDA neurons.

1. Tuberoinfundibular dopaminergic neurons

A substantial amount of evidence accumulated over the last five years has demonstrated that chronic treatment of male or female rats with estradiol attenuates TIDA neuronal activity. The suggestion was apparent in an early piece of work which indicated that injection of rats with estradiol for ten days depleted the hypothalamus of PIF (Ratner and Meites, 1964). Subsequently, the literature became quite difficult to interpret, because of the variety of estradiol-treated models, the methodological approaches used to evaluate TIDA neuronal activity, along with the complexity of action estradiol on these neurons.

Sub-acute treatment of ovariectomized rats with estradiol (24 hrs) suppresses the concentration of DA in the portal blood (Cramer et al., 1979) and may be related to the decline of portal blood DA on the day of proestrus during the estrous cycle (Ben-Jonathan et al., 1977). Interestingly, the mechanism by which short-term estrogen treatment suppresses the release of DA into hypophysial portal blood may involve the action of a metabolite of estradiol. 2-hydroxyestradiol, a catechol estrogen, but not estradiol, can inhibit the activity of tyrosine hydroxylase under <u>in vitro</u> conditions (Lloyd and Weisz, 1978; Lloyd and

Ebersole, 1980; Foreman and Porter, 1980). Since synthesis of DA is directly related to it's release, inhibition of tyrosine hysroxylase by 2-hydroxyestradiol may account for the decrease of DA into portal blood after short-term estradiol.

Longer treatment with estradiol (2-7 days) leads to an elevation in portal blood DA (Gudelsky et al., 1981), increased turnover (Eikenburg et al., 1977) and synthesis (Demarest et al,. 1984) of DA, and increased release of ^{3}H-DA during electrical stimulation of ME tissue after accumulation of ^{3}-DA (Gottschall and Meites, submitted). This augmented TIDA neuronal activity after longer estradiol treatment is a secondary action of the steroid; i.e., it is mediated via the hypothalamic and AP estradiol-induced release of prolactin. Hypophysectomy prior to estradiol administration did not result in any change in DA turnover (Eikenburg et al., 1977). Also, methods that increase circulating levels of prolactin, i.e., injection of bovine prolactin, transplantation of an AP under the kidney capsule or injection of drugs that enhance prolactin secretion, stimulate the release of DA into the portal blood (Gudelsky and Porter, 1980) and increase the turnover of DA in the ME (Hokfelt and Fuxe, 1980; Gudelsky et al., 1976; Gudelsky and Moore, 1977). Since estradiol exerts anti-dopaminergic effects on the lactotrophs of the AP (Lu et al., 1971; Raymond et al., 1978), elevated prolactin as a result of these treatments may augment the release of DA into the portal blood in an attempt to compensated for the anti-dopaminergic action of estradiol.

The first direct evidence that chronic estrogen treatment could reduce TIDA neuronal activity was that two weeks of estradiol treatment markedly decreased ME DA content and produced a substantial, but

non-significant, decline in mean DA turnover in the ME (Smythe and Brandstater, 1980; Dupont et al., 1981). The magnitude of the estradiol-induced hyperprolactinemia was related to the degree of reduction in ME content of DA (Smythe and Brandstater, 1980), which raised the possibility that the loss of DA content was due to the action of prolactin and not to estradiol directly. Furthermore, it was shown by pharmacological means, that there was a loss of TIDA neuronal control of prolactin secretion in animals which were hyperprolactinemic after chronic estrogen treatment (Smythe et al., 1982; Casaneuva et al., 1982). Sarkar et al., (1982) observed an abnormal histofluorescence of dopaminergic neurons in the arcuate nucleus and ME of female rats bearing estrogen-induced prolactinomas as indicated by the presence of distorted fibers and deposits of punctate autofluorescent material. The suggestion was made that chronic hyperprolactinemia could cause degeneration of TIDA neurons, since this pathology was also seen in old hyperprolactinemic rats and in those made hyperprolactinemic by implanting a transplantable prolactinoma. Whether or not estrogen and/or prolactin is neurotoxic to TIDA neurons has not been verified or confirmed. However, there is clearly a decrease in the function of TIDA neurons after long-term estradiol treatment. Chronic estrogen can diminish DA levels in portal plasma (Sarkar et al., 1984a), decrease the synthesis and turnover of DA (Demarest et al., 1984; Morgan et al., 1985; Terry et al., 1985), decrease medial basal hypothalamic tyrosine hydroxylase activity (Luine et al., 1977), attenuate AP DA content (DiPaola et al., 1985), reduce the electrically stimulated release of ^{3}-DA from ME tissue (Gottschall et al., 1986), and alter the serum prolactin response to central-acting dopaminergic drugs (Casaneuva et

al., 1982; Willoughby et al., 1983; Gottschall et al., 1986). The estradiol-induced decline in TIDA neuronal function occurs at a time when monoamine oxidase activity is low, since four weeks of estradiol treatment results in a 30% decrease in monoamine oxidase (type A) activity in the medial basal hypothalamus (Luine et al., 1977). The implication for the estrogen-induced loss of TIDA neuronal control of prolactin release is that the loss of the ability of prolactin to regulate it's own secretion in a negative feedback fashion may play a role in the later hypersecretion of prolactin after estrogen treatment and be involved in the development of prolactin secreting tumors. Moreover, if estradiol resulted in a loss of TIDA control of prolactin release that was permanent, it would clearly indicate a central role in the development and/or growth of prolactinomas.

Microprolactinomas are relatively common in humans with large adenomas occurring less frequently compared to rats (Frantz, 1984). These were initially recognized by pituitary enlargement (Forbes et al., 1954), and those without acromegalic symptoms were classified as "functionless" tumors of the pituitary. However, elevated serum prolactin levels were demonstrated initially with bioassay (Canfield and Bates, 1965) and later by radioimmunoassay (Frantz et al., 1972; Franks et al., 1977) in patients bearing "functionless" tumors. The hyperprolactinemia is responsible for symptoms of amenorrhea and galactorrhea in many of these patients. The two most often used therapies for these tumors are treatment with a dopaminergic agonist or surgery (Barbieri and Ryan, 1983). The observation that a majority of women with prolactin-secreting tumors developed their symptoms during or after oral contraceptive use suggests that exogenous estrogens may play

a role in the pathogenesis of these tumors in humans as well as in rats (Shearman and Fraser, 1977; Jaquet et al., 1978; Schlechte et al., 1980). However, other studies have found no correlation between the estrogen-containing oral contraceptives and the development of prolactinomas (Hulting et al., 1983; The Pituitary Adenoma Study Group, 1983). Therefore, this question remains. There is clear evidence that exogenous estrogens and the use of oral contraceptives elevates serum prolactin levels (Hagen et al., 1983). Interestingly, it appears that many hyperprolactinemic subjects have lost the central dopaminergic inhibition of prolactin secretion, as evidenced by an abnormal (attenuated) response pattern to central acting dopamninergic drugs. All of these same subjects can respond to a dopaminergic agonist acting at the level of the AP (Fine and Frohman, 1978; Muller et al., 1978; Sekiya et al., 1985).

2. Other neurotransmitter/neuropeptide systems affecting prolactin release

There is sparse information regarding other neurotransmitter function after chronic estradiol treatment. Chronic estradiol adminstration in vivo increased release of TRH from the hypothalaus incubated in vitro (Franks et al., 1984) and also decreased the content of VIP in the medial basal hypothalamus (Malletti et al., 1982). Estrogen given for three days decreased glutamic acid decarboxylase activity in the arcuate nucleus and anterior hypothalamic areas (Wallis and Luttge, 1980). Administration of serotonergic receptor agonists, zimelidine and quipazine, in combination with estradiol doubled AP weight compared to animals treated with estradiol alone. However, the

5-HT precursor, 5-HTP, did not increase, or the synthesis inhibitor PCPA did not decrease the estradiol-induced increase in AP weight (Walker and Cooper, 1985).

MATERIALS AND METHODS

I. Animals, Treatments and Blood Collections

Adult female Fischer 344 rats, an inbred strain, were used in all of the experiments. The animals weighed 160-180g each upon arrival and were housed in our animal facility for at least two weeks prior to experimentation. The rats were kept in temperature (25°C) and light (14 h light/10 h dark) controlled rooms. Ralston Purina Rat Chow (Ralston Purina Rat Chow (Ralston Purina Co., St. Louis MO) or Teklad Rat Chow (Harlan-Sprague-Dawley, Winfield IA) and tap water were provided ad libitum throughout the housing and experimental periods.

In each experiment, the rats were bilaterally ovariectomized prior to any treatment. At various periods after ovariectomy, animals were implanted with an empty or an estradiol-17-β (E_2)-containing Silastic capsule 10 mm in length (Dow Corning, Midland MI). The E_2 capsules were packed with 5-6 mg of crystalline E_2 (Sigma Chemical Co., St. Louis MO). The inner diameter of the capsules was 0.078" and the outer diamter was 0.125" and the ends were sealed with Silastic Medical Grade Elastomer using a stannous octoate catalyst (Dow Corning, Midland MI). Capsules were implanted subcutaneously on the dorsal side of the animal about 5 cm posterior from the skull. The length of E_2 administration and the duration of the withdrawal period after removal of E_2 are stated in the Materials and Methods section of each experiment. Drugs were administered by several different routes as stated in each Materials and

Methods section. Blood was collected by decapitation, by orbital sinus puncture under light ether anesthesia or chronically from a cannula in the right atrium. Cardia cannulae were made from Silastic tubing having an inside diameter of 0.025" and an outside diameter of 0.047". The length of the tubing from the silastic pad to the bevelled tip was 25-28 mm varying according to the size of the animal at the time of surgery. The saline-filled cannula was inserted into the right atrium of ether anesthetized animals via a small incision in the right external jugular vein (after the distal end of the jugular was tied off). The cannula was secured in place by suturing above and below the stabilization pad. The free end was passed underneath the skin and exited about 1 cm posterior to the base of the skull. The cannula were flushed with heparinized saline and tied. Rats were housed individually and allowed to recover for at least 2 days. On the day of the experiment, animals were moved to a room for the blood collection, and silastic tubing extentions with syringes were attached to the cannula. After a minimum two hour adaptation period, drug injections were given and samples were withdrawn into heparinized syringes from freely moving, non-disturbed animals. The samples were immediately centrifuged, plasma separated and stored at $-20^{o}C$ until assay. The erythrocytes were resuspended in equal volume amounts of sterile saline and reinjected into the animal after withdrawal of the next sample. Blood collected from animals by decapitation or orbital sinus puncture was allowed to clot overnight at $4^{o}C$ and serum was separated and frozen at $-20^{o}C$ until assayed.

II. Radioimmunoassay of Hormones

Serum and plasma prolactin, GH and LH were measured by radioimmunoassay (RIA). These assays were performed using RIA kits from the National Pituitary Agency of the NIADDK, except for rabbit anti-rat prolactin which was provided by Dr. C.L. Chen (University of Florida, Gainesville, FL). Bound hormone was separated from free hormone by IgSORB (Enzyme Center, Boston MA). Samples were assayed in duplicate and only those volumes which gave hormone values corresponding to the linear portion of the standard curve were used. Serum and plasma hormone concentrations were expressed in terms of NIADDK rPRL-RP-3, rGH-RP-1, and rLH-RP-1. The 50% bound dose and the minimum detectable dose for the prolactin, GH and LH RIAs were 0.9 ng, 0.8 ng, 5.9 ng and 0.09 ng, 0.05 ng, and 0.33 ng/tube, respectively. The coefficients of variation for each asay will be discussed in detail in Exp. IV of the "Experimental" section. In some of the experiments AP DNA content was measured according to Burton (1956).

III. Methods of Evaluating Tuberoinfundibular Dopaminergic Neuronal Activity

A. In Vitro Superfusion of Median Eminence Tissue After Accumulation of ^{3}H-Dopamine

After decapitation, the brain was quickly removed and placed on ice under the dissecting microscope. A few drops of ice cold Krebs-Henseleit buffer (see below) were placed on top of the hypothalamus, the median

eminence (ME) was visualized and dissected using a fine iris scissors according to the method of Cuello et al. (1973). The length and width of the ME tissue block were approximately 1.5 and 0.6 mm, respectively, and the ME weight was < 0.3 mg. It contained a small piece of pituitary stalk. MEs were kept on ice (< 5 min) until ready for accumulation. Four rats were used for each experiment (per day), two MEs for each experimental group. Tissues of ME were placed in 10 x 75 mm glass tubes (2/tube) containing Krebs-Henseleit buffer. The tissues were pre-incubated for 5 min at 37°C under constant oxygenation. The tissues were then transferred to one ml of buffer containing 0.36 μM ^{3}H-DA (Amersham, Chicago IL) and 0.1 μM desipramine (Sigma Chemical Co., St. Louis MO), and incubated for 20 min. The tissues were then rinsed with buffer and transferred to a superfusion apparatus similar to that described by Aceves and Cuello (1981), except that the inner chamber was smaller and had a narrow central tunnel 2 mm in diameter and length. The tubing used was polyethylene except in the pump where Silastic was used. The dead space in the tubing between the buffer reservoir and the chamber was 500 μl, and between the chamber and fraction collector was 100 μl. Superfusion was carried out by a peristaltic pump (STA, Buchler Instruments, Ft. Lee, NJ), at a rate of 300 μl/min. Superfusion medium was pre-warmed at 37°C under continuous oxygenation (95% O_2, 5% CO_2) and samples were collected at 2 min intervals using a Golden Retriever Fraction Collector (Instrument Specialty Co., Quincy MA).

Electrical stimulation was used to induce release of ^{3}H-DA from the median eminence tissue. Stimuli (bi-phasic square wave pulse for 15 sec, 6 mA magnitude 2 msec duration, at 20 hz) were applied by two silver electrodes and generated by two stimulators (Grass SD9, Grass

Instruments, Quincy MA), and were monitored on a calibrated oscilloscope (Type 564 storage oscilloscope, Tektronics Inc., Portland OR). At the end of the superfusion, MEs were homogenized in 0.5 ml of 0.1 N HCl. Radioactivity was determined in 400 μl aliquots of the superfusate samples and 250 μ l aliquots of the homogenized tissue sample. Tritium was counted using 10 ml of aqueous counting scintillant (Amersham Corp., Arlington Heights IL)) in a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Palo Alto CA).

The release of tritium was expressed as a fractional rate constant (FRC) per min which was calculated by dividing the amount released and double the amount of tritium content in the tissue at the start of the respective two minute period (Jaffe and Cuello, 1980). In all experiments, a 40 or 50 min period of spontaneous release was observed before release was steady. Electrical stimulation was applied for 15 sec either 1:30 or 1:45 sec after the beginning of a fraction. This allowed for stimulation-evoked release of tritium to be observed in two fractions after stimulation. Thus, the stimulation evoked release was calculated by subtracting the radioactivity in the two fractions before stimulation (baseline area) from the two fractions after stimulation (peak area). Under these conditions, the accumulation of ^{3}H-DA was temperature, time and Na^{+} dependent and reduced by unlabelled DA. The release of ^{3}H after electrical stimulation was magnitude and frequency dependent, almost completely blocked in Ca^{+} free media and partially blocked in the absence of Na^{+} (Sarkar et al., 1983). When TIDA neuronal "activity" or "function" are used in this text, it only refers to the ability of ME tissue to release radioactivity during electrecal stimulation after uptake of ^{3}H-DA. Since this method measures only

release from TIDA neuronal terminals, it's relationship to in vivo action potential spike frequency is not known.

1. Materials

[7,8-^{3}H]-DA (specific activity 41, 43 or 55 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Krebs-Henseleit medium contained gelatin (0.1%), ethylenediamine tetra acetic acid disodium salt (EDTA; 27 μM), ascorbic acid (130 μM), nialamide (Sigma Chemical Co., St. Louis MO; 12.5 μM), NaCl (134 mM), $CaCl_2$ (2 mM), KCl (5 mM), KH_2PO_4 (1.25 mM), $NaHCO_3$ (25 mM), $MgSO_4$ (1 mM) and glucose (10 mM), saturated with 5% CO_2, 95% O_2, pH 7.4. Medium was prepared fresh daily and the protocol for preparation is given in Appendix I.

B. Assessment of Prolactin Response After Administering Central-Acting Dopaminergic Drugs

The ability to evaluate TIDA neuronal function by neuropharmacological means is based on the observation that the "post-synaptic" biological action of TIDA neurons is a readily measurable event, i.e., the release of prolactin into the peripheral circulation. Consequently, assuming the lactotrophs have equal sensitivity to the dopaminergic inhibition of prolactin release, then central acting dopaminergic or anti-dopaminergic drugs should result in release (or inhibition of release) in direct proportion to the activity of TIDA neurons. For example, if TIDA neuronal activity is higher than normal, administration of morphine which inhibits TIDA neuronal activity results in a large release of prolactin into the blood. Conversely, if TIDA neuronal activity is low, administration of nomifensine, a drug which inhibits DA neuron reuptake, will cause only a small inhibition of prolactin

release.

The drugs used to evaluate TIDA neuronal activity by plasma prolactin responses were apomorphine hydrochloride (APO), a DA receptor agonist (Sigma Chemical Co., St. Louis MO), haloperidol (HALO), a DA receptor antagonist (McNeil Laboratories, Ft. Washington, PA), morphine sulfate (MOR), a drug which inhibits central TIDA neuron turnover (Mallenkrodt Labs, St. Louis MO), and nomifensine maleate (NOM), a catecholaminergic reuptake inhibitor (Hoechst-Roussel, Somerville NJ).

