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Pineal-mediated inhibition of prolactin cell activity: Investigation of dopaminergic involvement

Burns, Danny Michael, Ph.D.

The University of Arizona, 1989
PINEAL-MEDIATED INHIBITION OF PROLACTIN CELL ACTIVITY:
INVESTIGATION OF DOPAMINERGIC INVOLVEMENT

by
Danny Michael Burns

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF ANATOMY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1989
As members of the Final Examination Committee, we certify that we have read
the dissertation prepared by Danny Michael Burns
entitled Pineal-Mediated Inhibition of Prolactin Cell Activity:
Investigation of Dopaminergic Involvement

and recommend that it be accepted as fulfilling the dissertation requirement
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ABSTRACT

The purpose of these studies was to determine whether the inhibitory effects of short photoperiod exposure on prolactin cell activity in male Syrian hamsters and/or the inhibitory effects of melatonin treatment on the growth and activity of diethylstilbestrol- (DES) induced prolactinomas in Fisher 344 (F344) rats were possibly mediated through alterations in dopaminergic regulatory mechanisms. In both the hamster and the rat, changes in hypothalamic dopamine neuronal activity and changes in pituitary responsiveness to dopamine have been suggested as possible mechanisms in the prolactin-inhibitory effects of light deprivation or melatonin administration.

The present studies in the male Syrian hamster addressed two issues. First, it was of interest to determine if anterior pituitaries of long photoperiod-exposed male hamsters possess dopamine receptors, which are presumably necessary for responsiveness to dopamine. This was accomplished by analysis of $^3$H-spiperone binding to anterior pituitary membranes. Second, possible changes in pituitary sensitivity to dopamine were assessed by comparison of dose response curves for the inhibition by dopamine of prolactin release from hemipituitaries incubated in vitro from both long and short photoperiod-exposed animals over a series of time points from three to fifteen weeks.

In the second series of experiments, adult female F344 rats received daily injection of melatonin or saline vehicle. After two weeks, half of the animals were sacrificed for analysis of $^3$H-spiperone binding to anterior pituitary membranes,
measurement of hypothalamic dopamine turnover and analysis of in vitro pituitary sensitivity to dopamine. The remaining animals received subcutaneous implants containing DES and injections were continued on the same schedule until sacrifice four weeks later for measurement of the same parameters.

In both the hamster and rat models, treatments exerted profound inhibitory effects on indices of prolactin cell activity. However, these studies provide no evidence for the involvement of altered dopaminergic regulation in the production of such effects. Neither pituitary sensitivity to dopamine in vitro nor hypothalamic dopamine neuronal activity was enhanced by short photoperiod exposure or melatonin treatment. Prolactin-inhibitory effects of these treatments appear to be mediated through as yet unidentified dopamine-independent mechanisms.
CHAPTER 1

OVERVIEW OF THE LITERATURE

Neuroendocrine Regulation of Prolactin Cell Activity

A tonic inhibitory influence of the hypothalamus over PRL secretion is well documented (Weiner and Bethea, 1981). While PRL secretion is influenced by numerous substances found in the hypothalamus, it is generally accepted that the predominant influence is exerted by dopamine (DA) released from neurons of the hypothalamic tuberoinfundibular dopamine (TIDA) system (see Ben-Jonathan, 1985; Reymond and Porter, 1985).

TIDA System

Anatomy and Vascular Relationships

The hypophyseal portal system consists of a dense network of primary capillaries in the median eminence, pituitary stalk and neural lobe, supplied by the superior hypophyseal artery (Flerko, 1980). The primary capillary plexus is confluent with the sinusoids of the pars distalis via the long portal veins originating in the median eminence and the short portal veins originating in the neural lobe. Thus, the venous outflow of the median eminence represents the major blood supply of the anterior pituitary. Neurosecretory substances including dopamine are released from hypothalamic neurons in the median eminence, diffuse into capillaries of the primary plexus and are transported to the anterior pituitary by the long portal veins or via the neural lobe by the short portal veins (Flerko, 1980; Ben-Jonathan, 1985).
The perikarya of TIDA neurons are found in the arcuate and immediately adjacent periventricular nuclei of the medial basal hypothalamus (MBH), with projections to the median eminence and pituitary stalk. Abundant DA terminals may be found in the zona externa of the median eminence (ME), in close association with other neuron terminals, ependymal cells and pericapillary spaces of the hypophyseal portal vessels (Moore and Bloom, 1978). While numerous pharmacological studies had implicated DA as a prolactin inhibitory factor (discussed below), validation as a hypophysiotrophic hormone required the demonstration of its presence in portal blood at concentrations appropriately reflective of observed changes in PRL secretion (Ben-Jonathan, 1985). With the development of sensitive assay methods, it was determined that DA, but not epinephrine or norepinephrine, is present in hypophyseal portal plasma at levels higher than in the general systemic circulation, and in sufficiently high concentration to inhibit PRL release (Ben-Jonathan et al., 1977; Gibbs and Neill, 1978). In general, an inverse relationship has been found between DA release from the hypothalamus and PRL release from the pituitary (Reymond and Porter, 1985). A complex and dynamic interplay between DA and PRL and other hypothalamic substances and pituitary hormones, however, obscures any simple relationship between TIDA neuronal activity and pituitary PRL release.

Measurement of TIDA Neuronal Activity

The activity of TIDA neurons and the secretion of DA into hypophyseal portal plasma have been studied under a variety of experimental conditions (Moore and Demarest, 1982). Many of the biochemical methods employed for estimation of
activity in DA neuron populations are based upon the assumption that under steady-state conditions, the rate of DA synthesis within terminals must be equivalent to the rate of release. Thus, measurement of the rate of decline of DA following administration of the tyrosine hydroxylase inhibitor α-methyl-para-tyrosine (α-MPT) has been widely utilized as a method of estimating DA neuronal activity. Measurement of the rate of accumulation of dihydroxyphenylalanine (DOPA) following administration of decarboxylase inhibitors such as 3-hydroxybenzylhydrazine has been similarly employed. DA release has also been measured directly, both from hypothalami in vitro and by cannulation of long portal veins. Other methods of estimating DA neuronal activity such as direct electrical recording and measurement of DA metabolite accumulation have not proven applicable to the TIDA system (Moore and Demarest, 1982).

Special Properties of the TIDA System

The TIDA system differs from other DA systems in several ways (Moore and Demarest, 1982). As indicated above, TIDA terminals do not form true synapses but rather end in close approximation to pericapillary spaces of the hypophyseal portal system. Dihydroxyphenylacetic acid (DOPAC), a metabolite of DA, is found in low concentration in the median eminence compared to other brain regions containing DA terminals. This is probably related to the fact that DA released from TIDA terminals is rapidly carried away in the portal blood. In addition, a high affinity DA uptake mechanism found in nigrostriatal and mesocortical DA neurons is lacking in TIDA neurons. TIDA neurons also appear to lack autoreceptors. DA agonists decrease activity and DA antagonists increase activity in nigrostriatal DA
neurons, while local administration of agonists or antagonists has no effect on the rate of DA turnover in the median eminence (Moore and Demarest, 1982).

**TIDA Neurons and PRL Feedback Regulation**

A final important difference of the TIDA system from other DA systems is feedback regulation by PRL. The results of many different experimental approaches indicate a direct role for PRL in the regulation of TIDA neuronal activity. Intracerebroventricular (ICV) or subcutaneous administration of PRL results in increased DA turnover in the median eminence but not in other brain areas (Annunziato and Moore, 1978; Moore et al., 1980; Selmanoff, 1981) and also in increased release of DA into pituitary stalk blood (Gudelsky and Porter, 1980). Elevation of serum PRL levels by pituitary transplantation has a similar stimulatory effect on TIDA neuronal activity (Hohn and Wuttke, 1978; Morgan and Herbert, 1980). The acute administration of DA antagonist drugs has also been shown to result in increased ME DA synthesis (Perkins, et al., 1979), increased ME DA turnover (Moore et al., 1980) and increased release of DA into stalk blood (Gudelsky and Porter, 1980). Hypophysectomy (Perkins et al., 1979) or pretreatment with anti-serum to PRL (Gudelsky and Porter, 1980) reverses these effects, indicating that they are mediated through elevation of PRL levels. Demarest and co-workers (1984) have reported evidence for two distinct components of PRL-induced activation of TIDA neurons, a rapid "tonic" component and a delayed "induction" component. DA synthesis in the ME is first increased within four hours of elevation of PRL levels in the systemic circulation or cerebrospinous fluid (CSF), with a further increase seen after 12-16 hours. Only this delayed component can
be blocked by cyclohexamide, indicating the involvement of protein synthesis. The delayed component appears to represent an increase in the capacity of the rapid component, as appearance of the delayed component can be prevented if PRL levels are reduced by administration of bromocryptine 4 hours prior to measurement. It therefore appears that TIDA activity is determined by acute changes in circulating PRL with the capacity of this response determined by previous patterns of PRL concentrations (Demarest et al., 1984).

Gender and TIDA Activity

There are sex related differences in the activity of TIDA neurons. In general, the rates of ME DA synthesis and release to the hypophyseal portal blood are several fold greater in females than in males (Gudelsky and Porter, 1981). This may be an estrogen-dependent phenomenon. Administration of estrogen to males or to ovariectomized females results in increased synthesis and turnover of DA exclusively in the median eminence. This effect appears to be PRL-mediated as it is abolished by hypophysectomy (Elkenberg et al., 1977; Demarest and Moore, 1981). TIDA neurons in females are also more sensitive to changes in circulating PRL levels. Procedures that increase PRL levels result in greater increases in TIDA activity in females than in males and conversely, manipulations that result in lowered PRL levels decrease DA activity in the ME of females much more markedly than in males (Moore and Demarest, 1982).

Reports concerning alterations in TIDA activity during the estrus cycle have been variable. However, most investigators report a decrease in ME DA turnover on the afternoon of proestrus, coinciding with the proestrus PRL surge (Ben-
Jonathan, 1985). This finding is also consistent with the report of lowered levels of DA in portal plasma on proestrus compared to other days of the estrous cycle (Ben-Jonathan et al., 1977). In contrast, Gibbs and Neill (1978) did not observe a significant difference in stalk plasma DA concentrations between diestrus and proestrus.

Lactation and TIDA Activity

Suckling is the primary stimulus for the rapid discharge of PRL into the circulation during lactation in the rat. The increase in PRL becomes significant within 2 to 3 minutes and reaches plateau levels within about 30 minutes (Neill, 1980). This phenomenon appears to be a simple stimulus-bound neuroendocrine reflex. Biochemical experiments suggest that TIDA neuronal activity, but not activity in other DA pathways in the brain, is reduced in the lactating rat and is further reduced reflexly by suckling (Moore et al., 1983). There appears to be a lack of responsiveness of TIDA neurons to the stimulatory feedback of PRL in lactating animals. Administration of the DA antagonist drug haloperidol or ICV injection of PRL, treatments which result in increased DA turnover in the median eminence within 12-16 hours in control rats, have no effect on TIDA activity in lactating animals. The mechanism underlying this lack of responsiveness is unknown (Moore et al., 1983).

It may be that a decreased release of DA into hypophyseal portal blood is not solely responsible for suckling induced PRL release. It has been reported that stimulation of the mammary nerve induces a short-lasting decrease in DA levels in stalk blood, but that DA levels have returned to basal levels at the time of maximal
PRL release (DeGreef and Visser, 1981). Furthermore, isolated mammary nerve stimulation results in a several-fold increase in serum PRL levels, but only a slight (20%) decrease in stalk plasma DA concentration. DA infusion studies in α-MPT-treated animals indicate that similar diminution in DA concentrations results in more modest elevation of serum PRL levels (Neill, 1980). These studies are complicated, however, by the technical impossibility of measuring stimulus-bound PRL elevation and stalk plasma DA concentration in the same animal. The effects of anesthetic agents provide an additional layer of complication in interpretation. DeGreef and Visser (1981) observed an increase in thyrotropin releasing hormone (TRH) in pituitary stalk blood induced by mammary nerve stimulation, and suggest that a brief decline in DA concentration followed by an increase in TRH could account for the suckling induced rise in PRL release. Other substances with PRL releasing activity may also be involved in PRL regulation during lactation (Kiss et al., 1985).

Aging and TIDA Activity

It is well established that aging in both male and female rats is characterized by hyperprolactinemia and a decline in the concentration of DA in the median eminence and hypophyseal portal blood (Ben-Jonathan, 1985). This has been associated with decreased DA biosynthesis in TIDA neurons in aged rats (Demarest et al., 1982; Arita and Kimura, 1986). It does not, however, appear to be related to loss of TIDA neurons with aging, as the number of tyrosine hydroxylase-containing perikarya in the arcuate nuclei of young and aged rats is approximately the same (Ben-Jonathan, 1985). Despite the apparent decrease in TIDA function with aging, the concentration of DA in the adenohypophysis is
paradoxically increased in aged rats (Demarest et al., 1982). It has been suggested that a diminished ability of the anterior pituitary to respond to DA may account for the hyperprolactinemia of aging (Arita et al., 1984).

**Pituitary Site of Action**

Inhibition of PRL Release

Dopamine has been shown to inhibit the release of PRL from incubated pituitaries in a dose-dependent fashion, reversible by the addition of DA antagonists haloperidol or perphenazine (MacLeod et al., 1970; MacLeod and Lehmeyer, 1974). Caron and associates (1978) tested a wide variety of DA antagonist drugs for their ability to reverse DA-induced inhibition of PRL release from hemipituitaries in vitro. Their results indicate that the reversal of PRL inhibition produced by these drugs is dose-dependent, stereospecific and directly correlated with the ability of these compounds to compete for pituitary DA receptors. Dopaminergic agonists including apomorphine and the ergot alkaloids have also been shown to inhibit PRL release both in vivo (Clemens et al., 1974; Smalstig et al., 1974) and in vitro (Caron et al., 1978). Injection of L-dihydroxyphenylalanine (L-dopa) into rats bearing pituitary transplants results in a significant decline in serum PRL concentrations (Donoso et al., 1974). Pretreatment of the animals with a dopa decarboxylase inhibitor prevented the decline in PRL levels, indicating that conversion of L-dopa to dopamine is required to produce this effect. Drugs which have DA-releasing activity inhibit PRL release in vivo but not in vitro (Clemens and Shaar, 1980). Methylphenidate, which releases DA from storage pools, has little effect on basal PRL levels, but depresses the elevated serum PRL levels in rats treated with
α-MPT, which blocks DA synthesis. Similarly, amphetamine, which releases newly synthesized DA, reverses the rise in serum PRL seen after reserpine-induced depletion of DA stores (Clemens and Shaar, 1980).

The anterior pituitary contains measureable concentrations of DA (Saavedra et al., 1975). If this reflects DA released from TIDA neurons, transported to the anterior pituitary via the portal circulation, and subsequently bound to DA receptors or internalized within lactotrophs, then PRL cell activity might be correlated with anterior pituitary DA content (Demarest et al., 1982). Adenohypophyseal content of DA is inversely correlated with PRL release in rats during the estrous cycle, with lowest concentrations of DA occurring on the afternoon of proestrus, coincident with the proestrus PRL surge (Chiocchio et al., 1980). In lactating rats, separation from pups results in decreased levels of serum PRL and increased adenohypophyseal DA concentration while suckling induces a rapid rise in serum PRL and a significant decrease in DA concentration in the pars distalis (Chiocchio et al., 1979). A consistent inverse correlation between PRL release and anterior pituitary DA content in these studies contrasts with other reports. Ben-Jonathan and colleagues (1980) did not detect any changes in the concentrations of DA in the hypothalamus, anterior or posterior pituitary as a result of pup separation. Furthermore, a direct correlation between the hyperprolactinemia of aging and increased DA concentration in the adenohypophysis has been reported (Demarest et al., 1982). This discrepancy may be related to altered DA processing in the pituitaries of aged rats.
Despite the wealth of data indicating a physiological role for DA in the inhibition of PRL release from the anterior pituitary, it remains an unanswered question whether DA alone can account completely for tonic hypothalamic inhibition of PRL. In rats treated with α-MPT to diminish hypothalamic inhibition of PRL, systemic infusion of DA at rates resulting in DA concentrations in the blood which mimic measured concentrations in stalk plasma produces only a partial inhibition of the rise in serum PRL over baseline (Leong et al., 1983). Full suppression of α-MPT-induced PRL release requires DA infusion at rates which produce supraphysiological concentrations. In contrast, other studies utilizing similar methods have indicated that DA infusion at rates producing physiological DA concentrations reduces serum PRL to near baseline levels (Gibbs et al., 1979). These discrepancies may be explained, at least in part, by differences in the endocrine status of experimental animals. In the former case, where DA infusion failed to exert complete inhibition over α-MPT-stimulated PRL release, the animals were ovariectomized. In the latter case, the animals were left intact. Other lines of evidence indicate an important role of steroid milieu in modulation of pituitary responsiveness to DA. In the rat, alterations in the relative proportions of estradiol and progesterone result in alterations in both the ability of DA to inhibit PRL release from dispersed cells and in patterns of DA receptor binding in anterior pituitary membranes (Bression et al., 1985). In the rhesus monkey, DA exerts greater inhibition over PRL release in intact follicular phase and estrogen-treated stalk-transected animals than in untreated stalk-transected monkeys (Neill et al., 1981), suggesting a sensitizing role for estrogen over lactotroph responsiveness to DA in primates. Clear understanding of the relationship of estrogen to anterior pituitary
DA-responsiveness in the rat is complicated by PRL-releasing activity of estrogentic compounds in this species (vide infra).

Paradoxical Stimulation of PRL Release

Interestingly, Denef and colleagues (1984) have reported that the pattern of DA administration in superfused rat anterior pituitary cells significantly influences the character and magnitude of PRL release during DA administration and following withdrawal of DA. Continuous application of 0.1 to 1.0 nM DA results in rapid but unsustained inhibition of PRL release, followed by a gradual rise in secretion back to baseline levels. Continuous exposure to higher concentrations of DA (up to 10 nM) produces sustained inhibition. Withdrawal of 10 nM DA is followed by a rapid and marked rebound in PRL secretion, returning to baseline levels only after 40-50 minutes. This post-withdrawal release represents a stimulation of PRL secretion as the PRL mass released above baseline exceeds the mass of PRL inhibition below baseline during DA exposure. Pulsatile (4 minutes on, 4 minutes off) administration of DA results in even more dramatic stimulation of PRL release following withdrawal (Denef et al., 1984). Similarly, in stalk-sectioned female rhesus monkeys, brief interruptions in DA infusion result in large increases in serum PRL concentrations (Frawley and Neill, 1984).

Other studies also raise the possibility of a stimulatory role for DA on PRL release. It has been reported that at very low concentrations, apomorphine markedly stimulates PRL release from dispersed anterior pituitary cells in monolayer culture, while administration of higher concentrations results in the more well known inhibition of secretion (Denef, 1979). The inhibitory effect but not
the stimulatory effect is reversed by co-incubation with the antagonist domperidone, suggesting the possibility of distinct receptor populations. Furthermore, pre-treatment with estradiol enhances this stimulatory activity. Denef and co-workers (1984) have speculated that the stimulatory effects of DA, while still present, are masked by its inhibitory action at higher concentrations. This hypothesis might account for the rebound PRL surges seen following withdrawal of DA after brief or pulsatile exposures. Paradoxical stimulation of PRL synthesis and release of newly synthesized but not stored PRL by low dose DA has also been reported (Cheung et al., 1981). In healthy human volunteers, continuous infusion of DA at high concentrations produced a sustained significant decrease in serum PRL levels for at least 48 hours (Kaptein et al., 1980). Withdrawal of DA resulted in a rebound elevation in serum PRL concentration proportional to the degree of inhibition. These results suggest that the post-infusion rise in PRL titers represents the release of PRL accumulated during the period of infusion, and do not suggest the unmasking of stimulatory DA activity. This does not conflict with the results of Denef et al. (1984), however, as they reported a lack of post-withdrawal stimulation of PRL release if the period of continuous DA infusion exceeded 30 minutes.

Sensitivity to DA inhibition of PRL secretion is significantly enhanced in long term medial basal hypothalamus-lesioned rats versus short term lesioned and control rats (Cheung and Weiner, 1978). In these studies, significant inhibition of PRL release was observed at concentrations of DA as low as 10 nM in long term (two weeks) lesioned animals, while inhibition was only achieved in non-lesioned animals at 100-fold higher doses. A stimulatory effect of DA on PRL release was
seen in non-lesioned animals at 10 nM DA, and a slight stimulatory effect was seen in lesioned animals at still lower DA concentrations. This provides further evidence for a possible stimulatory role for DA in the regulation of PRL release and indicates that the acute ability of DA to regulate pituitary PRL release is modulated by previous patterns of pituitary exposure to DA.

Effects on PRL Synthesis and Newly Synthesized PRL

Early studies on the effects of DA on PRL release (MacLeod and Lehmeyer, 1974), indicated that the release of newly synthesized PRL is much more sensitive to inhibition by DA than the release of total or radioimmunoassayable PRL. Subsequent studies have provided further evidence for specific and perturbable effects of DA on the synthesis of PRL and inhibition of release of newly synthesized PRL. Dopamine and the DA agonists ergocryptine and bromoergocryptine have been shown to significantly reduce PRL synthesis in a time- and dose-dependent manner in dispersed anterior pituitary cells in monolayer culture (Maurer, 1980) and in vivo (Brocas et al., 1981), as estimated by measurement of incorporation of $^3$H-leucine into total PRL. This effect is specific for PRL, as total protein synthesis is unaffected. The effect is somewhat slow, achieving 50% reduction in PRL synthesis at approximately two days. Withdrawal of the DA agonist drugs results in a reversal of inhibition with a return to control rates of PRL synthesis within four days (Maurer, 1980).

It has also been reported that lesions in the medial basal hypothalamus result in increased sensitivity of the pituitary to the effects of DA on PRL synthesis and release of newly synthesized PRL (Cheung et al., 1981). Pituitaries from animals
bearing MBH lesions for two weeks release several-fold more newly synthesized PRL into incubation medium than pituitaries from non-lesioned animals, and this release is significantly inhibited in lesioned animals at concentrations of DA as low as 10 nM. Similar to earlier studies on the effects of DA on the release of radioimmunoassayable PRL, 100-fold higher concentrations were required to produce significant inhibition of release of newly synthesized PRL in non-lesioned animals, and a significant stimulatory effect on release was seen in non-lesioned animals at 10 nM. In non-lesioned animals, DA had no effect on the amount of total (media plus pituitaries) newly synthesized PRL, while all concentrations of DA from 10 nM to 1 μM significantly reduced the total amount of newly synthesized PRL in the lesioned group in a dose-related manner. Since these effects on synthesis were observed in short term (3 hours) incubations, this study appears to conflict with other reports concerning the time course of DA effects on PRL synthesis, as discussed above. However, the actual measurements made by Cheung et al. were of the amounts of labeled PRL present at the end of incubations, and not measurements of PRL synthesis per se. It may be that the observed decreases in amount of newly synthesized PRL represent increased rates of intracellular degradation of the hormone (vide infra) under conditions of release inhibition, and not real decreases in synthetic rates.

The DA-induced reduction in PRL synthesis appears to be mediated primarily at pre-translational levels. Treatment with ergocryptine or bromoergocryptine results in a reduction in the concentration of PRL messenger RNA (PRL mRNA), quantified by hybridization of total cell mRNA to clonal PRL DNA (PRL cDNA) (Maurer, 1980; Brocas et al., 1981). Ergocryptine-induced reductions in PRL
mRNA concentrations demonstrate dose-dependency which corresponds closely to dose response curves for inhibition of PRL synthesis. The time course of ergocryptine effects on PRL mRNA concentration likewise accompanies changes in PRL synthesis rates (Maurer, 1980). The relatively slow onset of PRL synthesis inhibition induced by DA agonists might therefore be explained by a long half-life of PRL mRNA.

PRL Storage and Degradation

Experimental evidence suggests that DA and DA agonists, in addition to their effects on the synthesis and release of PRL, also induce the intracellular degradation of PRL. Smith and Farquar (1966) first reported the incorporation of PRL secretory granules into lysosomes under conditions of PRL release inhibition. This observation has been confirmed and extended by Nansel and co-workers (1981), who examined the ultrastructural and biochemical effects of DA on lysosomal activity, both in vivo and in vitro. PRL cells in pituitary tissue from animals treated for 8 hours with L-dopa contained more lysosomes than PRL cells from vehicle-treated animals, and many of the lysosomes in L-dopa-treated rats appeared to have undergone fusion with PRL secretory granules. L-dopa treatment also resulted in an increase in β-glucuronidase (a lysosomal enzyme) activity in lysosome-containing subcellular fractions, whereas treatment with haloperidol or α-MPT resulted in decreased β-glucuronidase activity. Incubation of pituitary tissue with DA resulted in a dose-related increase in lysosomal enzyme activity, and yielded dose-response curves which were superimposable on curves for PRL
release inhibition. These effects were prevented by co-incubation with the DA antagonist cis-flupenthixol (Nansel et al., 1981).

Bromoergocryptine has been shown to reduce the accumulation of PRL in primary cultures of dispersed pituitary cells by more than 80% versus control cultures over a six day treatment period (Dannies and Rudnick, 1980). A similar time course and magnitude of effect has been observed for newly synthesized PRL (Maurer, 1980). Pulse-chase labeling experiments with $^3$H-leucine have indicated a relatively rapid decline in intracellular levels of newly synthesized PRL in bromoergocryptine-treated cultures (Dannies and Rudnick, 1980; Maurer, 1980), with the total amount of labeled hormone falling to approximately 50% of starting concentration over 8-12 hours of incubation. In control cultures a slower rate of hormone degradation was observed, producing a 25% decline in labeled PRL over the same time period (Maurer, 1980). Cyclohexamide was able to block bromoergocryptine-induced PRL degradation if both drugs were added simultaneously, but not in cultures pre-treated with bromoergocryptine. This indicates that protein synthesis is necessary for the initiation but not for the short term maintainance of hormone degradation, and further, that this process is inducible and not simply a passive result of increased cellular content of PRL (Maurer, 1980). Chloroquine, which inhibits lysosomal activity, attenuated the rate of PRL degradation in bromoergocryptine-treated cultures.

Functional Heterogeneity of PRL Pools

Evidences for differential effects of DA on newly synthesized versus total PRL are consistent with a number of reports suggesting the existence of heterogenous
intracellular pools of PRL within PRL-secreting cells. Experimental evidence suggests that minimally two separate PRL pools exist, a rapidly releaseable pool and a store with slower turnover rates (Swearingen, 1971; Walker and Farquar, 1980). The rapidly releasable pool consists largely of newly synthesized PRL, while the more slowly released pool contains a greater proportion of stored PRL. Thyrotropin-releasing normone (TRH) preferentially stimulates release of PRL from the slow turnover pool (Walker and Farquar, 1980). Based upon the observation that within the first minutes of suckling there is an apparent decrease in pituitary content of PRL which exceeds the amount of PRL released into the serum during the same period, Grosvenor and colleagues (1979) have suggested that PRL passes through two separate stages under conditions of stimulated release, a depletion or transformation stage and a release stage. The finding that TRH stimulates a greater release of PRL over baseline in recently suckled than in pup-deprived lactating rats (Grosvenor and Mena, 1980) provides further evidence for the hypothesis that PRL-releasing stimuli may induce the transformation of stored PRL to a more readily releasable pool. The processes of transformation and release also appear to be differentially regulated by DA. Again in lactating rats, bromoergocryptine administration prior to suckling prevents the suckling-induced increase in PRL release, but bromoergocryptine administered during a suckling period does not prevent the increased release associated with further suckling (Grosvenor et al., 1980).

It is also possible that these functionally heterogenous pools of PRL do not exist within the same mammotrophic cells. Walker and Farquar (1980) have reported evidence indicating the existence of heterogenous populations of PRL-
producing cells. In these studies, three distinct populations of mammotrophs could be distinguished based upon differential rates of labeling with $^3$H-leucine in pulse-chase experiments. In addition, the rates of loss of label due to release of $^3$H-PRL over time were not uniform among the three populations. The mammotrophic population which exhibited the most rapid synthetic rate also lost label at the fastest rate via release of this newly synthesized PRL (Walker and Farquar, 1980). It has also been reported that individual PRL-secreting cells demonstrate major differences in relative biopotency and immunopotency of released PRL, in that some cells release PRL with high biopotency but very low immunoreactivity, whereas the converse is true for other cells (Frawley et al., 1986), lending further support to the idea of functionally heterogenous populations of PRL cells.

Regulation of PRL Cell Proliferation

Several lines of evidence indicate an inhibitory role for DA over the rate of proliferation of PRL-producing cells of the anterior pituitary. Indirect evidence for such a role is provided by the observation that in female rhesus monkeys three weeks following pituitary stalk section, there is a several-fold increase in the numbers of immunostained mammotrophs as compared to intact controls, with no change in the numbers of gonadotrophs, corticotrophs or somatotrophs (Antunes et al., 1980). Similarly, in MBH-lesioned ovariectomized female rats, the volume percentage of PRL-containing cells in the anterior pituitary is increased over both intact controls and non-lesioned ovariectomized animals (Cronin et al., 1982). While it is tempting to speculate that these effects are related to elimination of the tonic inhibition exerted by hypothalamic DA, these studies do not address
dopaminergic control directly, and furthermore, the latter studies in MBH-lesioned rats do not differentiate between hyperplasia and hypertrophy of PRL-producing cells.

Numerous studies have addressed more directly the role of DA in regulation of mammotroph proliferation. While the majority of these studies have tested the effects of DA and dopaminergic drugs on estrogen-stimulated mammotroph proliferation, a few such studies have measured the effect of DA on various indices of proliferation in otherwise untreated pituitaries. The DA agonists bromoergocryptine (Jacobi and Lloyd, 1981; Komolov et al., 1985) and lisuride (Burdman et al., 1982) significantly decrease the uptake of \(^{3}\)H-thymidine by whole pituitary cell populations and more specifically decrease the numbers of tritium-labeled mammotrophs identified immunocytochemically. Conversely, the antagonists sulpiride (Kalbermann et al., 1979), haloperidol and pimozide (Jacobi and Lloyd, 1981) increase DNA synthesis in the rat anterior pituitary. These effects are accompanied, as expected, by inhibition of PRL release in the presence of agonists and increased PRL release in antagonist-treated animals. Similar results have been obtained in acute and short term estrogen-treated male and female rats. Dopamine (Pawlikowski et al., 1978) and bromoergocryptine (Davies et al., 1974; Lloyd et al., 1975; Pawlikowski et al., 1978) have been shown to significantly inhibit estrogen-stimulated DNA synthesis and mitoses in anterior pituitary cell populations from otherwise untreated male and female rats, while the antagonist sulpiride has been reported to enhance DNA polymerase activity in estrogen-treated male rats and pregnant rats (Jahn et al., 1980). Again it should be noted that these effects are concurrent with PRL release inhibition by agonists and enhancement of PRL
release by antagonists. Because of the consistent correlation between PRL-release inhibition and decreased cell proliferation, and conversely, between enhanced PRL release and increased proliferation, the hypothesis has been advanced that mammotroph replication is at least partially regulated on the basis of intracellular PRL concentration (Lloyd et al., 1975; Kalbermann et al., 1979; Burdman et al., 1982). Under this hypothesis, large intracellular stores of PRL inhibit mammotroph division while depletion of PRL stores through release sets in motion the machinery of cell division. Further support of this hypothesis comes from observations in rats in the last week of pregnancy (Kalbermann et al., 1979). During late pregnancy in rats, both serum concentrations and intramammotrophic concentrations of PRL are high, and DNA polymerase activity is low, compared to virgin controls. The DA antagonist sulpiride stimulates the acute release of PRL and increases DNA synthesis to control levels. Co-administration of bromoergocryptine reverses the effects of sulpiride while bromoergocryptine alone does not alter the rate of DNA synthesis compared to untreated pregnant rats. The authors suggest that bromocryptine alone does not inhibit DNA polymerase activity in these animals because intracellular PRL concentrations are already high enough to maximally inhibit this activity. The general conclusion of these various experiments is that DA modulates replication in mammotrophs at the pituitary level, although the precise mechanism of this effect remains unknown. While substantial indirect evidence supports the hypothesis that mammotrophic proliferation is inversely related to intracellular PRL stores, direct effects of DA independent of intracellular PRL concentration cannot be ruled out.
Dopamine Receptors

Classification

Dopamine (DA) exerts effects on its target cells by first binding to specific recognition sites located in the cell membrane. These recognition sites are complex proteins which interact with other membraneous macromolecules to form biologically functional ensembles, termed receptors (Ben-Jonathan, 1985). In their seminal review of the classification of DA receptors, Kebabian and Calne (1979) divided these receptors into two general categories based upon anatomical distribution, ligand specificity and primarily pharmacological/biochemical effects. The two categories were designated D1 and D2. More recent evidence also suggests the existence of a third class of DA receptors, the D3 receptor, which may actually represent an agonist high affinity state of the D1 receptor (Creese et al, 1983).

