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LOYOLA UNIVERSITY CHICAGO

EFFECTS OF PERINATAL EXPOSURE TO COCAINE ON SEXUALLY
DIFFERENTIATED GONADOTROPIN REGULATION IN RATS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF BIOLOGY

BY

JIHUN M. LEE

CHICAGO, ILLINOIS

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CHAPTER I

INTRODUCTION

Purpose

The illegal abuse of cocaine has become a major concern, particularly since the advent in the middle 1980's of "crack," a relatively pure and cheap alkaloidal form of the drug (Phibbs *et al.*, 1991; Van Dyke and Fox, 1990). The continued widespread use of cocaine created an increased use among women of reproductive age (Chavez *et al.*, 1989). It has been reported that in inner city hospitals as many as one out of four women who have given birth have used cocaine during pregnancy (Fackelman, 1991). Consequently, since cocaine can cross the placental blood barrier (Chasnoff, 1990), the effects of exposure of developing fetuses to cocaine have become of direct concern. Infants who have been exposed to cocaine during pregnancy have shown a high degree of irritability and tremulousness during postnatal week one (Chasnoff, 1990). Long-term effects from exposure to cocaine have been difficult to follow, primarily due to costs and socio-economic factors that may confound data. For example, the quality of pre- and post-natal care, nutrition, and social environment, including family and schooling, may all cloud observations of the effects of cocaine itself. Other confounding variables may include maternal age during pregnancy, variations in the method of cocaine administration, frequency of use, and multiple drug abuse. Because I can control a majority of these factors in animal studies, I may be better able to understand the effects cocaine has on the human

fetus by investigating them in an animal model in the laboratory.

Laboratory studies have shown that prenatal exposure to cocaine in rats has results in increased mortality and teratological abnormalities in rat pups (Church *et al.*, 1990b). This has particularly been the case with higher doses of cocaine exposure, around 80-100mg/kg body weight (Church *et al.*, 1990a, b). Studies concentrating on more subtle characteristics such as behavior, rather than on gross morphologic abnormalities, have shown that prenatal exposure to cocaine delays appearance of certain neurological indices such as righting reflex and cliff avoidance (Henderson and MacMillan, 1990, Van Dyke and Fox, 1990). Appearance of these reflex behaviors indicates maturation of the central nervous system; their delay, therefore, suggests effects of cocaine on neurological ontogeny.

The neurotransmitters that cocaine affects are also some of the ones that regulate gonadotropin secretion in adulthood (Gallo and Drouva, 1979; Cabrera *et al.*, 1993). Lauder has suggested that, in general, neurotransmitters (NTs) direct embryological development of the same systems that they will control in adulthood (Lauder and Krebs, 1986). It seems reasonable, therefore, that perinatal exposure to cocaine might alter gonadotropin regulation in adult rats. Gonadotropin regulation has been shown to be a sexually differentiated characteristic. For example, males exhibit tonic levels of the gonadotropin luteinizing hormone (LH), while females exhibit a cyclic pattern of LH secretion. Studies conducted by Dow-Edwards have shown that glucose utilization in the brain is a sexually differentiated characteristic in rats, and that its sexually differentiated nature is affected by cocaine (Dow-Edwards, 1988). The purpose of my experiments was to test the hypothesis that perinatal exposure to cocaine during the critical period of sexual differentiation of the brain will alter

normal sexually differentiated gonadotropin regulation. Specifically, I hypothesize that perinatal treatment with cocaine will induce LH-surges in males, but that in females they will either decrease in magnitude or remain the same. I also will measure various aspects of maternal metabolism, including maternal weight gain and nutritional intake, in addition to effects of perinatal exposure to cocaine on birth weight of pups, litter size, and offspring weight gain.

Literature Review

Effects of cocaine on neurotransmitters:

Nerve impulses travel down neurons in the form of action potentials. Once the electrical signal reaches the terminus, neurotransmitters (NTs) stored within vesicles located at the terminal end of the neuron are released. Once the NTs are released into the synaptic cleft, they can bind to specific receptors located on the postsynaptic neuron which will carry on the signal. NTs within the cleft can also be destroyed by certain enzymes or recycled by the presynaptic neurons via reuptake receptors. Cocaine acts at the level of the presynaptic neuron by blocking reuptake receptors (Dackis and Gold, 1985; Vathy *et al.*, 1993). The inability of presynaptic neurons to take up NTs results in an increased concentration of NTs within the synaptic cleft, causing hyperstimulation of postsynaptic neurons (Diagram 1). This hyperstimulation is associated with the euphoria that is experienced shortly after taking cocaine (Dackis and Gold, 1985). The NT primarily responsible for this experience of euphoria is dopamine (DA), a catecholamine NT that is influenced profoundly by cocaine