IV. Catecholamine Assay

The neuronal tissue in which catecholamines were measured was the neurointermediate lobe. Neurointermediate lobes were dissected and immediately sonicated in 250 µl of 0.1 N perchloric acid containing 5 mM glulathione. The homogenate was centrifuged and the supernatant stored frozen at -40°C until assay. The catecholamine assay was a modification of the radioenzymatic method of Cheng and Wooten (1980). Briefly, 50 µl samples were incubated with buffered COMT and ^{3}H-S-adenosyl methionine, a methyl donor (ICN Radiochemicals, Irvine CA) in a total volume of 100 µl. Both internal and external standards (2000-31.25 pg) were included in the assay. The DA and NE metabolites, methoxytryptamine and normetanephrine, respectively, were partially purified and concentrated by solvent extraction and thin layer chromatography. Amine content was determined by counting the chromatographic spots containing the ^{3}H-labelled metabolites in the liquid scintillation counter. The complete assay protocol including procedure for partial purification of

liver COMT can be found in Appendix II. Neurointermediate lobe DA and norepinephrine content were expressed as ng per mg protein as measured by the method of Bradford (1976) using a Bio-Rad kit (Richmond CA).

V. Statistics

The data from the in vitro superfusion was evaluated with the non-parametric Mann-Whitney U analysis. Much of the data revealed a significant F_{max} test for heterogeneity of variance and therefore were transformed logarithmically before parametric tests were performed. The remaining data were analyzed by analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test. In all cases, a $p \leq 0.05$ was considered significant (Steel and Torrie, 1980).

EXPERIMENTAL

I. Tuberoinfundibular Dopaminergic Neuronal Function: Effect of Chronic Estradiol Administration and Persistent Hyperprolactinemia After Removal of Chronic Estradiol

A. Objectives

Long-term estrogen administration in rats produces chronic hyperprolactinemia and development of prolactin-secreting adenomas. However, the effects of chronic estrogen treatment on hypothalamic dopaminergic control of prolactin release still remains unclear. There is evidence that long-term estrogen treatment can result in (a) cytopathological changes in the arcuate nucleus, including an increase in number of reactive glial cells and the appearance of axonal and dendritic degeneration (Brawer and Sonnenschein, 1976), (b) reduced neuronal catecholamine fluorescence in the ME and arcuate nucleus (Sarkar et al., 1982), (c) depletion of DA content from the ME and reduction of DA concentration in hypophysial portal blood (Casanueva et al., 1982; Sarkar et al., 1984), and (d) decreased turnover of DA and an attenuated ability to release ^{3}H-DA from ME tissue *in vitro* (Sarkar et al., 1984; Demarest et al., 1984).

The decline in TIDA activity during chronic estrogen treatment may result from loss of dopaminergic neurons of the arcuate nucleus (Sarkar et al., 1982) or loss in sensitivity of TIDA neurons to the increased levels of circulating prolactin (Demarest et al., 1984). The former suggests that a permanent decline in TIDA function may occur, and

persist even after removal of the estrogen treatment, whereas the latter suggests the possibility that TIDA function may be restored after estrogen removal.

To clarify this problem, the present study was undertaken to assess TIDA neuronal activity at the end of 4 weeks of estrogen treatment, and 4 weeks after removal of chronic estrogen treatment. TIDA neuronal activity was evaluated by two methods (a) in vitro superfusion and electrical stimulation of ME tissue after allowing for accumulation of ^{3}H-DA, and (b) testing the effectiveness of drugs that inhibit or stimulate prolactin release through dopaminergic or anti-dopaminergic mechanisms.

B. Materials and Methods

Animals were OVX prior to treatment and randomly divided into three groups: (a) OVX controls received (sc) an empty Silastic capsule 10 mm in length, for 4 weeks after which the capsule was removed for 4 weeks, (b) rats OVX for 4 weeks and then implanted with a Silastic capsule containing E_2 for 4 weeks, and (c) OVX rats implanted with an E_2 capsule for 4 weeks, followed by removal of the E_2 capsule for 4 weeks.

TIDA activity was estimated by determining the accumulation and release of ^{3}H from ME tissue after allowing for accumulation of ^{3}H-DA into the tissue as previously described in the Methods section. All experiments were performed beginning at 1100 hr. The rats were decapitated, trunk blood was collected for RIA of LH and prolactin, and ME tissue was dissected for superfusion. After a 40 min washout period, the tissue was electrically stimulated using field stimulation. The stimulation evoked release of ^{3}H was used as an index of TIDA activity.

Total uptake was expressed as cpm per μg ME protein as measured by the Bio-Rad assay.

The capacity of pharmacological agents which act either on TIDA neurons or on dopaminergic receptors on the AP to stimulate or inhibit prolactin release was assessed. The following three drugs were injected: apomorphine hydrochloride (APO) a DA receptor agonist; haloperidol (HALO), a DA receptor antagonist; and morphine sulfate (MOR), which inhibits central TIDA turnover (Deyo et al., 1979; Gudelsky and Porter, 1979).

Different animals were used for each of three experiments. The rats were implanted with an intra-atrial Silastic cannula under ether anesthesia as previously described. Two days later, the animals were adapted to the experimental room for two hours starting at 0800 hr. Blood samples were withdrawn 40 and 20 min prior to drug administration. After sc injection of APO (0.25 mg/kg, in saline) or iv injection of MOR (5 mg/kg, in saline), samples were withdrawn 15, 30, 60, and 90 min later. After sc administration of HALO (0.5 mg/kg, in 0.3% tartaric acid), blood samples were withdrawn 45, 90, 135, and 180 min later. Dosages were chosen which produce maximal effects on prolactin release in control male rats (Mueller et al., 1976). Serum and plasma prolactin, LH, and GH were measured by RIA. The DNA assay was performed by the method of Burton (1956).

Absolute values of the <u>in vivo</u> data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls' multiple comparison test. Since basal serum prolactin levels among groups were different, data were also expressed as ng prolactin/ml plasma/estimated DNA of prolactin-cells (μg) ratio. The Wilcoxson-Mann-Whitney analysis tested differences among

groups when data were expressed as a ratio. For the RIA data of Table 2, both the prolactin and LH results revealed a significant F_{max} test for heterogeneity of variance. Therefore, data were transformed logarithmically before performing the ANOVA and Student-Newman-Keuls multiple comparison test. The in vitro ME superfusion results were analyzed by the Wilcoxson-Mann-Whitney test.

C. Results

Figure 1 shows that at the end of 4 weeks of E_2 treatment (E) and after 4 weeks of withdrawal from E_2 (WD) there was a suppression of stimulation-evoked release of ^{3}H-DA from the ME, as compared to values in OVX controls (C). There was a trend for reduced ^{3}H-DA accumulation after 4 weeks of E_2 and after the 4 week withdrawal period which was not statistically significant.

The absolute values of plasma prolactin following administration of APO, HALO and MOR to the three treatment groups are shown in Table 1. APO, a DA receptor agonist, produced a significant decline in plasma prolactin values 30 and 90 minutes after injection in OVX controls, and a significant reduction in plasma prolactin levels at all time points after injection into rats treated with E_2 for 4 weeks and in rats after E_2 removal for 4 weeks. Injection of HALO, a DA receptor antagonist, produced an increase in circulating levels of prolactin in all groups. After administration of MOR, a drug which decreases TIDA activity, plasma prolactin was increased at 15 and 30 minutes but returned to baseline levels by 60 minutes in OVX control rats. Plasma prolactin increased only 30 minutes after MOR injection in animals at the end of 4 weeks of E_2 treatment. There was a significant increase in prolactin at

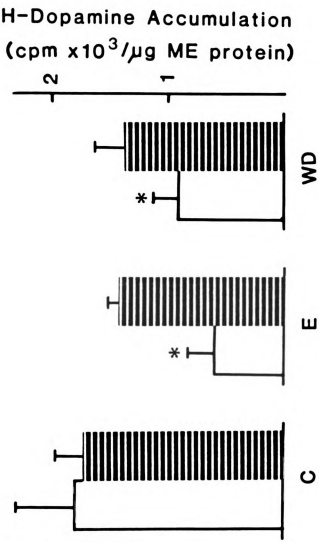

FIGURE 1.

Stimulation-evoked release of ^{3}H during electrical stimulation of median eminence tissue (open bars) and accumulation of ^{3}H-DA into median eminence (striped bars) in OVX control F344 rats (C), in rats treated with E_2 for 4 weeks (E), and in rats 4 weeks after withdrawal of chronic E_2 treatment (WD). Data are expressed as X + S.E.M. of 6 experiments (two median eminences/experiment) * = $p \leq 0.05$ compared to C.

Table 1. Effect of acute APO, HALO or MOR injection on plasma PRL levels (ng/ml) in F344 control rats, in rats treated with E_2 for 4 weeks and in rats 4 weeks after withdrawal of chronic E_2 treatment. Also shown for each group is total AP DNA content (µg) and estimated DNA (µg) of PRL-secreting cells.

	Group/Min	-40[a]	-20	+15	+30	+60	+90	TOT DNA (µg)	PRL-cell DNA (µg)
	(6) C	7.7±2.0[b]	7.4±1.0	6.8±1.8	2.7±0.4*	3.6±0.6	3.0±0.8*	104±6	31±2
APO	(9) E	1960±159	2247±197	565±155*	237±32*	168±13*	188±22*	531±44	318±26
	(10) WD	325±50	354±61	61±9*	30±4*	21±3*	23±3*	411±15	170±6
		-40	-20	+45	+90	+135	+180		
	(9) C	13.7±5.2	13.1±5.1	115.9±17.9*	105.8±13.8*	101.4±15.7*	81.8±12.0*	118±4	35±1
HALO	(9) E	847±73	1132±113	1847±135*	2042±139*	1959±140*	1667±140*	570±38	341±22
	(10) WD	271±52	257±37	652±74*	697±78*	635±84*	521±81	371±26	153±11
		-40	-20	+15	+30	+60	+90		
	(6) C	8.9±2.3	15.1±2.6	162.5±19.1*	98.2±16.2*	32.7±15.0	13.6±2.5	102±6	30±2
MOR	(8) E	1503±152	1581±211	2539±399	2741±529*	2584±395	2413±361	562±46	336±28
	(9) WD	207±22	321±35	547±87*	694±129*	659±78*	581±56*	358±23	148±10

[a] - signifies time before or time following drug injection
[b] - X ± S.E.M.; in OVX control rats (C), at the end of E_2 treatment (E) or after E_2 withdrawal (WD)
* - $p < 0.05$ significantly different from -20 min pre-injection sample

all time points after MOR administration in rats after E_2 removal for 4 weeks.

The prolactin response to pharmacological agents is also presented as the difference between the pre-injection and post-injection level of prolactin divided by an estimate of the portion of total AP DNA content which constitutes only the prolactin-secreting cells (Figure 2). Assuming blood volume and clearance of the hormone are similar among treatment groups, this ratio is an estimate which is proportional to the change in prolactin release per lactotroph in response to the drugs. This allows for evaluation of the prolactin response to the drug without the bias of differing basal plasma prolactin levels or the different number of lactotrophs among the treatment groups. The DNA attributed to the lactotrophs was calculated by first measuring total AP DNA content. For OVX F344 control and E_2-treated rats, Phelps and Hymer (1983) counted the number of immunocytochemically identifiable lactotrophs after separation of AP cells from red blood cells on a Ficoll-Hypaque gradient. When these authors used an E_2-treatment similar to ours, they observed AP enlargements comparable to those reported here and 59.8% of the AP cells were identified as lactotrophs. Only 29.8% lactotrophs were identified in OVX controls.

In our experiment, the rats showed a 31% decline in mean AP DNA content (539 µg to 375 µg; see Table 2) 4 weeks after the E_2-containing capsule was removed. Assuming that this decline was exclusively due to a loss of lactotrophs, the % loss of lactotrophs in rats after E_2 withdrawal for 4 weeks would be 0.598 x 0.310 = 0.185 or an 18.5% loss of lactotrophs (thus, the % of lactotrophs was 0.598 - 0.185 = 0.413 or 41.3%). The estimate used for the portion of AP DNA content due to the

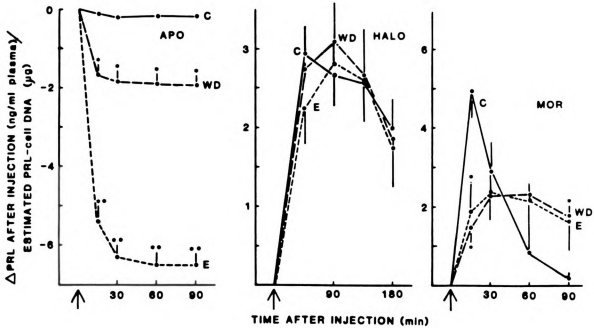

FIGURE 2.

Effects of acute administration of apomorphine (APO,left), haloperidol (HALO,center) and morphine (MOR,right) on stimulation or inhibition of prolactin (PRL) release in F344 control rats (C), in rats treated with E_2 for 4 weeks (E), and in rats 4 weeks after withdrawal of chronic E_2 treatment (WD). Data ($\bar{X} \pm$ S.E.M. of 6-10 animals/group) are expressed as the change in plasma prolactin after drug injection (ng/ml) divided by the estimated portion of total AP DNA content which constitutes PRL-secreting cells (μg). Arrow indicates the time of drug injection. * = $p \leq 0.05$ compared to C; • = $p \leq 0.05$ compared to WD.

lactotrophs was 29.8% for OVX controls, 59.8% for E_2-treated rats and 41.3% for rats withdrawn from E_2 for 4 weeks. The total DNA content and the estimated portion due to lactotrophs is shown in Table 1.

The inhibition of prolactin per μg prolactin-cell DNA after acute APO administration (Figure 2) was significantly greater in rats at the end of E_2 treatment (E) than in OVX controls (C) or in rats 4 weeks after E_2 removal (WD). Animals after removal of E_2 (WD) showed a greater prolactin inhibition per μg prolactin-cell DNA after APO than OVX controls. Blockade of DA receptors by administration of HALO resulted in similar increases in plasma prolactin in all three groups when the data were expressed per μg prolatin-cell DNA. Injection of MOR resulted in a rapid increase and then decline in prolactin release in OVX control rats (C). The prolactin rise in response to MOR was lower but more prolonged in E_2-treated rats (E) and in rats after withdrawal of E_2 (WD).

Since E_2-treated animals and animals after E_2 withdrawal exhibit a greatly enlarged AP, there is the possibility that the enlarged gland may impinge on blood flow between the median eminence and the AP. This could result in a smaller PRL response in the E_2-treated animals because of the limited blood flow in the portal vasculature, and reduce the passage of DA to the AP. To evaluate this possibility, the plasma GH response to acute MOR injection was also measured in the three treatment groups. Injection of 5 mg/kg MOR produced a significant increase in plasma GH (Figure 3) in animals after E_2 removal for 4 weeks, but MOR did not induce significant release of GH in control rats or in rats at the end of 4 weeks of E_2 treatment.

Table 2 shows that 4 weeks of E_2 treatment produced greater than a 6-fold increase in AP weight and a 65-fold rise in serum prolactin as

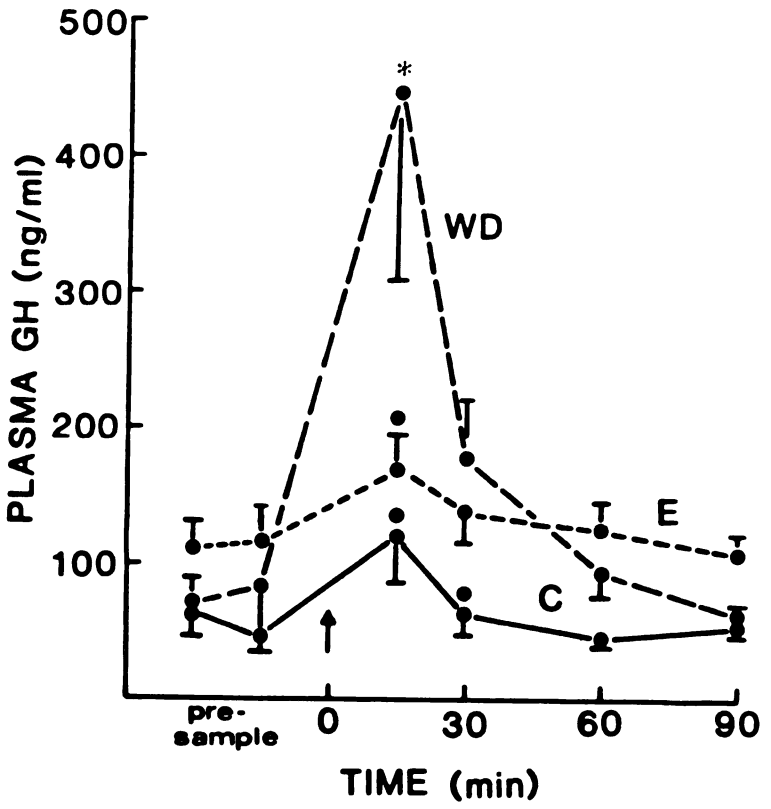

FIGURE 3.

Effect of morphine (MOR) on the concentration of plasma GH in OVX (C), E_2-treated (E) rats, and rats 4 weeks after withdrawal of chronic E_2 treatment (WD). Arrow indicates the time of drug injection. ($\bar{X} \pm$ S.E.M. of 7-10 animals/group) * = $p \leq 0.05$ compared to pre-injection GH level, • = $p \leq 0.05$ compared to WD.

Table 2. Effects of E_2-treatment and E_2-withdrawal on anterior pituitary (AP) weight and DNA content, and serum PRL and LH levels.