The prototype D1 receptor is located in the parathyroid gland. The hallmark of the D1 class of DA receptors is stimulation of adenylate cyclase activity by agonist occupancy (Kebabian and Calne, 1979). D1 receptors are also distinguished by the profile of pharmacological activity of a variety of drugs at these receptors. Dopamine is a complete agonist at D1 sites, with micromolar potency, while (-)-apomorphine exhibits only partial agonist activity. The phenothiazines and thioxanthenes are potent antagonists (nanomolar potency) at D1 receptors, as judged by their ability to inhibit dopamine-stimulated adenylate cyclase activity. In contrast, the butyrophenones are weak antagonists of DA-stimulated cAMP production, requiring micromolar doses, and the neurileptic benzamides exhibit
almost no inhibitory potency. Receptors with D1 characteristics have also been
demonstrated in the striatum (Creese et al., 1983).

The D2 class of DA receptors represents those receptors which are not
coupled to stimulation of adenylate cyclase activity. Agonist occupancy of D2 sites
results in either no change in adenylate cyclase activity or a decrease in cAMP
formation. Prototype D2 receptors are found in mammotrophs of the anterior
pituitary, where they are associated with dopamine-induced inhibition of prolactin
release (Kebabian and Calne, 1979). The pharmacological selectivity profile of D2
receptors is distinct from D1 receptors. Dopamine and (-)-apomorphine both
exhibit full agonistic activity at D2 sites. In addition, dopaminergic ergot alkaloids
such as bromocryptine are potent agonists at D2 receptors, whereas these
compounds act as antagonists or partial agonists at D1 sites. Regarding an-
tagons, the phenothiazines, thioxanthenes and butyrophenones all exhibit
nanomolar potency for reversal of dopamine-induced inhibition of PRL release
(Creese et al., 1983).

Putative D3 DA receptors have been demonstrated in striatum. These sites
are distinguished from D1 and D2 receptors by differential ligand specificity
(Creese et al., 1983). These sites differ from D1 receptors in that they exhibit high
(nanomolar) affinity for agonists such as dopamine and apomorphine. Like D1
receptors, D3 receptors exhibit high affinity for phenothiazines and thioxanthenes
but low affinity for butyrophenones. Agonist high affinity binding to D3 receptors
in membrane preparations is abolished by addition of exogenous GTP and is also
dependent upon the presence of divalent cations. Because of these characteristics
it has been suggested that the D3 receptor may actually represent an agonist high affinity state of the D1 receptor (Creese et al., 1983).

DA Receptors in the Anterior Pituitary

A variety of radiolabeled ligands, both agonists and antagonists, have been used to investigate dopaminergic binding in the anterior pituitary. The presence of saturable and reversible stereospecific DA receptors has been demonstrated by several groups in membrane preparations from rat (Brown et al., 1976; Cronin et al., 1978; Stefanini et al., 1980), bovine (Creese et al., 1977; Caron et al., 1978; Calabro and MacLeod, 1978; Creese et al., 1979) and ovine (Cronin et al., 1978) anterior pituitaries. The agonists dopamine (Calabro and MacLeod, 1978), dihydroergocryptine (Caron et al., 1978) and n-propylapomorphine (Kilpatrick et al., 1982) have been shown to bind with high affinity (nanomolar equilibrium dissociation constants) to anterior pituitary binding sites. As indicated above, high affinity agonist binding is a characteristic of D2 receptors. Competition for $^3$H-n-propylapomorphine binding demonstrates stereoselectivity. (+)-Butaclamol is several orders of magnitude more potent than (-)-butaclamol, as cis-flupenthixol is more potent than its trans-isomer (Sibley et al., 1982). The butyrophenones, which are reasonably selective D2 dopamine receptor antagonists (Creese et al., 1983), have been widely utilized both as tritium-labeled ligands in pituitary binding studies (Creese et al., 1977; Bression et al., 1985; Stefanini et al., 1980) and as pharmacological antagonists of dopamine-mediated events in the anterior pituitary (MacLeod and Lehmeyer, 1974). These compounds interact with membranes and cells of the anterior pituitary with high affinity and potency, also indicating the
presence of D2 receptors. Furthermore, there is no evidence to indicate that a stimulation of adenylate cyclase activity could account for the dopamine-mediated inhibition of PRL release (Kebabian and Calne, 1979). Dopamine has been shown to inhibit adenylate cyclase activity or cAMP accumulation in rat adenohipophyseal cells in culture (Giannattisio et al., 1981; Cronin et al., 1983) and in human PRL-secreting pituitary adenoma homogenates (DeCamilli et al., 1979).

Caron and colleagues (1978) utilized measurements of in vitro PRL secretion from dispersed rat AP cells incubated in the presence of a variety of catecholamines and dopaminergic and adrenergic drugs as an estimate of the biological potency of these compounds at pituitary target cells. These substances were then tested for their ability to displace $^3$H-dihydroergocryptine from binding sites in bovine AP membranes. The results demonstrate a strong correlation between potency for binding competition and the ability of agonists to inhibit PRL secretion or the ability of antagonists to reverse DA inhibition of PRL secretion. The rank order of potency of agonists was indicative of a dopaminergic process; apomorphine > dopamine > epinephrine ≥ norepinephrine > isoproterenol = phenylephrine (Caron et al., 1978).

In summary, the demonstration of high affinity binding and highly potent interactions of agonists and butyrophenone antagonists with anterior pituitary cells, and the absence of association between agonist effects and stimulation of adenylate cyclase activity indicate that pituitary DA receptors represent the D2 subtype. The criteria for demonstration of DA receptors, namely; biological potency of ligands, saturable and reversible stereospecific binding and correlation of
binding activity with biological activity (Cronin et al., 1980); have been satisfied in the anterior pituitary. To date, only the D2 subtype of DA receptor has been demonstrated in the adenohypophysis (Ben-Jonathan, 1985).

Binding Site Localization

The distribution of binding of \(^{3}\)H-dihydroergocryptine in sub-fractionated bovine anterior pituitary membranes closely parallels the distribution of adenylate cyclase activity, 5’nucleotidase activity and sodium-potassium ATP-ase activity, as demonstrated in binding studies with fractions centrifuged through discontinuous sucrose gradients. This suggests that DA binding sites in the anterior pituitary are associated with the plasma membrane fraction (Caron et al., 1978). Utilizing immunocytochemical methods, Goldsmith et al. (1979) have observed that the majority of specific haloperidol binding to dispersed rat anterior pituitary cells is confined to the plasmalemmal surface of PRL-secreting cells. In regions of high density binding, occasional internalization of peroxidase anti-peroxidase complexes could be visualized on PRL-containing cells. Low density binding was observed on presumptive somatotrophs and gonadotrophs, but areas of internalization were confined to immunocytochemically identified mammotrophs. As some binding was observed on non-PRL-containing cells, dopaminergic regulation of other pituitary hormones and/or DA-mediated paracrine regulation of PRL cannot be completely ruled out. The presence of high density dopaminergic binding sites on mammotrophs does however provide evidence for a direct action of DA on these cells (Goldsmith et al., 1979).
Biphasic binding of $^3$H-dopamine (Calabro and MacLeod, 1978) and the butyrophenones $^3$H-spiroperidol (Cronin and Weiner, 1979; DeLean et al., 1982) and $^3$H-domperidone (Bresson et al., 1985) has been reported in anterior pituitary membranes. Analysis of saturation curves for labeled antagonists and competition curves for agonists versus labeled antagonists by nonlinear regression and computer modeling techniques reveals the presence of two affinity states of the D2 dopamine receptor distinguished by both agonists and antagonists (Sibley et al., 1982; DeLean et al., 1982). Direct labeling of binding sites with the agonist $^3$H-n-propylapomorphine ($^3$H-NPA) reveals the presence of a single site with high (nmolar) affinity for agonist (Sibley et al., 1982). Direct labeling with the antagonist $^3$H-spiroperidol yields biphasic saturation curves best fit to a two-site model; a high affinity site with an equilibrium dissociation constant ($K_d$) in the picomolar range and a lower affinity site with nanomolar $K_d$ estimates (Cronin and Weiner, 1979; DeLean et al., 1982). Equilibrium binding capacity for labeled agonists is approximately half that for labeled antagonists in bovine (Sibley et al., 1982) and porcine (DeLean et al., 1982) pituitary membranes. Agonist or antagonist vs. labeled agonist competition curves are best fit to a single site model by computer-assisted nonlinear regression analysis (Sibley et al., 1982). Agonist low affinity sites are not revealed in saturation binding studies with most labeled agonists because these sites are insignificantly occupied at even the highest concentrations of ligand which can be utilized to good result. High levels of nonspecific binding at very high ligand concentrations reduce the signal to noise ratios at assay to unsatisfactory levels (DeLean et al., 1982). Competition curves for a variety of agonists vs.
\(^{3}\text{H}\)-spiroperidol are complex, mutiphasic and shallow, and are significantly better described by a two affinity state model. Parameter estimates indicate that agonists discriminate between two forms of the sites labeled by spiroperidol and that these sites are present in roughly equal proportions. The difference in affinities between the two sites, as estimated by \(K_d\) values, was roughly two orders of magnitude. Monophasic competition curves obtained in agonist vs. labeled agonist reveal the presence of a high affinity site which corresponds closely to the high affinity site observed in agonist vs. labeled antagonist competition studies (DeLean et al., 1982).

Guanine nucleotides have been shown to modulate ligand-receptor interactions in a variety of hormone receptor systems (Rodbell, 1980). The usual effect is a selective decrease in receptor affinity for agonist (Stadel et al., 1982). The interactions of dopaminergic agents with anterior pituitary receptors in the presence of guanine nucleotides have been investigated. Direct labeling of pituitary membrane binding sites by \(^{3}\text{H}\)-NPA can be completely inhibited in a dose-dependent fashion by addition of guanine nucleotides to incubations (Sibley et al., 1982). Among the compounds tested for this activity, the non-hydrolyzable guanosine triphosphate (GTP) analog guanyl-1-5'-phosphoimidoephosphate (GppNHp) is the most potent followed by GTP and GDP which exhibit almost equal potency. GMP and ATP are relatively ineffective. In the presence of effective doses of guanine nucleotides, agonist competition curves for \(^{3}\text{H}\)-spiperone are steepened and shifted to the right. Regression analysis reveals the presence of a single class of binding sites which correspond to the low affinity binding sites for agonist evidenced in agonist competition with \(^{3}\text{H}\)-spipiperone in the absence of guanine.
nucleotides (Sibley et al., 1982). The biphasic binding of $^3$H-spiperone observed in saturation analyses performed in the absence of guanine nucleotides is converted to monophasic high affinity binding in the presence of saturating doses of guanine nucleotides, with no change in binding capacity (DeLean et al., 1982). Antagonist vs. $[^3]$H- spiroperidol competition curves are shifted to the left by up to 10-fold in the presence of guanine nucleotides indicating increased affinity for antagonists (DeLean et al., 1982).

If agonist and antagonist prelabeled receptors are solubilized with the detergent digitonin, and then subjected to molecular weight exclusion high pressure liquid chromatography (HPLC) in order to resolve molecular species based upon differences in molecular weight, agonist prelabeled receptors elute as an apparently higher molecular weight species than antagonist prelabeled receptors (Kilpatrick and Caron, 1983). When unlabeled receptors are solubilized from membranes, they exhibit guanine nucleotide insensitive high affinity binding for antagonists and low affinity binding for agonists. Addition of guanine nucleotides to agonist prelabeled receptors results in loss of labeling, and rechromatography of agonist prelabeled receptors in the presence of guanine nucleotides results in complete reversal of the apparent increase in molecular weight. These data suggest the association of the agonist receptor complex with some other component, such as a guanine nucleotide regulatory protein (Kilpatrick and Caron, 1983). A similar agonist-associated increase in molecular weight has been reported for the $\alpha_2$-adrenergic receptor (Michel et al., 1981), which like the D2 receptor, is adenylate cyclase inhibitory, and for $\beta$-adrenergic receptors (Limbird and Lefkowitz, 1978).
Treatment of pituitary membranes with heat (incubation at 53° C) or the sulfhydryl alkylating agent n-ethylmaleimide (NEM) has an effect similar to treatment with guanine nucleotides. As estimated by both direct labeling and binding competition studies, these treatments induce a loss of high affinity agonist interactions but produce little or no measurable effect on antagonist binding (Kilpatrick et al., 1982). In the frog erythrocyte β-adrenergic system, nucleotide regulatory protein activity has been shown to be similarly thermolabile (Ross and Gillman, 1979) and NEM also eliminates the nucleotide sensitive high affinity agonist binding to these receptors (Stadel and Lefkowitz, 1979).

Based upon these observations, a model has been proposed for ligand interactions with pituitary dopamine receptors. The receptor appears to exist in two interconvertible affinity states which are modulated by guanine nucleotides, and which exhibit reciprocal affinities for agonists and antagonists (Delean et al., 1982; Wreggett and DeLean, 1984). This "ternary complex" model proposes that the ligand binding protein interacts reversibly with a guanine nucleotide binding protein, the uncoupled form of the receptor having high affinity for antagonists and low affinity for agonists, while the coupled form of the receptor exhibits high affinity for agonists and lowered affinity for antagonists.

Membrane Properties of PRL Cells

Excitability

The discovery of membrane excitability and spontaneous action potentials in a clonal line of PRL-secreting cells (Kikodoro, 1975) has led to a number of
investigations concerning the possible relationship between the membrane properties of PRL cells and their secretory activity, and further, the correlation between alterations in secretion and perturbations in excitability brought about by agents known to influence PRL cell activity. Unfortunately, owing to the intermingling of various cell types in the mammalian adenohypophysis, a literature regarding these properties in specifically identified non-tumorous PRL cells is all but non-existent. Nonetheless, a body of suggestive evidence has grown out of studies of membrane electrical activity in clonal PRL-secreting lines (Kikodoro, 1975; Dufy et al., 1979a,b; Armstrong and Matteson, 1985; Johansen et al., 1986) and normal PRL cells in the anatomically segregated pituitary of the teleost alewife fish (Taraskevich and Douglas, 1978). A final important contribution comes from a study of the electrical properties of surgically excised human PRL-secreting macroadenoma cells (Israel et al., 1985). Kikodoro (1975) first reported the presence of spontaneous and depolarization-inducible calcium-dependent action potentials in a clonal line of rat pituitary tumor cells (GH3/B6), and this observation has been confirmed in the same (Dufy et al., 1979a) and similar (Armstrong and Matteson, 1985; Johansen et al., 1986) cell lines, and in human PRL-secreting tumor cells (Israel et al., 1985). Reports disagree as to the relative importance of calcium and sodium currents in the depolarizing phase of PRL cell action potentials. In the alewife fish, tetrodotoxin (TTX) blockade of sodium channels markedly reduces the amplitude of action potentials, indicating a predominant sodium component and only a small calcium conductance component (Taraskevich and Douglas, 1978). In human macroadenoma cells TTX has no effect on the amplitude, threshold or duration of action potentials, but addition of calcium current
inhibitors completely blocks action potentials in these cells without affecting the resting membrane potential (Israel et al., 1985). Electrophysiological recordings from GH3 and GH3/B6 cells bathed in sodium-free medium indicate persistence of action potentials but with some reductions in amplitude and maximum rate of depolarization (Dufy et al., 1979a; Johansen et al., 1986). Calcium channel blockade results in a marked reduction in the amplitude of action potentials in GH3/B6 cells (Kikodoro, 1975; Dufy et al., 1979b). Thus, while the relative magnitude of the sodium channel component of PRL cell depolarization remains in question, all published reports to date agree as to the absolute requirement of calcium currents for this activity.

Effects of DA

Dopamine and bromoergocryptine have been reported to markedly reduce or abolish both spontaneous and depolarizing current-induced action potentials in alewife fish PRL cells (Taraskevich and Douglas, 1978), GH3 and subclone B6 cells (Dufy et al., 1979b; Johansen et al., 1986) and in human PRL-secreting tumor cells (Israel et al., 1985). In human tumor cells, exposure to DA at low concentration (nM) induces a strong hyperpolarizing response concomitant with a decrease in membrane resistance (Israel et al., 1985). In cells producing action potentials in response to small depolarizing current pulses, a brief exposure (50 msec) to DA induced a hyperpolarizing response persisting for several seconds, during which time action potentials were abolished, not reappearing until the cell returned to the previous resting potential. Action potentials could be maintained by voltage clamping at the original potential or produced during the hyperpolarization by
increasing the amplitude of the depolarizing current pulses, indicating that DA did not alter the threshold or interfere with the underlying ability of the cells to discharge action potentials. The DA effect appears to be mediated through D2 receptors as it could be blocked by the antagonists haloperidol, domperidone and spiroperidol but not by the D1 antagonist flupenthixol (Israel et al., 1985). It is also of interest to note that administration of a single pulse of TRH has been reported to induce a sustained burst of spiking activity in GH3/B6 cells (Dufy et al., 1979a).

While definitive answers concerning the physiological role of membrane electrical activity in stimulus secretion coupling in PRL-secreting cells await further studies in identified non-neoplastic mammalian cells, several observations can be made on the basis of existing data. First, spontaneous action potentials can be observed under conditions in which PRL cells secrete spontaneously. Second, these action potentials are abolished by DA and bromoergocryptine, which inhibit secretion. Third, the effects of DA and bromoergocryptine on both secretion and electrical activity can be reversibly inhibited by addition of D2 antagonist drugs. Fourth, TRH has a stimulatory effect of short latency on both secretion and action potential frequency in PRL-secreting cells. Finally, action potentials in PRL cells involve the opening of voltage-sensitive calcium channels. Dopamine-induced membrane hyperpolarization appears to inhibit activation of these channels, thereby decreasing the influx of calcium and possibly reducing the intracellular calcium available for exocytosis (Ben-Jonathan, 1985). Calcium influx has been implicated in PRL release under both basal (disinhibited) conditions and in response to a variety of secretagogues (vide infra).
Subcellular Mechanisms of DA Action

Hormones that exert their effects by interacting with cell surface receptors require intracellular "second messengers" for stimulus-response coupling. A variety of second messenger and subcellular pathways have been implicated in the regulation of PRL secretion. These include cyclic nucleotide regulatory mechanisms, calcium and calcium/calmodulin systems and polyphosphoinositide metabolism (Ben-Jonathan, 1985).

Cyclic Adenosine Monophosphate (cAMP)

By far the most widely studied of the second messengers to date has been cAMP. While earlier studies concerning the effects of DA on cAMP metabolism in the anterior pituitary yielded inconsistent results, the accumulated body of evidence provides strong support for a mechanistic interrelationship between DA and cAMP in the dopaminergic regulation of PRL release. DA attenuates adenylate cyclase activity and/or inhibits cAMP accumulation in primary cultures of anterior pituitary cells (Cronin and Thorner, 1982), in anterior pituitary homogenates (Giannattasio et al., 1981) and in membranes prepared from human prolactin-secreting pituitary adenomas (Camilli et al., 1979). The DA inhibition of cAMP accumulation is concentration dependent and occurs over concentrations similar to those producing suppression of PRL release (Swennen and Denef, 1982). The effects of DA on both cAMP levels and on PRL release are blocked by a variety of DA antagonist drugs with very similar rank orders of potency. Furthermore, the cAMP-inhibitory activity of DA appears to be located on lactotrophs, as this activity
is absent in lactotroph-poor cell populations and greatest in lactotroph-enriched populations (Swennen and Denef, 1982).

The DA agonist drugs bromoergocryptine (Cronin and Thorner, 1982) and apomorphine (Giannattasio et al., 1981, Swennen and Denef, 1982) also exert concentration-dependent inhibitory effects over cAMP accumulation. Furthermore, the ability of bromoergocryptine to inhibit both PRL release and intracellular cAMP accumulation can be blocked by preincubation with pertussis toxin (Cronin et al., 1983), which is believed to exert its main effects through ADP-ribosylation of a sub-unit of the protein Ni. Ni in turn is believed to be involved in coupling of hormone-mediated inhibition of adenylate cyclase activity or "negative signal transduction" (Kurose et al., 1983). In contrast to these studies, other published reports indicate a lack of correlation between the ability of DA to inhibit cAMP accumulation and its inhibitory effect on PRL release (Ray and Wallis, 1979; 1981). No clear-cut explanation exists for these discrepancies.

Cholera enterotoxin, which stimulates adenylate cyclase activity in a variety of systems including the anterior pituitary, increases the rate of PRL release in primary cultures of pituitary cells. This effect can be blocked, at least in short term incubations, by the addition of bromoergocryptine (Tam and Dannies, 1981). Other agents which mimic cAMP or produce increases in cAMP also stimulate PRL release, and this effect may also be blocked by DA or bromoergocryptine in short term incubations (Tam and Dannies, 1981; Delbeke and Dannies, 1985). Forskolin, which directly activates adenylate cyclase, is a potent stimulator of PRL release. Pre-incubation with DA prior to addition of forskolin completely prevents the
increase in PRL release but only partially blocks the rise in cAMP concentrations, which remain elevated by more than an order of magnitude over controls (Delbeke et al., 1986). This has been interpreted to indicate that while increases in cAMP activity result in increased release of PRL, such increases in cAMP are not alone capable of completely reversing the effects of DA and its agonists on PRL release, and thus, DA must exert part of its effects through mechanisms not directly involving acute regulation of PRL release by cAMP (Tam and Dannies, 1981; Ray and Wallis, 1981; Delbeke and Dannies, 1985)

Calcium Messenger Systems

A substantial body of evidence also exists regarding the involvement of calcium messenger systems in dopaminergic regulation of PRL secretion. As for many other hormone systems, the secretion of PRL is calcium dependent (Ben-Jonathan, 1985). Evidence suggests that both the influx of extracellular calcium and the mobilization of intracellular calcium stores participate in this process. In an ultrastructural study (Yancey et al., 1980), inhibition of PRL release by DA resulted in increased sequestration of calcium in the mitochondria and Golgi apparatus, whereas stimulation of PRL release with monobutyryl cAMP resulted in a decline in mitochondrial stores. Thyrotropin-releasing hormone- (TRH) stimulated PRL release follows a biphasic spike and plateau pattern which is paralleled by a spike and plateau pattern of increase in cytosolic free calcium concentration (Albert and Tashjian, 1984). The initial spike can be prevented by depletion of intracellular calcium stores but not by calcium channel blockade whereas the plateau phase is largely prevented by calcium channel inactivators nifedipine or verapamil (Albert
and Tashjian, 1984; Gershengorn and Thaw, 1985). PRL release is inhibited by the removal of calcium from the medium or by the addition of calcium channel blockers (Thorner et al., 1980). PRL release is stimulated by maitotoxin, a calcium channel activator (Schettini et al., 1984), and by divalent cation ionophores in the presence but not in the absence of calcium (Tam and Dannies, 1980). In the latter study, preincubation with DA, bromoergocriptine or dihydroergocryptine inhibited ionophore-induced PRL release in a concentration-dependent manner, and this effect could be blocked by the addition of dopaminergic antagonists. Utilizing a trapped fluorescent indicator for calcium, Schofield (1983) reported that administration of DA induced a transient fall in cytoplasmic calcium concentrations in lactotroph-enriched anterior pituitary cell fractions. Conversely, Tam and Dannies (1980) reported that bromocriptine had no effect on the rates of calcium uptake, either in the presence or absence of calcium ionophore, indicating that dopaminergic agonists interfere with calcium-induced PRL release at a step following calcium uptake. Thus, DA appears to affect PRL release mechanisms related to calcium at steps both before and after calcium influx and mobilization.

As an intracellular messenger, calcium functions through two distinct branches (Rasmussen and Barrett, 1984). The first is the calmodulin branch, which is activated by a transient rise in cell cytosol calcium concentration, and includes calmodulin as well as other calcium receptor proteins and calcium-activated enzymes. The second is the C-kinase branch, which is the calcium- and phospholipid-dependent protein kinase which is activated by diacylglycerol (Rasmussen and Barrett, 1984). The turnover of inositol phospholipids appears to an early and key event in calcium-mediated signal transduction (Nishizuka, 1984). Evidence
suggests that DA is capable of interacting with both limbs of the calcium second messenger system. Treatment of rats with α-MPT results in increased serum PRL levels and increased incorporation of $^{32}$P into phosphatidyl inositol (PI) in subsequent pituitary incubations (Canonico et al., 1983). This effect is counteracted by addition of DA to incubation media. Similarly, pre-treatment with bromocriptine resulted in significant depression of pituitary PI turnover in vitro (Canonico et al., 1983). Drugs which block the activity of calmodulin have been shown to inhibit both basal and cAMP-stimulated PRL release, indicating a role for calmodulin in PRL release which is intertwined with cAMP (Schettini et al., 1983a; 1983b).

**PRL Cell Regulation by Other Neurotransmitters and Hormones**

The following discussion represents a brief overview of substances, which, in addition to DA, have been implicated as possible neuromodulators of PRL cell activity. Particular emphasis has been placed upon reported interrelationships with the TIDA system.

**Opiates and Opioid Peptides**

Endogenous opioid peptides have been implicated in the regulation of PRL secretion. Administration of an opiate antagonist such as naloxone or naltrexone has been shown to block the proestrus surge of PRL in the rat (Leiri et al., 1980) as well as stress-induced PRL release (Grandison and Guidotti, 1977; Van Vugt et al., 1978). Naloxone administration in vivo has also been shown to reduce circulating levels of PRL in otherwise untreated rats (Bruni et al., 1977; Shaar et al., 1977), whereas this compound has no effect on PRL release from pituitaries
incubated in vitro (Shaar et al., 1977; Cheung 1984). Systemic administration of morphine or opiate-like peptides is reported to increase PRL secretion, and this stimulatory effect is reversed by naloxone (Rivier et al., 1977; Shaar et al., 1977; Bruni et al., 1977; Van Vugt and Meites, 1980; Dupont et al., 1980; Spies et al., 1980). The primary site of action of these various compounds on PRL release appears to be at the hypothalamus or higher levels, as most studies indicate a lack of direct stimulatory effect at the anterior pituitary (Grandison and Guidotti, 1977; Rivier et al., 1977; Login and MacLeod, 1979). In contrast, other investigators have reported a slight stimulatory effect of opioid peptides on PRL release at the level of anterior pituitary cells (Lien et al., 1976; Matsushita et al., 1982). In addition, En jalbert and co-workers (1979) reported that while morphine, ß-endorphin or met-enkephalin did not directly stimulate PRL release from pituitaries, these compounds could reverse DA-induced inhibition of PRL release in vitro, a finding confirmed by Cheung (1984). However, because this effect is transitory and only occurs at concentrations of ß-endorphin which appear to be supraphysiological, the biological relevance of direct pituitary effects remains questionable (Cheung, 1984).

Substantial evidence exists pointing to a central site of action for the opiates on PRL release, and in particular, a hypothalamic site of action. Intracerebroventricular (ICV) administration of morphine derivatives which do not cross the blood brain barrier results in increased serum PRL levels whereas peripheral administration of these compounds is ineffective (Panerai et al., 1981). Local administration of opioid peptides into the mediobasal hypothalamus results in antagonist reversible stimulation of PRL release from the anterior pituitary
(Grandison et al., 1980; Haskins et al., 1981), while, as discussed above, most studies point to a lack of direct effect of these compounds at the anterior pituitary. In addition, there may be sexually dimorphic responses to specific opioid receptor population activation in the rat. In ovariectomized females, ICV administration of selective $\mu$ receptor agonists results in naloxone-reversible stimulation of PRL release, administration of $\delta$ selective agonists has no effect on PRL release, and administration of $\kappa$ selective agonists exerts a variable effect on PRL release which is not affected by naloxone (Leadem and Yagenova, 1987). The reasonably selective $\kappa$ agonist dynorphin exerts similarly variable effects on PRL release in ovariectomized females (Leadem and Kalra, 1985), while in male rats, dynorphin and the highly selective $\kappa$ agonist U50,488H have been reported to stimulate PRL release (Van Vugt et al., 1981; Krulich et al., 1986).

Administration of morphine or $\beta$-endorphin inhibits the turnover (Van Vugt et al., 1979; Alper et al., 1980; Van Loon et al., 1980; Reymond et al., 1983) and release (Haskins et al., 1981; Reymond et al., 1983; Arita and Porter, 1984) of DA from TIDA neurons in the mediobasal hypothalamus, while DA turnover in the nigrostriatal and mesolimbic DA neurons is increased (Alper et al., 1980; Demarest and Moore, 1981). Arita and Porter (1984) demonstrated that while the increases in PRL release produced by either DA synthesis blockade or morphine treatment were quantitatively similar, replacement of DA by intravenous infusion completely reversed the effect of DA synthesis blockade, but only partially suppressed PRL secretion in morphine treated animals, suggesting the involvement of hypothalamic factors in addition to dopamine.
5-Hydroxytryptamine (5HT)

The neurotransmitter serotonin (5HT) has also been implicated in the regulation of PRL. The administration of 5HT, the 5HT agonist quipazine or the 5HT precursor 5-hydroxytryptophan (5HTP) results in a large, albeit transitory, increase in PRL release, and this effect is blocked by pretreatment with the 5HT antagonist methysergide (Clemens et al., 1977; Lamberts and MacLeod, 1978; Lawson and Gala, 1978; Pilotte and Porter, 1979). The effects of these various treatments appear to be mediated at the hypothalamic level as neither 5HT nor 5HTP has a direct effect on PRL release in vitro (Lamberts and MacLeod, 1978), and the effect of quipazine is abolished by lesion of the mediobasal hypothalamus (Krulich et al., 1979). It should be noted that the use of methysergide as a 5HT blocker is problematic in these studies as some evidence suggests that methysergide or its metabolite methergine may activate pituitary DA receptors, thereby exerting a direct inhibitory effect over PRL release (Lamberts and MacLeod, 1978; Krulich et al., 1979).