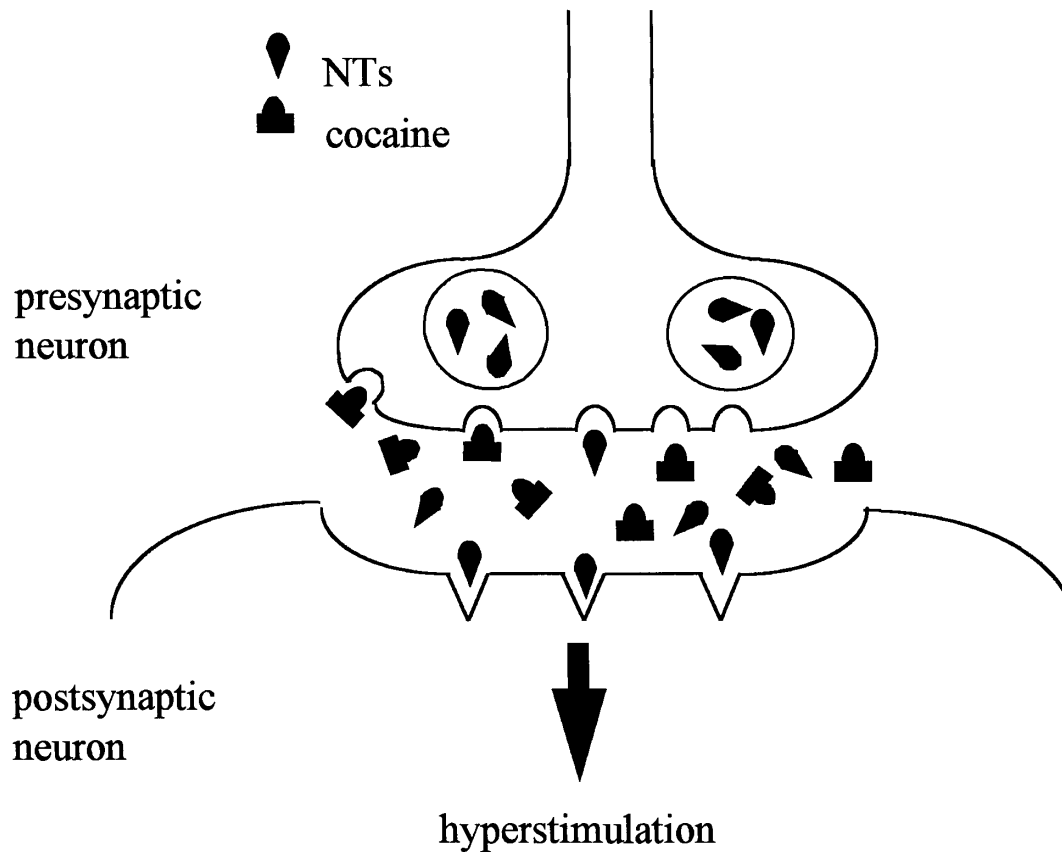


Diagram 1. Effects of cocaine on neurotransmitters. Cocaine blocks reuptake receptors at the level of presynaptic neurons. The increased concentrations of NTs within the synaptic cleft cause hyperstimulation of postsynaptic neurons.

and plays an important role in reward states. The euphoric state due to hyperstimulation is only transitory and is soon replaced by a state of dysphoria also known as “crashing.” “Crashing” from cocaine is thought to be due to depletion of NTs, particularly dopamine. The increased concentration of NTs in the synaptic cleft increases the chance of diffusion into surrounding blood vessels. This depletion may be partially offset by increased production of NTs by presynaptic neurons due to homeostatic regulation, but it is hypothesized that during chronic cocaine abuse the increased production cannot completely compensate for the more rapid depletion, which results in a progressive dysphoric state (Dackis and Gold, 1985).

Recently, researchers have found evidence that supports the idea of homeostatic regulation of DA levels, and the key role DA transporters play in that regulation. Giros *et al.* created a strain of "knock out" mice which lack the gene for the dopamine transporter (reuptake receptor) (Giros *et al.*, 1996). The mice, lacking the dopamine transporter gene, were hyperactive as expected. These mice also had lower levels of DA compared to normal strains of mice. Low levels of DA in the "knock out" mice were consistent with lower levels of tyrosine hydroxylase, the rate-limiting enzyme in DA synthesis. Down-regulation of DA release from the presynaptic neurons appears to serve as a mechanism to compensate for the inability of these mice to take up DA. It is also interesting that the “knock out” mice were hyperactive even in the face of low levels of DA. This suggests that the extent of depletion of NTs responsible for the dysphoric state is almost complete. Furthermore, intraperitoneal injection of 40mg/kgbw cocaine had no significant effect on locomotor activity in these mice. Normal mice, however, produced a 6- to 8-fold increase in locomotor activity upon

the same stimulant, providing strong evidence for the role DA and its reuptake receptor play in the action of cocaine (Giros *et al.*, 1996).

The study of Giros *et al.* (1996) concentrated on DA's role in the addictive action of cocaine. It should be noted that the DA transporter is member of a family of Na/Cl dependent transporters whose members also include transporters specific for norepinephrine (NE) and serotonin (5-HT), the two other NTs most strongly affected by cocaine. Although members of this family of transporters do display substrate specificity, there is some overlap, which would explain cocaine's ability to bind to all three transporters.