	AP weight (mg)	AP DNA (μg)	AP DNA (μg/mg)	serum PRL (ng/ml)	serum LH (ng/ml)
1) OVX controls[a]	10.0±0.3	112±4	11.3±0.4	26±4	535±77
2) E_2-treated	64.5±4.7*	539±37*	8.1±0.3*	1695±208*	29±4*
3) E_2-withdrawal	30.0±1.7*	375±24*	12.3±0.4	247±26*	161±22*

[a] X ± S.E.M. of 11-12 animals/group; Group 1 was OVX for 8 weeks, Group 2 received E_2 for 4 weeks, Group 3 received E_2 for 4 weeks after which E_2 was withdrawn for 4 weeks

* $p \leq 0.05$ compared to Group 1

compared to non-E_2-treated control rats. Both AP weight and serum prolactin declined by the end of 4 weeks of E_2 withdrawal (Group 3), but remained elevated when compared with OVX controls. AP DNA content increased from 112 μg in OVX controls to 539 μg after 4 weeks of E_2 treatment, and remained elevated (3x) above control values 4 weeks after E_2 withdrawal. When DNA content was expressed per mg AP tissue weight, E_2-treated rats showed reduced DNA content when compared with control values. Four weeks of E_2 treatment significantly suppressed the LH response to OVX. This suppression of LH release persisted 4 weeks after removal of the E_2.

D. Discussion

The results presented here confirm previous reports that chronic E_2 treatment depresses TIDA neuronal activity (Casanueva et al., 1982; Sarkar et al., 1984; Demarest et al., 1984), and indicate that after removal of long-term E_2 treatment in F344 OVX rats, TIDA activity remains depressed. Attenuated TIDA neuronal activity is clearly indicated by the observation that at the end of E_2 treatment or even 4 weeks after E_2 removal, the electrically-stimulated release of ^{3}H-DA from ME tissue was decreased when compared to rats not treated with E_2.

The plasma prolactin response to APO, HALO and MOR was expressed as the ratio of the change in plasma prolatin after injection of the drug divided by an estimated portion of total AP DNA content which is contained only in the lactotrophs. In both E_2-treated groups, the estimates of prolactin-cell DNA are presumed to be low. E_2-treatment results in low prolactin content in the lactotrophs (per cell) and therefore it is probable that a portion of the lactotrophs were not

immunocytochemically identified as such. These possibly low estimates of prolactin-cell DNA are conservative in the sense that, after drug injection, they allow for greater prolactin responses in E_2-treated rats and rats after withdrawal from E_2. Decreased TIDA activity after E_2-treatment and after E_2-withdrawal is supported by the pharmacological evidence that acute administration of MOR to E_2-treated rats resulted in a slower and attenuated increase in plasma prolactin when compared with OVX control rats. Chronic E_2 has been shown to elevate hypothalamic opiate binding sites (Wilkinson et al., 1985) which would be expected to permit a larger increase in prolactin in E_2-treated animals after MOR. However, E_2-treated rats showed a smaller rise in prolactin compared to controls which further suggests a depressed TIDA function. Depression of TIDA neurons (e.g., by MOR) which have a low basal activity, i.e., E_2-treated rats and rats after E_2 withdrawal, increased prolactin to a lesser degree than rats with a higher TIDA activity, i.e., controls. After HALO injection, all groups showed similar increases in circulating prolactin levels, suggesting that a maximal blockade of DA action on the lactotrophs permitted a similar release of prolactin/ g prolactin-cell DNA in all three treatment groups. APO produced a greater inhibition of prolactin release in both E_2-treated groups as compared to controls, when the data are expressed as per lactotroph.

There are potential problems in the evaluation of TIDA activity <u>in vivo</u> using lactotrophic responses to DA agonists and antagonists, especially in animals with enlarged AP's. A restricted blood flow in the portal vasculature or genesis of systemic arteries to the pituitary gland (Elisa and Weiner, 1984) as a result of AP growth, could decrease the concentration of DA reaching the AP lactotrophs. This should

effectively reduce all the releasing/release-inhibiting factor levels from the hypothalamus that regulate AP function. The combination of decreased release of DA from TIDA neurons, together with the possible limited reduction in portal blood flow and development of new vascularization to the AP, could greatly decrease the inhibitory hypothalamic influence on prolactin secretion. Thus, the reduced prolactin release in E_2-treated animals after injection of MOR could be partially the result of less DA reaching the AP because of these physical factors and not due to diminished TIDA activity. To evaluate this possibility, the ability of MOR to increase GH was measured in controls, in E_2-treated rats and in rats after E_2 withdrawal. MOR is known to increase GH by a central action on neurotransmitters, growth hormone releasing factor and somatostatin (Sonntag et al., 1983). In the present study, MOR increased plasma GH to levels similar in control and E_2-treated animals; however a 4-fold greater increase in GH was observed in animals 4 weeks after withdrawal of E_2-treatment. If reduced portal blood flow and dilution of portal blood DA due to new vascularization were effective in reducing hypothalamic influence on the AP, the time course for the MOR-induced GH release among treatment groups should be similar to the time course of prolactin release. However, the time course for GH release was very different from prolactin, suggesting that the central nervous system mechanisms responsible for MOR-induced GH release were not significantly affected by altered portal blood flow. This interpretation should be viewed with caution, since the influence of E_2 on the hypothalamic and AP mechanisms effecting GH release is not well understood at present.

In summary, it is clear that TIDA neuronal function, as evaluated

directly by *in vitro* superfusion release of ^{3}H-DA and indirectly by the plasma prolactin response to MOR, a drug which inhibits TIDA neuronal activity, is diminished in E_2-treated rats and also in rats 4 weeks after removal of the chronic E_2 treatment.

II. Evidence for a Permanent Decline in Tuberoinfundibular Dopaminergic Neuronal Function After Chronic Estrogen Treatment

A. Objectives

It has been suggested that long-term estrogen exposure can cause damage to TIDA neurons in female rats (Sarkar et al., 1982; Sarkar et al., 1984). If estrogen does result in permanent TIDA neuronal damage, the depressive effects on TIDA neuronal function in the presence of elevated estrogen levels should remain long after removal of the estrogen. The objective of this study, therefore, was to assess TIDA neuronal activity long after removal of chronic E_2 treatment. TIDA neuronal activity was evaluated by *in vitro* superfusion of median eminence tissue and by testing the ability of TIDA neurons to alter prolactin secretion in response to pharmacological manipulation, 26 weeks after removal of chronic estrogen treatment.

B. Materials and Methods

Animals were ovariectomized immediately prior to implanting (sc) an empty or E_2 containing Silastic capsule The capsules were removed after 4 weeks of treatment. Experiments were performed 26 weeks after removal of the capsules. In other experiments, animals were implanted with a capsule for 4 weeks, the capsule was then removed, and 26 weeks later the rats received E_2 for 3 days. The capacity of the following four drugs to alter prolactin release was assessed in control and E_2-treated groups and used to evaluate TIDA neuron activity: 1) apomorphine

hydrochloride (APO), a DA receptor agonist; 2) morphine sulfate (MOR), which inhibits central TIDA turnover; 3) haloperidol (HALO), a DA receptor antagonist; and 4) nomifensine maleate (NOM), which blocks the uptake of DA into catecholaminergic neuronal terminals. Different groups of animals were used for each experiment. Blood samples were withdrawn by the retro-orbital technique under light ether anesthesia. After taking a pre-sample 30 min before drug injection, two blood samples were withdrawn at various times following drug adminstration via the following routes and dosages: APO, 0.25 and 0.01 mg/kg sc; HALO, 0.5 mg/kg, sc; MOR, 5 mg/kg, ip; NOM, 10 mg/kg, ip.

All superfusion experiments were performed at 1100 hr. Animals were decapitated and blood collected for prolactin and LH RIA. The AP was removed, weighed, and homogenized in 2 ml 0.01 M phosphate buffered saline for later DNA assay. The ME superfusion has been described previously in full Methods section. One estimation of TIDA neuronal activity was the ability of the neurons to increase the radioactivity released in response to electrical stimulation termed the stimulation-evoked release. Total accumulation of ^{3}H-DA was also used as an index of TIDA function and was expressed as cpm per μg ME protein as measured by the Bio-Rad assay. Statistical analysis was performed as in Experiment I.

C. Results

The ability of the dopaminergic agonist APO to inhibit prolactin secretion in OVX controls and in rats long after E_2 withdrawal is shown in Table 3. Injection of 0.25 mg/kg APO reduced serum prolactin in both groups for at least 90 min. However, after 0.01 mg/kg APO, prolactin

Table 3. Ability of APO to inhibit PRL secretion in OVX controls and 26 weeks after withdrawal from chronic E_2 treatment.

	OVX controls[a] (n = 6) ng PRL/ml serum		
	Pre-sample	+ 30 min	+ 90 min
APO (0.25 mg/kg)	40.8 ± 8.3[b]	5.0 ± 1.7*	13.2 ± 2.1*
APO (0.01 mg/kg)	38.2 ± 9.3	25.8 ± 9.7	34.4 ± 9.7
AP DNA (µg)	115 ± 6	estimated PRL-cell DNA (µg)	34 ± 2

	26 weeks after removal E_2 (n = 4) ng PRL/ml serum		
	Pre-sample	+ 30 min	+ 90 min
APO (0.25 mg/kg)	97.4 ± 25.4	6.8 ± 1.2*	8.7 ± 1.7*
APO (0.01 mg/kg)	103.4 ± 19.3	41.7 ± 3.8*	108.6 ± 17.9
AP DNA (µg)	312 ± 42	estimated PRL-cell DNA (µg)	111 ± 15

[a] animals were OVX and received either an empty or E^2-containing silastic capsule for 4 weeks; 26 weeks later the capsules were removed and experiments were performed. APO was injected sc and blood samples were taken at times indicated (min)

[b] X ± S.E.M.

* $p \leq 0.05$ compared to pre-injection PRL level

release was significantly suppressed only in the animals after E_2 withdrawal and not in the controls. Table 4 shows the prolactin response after MOR, HALO or NOM administration. Injection of MOR significantly elevated serum prolactin in OVX control rats but not in animals after E_2 withdrawal. HALO, a dopaminergic receptor antagonist produced a significant elevation in serum prolactin in both treatment groups. Injection of NOM, a drug which blocks the re-uptake of DA in catecholaminergic neuronal terminals, significantly reduced serum prolactin in OVX controls but not in rats after E_2-withdrawal.

In an attempt to compensate for the differing basal prolactin levels, the serum prolactin response to the above pharmacological agents is presented in Figures 4 and 5 as the change in serum prolactin concentration after injection of the drug, divided by an estimate of the prolactin-cell DNA content. Since basal prolactin levels are different between OVX control rats and rats after E_2-withdrawal, it is difficult to evaluate changes in serum prolactin when the drugs are acting on different numbers of lactotrophs present. Therefore, an estimate of prolactin-cell DNA was made, and the change in serum prolactin was expressed as a ratio of this prolactin-cell DNA level. The AP's of OVX F344 control animals were previously reported to have 29.8% lactotrophs and F344 OVX rats treated with E_2 for similar periods were reported to have 59.8% lactotrophs (Phelps and Hymer, 1983). In our experiment, rats 26 weeks after removal of E_2 showed a 40.2% decline in total AP DNA content compared to animals at the end of the 4 week E_2 treatment (Gottschall, et al., 1986). If this decline is due exclusively to a loss of lactotrophs, then the % loss of lactotrophs would be 0.598 x 0.402 = 0.241 or a 24% loss. Therefore, the % lactotrophs remaining in the AP

Table 4. Effect of MOR, HALO and NOM on serum PRL (ng/ml) in OVX controls and 26 weeks after withdrawal from chronic E_2 treatment

	OVX controls[a] ng PRL/ml serum			26 weeks after removal of E_2 ng PRL/ml serum		
	Pre	+ 0.5	+ 1.5	Pre	+0.5	+1.5
MOR (5 mg/kg)	25.2 ± 9.5	64.3 ± 9.6*	32.5 ± 6.8	83.9 ± 15.3	139.9 ± 22.8	123.4 ± 21.4
	µg DNA 112 ± 15	estimated PRL-cell DNA 33 ± 5		µg DNA 319 ± 30	estimated PRL-cell DNA 114 ± 11	
	Pre	+ 1	+ 18	Pre	+ 1	+ 18
HALO (0.5 mg/kg)	9.0 ± 2.5	65.7 ± 8.1*	39.1 ± 14.4	77.8 ± 20.2	198.5 ± 34.6*	150.7 ± 29.8
	µg DNA 110 ± 15	estimated PRL-cell DNA 33 ± 4		µg DNA 338 ± 20	estimated PRL-cell DNA 120 ± 7	
	Pre	+ 1	+ 3	Pre	+ 1	+ 3
NOM	32.5 ± 9.6	6.7 ± 3.5*	9.9 ± 5.3	149.8 ± 24.9	91.4 ± 22.1	84.6 ± 16.2
	µg DNA 113 ± 10	estimated PRL-cell DNA 34 ± 3		µg DNA 318 ± 27	estimated PRL-cell DNA 114 + 10	

[a] see Table 3 for details of treatment, except that blood sampling times following drug injection are in (h); (X ± S.E.M.; n = 5-12

* $p \leq 0.05$ compared to pre-injection PRL levels

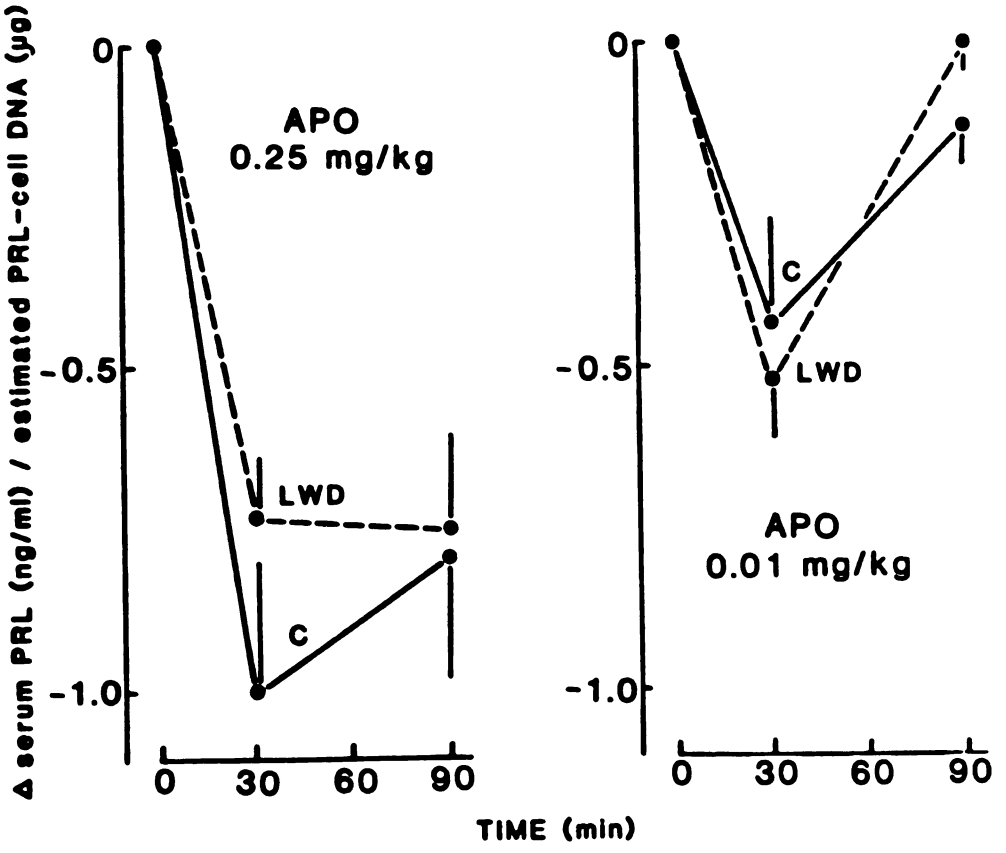

FIGURE 4.

The ability of apomorphine (APO; 0.25 left and 0.01 mg/kg right) to inhibit prolactin (PRL) secretion in F344 OVX controls (C) and after long-withdrawal (26 weeks) from chronic E_2 treatment (LWD). The data are expressed as the change in serum prolactin after APO injection (ng/ml) divided by an estimate of the portion of AP DNA content which constitutes PRL-secreting cells (μg). Zero time is the time of APO injection. $p > 0.05$ at each time point when C is compared to LWD. There were no significant differences in serum prolactin between treatment groups at either dose of APO.