Evidence exists pointing to an interaction between the endogenous opioids and 5HT. The PRL-releasing effects of quipazine and 5HTP are partially but significantly reversed by co-administration of naloxone (Meites et al., 1979). Similarly, the opioid peptide met-enkephalin and an amide analog (D-Met2-Pro5)-enkephalinamide (EKNH2) have been shown to induce a rise in serum PRL which is significantly reversed by the 5HT antagonists metergoline and methysergide (Spampinato et al., 1979). Furthermore, EKNH2 stimulation of PRL release could be almost completely abolished by chemical ablation of 5HT terminals with
5,6-dihydroxytryptamine. Together, these studies suggest that hypothalamic 5HT neurons may mediate the effects of opioids on PRL release.

Increased serotonergic tone could stimulate PRL release through either suppression of TIDA neuronal activity or through stimulation of the secretion of a PRL-releasing factor (PRF). In fact, evidence exists in support of both of these possibilities. Clemens and co-workers (1978) found that the administration of 5HTP to animals pretreated with the 5HT reuptake inhibitor fluoxetine resulted in an increase in PRL release that was considerably greater than that observed in animals treated with the DA synthesis blocker α-MPT or a variety of dopaminergic antagonist drugs, suggesting that removal of dopaminergic inhibition could not account for the total mass of serotonin-induced release. Similarly, pretreatment with quipazine abolishes any further stimulation of PRL release by the opioid analog EKNH2 but not by the DA blocker domperidone (Spampinato et al., 1979). In the same studies, disruption of catecholamine transmission with α-MPT did not qualitatively modify the EKNH2-induced stimulation of PRL release. In a contrasting study, Lawson and Gala (1978) reported that pretreatment with apomorphine completely blocked the PRL-releasing activity of 5HT, 5HTP and quipazine, and further, that the DA antagonist pimozide did not exert additive effects on PRL release in the presence of a maximally effective dose of 5HT. Differences in results among these studies may be related to the ability of the serotonergic or dopaminergic system to overwhelm or mask the other under conditions of maximal activity, or to differences in the physiological state of experimental animals. There is also some direct evidence for the existence of a serotonin-stimulated PRF. Garthwaite and Hagen (1979) found that methanol extracts of plasma and hypothalami from
rats treated in vivo with 5-HTP and fluoxetine released PRL from hemipituitaries in vitro in a dose-dependent fashion, while similar extracts from animals in which serum PRL concentrations had been elevated by administration of the DA antagonist drug chlorpromazine did not exhibit PRL-releasing activity. Interestingly, Johnston and co-workers (1986) found that removal of the neurointermediate pituitary lobe (NIL) abolished the PRL response to 5HTP. These authors postulate that the NIL may serve as a point of integration for serotonergic influences on PRL secretion, perhaps through release of a PRF.

Regarding the effect of 5HT on the activity of TIDA neurons, intracerebroventricularly administered 5HTP has been shown to significantly diminish the concentration of DA in hypophysial portal plasma, concommitant with increased PRL levels in central venous plasma (Pilotte and Porter, 1981). In further support of a modulatory role for 5HT over TIDA activity, Demarest and Moore (1981) found that administration of the 5HT antagonist metergoline or selective chemical destruction of 5HT neurons could block the inhibitory effects of morphine on DA turnover in the hypothalamic median eminence. However, in the former study (Pilotte and Porter, 1981), intravenous infusion of DA at rates yielding very high concentrations of DA in arterial plasma failed to prevent the 5HTP-induced PRL release, leading these authors to postulate that serotonergic modulation of PRF activity rather than suppression of dopaminergic activity, was primarily responsible for the observed increases in PRL release. A collective interpretation that may be drawn from these various studies is that increased serotonergic activity in the hypothalamus stimulates PRL release either through inhibition of TIDA
neuronal activity and/or by other DA-independent mechanisms, possibly including stimulation of PRF secretion.

Thyrotropin-Releasing Hormone (TRH)

The most widely studied of hypothalamic substances with PRL-releasing activity is the tripeptide thyrotropin-releasing hormone (TRH). In clonal rat pituitary cells (GH3), TRH has been demonstrated to stimulate transcription of the PRL gene (Murdoch et al., 1983), elevate PRL mRNA levels (White and Bancroft, 1983) and to stimulate the synthesis (Yajima and Saito, 1983) and release (Tashjian et al., 1983) of PRL. Further, in most mammals studied to date, TRH has been reported to be a potent secretagogue for PRL, although in the rat it has demonstrated only minor effects on PRL release by normal pituitaries both in vivo and in vitro (Vale et al., 1977). In male rats and in ovariectomized rats, estrogen appears to exert a permissive effect over TRH-induced PRL release (Mueller et al., 1973; Rivier and Vale, 1974; DeLean et al., 1977; Jacques and Gala, 1979), and a PRL stimulatory effect of TRH has been reported in proestrus and lactating rats (Blake, 1974). DeLean and co-workers (1977) found that estradiol administration to male rats increased the number of pituitary TRH receptors concomitant with the development of detectable serum PRL response to TRH. In lactating rats, most evidence indicates that acute administration of TRH is not effective in elevating serum PRL concentrations (Fagin and Neill, 1981; Plotsky and Neill, 1982a) unless the injections are given following a brief period of suckling (Grosvenor and Mena, 1980). The suckling stimulus has been shown to induce a brief but profound decrease in secretion of DA into hypophyseal portal plasma (Plotsky and Neill,
In an attempt to duplicate this phenomenon experimentally, Plotsky and Neill (1982a) treated animals with \( \alpha \)-MPT to abolish endogenous dopaminergic transmission, and infused the animals with DA at a rate which reproduced the level of inhibition of PRL release typically observed in lactating animals. Administration of TRH did not produce significant increases in serum PRL in control animals, in animals treated with \( \alpha \)-MPT alone or in DA-infused animals treated with \( \alpha \)-MPT. However, if TRH was administered after a brief interruption of DA infusion in \( \alpha \)-MPT treated animals, a PRL surge was produced (Plotsky and Neill, 1982a). Essentially the same results have been obtained in an in vitro model of this system (Fagin and Neill, 1981). Taken together, these studies provide indirect evidence that in face of the brief decrease in DA release evoked by suckling, TRH and/or other PRF activity may play a physiological role in PRL regulation during lactation. At the molecular level, it has been shown that TRH increases cAMP accumulation in mammotroph-rich cell fractions, and that DA antagonizes this effect (Barnes et al., 1978). TRH also increases cAMP accumulation in thyrotroph-rich fractions, but this is not reversed by DA. Further evidence supporting a physiological role for TRH in PRL regulation is provided by the finding that administration of anti-serum to TRH to female rats blocks the proestrus PRL surge (Koch et al., 1977). Arguing against a physiological role for TRH in PRL regulation, at least in lactation, are the observations that while acute administration of TRH to lactating females results in a rapid increase in both PRL and thyroid stimulating hormone (TSH) release, suckling itself produces an increase in TSH release which occurs several minutes after a more immediate increase in serum PRL (Blake, 1974). Furthermore, at least one group has found no effect of TRH on PRL release in lactating rats, even when
administered following suckling (Riskind, et al., 1984). Thus a role for TRH in the normal regulation of PRL in the rat remains questionable.

Gamma-aminobutyric Acid (GABA)

Gamma-aminobutyric acid (GABA) is also a putative neuromodulator of PRL cell activity. Morphologically, dense networks of GABAergic fibers have been observed throughout the hypothalamus including the median eminence (Vincent et al., 1982; Perez de la Mora et al., 1981). Accumulated evidence indicates both a stimulatory and inhibitory role for this neurotransmitter in PRL regulation. Central (intracerebroventricular or intrahypothalamic) administration of GABA or the GABA<sub>A</sub> agonist muscimol results in stimulation of PRL secretion (Locatelli et al., 1979; Pass and Ondo, 1977) while intravenous administration of GABA or muscimol suppresses serum PRL levels (Locatelli et al., 1979; Schally et al., 1977; Grandison and Guidotti, 1979). The latter effect can be blocked by i.v. administration of bicuculline methiodide, a GABA antagonist which does not cross the blood brain barrier (Grandison and Guidotti, 1979), indicating a peripheral site of action for the PRL inhibitory activity of GABA. Binding sites for GABA have been demonstrated in the anterior pituitary (Grandison and Guidotti, 1979), and several studies have demonstrated a direct inhibitory effect of GABA and GABA<sub>A</sub> agonists on PRL release and synthesis by the anterior pituitary in vitro (Schally et al., 1977; Enjalbert et al., 1979; Grossman et al., 1981; Loeffler et al., 1986).

Evidence exists suggesting that the central PRL-stimulatory effect of GABA is mediated, at least in part, through modulation of TIDA neuronal activity. Anatomically, GABA neurons and tyrosine hydroxylase-containing neurons are co-local-
ized in the basal hypothalamus-arcuate region (Everitt et al., 1984). In hypothalamic microinjection studies, Ondo and Dom (1986) found that microinfusion of muscimol into the arcuate nucleus, but not into other nearby sites, produced a significant rise in serum PRL levels. Finally, i.v. injection of muscimol has been reported to decrease the rate of DA turnover in the mediobasal hypothalamus (Fuchs et al., 1984).

Estrogens and PRL Cell Activity

Estrogens have been reported to affect essentially every aspect of PRL cell physiology and regulation. Because of such global effects, in many cases it has proven difficult to precisely "compartmentalize" effects. For example, owing to the complex interplay between the rates of synthesis, release and intracellular degradation of PRL, a precise modulatory role for estrogens on these processes individually is difficult to establish. Similar comments could be made regarding the apparent interrelationship between intracellular PRL content and mitotic rates. For purposes of discussion, however, it is useful to treat these various interrelated processes individually.

PRL Cell Proliferation

The theoretical question of a relationship between estrogens and cell division in the anterior pituitary was first raised more than forty years ago by Hunt, who reported that mitotic activity in the adenohypophysis changed rhythmically during the estrous cycle, with maximum activity occurring with a lag time of 12-24 hours following presumptive serum estrogen titer maxima (Hunt, 1943). Hyperplastic and
hypertrophic effects of estrogens on PRL cells have since been demonstrated in the work of Gersten and Baker (1970). In these studies, intrahypophyseal estrogen implants induced hyperplasia and hypertrophy of immunocytochemically defined PRL cells. The effects were limited to the lobe ipsilateral to the implant, and further limited to those portions of the lobe vascularly distal to the implant, pointing to a direct action of estrogens at the pituitary level. Indeed, a direct stimulation by estrogens of mammotroph proliferation has been shown in primary cultures of pituitary cells (Lieberman et al., 1982). Numerous reports exist in the literature which further substantiate, although indirectly in many cases, a stimulatory role for estrogens on mammotroph proliferation (Davies et al., 1974; Maurer and Gorski, 1977; Pawlikowski et al., 1978; Maurer, 1978; Maurer, 1979; Wiklund and Gorski, 1982; Corenblum et al., 1982).

PRL Cell Morphology

Estrogen treatment also alters the morphology of PRL cells (see Dannies, 1985). Observed changes include increased cell size, hypertrophy of the Golgi apparatus, increased volume of rough endoplasmic reticulum (RER), alterations in the configuration of RER into whorls or long rows parallel to the plasma membrane and changes in the size distribution and number of PRL secretory granules. The intuitive picture which emerges is that of a cell tooled for the production and release of PRL.
It had been postulated for many years before direct evidence was forthcoming that estrogens stimulated the activity of PRL-secreting cells. Nicoll and Meites (1962) published the first major report providing such evidence. In these experiments, pituitary explants were incubated in vitro in the presence or absence of estradiol for several days, and it was demonstrated by bioassay that the medium from estrogen-exposed pituitaries contained 45% more PRL activity than control medium. It should be noted that these studies provided the first evidence of a direct effect of estrogens on the secretion of a pituitary hormone (Nicoll and Meites, 1962). Studies involving observation and perturbation of circulating hormone patterns in the rat estrous cycle provide further evidence of a physiological PRL-stimulatory role for estrogens. In the rat estrous cycle there is a surge in serum PRL titers on the afternoon of proestrus, occurring only a few hours after peak serum estrogen concentrations (Butcher et al., 1974). Administration of antiserum to estradiol effectively blocks the proestrus PRL surge, while co-administration of diethylstilbestrol (DES) with the antiserum reverses this inhibitory effect (Nicoll et al., 1971).

A substantial literature has accumulated which clearly demonstrates the ability of estrogens to stimulate the synthesis of PRL through a direct action at the pituitary level, and many of the underlying mechanisms of this stimulation have been addressed experimentally. An additional body of evidence suggests that estrogens also exert effects over PRL cell activity mediated through neuroen-
doctrine mechanisms. It has been demonstrated that the administration of estrogens in vivo elevates serum PRL levels and stimulates the incorporation of radiolabeled leucine into newly synthesized PRL by pituitaries subsequently cultured in vitro (MacLeod et al., 1969; Maurer and Gorski, 1977). These effects occur in intact or castrated male or female rats, and may be observed as early as 24 hours following estrogen administration (Maurer and Gorski, 1977). Subsequent studies have shown that incubation of rat pituitary cells in culture with estradiol causes a similar increase in de novo PRL synthesis as measured by leucine incorporation (Lieberman et al., 1978; Lieberman et al., 1982). This effect has also been observed in monkey (Bethea, 1986) and ovine (Vician et al., 1979) pituitary cell cultures. Studies utilizing a variety of estrogenic agonists, partial agonists and antagonists have yielded results consistent with the hypothesis that the stimulation of PRL production in vitro by estrogens is mediated through an interaction with the estrogen receptor (Lieberman et al., 1983; Jordan and Lieberman, 1984). While these studies indicate that estrogens stimulate PRL production predominantly through a direct action on the pituitary, they do not rule out the possibility of other hypothalamic/neuroendocrine effects of estrogens in vivo. Furthermore, changes in rates of leucine incorporation after several days in culture may reflect, in addition to changes in rate of synthesis by pre-existing cells, an increase in the number of PRL cells. Lieberman and colleagues (1982) found that in their primary culture system, the estrogen-stimulated increase in PRL synthesis was much greater than the increase in the number of mammotrophs over the period of incubation, and in immature female rats, co-treatment with DNA synthesis inhibitors only partially blocked the ability of estrogens to elevate serum PRL and to stimulate PRL
synthesis (Maurer, 1979). These studies indicate that mammotroph proliferation may contribute to, but is not, the major underlying mechanism in the stimulation of PRL production by estrogens.

With the development of technologies, studies concerning the effects of estrogens on PRL synthesis were extended to include effects on PRL mRNA levels. Estrogen treatment has been shown to increase both PRL mRNA activity as measured in a cell-free translational system (Seo et al., 1979) and PRL mRNA levels as measured by hybridization to a complementary DNA (cDNA) probe (Seo et al., 1979; Ryan et al., 1979). Rat pituitary cells in culture with estradiol also respond with specific increases in PRL mRNA levels (Lieberman et al., 1982). A direct effect of estradiol on PRL messenger activity in cultured ovine pituitary cells has also been reported (Vician et al., 1979). In the majority of these studies, close agreement has been found between increases in PRL mRNA levels and increases in PRL synthesis (Ryan et al., 1979; Vician et al., 1979; Lieberman et al., 1982), indicating that estrogens affect PRL synthesis primarily at pre-translational levels. Seo and colleagues (1979), on the other hand, reported that while a single injection of estradiol in male rats produced increases in PRL mRNA levels within 12 hours, the rate of PRL synthesis was not affected during the first 24 hours. In the same studies, however, chronic stimulation with estradiol over 1 through 4 weeks produced increases in PRL synthesis and messenger activity that were quantitatively indistinguishable. These authors concluded that estrogens exert effects on PRL synthesis at pre-translational levels, and hypothesized that changes may also be induced in the translational process. Maurer (1982) reported that estrogen treatment in vivo stimulated transcription of the PRL gene, and further, that at least
during the first hour following estradiol injection, the increased rate of synthesis of PRL mRNA precursor sequences was paralleled by increasing levels of nuclear resident estradiol receptors, leading to the hypothesis that the estrogen receptor may modulate PRL gene transcription by interacting with nucleotide sequences at or near the PRL gene. Shull and Gorski (1985) have described a biphasic in vivo effect of estrogens on transcription of the PRL gene, a rapid stimulation measurable from 30 minutes to 2 hours following injection, and a second phase of increased transcription occurring from hour 6 through hour 24. The first phase appears to be independent of de novo protein synthesis while the second phase is blocked by prior administration of protein synthesis inhibitors.

Reported effects of estrogens on PRL mRNA accumulation occurring within the first 24 hours, and certainly within the first hour (Vician et al., 1979; Maurer, 1982; Shull and Gorski, 1985), lend strong support to the concept that increases in PRL synthesis induced by estrogens are the result of increased activity in pre-existing PRL cells, and are not dependent on cell division. This issue has been addressed more directly in experiments utilizing combined immunocytochemical and in situ cDNA hybridization methods. These studies confirm that estrogen administration increases PRL mRNA accumulation in individual cells, in addition to stimulating PRL cell proliferation (Nogami et al., 1985; Yamamoto et al., 1986).

Perturbation of PRL Cell Regulation

**TRH Sensitivity.** Several lines of evidence indicate that estrogens, in addition to direct stimulation of PRL gene transcription, may alter the activity of PRL-secreting cells by modulating the responsiveness of these cells to other PRL-regulating
hormones. It has been reported that in ovariectomized rats, administration of TRH does not elevate circulating PRL levels unless the rats are also treated with estrogen (Stevens and Lawson, 1979). Similarly, the addition of estradiol to anterior pituitary cell cultures results in a dramatic increase in TRH-stimulated PRL release (Raymond et al., 1978; LaBrie et al., 1980). DeLean and co-workers (1977) found that the administration of estrogens \textit{in vivo} increased the total number of TRH receptors in anterior pituitary homogenates. While it cannot be determined from this study whether TRH receptors on mammotrophs and/or thyrotrophs are affected, estrogens have also been shown to increase the number of TRH receptors on tumor-derived mammotrophic cells, and this effect is blocked by addition of the anti-estrogen tamoxifen (Gershengorn et al., 1979). Thus, it appears that estrogens, through an interaction with the estrogen receptor, increase pituitary responsiveness to the PRL-releasing effect of TRH by increasing the total number of pituitary TRH receptors.

\textbf{DA Sensitivity.} In addition to modulation of the PRL-stimulatory effects of TRH, many studies have indicated that estrogens interfere with PRL-inhibitory dopaminergic mechanisms at the pituitary level. Estrogen administration \textit{in vivo} has been shown to decrease the inhibitory effect of DA on PRL release from pituitaries incubated \textit{in vitro} (Gudelsky et al., 1981) and to partially block the inhibitory effect of exogenous DA on morphine-stimulated PRL release \textit{in vivo} (Ferland et al., 1979). The addition of estradiol to pituitary cell cultures also results in a decreased inhibitory effect of DA and a variety of dopaminergic agonist drugs on both basal and TRH-stimulated PRL release (Reymond et al., 1978; LaBrie et al., 1980). West and Dannies (1980) found that in primary cultures of rat pituitary
cells, the addition of estradiol resulted in both a decreased maximal inhibition of PRL release by dihydroergocriptine and an increase in the half-maximal inhibitory concentration (IC\textsubscript{50}) for this drug. Maurer (1980) reported that estradiol blocked the ability of ergocriptine to inhibit both PRL mRNA levels and the synthesis of PRL. From these studies, it appears that estrogens interfere with the ability of DA to inhibit both the synthesis and release of PRL, and that these effects are mediated, at least in part, through a direct action on the pituitary.

It has also been suggested that estrogens may decrease the ability of PRL cells to degrade stored hormone in response to DA. Nansel and co-workers (1981) found that pituitaries harvested from estrous as compared to diestrous rats showed diminished responsiveness to DA in terms of both PRL release and DA-mediated stimulation of /\beta/-glucuronidase (a lysosomal enzyme) activity. While these changes in DA sensitivity could be abolished by ovariectomy during diestrus, administration of estradiol to ovariectomized animals resulted in a marked decrease in pituitary responsiveness to DA, similar to pituitaries from estrous animals.

Anterior Pituitary DA Receptors

As discussed in a previous section, the effects of DA on PRL cell activity are mediated through binding to cell surface receptors. A change in the total number of DA receptors or their affinity for agonists might be a mechanism for modulating the sensitivity of PRL cells to the effects of DA. Possible effects of estrogens on DA binding parameters have been addressed experimentally, with conflicting results. Evidence suggests that the number of pituitary DA receptors is regulated
during the estrus cycle. Heiman and Ben-Jonathan (1982a) found that pituitary binding capacity for $^3$H-spiperone flutuated throughout the estrus cycle, with the greatest capacity occuring on the afternoon of proestrus, coincident with the PRL surge. Binding site concentrations declined during estrus through diestrus I, with nadir values on diestrus II through the morning of proestrus. Pasqualini et al. (1984) have also examined DA receptors during the estrus cycle. While this work confirms the fluctuation in numbers of pituitary binding sites for $^3$H-spiperone, the pattern of change observed was quite different. In general, an inverse correlation was found between binding capacity and serum PRL concentrations at all times during the four day cycle. Specifically, nadir values for binding site concentration were observed on the afternoon of proestrus, in contrast to the peak values reported by Heiman and Ben-Jonathan at this time point. The reason for the discrepancy between these studies is not clear, and unfortunately it is not possible to reasonably interpret from these conflicting results whether ovarian steroids, portal plasma DA concentrations and/or PRL titers are exerting direct regulatory effects on pituitary DA receptors. However, it appears that modulation of DA receptors during the estrus cycle may be a physiological mechanism in the regulation of PRL secretion patterns, and direct effects of estrogens on DA binding site populations cannot be ruled out. Short term administration of estradiol to ovariectomized rats has been reported to decrease (Cronin et al., 1980; Heiman and Ben-Jonathan, 1982b) or to have no effect (DiPaolo et al., 1979; Pilotte et al., 1984) on the number of DA receptors in the anterior pituitary.

Bression and colleagues (1983; 1985) have reported the presence of biphasic saturation binding curves for $^3$H-domperidone in anterior pituitary
membranes from intact female rats. This indicates either the presence of two separate populations of binding sites, or more likely, the presence of a single receptor population existing in two affinity states, as previously discussed. To briefly review, under the ternary complex model (Wreggett and DeLean, 1984), the D2 dopamine receptor is postulated to exist in two pharmacologically distinguishable affinity states which exhibit reciprocal affinities for agonist versus antagonist drugs. Thus, the site exhibiting high affinity for antagonist ligands corresponds to the low affinity binding site for agonists, and the site exhibiting low affinity for antagonists represents a high affinity binding site for agonists.

In a complex and interesting series of experiments, Bression et al. (1983; 1985) have examined the effects of ovarian steroid milieu on these two classes of binding sites. High affinity domperidone binding sites (agonist low affinity sites) were not affected by any treatment. Conversely, sites exhibiting lower affinity for domperidone were perturbed by a variety of treatments. Chronic estrogenization of intact animals increased the maximum number of these sites by more than 50%, while ovariectomy resulted in a complete disappearance of antagonist low affinity binding (Bression et al., 1983). In ovariectomized rats, replacement of estrogen and progesterone, but not estrogen alone, resulted in restoration of low affinity antagonist binding (Bression et al., 1985). Interestingly, estrogen and progesterone also exert effects in vitro on the characteristics of domperidone binding to pituitary membranes harvested from intact or ovariectomized rats (Bression et al., 1985).
Furthermore, domperidone binding patterns in these studies correlated with the ability of DA to inhibit the release of PRL from perifused pituitaries. Membranes harvested from intact rats exhibited biphasic binding for domperidone. The addition of estradiol to incubations resulted in the disappearance of low affinity binding sites for this antagonist drug. This result was paralleled in pituitary perifusion studies in the presence of various concentrations of DA, such that in the absence of estradiol, pituitaries from intact animals showed a biphasic dose-dependent inhibition of PRL release by DA, whereas the addition of estradiol to perifusion media resulted in the elimination of high affinity (low dose) inhibition of PRL release by DA. To the point, the disappearance of antagonist low affinity binding to membranes co-incubated with estradiol directly correlated with the loss of high affinity inhibition by DA of PRL release from pituitaries perifused with estradiol-containing media. Similarly, the addition of progesterone to membranes harvested from ovariectomized rats resulted in restoration of low affinity domperidone binding, and addition of progesterone to perifusion media restored biphasic inhibition of PRL release by DA from pituitaries of ovariectomized animals (Bression et al., 1985). These authors suggest that pituitary dopamine receptors may be regulated at the membrane level by ovarian steroids, such that when estrogens are in relative excess, agonist high affinity interactions are diminished or eliminated, and that a balanced ratio of estrogen and progesterone is necessary for the presence of such interactions. This modulation of DA receptors may explain part of the antidopaminergic effect of estrogens on PRL cells.

Estrogens may also alter the subcellular processing of DA subsequent to cell surface binding. Gudelsky and colleagues (1981) found that estrogen treatment
reduced by 40% the amount of DA associated with PRL secretory granules. These authors have hypothesized that part of the ability of estrogens to desensitize PRL cells to inhibition by DA may be mediated through a reduction in the capacity of these cells to internalize DA and incorporate it into secretory granules.

Effects on Central Regulation of PRL

In addition to direct effects at the pituitary level, estrogens administered in vivo also exert effects over PRL cell activity mediated through the central nervous system and hypothalamic/pituitary axis. In ovariectomized rats, frontal deafferentation of the hypothalamus largely blocks the serum PRL elevating effect of estradiol injections (Calagaris and Taleisnik, 1976). Lesions of the medial basal part of the suprachiasmatic area or more discrete lesions of the suprachiasmatic nuclei (SCN), medial preoptic area (MPO) or ventromedial hypothalamic nuclei (VMH) in ovariectomized rats also block estrogen-induced PRL surges (Kawakami et al., 1980; Kawakami and Arita, 1981; Pan and Gala, 1985a), while lesions of the dorsomedial hypothalamic nuclei (DMH) increase the magnitude of these afternoon surges (Pan and Gala, 1985a). Furthermore, while ovariectomy on the first day of diestrus prevents the normal PRL surge on the afternoon of proestrus, this surge can be maintained by systemic administration of polyestradiol phosphate (PEP) or by bilateral implantation of estradiol in the MPO or VMH, but not other brain areas (Pan and Gala, 1985b). Collectively, these studies indicate that the MPO, SCN, VMH, DMH and other rostral brain areas may all participate in the regulation of estrogen-induced PRL surges, and lend support to the hypothesis that the MPO and/or VMH may serve as estrogen feedback sites for production of
these surges. Johnson and Crowley (1984) found that the PRL-elevating effects of estradiol are associated with increased 5-HT turnover in the MPO and VMH, and that administration of the opiate antagonist nalmetrene blocks both these effects. These authors concluded that acute estrogen-induced elevations of serum PRL are produced by activation of an endogenous opioid neuronal system which in turn activates transmission in specific serotonergic projections to the hypothalamus.

There is also evidence that central noradrenergic neurons participate in the regulation of PRL secretion by estrogens. The turnover of norepinephrine (NE) in the median eminence is increased on the afternoon of proestrus (Rance et al., 1981), coincident with the proestrus PRL surge, and lesion of the predominant noradrenergic projection to the hypothalamus (ventral noradrenergic tract) completely blocks this surge (Langelier and McCann, 1977). Also, in ovariectomized rats, administration of NE synthesis blockers prevents the estrogen-induced afternoon rise in plasma PRL levels (Carr et al., 1977). These studies suggest that increased impulse traffic along noradrenergic projections to the hypothalamus is involved in the afternoon PRL surges observed on proestrus and in estrogen-treated ovariectomized rats.

Estrogens have also been reported to alter dopaminergic activity in the hypothalamus. In long term ovariectomized rats, the DA content of the median eminence is increased, and this is reversed by estrogen replacement (Gudelsky et al., 1977). Eikenburg and co-workers (1977) found that several days of estrogen treatment increased DA turnover in tuberoinfundibular neurons, and that this
apparent increase in dopaminergic transmission did not occur in hypophysectomized animals. In a contrasting report, estradiol injections were found to acutely decrease DA concentrations in the hypophyseal portal blood in rats subjected to adrenalectomy and ovariectomy on the day of proestrus (Cramer et al., 1979). Crowley (1982) also observed decreased DA turnover in the median eminence three hours following estradiol injections in ovariectomized rats, while longer term estrogen treatment resulted in elevated TIDA activity. These results suggest two separate effects of estrogen on TIDA neuronal activity. Acute elevation of circulating estrogen concentrations appears to decrease DA release from TIDA neurons, while longer periods of elevated estrogen levels lead to increased release of DA from these neurons, probably as a result of PRL feedback mechanisms.

Membrane Effects

Most of the effects of estrogens on PRL cell activity discussed thus far appear to be mediated through an interaction with the estrogen receptor. However, evidence exists which suggests that estrogens may also exert non-receptor-mediated effects on pituitary cell membranes. As discussed in a previous section, action potentials have been detected in both normal and clonal (GH) pituitary cells. It has been demonstrated that 17β-estradiol induces a burst of calcium-dependent action potentials within 1 minute in GH cells in culture (Dufy et al., 1979a), and further, that estradiol is able to reverse the inhibitory effect of DA on TRH-induced action potentials (Dufy et al., 1979b). Because these effects occur with such short latencies, it seems unlikely that they are mediated through estrogen-receptor-genomic interactions. The observation by Bression et al. (1985) that incubation of
anterior pituitary membranes alone with estradiol resulted in altered dopaminergic binding, further supports the concept of direct membrane level effects of estrogens on PRL cell regulation.

The Pineal Gland and PRL Cell Activity

Overview

The pineal gland of rodents has been shown in numerous studies to mediate profound changes in the neuroendocrine-reproductive axis induced by altered photoperiodic environment (for review, see Reiter, 1974; 1980). When adult Syrian hamsters are exposed to short photoperiod (less than 12.5 hours of light/day) or blinded, gonadal function is suppressed and the reproductive organs involute. This effect is largely preventable by prior removal of the pineal gland or by interruption of the picaresque neural pathway which provides the mammalian pineal with photic information. Information concerning environmental light:dark cycles is detected by the retinæ and transferred to the pineal over a complex chain of neurons involving the optic nerves, retinohypothalamic tracts, suprachiasmatic nuclei, multisynaptic hypothalamospinal projections and elements of the sympathetic nervous system including the superior cervical ganglia and ultimately the post-ganglionic nervi conarii. In general, light inhibits pineal synthetic and secretory activity whereas darkness activates pineal endocrine activities.

Potential pineal hormones are generally divided into two classes, the peptides and the indolamines. The indolamine melatonin, which may be the factor responsible for pineal effects on reproduction, is undoubtedly the most widely studied of
pineal hormones. Pineal content and circulating levels of melatonin increase during the dark phase of the light:dark cycle. Strong support for antigonadotrophic effects of melatonin was engendered by the discovery that appropriately timed administration of exogenous melatonin results in gonadal repression reminiscent of that produced by light deprivation (Tamarkin et al., 1976; 1977). For antigonadotrophic efficacy, exogenous melatonin must be administered late in the photoperiod and the presence of an intact pineal is also required (Reiter et al., 1976). In pinealectomized hamsters, three daily injections of melatonin also mimic the effects of light deprivation on reproductive function (Tamarkin et al., 1977). The precise mechanism of reproductive suppression by melatonin is unknown, but such experimental evidence indicates that exogenous melatonin, administered in an appropriate pattern, can duplicate most if not all of the effects of short photoperiod on reproductive function in hamsters. The preceding paragraphs represent a very brief overview of many years of investigation, which has been exhaustively reviewed (Reiter, 1974; 1980; 1981).

Effects on PRL Cell Activity

The effects of light deprivation on reproductive function in hamsters are accompanied by changes in PRL cell activity. Circulating PRL levels are reduced, reaching nadir values after approximately 6-12 weeks (Reiter and Johnson, 1974a; 1974b; Benson and Matthews, 1980; Borer et al., 1982). Pituitary content of immunoreactive (RIA) PRL is also markedly reduced by blinding or short photoperiod exposure (Reiter and Johnson, 1974a; 1974b) as is the release of RIA-PRL in vitro (Orstead and Benson, 1980; Blask et al., 1982; Steger et al., 1983).
Finally, light deprivation inhibits the synthesis of PRL, as measured by $^3$H-leucine incorporation into PRL molecules in vitro (Blask et al., 1986; Orstead and Blask, 1987). Daily afternoon injections of melatonin also inhibit PRL cell activity to a similar magnitude as light deprivation (Reiter et al., 1976; Tamarkin et al., 1976).

In contrast to hamsters, the laboratory rat is generally unresponsive to reproductive effects of blinding or short photoperiod exposure. However, certain experimental manipulations, referred to as sensitizing or potentiating factors, increase the sensitivity of the reproductive systems of rats to the suppressive effects of the pineal (see Reiter, 1974; 1980). Potentiating factors include neonatal androgenization, restriction of food intake and olfactory bulbectomy. Olfactory bulbectomy alone exerts minimal effects on reproductive physiology, but aspiration of the olfactory bulbs in combination with blinding results in severely delayed sexual maturation in pre-pubertal rats and reproductive system regression in mature animals (Reiter, 1972; 1973; Reiter et al., 1969; 1970). The potentiating effect of anosmia appears to involve sensitization to the pineal hormone melatonin, as daily afternoon administration of melatonin mimics the effects of light deprivation in anosmic rats (Blask and Nodelman, 1979). Changes in reproductive physiology in blind anosmic rats are accompanied by decreased storage, synthesis and release of PRL (Leadem and Blask, 1981; 1982a), decreased total anterior pituitary DNA content and a proportionately decreased number of immunocytochemically identified PRL cells (Leadem and Blask, 1982a; 1982b; 1982c). Combined blinding and anosmia has also been shown to exert profound inhibitory effects on the hyperplasia and hypertrophy of PRL cells that occurs at the time of puberty (Leadem and Blask, 1984).
Involvement of Dopaminergic Mechanisms

Inhibitory effects of melatonin on PRL cells may involve alterations in dopaminergic regulatory mechanisms. The PRL-inhibitory effects of melatonin injections in anosmic rats are blocked by co-administration of the DA antagonist pimozide (Blask et al., 1980). Placement of male hamsters in short photoperiod results in a transient increase in hypothalamic DA turnover after 3 to 6 weeks (Benson, 1987) followed by a decline after 9 to 12 weeks to below that seen at the start of the experiment (Steger et al., 1982; 1984; Benson, 1987) with a return to starting levels after 15 weeks (Steger et al., 1982). PRL release in vitro from pituitaries of short photoperiod-exposed male hamsters is significantly reduced after 4 weeks, declines to nadir levels by 11 weeks and returns to long photoperiod control levels by 15 weeks (Steger et al., 1983). Thus, PRL cell activity appears to decline at early time points co-incident with increased hypothalamic DA turnover, while after 11 weeks both PRL cell activity and DA turnover are significantly depressed. Such a result might be explained by postulating an early transient involvement of the DA system in initiating PRL cell inhibition, with maintenance of inhibition by other unidentified mechanisms. Very low circulating levels of PRL would eliminate the feedback drive for DA release from TIDA terminals, as evidenced by diminished turnover at intermediate time points. Steger et al. (1983) reported that incubation in the presence of DA (5 x 10^{-8} M) had no effect on RIA-PRL release from hemipituitaries of short photoperiod-exposed animals at 9 days, while the same dose elicited significant inhibition of PRL release at the 4 and 11 week time points. No effect of this concentration of DA was seen in long photoperiod controls at any time, leading these authors to hypothesize that short photoperiod
exposure results in increased pituitary sensitivity to the PRL-inhibitory effects of DA, and that this might explain sustained inhibition of PRL cell activity when DA turnover is diminished. However, Blask and Orstead (1986) found that similar incubations in the presence of $5 \times 10^{-7}$ or $5 \times 10^{-5}$ M DA resulted in marked inhibition of the release of both RIA-PRL and $^3$H-PRL (newly synthesized) from pituitaries of long photoperiod-exposed male hamsters. There was no effect of DA on total PRL synthesis. In female hamsters, RIA-PRL release \textit{in vitro} was inhibited by DA to a similar degree, while the release of newly synthesized PRL was only slightly reduced by DA (Orstead and Blask, 1987). Again, total PRL synthesis was unaffected by DA. In blind-anosmic rats, there is a transient increase in DA neuron activity after 1 week that precedes the inhibition of PRL secretion, with a return to control turnover levels by 4 weeks, while serum PRL levels remain significantly depressed at 4 and 8 weeks (Leadem et al., 1988). There was no apparent change in pituitary sensitivity to DA in these animals, as determined by single dose ($5 \times 10^{-7}$ M) testing of PRL release inhibition \textit{in vitro}. Finally, melatonin inhibits stimulated release of dopamine from brain slices \textit{in vitro}, with maximal inhibitory activity in the hypothalamus (Zisapel et al., 1982). In summary, experimental evidence indicates that the inhibitory effects of light deprivation and melatonin on PRL cell activity may involve alterations in dopaminergic control mechanisms, both at the hypothalamic and pituitary level.
Pituitary PRL-Secreting Adenomas

Estrogens and Tumorigenesis

The stimulatory effects of estrogens on essentially every phase of PRL cell activity have been discussed in a previous section. In accordance with these properties, the chronic administration of estrogens induces the growth of PRL-secreting pituitary adenomas in rats (see Dannies, 1985). The degree and rapidity of tumorigenesis varies widely among rat strains. The potent estrogenic compound diethylstilbestrol (DES) produces a rapid and profound growth of anterior pituitary tumors in Fisher 344 (F344) rats (Dunning et al., 1947; Wiklund et al., 1981). Wet pituitary weight is significantly increased by 2 weeks, and increases to 6 to 10 times normal by 8 weeks (Wiklund et al., 1981). These tumors arise almost exclusively from the hypertrophy and hyperplasia of PRL cells (Clifton and Meyer, 1956; DeNicola et al., 1978; Bartke et al., 1984), with concurrent increases in serum PRL levels, PRL synthesis and pituitary DNA content (Kaplan and DeNicola, 1976; Wiklund et al., 1981). As little as 14 days of exposure to DES may be sufficient for adenoma induction. In one series of experiments (Morgan et al., 1985a), animals were implanted with DES-containing capsules for 3, 7, 14 and 30 days, at which time the capsules were removed. Serum PRL was measured at the time of capsule removal. Twenty days after capsule removal, serum PRL was measured again, along with pituitary PRL content and in vitro release. At the time of capsule removal, serum PRL levels were significantly increased by 3 days of DES exposure and markedly increased in animals bearing implants for 30 days. Twenty days after removal of capsules, serum PRL levels remained elevated in 30 day DES-treated
animals, and furthermore, in vitro release of PRL was markedly increased in 14 and 30 day DES-treated rats. Pituitary PRL content in 30 day DES-treated animals remained increased by 4-fold over controls 20 days after capsule removal. In a second series of experiments, Morgan et al. (1985b) found that in animals exposed to DES for 2 months, anterior pituitary weights and serum PRL levels remained elevated for at least 8 months following capsule removal. These results appear to indicate a graded transition from estrogen-dependent hyperplasia to frank neoplasia between 14 and 60 days of DES exposure. However, as discussed below, more long term effects on TIDA neurons may also play a role in this persistent hyperprolactinemia.

Relationship to Dopaminergic Regulation of PRL Cells

While the precise mechanism of DES induction of PRL-secreting tumors is not known, it may be related to perturbation of dopaminergic PRL-regulatory mechanisms. As discussed previously, acute administration of estrogens results in a brief decline in DA release into the hypophyseal portal circulation (Cramer et al., 1979; Crowley, 1982), followed by a secondary increase in TIDA activity within 24 hours (Crowley, 1982). Increased activity of TIDA neurons following estrogen administration is probably attributable to feedback effects of increased circulating PRL levels, as hypophysectomy abolishes this effect (Eikenberg et al., 1977).

Long term estrogen treatment exerts somewhat more complex effects on TIDA neurons. There is general agreement in published reports that long term estrogenization results in decreased steady state DA concentrations in the median eminence (Sarkar et al., 1982; Demarest et al., 1984; Terry et al., 1985; Morgan et
al., 1985). Sarkar et al. (1982; 1984) have attributed this to a toxic effect of estrogen and/or the attendant hyperprolactinemia on dopaminergic neurons, based upon their observations of cytopathological changes in the arcuate nucleus and reduced stimulated release of DA from median eminence in vitro. These effects were noted after several months of estrogen treatment. Demarest et al. (1984) examined the effects of 6 to 18 days of estrogen treatment on TIDA function. They found that exposure to estrogen for 6 days resulted in increased DA neuron activity, while at 18 days TIDA activity was decreased, while serum PRL levels were greatly increased. In another group of animals, estrogen containing capsules were left in place for 18 days and then removed. Eighteen days following capsule removal, circulating PRL levels had returned to normal while TIDA activity was increased to levels greater than controls. Furthermore, animals bearing capsules for 18 days showed diminished response of TIDA neurons to intracerebroventricularly administered PRL compared to controls, while 18 days after capsule removal, TIDA activity was stimulated by exogenous PRL to a greater degree than controls (Demarest et al., 1984). These authors suggest that TIDA neurons are less responsive to elevated PRL levels in the presence of estrogens, but that this is a reversible effect, as evidenced by rebound hypersensitivity of dopaminergic neurons following withdrawal of estrogen. This hypothesis is supported by reports of Morgan et al. (1985a; 1985b), who found that TIDA activity was not increased above control levels in rats implanted continuously with DES for 52 days, but that TIDA activity was significantly increased up to 8 months following capsule removal from animals treated with DES capsules for 2 months. These results indicate that TIDA neurons show diminished responsiveness to hyperprolactinemia in the
presence of DES, but that this effect is reversed following withdrawal of DES. Demarest et al. (1984) have suggested that DA synthetic rates in TIDA neurons are not as tightly coupled to steady state concentrations as in mesotelencephalic DA neurons, such that changes in steady state concentrations may be more indicative of altered synthesis/release dynamics rather than neurotoxicity. However, Casanueva et al. (1982) and Cocchi et al. (1985) have reported that rats chronically treated with estrogens failed to demonstrate diminished serum PRL concentrations following administration of nomifensine, an activator of central dopaminergic activity, up to seven months following discontinuation of estrogen treatment, suggesting a persistent defect in TIDA function. A simple relationship between TIDA function and PRL release inhibition from adenomatous pituitaries may be obscured, however, by neovascularization of tumor tissue, such that tumorous tissue derives less of its blood supply from the hypophyseal portal system (Elias and Weiner, 1983).

It also bears repeating that estrogens have been reported to antagonize PRL-inhibitory actions of DA at the anterior pituitary level (LaBrie et al., 1980; West and Dannies, 1980; Maurer, 1980), and to alter anterior pituitary DA receptor populations (Heiman and Ben-Jonathan, 1982; Bression et al., 1983; 1985). Estrogen-induced pituitary tumors in general, and DES-induced tumors in F344 rats in particular, appear to retain the ability to respond to DA receptor occupancy, however, as evidenced by tumor involution and reduced serum PRL levels elicited by treatment with the potent dopaminergic agonist bromocriptine (DiPaolo and Falardeau, 1984; Miyazaki et al., 1985; Eljarmak et al., 1985). Furthermore, DiPaolo and Falardeau (1984) reported the presence of DA receptors in estradiol-induced
pituitary tumors with binding site concentration and affinity which were indistinguishable from normal pituitary.

Melatonin and Tumor Inhibition

Leadem (1984; 1985) first reported that combined blinding and anosmia resulted in inhibition of DES-induced pituitary tumors in F344 rats, as reflected by decreases in pituitary weight, pituitary DNA content, serum PRL and \textit{in vitro} PRL synthesis and total radioimmunoassayable PRL. It was subsequently demonstrated that pinealectomy completely blocked the inhibitory effects of blinding and anosmia on DES-stimulated tumor activity, indicating that pineal gland is the primary mediator of these effects (Leadem, 1986). In subsequent studies assessing the individual effects of olfactory bulbectomy, blinding and pinealectomy it was serendipitously discovered that blinding alone was equally as effective as combined blinding and anosmia in inhibiting the growth of DES-induced adenomas (Leadem and Burns, 1987; 1988). Olfactory bulbectomy or pinealectomy alone had no effect on tumor activity. Moreover, daily afternoon injection of 50 $\mu$g melatonin mimicked the effects of blinding on tumor growth, and this was not dependent upon nor altered by olfactory bulbectomy (Leadem and Burns, 1987; 1988). Surprisingly, constant release implants of melatonin were also effective in inhibiting tumor growth. Given the important role of DA in normal PRL cell regulation and the experimental evidence suggesting that altered TIDA function (Benson, 1987; Leadem \textit{et al}., 1988) and/or changes in pituitary sensitivity to DA (Steger \textit{et al}., 1989) are possible mechanisms in the PRL-inhibitory effects of light deprivation on non-tumorous pituitaries in the hamster and rat, it is possible that the effects of
blinding or melatonin treatment on DES-induced tumors are mediated through changes in dopaminergic regulatory mechanisms.
CHAPTER 2

STATEMENT OF THE PROBLEM AND SPECIFIC AIMS

The pineal gland of the male Syrian hamster exerts profound inhibitory effects on PRL cell activity when these animals are exposed to short photoperiods (less than 12.5 hours of light/day). Appropriately timed daily injections of melatonin inhibit PRL cell activity to a similar magnitude as light deprivation. While the laboratory rat is generally unresponsive to PRL-inhibitory effects of light deprivation or exogenous melatonin, similar effects can be elicited by additional experimental manipulations referred to as potentiating or sensitizing factors. Furthermore, the Fisher 344 (F344) strain, unlike other rat strains, appears to be inherently (i.e. in the absence of potentiating factors) sensitive to photoperiod effects on the neuroendocrine-reproductive axis. This characteristic is particularly serendipitous given the exquisite sensitivity of the F344 strain to induction of pituitary PRL-secreting adenoma growth by chronic estrogenization. In this unique strain, blinding alone produces strong inhibition of PRL-secreting adenoma growth and synthetic and secretory activity induced by subcutaneous implantation of capsules containing the potent estrogenic compound diethylstilbestrol (DES). Moreover, this effect can be mimicked by exogenous administration of melatonin. Thus, in the Syrian hamster and in the F344 rat, it is possible to investigate the mechanisms of pineal/pineal hormone related effects on PRL cell activity under paradigms where
interpretations are not encumbered by potential side effects of multiple surgical, dietary or endocrine manipulations.

A very large body of evidence accumulated over many years has demonstrated the physiological importance of dopamine released from terminals in the hypothalamic median eminence in regulation of pituitary PRL cell activity. In contrast, only a few published reports exist which examine the possible nexus between pineal/pineal hormone effects on PRL cells and altered dopaminergic PRL-regulatory mechanisms. In general, such studies as do exist suggest that enhanced release of dopamine from hypothalamic terminals and/or increased pituitary responsiveness to dopamine underly, at least in part, the observed effects of light deprivation on PRL cell activity.

The major issue addressed in this dissertation is the possible involvement of dopaminergic mechanisms in PRL cell inhibition elicited by short photoperiod exposure or melatonin administration. Such possible involvement is broadly addressed by: 1) measurement of dopamine receptors in pituitary membranes of male hamsters 2) assessment and comparison of in vitro dopamine sensitivity of pituitaries from long vs. short photoperiod-exposed male hamsters at several time points during the development and maintenance of PRL cell inhibition induced by short photoperiods 3) assessment and comparison of in vitro dopamine sensitivity of pituitaries, pituitary dopamine receptors and hypothalamic dopamine neuron activity in F344 rats treated with melatonin vs. saline vehicle both before and after adenoma induction by subcutaneous implantation of DES-containing capsules. The specific questions addressed in this dissertation, some of which include issues
tangential to the main issue of dopaminergic involvement in production of effects
(i.e. PRL cell inhibition induced by short photoperiod or melatonin administration)
are as follows:

1) Given that the pituitaries of long photoperiod-exposed male hamsters
may not respond to the PRL-inhibitory effects of dopamine, and
given that the actions of dopamine are presumably mediated
through first binding to cell surface receptors, do the pituitaries of
these animals express dopamine receptor populations as ob-
served in other mammalian species? (Chapter 3)

2) Does dopamine effectively inhibit the release of PRL in vitro from
pituitaries of long photoperiod-exposed male hamsters? (Chapter
3)

3) As measured by dose response modeling of PRL release inhibition, is
pituitary sensitivity to dopamine enhanced over the time course of
PRL cell inhibition induced by short photoperiod, compared to
long photoperiod controls? (Chapter 3)

4) Does the daily afternoon injection of melatonin for a period of two
weeks alter indices of PRL cell activity in otherwise untreated F344
rats? (Chapter 4)
5) Is the daily afternoon injection of melatonin effective in inhibiting the
growth and activity of DES-induced PRL-secreting adenomas in
the F344 rat? (Chapter 4)

6) Does the daily administration of melatonin enhance pituitary sensitivity
to dopamine in otherwise untreated F344 rats? (Chapter 4)

7) Does the daily administration of melatonin enhance pituitary sensitivity
to dopamine in F344 bearing PRL-secreting adenomas induced
by 4 weeks of exposure to DES? (Chapter 4)

8) Does daily afternoon melatonin administration alter pituitary dopamine
receptor populations in otherwise untreated F344 rats? (Chapter 4)

9) Does melatonin administration in vitro alter agonist vs. antagonist com-
petition behaviour at pituitary dopamine receptors? (Chapter 4)

10) Are pituitary DA receptor populations altered in DES-induced
adenomas, and if so, is this effect augmented or reversed by daily
administration of melatonin? (Chapter 4)

11) Are agonist vs. antagonist interactions at dopamine receptors altered
in DES-induced adenomas? (Chapter 4)

12) Does the daily administration of melatonin alter steady-state
dopamine concentrations in the hypothalamic median eminence
of otherwise untreated F344 rats? (Chapter 4)
13) Does daily melatonin administration of melatonin alter steady-state dopamine concentrations in the hypothalamic median eminence of F344 rats with DES-induced pituitary adenomas? (Chapter 4)

14) Does melatonin administration alter the activity of hypothalamic dopamine neurons in otherwise untreated F344 rats? (Chapter 4)

15) Does melatonin administration alter the activity of dopamine neurons in F344 rats with DES-induced PRL-secreting adenomas? (Chapter 4)
CHAPTER 3

EFFECTS OF SHORT PHOTOPERIOD ON PROLACTIN CELL ACTIVITY IN MALE SYRIAN HAMSTERS: ASSESSMENT OF PITUITARY RESPONSIVENESS TO DOPAMINE

Abstract

The purpose of the present study was to determine whether the inhibitory effects of short photoperiod exposure on prolactin (PRL) cell activity in male Syrian hamsters were possibly mediated through changes in anterior pituitary responsiveness to dopamine (DA). Previously published results (Steger et al., 1983) suggested that DA (5 x 10^{-8} M) did not alter in vitro PRL release from pituitaries of long photoperiod-exposed male hamsters and further, that decreased circulating PRL levels and decreased ability of pituitaries to secrete PRL in vitro observed in short photoperiod-exposed male hamsters were attributable to the development of pituitary responsiveness to DA. It was therefore of interest to determine if pituitary membranes from long photoperiod-treated animals exhibited typical D2 DA receptor populations as have been reported for a variety of mammalian species. This was accomplished by analysis of saturation binding behaviour of the DA antagonist drug \(^{3}\)H-Spiperone to anterior pituitary membranes harvested from long photoperiod- (14:10 Light:Dark cycle) exposed male hamsters. Nonspecific binding was determined in the presence of 5 \(\mu\)M (+)-butaclamol. To further examine the relationship between pituitary DA sensitivity and short photoperiod-induced PRL inhibition, adult male hamsters were housed in either long (14:10 L:D) or short (10:14 L:D) photoperiod and animals representing both treatment groups were
sacrificed after 3, 7, 11 and 15 weeks of treatment. Anterior pituitaries were removed and hemisected and the resulting halves were incubated for 3 hours at 37°C in medium containing DA (5 x 10^{-11} - 5 x 10^{-7} M) and ascorbic acid (5.6 x 10^{-4} M) or medium containing ascorbic acid alone. Thus, the hemipituitary of one animal served as the vehicle-treated control for the corresponding half incubated with one of the concentrations of DA. At the end of the incubation period, media were saved for subsequent PRL radioimmunoassay. Pituitary sensitivity to DA was assessed by modeling the proportional inhibition of release of radioimmunoassayable PRL (RIA-PRL) as a function of DA concentration, yielding estimates of the half-maximal effective concentration ($IC_{50}$) and maximal magnitude of response (maximum inhibition) for both treatment groups at each time point.

Analysis of saturation binding data revealed the presence of D2-like binding sites in anterior pituitary membranes of long photoperiod-exposed animals, and these binding sites could be resolved by nonlinear modeling into 2 distinct subpopulations with equilibrium dissociation constant values of $0.07 \pm 0.016$ nM ($K_H$) and $4.03 \pm 1.41$ nM ($K_L$) and corresponding binding site concentrations of $41.9 \pm 2.3$ (BH) and $91.2 \pm 18.1$ (BL) femtomoles per mg protein (fmol/mg). Short photoperiod exposure resulted in decreased circulating PRL levels, decreased total pituitary content of RIA-PRL and diminished PRL release from incubated pituitaries by 7 weeks, with further decreases at 11 and 15 weeks as compared to long photoperiod controls. However, dose response analysis indicated that DA was highly and equally effective in inhibiting PRL release \textit{in vitro} from the pituitaries of both long and short photoperiod-exposed animals and sensitivity to DA was not altered over the time course of development of diminished PRL cell activity induced
by exposure to short photoperiods. Inhibition of PRL release occurred with apparent high affinity in both treatment groups, with IC\(_{50}\) values in the nanomolar range. Maximal effectiveness of DA in inhibiting PRL release was also unaffected by treatments. From these data we conclude that: 1) the pituitaries of long photoperiod-exposed male hamsters contain dopaminergic binding sites with characteristics comparable to pituitary D2 DA binding sites as reported in other mammalian species; 2) these binding sites are associated with a functional dopamine receptor complex, as indicated by the ability of DA to inhibit the release of PRL \textit{in vitro} from pituitaries of long photoperiod-treated animals; 3) in contrast to earlier reports, short photoperiod-induced PRL inhibition is not associated with enhanced pituitary sensitivity to the inhibitory effects of dopamine.

\textbf{Introduction}

The pineal gland of rodents has been shown in numerous studies to mediate profound changes in the neuroendocrine-reproductive axis induced by altered photoperiodic environment (see Reiter, 1974; 1980). When adult male Syrian hamsters are exposed to short photoperiod (less than 12.5 hours of light/day), gonadal function is suppressed and the reproductive organs involute. The effects of light deprivation on reproductive function are accompanied by changes in PRL cell activity. Circulating PRL levels are reduced, reaching nadir values after approximately 6-12 weeks (Reiter and Johnson, 1974a; 1974b; Benson and Matthews, 1980). Pituitary content of radioimmunoassayable PRL (RIA-PRL) is also markedly reduced by light deprivation (Reiter and Johnson, 1974a; 1974b) as is the release of RIA-PRL \textit{in vitro} (Orstead and Benson, 1980; Blask et al., 1982;
Steger et al., 1983). Light deprivation also inhibits the synthesis of PRL, as measured by $^3$H-leucine incorporation into PRL molecules in vitro (Blask et al., 1986; Orstead and Blask, 1987).

It is well recognized that dopamine (DA) secreted into the hypophyseal portal circulation by tuberoinfundibular (TIDA) neurons in the hypothalamus is an important physiological regulator of PRL cell activity in mammals (see Neill, 1980). DA is believed to exert its effects by binding to D2 dopamine receptors on the surface of PRL cells (Kebabian and Calne, 1979) to inhibit the release (Caron et al., 1978) and synthesis (Maurer, 1980) of PRL and to inhibit the proliferation of PRL cells (Jacobi and Lloyd, 1981). Such studies regarding the role of hypothalamic DA in the regulation of PRL cell activity have been performed predominantly using the laboratory rat as the animal model. Relatively few published reports exist regarding the physiological role of DA in PRL cell regulation in the hamster. Steger et al. (1983) reported that incubation in the presence of DA ($5 \times 10^{-8}$ M) had no effect on RIA-PRL release from hemipituitaries of short photoperiod-exposed male hamsters after 9 days of treatment, while the same dose elicited significant inhibition of PRL release at 4 and 11 week time points. No effect of this concentration of DA was seen in long photoperiod controls at any time point, leading these authors to conclude that short photoperiod exposure in these animals results in the development of enhanced pituitary sensitivity to the PRL-inhibitory effects of DA. In contrast, Blask and Orstead (1986) found that similar incubations in the presence of $5 \times 10^{-7}$ or $5 \times 10^{-5}$ M DA resulted in marked inhibition of the release of both RIA-PRL and $^3$H-PRL (newly synthesized) from the pituitaries of long photoperiod-exposed male hamsters. There was no effect of DA on total PRL
synthesis in vitro. In female hamsters, RIA-PRL release in vitro was inhibited by DA to a similar degree, while the release of $^{3}$H-PRL was only slightly reduced by DA (Orstead and Blask, 1987). As in the male, total PRL synthesis was unaffected by DA.

Evidence also exists which suggests the involvement of altered hypothalamic dopamine neuronal activity in short photoperiod-induced PRL inhibition. Placement of male hamsters in short photoperiod results in a transient increase in hypothalamic DA turnover after 3 to 6 weeks (Benson, 1987) followed by a decline after 9 to 12 weeks to below that seen at the start of the experiment (Steger et al., 1982; 1984; Benson, 1987) with a return to starting levels after 15 weeks (Steger et al., 1982). PRL release from pituitaries of short photoperiod-exposed male hamsters has been reported to be significantly reduced after 4 weeks, declining to nadir values by 11 weeks and subsequently returning to long photoperiod control levels by 15 weeks (Steger et al., 1983). Thus, PRL cell activity appears to decline at early time points co-incident with increased hypothalamic DA turnover, while after 11 weeks both PRL cell activity and DA turnover are depressed. Such a result might be explained by postulating an early transient involvement of increased DA turnover in initiating PRL cell inhibition, with subsequent maintenance of inhibition by either enhanced pituitary sensitivity to DA or by other unidentified DA-independent mechanisms. In either case, observed decreases in DA turnover co-incident with diminished PRL cell activity may be explained on the basis of diminished feedback drive on hypothalamic DA neurons owing to lowered circulating PRL levels.
Because no published reports existed regarding dopaminergic binding in the anterior pituitary of the Syrian hamster, and because of the reported lack of responsiveness to DA in long photoperiod-exposed males, it was of interest to determine if pituitary D2 DA receptors, as characterized in several mammalian species (see Cronin, 1982), were present in the pituitaries of long photoperiod-exposed male hamsters. This was accomplished by analysis of specific binding saturation isotherms for binding of the butyrophenone $^3$H-spiperone to anterior pituitary membranes. In order to further investigate the possible relationship between exposure to short photoperiods and altered pituitary sensitivity to DA, the potency of DA for inhibition of RIA-PRL release from pituitaries in vitro was determined for both long and short photoperiod treatment groups at each of a series of time points (3, 7, 11 and 15 weeks) during the course of development of PRL cell inhibition induced by short photoperiods. The potency of DA was assessed by estimation of half-maximal inhibitory concentration ($IC_{50}$) and maximal effect (expressed as % maximum inhibition) by nonlinear modeling of inhibition data obtained over a wide range of DA concentrations ($5 \times 10^{-11} - 5 \times 10^{-7}$ M).

Materials and Methods

Animals

Young adult male Syrian hamsters (91-100 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). After a 2 week period of acclimatization in long photoperiod (14:10 Light:Dark cycle), half of the animals (120) were transferred into specially constructed light tight chambers in which short photoperiod (10:14 L:D) was maintained. Hamsters were housed in clear plastic
cages on metal shelves in chambers with a source of cool white light (General Electric, NOF2T12-CW) mounted 25 cm above tops of cages. Food and water were provided ad libitum and air flow was held at approximately 30 ft³/min with ambient temperature maintained at 22° C. Groups of 30 animals from both photoperiod treatment groups were sacrificed by rapid decapitation at 3, 7, 11 and 15 weeks following initiation of photoperiod treatments. Truncal blood was collected for measurement of circulating PRL levels and pituitaries were removed and processed for incubation in vitro for DA sensitivity studies. For ³H-spiperone binding studies, an additional 3 groups of 35 animals each were maintained in long photoperiod for 2 weeks of acclimatization prior to sacrifice for collection of anterior pituitaries.

Radioimmunoassays

Sera, incubation media and incubated pituitaries were assayed for immunoreactive prolactin (RIA-PRL) using the procedure of Soares et al. (1983). All samples were assayed in duplicate. For details, see Appendix D. Hamster PRL and hamster PRL antisera were supplied by Dr. Frank Talamantes, Department of Biology, Thimann Laboratories, University of California, Santa Cruz, California. Sheep anti-rabbit gamma globulins were kindly supplied by Dr. Mark Wise, University of Arizona, Tucson, Arizona.

In Vitro Pituitary Incubations

Incubation procedures are presented in detail in Appendix B of this dissertation. Briefly, at the time of sacrifice, anterior pituitaries were removed, weighed
and hemisected. Incubations were subsequently carried out such that one hemipituitary served as the vehicle-treated control for the other half incubated in the presence of DA. Individual hemipituitaries were placed in small glass vials containing 0.5 ml minimal essential medium (GIBCO) with penicillin (5000 U), Fungizone (12.5 μg), streptomycin (5000 μg) and ascorbic acid (5.6 x 10^-4 M). The pituitaries were then preincubated for 30 minutes in a Dubnoff Shaking Metabolic Incubator (37° C, gassed with 95% O₂:5% CO₂). After 30 minutes, the media were aspirated and discarded and 0.5 ml of fresh medium was added to each vial. For each hemipituitary to be incubated in the presence of DA, 5 μl of medium containing DA prepared in advance to yield one of 5 concentrations (5 x 10^-11, 5 x 10^-10, 5 x 10^-9, 5 x 10^-8 or 5 x 10^-7 M) was added to the incubation vial. Bench stock DA solutions were freshly prepared every 30 minutes. Incubations were carried out for an additional 3 hours, at which time incubation media were aspirated and frozen, and incubated hemipituitaries were weighed, homogenized in 0.5 ml 0.1 M PBS and frozen. The PRL content of incubation media and incubated hemipituitaries were subsequently determined by radioimmunoassay. For dopamine sensitivity studies, the inhibition of RIA-PRL release elicited by dopamine over a range of concentrations was analyzed by nonlinear modeling (see Appendix A) in order to obtain estimates of percent maximum inhibition and half-maximal effective concentration (designated IC₅₀) for both treatment groups at each time point. The proportional inhibition of PRL release, which served as the dependent variable in regression analysis, was calculated by dividing the PRL concentration (ng RIA-PRL/mg tissue) in the medium from each DA-treated hemipituitary by the medium PRL concentration from the corresponding half incubated in the absence of DA.
Control (DA-absent) incubations were additionally used to measure RIA-PRL release and pituitary content for direct comparison between treatment groups.