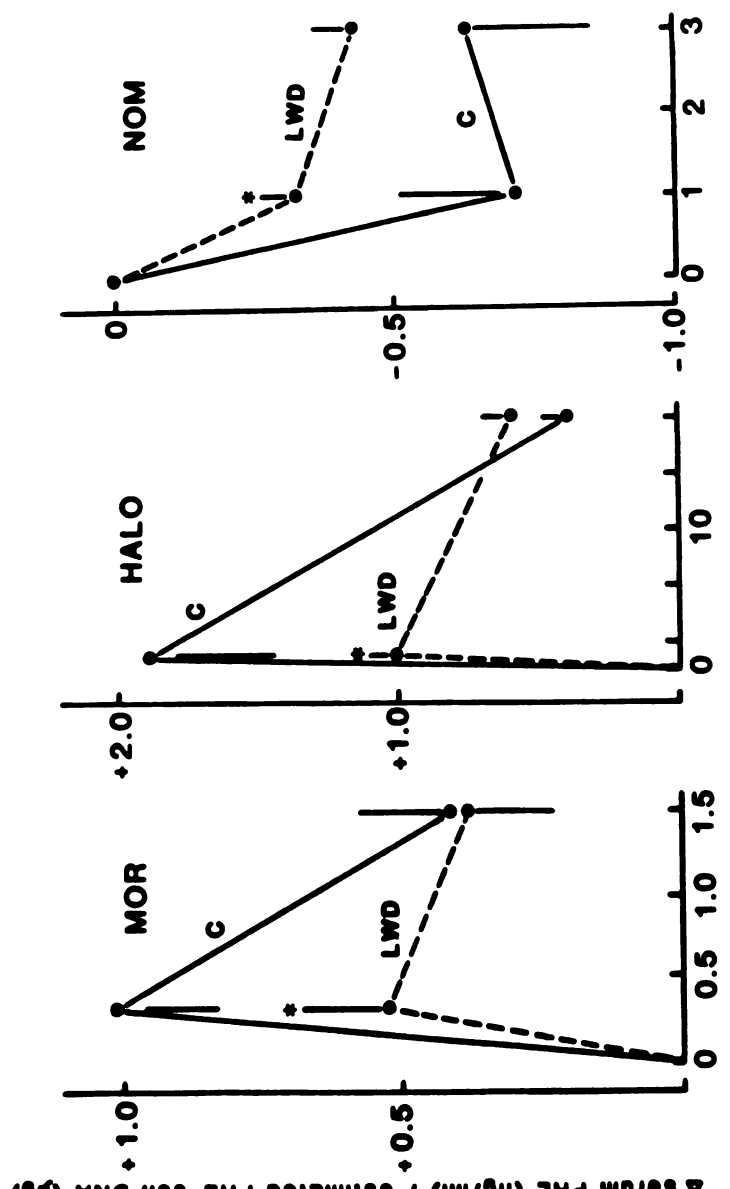

FIGURE 5.

Effect of morphine (MOR;left), haloperidol (HALO;center) or nomifensine (NOM;right) on prolactin secretion in F344 OVX controls (C) and after long-withdrawal (26 weeks) from chronic E_2 treatment (LWD). The data are expressed as the change in serum prolactin after drug injection (ng/ml) divided by an estimate of the portion of AP DNA content which constitutes PRL-secreting cells (μg). Zero time is the time of drug injection. * = $p \leq 0.05$ at each time point when C is compared to LWD.

would be 0.598 - 0.241, or 35.7%. The total DNA content and estimated lactotroph DNA content for each group are presented in Tables 3 and 4.

Figure 4 shows the serum prolactin response, expressed as estimated lactotroph DNA to two different doses of APO in OVX and E_2-withdrawn F344 rats. There were no significant differences between the two groups at any time point or at either dose. However, the prolactin response/estimated lactotroph DNA after MOR, HALO or NOM administration (Figure 5) was consistently smaller in rats previously treated with E_2 than in OVX control rats.

Long after (26 weeks) withdrawal of 4 weeks of E_2 treatment, AP weight and DNA content were still elevated over OVX control levels (Table 5). E_2-treated animals showed persistent hyperprolactinemia (about 4 fold over controls) but serum LH levels were not different from controls. As a result of the 3-day E_2 challenge, AP weight significantly increased in rats after E_2 withdrawal (18.8 mg to 30.8 mg) but not in controls even though both groups had similar prolactin levels after the 3-day E_2 treatment. The E_2 challenge suppressed LH levels to a lesser degree in rats treated with E_2 26 weeks earlier with E_2 than in OVX control animals.

In assessing TIDA neuronal activity 26 weeks after withdrawal from 4 weeks of E_2 treatment (LWD) <u>in vitro</u> using the superfusion method, stimulation-evoked release and total accumulation of ^{3}H-DA was not different from OVX controls (C; Figure 6). The hyperprolactinemia invoked by the 3-day E_2 challenge was reflected by a 2-fold increased stimulation-evoked release in OVX control rats (C + E_2). As a result of the 3 days of E_2 treatment, there was also an increase in serum prolactin in animals previously give E_2 (LWD + E_2). However, the

Table 5. AP weight, AP DNA content and serum PRL and LH in OVX control F344 rats, in rats 26 weeks after E_2 withdrawal, and in both groups after a 3-day E_2 challenge.

	AP weight (mg)	AP DNA (μg)	Serum PRL	Serum LH
OVX control[a] (C)	10.7 ± 0.3	113 ± 10	14 ± 3	669 ± 114
E_2 withdrawal (LWD)	18.8 ± 1.4*	322 ± 29*	57 ± 11*	567 ± 81
OVX control + E_2 challenge (C + E_2)	12.3 ± 0.8	108 ± 8	304 ± 119**	47 ± 12**
E_2 withdrawal + E_2 challenge (LWD + E_2)	30.8 ± 1.6*'**	376 ± 32*	340 ± 43**	176 ± 30*'**

[a]OVX controls were OVX for 30 weeks (C); E_2 withdrawal received E_2 for 4 weeks after which E_2 was removed and animals decapitated 26 weeks later (LWD); OVX control + E_2 challenge were OVX for 30 weeks and then received an E_2 casule for 3 days (C + E_2), E_2 withdrawal + E_2 challenge received E_2 for 4 weeks, withdrawn for 26 weeks and then received an E_2 capsule for 3 days (LWD + E_2)

* = $p \leq 0.05$ compared to OVX control or OVX control + E_2 challenge

** = $p \leq 0.05$ compared to respective group before E_2 challenge.

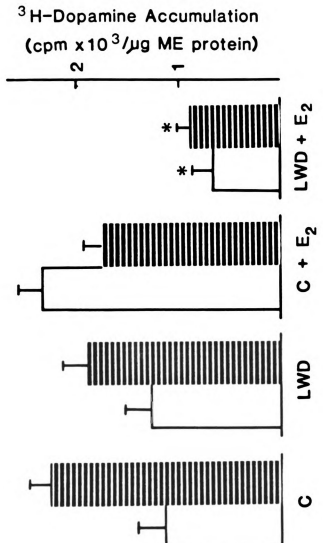

FIGURE 6.

Stimulation-evoked release of ^{3}H during electrical stimulation of median eminence tissue (open bars) and accumulation of ^{3}H-DA into median eminence (striped bars) in OVX control rats (C), in rats 26 weeks after removal of E_2 (LWD) and E_2 challenge in control (C + E_2) and E_2-withdrawn (LWD + E_2) rats. See Table 5 for details of treatment. * = $p < 0.05$ compared to control group that did not recieve chronic E_2.

hyperprolactinemia in these animals did not alter stimulation-evoked release and actually decreased ^{3}H-DA accumulation (Figure 6).

D. Discussion

The present study demonstrates that permanent alterations occur in TIDA neuronal function after E_2 treatment for 4 weeks in F344 rats. This is supported by the failure of TIDA neurons in E_2-treated F344 rats to respond similarly to non-E_2 treated animals to most stimuli used in this study. Even though there was no significant difference in the stimulation evoked release of ^{3}H-DA from ME tissue *in vitro* between control rats and rats 26 weeks after E_2 withdrawal, there was a 4-fold difference in serum prolactin levels between these groups. It is well established that serum prolactin levels are important in regulating TIDA activity via a short-loop feedback mechanism (Moore and Demarest, 1982). Since animals given 4 weeks of E_2 treatment still had a 4-fold greater serum prolactin concentration 26 weeks later than rats not given E_2, it might be expected that the hyperprolactinemic animals would show increased TIDA activity. However, there was no measurable difference in TIDA activity in the E_2-treated and control groups, suggesting that in the E_2-treated animals, there was a failure of the TIDA neurons to respond to the increased serum prolactin levels.

The prolactin responses to the dopaminergic and anti-dopaminergic drugs used in the present study also demonstrate that TIDA activity was decreased after E_2 removal. APO, a dopaminergic agonist, when given at a low dose (0.01 mg/kg) did not produce a significant decrease in serum prolactin in control rats, but decreased prolactin levels in rats after E_2 withdrawal. This suggests that animals previously treated with E_2 are

at least as sensitive to APO as OVX controls, and confirms previous reports of the effects of DA agonists on prolactin secretion in other chronic E_2 treatment models (Smythe and Brandstater, 1980; Willoughby et al., 1983). Injection of MOR, a drug which decreases TIDA activity, significantly raised prolactin only in OVX controls and not in the E_2-treated animals. When the change in serum prolactin was expressed as per µg estimated lactotroph DNA, OVX control rats exhibited a 2-fold greater prolactin rise than E_2 treated rats. This suggests that there was a smaller MOR-induced inhibition of TIDA neurons in E_2 treated animals, possibly due to reduced activity of the neurons. Both groups responded to a single injection of HALO with a significant increase in serum prolactin as compared to pre-injection levels. However, when expressed as µg prolactin-cell DNA, OVX controls exhibited a 2-fold greater increase in prolactin than E_2 treated animals. Reduced TIDA neuronal activity long after withdrawal from E_2 probably accounts for this reduced response. Acute administration of NOM, a drug that inhibits DA reuptake, also resulted in a reduced prolactin response in rats long after E_2 withdrawal. All of these data indicate that TIDA responsiveness is permanently attenuated long after removal of chronic E_2 treatment in F344 rats.

The 3-day E_2 treatment in OVX control rats doubled the evoked release of ^{3}H-DA and increased serum prolactin from 14 $\pm$ 3 ng/ml before the E_2 challenge to 304 $\pm$ 119 ng/ml at the end of 3 days of E_2 treatment. However, 3 days of E_2 administration had no significant effect on evoked ^{3}H-DA release and decreased ^{3}H-DA accumulation in the rats previously treated with E_2 for 4 weeks. E_2 also increased serum prolactin from 57 $\pm$ 11 to 340 $\pm$ 43 ng/ml. At least part of the action

of E_2 in stimulating prolactin release in vivo appears to occur by antagonizing the inhibitory effect of DA at the AP level (Raymond et al., 1978). In the control rats, E_2 may have removed dopaminergic inhibition from the lactotrophs and greatly increased serum prolactin levels. In rats previously given E_2 for 4 weeks, TIDA activity was not augmented by 3 days of E_2 treatment, and E_2 may have been acting on the AP in the presence of low DA concentrations. E_2 action on lactotrophs in the presence of little dopaminergic inhibition may account for the large increase in AP weight without a concomitant large increase in serum prolactin levels. Old rats are known to show deficient TIDA neuronal function, and exhibit relatively small increases in prolactin secretion in response to E_2 administration as compared to young rats (Shaar et al., 1975).

The mechanism(s) responsible for the decrease in ^{3}H-DA accumulation after 3 days of E_2 administration in rats previously treated with E_2 for 4 weeks is unknown. It has been observed that E_2 can inhibit ^{3}H-DA accumulation in hypothalamic tissue slices when E_2 was included in the incubation medium in vitro but not when injected in vivo (Endersby and Wilson, 1974).

Acute E_2 depresses LH secretion in OVX rats. This negative feedback effect of E_2 on LH secretion is partly exerted at the hypothalamic level. The acute E_2 treatment in F344 rats treated for 4 weeks with E_2 26 weeks previously, reduced LH by a smaller absolute amount than in animals not given E_2 earlier. This suggests a deficiency in the negative feedback control of LH in the chronically E_2 treated rats and is consistent with reports concerning the regulation of LH after different regimens of chronic E_2 treatment (Finch et al., 1984). The E_2-induced

deficiency in the central mechanisms controlling LH secretion is associated with an increase in glial reactivity in the arcuate nucleus and may mimic the age-related decline in hypothalamic mechanisms which control reproductive function.

The permanent attenuation in TIDA neuronal function after 4 weeks of E_2 treatment in Fischer 344 rats may not only be due to a direct action of E_2 or to the resultant hyperprolactinemia, but also to physical compression on the medial basal hypothalamus by the grossly enlarged pituitary that occurs at the end of E_2-treatment.

III. Bromocryptine Prevents the Decline in Tuberoinfundibular Dopaminergic Neuronal Function After Removal of Chronic Estrogen Treatment

A. Objectives

Decreased function of TIDA neurons up to 6 months after removal of prolonged (4 weeks) E_2 treatment has been observed in female F344 rats. An attempt will be made to elucidate the mechanism(s) by which chronic E_2 treatment in F344 rats results in apparently permanent deficiencies in TIDA neuronal function. The specific questions to be answered were: (a) when E_2-induced AP growth and hyperprolactinemia are inhibited by simultaneous administration of a dopaminergic agonist, is TIDA neuronal activity altered?, and (b) if TIDA neuronal activity is altered when E_2 is administered together with the DA agonist, is this a permanent effect or can it be reversed after treatment is discontinued?

B. Materials and Methods

Rats were divided into four groups as follows: (1) OVX controls implanted with an empty Silastic capsule sc for 30 days and given daily vehicle injections (2) rats given daily bromocryptine injections for 30 days (Sandoz, Hanover, NJ; 3 mg/kg, sc, 50% ethanol in saline vehicle) and implanted with an empty capsule, (3) rats given an E_2-filled capsule (10 mm in length) and daily vehicle injections, and (4) rats given bromocryptine daily together with an E_2-filled capsule. At the end of the 30-day treatment period TIDA neuronal function was evaluated *in vitro*. Other animals were treated the same as above except that

treatment was discontinued after 30 days, and the rats were left untreated for 30 more days before acute experiments were performed to test TIDA neuronal function.

In each group, TIDA activity was measured using an in vitro superfusion technique after allowing for accumulation of ^{3}H-DA into median eminence (ME) tissue, as previously described. The tissue was electrically stimulated for 15 sec, 50 and 80 min after the beginning of the superfusion, and the release was expressed as a stimulation-evoked release. At sixty minutes after the beginning of superfusion, nomifensine maleate (10 μM; Hoechst-Roussel, Somerville, NJ), was added to the medium to evaluate ^{3}H release from the ME tissue after inhibition of DA reuptake (Gerhards et al., 1974; Hurt et al., 1974). Experiments were performed starting at 1100 hr. Animals were decapitated, blood collected, serum separated and frozen, and ME tissue was dissected for superfusion. The AP was separated from the neurointermediate lobe, weighed and homogenized in 2 ml of 0.01 M phosphate buffered saline (pH 7.6) and frozen for later measurement of AP prolactin and DNA content. Serum and AP prolactin were measured by RIA. The catecholamine assay was a modification of the radioenzymatic method of Cheng and Wooten (1980). Neurointermediate lobes were dissected and homogenized in 250 μl of 0.1 N $HClO_4$ containing 5 mM glutathione. The homogenate was centrifuged, and the supernatant was stored frozen at -40°C until assay. The DA and norepinephrine (NE) contents were expressed as ng catecholamine per mg neurointermediate lobe protein as measured by the Bio-Rad assay. AP DNA content was measured using the method of Burton (1956).

The AP weight, serum and AP prolactin concentrations, and AP DNA

content revealed a significant F_{max} test for heterogeneity of variance. The data were transformed logarithmically before performing analysis of variance and Student-Newman-Keuls multiple comparison test. Data expressed as a ratio were analyzed by the Wilcoxson-Mann-Whitney test.

C. Results

Thirty days of E_2 treatment of OVX F344 rats (E) increased AP weight 10-fold, serum prolactin 140-fold, AP prolactin content 15-fold and AP DNA content about 6-fold as compared to values in non-E_2-treated controls (C; Figure 7). Bromocryptine given alone for 30 days (B) reduced serum prolactin and AP prolactin content but did not alter AP weight and DNA content when compared to control values. When bromocryptine was injected daily in E_2-treated animals (BE), it completely inhibited the E_2-induced increase in serum prolactin and AP DNA content and resulted in only a modest increase in AP weight as compared to OVX controls. However, bromocryptine did not significantly alter the E_2-induced increase in AP prolactin content. Figure 8 shows the mean fractional rate constants in the superfusion experiments in the four treatment groups after allowing for accumulation of ^{3}H-DA into ME tissue and the response to electrical stimulation in the absence and presence of 10 μM nomifensine. Stimulation-evoked release of ^{3}H in the absence of nomifensine (open bars, Figure 9) was significantly reduced in both E_2-treated groups (E and BE) when compared to controls (C), and there was a trend for a reduced stimulation-evoked release in bromocrypine only (B)-treated animals. The controls (C) and bromocryptine alone (B)-treated animals exhibited an increased stimulation-evoked release in the presence, as compared to the absence of 10 μM nomifensine

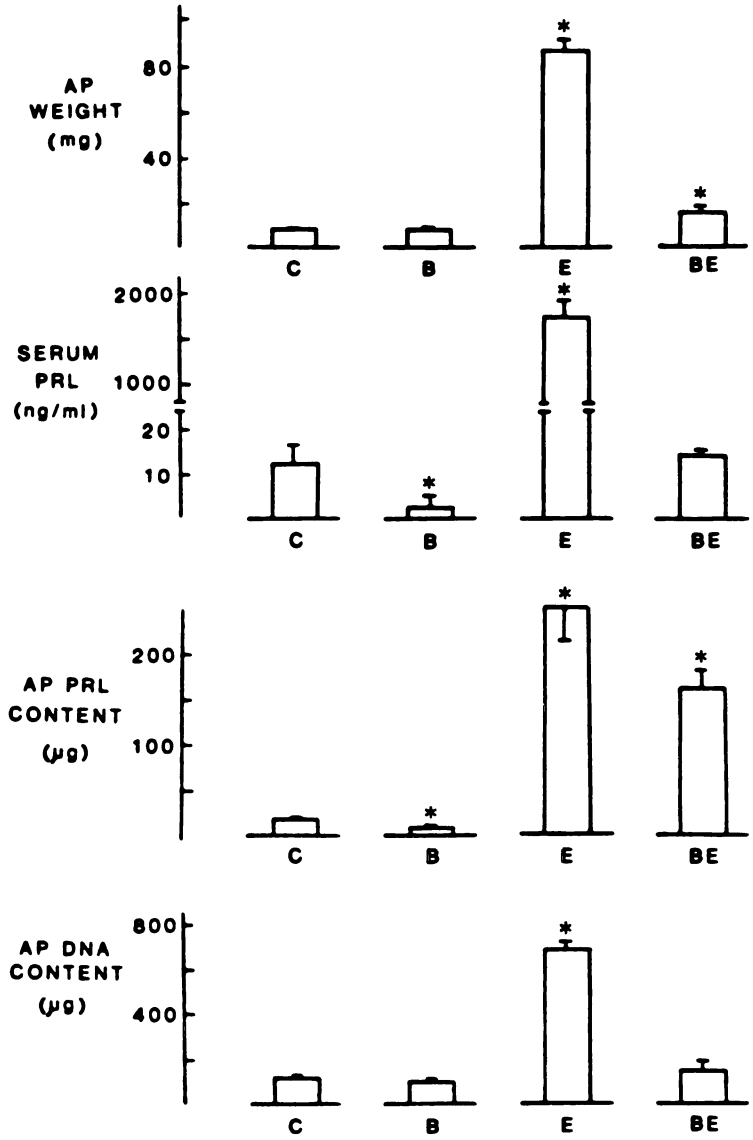

FIGURE 7.