**Binding Assays**

Anterior pituitary membranes were prepared according to the methods of Bression et al. (1985) with slight modifications (for details, see Appendix G). Animals were killed by rapid decapitation. Anterior pituitaries were removed, rinsed in cold saline and weighed. The tissue was then homogenized (1:10, weight:volume) in ice cold 50 mM Tris-HCl buffer, pH 7.4, with a hand-held teflon/glass tissue grinder. The homogenate was centrifuged at 800 x g for 10 minutes (4°C) and the resulting supernatant was centrifuged at 22,500 x g for 20 minutes (4°C). The resulting pellet was washed by rehomogenization in 10 volumes Tris buffer followed by centrifugation at 22,500 x g for 20 minutes (4°C). The final pellet was then suspended by gentle homogenation in incubation buffer (50 mM Tris HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 μM EDTA, 0.1% Ascorbate and 5 μM pargyline) at a concentration of 25 mg (wet tissue weight equivalents)/ml.

³H-Spiperone (23-27 Ci/mmol, New England Nuclear) binding to anterior pituitary membranes was assessed by a rapid filtration technique. ³H-Spiperone was incubated with membrane preparations for 30 minutes at 27°C. Incubation tubes received 200 μl of membrane preparation and either 50 μl of incubation buffer containing (+)-butaclamol (5 μM final concentration, nonspecific binding tubes) or 50 μl incubation buffer alone (total binding tubes). Binding reactions were initiated by addition of 250 μl ³H-spiperone diluted in incubation buffer. At the end of the
incubation, 5 ml of ice cold incubation buffer were added and the membranes were harvested by filtration through glass microfiber filters (GF/C, Whatman) under vacuum. Incubation tubes were rinsed with an additional 5 ml buffer which was also filtered. Filters were then washed twice with 5 ml aliquots of incubation buffer. The filtration and washing procedure took less than 10 seconds. Filters were placed into scintillation vials along with 10 ml aqueous counting scintillant (ACS, Amersham) for counting of radioactivity by scintillation spectrometry. Using external standard ratios, CPM were converted to DPM for final calculations. Specific binding was defined as total binding (determined in triplicate) minus nonspecific binding (determined in duplicate).

Statistics

Pituitary weight, serum RIA-PRL and RIA-PRL concentration in incubation media and pituitary homogenates were analyzed by two-way analysis of variance. If interaction effects (time x treatment) were significant, further analysis was performed by one-way ANOVA using Fischer's Least Significant Difference Test for post-hoc comparisons. ANOVAs were performed using SAS 6.02 running on an IBM-PC. A p value of less than 0.05 was considered significant.

Nonlinear modeling methods are described in detail in Appendix A. Model fitting was performed using the SPSS-X program CNLR (Constrained Nonlinear Regression) running on a VAX/VMS computer at the Arizona Cancer Center. Testing for significant differences between multiple models was accomplished using partial F-tests (DeLean et al., 1982). A model for two binding sites was retained only when it fitted the data significantly better (p < 0.05) than a model for
Parameter estimates were tested for significance of difference by pairwise t-tests using the pooled variance. A p value of less than 0.05 was considered significant.

Presentation of Data

In order to underscore the temporal form of effects, and for the sake of clarity and completeness, results will frequently be presented in both graphic and tabular form. This is intended to be understood as duplicate reporting of the same data in two useful forms and not as additional data.

Experimental Design

Experiment #1. For saturation binding studies, 3 separate groups of 35 adult male Syrian hamsters were acclimatized to long photoperiod for 2 weeks and then killed by rapid decapitation. At the time of sacrifice, anterior pituitaries were removed, weighed and processed for binding assays (see Materials and Methods section and Appendix G). Membrane suspensions were incubated in the presence of increasing concentrations of $^3$H-spiperone (0.05 - 5.25 nM). Non-specific binding was determined in the presence of excess unlabeled competitor. Specific binding data thus generated were modeled as a function of $^3$H-spiperone concentration to obtain estimates of binding site concentration(s) ($B_{max}$) and equilibrium dissociation constant(s) ($K_d$). Final estimates presented represent the mean of 3 separate binding assays.

Rationale. As discussed above, conflicting results exist in the literature concerning the ability of DA to inhibit the release of PRL in vitro by pituitaries
harvested from long photoperiod-exposed male hamsters. The effects of DA on PRL-secreting cells of the anterior pituitary are believed to be mediated through binding to D2 DA receptors (Ben-Jonathan, 1985). High affinity interaction with butyrophenones is a characteristic of D2 DA receptors (Kebabian and Calne, 1979; Creese et al., 1983) and the butyrophenone DA antagonist $^{3}$H-spiperone has been utilized to identify and characterize D2 dopaminergic binding sites in anterior pituitary membranes in a variety of mammalian species (Cronin, 1982). Because of the reported lack of responsiveness to the PRL-inhibitory effects of DA in long photoperiod-exposed male hamsters and because DA binding in the pituitaries of these animals has not been previously investigated, it was of interest to determine if such binding sites, presumably necessary for responsiveness to DA, could be detected.

**Experiment #2** Adult male Syrian hamsters were housed in either long (14:10 L:D) or short (10:14 L:D) photoperiod. After 3, 7, 11 and 15 weeks of photoperiod treatment, 30 animals from each group were killed by rapid decapitation. Trunk blood was collected for determination of circulating RIA-PRL levels and anterior pituitaries were removed, weighed and hemisected for incubation in vitro in the presence of one of 5 concentrations of DA. One hemipituitary from each pair was incubated in the absence of DA. At the end of the experiment, the content of RIA-PRL in incubation media and incubated pituitaries was determined for between-groups comparisons and construction of DA dose response curves.

**Rationale.** As previously discussed, several lines of experimental evidence have suggested the involvement of altered dopaminergic regulation of PRL cell
activity in short-photoperiod-induced PRL inhibition. Specifically, after several weeks of short photoperiod exposure, it has been reported that circulating PRL levels and the ability of pituitaries to release PRL in vitro are both depressed, concurrent with diminished hypothalamic DA turnover (Steger et al., 1982; 1983; Benson, 1987). This effect might be explained by postulating either the development of enhanced pituitary sensitivity to the PRL-inhibitory effects of DA in short photoperiod-exposed animals or other unidentified DA-independent PRL-inhibitory mechanisms. The purpose of this experiment was to test the hypothesis that exposure to short photoperiods results in increased pituitary sensitivity to DA. This was accomplished by estimating DA dose response constants (IC$_{50}$ and % maximum inhibition) for the inhibition of PRL release from hemipituitaries in vitro and comparing such estimates between short photoperiod-treated animals and long photoperiod controls over a series of time points from 3 to 15 weeks. Changes in sensitivity would be detected as shifts in IC$_{50}$ and/or % maximum inhibition values for the inhibition of RIA-PRL release in vitro by DA.

Results

Experiment #1

Saturation Binding of $^3$H-Spiperone

In three separate binding assays, analysis of specific binding data by least squares modeling revealed the presence of two distinct classes of binding sites for $^3$H-spiperone in anterior pituitary membranes from long photoperiod-exposed male hamsters (Fig. 1, Table 1).
Figure 1. Saturation binding of $[^3$H$]$-spiperone to male hamster anterior pituitary membranes. Membranes were incubated with various concentrations of $[^3$H$]$-spiperone (0.05 - 5.25 nM) for 30 minutes at 27°C. Nonspecific binding was determined in the presence of 5 µM (+)-butaclamol. Specific binding was defined as total binding minus nonspecific binding. Results shown were pooled from 3 separate binding assays. Data were analyzed by non-linear modeling. For details see Table 1. Panel A, Saturation isotherms; Panel B, Transformation of saturation isotherm by the method of Scatchard. Dashed lines represent resolution of high and low affinity binding sites as estimated by modeling.
Table 1. Parameters for $[^3H]$-spiperone binding to anterior pituitary membranes from male hamsters exposed to long (14:10 Light:Dark) photoperiod.

<table>
<thead>
<tr>
<th>KH (nM)</th>
<th>BH (fmol/mg)</th>
<th>KL (nM)</th>
<th>BL (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.070 ± 0.016</td>
<td>41.9 ± 2.3</td>
<td>4.03 ± 1.41</td>
<td>91.2 ± 18.1</td>
</tr>
</tbody>
</table>

The high-affinity (KH) and low-affinity (KL) equilibrium dissociation constants and corresponding binding site concentrations (BH and BL) are expressed as the mean ± SEM (n = 3). Saturation binding data were analyzed by non-linear regression modeling under both one-site and two-site model hypotheses. The two-site binding model provided significant improvement in goodness-of-fit to the data (p < 0.01).

Experiment #2

Anterior Pituitary Weights

Short photoperiod exposure had no effect on pituitary weight at any time during the course of the experiment (Fig. 2, Table 2).

Serum PRL Levels

Compared to long photoperiod controls, circulating PRL levels in short photoperiod-treated animals were significantly (p < 0.05) reduced by week 7, declined further by week 11 and remained at very low levels through week 15 (Fig. 2, Table 2).

Dopamine Sensitivity In Vitro

IC$_{50}$ values for the inhibition by DA of RIA-PRL release were in the nM range (3.47 - 9.57 nM) for both photoperiod groups at all time points and the % maximum
Table 2. Serum RIA-PRL and anterior pituitary weights from male hamsters after 3, 7, 11 and 15 weeks of exposure to either long (14:10 Light:Dark) or short (10:14 L:D) photoperiod.

<table>
<thead>
<tr>
<th>Week</th>
<th>Photoperiod</th>
<th>Serum RIA-PRL (ng/ml)</th>
<th>Pituitary Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>14:10</td>
<td>4.57 ± 0.55</td>
<td>2.13 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>10:14</td>
<td>4.82 ± 0.59</td>
<td>2.17 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>14:10</td>
<td>4.94 ± 0.49</td>
<td>2.21 ± 0.08</td>
</tr>
<tr>
<td>7</td>
<td>10:14</td>
<td>3.35 ± 0.39*</td>
<td>2.14 ± 0.05</td>
</tr>
<tr>
<td>11</td>
<td>14:10</td>
<td>5.22 ± 0.61</td>
<td>2.47 ± 0.07</td>
</tr>
<tr>
<td>11</td>
<td>10:14</td>
<td>0.81 ± 0.02*</td>
<td>2.20 ± 0.06</td>
</tr>
<tr>
<td>15</td>
<td>14:10</td>
<td>4.45 ± 0.36</td>
<td>2.03 ± 0.05</td>
</tr>
<tr>
<td>15</td>
<td>10:14</td>
<td>0.55 ± 0.02*</td>
<td>2.09 ± 0.06</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM (n = 27-30). (* p < 0.05 vs. long photoperiod).
Figure 2. Anterior pituitary weights (panel A) and serum RIA-PRL (panel B) from male hamsters after 3, 7, 11 or 15 weeks of exposure to either long (14:10 Light:Dark) or short (10:14 L:D) photoperiod. See Table 2 for details. (*, p < 0.05 vs. long photoperiod).
Table 3. Parameters for the inhibition by dopamine of RIA-PRL release from incubated hemipituitaries harvested from male hamsters after 3, 7, 11 and 15 weeks of exposure to long (14:10 Light:Dark) or short (10:14 L:D) photoperiod.

<table>
<thead>
<tr>
<th>Week</th>
<th>Photoperiod</th>
<th>IC$_{50}$ (nM)</th>
<th>Max Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>14:10</td>
<td>$3.47 \pm 2.62$</td>
<td>$76.7 \pm 13.7$</td>
</tr>
<tr>
<td>3</td>
<td>10:14</td>
<td>$9.57 \pm 7.82$</td>
<td>$86.0 \pm 14.1$</td>
</tr>
<tr>
<td>7</td>
<td>14:10</td>
<td>$7.55 \pm 4.87$</td>
<td>$77.1 \pm 9.8$</td>
</tr>
<tr>
<td>7</td>
<td>10:14</td>
<td>$5.22 \pm 4.03$</td>
<td>$87.2 \pm 13.0$</td>
</tr>
<tr>
<td>11</td>
<td>14:10</td>
<td>$6.77 \pm 4.60$</td>
<td>$86.2 \pm 11.5$</td>
</tr>
<tr>
<td>11</td>
<td>10:14</td>
<td>$5.68 \pm 3.51$</td>
<td>$95.8 \pm 10.8$</td>
</tr>
<tr>
<td>15</td>
<td>14:10</td>
<td>$6.81 \pm 2.56$</td>
<td>$92.4 \pm 6.8$</td>
</tr>
<tr>
<td>15</td>
<td>10:14</td>
<td>$7.40 \pm 2.76$</td>
<td>$98.4 \pm 7.2$</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± asymptotic standard error as estimated from data analysis by non-linear regression modeling. There were no significant differences.

Figure 3. (following page) Dose-response curves for the inhibition by dopamine of RIA-PRL release from incubated hemipituitaries harvested from male hamsters after 3, 7, 11 or 15 weeks of exposure to either long (14:10 Light:Dark) or short (10:14 L:D) photoperiod. At the time of sacrifice, anterior pituitaries were removed and bisected for incubation in either the presence of various concentrations of dopamine ($5 \times 10^{-11} - 5 \times 10^{-7}$ M) or the vehicle for dopamine administration, such that one hemipituitary served as the vehicle control for its corresponding half incubated with one of the concentrations of dopamine. Data were analyzed by non-linear regression modeling for estimates of IC$_{50}$ and percent maximum inhibition. Resulting model predictions are represented by solid lines for long photoperiod groups and dashed lines for short photoperiod groups. For details see Table 3.
Table 4. RIA-PRL in the incubation media and incubated hemipituitary after a 3 hour incubation, from male hamsters following 3, 7, 11 and 15 weeks of exposure to either long (14:10 Light:Dark) or short (10:14 L:D) photoperiod.

<table>
<thead>
<tr>
<th>Week</th>
<th>Photoperiod</th>
<th>Media (ng/pituitary)</th>
<th>Pituitary (ng/pituitary)</th>
<th>Total (ng/pituitary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>14:10</td>
<td>4159.4 ± 271.4</td>
<td>2942.5 ± 151.0</td>
<td>7167.8 ± 346.1</td>
</tr>
<tr>
<td>3</td>
<td>10:14</td>
<td>3526.6 ± 274.0</td>
<td>2622.8 ± 243.4</td>
<td>6349.4 ± 335.9</td>
</tr>
<tr>
<td>7</td>
<td>14:10</td>
<td>3404.4 ± 349.9</td>
<td>2642.9 ± 136.4</td>
<td>6047.7 ± 381.0</td>
</tr>
<tr>
<td>7</td>
<td>10:14</td>
<td>2062.5 ± 304.6*</td>
<td>1771.3 ± 286.5*</td>
<td>3833.8 ± 553.2*</td>
</tr>
<tr>
<td>11</td>
<td>14:10</td>
<td>3314.3 ± 244.8</td>
<td>3050.6 ± 177.8</td>
<td>6364.9 ± 343.5</td>
</tr>
<tr>
<td>11</td>
<td>10:14</td>
<td>778.2 ± 98.0*</td>
<td>747.1 ± 83.6*</td>
<td>1525.4 ± 163.9*</td>
</tr>
<tr>
<td>15</td>
<td>14:10</td>
<td>3727.5 ± 154.6</td>
<td>3177.5 ± 308.0</td>
<td>6905.4 ± 392.8</td>
</tr>
<tr>
<td>15</td>
<td>10:14</td>
<td>487.1 ± 49.8*</td>
<td>338.4 ± 36.4*</td>
<td>825.5 ± 80.2*</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM (n = 27-30). (*, p < 0.05 vs. long photoperiod).
Inhibition ranged from 76.7 - 98.4% (Fig. 3, A-D; Table 3). There were no significant differences.

**RIA-PRL Release In Vitro**

Short photoperiod exposure resulted in reduced ability of pituitaries to release PRL in vitro, with a time course paralleling that of circulating PRL levels. Medium PRL concentration following a three hour incubation was significantly (p < 0.05 vs. long photoperiod controls) reduced by 7 weeks, declined further by 11 weeks and remained at low levels through 15 weeks (Fig. 4, Table 4). PRL content
of incubated pituitaries and total (medium + pituitary) RIA-PRL were also reduced by exposure to short photoperiods over an identical time course (Fig. 4, Table 4).

**Discussion**

In the present study, transfer of hamsters from long to short photoperiod led to inhibition of PRL cell activity by 7 weeks and this effect was maintained throughout the remainder of the experiment (to 15 weeks). Circulating PRL levels and PRL content of incubated pituitaries and incubation media were depressed by short photoperiod exposure over the same time course. These observations are generally in good agreement with previous reports (see Introduction for references), although Orstead and Benson (1980) and Goldman et al. (1981) have reported significant reduction in circulating PRL levels at 3 weeks, at which time in the present study there was no difference. Additionally, Steger et al. (1983) observed a non-significant trend toward diminished PRL release in vitro by 9 days of short photoperiod exposure, significantly decreased PRL release at 27 and 77 days, followed by a return to long photoperiod control levels by 105 days (15 weeks). In our studies, there was no apparent change in PRL release in vitro at 3 weeks, and at 15 weeks PRL release levels in vitro remained at extremely low levels. At least two possible explanations exist for these discrepancies. First, at least in terms of reproductive effects, it has been suggested that over the past 20 years, the latency to onset of changes induced by short photoperiod has progressively increased in the hamster (Reiter, 1980). This observation, if it can be appropriately extended to include effects on PRL cell activity, might explain discrepancies in time course to effects. Second, in the previous reports cited, PRL was most often
measured in a heterologous (rat) radioimmunoassay, whereas we used a homologous RIA procedure (Soares et al., 1983). In any case, as stated above, qualitative and quantitative changes observed in this study generally support previous reports of short photoperiod-induced inhibition of PRL cell activity in the hamster.

The major issue addressed in this series of experiments is the role of altered pituitary responsiveness to the PRL-inhibitory effects of DA in the mechanism of short photoperiod-induced PRL cell inhibition. This was assessed by measuring DA binding site populations in anterior pituitary membranes of long photoperiod exposed animals, as an indication of capacity to respond to DA, and by comparing the sensitivity of pituitary tissue from long vs. short photoperiod-exposed animals to the PRL release-inhibiting effect of DA in vitro.

Least squares modeling of $^{3}$H-spiperone binding revealed the presence of two classes of binding sites, quantitatively and qualitatively similar to results of previous studies in rat (Cronin and Weiner, 1979; Bression et al., 1983; 1985) and porcine (DeLean et al., 1982a) anterior pituitary membranes. The presence of two distinguishable classes of binding sites suggests either the presence of two discrete receptors or, as is widely accepted, the presence of a single receptor existing in two affinity states regulated by ligand and guanine nucleotide interactions (Sibley et al., 1982; DeLean et al., 1982a; Wreggett and DeLean, 1984). The presence of these binding sites in pituitaries of long photoperiod-exposed male hamsters indicates that these animals possess the macromolecular machinery presumed to be necessary for responsiveness to DA. Furthermore, these binding
sites appear to represent functional DA receptors, as evidenced by the ability of DA to inhibit PRL release \textit{in vitro}, with IC$_{50}$ values suggestive of high affinity interaction (nM range).

While PRL cell activity was unequivocally inhibited in temporal relationship to short photoperiod exposure, there was no change in pituitary sensitivity to DA in short photoperiod-treated animals at any time point measured. The finding of reduced PRL cell activity despite unaltered DA sensitivity at times when hypothalamic DA turnover is reported to be lowered (Steger et al., 1982; Benson, 1987) appears to indicate the involvement of DA-independent PRL-inhibitory mechanisms in this phenomenon. The potential site(s) or form of such effects cannot be inferred from these experiments.

The observation that DA was equally effective in inhibiting PRL release from pituitaries of both long and short photoperiod-exposed animals is supported by the previous report of Blask and Orstead (1986). These findings are at odds, however, with the finding of Steger et al. (1983) that 5 x 10$^{-8}$ M DA was not effective in inhibiting PRL release from pituitaries of long photoperiod-treated males and that sensitivity to this concentration of DA was expressed only after several weeks of short photoperiod treatment. Modeling of dose response results in our studies indicate that 5 x 10$^{-8}$ M DA, under these similar incubation conditions, would be predicted to elicit greater than 50% inhibition of PRL release. While the reasons for this discrepancy are not evident, differences in ascorbate concentration in the incubation media may account for at least part of the difference. Ascorbic acid, a reducing agent, is routinely added to incubation medium in studies involving DA.
in order to protect DA from oxidation. While ascorbic acid alone does not affect PRL release, it has been reported that ascorbic acid may potentiate the ability of DA and apomorphine to inhibit PRL release (Stirling and Shin, 1988). In our studies and in the study of Blask and Orstead, ascorbic acid was present at $5.6 \times 10^{-4}$ M (0.01%) whereas in the study of Steger et al. the ascorbate concentration was an order of magnitude lower (0.001%). This explanation is not entirely satisfactory as Stirling and Shin report that the minimal effective concentration of ascorbic acid for producing this effect is more than an order of magnitude lower still.

In summary, the results of the present study support the following conclusions: 1) binding sites with the characteristics of D2 DA receptors, as reported in other mammalian species, are present in the pituitaries of long photoperiod-exposed male hamsters; 2) these binding sites are associated with a functional DA receptor complex, as evidenced by the ability of DA to inhibit PRL release from pituitaries of long photoperiod-exposed animals, with dose response constants in the nanomolar range; 3) in contrast to earlier reports, short photoperiod-induced PRL cell inhibition is not mediated through enhanced pituitary sensitivity to PRL-inhibitory effects of DA. PRL cell inhibition elicited by exposure to short photoperiod appears to involve as yet unidentified DA-independent mechanisms.
CHAPTER 4

INHIBITION BY MELATONIN OF PROLACTIN-SECRETING ADENOMA GROWTH AND SECRETORY ACTIVITY IN FISHER 344 RATS: EFFECTS ON DOPAMINERGIC REGULATORY MECHANISMS

Abstract

The purpose of the present study was to determine whether the inhibitory effects of melatonin on the growth and secretory activity of diethylstilbestrol- (DES-) induced prolactin- secreting adenomas of the anterior pituitary were possibly mediated through changes in dopaminergic regulatory mechanisms. Adult female Fisher 344 (F344) rats received daily injections of melatonin (50 μg) or saline vehicle. Following two weeks of injections, half of the animals were sacrificed for measurement of hypothalamic dopamine (DA) turnover and in vitro sensitivity to DA. The remaining animals received subcutaneous implants of 5 mg DES. Injections continued on the same schedule until sacrifice 4 weeks later for measurement of the same parameters. The binding behaviour of the DA agonist (-)- apomorphine and the antagonist drugs ³H-spiperone and (+)- butaclamol to anterior pituitary membranes was also assessed before and after DES exposure.

Melatonin treatment resulted in significant reversal of DES- induced growth and secretory activity of pituitary adenomas, confirming our previous observations. After two weeks of melatonin treatment, at the time of implantation of DES capsules, TIDA activity was not affected compared to vehicle-injected controls. Following 4 weeks of DES exposure, DA neuronal activity was higher in the vehicle-treated group than in the melatonin- treated group, presumably reflecting the positive
feedback effects of high circulating PRL levels observed in vehicle-treated animals on TIDA neurons. PRL cell sensitivity to DA inhibition was assessed by measuring PRL release from pituitary fragments incubated in the presence of increasing concentrations of DA or vehicle. No differences were observed at either time regarding 50% effective concentrations (IC$_{50}$) or maximal effectiveness of DA for inhibiting the release of radioimmunoassayable or newly synthesized PRL. DES exposure resulted in decreased number and affinity of pituitary binding sites for $^3$H-spiperone, and this was not reversed by melatonin treatment. DES treatment also resulted in the loss of high affinity binding sites for apomorphine. From these data we conclude that inhibitory effects of melatonin on DES-induced prolactinomas are neither mediated through increased release of DA from the hypothalamus nor through enhanced sensitivity of PRL cells to the effects of endogenous DA.

**Introduction**

The potent estrogenic compound diethylstilbestrol (DES) produces a rapid and profound growth of anterior pituitary tumors in Fisher 344 (F344) rats (Dunning et al., 1947; Wiklund et al., 1981). These tumors arise almost exclusively from the hypertrophy and hyperplasia of prolactin (PRL) cells (Kaplan and DiNicola, 1976; DiNicola et al., 1978; Wiklund et al., 1981), and serum PRL levels are strikingly increased in tumor-bearing animals (DiNicola et al., 1978; Leadem and Burns, 1987).

It is well recognized that dopamine (DA) secreted into the hypophyseal portal circulation by tuberoinfundibular (TIDA) neurons in the hypothalamus is an impor-
tant physiological regulator of PRL cell activity (see Neill, 1980). DA acts at D2 dopamine receptors on the surface of PRL cells (Kebabian and Caine, 1979) to inhibit the release (Caron et al., 1978) and synthesis (Maurer, 1980) of PRL and to inhibit the proliferation of PRL cells (Jacobi and Lloyd, 1981).

While the mechanism of DES induction of PRL-secreting tumors is not precisely known, it may be related to suppression of TIDA neuronal activity (Sarkar et al., 1982; Morgan et al., 1985a). Casanueva et al. (1982) found that rats chronically treated with estrogens failed to demonstrate inhibition of PRL release after injection of nomifensine, an activator of central dopaminergic activity. Estrogens have also been reported to antagonize PRL-inhibitory actions of DA at the anterior pituitary level (LaBrie et al., 1980; West and Dannies, 1980; Maurer, 1980), and to alter pituitary DA receptor populations (Heiman and Ben-Jonathan, 1982; Albaladejo, et al., 1984; Bression et al., 1983; 1985).

These tumors appear to retain the ability to respond to DA receptor occupancy, however, as evidenced by tumor involution and reduced serum PRL levels elicited by treatment with the potent dopaminergic agonist bromocriptine (Miyazaki et al., 1985; Eljarmak et al., 1985).

The pineal gland of rodents has been shown in numerous studies to mediate profound changes in the neuroendocrine-reproductive axis induced by light deprivation (see Reiter, 1980). The laboratory rat is generally unresponsive to reproductive effects of blinding or short photoperiod exposure, but can be sensitized to light deprivation by neonatal androgenization, underfeeding or olfactory bulbectomy (Reiter, 1974). The potentiating effect of olfactory bulbectomy appears to
involve sensitization to the pineal hormone melatonin, as daily afternoon administration of melatonin mimics the effects of light deprivation in anosmic animals (Blask and Nodelman, 1979). Changes in reproductive physiology are accompanied by decreased storage, synthesis and release of PRL (Leadem and Blask, 1981; 1982a), decreased total anterior pituitary DNA content and a proportionately decreased number of immunocytochemically identified PRL cells (Leadem and Blask, 1982a; 1982b; 1982c). The pineal has also been shown to exert profound inhibitory effects on the hyperplasia and hypertrophy of PRL cells that occurs at the time of puberty (Leadem and Blask, 1984). PRL cell proliferation during this period of development is thought to result from rising estrogen titers (Maurer, 1979).

Inhibitory effects of melatonin on PRL cells may involve alterations in dopaminergic control mechanisms. The PRL-inhibitory effects of melatonin injections in anosmic rats are blocked by co-administration of the DA antagonist pimozide (Blask et al., 1980). Furthermore, inhibitory effects of the pineal on PRL cells of blind-anosmic Sprague-Dawley rats (Leadem et al., 1988) and male Syrian hamsters (Benson, 1987) are accompanied by increased hypothalamic dopamine turnover.

Leadem (1984; 1985) first reported that combined blinding and anosmia resulted in inhibition of DES-induced pituitary tumors in F344 rats, as reflected by decreases in pituitary weight, pituitary DNA content, serum PRL, PRL synthesis and total radioimmunoassayable PRL (RIA-PRL) in vitro. Surprisingly, it was subsequently discovered that blinding or melatonin administration alone were
equally as effective as these treatments combined with olfactory bulbectomy in inhibiting DES-induced prolactinoma growth in F344 rats (Leadem and Burns, 1987; 1988). In the present study, we have set out to investigate the possible involvement of altered dopaminergic regulation of PRL cells as a mechanism underlying the inhibitory effects of melatonin on DES-induced prolactinoma growth and secretory activity. To do this, we measured the effects of melatonin treatment on TIDA neuronal activity and pituitary responsiveness to dopamine both before and after exposure to DES.

**Materials and Methods**

**Animals**

All animals used in these experiments were adult female F344 rats (Charles River Laboratories, CDF(F-344)/CrlBR, 126-150 g). Animals were housed in clear plastic cages (4-6 animals/cage) in a 14:10 light:dark photoperiodic cycle (lights on 0600 h) at 23° C, and provided with lab chow and tap water ad libitum.

**Preparation of Diethylstilbestrol (DES) Implants**

DES implants were prepared according to the method of Wiklund et al. (1981). Silastic tubing (0.062* x 0.125") was cut into 15 mm lengths and one end sealed with Silastic adhesive and allowed to cure overnight. Each capsule was then filled with 25 \( \mu l \) of a solution of 200 mg/ml DES in 100% ethanol. The ethanol was evaporated in a 50° C oven for 2 hours. Finally, the open end of the capsule was sealed with Silastic adhesive and allowed to cure for at least 48 hours before
implantation. Capsules were implanted subcutaneously in the interscapular region of animals anesthetized with ether.

Radioimmunoassays

Sera, incubation media and incubated pituitaries were assayed for immunoreactive prolactin (RIA-PRL) using a kit supplied by the National Hormone and Pituitary Program using rat PRL RP3 as a standard. All samples were assayed in duplicate. For details, see Appendix E.

In Vitro Pituitary Incubations

Incubation protocols and disc gel electrophoresis procedures for measurement of $^3$H-PRL are presented in detail in Appendices B and F, respectively, of this dissertation. Briefly, at the time of sacrifice, anterior pituitaries were removed, weighed and divided into fragments of 5-7 mg. Pituitary fragments from each treatment group were pooled and subsequently placed in vials containing 0.5 ml minimal essential medium (GIBCO) with penicillin (5000 U), Fungizone (12.5 µg), streptomycin (5000 µg), ascorbic acid (5.6 x 10^{-4} M) and 10 µCi $^3$H-leucine (50 Ci/mmol, Research Products International). The pituitaries were then incubated in the presence of dopamine (5 x 10^{-5} - 5 x 10^{-11} M) for 3 hours in a Dubnoff Shaking Metabolic Incubator (37° C, gassed with 95% O$_2$:5% CO$_2$). Pituitary fragments from each treatment group were also incubated in the absence of added dopamine, serving as controls for dopamine effects. After 3 hours, media were aspirated and frozen, and incubated pituitary tissue was weighed, homogenized in 0.5 ml 0.1 M PBS and frozen. RIA-PRL and $^3$H-PRL contents of incubation media and incubated
pituitaries were determined by radioimmunoassay and disc gel electrophoresis/scintillation spectrometry, respectively. For dopamine sensitivity studies, the inhibition of RIA-PRL and $^3$H-PRL release elicited by dopamine over a range of concentrations was analyzed by nonlinear modeling (see Appendix A) in order to obtain estimates of percent maximum inhibition and 50% maximum effective concentration (designated IC$_{50}$) for each treatment group. The proportional inhibition of PRL release, which served as the dependent variable in regression analysis, was calculated by dividing the PRL concentration (ng RIA-PRL or DPM $^3$H-PRL, per mg tissue) in the medium from DA-treated pituitary fragments by the corresponding mean PRL concentration in media from pituitary tissue incubated in the absence of DA. Control (DA-absent) incubations were additionally used to determine RIA-PRL and $^3$H-PRL release and pituitary content for direct comparison between treatment groups.

Dopamine Depletion and Turnover

Reverse-phase, high performance liquid chromatography with electrochemical detection was used for quantification of hypothalamic DA concentrations, according to the method of Gregory et al. (1985). For a detailed description of the methods used, see Appendix H of this dissertation.

In order to estimate DA depletion and turnover kinetics, animals in each treatment group were injected intraperitoneally with 250 mg/kg of the tyrosine hydroxylase inhibitor $\alpha$-methyl-para-tyrosine ($\alpha$-MPT) at 45 and 90 minutes prior to sacrifice. Additional animals from each group received saline injections and were killed at various times throughout the procedure. At the time of sacrifice, the
hypothalamic M.E. from each animal was dissected and immediately sonicated in 70% ethanol for subsequent DA assay. The rate constant of DA depletion (k) and DA turnover rate (\( K \)) were estimated by nonlinear modeling of the DA depletion curve generated by plotting DA concentration as a function of time after \( \alpha \)-MPT injection (Brodie et al., 1966; see Appendix A of this dissertation for further details). Saline-injected animals were used for estimation of steady-state (time 0) concentrations.

**Binding Assays**

Anterior pituitary membranes were prepared according to the methods of Bression et al. (1985) with slight modifications. Animals were killed by rapid decapitation. Anterior pituitaries were removed, rinsed in cold saline and weighed. The tissue was then homogenized (1:10, weight:volume) in ice cold 50 mM Tris-HCl buffer, pH 7.4, with a hand-held teflon/glass tissue grinder. The homogenate was centrifuged at 800 x g for 10 minutes (4° C) and the resulting supernatant was centrifuged at 22,500 x g for 20 minutes (4° C). The resulting pellet was washed by rehomogenization in 10 volumes Tris buffer followed by centrifugation at 22,500 x g for 20 minutes (4° C). The final pellet was then suspended by gentle homogenization in incubation buffer (50 mM Tris HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 \( \mu \)M EDTA, 0.1% Ascorbate and 5 \( \mu \)M pargyline) at a concentration of 25 mg (wet tissue weight equivalents)/ml.

\(^3\)H-Spiperone (23-27 Ci/mmol, New England Nuclear) binding to anterior pituitary membranes was assessed by a rapid filtration technique. \(^3\)H-Spiperone was incubated with membrane preparations for 30 minutes at 27° C. Incubation
tubes received 200 \mu l of membrane preparation and either 50 \mu l of incubation buffer containing (\(+\))-butaclamol (5 \mu M final concentration, nonspecific binding tubes) or 50 \mu l incubation buffer alone (total binding tubes). For binding competition studies, 50 \mu l of incubation buffer containing various concentrations of (\(+\))-butaclamol or (\(-\))-apomorphine were added at this step. Binding reactions were initiated by addition of 250 \mu l \(^3\)H-spiperone diluted in incubation buffer. At the end of the incubation, 5 ml of ice cold incubation buffer were added and the membranes were harvested by filtration through glass microfiber filters (GF/C, Whatman) under vacuum. Incubation tubes were rinsed with an additional 5 ml buffer which was also filtered. Filters were then washed twice with 5 ml aliquots of incubation buffer. The filtration and washing procedure took less than 10 seconds. Filters were placed into scintillation vials along with 10 ml aqueous counting scintillant (ACS, Amersham) for counting of radioactivity by scintillation spectrometry. Using external standard ratios, CPM were converted to DPM for final calculations. Specific binding was defined as total binding (determined in triplicate) minus nonspecific binding (determined in duplicate).

**Statistics**

Pituitary weight, serum RIA-PRL and both RIA-PRL and \(^3\)H-PRL concentration in incubation media and pituitary homogenates were analyzed by two-way analysis of variance. If interaction effects (time x treatment) were significant, further analysis was performed by one-way ANOVA using Fischer’s Least Significant Difference Test for post-hoc comparisons. ANOVAs were performed using SAS 6.02 running on an IBM-PC. A p value of less than 0.05 was considered significant.
Nonlinear model fitting was performed using the SPSS-X program CNLR (Constrained Nonlinear Regression) running on a VAX/VMS computer at the Arizona Cancer Center. Testing for significant differences between multiple models was accomplished using partial F-tests (DeLean et al., 1982). A model for two binding sites was retained only when it fitted the data significantly better (p < 0.05) than a model for one site. Parameter estimates were tested for significance of difference by pairwise t-tests using the pooled variance. A p value of less than 0.05 was considered significant.

Presentation of Data

In order to underscore the underlying form of responses, and for the sake of clarity and completeness, results will frequently be presented in both graphic and tabular form. This is intended to be understood as duplicate reporting of the same data in two useful forms and not as additional data.

Experimental Design

Experiment #1. Daily afternoon (1600 - 1800 h) subcutaneous injections of either 50 µg melatonin in 0.1 ml normal saline or 0.1 ml saline vehicle alone were initiated 2 weeks prior to implantation of DES-containing capsules. Injections were continued on this schedule throughout the course of the experiment. On the day before capsule implantation (referred to as Week 0), half of the animals from both injection groups were killed by rapid decapitation. Trunk blood was collected for determination of circulating RIA-PRL and anterior pituitaries were removed, weighed and processed for incubation in vitro with ³H-leucine in the presence of
various concentrations of DA. The remaining animals were implanted with DES-containing capsules and killed 4 weeks later (referred to as Week 4). Tissues were processed exactly as described for Week 0 animals. At the end of the experiment, the content of RIA-PRL and $^3$H-PRL in incubation media and incubated pituitaries was determined for between-groups comparisons and construction of DA dose response curves.

**Rationale.** Daily afternoon injections of 50 $\mu$g of melatonin have been shown to be effective in inhibiting the growth of DES-induced prolactinomas in F344 rats (Leadem and Burns, 1987). It is possible that melatonin exerts this effect by direct interaction with the dopaminergic control of PRL cells. The purpose of this experiment is to test the hypothesis that melatonin treatment inhibits the growth of DES-induced prolactinomas by increasing the sensitivity of PRL cells to the inhibitory effects of DA. Such changes in sensitivity would be detected as shifts in IC$_{50}$ values for the inhibition of release of either stored (radioimmunoassayable) or newly synthesized ($^3$H-) PRL, or both. Furthermore, these changes might be manifested as either increases in baseline sensitivity to DA or as a reversal of DES-induced decreases in sensitivity.

**Experiment #2.** For saturation binding studies, in vivo treatments were carried out exactly as described above for experiment #1. At the time of sacrifice, anterior pituitaries were removed, weighed and processed for binding assays (see Materials and Methods section and Appendix G). Membrane suspensions were incubated in the presence of increasing concentrations of $^3$H-spirperone (0.05 - 4.1 nM) and specific binding data thus generated were modeled as a function of
$^{3}$H-spiperone concentration to obtain estimates of binding site concentration(s) ($B_{\text{max}}$) and equilibrium dissociation constant(s) ($K_d$) for each treatment group.

**Rationale.** It has been reported that treatment with estrogenic compounds alters dopaminergic binding to anterior pituitary membranes (Heiman and Ben-Jonathan, 1982), and that these changes are correlated with diminished sensitivity to DA (Bression et al., 1985). The purpose of this experiment is to test the hypothesis that melatonin treatment changes the ability of pituitary membranes to bind the DA antagonist drug spiperone in a manner consistent with increased ability to bind and thus respond to DA. Again, such changes could be manifest as changes in the inherent affinity and/or capacity of receptors, or as a reversal of DES-induced alterations in these parameters.

**Experiment #3.** In these studies, the abilities of the antagonist (+)-butaclamol and the agonist (-)-apomorphine to displace $^{3}$H- spiperone binding to anterior pituitary and caudate nucleus membranes were assessed in 2 groups of animals. The first group of animals were utilized untreated. Following shipment, these animals were allowed 2 weeks to acclimatize to their surroundings before sacrifice. The second group of animals (following acclimatization) were implanted with DES-containing capsules and sacrificed 5 weeks later. All animals were killed by rapid decapitation. Anterior pituitaries were removed and processed for binding assays. Caudate nuclei were dissected and processed in exactly the same manner. Membrane suspensions were incubated in the presence of a constant amount of $^{3}$H-spiperone (2.5 nM) along with varying concentrations of competing drugs. A second set of membrane suspensions from untreated animals were incubated in the presence of a constant amount of $^{3}$H-spiperone and varying
concentrations of (-)-apomorphine plus melatonin (10 nM). Specific binding data were modeled as a function of competing ligand concentration to obtain estimates of the apparent dissociation constant(s) (K') of the competing ligand and, for 2 site models, the relative proportions of binding sites of each type.

Rationale. Dopamine receptors of the anterior pituitary gland are postulated to exist in 2 interconvertible affinity states having reciprocal affinities for agonists and antagonists (Sibley et al., 1982; Wreggett and DeLean, 1984). In a variety of receptor systems, agonist drugs and antagonist drugs differ in their ability to distinguish between (or possibly induce) different receptor subtypes (see DeLean et al., 1982b). In the anterior pituitary, while most investigators report that 3H-spiperone labels a single class of dopamine receptors, other reports suggest the existence of 2 classes of pituitary binding sites which are differentially labeled by 3H-spiperone (Cronin, 1982; DeLean et al., 1982a). Even under conditions where direct labeling with 3H-spiperone reveals the presence of only a single class of binding sites, (-)-apomorphine competition curves for 3H-spiperone binding are biphasic and can be resolved into high and low affinity binding sites (Sibley et al., 1982). It is therefore possible in binding competition studies to exploit the greater selectivity of agonists such as (-)-apomorphine to reveal the presence of mixed receptor subpopulations when such distinctions cannot be determined by direct labeling with 3H-antagonists.

Bression and colleagues (1985) have reported that estrogen treatment results in parallel changes in both pituitary dopaminergic binding and pituitary responsiveness to DA, from biphasic to monophasic (low affinity for agonist) states. These effects on binding were induced by co-incubation of membrane
suspensions with estradiol, suggesting a direct membrane-level interaction. Such a shift in affinity states might account for the induction of prolactinomas by DES. Conversely, melatonin treatment might increase pituitary sensitivity to DA by increasing the proportion of agonist high affinity sites. The purpose of this experiment was to test the following hypotheses:

1) DES treatment results in a loss of pituitary dopamine receptor subtypes exhibiting high affinity for agonists.

2) Melatonin treatment increases the proportion of pituitary binding sites exhibiting high affinity for agonists.

**Experiment #4.** For dopamine depletion and turnover studies, in vivo treatments were carried out exactly as described above for experiment #1. Animals received intraperitoneal injections of α-MPT 45 and 90 minutes before sacrifice by rapid decapitation. Hypothalamic median eminences were dissected and immediately sonicated in 70% ethanol for subsequent determination of DA content. Trunk blood was collected for measurement of circulating RIA-PRL and anterior pituitaries were removed and weighed. Dopamine depletion rate constants (k) and turnover rates (K) were estimated by modeling M.E. DA concentration as a function of time following α-MPT injection. These estimates of dopamine depletion kinetics during synthesis blockade are assumed to reflect underlying rates of DA release in vivo, such that increased depletion rate constants and turnover rates are indicative of increased activity of dopaminergic neurons.

**Rationale.** Melatonin treatment might exert its effect on prolactinoma growth by increasing the activity of hypothalamic TIDA neurons. In support of this
possibility, hypothalamic DA turnover is increased in male hamsters 3-6 weeks after placement in short photoperiod (Benson, 1987) and increased in blind-anosmic male rats by 1 week following surgery (Leadem et al., 1988). The purpose of this experiment is to test the hypothesis that melatonin treatment results in increased activity of dopaminergic neurons in the hypothalamus, as reflected by increases in DA depletion rate constants and/or turnover rates.

Results

Experiment #1

Anterior Pituitary Weights

At 4 weeks, DES exposure resulted in a 230% increase in anterior pituitary weights of saline-injected animals (Fig. 5A, Table 5). Melatonin treatment resulted in significant (p < 0.05) inhibition of DES-induced tumor growth, but pituitary weights were still significantly elevated over Week 0 animals. Melatonin injections did not affect anterior pituitary weight in Week 0 rats.

Serum PRL Levels

Changes in serum RIA-PRL titers paralleled changes in anterior pituitary weight (see Fig. 5B, Table 5). In saline-treated animals, DES treatment resulted in a 30-fold increase in serum PRL levels. Melatonin treatment reversed this increase by 59% (p < 0.05). Serum PRL levels in Week 4 melatonin-treated animals were still significantly increased over Week 0 saline-treated rats. Melatonin treatment had no effect on serum PRL titers at Week 0.
Figure 5. The effect of daily afternoon injections of melatonin on DES-induced changes in pituitary weight (Panel A) and circulating PRL levels (Panel B) in adult female F344 rats. Daily subcutaneous injections of either melatonin or saline vehicle were initiated 2 weeks prior to subcutaneous implantation of silastic capsules containing 5 mg DES and continued throughout the course of the experiment. On the day before capsule implantation (Week 0), half of the animals from each treatment group were killed by rapid decapitation. Trunk blood was collected for determination of circulating PRL levels and anterior pituitaries were removed, weighed and further processed for determination of in vitro DA sensitivity. Animals receiving DES-containing implants were maintained in 14:10 (Light:Dark) photoperiod for 4 weeks, at which time they were sacrificed and tissues were processed in the same manner as described for Week 0 animals. (a, p < 0.05 vs. Week 0 Saline; b, p < 0.05 vs. Week 4 Saline). For details see Table 5.
Table 5. Serum RIA-PRL and anterior pituitary weights from female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Serum RIA-PRL (ng/ml)</th>
<th>Pituitary Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>4.81 ± 1.30</td>
<td>11.70 ± 0.39</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>2.14 ± 0.50</td>
<td>11.29 ± 0.33</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>154.72 ± 15.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.73 ± 1.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>63.82 ± 9.02&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>18.04 ± 0.99&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM (n = 35-42). Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. (a, p < 0.05 vs. Week 0 Saline-treated animals; b, p < 0.05 vs. Week 4 Saline-treated animals).
Dopamine Sensitivity In Vitro

IC$_{50}$ values for the inhibition by DA of RIA-PRL release were in the high nM range (69.9 - 151.8 nM) for all treatment groups and the % maximum inhibition ranged from 49.2 - 54.9% (Fig. 6A,B, Table 6). There were no significant differences.

There were also no differences between groups in IC$_{50}$ values or % maximum effect for the inhibition of $^3$H-PRL release (Fig. 6C,D, Table 7). It should be noted that IC$_{50}$ values for $^3$H-PRL release were somewhat lower (18.0 - 44.1 nM) than the corresponding estimates for RIA-PRL, and the % maximum effect was larger (81.4 - 86.3%).

Table 6. Parameters for the inhibition by dopamine of RIA-PRL release from incubated pituitary fragments (3 hour incubation) harvested from female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>IC$_{50}$ (nM)</th>
<th>Max Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>73.9 ± 55.5</td>
<td>54.9 ± 5.6</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>69.9 ± 54.2</td>
<td>49.2 ± 7.2</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>72.9 ± 59.7</td>
<td>51.6 ± 7.2</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>151.8 ± 101.7</td>
<td>52.9 ± 5.8</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± asymptotic standard error as estimated from data analysis by non-linear regression modeling (regression n = 35-42). Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. There were no significant differences.
Table 7. Parameters for the inhibition by dopamine of $^3$H-PRL (newly synthesized) release from incubated pituitary fragments (3 hour incubation) harvested from female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>IC$_{50}$ (nM)</th>
<th>Max Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>24.3 ± 16.1</td>
<td>81.4 ± 8.0</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>44.1 ± 31.9</td>
<td>83.7 ± 12.0</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>18.0 ± 10.1</td>
<td>84.3 ± 6.9</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>27.9 ± 13.3</td>
<td>86.3 ± 6.1</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± asymptotic standard error as estimated from data analysis by non-linear regression modeling (regression n = 35-42). Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. There were no significant differences.

Figure 6. (following page) Dose-response data for the inhibition by DA of the release of RIA-PRL and $^3$H-PRL (newly synthesized) from incubated pituitary fragments harvested from adult female F344 rats treated as described in the legend accompanying Figure 5. At the time of sacrifice, anterior pituitaries were removed, weighed and divided into fragments for incubation in the presence of $^3$H-leucine and in the presence of either various concentrations of DA $(5 \times 10^{-11} - 5 \times 10^{-6} \text{ M})$ or the vehicle for DA administration. PRL-release inhibition data were subsequently analyzed by non-linear model fitting for estimates of IC$_{50}$ and percent maximum inhibition. Resulting model predictions are represented by solid lines for saline-treated groups and dashed lines for melatonin-treated groups. There were no significant effects of melatonin injections on the ability of DA to inhibit PRL release. For details see Table 6 (RIA-PRL) and Table 7 ($^3$H-PRL).
RIA-PRL In Vitro

Expressed as ng RIA-PRL/mg pituitary (i.e. unit activity), there were no differences in RIA-PRL levels in incubation media or incubated pituitaries in any of the treatment groups (Fig. 7A, Table 8). Similarly, total (medium + pituitary) RIA-PRL remained unchanged. If RIA-PRL levels are expressed as ng/pituitary, a pattern emerges which parallels changes in anterior pituitary weight and serum PRL levels. Because these values were back-calculated by group means for pituitary weight, they do not reflect co-variance of pituitary weight, and statistical comparisons were not considered appropriate. These values are included as a reflection of the underlying response in vivo (Fig 7B, Table 8).

$^{3}$H-PRL In Vitro

Expressed as DPM/mg pituitary, $^{3}$H-PRL levels of incubation media and incubated pituitaries from DES-treated animals approached (0.10 > p > 0.05) significant elevation over Week 0 levels. Total (medium + pituitary) $^{3}$H-PRL levels were significantly (p < 0.05) increased by DES treatment and this effect was not reversed by melatonin treatment (Fig. 8A, Table 9).

Data are also shown expressed as DPM/pituitary (Fig. 8B, Table 9). These are included as a reflection of the underlying form of response in vivo. Statistical analysis was not performed.
Figure 7. RIA-PRL in pituitary, media and total (pituitary + media) from incubated pituitary fragments harvested from adult female F344 rats following treatment as described in the legend accompanying Figure 5. Incubations were carried out as described in the legend for Figure 6. Results shown here are for pituitary fragments incubated in the absence of DA. Panel A, results expressed as ng RIA-PRL/mg pituitary ± SEM (n = 10-12). There were no significant differences. Panel B, results expressed as ng RIA-PRL/pituitary (back-calculated by group means for pituitary weight). Because these values do not reflect co-variance of pituitary weight, statistical analysis was not performed. For details see Table 8.
Table 8. RIA-PRL in the incubation media and incubated pituitary fragments after a 3 hour incubation, from female F344 rats treated as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Media (ng)</th>
<th>Pituitary (ng)</th>
<th>Total (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Saline</td>
<td>1971.4 ± 287.3</td>
<td>2698.3 ± 551.5</td>
<td>4669.7 ± 741.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(25037.2 ± 1025.5)</td>
<td>(34268.5 ± 1965.5)</td>
<td>(59305.7 ± 2642.7)</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>2327.6 ± 285.3</td>
<td>2515.7 ± 270.5</td>
<td>4843.3 ± 400.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(28606.3 ± 1001.1)</td>
<td>(30881.3 ± 948.6)</td>
<td>(59524.5 ± 1403.3)</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>1544.1 ± 122.6</td>
<td>2520.4 ± 183.1</td>
<td>4064.5 ± 273.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(41274.6 ± 633.9)</td>
<td>(67371.9 ± 946.9)</td>
<td>(108646.5 ± 1413.8)</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>1943.8 ± 101.5</td>
<td>3133.9 ± 304.1</td>
<td>5077.7 ± 383.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(35066.3 ± 388.8)</td>
<td>(56537.0 ± 1291.8)</td>
<td>(91603.3 ± 1628.9)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM (n = 10-12). Values without parentheses are expressed as ng/mg pituitary. Those in parentheses are expressed as ng/pituitar' (back-calculated by group means for pituitary weight). Week refers to the number of weeks following Implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule Implantation. There were no significant differences.
Figure 8. \(^{3}H\)-PRL (newly synthesized) in pituitary, media and total (pituitary + media) from incubated pituitary fragments harvested from adult female F344 rats following treatment as described in the legend accompanying Figure 5. Incubations were carried out as described in the legend for Figure 6. Results shown here are for pituitary fragments incubated in the presence of \(^{3}H\)-leucine and the absence of DA. Panel A, DPM of \(^{3}H\)-leucine incorporated into PRL expressed as DPM/mg pituitary ± SEM (n = 10-12). (*, p < 0.05 vs. Week 0 Saline). Panel B, DPM of \(^{3}H\)-leucine incorporated into PRL expressed as DPM/pituitary (back-calculated by group means for pituitary weight). Because these values do not reflect co-variance of pituitary weight, statistical analysis was not performed. For details see Table 9.
Table 9. $^3$H-PRL in the incubation media and incubated pituitary fragments after a 3 hour incubation, from female F344 rats treated as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Media (DPM)</th>
<th>Pituitary (DPM)</th>
<th>Total (DPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>345.8 ± 41.3</td>
<td>586.1 ± 60.4</td>
<td>931.9 ± 78.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4392.2 ± 147.0)</td>
<td>(7442.9 ± 215.4)</td>
<td>(11835.1 ± 280.7)</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>330.9 ± 39.2</td>
<td>421.5 ± 87.3</td>
<td>752.3 ± 111.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4066.4 ± 137.3)</td>
<td>(5179.8 ± 306.1)</td>
<td>(9246.1 ± 392.4)</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>447.7 ± 27.6</td>
<td>797.2 ± 82.6</td>
<td>1244.9 ± 88.5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11965.9 ± 142.9)</td>
<td>(21309.2 ± 426.9)</td>
<td>(33275.4 ± 457.8)</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>474.9 ± 34.2</td>
<td>751.3 ± 89.8</td>
<td>1234.6 ± 112.5*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8566.8 ± 145.4)</td>
<td>(13553.3 ± 381.6)</td>
<td>(22272.0 ± 477.7)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM (n = 10-12). Values without parentheses are the DPM of $^3$H-Leucine incorporated into PRL, expressed as DPM/mg pituitary. Those in parentheses are expressed as DPM/pituitary (back-calculated by group means for pituitary weight). Week refers to the number of weeks following Implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. (*, p < 0.05 vs. Week 0 Saline-treated animals).
Experiment #2

Anterior Pituitary Weights

Treatment effects on anterior pituitary weights were essentially identical to those reported for experiment #1. DES treatment resulted in a large (270%) increase in pituitary weight and this DES-induced increase was significantly inhibited (p < 0.05) by melatonin treatment (Fig. 9, Table 10). Anterior pituitary weights of Week 4 melatonin-treated animals remained elevated over those of Week 0 rats. There was no effect of melatonin at Week 0.

Table 10. Anterior pituitary weights of female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Pituitary Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>10.06 ± 0.31</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>9.41 ± 0.27</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>27.47 ± 1.05(^a)</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>18.78 ± 1.64(^a,b)</td>
</tr>
</tbody>
</table>

Weights are expressed as the mean ± SEM (n = 12-15). Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. (\(^a\), p < 0.05 vs. Week 0 Saline-treated animals; \(^b\), p < 0.05 vs. Week 4 Saline-treated animals)
Figure 9. The effect of daily afternoon melatonin injections on DES-induced changes in anterior pituitary weight in adult female F344 rats. Daily subcutaneous injections of either melatonin or saline vehicle were initiated 2 weeks prior to subcutaneous implantation of silastic capsules containing 5 mg DES and continued throughout the course of the experiment. On the day before capsule implantation (Week 0) half of the animals from each treatment group were killed by rapid decapitation. Anterior pituitaries were removed, weighed and processed for [³H]-spiperone binding studies. Animals receiving DES-containing implants were maintained in 14:10 (Light:Dark) photoperiod for 4 weeks, at which time they were sacrificed and pituitaries were processed in the same manner as for Week 0 animals. For details see Table 10. (a, p < 0.05 vs. Week 0 Saline; b, p < 0.05 vs. Week 4 Saline).

Saturation Binding of [³H]-Spiperone

Specific binding data for all treatment groups were best described by a model for a single binding site (Figs. 10,11,12). DES treatment resulted in a significant (p < 0.05) decreases in receptor affinity (increased Kd) and concentration (Bmax). This effect was not reversed by melatonin (Table 11). Melatonin treatment did not affect binding parameters at Week 0. Scatchard transformations of specific binding for all groups are presented in Figure 12.
Table 11. Equilibrium dissociation constant (kd) and binding site concentration (B_{max}) for \[^{3}H\]-spiperone binding to anterior pituitary membranes from female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Kd  (nM)</th>
<th>B_{max} (pmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>0.152 ± 0.012</td>
<td>5.83 ± 0.12</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>0.196 ± 0.028</td>
<td>5.25 ± 0.20</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>0.393 ± 0.113*</td>
<td>3.76 ± 0.33*</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>0.467 ± 0.141*</td>
<td>3.48 ± 0.33*</td>
</tr>
</tbody>
</table>

Values are the mean ± asymptotic standard error as estimated from analysis of saturation binding data by non-linear regression modeling. Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. (*, p < 0.05 vs. Week 0 Saline-treated animals).

Figure 10. (following page) Analysis of saturation binding of \[^{3}H\]-spiperone to anterior pituitary membranes from adult female F344 rats treated as described in the legend accompanying Figure 9. Membranes were incubated with various concentrations of \[^{3}H\]-spiperone (0.05 - 3.6 nM) for 30 minutes at 27°C. Nonspecific binding was determined in the presence of 5 μM (+)-butaclamol. Specific binding was defined as total binding minus nonspecific binding. Data were analyzed by non-linear model fitting for estimates of equilibrium dissociation constant (Kd) and binding site concentration (B_{max}). For details see Table 11. Panel A, Saturation Isotherms for Week 0 Saline; Panel B, Scatchard transformation of specific binding for Week 0 Saline; Panel C, Saturation Isotherms for Week 0 Melatonin; Panel D, Scatchard transformation of specific binding for Week 0 Melatonin.

Figure 11. (page 130) Analysis of saturation binding of \[^{3}H\]-spiperone to anterior pituitary membranes from adult female F344 rats treated as described in the legend accompanying Figure 9. Membranes were incubated with various concentrations of \[^{3}H\]-spiperone (0.05 - 4.1 nM) for 30 minutes at 27°C. Nonspecific binding was determined in the presence of 5 μM (+)-butaclamol. Specific binding was defined as total binding minus nonspecific binding. Data were analyzed by non-linear model fitting for estimates of equilibrium dissociation constant (Kd) and binding site concentration (B_{max}). For details see Table 11. Panel A, Saturation Isotherms for Week 4 Saline; Panel B, Scatchard transformation of specific binding for Week 4 Saline; Panel C, Saturation Isotherms for Week 4 Melatonin; Panel D, Scatchard transformation of specific binding for Week 4 Melatonin.
Fig. 11: A

Saline, Week 4

Melatonin, Week 4

Bs/FA (pmol/gm tissue)

[3H]-Spiperone (nM)

Bs (pmol/gm)

[3H]-Spiperone (nM)

Bs/FA (pmol/gm tissue)

Bs (pmol/gm)
Experiment #3

In anterior pituitary and caudate nucleus membranes from untreated rats, competition curves for (-)-apomorphine vs. $^3$H-spiperone were multiphasic and best described ($p < 0.05$) by a model for two classes of binding sites (Figs. 13,14, Table 12). After 5 weeks of DES treatment, (-)-apomorphine competition curves in the anterior pituitary were altered such that they were best described by a model for a single class of binding sites, which corresponded to the lower affinity site ($\mu$M apparent dissociation constant) observed in pituitaries of untreated animals (Fig. 13, Table 13). In caudate membranes from DES-treated animals, apomorphine
competition for \(^3\)H-spiperone binding remained best fit by a model for interaction at two classes of binding sites.

The characteristics of (-)-apomorphine competition for \(^3\)H-spiperone binding were not altered by co-incubation in the presence of 10 nM melatonin, in either anterior pituitary or caudate membranes from untreated animals (Fig. 14, Table 12). In these analyses, least squares fits were obtained for models which included a parameter which estimated the contribution of melatonin to the overall effect. The estimated value of this parameter was not significantly different from zero in either tissue.

As expected, competition curves for the antagonist (+)-butaclamol vs. \(^3\)H-spiperone were best described by a model for interaction at a single class of high affinity binding sites in both pituitary and caudate membranes, and this was not affected by DES treatment.