Effects of 30 days of vehicle injections (C), bromocryptine (B), estradiol-17-β (E) or bromocryptine and estradiol treatment (BE) in OVX F344 rats on AP weight, serum prolactin concentration, and AP prolactin and DNA content. (X ± S.E.M., n = 12). Note that estradiol-induced increases in the parameters were reduced or prevented by concommitant bromocryptine treatment. * = $p < 0.05$ compared to C values.

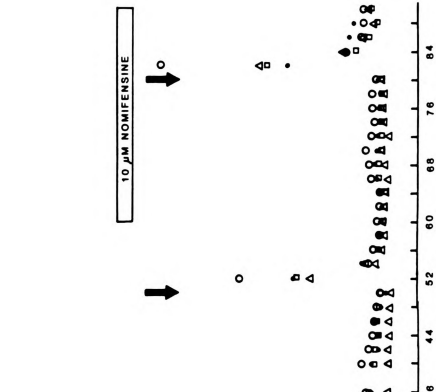

FIGURE 8.

Fractional release of ^{3}H during superfusion of median eminence tissue after allowing for accumulation of ^{3}H-DA in bromocryptine and/or estradiol-17-β treated OVX F344 rats (see Figure 7 for details of treatment). Fractions were collected every two minutes and arrows indicate electrical stimulation (15 sec) of the median eminence tissue in the absence or presence of 10 μM nomifensine. Data are presented as the mean (n = 6) fractional rate constant.

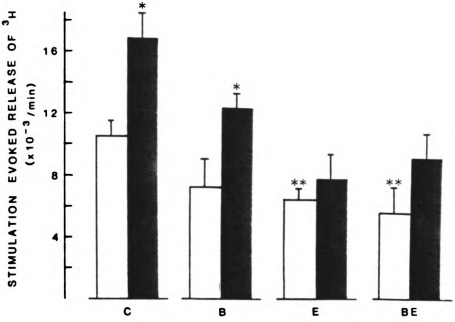

FIGURE 9.

Stimulation evoked release of ^3H (X $\pm$ S.E.M., n = 6) before (open bars) and during infusion of 10 μM nomifensine (closed bars) in bromocryptine and/or estradiol-17- β treated OVX F344 rats. (See Figure 7 for details of treatments.) Note that in both estradiol-treated groups (E and BE) stimulation-evoked release of ^3H was reduced as compared to controls and response to nomifensine was absent. ** = $p < 0.05$ compared to C values, * = $p < 0.05$ compared to evoked release in absence of NOM.

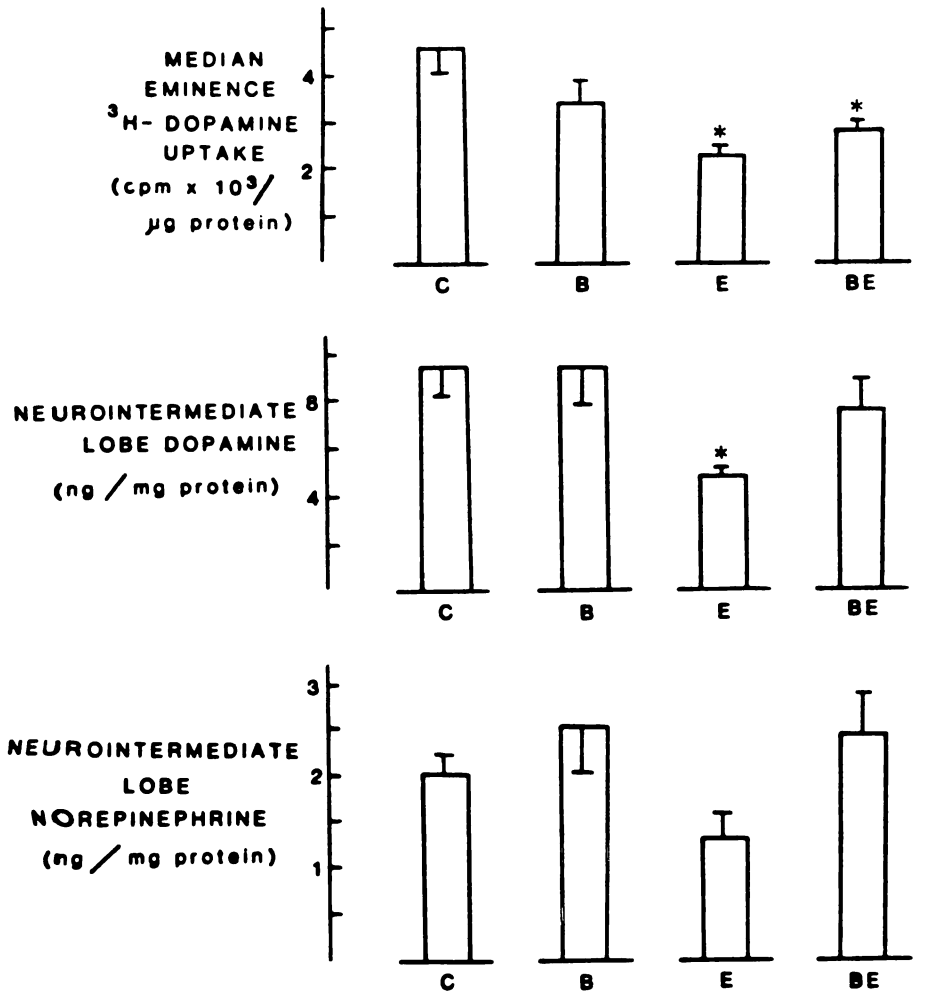

FIGURE 10.

Median eminence ^{3}H-DA uptake (X ± S.E.M., n = 6) and neurointermediate lobe DA and NE content (X ± S.E.M., n =12) in bromocryptine and/or estradiol-17-β treated OVX F344 rats. (See Figure 7 for details of treatment.) Note that estradiol treatment alone (E) reduced neurointermediate lobe DA content. * = $p < 0.05$ compared to C values.

(solid bars, Figure 9). However, neither E_2-treated group (E and BE) responded significantly to nomifensine. Accumulation of ^{3}H-DA into ME neuron terminals and neurointermediate lobe DA and NE content for each experimental group are shown in Figure 10. In E_2-treated animals (E and BE), ME ^{3}H-DA accumulation was reduced when compared to controls (C). Neurointermediate lobe DA content was reduced about 50% in animals given only E_2 (E), but not E_2 and bromocryptine, as compared to OVX control values (C).

Figures 11-14 show the results 30 days after withdrawal of the different treatments. Figure 11 demonstrates that 30 days after E_2 withdrawal (EW), AP weight, serum prolactin levels, AP prolactin and DNA content fell markedly but remained higher than the control animals (CW). The decreases in serum and AP prolactin levels observed during treatment with bromocryptine alone were not maintained after drug removal. Bromocryptine administered during E_2 treatment completely prevented the E_2-induced increases in serum prolactin, AP prolactin and DNA content, and AP weight at the end of the withdrawal period. Figure 12 shows the mean fractional rate constants during ME superfusion of the four groups after withdrawal of treatments, and Figure 13 shows the stimulation-evoked release of ^{3}H before and after nomifensine infusion. Only in animals after withdrawal from E_2 (EW) was there a reduced stimulation-evoked release (Figure 13, open bars) and no significant response to nomifensine (Figure 13, closed bars), as compared to controls. The reduction observed in the presence of combined bromocryptine and E_2 treatment was not present 30 days after removal of treatment (BEW). In fact, bromocryptine and E_2-treated animals responded to NOM infusion similarly to controls at the end of the 30 day

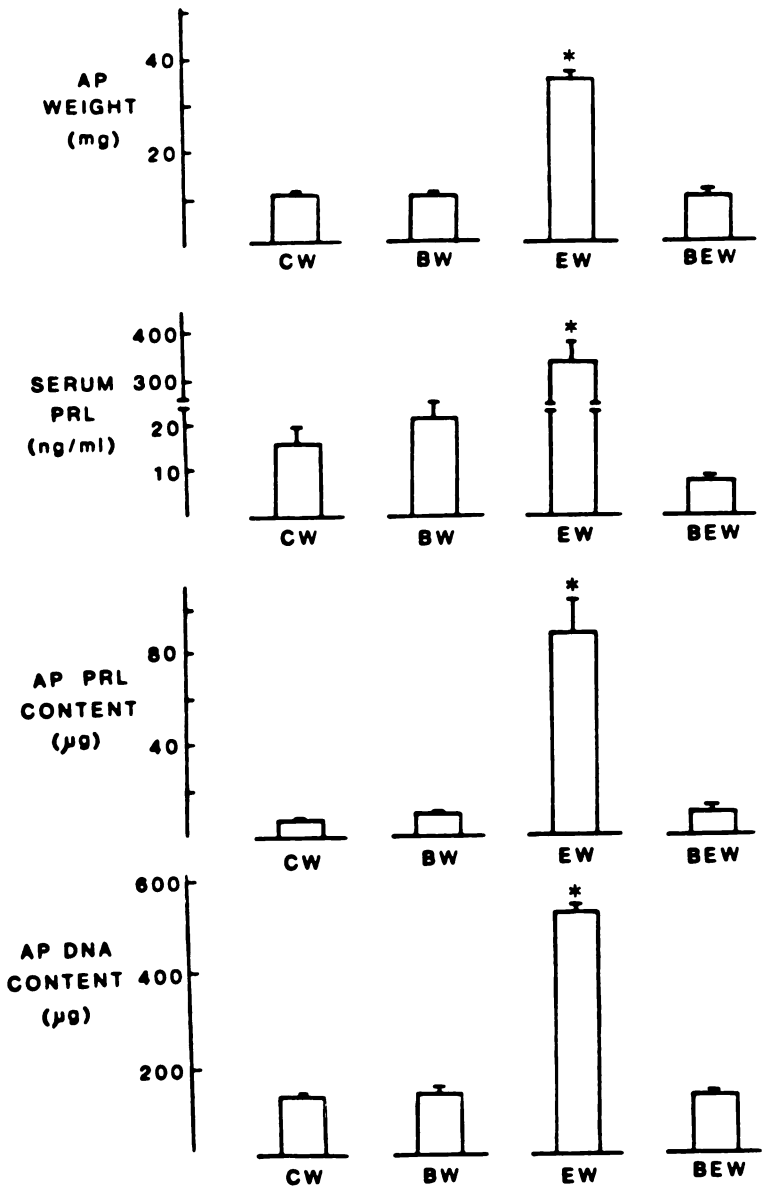

FIGURE 11.

F344 OVX rats were treated for 30 days with vehicle (CW), bromocryptine (BW), estradiol-17- β (EW) or bromocryptine and estradiol (BEW) and thereafter treatment was discontinued for 30 days before experiments were performed. The effects shown are of the different treatments on AP weight, serum prolactin levels, AP prolactin and DNA content. ($\bar{X} \pm$ S.E.M.,n = 12). Note that 30 days after withdrawal of estradiol (EW), all parameters were still increased over controls. * = $p < 0.05$ compared to CW values.

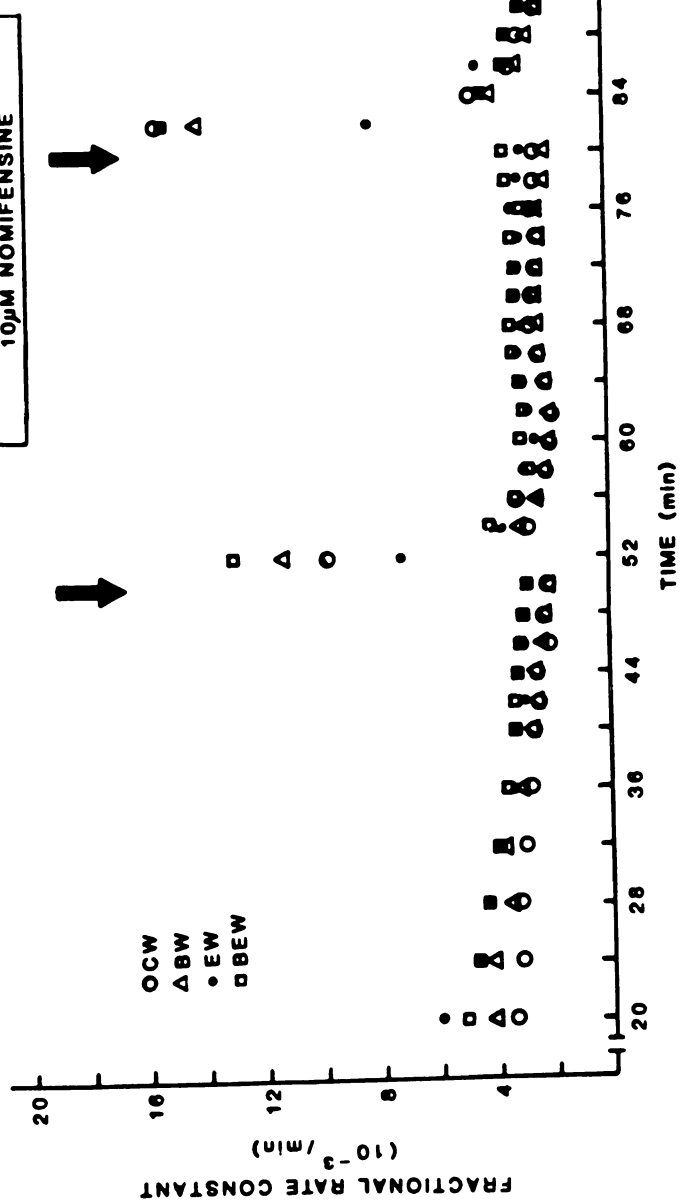

FIGURE 12.

Fractional release of ^{3}H during superfusion of median eminence tissue after allowing for accumulation of ^{3}H-DA in bromocryptine and/or estradiol-17-β treated and recovered (30 days) OVX F344 rats. (See Figure 11 for details of treatments). Fractions were collected every two minutes and arrows indicate electrical stimulation (15 sec) of the median eminence tissue in the absence or presence of 10 μM nomifensine. Data are presented as the mean (n = 6) fractional rate constant.

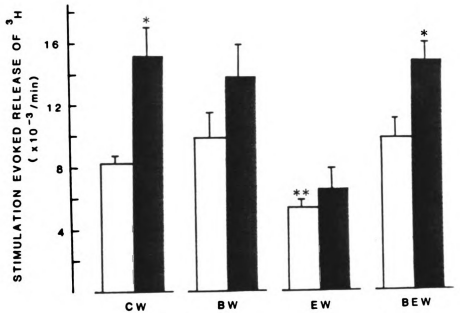

FIGURE 13.

Stimulation evoked release of ^{3}H (X $\pm$ S.E.M.,n = 6) before (open bars) and during infusion of 10μ M nomifensine (closed bars) in bromocryptine and/or estradiol-17-β treated and recovered (30 days) OVX F344 rats. (See Figure 11 for details of treatments.) Note values in bromocryptine and estradiol-treated animals during (Figure 9) and after withdrawal of treatment (BEW). ** = $p < 0.05$ compared to CW values. * = $p < 0.05$ compared to evoked release in absence of nomifensine.