Figure 13. (following page) Competition for \(^3\)H-spiperone binding to anterior pituitary and caudate nucleus membranes from untreated female F344 rats and female F344 rats bearing DES-containing implants for 5 weeks. Increasing concentrations of (+)-butaclamol or (-)-apomorphine were incubated with membranes in the presence of 2.25 - 2.5 nM \(^3\)H-spiperone. Data were analyzed by non-linear model fitting under both one-site and two-site hypotheses. For details see Tables 12 and 13. Panel A, inhibition of \(^3\)H-spiperone binding to anterior pituitary membranes from untreated animals; Panel B, inhibition of \(^3\)H-spiperone binding to anterior pituitary membranes from animals bearing DES-containing implants; Panel C, comparison of binding inhibition in anterior pituitary vs. caudate nucleus from DES-treated animals; Panel D, Comparison of best-fit models for (-)-apomorphine competition for \(^3\)H-spiperone binding to anterior pituitary and caudate nucleus membranes from untreated vs. DES-treated animals.
Fig. 13: A

Pituitary

$\frac{B}{B_0}$ vs. Log [Competing Drug]

- (−)-Apomorphine
- (−)-Apomorphine, Pituitary
- (−)-Apomorphine, Caudate
- Butaclamol, Pituitary
- Butaclamol, Caudate

Fig. 13: B

Pituitary DES

$\frac{B}{B_0}$ vs. Log [Competing Drug]

- (−)-Apomorphine
- Pituitary, untreated
- Pituitary, 5 weeks DES
- Caudate, untreated
- Caudate, 5 weeks DES

Fig. 13: C

DES

$\frac{B}{B_0}$ vs. Log [Competing Drug]

- (−)-Apomorphine
- Pituitary, untreated
- Pituitary, 5 weeks DES
- Caudate, untreated
- Caudate, 5 weeks DES
Figure 14. The acute effect of melatonin on (-)-apomorphine competition for [3H]-spiperone binding to anterior pituitary and caudate nucleus membranes from untreated adult female F344 rats. Increasing concentrations of (-)-apomorphine were incubated with membranes and [3H]-spiperone (2.25 nM) in the presence or absence of 10 nM melatonin. Data were analyzed and compared by non-linear regression modeling. There were no significant effects of co-incubation with melatonin. Solid lines represent best-fit models for apomorphine alone. Dashed lines represent best-fit models for all data. For details see Table 12. Panel A, anterior pituitary; Panel B, caudate nucleus.
Table 12. Parameters from inhibition studies of $[^3H]$-spiperone binding to anterior pituitary and caudate nucleus membranes harvested from untreated female F344 rats.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Drug</th>
<th>$K'_H$ (nM)</th>
<th>$B_H$ (%)</th>
<th>$K'_L$ (nM)</th>
<th>$B_L$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td>(+)-butaclamol</td>
<td>4.8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(-)-apomorphine</td>
<td>11.4</td>
<td>26</td>
<td>4772</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>(-)-apomorphine + melatonin (10 nM)</td>
<td>28.3</td>
<td>29</td>
<td>6596</td>
<td>71</td>
</tr>
<tr>
<td>Caudate</td>
<td>(-)-apomorphine</td>
<td>45.8</td>
<td>23</td>
<td>5030</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>(-)-apomorphine + melatonin (10 nM)</td>
<td>130.4</td>
<td>28</td>
<td>5942</td>
<td>72</td>
</tr>
</tbody>
</table>

Values are expressed as the mean estimate of high-affinity ($K'_H$) and low-affinity ($K'_L$) apparent dissociation constants, and the percentage of binding sites of the high-affinity ($B_H$) and low-affinity ($B_L$) type, as determined by non-linear regression modelling. Data were analyzed under both one-site and two-site model hypotheses. Parameter estimates for two sites are presented only in those cases where the two-site model produced significant improvement in goodness-of-fit.
Table 13. Parameters from inhibition studies of \(^{3}\text{H}\)-spiperone binding to anterior pituitary and caudate nucleus membranes harvested from female F344 rats 5 weeks following implantation of DES-containing capsules.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Drug</th>
<th>(K'H) (nM)</th>
<th>(B_H) (%)</th>
<th>(K'L) (nM)</th>
<th>(B_L) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td>(+)-butaclamol</td>
<td>12.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(-)-apomorphine</td>
<td>2527</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Caudate</td>
<td>(+)-butaclamol</td>
<td>7.8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>(-)-apomorphine</td>
<td>79.3</td>
<td>41</td>
<td>3345</td>
<td>59</td>
</tr>
</tbody>
</table>

Values are expressed as the mean estimate of high-affinity (\(K'H\)) and low-affinity (\(K'L\)) apparent dissociation constants, and the percentage of binding sites of the high-affinity (\(B_H\)) and low-affinity (\(B_L\)) type, as determined by non-linear regression modeling. Data were analyzed under both one-site and two-site model hypotheses. Parameter estimates for two sites are presented only in those cases where the two-site model produced significant improvement in goodness-of-fit.
Experiment #4

Serum Prolactin Levels and Anterior Pituitary Weights

Effects on serum RIA-PRL titers and anterior pituitary weights paralleled those observed in experiments 1 and 2. DES treatment resulted in significant increases in serum PRL levels and pituitary weights. This was effectively reversed in melatonin-treated animals, although both values remained elevated over those for Week 0 rats. There was no significant effect of melatonin on serum PRL or anterior pituitary weight at Week 0 (Fig. 15, Table 14).

Table 14. Serum RIA-PRL and anterior pituitary weights from female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Serum RIA-PRL (ng/ml)</th>
<th>Pituitary Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>2.04 ± 0.57</td>
<td>9.06 ± 0.22</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>2.22 ± 0.51</td>
<td>7.10 ± 0.29</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>294.85 ± 67.54\textsuperscript{a}</td>
<td>25.67 ± 0.95\textsuperscript{a}</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>147.85 ± 36.78\textsuperscript{a,b}</td>
<td>19.78 ± 1.43\textsuperscript{a,b}</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SEM (for serum RIA-PRL, n = 6; for pituitary weight, n = 18). Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. (a, p < 0.05 vs. Week 0 Saline-treated animals; b, p < 0.05 vs. Week 4 Saline-treated animals).
Figure 15. The effect of daily afternoon melatonin injections on DES-induced changes in pituitary weight (Panel A) and circulating PRL levels (Panel B) in adult female F344 rats. Daily subcutaneous injections of either melatonin or saline vehicle were initiated 2 weeks prior to subcutaneous implantation of silastic capsules containing 5 mg DES and continued throughout the course of the experiment. On the day before capsule implantation (Week 0), half of the animals from each treatment group were utilized for measurement of hypothalamic DA turnover. At the time of sacrifice for median eminence dissection, trunk blood was collected for determination of circulating PRL levels and anterior pituitaries were removed and weighed. Animals receiving DES-containing implants were maintained in 14:10 (Light:Dark) photoperiod for 4 weeks, at which time they were sacrificed and tissues were processed in the same manner as for Week 0 animals. (a, p < 0.05 vs. Week 0 Saline; b, p < 0.05 vs. Week 4 Saline). For details see Table 14.
Hypothalamic Dopamine Content, Depletion and Turnover

Steady-state (initial) DA concentration in hypothalamic median eminence was significantly \((p < 0.05)\) decreased by DES treatment. Melatonin treatment did not reverse this effect nor was there a significant effect of melatonin on the steady-state concentration at Week 0 (Fig. 16, Table 15).

While DA turnover rates \((K)\) were not significantly altered by treatments, the rate constant of DA depletion \((k)\) was increased in Week 4 saline-treated animals by 2-fold \((p < 0.05)\) over Week 0 saline-treated animals. Melatonin treatment completely reversed this effect at 4 weeks (Fig. 17, Table 15).
Figure 17. Decline in hypothalamic median eminence (M.E.) DA concentrations at 45 and 90 minutes following intraperitoneal injection of the tyrosine hydroxylase inhibitor α-methylparatyrosine (α-MPT). Time 0 animals received saline injections and were sacrificed at various times throughout the course of the procedure. Animals were treated as described in the legend for Figure 15. Data were analyzed by non-linear model fitting for estimates of rate constant of decline (k) and turnover rate (K). Resulting model predictions are represented by solid lines for saline-treated groups and dashed lines for melatonin-treated groups. For details see Table 15. Panel A, Week 0; Panel B, Week 4.
Table 15. Parameters for α-MPT-induced DA depletion from the hypothalamic median eminence (M.E.) of female F344 rats following treatment as indicated.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Initial [DA] (pg/M.E.)</th>
<th>Rate Constant (k) (min⁻¹)</th>
<th>Turnover Rate (K) (pg/M.E. · min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Saline</td>
<td>4703.0 ± 909.1</td>
<td>0.014 ± 0.004</td>
<td>64.6 ± 21.8</td>
</tr>
<tr>
<td>0</td>
<td>Melatonin</td>
<td>3948.8 ± 420.9</td>
<td>0.011 ± 0.002</td>
<td>44.6 ± 10.8</td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td>2278.2 ± 421.5*</td>
<td>0.032 ± 0.006*</td>
<td>73.3 ± 16.1</td>
</tr>
<tr>
<td>4</td>
<td>Melatonin</td>
<td>2143.9 ± 324.9*</td>
<td>0.019 ± 0.004</td>
<td>41.8 ± 10.4</td>
</tr>
</tbody>
</table>

Initial (steady state) DA concentrations are expressed as the mean ± SEM (n = 6). Rate Constants and Turnover Rates are expressed as the mean ± asymptotic standard error as estimated from non-linear regression modeling (regression n = 16-18). Week refers to the number of weeks following implantation of DES-containing capsules. Animals received daily afternoon injections of either melatonin or vehicle beginning two weeks prior to capsule implantation. (*, p < 0.05 vs. Week 0 Saline-treated).
Discussion

These studies confirm our previous report that daily afternoon administration of melatonin effectively inhibits the growth of DES-induced PRL-secreting pituitary tumors in F344 rats (Leadem and Burns, 1987). This effect appears to be specific for PRL cells, as the hyperprolactinemia associated with these tumors is also significantly inhibited by melatonin treatment.

Expressed as DPM of $^3$H-PRL/mg pituitary, PRL synthesis in vitro was increased in DES-treated animals and this effect was not reversed by melatonin. This provides further evidence that the inhibitory effects of melatonin on tumor growth are specific for PRL cells. Given that these tumors are composed largely of PRL cells (DeNicola et al., 1978), if the inhibitory effect of melatonin on tumor size is accomplished through a decrease in the number and/or size of individual PRL cells, then overall tumor activity (as reflected in pituitary weight and serum PRL levels) should decrease while unit tumor activity (as reflected by PRL synthesis per milligram of tissue) should not be affected. This was precisely the pattern observed for anterior pituitary weights, circulating PRL levels and both $^3$H-PRL and RIA-PRL concentrations in vitro.

The major issue addressed in these studies is the role of dopaminergic control mechanisms in the suppression of tumor growth and secretory activity by melatonin. This was assessed in terms of in vitro sensitivity to PRL release inhibition by DA, binding properties of dopaminergic drugs to anterior pituitary membranes and activity of tuberoinfundibular dopamine neurons.
Melatonin treatment did not alter IC₅₀ values or maximal effectiveness of DA on inhibition of the release of either immunoreactive or newly synthesized PRL suggesting that increased dopamine sensitivity is not a major component in the inhibition of tumor cell activity by melatonin. In agreement with earlier studies in the normal anterior pituitary (MacLeod and Lehmeyer, 1974) newly synthesized PRL was apparently more sensitive to the effects of DA than RIA-PRL in terms of both IC₅₀ and maximum effect in all treatment groups. This observation is thus extended to include DES-induced pituitary tumors, indicating some parallelism between these tumors and the normal pituitary regarding compartmentalization of PRL pools and their regulation by DA.

The finding that DES treatment altered anterior pituitary dopaminergic binding parameters is also generally supported by previous reports (Heiman and BenJonathan, 1982; Bression et al., 1983,1985). Because of differences in the interactions of agonists vs. antagonists with dopamine receptors, the observation that DES treatment resulted in reduced affinity and binding capacity of ³H-spiperone in anterior pituitary membranes must be interpreted with caution. Decreased receptor density could be due to actual decreases in the number of binding sites per cell, decreased numbers of cells or dilution of plasmalemmal membranes with other membraneous organelles. Still, alterations in DA receptor populations, with concomitant reductions in ability to bind DA might be a mechanism in DES-induced tumor development. The loss of high affinity binding sites for (-)-apomorphine with DES treatment, as evidenced in binding competition studies, provides more direct evidence for such a hypothesis. The possibility also exists that observed differences in binding parameters are an effect of, rather than the cause
of tumor development. Elevated circulating PRL levels in DES-treated animals may exert feedback effects on the levels of DA or other neuroendocrine regulatory hormones reaching the anterior pituitary. The results of dopamine turnover studies, as discussed below, lend support to this idea. Alterations in binding could then be viewed as the result of alterations in endocrine milieu. In any case, melatonin treatment did not reverse the effects of DES on binding, further suggesting that the inhibitory effects of melatonin on tumor activity are not the result of enhanced sensitivity of PRL cells to endogenous dopamine.

Effects on hypothalamic DA were two-fold. First, the steady-state concentration of DA in the median eminence was reduced in both saline- and melatonin-treated animals exposed to DES. This effect has several possible explanations. As has been previously suggested, decreased hypothalamic DA content may be due to toxic effects on TIDA neurons of chronic estrogenization and/or the resulting hyperprolactinemia, or due to an inability of DA synthesis to keep pace with its release as enhanced by continuous feedback drive (Sarkar et al., 1982; 1984; Morgan et al., 1985a). Because of the rapid growth of tumors in this model, direct mechanical trauma to the basal hypothalamus is also conceivable. Second, the rate constant of DA depletion following α-MPT injection in saline-treated animals exposed to DES was double that of controls, presumably reflecting an increased rate of DA release in these animals. It was originally hypothesized that increased activity of TIDA neurons might be a mechanism in inhibition of these tumors by melatonin. At Week 0, melatonin treatment had no effect on median eminence DA content or depletion and after 4 weeks of DES exposure, the rate constant of depletion in melatonin-treated animals was in fact decreased to a level indistin-
guishable from controls. It seems likely that the increased rate of release in saline-treated DES animals was due to feedback effects of dramatically elevated serum PRL titers on TIDA neurons, and that the reversal of this effect by melatonin is simply a reflection of the effectiveness of melatonin treatment for reducing the secretory activity of DES-induced tumors. This study provides no evidence that melatonin increases DA release from TIDA neurons as an underlying mechanism in tumor inhibition. However, the possibility of enhanced DA release at some time between Week 0 and Week 4 cannot be ruled out. It is possible that the inhibition of hyperprolactinemia observed in Week 4 melatonin-treated animals resulted in diminished feedback drive on TIDA neurons, thereby over-riding or masking underlying direct effects of melatonin on TIDA neuronal activity. Previous evidence in the rat and hamster has suggested that the PRL inhibitory effects of light deprivation are mediated, at least in part, through increased DA turnover rates in the hypothalamus (Steger et al., 1982; Benson, 1987; Leadem et al., 1988). The DA turnover parameter is defined as the product of the steady-state concentration and the rate constant of depletion \( K = [DA]_0 k \). All other things being equal, a change in either the steady-state concentration or the rate constant is reflected as a change in the turnover rate. However, if the steady-state concentration and the rate constant are altered simultaneously and in reciprocal directions, then the value of the turnover rate can remain relatively unchanged. In these studies, Week 4 saline-treated animals exhibited significantly diminished steady-state concentration and a significantly enhanced rate constant, compared to controls, while the turnover rate was unchanged. As it has been suggested that steady-state concentrations in TIDA neurons are not as tightly coupled to synthesis and activity as
in other DA neuron systems (Demarest et al., 1984), for purposes of interpretation in the present study, the rate constant has been regarded as a more important indicator of TIDA activity.

The finding that biological responsiveness to DA in vitro was not altered by DES or melatonin treatment, while pituitary membrane binding parameters were altered by DES in a manner suggesting decreased affinity for agonists, reinforces the need for caution in interpretation of ligand binding data. It is clearly difficult to draw directly parallel conclusions between biochemical measurements in isolated membranes and biological endpoints in the more complex milieu of organ culture. The possibility must be considered, however, that genuine effects of DES on DA receptor populations did not result in detectable changes in DA sensitivity, as estimated in static incubations of pituitary fragments. This could be explained by media "auto-conditioning" (accumulation of PRL or other cell products) or by relative inaccessibility of the central area of tissue fragments to dopamine and/or oxygen.

The results of the present study support the conclusion that the effects of melatonin on the growth and activity of PRL-secreting tumors are neither mediated through increased sensitivity of PRL cells to the inhibitory effects of endogenous dopamine, nor through increased activity of tuberoinfundibular dopaminergic neurons. Possible effects of melatonin on other neuroendocrine regulatory systems or direct effects of melatonin on tumor cells require further studies.
The major issue addressed in this dissertation is the possible involvement of altered dopaminergic regulation in PRL cell inhibition induced by short photoperiod exposure or melatonin administration. The results indicate that PRL inhibitory effects elicited by these treatments are not associated with either enhanced release of dopamine from hypothalamic terminals or with enhanced pituitary responsiveness to the PRL-inhibitory effects of dopamine. Experimental answers to specific questions posed at the beginning of this dissertation are as follows:

Q. Given that the pituitaries of long photoperiod-exposed male hamsters may not respond to the PRL-inhibitory effects of dopamine, and given that the actions of dopamine are presumably mediated through first binding to cell surface receptors, do the pituitaries of these animals express dopamine receptor populations as observed in other mammalian species? (Chapter 3)

A. Yes. Analysis of $^3$H-spiperone binding data revealed the presence of two classes of binding sites in anterior pituitary membranes of these animals.
Q. Does dopamine effectively inhibit the release of PRL \textit{in vitro} from pituitaries of long photoperiod-exposed male hamsters? (Chapter 3)

A. \textbf{Yes.} RIA-PRL release from pituitaries of long photoperiod-exposed male hamsters was inhibited by DA in a dose-dependent fashion, with IC$_{50}$ values indicative of a high affinity interaction.

Q. As measured by dose response modeling of PRL release inhibition, is pituitary sensitivity to dopamine enhanced over the time course of PRL cell inhibition induced by short photoperiod, compared to long photoperiod controls? (Chapter 3)

A. \textbf{No.} Comparison of IC$_{50}$ and \% maximum inhibition estimates for long vs. short photoperiod-exposed groups indicated no difference in pituitary sensitivity to DA at any time point tested.

Q. Does the daily afternoon injection of melatonin for a period of two weeks alter indices of PRL cell activity in otherwise untreated F344 rats? (Chapter 4)

A. \textbf{No.} There were no differences in anterior pituitary weights, serum PRL levels, total RIA-PRL, content of RIA-PRL in incubated pituitaries, RIA-PRL release \textit{in vitro}, PRL synthesis or release of $^{3}$H-PRL \textit{in vitro} in melatonin vs. saline-injected animals after two weeks of treatment.
Q. Is the daily afternoon injection of melatonin effective in inhibiting the growth and activity of DES-induced PRL-secreting adenomas in the F344 rat? (Chapter 4)

A. Yes. Melatonin treatment initiated two weeks prior to implantation of DES-containing capsules resulted in significant reductions in DES-induced increases in anterior pituitary weight and serum PRL levels as compared to saline vehicle-treated controls.

Q. Does the daily administration of melatonin enhance pituitary sensitivity to dopamine in otherwise untreated F344 rats? (Chapter 4)

A. No. IC₅₀ and % maximum inhibition values for the inhibition by DA of RIA-PRL and ³H-PRL release in vitro were identical for melatonin vs. saline-treated animals following two weeks of treatment.

Q. Does the daily administration of melatonin enhance pituitary sensitivity to dopamine in F344 rats bearing PRL-secreting adenomas induced by 4 weeks of exposure to DES? (Chapter 4)

A. No. While melatonin treatment effectively reversed DES-induced increase in pituitary weight and serum PRL levels, DA was equally effective in inhibiting the release in vitro of both RIA-PRL and ³H-PRL from pituitaries of melatonin and saline-treated animals exposed to DES. Furthermore, regression coefficients (IC₅₀ and %
maximum inhibition) for tumor-bearing animals were no different from those for Week 0 (DES-unexposed) animals.

Q. Does daily afternoon melatonin administration alter pituitary dopamine receptor populations in otherwise untreated F344 rats? (Chapter 4)

A. **No.** Estimates of \( k_d \) and \( B_{\text{max}} \) for the binding of \(^3\text{H}\)-spiperone to anterior pituitary membranes were not altered by two weeks of melatonin treatment, as compared to saline-injected controls.

Q. Does melatonin administration *in vitro* alter agonist vs. antagonist competition behaviour at pituitary dopamine receptors? (Chapter 4)

A. **No.** Addition of melatonin (\( 10^{-8} \) M) to incubations did not alter the ability of (-)-apomorphine to inhibit the binding of \(^3\text{H}\)-spiperone to anterior pituitary membranes.

Q. Are pituitary DA receptor populations altered in DES-induced adenomas, and if so, is this effect augmented or reversed by daily administration of melatonin? (Chapter 4)

A. **Yes.** Both the affinity and binding capacity of anterior pituitary membranes for \(^3\text{H}\)-spiperone were significantly reduced in DES-induced adenomas, as compared to binding parameters in pituitary membranes of Week 0 (DES-unexposed) animals. Melatonin treatment did not augment or reverse this effect.
Q. Are agonist vs. antagonist interactions at dopamine receptors altered in DES-induced adenomas? (Chapter 4)

A. Yes. The biphasic curve for (-)-apomorphine inhibition of $^3$H- spiperone binding to anterior pituitary membranes observed in Week 0 animals was converted to a monophasic (low affinity) curve in Week 4 (DES-treated) animals.

Q. Does the daily administration of melatonin alter steady-state dopamine concentrations in the hypothalamic median eminence of otherwise untreated F344 rats? (Chapter 4)

A. No. Steady-state ([DA]$_0$) concentrations in hypothalamic median eminence were not different in Week 0 melatonin vs. Week 0 saline-treated animals.

Q. Does daily melatonin administration of melatonin alter steady-state dopamine concentrations in the hypothalamic median eminence of F344 rats with DES-induced pituitary adenomas? (Chapter 4)

A. No. While DES treatment resulted in significant reduction of hypothalamic M.E. steady-state DA concentrations, as compared to Week 0 animals, this effect was not reversed by melatonin treatment.
Q. Does melatonin administration alter the activity of hypothalamic dopamine neurons in otherwise untreated F344 rats? (Chapter 4)

A. No. Neither the DA turnover rate (K) nor the rate constant (k) of DA depletion was altered by melatonin treatment, as compared to saline-treated controls, at Week 0.

Q. Does melatonin administration alter the activity of dopamine neurons in F344 rats with DES-induced PRL-secreting adenomas? (Chapter 4)

A. Yes. Four weeks following implantation of DES-containing capsules, the rate constant (k) of DA depletion from hypothalamic M.E. following α-MPT administration was significantly reduced in melatonin-treated animals, compared to saline-injected controls. The most likely explanation for this result is reduced feedback drive on TIDA neurons owing to the efficacy of melatonin treatment for reversing DES-induced increases in serum PRL concentrations.
APPENDIX A

NON-LINEAR MODELING

The purpose of non-linear regression analysis is to obtain best-fit (least squares) coefficient estimates for models which are non-linear in one or more parameters. Non-linear modeling is indicated when observed data are not adequately described by simple linear models (as objectively determined by estimation of pure experimental error and lack-of-fit error mean square probabilities). The fitting of non-linear models is little more than a problem of computational labor, a problem which has been largely removed by the widespread availability of digital computers and programs specifically designed for this purpose. The problem which remains is arriving at appropriate and meaningful models. In some cases, widely accepted theoretical models are available. In such cases, systems can typically be represented and compared by estimating model constants and their variances. Even in cases where much less is known about underlying mechanisms, non-linear modeling can be used to obtain relevant information about a system of interest by means of equations which reflect the main features of the system (i.e. maxima, minima, rate constants). Generally these take the form of time- or mass-dependent differential equations. As a "rule of thumb", the simplest form of a model which adequately describes a system is the appropriate model, and more complex (i.e. multiple site) models should only be used when they provide significant improvement in goodness-of-fit. In situations where multiple site inter-
actions are theoretically possible, this provides an objective method for discrimination.

The preceding comments are largely attributable to Box, Hunter and Hunter in their excellent text, *Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building* (John Wiley & Sons, New York, 1978).

In no case in this dissertation was it necessary to build models from the ground up. For ligand interactions with binding sites, uniform models have been developed based on detailed theoretical relationships. In dopamine turnover studies, the model used for estimation of depletion rate constants and turnover rates is an exact algebraic equivalent of the original logistic model proposed by Brodie *et al.* (1966). The model for prolactin release inhibition by dopamine belongs to a family of equations which are widely used for estimation of 50% response levels. The specific version used also includes a parameter which models the maximum range of response in the dependent variable.

**MODEL FITTING**

Models were fitted to obtain estimates of parameter values using the SPSS-X program CNLR (Constrained Nonlinear Regression) running on a VAX/VMS computer at the Arizona Cancer Center. In the same analyses, parameter variances and residual sums of squares were computed and saved for post-hoc comparisons.
MODEL EQUATIONS

Ligand Binding Saturation Studies:

One Site

\[ B_s = \frac{B_{\text{max}}[L]}{([L] + K_d)^{-1}}, \]

where:

- \( B_s \) = specific binding of \(^3\)H-ligand at concentration [L].
- \( B_{\text{max}} \) = total binding site concentration.
- \([L]\) = free \(^3\)H-ligand concentration.
- \( K_d \) = equilibrium dissociation constant.

Two Site

\[ B_s = \frac{B_{\text{max}1}[L]}{([L] + K_{d1})^{-1}} + \frac{B_{\text{max}2}[L]}{([L] + K_{d2})^{-1}}, \]

where:

- \( B_{\text{max}1}, B_{\text{max}2} \) = concentrations of binding sites of type \( K_{d1} \) and \( K_{d2} \), respectively.
- \( K_{d1}, K_{d2} \) = distinct equilibrium dissociation constants (i.e. high affinity, low affinity).
Ligand Binding Competition Studies:

One Site

\[ \frac{B_i}{B_0} = \left(1 + \left[ I \right]/k' \right)^{-1} \], where:

- \( B_i \) = specific binding of \(^3\)H-ligand in the presence of competing unlabeled ligand.
- \( B_0 \) = specific binding of \(^3\)H-ligand in the absence of competitor.
- \([I]\) = molar concentration of competing ligand.
- \( k' \) = apparent dissociation constant of competing ligand.

Two Site

\[ \frac{B_i}{B_0} = p(1 + [I]/k_1)^{-1} + (1-p)(1 + [I]/k_2)^{-1} \], where:

- \( p \) = proportion of sites of type \( k_1 \).
- \( k_1, k_2 \) = distinct apparent dissociation constants (i.e. high affinity, low affinity).
**Prolactin Release Inhibition Studies:**

\[ \frac{R_l}{R_0} = r(1 + [DA]/IC_{50})^{-1} + (1-r), \]

where:

- \( R_l \) = PRL release in the presence of dopamine at concentration \([DA]\).
- \( R_0 \) = PRL release in the absence of dopamine (vehicle control).
- \( r \) = range of rational \((R_l/R_0)\) response, from \((1-r)\) to 1.
- \([DA]\) = molar concentration of dopamine.
- \( IC_{50} \) = dopamine concentration predicted to produce 50% of possible response.

**Dopamine Turnover Studies:**

\[ [DA]_t = \left( \frac{K \times \exp^{kt}}{k} \right), \]

where:

- \([DA]_t\) = dopamine concentration at time \(t\).
- \( K \) = dopamine turnover rate.
- \( k \) = dopamine depletion rate constant.

**MULTIPLE MODEL RESOLUTION**

Testing for significant improvement in goodness-of-fit by increasing complexity of models (such as two site vs. one site binding models) was accomplished using partial F-tests (DeLean et al., 1982):

\[ F = \frac{(SS_1 - SS_2)/(df_1 - df_2))/(SS_2/df_2), \]

where \( SS_1 \) and \( SS_2 \) are the residual sums of squares of the less complex and more complex models, respectively, and \( df_1 \) and \( df_2 \) are the corresponding regression degrees of freedom.
degrees of freedom (number of data points minus the number of estimated parameters). F is distributed as an F distribution with df1-df2 numerator and df2 denominator degrees of freedom. A probability point of less than 0.05 was considered significant.
APPENDIX B

INCUBATION OF HEMIPITUITARIES AND PITUITARY FRAGMENTS IN VITRO AND $^3$H-LEUCINE LABELING OF NEWLY SYNTHESIZED PROLACTIN

These procedures may be utilized to answer several experimental questions. The simplest form of the question is, for a single incubated hemipituitary or pituitary fragment, "During the period of incubation, how much immunoreactive PRL or $^3$H-PRL was released into the incubation medium, and how much remains in the pituitary tissue at the end of the incubation?"

Newly synthesized prolactin is radioactively labeled by incubation in the presence of $^3$H-leucine, such that as proteins are synthesized in vitro, $^3$H-leucine is incorporated into the peptide chain. $^3$H-PRL in the incubation media or pituitary homogenates can then be separated from other proteins by polyacrylamide gel electrophoresis and semi-quantified by scintillation spectrometry (see APPENDIX F).

Immunoreactive PRL in incubation media and pituitary homogenates is quantified by PRL radioimmunoassay (see APPENDIX D and APPENDIX E).

Thus, in the simplest form, these assays may be used to compare the effect of in vivo treatments on the storage and release of immunoreactive PRL or to compare relative rates of PRL synthesis and release of newly synthesized PRL between treatment groups. An important underlying assumption for these comparisons is that activity in vitro reflects activity in vivo. In addition, dopamine (or
other agents hypothesized to perturb PRL physiology) may be added to incubations, for subsequent dose response analysis of sensitivity to these agents. Similarly, for comparison of sensitivity between treatment groups, the assumption is made that that in vitro "physiology" reflects the effects of in vivo treatments.

Procedure: Rat Studies

1. One half hour before pituitaries are harvested, bubble 95% O₂-5% CO₂ through minimal essential medium (GIBCO) containing penicillin (5000 U/100 ml), Fungizone (12.5 μg/100 ml), streptomycin (5000 μg/100 ml) and ascorbic acid (5.6 x 10⁻⁴ M). Before beginning incubations, add 20 μCi/ml of ³H-leucine (L-(3,4,5-³H-leucine, 50 Ci/mm, Research Products International) to an appropriate volume (0.5 ml per incubation vial) of medium.

2. Kill animals by rapid decapitation. Expose the pituitary and remove the posterior lobe. Remove and weigh the anterior pituitary. Divide the anterior pituitary into 5-7 mg fragments and pool fragments in a vial containing pre-gassed medium on ice. For dose-response studies, incubations were subsequently carried out such that pooled fragments were randomly selected for incubation in the presence of either media containing 5.6 x 10⁻⁴ M ascorbic acid alone or media containing 5.6 x 10⁻⁴ M ascorbic acid plus dopamine at one of several concentrations (5 x 10⁻⁵ M - 5 x 10⁻¹¹ M).

3. Place the pituitary fragment in a small glass vial (14.5 x 45 mm, Kimble) containing 0.5 ml medium with 20μCi/ml ³H- leucine.
4. Place the vial in a Precision Dubnoff Shaking Metabolic Incubator (humidified, 37° C, 95% O₂ - 5% CO₂ on an incubator setting of 7-9 arbitrary units of gas flow, shaking at 20-30 cycles per minute).

5. For pituitaries to be incubated in the presence of dopamine, add 5 μl of medium containing dopamine, prepared in advance to yield the desired concentration.

6. Record the time. Incubate each pituitary fragment for 3 hours.

7. After 3 hours of incubation, remove the vial from the incubator and record the time.

8. Remove the media from the vial with a pasteur pipet and save in a 12 x 75 mm plastic tube.

9. Weigh the pituitary fragment and place it in a 12 x 75 mm plastic tube containing 0.5 ml 0.01 M PBS. Homogenize by sonication.

10. Freeze all samples (media and pituitaries) until radioimmunoassay (see APPENDIX E) or polyacrylamide gel electrophoresis (see APPENDIX F).

Procedure: Hamster Studies

1. One half hour before pituitaries are harvested, bubble 95% O₂-5% CO₂ through minimal essential medium (GIBCO) containing penicillin (5000 U/100 ml), Fungizone (12.5 μg/100 ml), streptomycin (5000 μg/100 ml) and ascorbic acid (5.6 x 10⁻⁴ M).

2. Kill animals by rapid decapitation. Expose the pituitary, remove posterior lobe, and bisect the pituitary in situ. For dose-response studies, incubations were
subsequently carried out such that one hemipituitary served as the vehicle control for the other half incubated with one of the concentrations of dopamine.