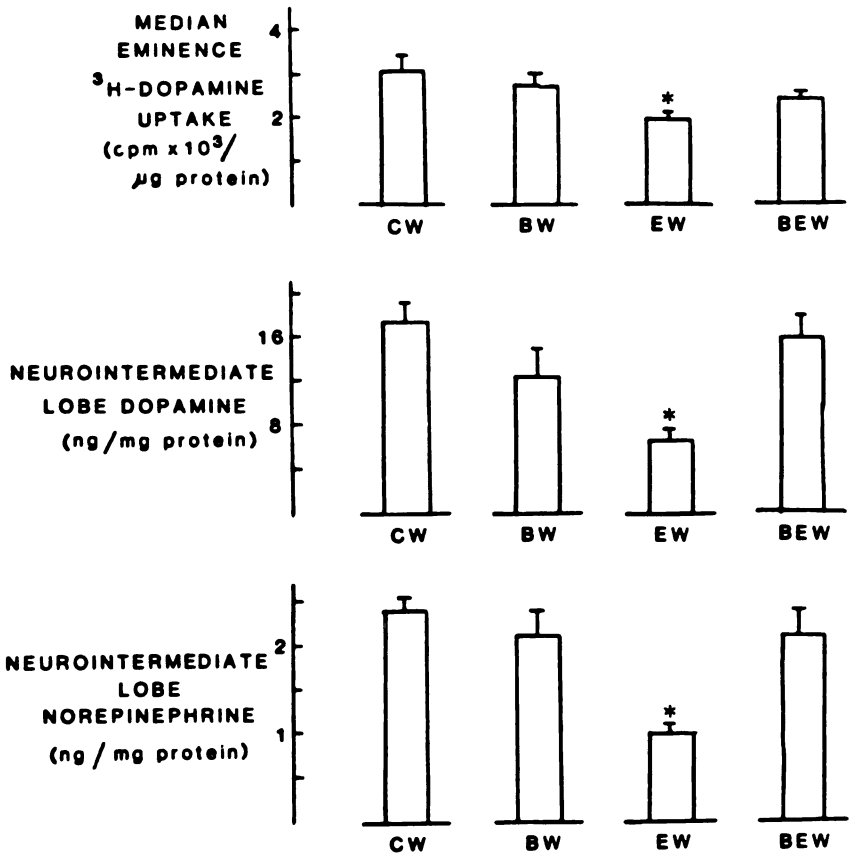

FIGURE 14.

Median eminence ^{3}H-DA uptake (X ± S.E.M.,n = 6), neurointermediate lobe DA and norepinephrine content (X ± S.E.M.,n = 12) in bromocryptine and/or estradiol-17-β treated and recovered (30 days) OVX F344 rats. (See Figure 11 for details of treatments.) Note reduction in neurointermediate DA and NE content only after withdrawal of estradiol treatment (E). * = $p < 0.05$ compared to CW values.

withdrawal period. Accumulation of ^{3}H-DA in ME tissue and neurointermediate lobe DA and NE content (Figure 14) were signficantly reduced only in animals 30 days after removal of E_2 (EW).

D. Discussion

The present results confirm that chronically elevated levels of circulating E_2 in F344 rats can produce long-lasting deficiencies in TIDA neuronal function (Gottschall et al., 1986), and also show that this deficiency can be prevented by simultaneous administration of the dopaminergic agonist, bromocryptine. Bromocryptine inhibited the E_2-induced increases in AP weight and prolactin secretion. Although bromocryptine did not prevent the decline in TIDA function in the presence of E_2, it was able to reverse the deficits in TIDA neuronal function induced by E_2 after E_2 treatment was discontinued. Since bromocryptine prevented the increases in AP weight and prolactin secretion in the E_2 treated animals, it appears that the the secondary effects of E_2, ie. the large increase in AP weight and/or hyperprolactinemia, are mainly responsible for the decline in TIDA neuronal function rather than a direct action of E_2 on TIDA neurons.

In the presence of the elevated E_2 levels produced by the Silastic implant, the stimulation-evoked release of ^{3}H was reduced after ^{3}H-DA accumulation, and the responsiveness to nomifensine was absent when bromocryptine was administered concomittantly. This indicates that even when prolactin levels are low, E_2 can still significantly decrease TIDA neuronal activity. After injection of ^{3}H-E_2, E_2-concentrating perikarya have been localized in the arcuate nucleus by autoradiography, within neurons that contained immunoreactive tyrosine hydroxylase (Sar, 1984).

Implantation of E_2 in the ME resulted in marked increases in serum prolactin 25 days later (Nagasawa et al., 1969). Moreover, short-term E_2 can increase DA turnover in hypophysectomized rats (Fuxe et al., 1981). Many other studies have demonstrated a role for E_2 in regulating TIDA neuronal function, but suggested (and many have demonstrated) that its action was exerted indirectly via prolactin (Eikenburg et al., 1977; Gudelsky et al., 1981; Moore and Demarest, 1982). However, our results indicate that chronic E_2 may have a direct action in depressing the activity of TIDA neurons. Bromocryptine alone has previously been shown to reduce TIDA neuronal activity either by specifically binding to dopaminergic autoreceptor sites on terminals of TIDA neurons (Sarkar et al., 1983), or by diminishing prolactin levels and thereby lessening the short-loop feedback of prolactin on the neurons (Demarest et al., 1985b). Since bromocryptine alone did not significantly reduce TIDA neuronal function in OVX F344 rats, the major factor depressing TIDA neuronal function after combined bromocryptine and E_2 treatment was probably the increased E_2 levels.

E_2 administration for 30 days in F344 OVX rats resulted in AP weights over 80 mg and serum prolactin levels of about 1500 ng/ml. It was observed that the large AP compressed the basal hypothalamus and may have damaged neurons of the arcuate nucleus. This receives support from the observation that DA content was decreased in the neurointermediate lobe after treatment with E_2 alone, but not after E_2 and bromocryptine. Tuberohypophyseal dopaminergic neurons also have their origins in the arcuate nucleus (Björkland et al., 1973), and decreased DA content in the terminals of these neurons are suggestive of damage, since short-term E_2 or prolactin treatment have been shown not to change

(Demarest et al., 1984; Moore and Demarest, 1982) or increase (Barden et al., 1982) tuberohypophyseal DA content.

Therefore, the permanent action of E_2 on TIDA neuronal function may occur from the E_2-induced AP growth and hyperprolactinemia, and as a direct effect of E_2. The decline in neurointermediate lob catecholamine levels suggests that physical compression on the hypothalamus from the large AP may produce significant effects.

IV. Pulsatile Release Patterns of Prolactin, Luteinizing Hormone and Growth Hormone: Effect of Estradiol-Induced Anterior Pituitary Growth

A. Objectives

Since blockade of estradiol-induced AP growth and hyperprolactinemia by bromocryptine allows functional recovery of TIDA neurons after estradiol withdrawal (Experiment III), the possibility exists that the deficiency in TIDA neurons after chronic estradiol treatment may result, at least partly, from the secondary effects of physical compression by the enlarged AP on the medial basal hypothalamus (MBH) or from the chronic hyperprolactinemia. If non-selective damage occurs when the enlarged AP compresses the MBH, functional alterations may also occur in other neuronal systems of the MBH that regulate AP hormone release. The episodic release of LH and GH are believed to be controlled mainly by neurons that originate in the pre-optic area and MBH and terminate in the ME (Kalra and Kalra, 1983; Jansson et al., 1985). Therefore, it was of interest to determine if chronic estradiol administration can permanently alter the functional control of LH and GH by observing the pulsatile pattern of their release. We also measured the pulsatile release of prolactin to determine if the loss of dopaminergic control of prolactin secretion would diminish prolactin pulses.

B. Materials and Methods

All animals were OVX and some rats received an empty or estradiol-17-β (E_2;Sigma Chemical Co., St. Louis, MO) filled Silastic capsule (10

mm length, Dow Corning, Midland, MI;). After 30 days, the capsules were removed and the animals housed until blood withdrawal 120 days later. Others received an E_2-containing capsule 120 days after OVX and 30 days later, they were subjected to blood withdrawal. Two days before the end of treatment, animals under ether anesthesia were fitted with a Silastic indwelling cannula through the right jugular vein into the right atria. On the experimental day, animals were brought into the experimental room, and silastic tubing extensions with syringes were attached to the cannula for blood withdrawal. After a two-hour adaptation period, 0.6 ml blood samples were drawn into heparinized syringes every 20 minutes for three hours starting at 1130 h. Samples were assayed for prolactin, LH, and GH at one volume in duplicate except for GH in which all samples were assayed at 10 and 30 µl in duplicate. For prolactin 1, 10, and 30 µl was used in the assay for E_2-treated, E_2-withdrawn and OVX control animals, respectively. For LH, 50 µl was used for E_2-treated rats, and 20 µl used for E_2-withdrawn and OVX control animals.

Part of the variation of hormone levels in plasma is inherent in the error of the assay system. Therefore, the intra-assay variation for each hormone was determined by calculating the coefficient of variation (CV) from two or three plasma pools of varying hormone levels, to determine if absolute hormone level or sample volume significantly affected the variation. Twelve samples of pooled plasma were assayed in duplicate to calculate the CV for each hormone level. The CVs are given in Table 6.

The peak of a secretory pulse was defined as an increase in hormone concentration of greater than 20% from nadir to zenith according to the criteria of Santen and Bardin (1973). This criteria of 20% is greater than 2x the CVs listed in Table 6 for each level of prolactin, LH, and

Table 6. Effect of varying pooled plasma concentrations of AP hormones and varying sample volumes on within-assay coefficient of variation

Hormone Level	Sample Volume (µl)	Binding (%)	CV (%)
high prolactin	1	61-65	7.4
intermediate prolactin	10	49-51	4.8
low prolactin	30	66-69	6.9
high LH	20	25-28	6.1
intermediate LH	20	51-54	6.6
low LH	50	75-78	7.8
high GH	10	49-52	2.9
low GH	30	78-83	8.0

GH. The nature of pulsatile release was assessed by the following parameters (Ambrosi et al, 1985):

- the mean of all samples withdrawn from all the subjects of particular a treatment group
- the coefficient of variation from the mean value for each subject
- the number of secretory pulses per 3 h for each subject
- the absolute and percent increment of each pulse from all the subjects of a particular treatment group.

The data from each parameter were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test. Data which showed a significant heterogeneity of variance were first logarithmically transformed before statistical analysis. $p \leq 0.05$ was chosen as the level of statistical significance.

C. Results

The data in Figures 15 and 16 were selected as representative examples of the pulsatile release of prolactin, LH, and GH in OVX controls (OVX), animals which received E_2 for 30 days (E) and in animals 120 days after discontinuing the 30 day E_2 treatment (WD). Prolactin levels are presented in both figures to show the magnitude of hyperprolactinemia in the E_2-treated individuals, in addition to the pulsatile release of prolactin. Tables 7-9 present the parameters used to evaluate the pulsatile pattern of prolactin, LH, and GH, respectively.

The mean prolactin level and the absolute amplitude of the prolactin secretory peaks were greatest in animals at the end of E_2 treatment and remained significantly above control levels 120 days after removal of E_2 (Table 7). However, the frequency and percent amplitude of the prolactin

pulses decreased after E_2 treatment, and continued to be lower than OVX controls 120 days after E_2 administration. The CV, for prolactin pulses, an indication of total variation across the ten samples, was reduced compared to OVX controls at the end of E_2 treatment and was even significantly lower 120 days after E_2 treatment compared to animals during steroid administration.

In contrast to the E_2-induced increase in prolactin levels, mean plasma levels of LH and the absolute amplitude of LH pulses were dramatically decreased as a result of E_2 treatment (Table 8). After the 120-day withdrawal period, LH levels and the amplitude of the LH pulses were depressed compared to OVX controls but greater than at the end of E_2 treatment. Treatment for 30 days with E_2 did not significantly change the CV, or the percent amplitude of the pulses compared to OVX controls. Interestingly, in animals which were still hyperprolactinemic, 120 days after chronic E_2 treatment, the CV of the LH values was significantly greater than in OVX controls. The measurable frequency of LH pulses was not altered as a result of either E_2-treatment regimen compared to OVX animals.

Treatment with E_2 for 30 days significantly increased mean plasma GH levels, 4-fold, however, after withdrawal of E_2, mean GH values returned to control values (Table 9). The CV, the frequency of GH pulses, and the percent amplitude of the GH pulses were lower at the end of E_2 treatment compared to control values. Although 120 days after withdrawal of treatment, the CV and the percent amplitude of the GH pulses remained lower than OVX control values, the frequency of GH pulses returned to control values. Chronic E_2 treatment did not significantly affect the absolute magnitude of the GH pulses, although there was a trend toward

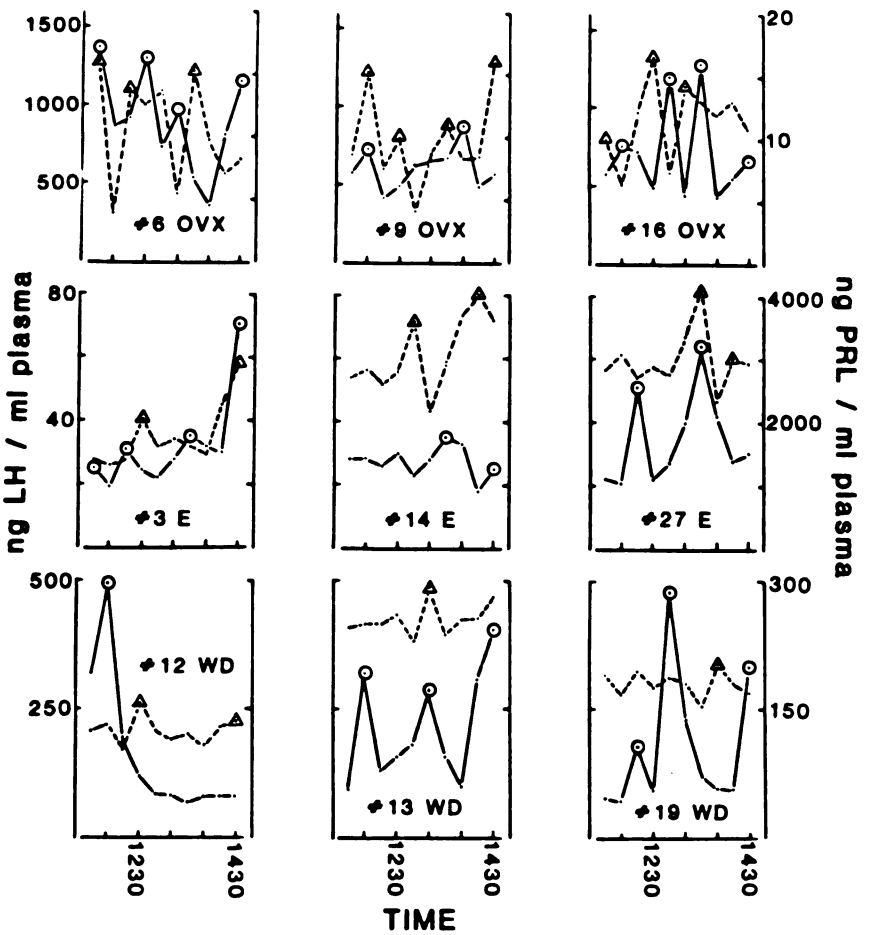

FIGURE 15.

Pulsatile release pattern of LH (solid lines, open circles signify pulse peaks) and prolactin (dashed lines, open triangles signify pulse peaks) in OVX controls (OVX), rats treated with estradiol for 30 days (E), and rats which received estradiol for 30 days, estradiol withdrawn and blood drawn 120 days later (WD). Shown are representative patterns from three selected individuals from each treatment group.

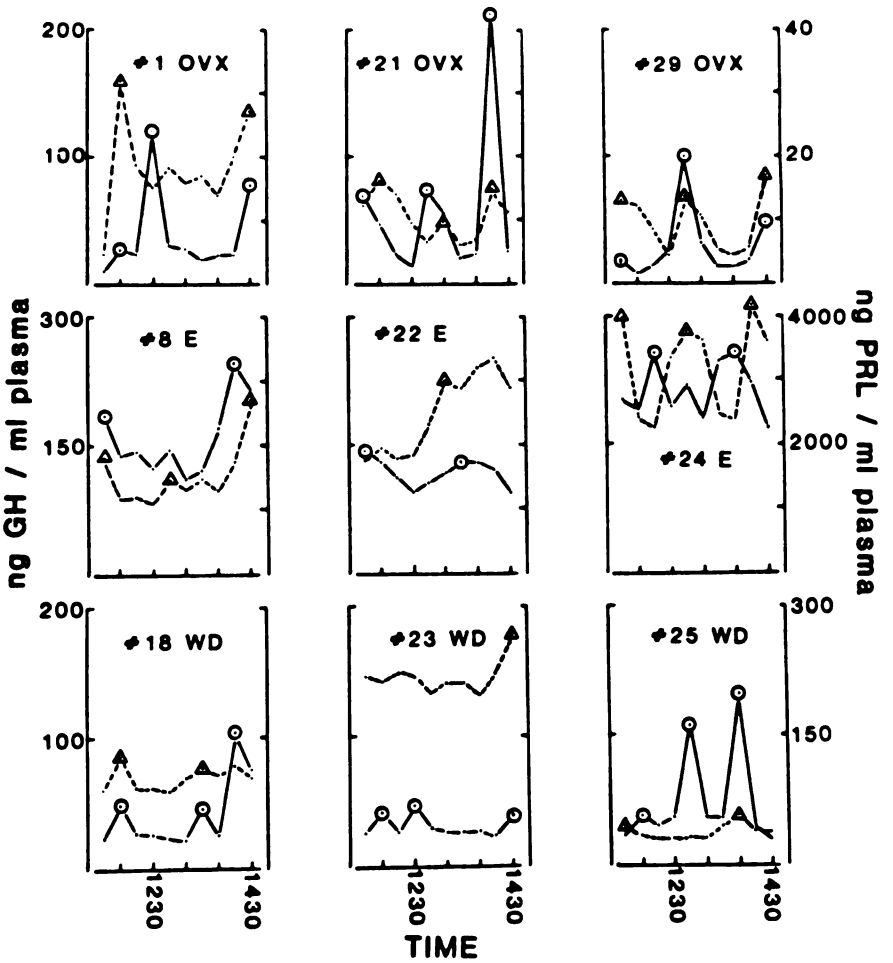

FIGURE 16.

Pulsatile release of GH (solid lines, open circles signify pulse peaks) and prolactin (dashed lines, open triangles signify pulse peaks) in OVX controls (OVX), rats treated with estradiol for 30 days (E) and rats which received estradiol for 30 days, estradiol withdrawn and blood drawn 120 days later (WD). Shown are representative patterns from three selected individuals from each treatment group.

Table 7. Effect of chronic estradiol treatment and 120 days after estradiol was discontinued on parameters of pulsatile prolactin secretion.

	N	Mean Prolactin (ng/ml)	CV	Secretory peaks		
				Frequency /3h	Amplitude	
					ng/ml	%
OVX controls	9	12 ± 1 (90)	34 ± 2 (9)	3.2 ± 0.2 (9)	8 ± 1 (29)	51 ± 3 (29)
Chronic E_2	8	2459 ± 278* (80)	20 ± 2* (8)	2.1 ± 0.3* (8)	1039 ± 126* (17)	36 ± 2* (17)
120 days after chronic E_2	6	151 ± 10*,** (60)	12 ± 1*,** (6)	1.5 ± 0.2* (6)	38 ± 8*,** (9)	27 ± 2* (9)

See Materials and Methods for details of the estradiol administration; parentheses indicate number from which mean was calculated
* = p 0.05 compared to OVX control level
** = p 0.05 compared to chronic estradiol level

Table 8. Effect of chronic estradiol treatment and 120 days after estradiol was discontinued on parameters of pulsatile luteinizing hormone (LH) secretion.

	N	Mean LH (ng/ml)	CV	Secretory peaks		
				Frequency /3h	Amplitude	
					ng/ml	%
OVX controls	9	792 ± 44 (90)	33 ± 3 (9)	2.8 ± 0.4 (9)	530 ± 56 (25)	47 ± 4 (25)
Chronic E_2	8	28 ± 1 * (80)	25 ± 4 (8)	2.5 ± 0.4 (8)	14 ± 3 * (20)	35 ± 3 (20)
120 days after chronic E_2	6	232 ± 20 *,** (60)	59 ± 8 *,** (6)	2.7 ± 0.4 (6)	229 ± 23 *,** (16)	55 ± 5 ** (16)

See Materials and Methods for details of the estradiol administration; parentheses indicate number from which mean was calculated

* = p 0.05 compared to OVX control level

** = p 0.05 compared to chronic estradiol level

Table 9. Effect of chronic estradiol treatment and 120 days after estradiol was discontinued on parameters of pulsatile growth hormone (GH) secretion.

	N	Mean GH (ng/ml)	CV	Secretory peaks		
				Frequency /3h	Amplitude	
					ng/ml	%
OVX controls	9	37 ± 4 (90)	93 ± 13 (9)	2.9 ± 0.3 (9)	56 ± 12 (26)	63 ± 4 (26)
Chronic E_2	8	148 ± 5* (80)	19 ± 2* (8)	1.6 ± 0.2* (8)	59 ± 8 (17)	31 ± 2* (13)
120 days after chronic E_2	6	41 ± 3** (60)	47 ± 10* (6)	2.8 ± 0.3** (6)	32 ± 8 (13)	45 ± 5* (17)

See Materials and Methods for details of the estradiol administration; paraentheses indicate number from which mean was calculated
* = p 0.05 compared to OVX control level
** = p 0.05 compared to chronic estradiol level

reduced GH pulse amplitude after E_2 withdrawal.

Upon autopsy of rats at the end of the 30 day E_2 treatment, it was observed that the large AP compressed the basal hypothalamus. Visual inspection of the hypothalamus-AP 120 days after the removal of the E_2 treatment, revealed hypothalami that showed little or no compression of the MBH. In all E_2-treated animals the pituitary stalk remained grossly intact.

D. Discussion

The results of this study show that long term E_2 treatment to F344 rats diminishes the frequency of prolactin pulses and that this decrease remains for at least 120 days after removal of E_2. Experiment II showed that chronic E_2 administered to F344 rats attenuates TIDA neuronal activity even up to 26 weeks after the E_2 treatment was discontinued. The reduction of prolactin pulses and TIDA neuronal activity after chronic E_2 treatment suggests that TIDA neurons are responsible for the pulsatile secretion of prolactin although presently there are no data to support this claim.

The negative feedback of E_2 on LH secretion in OVX rats has been well characterized (Kalra and Kalra, 1983) by decreases in mean LH level and pulse amplitude, in agreement with the present results. Most previous reports on LH pulses have used short-term estradiol treatment and found that estradiol decreases (Akema et al., 1983; Weick and Noh, 1984) LH pulse frequency. Although LH pulse frequency in this report also remains unchanged after E_2 treatment, the diminished mean LH concentration at the end of the E_2 treatment was still present 120 days after removal of E_2. The fact that animals long after E_2 withdrawal were

still hyperprolactinemic may account for the low LH levels observed, since high prolactin can decrease serum LH (Meites et al., 1972). However, animals after withdrawal of E_2 showed a CV and percent pulse amplitude of LH that was greater than in OVX controls, suggesting a functional neuronal regulatory system for LH that is qualitatively not different from OVX control rats.

Chronic estradiol treatment increases mean GH levels, apparently by acting directly on the AP (Jansson et al., 1983), although 120 days after E_2 was removed, mean GH concentrations were similar to control levels. Thirty days of E_2 treatment decreased the frequency of GH pulses but the frequency returned to control values after E_2 was removed. The absolute amplitude of GH pulses did not decrease during or following E_2 treatment, but reductions in percent amplitude and CV occurred as a result of E_2 treatment when compared with controls. Therefore, the relative pulse amplitude (baseline compared to peak values) appears to diminish during and after chronic E_2 treatment. Nevertheless, since no significant changes were observed in pulse frequency or absolute pulse amplitude 120 days after E_2 was removed, the neuronal systems regulating GH secretion appear to be functioning, in a qualitative fashion, not different from OVX controls. Since the relative pulse amplitude was reduced by E_2, the large AP may be responsible for a quantitative change in the mechanism(s) that regulate GH pulse height.

GENERAL DISCUSSION

The data presented in this thesis indicate that chronic E_2 treatment in F344 rats reduced the hypothalamic inhibitory control that DA exerts on prolactin secretion. Of greater significance, the diminished dopaminergic function persisted for up to 26 weeks after E_2 treatment was discontinued. The apparently "permanent" decline in TIDA activity may not be a primary effect of increased circulating E_2 since bromocryptine, prevented the E_2-induced AP hypertrophy and hyperprolactinemia, and thereby prevented the apparently "permanent" decline in TIDA activity. The final experiment showed that chronic E_2 treatment decreased the pulsatile secretion of prolactin secretion and continued to do so long after E_2 was removed, whereas it did not significantly alter the pattern of LH or GH secretion after removal of E_2.

Although other studies have demonstrated reduced TIDA activity in the presence of elevated estrogen levels (Smythe and Brandstater, 1980; Casaneuva et al., 1982; Sarkar et al., 1982; Sarkar et al., 1983a), this is the first evidence that reduced TIDA neuronal activity is maintained long after discontinuing the E_2 treatment. One group has shown reduced prolactin responsiveness to central-acting dopaminergic drugs long after a single injection of a large-dose injection of estrogen (Willoughby et al., 1984). These authors speculated that the reason for the decreased prolactin response after the drug injection was that the mass of the

release. Even though this suggestion may be partly valid, in rats with very large APs, i.e., at the end of E_2 treatment, this is not the case in the present study 4 or 26 weeks after E_2 was removed, when AP size declined more nearly to that of non-E_2-treated rats. Also, the release of ^{3}H-DA from the ME was measured directly and found to be reduced long after E_2 was removed.

It is interesting that other groups, using female Long-Evans rats and Fischer 344 male rats chronically treated with estrogen have reported a decline in TIDA function at the end of estrogen treatment but after estrogen was removed there was a rebound increase in TIDA neuronal synthesis (Demarest et al., 1984; Morgan et al., 1985). The estrogen-induced AP hypertrophy was considerably smaller than observed in the present studies. It was suggested that enhanced TIDA function after removal of estrogen may be a factor in the subsequent involution of the hyperplastic AP. Since, in the present study, there was no enhanced TIDA function in Fischer 344 female rats after discontinuing E_2 treatment, the low TIDA neuronal activity may have been responsible for the persistent hyperprolactinemia and pituitary hyperplasia observed 4 and 26 weeks after removal of E_2.

The apparently "permanent" damaging action of E_2 on TIDA neurons was demonstrated here by a lowered prolactin response to central-acting dopaminergic drugs, i.e., morphine and nomifensine, and by the absence of TIDA neuronal response to short-term E_2 treatment 26 weeks after withdrawal of the E_2 treatment. At 26 weeks after chronic E_2, AP weight was still elevated about 2-fold, AP DNA content about 3-fold and serum prolactin was about 4-fold greater than OVX control levels. The failure of TIDA neurons to increase their function in the presence of the

hyperprolactinemia still present after removal of E_2, may be responsible for the persistent and apparently permanent hypertrophy and hyperplasia of the lactotrophs of the AP gland.

Since chronic E_2 treatment results in an apparent permanent decline in TIDA neuronal function, the possibility exists that E_2 directly, or a secondary action of E_2, is degenerating TIDA neurons. It has been reported previously in rats that chronic estrogen treatment produces a lesion of the arcuate nucleus characterized by a hyperreactive glial response and a permanent loss of reproductive function even when AP weight and serum prolactin levels were only moderately elevated (Brawer et al., 1983). The neurochemical nature of the neurons undergoing degeneration were not determined. Whether the length and magnitude of the E_2 treatment used in the present studies was sufficient to produce a similar lesion is unknown. The present experiments have the confounding presence of a very large AP at the end of E_2 treatment that physically compressed the MBH and may have mechanically damaged TIDA neurons; there was also an extreme hyperprolactinemia present at the end of E_2 administration, which has been suggested to damage TIDA neurons (Sarkar et al., 1984b).

When bromocryptine was administered during E_2 treatment to inhibit AP growth and hyperprolactinemia, TIDA activity was still depressed when compared with control animals. When all treatment was discontinued for 30 days, animals previously treated only with E_2 showed reduced TIDA function, whereas in animals treated with bromocryptine and E_2 for 30 days, after which treatment was removed for 30 days, TIDA function had returned to control levels. Therefore, E_2 alone can alter TIDA neuronal activity, independent of AP size and serum prolactin levels. This

observation is in contrast to a study which showed that shorter-term estrogen treatment in hypophysectomized rats did not alter TIDA neuronal function (Eikenburg et al., 1977). More importantly, E_2 given together with bromocryptine, resulted in normal AP weight and serum prolactin levels, but did not permanently affect TIDA neuronal function, ie. TIDA function was not different from non-E_2 treated controls after removal of E_2. This suggests that either the enlarged AP or hyperprolactinemia was responsible for the permanent effect of E_2 on TIDA neuronal activity. Mechanical damage to TIDA neurons in animals with large APs was further indicated by a persistent decline in neurointermediate lobe catecholamine content which is not believed to be significantly influenced (Demarest et al., 1984; Moore and Demarest, 1982) or even increased (Barden et al., 1982) by E_2 or prolactin. These results are in agreement with a study showing that the permanent decline in TIDA neuronal function after chronic E_2 is strain-specific in the Fischer rat and does not occur in the Long-Evans rat strain (Riegle et al., 1985).

Experiment IV attempted to confirm the view that physical compression of the MBH was responsible for the loss of TIDA neuronal function. Since the hypothalamic peptides regulating the release of other AP hormones are also secreted by neurons located in the MBH, it would be expected that if TIDA neurons are damaged by the large AP, that growth hormone releasing factor and somatostatin neurons or GnRH neurons which regulate GH and LH release, respectively, would also be damaged. All three of these AP hormones--prolactin, GH, and LH--are normally released in an episodic manner, believed to result from the episodic nature of the releasing factor/release-inhibiting factor secretion. Only the frequency of prolactin pulses, and not the frequency of GH and LH

pulses, was reduced long after the removal of E_2. However, the magnitude of the pulses, particularly GH pulses, was reduced long after E_2 was removed. Therefore, the qualitative nature of the prolactin pulses was influenced long after E_2 was removed, but only the magnitude of GH and LH pulses were permanently affected by E_2 administration. TIDA neurons may be more sensitive to the damaging effects of an enlarged AP than the peptidergic neurons which regulate GH and LH secretion.

A mechanism which might account for the loss of TIDA neurons is the unique O_2 requirement for neurotransmitter synthesis in catecholaminergic neurons. The physical compression on the MBH and the large, increased blood flow to the growing AP after E_2 treatment may reduce blood flow and make this area relatively hypoxic. Moderately reduced O_2 levels have been shown to decrease synthesis in other catecholaminergic systems (Davis, 1977; Robin, 1980). The rate-limiting enzyme in catecholamine synthesis, tyrosine hydroxylase, requires O_2 for its activity (Davis and Carlsson, 1973). Since it has been shown that E_2 can reduce activity in these neurons, the combination of reduced synthesis by hypoxia and physical compression by the enlarged AP, could dramatically diminish the activity of TIDA neurons. Catecholaminergic neurons may also undergo a "disuse atrophy", whereby axons degenerate when activity is very low. The physical compression and "disuse atrophy" may result in a permanent degeneration of TIDA neurons, which cannot regenerate when the AP returns to near normal size.

Although the knowledge of the mechanism(s) by which long-term estrogen treatment influences TIDA neuronal function is beginning to emerge, it is far from complete. It was observed in these experiments that increased circulating E_2 can reduce TIDA neuronal activity whether

or not the animal is hyperprolactinemic or has a large AP. It will be important to determine the location of estrogen action in the CNS. Does estrogen influence TIDA neurons directly or does it act on neurons in an afferent pathway that impinges on TIDA neurons? Although there is good evidence for the presence of dense estrogen-binding sites in TIDA neurons (Sar, 1984), suggestive of a direct effect of estrogen on TIDA neurons, a recent observation indicated that administration of the opiate antagonist, naloxone, can reverse the depression on TIDA neuronal function produced by chronic estrogen-treatment (K. T. Demarest, personal communication).

At least three recent studies have demonstrated the reversible action of chronic estrogen treatment on TIDA neuronal function, ie. a return of TIDA function after chronic estrogen was withdrawn from the animal, and have strongly suggested that estrogen does not exert any permanent depression on TIDA neurons (Demarest et al., 1984; Morgan et al., 1985a; Morgan et al., 1985b). All of these studies, used a high dose of estrogen for a period of less than 8 weeks. It would be of interest to administer estrogen at very low levels for a long period of time, similar to that which occurs in recurrent estrous cycles, and observe whether TIDA neuronal activity is depressed by the estrogen treatment. This E_2 treatment regimen would be similar to the experiments of Brawer et al. (1978; Brawer et al., 1983) who observed only a moderate AP hypertrophy, yet animals had the characteristic arcuate nucleus lesion after the long-term E_2 treatment. TIDA neuronal activity was not measured in the experiments of Brawer et al. (1978;1983).

Even after a variety of different estrogen treatments in rats and mice (Brawer and Finch, 1983; Finch et al., 1984), glial hyperreactivity

was seen in the hypothalamic arcuate nucleus, indicative of neuronal degeneration. Although it has been 10 years since this very important observation was first reported (Brawer and Sonnenschein, 1976), the neurochemical nature of the degenerating neurons has yet to be determined. A careful immunocytochemical study characterizing possible neurotransmitter systems invloved in the lesion could be extremely helpful. In conclusion, even though E_2-induced AP hypertrophy may be involved in the permanent decline in TIDA neuronal function after removal of E_2 in F344 rats, there may be other nervous system actions that result from chronic E_2 in the absence of a large AP which may have important consequences on the regulation of AP function.

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APPENDICES

APPENDIX I

BASIC METHOD FOR UPTAKE AND RELEASE OF [^{3}-H]-DOPAMINE FROM MEDIAN EMINENCE TISSUE

1) After decapitation, and brain removal, the brain is placed on ice under the dissecting microscope. A few drops of cold Krebs-Henseleit buffer (KHB) are placed on top of the hypothalamus and the median eminence dissected out according to Cuello (Nature 1973, 2436: 465). Median eminences are placed in ice cold KHB after dissection. Tissue is then pre-incubated in fresh KHB at 37°C under 95% O_2 and 5% CO_2 for 5 min. No more than 2 ME's/tube.

2) Tissues are transferred to 1 ml of buffer containing about 2 x 10^{-7} M ^{3}H-DA. This is 20 µl of Amersham 46 Ci/mmol; 295 mCi/mg [7,8-^{3}H] dopamine (Code tRK.582). Be sure to blow off ethanol and acetic acid first (^{3}H-DA is stored in this), before adding 1 ml KHB. Drugs can be added to KHB at this point to effect the uptake process. Uptake is linear to 5 min. For superfusion experiments, uptake time is 20 min.

3) Following uptake, ME tissue is rinsed with fresh KHB and <u>very carefully</u> placed into the superfusion chambers. (Two ME's/chamber minimum).

4) Superfusion flow rate is 300 µl/min, and dead volume should be kept to a minimum. Spontaneous release is not stable for at least 40 min. ^{3}H is collected in two min fractions.

5) Physiological induced release (depolarization) can be accomplished by two methods. Electrical stimuli (biphasic square wave pulses) can be applied using chambers which contain silver electrodes and generated by two stimulators. The stimulus is monitored on a calibrated oscilliscope (see diagram). With two chambers, the stimulus can be applied to a particular chamber during the last 15 sec of a particular fraction, and then to other chamber during the first 15 sec of the next fraction. This allows for the major overflow of ^{3}H to appear in one fraction. Stimulation is usually 15 sec in duration. Based on our system, 6 mA amplitude, 20 hz frequency and 2 msec duration gives a maximal response.

   A second method of neuronal depolariztion is by infusion of hypertonic K^{+}. 40 mM K^{+} is sufficient for a good response. Be sure to lower Na^{+} concentration accordingly to eliminate osmotic effects.