3. Place the hemipituitary in a small glass vial (14.5 x 45 mm, Kimble) containing 0.5 ml pre-gassed medium.

4. Place the vial in a Precision Dubnoff Shaking Metabolic Incubator (humidified, 37°C, 95% O₂ - 5% CO₂ on an incubator setting of 7-9 arbitrary units of gas flow, shaking at 20-30 cycles per minute).

5. Record the time that the vial was placed in the incubator.

6. Pre-incubate each hemipituitary for 30 minutes.

7. After 30 minutes of pre-incubation, remove the media with a pasteur pipet and replace with 0.5 ml of fresh medium. For hemipituitaries to be incubated in the presence of dopamine, add 5 μl of medium containing dopamine, prepared in advance to yield the desired concentration (5 x 10⁻⁷ M - 5 x 10⁻¹¹ M).

8. Record the time. Incubate each hemipituitary for 3 hours.

9. After 3 hours of incubation, remove the vial from the incubator and record the time.

10. Remove the media from the vial with a pasteur pipet and save in a 12 x 75 mm plastic tube.

11. Weigh the hemipituitary and place it in a 12 x 75 mm plastic tube containing 0.5 ml 0.01 M PBS. Homogenize by sonication.
12. Freeze all samples (media and pituitaries) until radioimmunoassay (see APPENDIX D).
APPENDIX C

IODINATION OF PROLACTIN

The purpose of this procedure is to radioactively label prolactin by the addition of $^{125}$I to tyrosine residues in the molecule. The presence of $^{125}$I-PRL can then be detected by gamma spectrometry.

This method can be used for iodination of either hamster or rat prolactin.

Procedure

1. Soak Sephadex G-75 in 0.01 M PBS (10 gm/200 ml) overnight at 4° C. Bring to room temperature and deaerate under vacuum before use.

2. Use a disposable 10 ml glass pipet for the column. Add a small glass bead to retain the Sephadex in the column.

3. Pour the Sephadex G-75 into the column to a height of approximately 20 cm and perfuse with 0.01 M PBS for 1 hour.

4. Coat the column with 2 ml of 2% BSA (w/v) in 0.01 M PBS, then wash the column with 10 ml 0.01 M PBS.

5. Add the following to a 400 μl disposable reaction vial:

   2.5 μg of iodination grade PRL dissolved in 25 μl 0.01 M sodium bicarbonate (pH 8.75).

   100 μl 0.5 M PBS.
1.0 mCi Na\textsuperscript{125}I in 10 \mu l 0.5 M PBS.

12.5 \mu g chloramine-T (Sigma) in 25 \mu l 0.01 M PBS. Vortex lightly for 30 sec.

250 \mu g sodium metabisulfite in 100 \mu l 0.01 M PBS. Vortex 15 sec.

50 \mu l 5\% BSA (w/v) in 0.01 M PBS. Vortex 10 sec.

6. Carefully layer the contents of the reaction vial onto the column and gently wash the column 3 times with 500 \mu l 0.01 M PBS.

7. Elute the column with 0.01 M PBS, collecting 50 fractions of 15 drop aliquots in 13 x 100 mm glass tubes containing 50 \mu l 2\% BSA (w/v) in 0.01 M PBS.

8. Count 10 \mu l aliquots of each tube in a gamma counter and plot counts/tube vs. fraction number. Two distinct peaks should appear. The first peak should occur in the column volume and should contain the intact iodinated PRL. Save (store at -20\degree C) fractions from the back side of this peak through the trough. The second peak, which occurs in the void volume and represents unincorporated Na\textsuperscript{125}I, should be appropriately discarded.
Buffers and Reagents

0.5 M PHOSPHATE BUFFER

Monobasic Sodium Phosphate 16 ml
(68.99 g/l)
Dibasic Sodium Phosphate 84 ml
(70.98 g/l)

0.01 M PHOSPHATE BUFFERED SALINE (PBS)

0.5 M Phosphate Buffer 20 ml
Sodium Chloride 8.77 g
Sodium Azide 100 mg
Sodium EDTA 3.72 g

QS to 1 L with DDW

Adjust pH to 7.6 with Sodium Hydroxide

CHLORAMINE-T (Sigma)

5 mg in 10 ml 0.01 M PBS.

Store in dark in dessicator and avoid light as possible while weighing.

Prepare no more than 10 minutes before use.

SODIUM METABISULFITE

25 mg in 10 ml 0.01 M PBS.
2% BSA-PBS

Lyophilized Bovine Serum Albumin 2 g
0.01 M PBS 100 ml
APPENDIX D

HAMSTER PROLACTIN RADIOIMMUNOASSAY

The purpose of this procedure is to quantify hamster prolactin in samples of serum, incubation media or pituitary homogenates. It is based on the procedure of Soares et al. (1983). Hamster PRL and hamster PRL antiserum were supplied by Dr. Frank Talamantes, Department of Biology, Thimann Laboratories, University of California, Santa Cruz, California. Sheep anti-rabbit gamma globulins were kindly supplied by Dr. Mark Wise, University of Arizona, Tucson, Arizona.

Procedure

Assay Tube Preparation

Label a sufficient number of 12 x 75 mm glass tubes. There are 5 sets of tubes in the assay:

1. TOTAL COUNT TUBES (6 Replicates)

100 µl of $^{125}\text{I}$-PRL (10,000 CPM/100 µl 0.1% BSA- PBS). This set is prepared on the Day 2 of the assay, when $^{125}\text{I}$- PRL is added to all assay tubes.
2. NONSPECIFIC BINDING TUBES (6 Replicates)

100 μl 0.1% BSA-PBS (Day 1)

100 μl 3% NRS-PBS (Day 1)

100 μl 125I-PRL (10,000 CPM/100 μl 0.1% BSA- PBS) (Day 2)

100 μl sheep anti-rabbit gamma globulins (SARGG) (1:2 dilution in 0.01 M PBS) (Day 3)

3. ZERO (PRL) TUBES (10 Replicates)

100 μl 0.1% BSA-PBS (Day 1)

100 μl rabbit anti-hamster PRL antiserum (1:30,000 dilution in 3% NRS-PBS) (Day 1)

100 μl 125I-PRL (10,000 CPM/100 μl 0.1% BSA- PBS) (Day 2)

100 μl SARGG (1:2 dilution in 0.01 M PBS) (Day 3)

4. STANDARD CURVE TUBES (3 Replicates per Standard Curve Mass)

100 μl 0.1% BSA-PBS containing 0.1 - 1.0 ng of hamster PRL (See "Construction of Hamster PRL Standard Curve," below) (Day 1)

100 μl rabbit anti-hamster PRL antiserum (1:30,000 dilution in 3% NRS-PBS) (Day 1)

100 μl 125I-PRL (10,000 CPM/100 μl 0.1% BSA- PBS) (Day 2)

100 μl SARGG (1:2 dilution in 0.01 M PBS) (Day 3)
5. SAMPLE (UNKNOWN) TUBES (2 or 3 Replicates per Sample)

50 μl sample (diluted in 0.1% BSA-PBS, as indicated by test assay) (Day 1)
50 μl 0.1% BSA-PBS (Day 1)
100 μl rabbit anti-hamster PRL antiserum (1:30,000 dilution in 3% NRS-PBS) (Day 1)
100 μl 125I-PRL (10,000 CPM/100 μl 0.1% BSA-PBS) (Day 2)
100 μl SARGG (1:2 dilution in 0.01 M PBS) (Day 3)

Protocol

Day 1

1. Dilute samples in 0.1% BSA-PBS. A test assay is generally necessary to determine appropriate dilutions such that the PRL mass within sample tubes falls between the extremes of the standard curve. Add 50 μl of the diluted sample to 50 μl of 0.1% BSA-PBS.

2. Prepare the standard curve, as described below.

3. Add 100 μl 0.1% BSA-PBS and 3% NRS-PBS to the NONSPECIFIC BINDING TUBES.

4. Add 100 μl rabbit anti-hamster PRL antiserum ("first antibody") diluted 1:30,000 in 3% NRS-PBS to the ZERO TUBES, STANDARD CURVE TUBES and SAMPLE TUBES. Do not add the first antibody to TOTAL COUNT TUBES or NONSPECIFIC BINDING TUBES.
5. Vortex tubes lightly, cover and incubate at room temperature for 24 hours.

**Day 2**

6. Add 100 μl \[^{125}\text{I}]\text{-PRL} (10,000 CPM/100 μl 0.1% BSA- PBS) to all tubes.

7. Vortex lightly, cover and incubate at room temperature for 24 hours.

**Day 3**

8. Add 100 μl sheep anti-rabbit gamma globulins ("second antibody," 1:2 dilution in 0.01 M PBS) to all tubes except TOTAL COUNT TUBES.

9. Vortex tubes lightly, cover and incubate at room temperature for 24 hours.

**Day 4**


11. Spin all other tubes at 2400 rpm for 25 min at 4° C.

12. The pellet appears as a thin white film at the bottom of the tubes. Carefully aspirate the supernatant, and along with the TOTAL COUNT TUBES, count in a gamma counter for 1 min.
Buffers and Reagents

0.01 M PHOSPHATE BUFFERED SALINE (PBS)

Dibasic Sodium Phosphate 1.42 g
Sodium Chloride 8.77 g
Sodium EDTA 3.72 g
Sodium Azide 1.00 g

QS to 1 L with DDW
Adjust pH to 7.6 with Sodium Hydroxide

0.1% BSA-PBS

Lyophilized Bovine Serum Albumin 1.00 g

0.01 M PBS 1000 ml

3% Normal Rabbit Serum-PBS (NRS-PBS)

Normal Rabbit Serum 3 ml

(Gibco 200-6120)

QS to 100 ml with 0.01 M PBS
Hamster Prolactin (hPRL) Standard Curve

Stock hPRL Standards:

Prepare 700 μl aliquots of 10 ng hPRL/ml of 0.1% BSA-PBS.

Immediately before construction of the standard curve, prepare a second dilution of hPRL standard, as follows: Add 100 μl of 10 ng/ml stock to 400 μl 0.1% BSA-PBS yielding a 2 ng/ml stock solution.

Both the 10 ng/ml and the 2 ng/ml hPRL stock solutions will be used in construction of the standard curve.

Table 16. Construction of hPRL Standard Curve.

<table>
<thead>
<tr>
<th>hPRL Mass (ng)</th>
<th>hPRL (2 ng/ml) (μl)</th>
<th>hPRL (10 ng/ml) (μl)</th>
<th>0.1% BSA-PBS (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>5</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>0.02</td>
<td>10</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>0.05</td>
<td>25</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>0.10</td>
<td>50</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>25</td>
<td>75</td>
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<td>50</td>
</tr>
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<td>1.00</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Make 3 Replicates for Each Point on the Curve.
APPENDIX E

RAT PROLACTIN RADIOIMMUNOASSAY

The purpose of this procedure is to quantify rat prolactin (rPRL) in samples of serum, incubation media or pituitary homogenates. Samples were assayed for immunoreactive rPRL using a kit supplied by the National Hormone and Pituitary Program using PRL-RP3 as a standard. Sheep anti-rabbit gamma globulins were kindly supplied by Dr. Mark Wise, University of Arizona, Tucson, Arizona.

PROCEDURE

Assay Tube Preparation

Label a sufficient number of 12 x 75 mm glass tubes. There are 5 sets of tubes in the assay:

1. TOTAL COUNT TUBES (6 Replicates)
   
   100 µl of $^{125}$I-PRL (30,000 CPM/100 µl 0.1% BSA- PBS). This set is prepared on the Day 2 of the assay, when $^{125}$I- PRL is added to all assay tubes.
2. NONSPECIFIC BINDING TUBES (6 Replicates)

400 µl 1% BSA-PBS (Day 1)

200 µl 3% NRS-PBS (Day 1)

100 µl ¹²⁵I-PRL (30,000 CPM/100 µl 0.1 % BSA- PBS) (Day 2)

100 µl sheep anti-rabbit gamma globulins (SARGG)(1:2 dilution, 2% NRS-PBS) (Day 3)

3. ZERO (PRL) TUBES (10 Replicates)

400 µl 1% BSA-PBS (Day 1)

200 µl rabbit anti-rPRL antiserum (1:2500 dilution in 3% NRS-PBS) (Day 1)

100 µl ¹²⁵I-PRL (30,000 CPM/100 µl 0.1% BSA- PBS) (Day 2)

100 µl SARGG (1:2 dilution, 2% NRS-PBS) (Day 3)

4. STANDARD CURVE TUBES (3 Replicates per Standard Curve Mass)

400 µl 1% BSA-PBS containing 0.025 - 5.0 ng of rat PRL (See "Construction of Rat PRL Standard Curve," below) (Day 1)

200 µl rabbit anti-r PRL antiserum (1:2500 dilution in 3% NRS-PBS) (Day 1)

100 µl ¹²⁵I-PRL (30,000 CPM/100 µl 0.1% BSA- PBS) (Day 2)

100 µl SARGG (1:2 dilution, 2% NRS-PBS) (Day 3)
5. SAMPLE (UNKNOWN) TUBES (2 or 3 Replicates per Sample)

400 µl sample (diluted in 1% BSA-PBS, as indicated by test assay) (Day 1)
200 µl rabbit anti-rPRL antiserum (1:2500 dilution in 3% NRS-PBS) (Day 1)
100 µl $^{125}$I-PRL (30,000 CPM/100 µl 0.1% BSA- PBS) (Day 2)
100 µl SARGG (1:2 dilution, 2% NRS-PBS) (Day 3)

Protocol

Day 1

1. Dilute samples in 1% BSA-PBS. A test assay is generally necessary to determine appropriate dilutions such that the PRL mass within sample tubes falls between the extremes of the standard curve.

2. Prepare the standard curve, as described below.

3. Add 400 µl 1% BSA-PBS and 200 µl 3% NRS-PBS to the NONSPECIFIC BINDING TUBES.

4. Add 200 µl rabbit anti-rat PRL antiserum ("first antibody") diluted 1:2500 in 3% NRS-PBS to the ZERO TUBES, STANDARD CURVE TUBES and SAMPLE TUBES. Do not add the first antibody to TOTAL COUNT TUBES or NONSPECIFIC BINDING TUBES.

5. Vortex tubes lightly, cover and incubate at room temperature for 24 hours.
Day 2

6. Add 100 μl $^{125}$I-PRL (30,000 CPM/100 μl 0.1% BSA- PBS) to all tubes.

7. Vortex lightly, cover and incubate at room temperature for 24 hours.

Day 3

8. Add 100 μl sheep anti-rabbit gamma globulins ("second antibody," 1:2 dilution in 2% NRS-PBS) to all tubes except TOTAL COUNT TUBES.

9. Vortex tubes lightly, cover and incubate at room temperature for 24 hours.

Day 4


11. Spin all other tubes at 2400 rpm for 25 min at 4° C.

12. The pellet appears as a thin white film at the bottom of the tubes. Carefully aspirate the supernatant, and along with the TOTAL COUNT TUBES, count in a gamma counter for 1 min.
Buffers and Reagents

0.5 M Phosphate Buffer

0.5 M Monobasic Sodium Phosphate 16 ml
(68.99 g/l)

0.5 M Dibasic Sodium Phosphate 84 ml
(70.98 g/l)

0.01 M PHOSPHATE BUFFERED SALINE (PBS)

0.5 M Phosphate Buffer 20 ml
Sodium Chloride 8.77 g
Sodium Azide 100 mg

QS to 1 L with DDW

Adjust pH to 7.6 with Sodium Hydroxide

1% BSA-PBS

Lyophilized Bovine Serum Albumin 1.00 g

0.01 M PBS 100 ml

0.1% BSA-PBS

Lyophilized Bovine Serum Albumin 1.00 g

0.01 M PBS 1000 ml
3% Normal Rabbit Serum-PBS (NRS-PBS)

Normal Rabbit Serum 3 ml
(Gibco 200-6120)
QS to 100 ml with 0.01 M PBS

2% Normal Rabbit Serum-PBS (NRS-PBS)

Normal Rabbit Serum 2 ml
QS to 100 ml with 0.01 M PBS
**Rat Prolactin (rPRL) Standard Curve**

Stock rPRL Standards:

Prepare 25 μl aliquots of 10 μg rPRL/ml of 1% BSA-PBS.

Immediately before construction of the standard curve, prepare 2 dilutions of rPRL stock, as follows: Add 10 μl of 10 μg/ml stock to 990 μl 1% BSA-PBS yielding a 100 ng/ml stock solution; Subsequently, add 75 μl of 100 ng/ml stock to 2925 μl 1% BSA-PBS yielding a 2.5 ng/ml stock solution.

Both the 100 ng/ml and the 2.5 ng/ml hPRL stock solutions will be used in construction of the standard curve.
Table 17. Construction of rPRL Standard Curve.

<table>
<thead>
<tr>
<th>rPRL Mass (ng)</th>
<th>rPRL (2.5 ng/ml) (μl)</th>
<th>rPRL (100 ng/ml) (μl)</th>
<th>1% BSA-PBS (μl)</th>
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<td>0.025</td>
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</tr>
<tr>
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<td>380</td>
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<td>360</td>
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</tr>
<tr>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2.50</td>
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<td>375</td>
</tr>
<tr>
<td>5.00</td>
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<td>50</td>
<td>350</td>
</tr>
</tbody>
</table>

Make 3 Replicates for Each Point on the Curve.
APPENDIX F

POLYACRYLAMIDE GEL ELECTROPHORESIS OF $^3$H-PROLACTIN

The purpose of this procedure is to isolate $^3$H-Prolactin from other proteins contained in samples of incubation media or pituitary homogenates, for semi-quantification of newly synthesized prolactin. If pituitaries are incubated in the presence of $^3$H-leucine (see APPENDIX), proteins which are synthesized during the period of incubation will incorporate $^3$H-leucine into the peptide chain. Newly synthesized protein molecules are thereby labeled with a radioactive tracer. In this procedure, separation of proteins is based upon differences in charge, which result in differential rates of migration on tubular gels through an electric field. By adding a relatively large "spike" of non-radioactive PRL to at least one pair of samples in a given run and staining all proteins with a suitable dye, it is possible to identify prolactin bands, which can then be cut out of the gels and appropriately prepared for scintillation counting. Relative changes in radioactivity of PRL bands are then taken as relative changes in amount of newly synthesized PRL.
Procedure

Gel Preparation

1. Place clean 5 mm ID x 75 mm glass tubes in rubber grommets and mount in polymerization rack.

2. Prepare gel solution by combining in a small beaker:

   For 12 Gels
   - 2 ml Solution A
   - 4 ml Solution C
   - 2 ml DDW
   - 8 ml Solution G

   For 18 Gels
   - 3 ml Solution A
   - 6 ml Solution C
   - 3 ml DDW
   - 12 ml Solution G

   Mix well by trituration with a pasteur pipet.

3. With a pasteur pipet, fill each tube to within 5-10 mm of the top.

4. After filling tubes, gently layer a small amount of distilled water on top of the gel solution to eliminate the meniscus and form a flat surface on the polymerizing gel.

5. Allow the gels to polymerize for 30-45 minutes.

Sample Preparation

6a. Incubation Media: Add 40 μl of 10% sucrose solution to 200 μl aliquots of sample for a total volume of 240 μl. Load 100 μl onto each of two gels.
6b. **Pituitary Homogenates:** In a typical experiment, pituitaries are homogenized in 500μl 0.01 M PBS immediately following incubations, and subsequently stored at -30°C for future assay. After thawing, rehomogenize samples by sonication and add 20μl Triton-X 100 to 200μl aliquots. Mix and let stand for 15 min at room temperature. Centrifuge at 3200 rpm for 5 min. Carefully transfer supernatants to clean vials and add 50μl 10% sucrose. Load 100μl onto each of 2 gels.

**Loading Samples onto Gels**

7. **After** the gels have polymerized, remove the rubber grommets and shake off the water layer. Fit the gels into the upper chamber of the electrophoresis unit.

8. Fill the lower chamber with enough Tris-glycine buffer to cover the lower end of the gels when the unit is assembled.

9. Assemble the electrophoresis unit and fill the upper chamber with Tris-glycine buffer containing 5 ml/liter tracking dye. Displace air bubbles from tops of gels by gently dispensing upper buffer from a pasteur pipet.

10. **With** a Hamilton syringe, carefully layer samples into the wells formed by the top of the gel and the sides of the glass tube. Each sample should be run in duplicate. At least 2 blank gels should be included in each run and processed through the entire procedure to allow for subtraction of background contamination counts from sample gels. Spike the blank gels with 100μl PRL standard in 0.01 M PBS to which 10% sucrose has been added.
**Electrophoresis**

11. After all samples are loaded, connect the electrodes to the power supply.

12. Set the power supply for constant current at 4 mA/gel.

13. When the tracking dye front reaches a few millimeters from the bottom of the gel, turn off the power supply and begin the staining procedure.

**Staining**

14. Remove the gels from the glass tubes with distilled water ejected from a 50 ml syringe with a blunted 20 gauge needle. Insert the needle between the gel and the tube wall while rotating the tube. The gel should slide out of the tube.

15. Place the gels in 12 x 75 mm tubes and fill with specimen stain. Stain gels for at least one hour.

16. Remove unbound stain from gels by diffusion destaining in 7% acetic acid. Destain until protein bands are clearly visible.

**Cutting the Gels**

17. Place the gels on a light box and line up the dye bands. Cut out the bands corresponding to the band produced by the PRL spike.

18. Cut each gel segment lengthwise and place both halves in a scintillation vial containing 1 ml 92% NCS. Let stand at room temperature for 24 hours and add 10 ml Aqueous Counting Scintillant to each vial. Let stand at room temperature
for 48 hours and count in a scintillation spectrometer windowed to appropriate energies. Use external standards ratio to convert to DPM.

Buffers and Reagents

SOLUTION A

1 N HCl 48 ml
Tris 36.3 g
Tetramethylethylenediamene 0.36 ml
QS to 100 ml with DDW
Adjust pH to 8.8 - 9.0

SOLUTION C

Acrylamide 28.0 g
Bis Acrylamide 0.735 g
QS to 100 ml with DDW

SOLUTION G

Ammonium Peroxydisulfate 0.14 g
QS to 100 ml with DDW
TRIS-GLYCINE BUFFER

Tris 3.0 g
Glycine 14.1 g
QS to 1 L with DDW

TRACKING DYE

0.005% Bromphenol Blue

SPECIMEN STAIN

Aniline Black 1 g
QS to 200 ml with 7% Acetic Acid
APPENDIX G

LIGAND BINDING STUDIES

ANTERIOR PITUITARY DOPAMINE RECEPTORS

The purpose of these studies is to analyze quantitatively and qualitatively the interactions of dopaminergic drugs with anterior pituitary dopamine binding sites. The types of binding studies used in this dissertation fall into two general types: saturation binding and competition binding. In saturation binding studies membrane preparations are incubated in the presence of increasing concentrations of radioactively labeled ligand. Analysis of specific binding curves generated in this type of assay yields information about the concentration of binding sites and the affinity of binding sites for the tracer ligand. By use of appropriate modeling techniques (see APPENDIX A), it is also possible to detect and quantify multiple classes of binding sites based on their different affinities for ligand. In competition or inhibition binding, a constant amount of tracer ligand is incubated along with membranes in the presence of increasing concentrations of competing unlabeled agonist or antagonist drugs. These studies provide information about the relative affinities of the binding sites for these drugs vs. the tracer ligand, and can also be used to detect and analyze the presence of multiple classes of binding sites. By analyzing the combined results of these types of binding studies it is possible to draw inferences about the effects of various treatments on the ability of studied tissues to bind (and potentially respond to) the hormones or drugs of interest.
Anterior pituitary dopamine binding was analyzed in these studies to determine if differences in PRL cell activity between treatment groups might be accounted for by altered dopamine binding parameters at the anterior pituitary level.

**PROCEDURE**

**Membrane Preparation**

1. Kill animals by rapid decapitation. Remove anterior pituitaries and rinse in cold saline. Record pituitary weights.

2. Homogenize pituitaries in 10 volumes ice cold 50 mM Tris HCl buffer (pH 7.4) in a hand held teflon glass tissue grinder.

3. Centrifuge homogenate at 800 x g for 10 minutes (4° C).

4. Collect supernatant and centrifuge at 22,500 x g for 20 minutes (4° C).

5. Discard supernatant and wash pellet by resuspension in 50 mM Tris buffer followed by a second centrifugation at 22,500 x g for 20 minutes.

6. Discard supernatant. Resuspend final pellet at 25 mg (wet tissue weight equivalents)/ml of incubation buffer (50 mM Tris HCl containing 120 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl₂, 1.0 mM MgCl₂, 10 μM Sodium EDTA, 0.1% ascorbic acid and 5 μM pargyline, pH 7.4).
**Incubations**

Membrane suspensions were incubated for 30 minutes at 27°C with various concentrations of \( ^3 \text{H} \)-spiperone (23-27 Ci/mmol, New England Nuclear) or with a single concentration of \( ^3 \text{H} \)-spiperone in the presence of increasing concentrations of the antagonist (+)-butaclamol or the agonist (-)-apomorphine. Nonspecific binding was determined in the presence of 5 \( \mu \text{M} \) (+)-butaclamol. Specific binding was defined as total binding (measured in triplicate) minus nonspecific binding (measured in duplicate).

1. Add 200 \( \mu \text{l} \) aliquots of 25 mg/ml membrane suspension to 13 x 100 mm glass tubes.

2. To nonspecific binding tubes add 50 \( \mu \text{l} \) (+)-butaclamol stock solution diluted in incubation buffer to yield a final concentration of 5 \( \mu \text{M} \). Add 50 \( \mu \text{l} \) incubation buffer to total binding tubes. For binding competition studies, add drugs in 50 \( \mu \text{l} \) aliquots diluted to appropriate concentrations in incubation buffer. Vortex lightly.

3. Initiate binding reaction by addition of 250 \( \mu \text{l} \) \( ^3 \text{H} \)-spiperone diluted in incubation buffer to desired concentration(s).

4. Gently vortex and place tube in Precision Dubnof Shaking Metabolic Incubator (27°C, 10-20 cycles per minute). Record the time.

5. At the end of 30 minutes incubation, stop binding reactions by dilution with 5 ml ice cold incubation buffer followed by vacuum filtration through glass microfiber filters (GF/C, Whatman). Rinse the tube with 5 ml buffer and filter.
Wash the filter with buffer (2 x 5 ml). The dilution, filtration and washing steps should be executed as quickly as possible to avoid dissociation.

6. Place filters in scintillation vials. Add 10 ml aqueous counting scintillant (ACS, Amersham).

7. Count in a scintillation spectrometer. Include duplicate 250 μl aliquots of each concentration of $^3$H- spiperone used, in 10 ml ACS, for measurement of total counts.

**Analysis**

Using external standards ratio, convert CPM to DPM for final calculations. Analyze data by nonlinear modeling (see APPENDIX A) for estimates of binding parameters, their variances and residuals.
Buffers and Reagents

50 mM Tris HCl Buffer

Tris HCl 12.11 g

QS to 2 liters with DDW.

Adjust pH to 7.4.

Incubation Buffer

Tris HCl 12.11 g
NaCl 14.02 g
KCl 0.75 g
CaCl₂ 0.59 g
MgCl₂ 0.41 g
EDTA (sodium) 6.7 mg
Ascorbic Acid 2.0 g
Pargyline 1.6 mg

QS to 2 liters with DDW

Adjust pH to 7.4.
APPENDIX H

HYPOTHALAMIC DOPAMINE ASSAY

The purpose of this procedure is to extract and measure dopamine in hypothalamic median eminence for subsequent estimates of tuberoinfundibular dopamine (TIDA) neuronal activity in vivo. In order to estimate dopamine turnover rates the tyrosine hydroxylase inhibitor $\alpha$-methylparatyrosine ($\alpha$-MPT) is injected at time 0 and the animals are sacrificed 45 and 90 minutes later. Rats in the same experimental group are injected with saline and sacrificed at various times throughout the procedure for determination of steady-state (initial) dopamine concentration. Dopamine depletion rate constants and turnover rates are then estimated by non-linear modeling (see APPENDIX A) of the depletion curve generated by plotting median eminence dopamine concentrations as a function of time after $\alpha$-MPT injection. These estimates of dopamine depletion kinetics during synthesis inhibition are assumed to reflect the underlying rate of dopamine release in vivo (Brodie et al., 1966).

Procedure

1. Add 5 ng 3,4-dihydroxybenzylamine (DHBA) in 100 $\mu$l 70% ethanol to each sample vial. Keep on ice protected from light. DHBA serves as an internal standard to account for differences in relative dopamine recovery between samples.
2. Weigh an animal and administer an intraperitoneal injection of α-MPT (250 mg/kg body weight) or normal saline (0.1 ml). Record the time of injection. Allow a 3-5 minute interval between animals.

3. After 45 or 90 minutes, as appropriate, kill the animal by rapid decapitation. Remove the brain and dissect out the hypothalamic median eminence.

4. Place the median eminence in a vial containing 5 ng DHBA in 100 μl 70% ethanol and homogenize by sonication. After all samples have been collected, store at -20°C for a minimum of 2 days.

5. After 2 days, centrifuge at 10,000 x g for 20 minutes. Transfer the supernatants to clean vials and evaporate under nitrogen.

6. Dried extracts may be stored at -20°C for chromatographic separation and electrochemical detection/quantification at a later date.

7. For injection into chromatography apparatus, reconstitute samples in 100 μl 0.2 M formic acid.

**Liquid Chromatographic System**

Reverse-phase, high performance liquid chromatography (HPLC) with electrochemical detection was used for quantification of dopamine. The HPLC system consisted of a chromatography pump (Waters M-6000A) linked to a sample injector from Waters (model UGK). A reverse-phase, Ultrasphere ODS (5 μm particle size) column made by Beckman was used with a model LC-4B electrochemical detector (Bioanalytical Systems). The detector was equipped with a glassy-carbon
electrode with a potential maintained at +650 mV (0.2 nA offset and 1.0 nA range sensitivity) versus a silver-silver chloride reference electrode. The signal from the electrode was recorded and integrated with a Hewlett-Packard model 3390A integrator.

The column eluent consisted of 7% CH₃CN in an octanesulfonate buffer. Flow rate through the column was maintained at 1.5 ml/min.

Standards and Calculations

Standard solutions of dopamine at 25, 50 and 100 pg/μl are prepared in 100 μl 70% ethanol containing 5 ng DHBA. Solutions are evaporated under nitrogen and reconstituted in 100 μl 0.2 M formic acid for injection into the chromatography apparatus. After electrochemical detection and integration, relative recovery of dopamine is determined for dopamine standards and samples by dividing the area under the dopamine spike by the area under the DHBA spike. If the dopamine/DHBA ratios for standard solutions are plotted as a function of standard concentrations and analyzed by linear regression, it is then possible to determine by extrapolation the pg amount of dopamine in samples.
Buffers and Reagents

α-MPT

α-methylparatyrosine (methyl ester) 1125 mg
Normal Saline 3.75 ml
Dissolve by gentle warming.

0.2 M Formic Acid

Formic Acid (25 N) 8 ml
QS to 1 liter with DDW.

Octanesulfonate Buffer

Octane Sulfonic Acid (sodium salt) 2.16 g
Potassium Phosphate 13.60 g
EDTA (free acid, 0.1 M) 10 ml
Glatial Acetic Acid 5 ml
QS to liter with DDW.

Adjust pH to 4.0 with potassium hydroxide.
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