6) When studying ionic mechanisms of ^{3}H-DA uptake and ^{3}H-DA release, Tris and sucrose are used to replace $NaHCO_3$ and NaCl respectively in Na^{+} free KHB. For Ca^{++} free media, EGTA is used to replace $CaCl_2$.

7) After superfusion, median eminences are homogenized in 0.5 ml of 0.1N HCl, centrifuged and supernatant radioactivity is quantified. Calculation of ^{3}H efflux is performed by dividing the amount of ^{3}H released into a fraction by the tissue content of ^{3}H at the end of

that fraction. This value is then divided by 2 to come up with an efflux fractional rate constant (EFRC)/min.

EFRC = radioactivity released in fraction / radioactivity in tissue divided by 2.

Preparation of Krebs-Henseleit Medium for Median Eminence Superfusion after Uptake of [^{3}H]-Dopamine

Chemical	M.W.	Final M	Amount/Liter	Amount/400 ml
β-D-glucose	180.2	10 mM	1.80 g	720 mg
$NaHCO_3$	84.0	25 mM	2.10 g	840 mg
EDTA	372.2	27 μM	10.05 mg	4 mg
Ascorbic Acid	176.1	130 μM	22.89 mg	9 mg

CSF (artificial)	--	--	--	20 ml
$CaCl_2$	111.0	200 mM (stock) (2.948 g/100 ml)	10 ml	4 ml
Gelatin	--	0.1%	1 g	400 mg

Artificial CSF consists of:

Chemical	M.W.	Final M	Amount/Liter (final)	Amount/500 ml M in 20 ml
NaCl	58.4	134 mM	7.83 g	78.3 g
KCl	74.6	5 mM	0.37 g	3.7 g
KH_2PO_4	136.1	1.25 mM	0.17 g	1.7 g
$MgSO_4 \cdot 7H_2O$	246.5	1 mM	0.25 g	2.5 g

20 ml is then added to 400ml KHB to give final molarity

Artificial CSF which will Contain

a Final $[K^+]$ of 40 mM (in final 400 ml)

Chemical	M.W.	Final M	Amount/Liter (final)	Amount/500 ml M in 20 ml
NaCl	58.4	99 mM	5.8 g	57.8 g
KCl	74.6	40 mM	3.1 g	31.4 g
KH_2PO_4	136.1	1.25 mM	0.17 g	1.7 g
$MgSO_4 \cdot 7H_2O$	246.5	1 mM	0.25 g	2.5 g

20 ml is then added to 400 ml KHB to give final M

To finish solution nialamide is added to

the final 400 mls to give [nialamide] of 12.5 μM

nialamide = 298.6 MW = 1.5 mg/400 mls

If desipramine (DMI) is required for uptake 0.1 μM

MW of HCl is 302.77 and 12 g/400 mls = 0.4 M

0.030 mg/ml = 0.1 mM DMI in saline

1) make up 0.1 mM DMI in saline

2) add 10 1 of 0.1 mM DMI to 10 mls of media to give 0.1 μM DMI

APPENDIX II

RADIOENZYMATIC ASSAY FOR CATECHOLAMINES

Principle: Catecholamines (CA), incubated with partially purified catechol-o-methyl transferase (COMT), the methyl donor S-adenosyl methionine (SAM) and Mg^{++} under suitable conditions will result in the transfer of methyl groups from SAM to the m-hydroxyl group of the CA. Dopamine will be converted to 3-methoxy-tyramine (MT), norepinephrine to normetanephrine (NNM) and epinephrine to metanephrine (MN). These three methylated CAs can then be separated by thin-layer chromatography (TLC). Since a labelled methyl donor is used, (^{3}H-SAM), these three CA can be quantitated by the use of liquid scintillation counting.

General Procedure

Samples of nervous tissue or plasma are incubated with COMT and ^{3}H-SAM in an appropriate buffer with the result the conversion of CA to their ^{3}H-methylated products. The products are isolated from the incubation media by an organic extraction after elevating pH (^{3}H-methylated products will enter organic phase and unreacted ^{3}H-SAM will remain in aqueous phase). The ^{3}H-methylated CA products are concentrated back to the aqueous phase in an acid extract. A portion of the acetic acid is then spotted on a TLC plate and ^{3}H-MT, ^{3}H-NMN and ^{3}H-MN separated and counted.

I. Methylation of CA

1) Sample preparation: For brain tissue, either whole brain is frozen on dry ice for later dissection or tissue is dissected immediately and frozen. Tissue is then placed in appropriate amount of 0.1 N $HClO_4$ with 5 mM glutathione (GSH). Samples are then homogenized (or sonified), spun down at 2500 rpm (RCB-2) and the supernatant used for the assay. Be sure to save aliquot of homogenate for protein assay. Recommended volume of 0.1 N $HClO_4$ with GSH is as follows:

Tissue	0.1 N $HClO_4$ w/ GSH
whole hypothalamus	4 ml
preoptic area / anterior hypothalamus (POA/AHA)	1 ml
median eminence	0.5 ml

2) Standard preparation: All standards are prepared with 0.1 N $HClO_4$ with GSH. Internal standards (standard plus tissue homogenate), rather than external standards are used because recovery is low and there is significant inhibition of the reaction by the sample (especially plasma). Standards are prepared such that a concentration of DA and NE are run with the same sample.

Standard Curve for CA Assay

For nervous tissue samples, only DA and NE are required for the standards. For plasma, use DA, NE and E. Standards run from 1 ng to 31.25 pg. Easiest for nervous tissue to prepare each standard amount for DA and NE per 5 µl, separately, and combine to give amount per 10 µl.

e.g., weighed 42.3 µg DA we want 1 ng/5 µl = 200ng/ml

and q.s. with 10 ml $HClO_4$ with GSH

42,300 ng DA/10 ml = 4230 ng DA/ml = 423 ng DA/100 µl

if we take 100 µl, we have 423 ng DA/"x" = 200 ng DA/ml, therefore x = 2.11 ml

q.s. 100 µl to 2.11 ml and this is equal to 1 ng/5 µl, dilute 1:1 to get 500 pg/5 µl, 250 pg/5 µl, 125 pg/5 µl, 62.5 pg/5 µl and 31.25 pg/ 5 µl

Do the same for NE and combine DA and NE 1:1 to give standards amount/10 µl

3) Prepare buffer for incubation:

	for 19 samples
2 M Tris (pH 9.1)	500 µl
0.25 M EGTA (pH 9.1)	100 µl
1 M $MgCl_2$	150 µl
0.25 M GSH	100 µl
H_2O	150 µl

4) Prepare reaction mix:

	for 19 samples
buffer for incubation	400 µl
^{3}H-SAM (a)	200 µl (20 Ci)
COMT (b)	200 µl

(a) ^{3}H-SAM from ICN comes as 1 mCi/ml
200 µl of ^{3}H-SAM from the bottle is diluted to 200 µl with 180 µl H_2O
(b) isolation of COMT from rat liver is described

5) Pipet assay: Use 13 x 100 mm cultures tubes and be sure to add each component directly to bottom of tube
A. for nervous tissue:

	Reagent Blank	Internal Standard	Sample
0.1 M $HClO_4$ w/ GSH	60 µl	--	10 µl
CA standard	--	10 µl	--
sample homogenate	--	50 µl	50 µl
reaction mix	40 µl	40 µl	40 µl

B. for plasma

0.1 N $HClO_4$ w/ GSH	10 µl	--	10 µl
dialyzed plasma (a)	50 µl	--	--
CA standard	--	10 µl	--
sample plasma	--	50 µl	50 µl
reaction mix	40 µl	40 µl	40 µl

(a) Dialyzed plasma was prepared by dialyzing plasma against two changes of saline for 24 hrs. Theoretically, it contains no CA.

6) Incubate, well-parafilmed tubes in Dubonoff shaking incubator at 37°C for 75 min.

II. Extraction of ^{3}H-methylated CA

7) Place all tubes on ice; add to each tube 55 µl of the following mixture:

	for 19 tubes
0.45 M borate buffer (pH 10.5) (a)	1 ml
"carrier" (b)	0.1 ml

(a) may require heating to go into solution
(b) "carrier" is mixture of non-radioactive MT, MN and NMN, each 10 mg/ml in 0.01 N HCl. This reduces loss of tritiated MT, MN and NMN during processing and to allow visualization after TLC.

8) Add to each tube 2 ml of toluene: iso-amyl-alcohol (3:2); (24 ml : 16 ml for 19 tubes)

9) Vortex 1 min, centrifuge 2500-3000 rpm, 5 min

III. Back extraction

10) Transfer organic phase into 15 ml/conical centrifuge tubes which contain 40 1 0.1 N acetic acid in the bottom tip (pasteur pipets work well for transfer). Important to completely exclude the aqueous phase since this contains a large amount of unreacted ^{3}H-SAM.

11) Vortex 1 min, centrifuge 2500-3000 rpm, 5 min

IV. TLC

12) Carefully discard organic phase using suction

13) Spot 25 1 of the acetic acid extract onto Whatman silica gel plates with preabsorption area. After complete dryness, develop for about 3 hours in a solvent system composed of:

	Ratio	for 1 chamber
tertiary amyl alcohol	10	40 ml
toluene	4	16 ml
methylamine (40%)	5	20 ml

14) Remove plate, allow to dry under hood, and place under UV lamp for a minimum of 4 hours, preferably overnight.

- if TLC plates without pre-absorption area are used, spot one-half of sample at a time and allow for complete dryness between them

- the developing system requires saturation, e.g., use tape on the chamber lid or vacuum grease with a heavy object on top

- under the above conditions, R_f is as follows: MT = 0.67, MN = 0.53, NMN = 0.47. There is no cross-over between MT and NMN so if only DA and/or NE are present in the sample (e.g., brain tissue) no correction is needed. With other combinations of CA products, there is cross-over, and the percent cross over should be determined using the standards and the results corrected accordingly.

V. Liquid scintillation (LS) counting

15) When the carrier spots are clearly visible, scrape the spots into LS vials.

16) Add to each vial 1 ml of mixture of ethyl acetate: glacial acetic acid: H_2O (3:3:1)

17) After 30 min, add 10 ml of ACS (Amersham), shake, and count.

VI. Calculations

For nervous tissue:

1. Since DA and NE come as HCl, the weight due to the HCl must be subtracted off the standard amounts; e.g., DA MW = 153.18; DA HCl MW = 189.64 19.2% is due to HCl

   1000 pg DA HCl = 808 pg DA
   500 pg DA HCl = 404 pg DA
   250 pg DA HCl = 202 pg DA
   125 pg DA HCl = 101 pg DA
   62.5 pg DA HCl = 50.5 pg DA
   31.25 pg DA HCl = 25.25 pg DA

   NE MW 169.18 17.7% is due to HCl
   NEHCl MW 205.64

   1000 pg NE HCl = 823 pg NE
   500 pg NE HCl = 411.5 pg NE
   250 pg NE HCl = 205.7 pg NE
   125 pg NE HCl = 102.9 pg NE
   62.5 pg NE HCl = 51.4 pg NE
   31.25 pg NE HCl = 25.7 pg NE

2. Plot standard curve after subtracting cpms of reagent blank and cpms of sample used for the standard from each standard point

3. Calculate best straight line using linear regression analysis. Be sure that 0,0 is included as a standard value.

4. Subtract reagent blank cpms from each sample and calculate pg from regression equation

5. Multiply by tissue dilution factor to get total CA in whole tissue

6. Can express per tissue protein, or wet weight or even DNA

- same as above for plasma except CA usually expressed as pg/ml plasma

- minimum detectable dose is taken to be 2x the cpms of the reagent blank and is usually 25-35 pg/tube.

Reagents and Materials for CA Assay

- 0.1 N $HClO_4$ plus 5 mM GSH
- CA stds DA HCl
  NE HCl
- sample for internal standard
- reaction mixture
  2 M Tris-buffer (pH 9.1)
  0.25 M EGTA (pH 9.1)
  1 M $MgCl_2$
  0.25 M GSH
  H_2O
- ^3H-SAM (ICN)
  1 mCi/1.0 ml in H_2SO_4/EtOH (9:1), 14 Ci/mmol
- carrier mixture
  3 methoxytyramine, normetanephrine, metanephrine each at 10 mg/ml in 0.01 N HCl
- 0.45 M borate buffer (pH 10.5)
- toluene:isoamylalcohol
- 0.1 N acetic acid
- solvent system for TLC
  tertiary amyl alcohol:toluene:methylamine (40%)

1- Tris HCl 158 g/mole x 2 moles/l = 316 g/l x 0.050 l =

15.8 g in 50 ml

Tris base 121.1 g/mole x 2 moles/l = 242.2 g/l x 0.50 l =

12.12 g in 50 ml

pH 2M Tris base with 2M Tris HCl to pH 9.1

2- 50 ml 0.25 M EGTA

380.4 g/mole x 0.25 mole/l x 0.050 l = 4.75 g in 50 ml

pH to 9.1 w/ NaOH

3- 1 M $MgCl_2$ we have $MgCl_2 \cdot 6H_2O$ MW 203.3

203.3 g/mole x 1 mole/1 x 0.05 1 = 101.g in 50 ml

4- 0.25 M GSH

307.3 g/mole x 0.25 mole/1 x 0.01 1 = 0.76 g in 10 ml

5- 0.45 M Borate (pH 11) $NaBO_4 \cdot 10\ H_2O$

381.37 g/mole x 0.45 mole/1 x 0.05 1 = 8.58 g in 50 ml

pH to 10.5 w/ NaOH and heat until it goes in

6- 0.01 N HCl we have 2 N HCl

we want 50 ml 0.25 ml of 2 N HCl in 49.75 ml

weigh 30 mg each of 3-MT, NM & M and add 3 ml 0.01 N HCl. Store in brown bottle.

7- we want 0.1 N $HClO_4$ plus 5 mM glutathione

MW $HClO_4$ = 100.46

100.46 g/mole x 0.1 mole/1 x 0.5 1 = 5.023 g $HClO_4$ in 500 ml

we have 70% $HClO_4$ 70 g/100 ml = 5.023/"x" ; we need 7.17 ml $HClO_4$ in 500 ml H_2O

MW GSH = 307.3 g/mole

307.3 g/mole x 0.005 mole/1 x 0.5 1 = 0.768 g or 768 mg

Procedure for COMT Purification

1) Everything performed at 4°C; 50 g of rat liver is homogenized in 200 ml 0.154 M KCl.
2) Centrifuge for 30 min at 28,000 x g
3) Filter supernatant through cotton gauze and titrate to pH 5 with 1.0 M acetic acid
4) Centrifuge at 12,000 x g for 20 min
5) Save supernatant and measure out 150 ml
6) Add 26 g of ammonium sulfate (0-30%), stir and centrifuge at 12,000 x g for 20 min. Save supernatant
7) Add 17 g of ammonium sulfate to supernatant (30-50%). Stir, centrifuge at 12,000 x g for 20 min and save pellet
8) Add 25 ml of 1mM phosphate buffer (pH 7.0) to pellet, suspend it, and dialyze against two changes of 2 liters of phosphate buffer (pH 7.0) containing dithiothreitol at 0.1 mM
9) Centrifuge dialyzed solution at 12,000 x g for 30 min to remove precipitate
10) Titrate supernatant to pH 8.1 with 2M Tris buffer (pH 8.2)
11) Add sufficient dithiothreitol and pargyline to make final concentrations of 5 mM and 0.1 mM respectively
12) Aliquot (500 µl/tube), ended with 32 ml enzyme)

Reagents Needed for COMT Purification

- 400 ml KCl 0.154 M
- 1 M acetic acid
- ammonium sulfate 26 g and 17 g
- 25 ml 1mM phosphate buffer
- 3 liter 1mM phosphate buffer with 0.1 mM dithiothreitol
- dithiothreitol and pargyline were added to final solution to make final concentrations of 5 mM and 0.1 mM respectively

1- To make up 1 mM or 0.001 M phosphate buffer

pKa_2 of PO_4 = 7.2

$7.0 = 7.2 + \log \frac{[HPO_4^{-2}]}{[H_2PO_4^{-}]}$; x = $[HPO_4^{--}]$, y = $[H_2PO_4^{-}]$

Ratio = 0.631

since we want 4 1

0.01 M x 4 1 = 0.004 moles

$\underline{x} = 0.631$ also x + y = 0.004

y $\quad$ 0.631 y + y = 0.004

x = 0.631 $\quad$ 1.631 y = 0.004

y = .0024 moles

x = 0016 moles

0.0016 mole $[HPO_4^{-2}]$ dibasic x 141.96 g/mole = 0.2271 g

0.0024 mole $[H_2PO_4^{-}]$ monobasic x 138.01 g/mole = 0.3312 g

2- 0.154 moles KCl/1 x 0.4 1 = 0.0616 moles

74.55 g KCl/mole x 0.0616 moles = 4.59 g in 400 ml

3- 100 ml 1M AcA 60.05 g/mole

60.05 g/liter $\quad$ 6.00 g/100 ml H_2O

6.00 ml/100 ml H_2O

4- 4 1 PO_4 buffer $\quad$ 0.1 mM DTT $\quad$ 154.25 g/mole x 0.0001 moles/1

= 0.01542 g/1 x 4 1 = 0.0617 g

30 mls enzyme

pargyline MW 195.7

dithiothreitol MW 154.2

0.005 M dithiothreitol x 0.03 l = 0.00015 moles

154.2 g/mole x 0.00015 mole = 0.02313 g or 23.1 mg

0.001 M pargyline x 0.03 l = 0.000003 moles

195.7 g/mole x 0.000003 = 0.00058 g or 0.58 mg

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