The purpose of my experiment was to determine the effect of perinatal exposure to cocaine on sexual differentiation of the brain in rats. NE, 5-HT and DA are important in sexually differentiated functions of the brain in adults and are also thought to be important during development of those functions. It has been postulated that NTs may act as a signal for synaptogenesis in target neurons during development of the brain (Lauder, 1983; Dow-Edwards, 1988; Raum *et al.*, 1990). Development of sexually differentiated characteristics in the brains of rats occurs between a few days before birth (prenatally) and a few days after birth (postnatally). This period is called the critical period of sexual differentiation of the brain. Therefore, exposure to cocaine around the time of birth (perinatally) may irreversibly alter normal sexually differentiated functions in adults.

Sexual differentiation of the rat brain:

Sexual differentiation of the rat brain seems to be under steroidal influence similar to that responsible for sexual differentiation of the genital tract. Development of the female

or male brain appears to depend on testosterone, which acts like a switch. In the presence of testosterone the brain develops along a male pattern, and in the absence of testosterone the brain develops along a female pattern. Treating female rat pups with testosterone during the critical period for sexual differentiation of the brain causes them to exhibit male behaviors as adults. Orchidectomy of male rat pups during the critical period results in male adult rats exhibiting female behaviors (reviewed by Dohler, 1991). Although testosterone is the androgenizing hormone for the internal genitalia, estradiol is the hormone directly responsible for masculinization of the brain. Testosterone taken up by the brain is converted to estradiol by aromatase (Gorski, 1985). Recent studies conducted by McCarthy *et al.* have shown the masculinizing effect of estrogen using a new method. By infusing an antisense oligonucleotide for estrogen receptor mRNA into the ventromedial hypothalamus in 3-day-old female rats in combination with an injection of testosterone, McCarthy *et al.* were successfully able to attenuate the androgenizing effects of testosterone (McCarthy *et al.*, 1993).

If estrogen is the masculinizing hormone, it would seem likely that females would be exposed to these masculinizing effects since during this period circulating estrogens are present. In fact, substantial amounts of placental estrogens are present at this time in fetuses of both sexes. The placental estrogens, however, are tightly bound to alpha-fetoproteins and are thus unable to diffuse into the brain and cause masculinization in either sex (Gorski, 1985).

Researchers have shown that prenatal exposure to cocaine can reduce hypothalamic uptake of estradiol (Raum *et al.*, 1990). Therefore, reduced availability of estradiol may

prevent development of male-like patterns. In fact, Raum and his colleagues have shown that perinatal exposure to cocaine can reduce the scent-marking behavior typical of males (Raum *et al.*, 1990). Therefore, I hypothesized that perinatal exposure to cocaine would prevent development of male-typical LH regulation and induce the capacity to have steroid-induced LH surges, typical of females.

Sexually differentiated structures of the brain:

Sexual differentiation of the brain can be categorized into the development of structural, behavioral, or physiological differences. A key structural difference between male and female rats was found by Gorski *et al.* in 1978. Within the preoptic area (POA) of the rat brain there is a nucleus, or collection of cell bodies, that is significantly larger in males than in females. This region was termed the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski *et al.*, 1978; Gorski, 1985). From further studies on the SDN, Gorski *et al.* were able to demonstrate that female pups perinatally exposed to testosterone developed SDNs of increased size. Therefore, it appears that the SDN-POA volume is determined by the hormonal environment during the critical period for sexual differentiation of the brain (Gorski, 1985).

The structural differences found between the SDNs of males and females may be responsible for some behavioral differences exhibited between the sexes. Studies conducted by Houtsmuller *et al.* (1994) have shown that perinatal treatment with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione significantly reduced the size of the SDN-POA in males and reduced the frequency of male sexual behaviors in adult offspring. Furthermore, the size of

the SDN-POA and the frequency of male sexual behavior were shown to be positively and significantly correlated. By inhibiting aromatase, the researchers appear to have been successful in preventing the conversion of testosterone to estradiol, thus preventing the masculinizing effects of testosterone (Houtsmuller *et al.*, 1994) on both the SDN-POA and the behavioral consequences of its development.

Another structure of the brain that has been found to be sexually differentiated in rats is the anteroventral periventricular nucleus (AVPV) of the preoptic region. This nucleus, unlike the SDN-POA, is larger in females than in males. One of the functional roles of the AVPV appears to be participation in the regulation of gonadotropin-releasing hormone (GnRH) secretion. By introducing electrolytic lesions of the AVPV, researchers were able to induce persistent estrus in female rats and to eliminate the preovulatory surge of LH. Perinatal manipulation of steroids altered AVPV volume in the opposite way from SDN-POA volume (Gu and Simerly, 1994).

The exact mechanism by which steroids influence SDN-POA and AVPV volumes is still unknown. Steroids may increase neurogenesis, stimulate migration of neurons, or prevent death of existing neurons (Gorski, 1985). As stated earlier, the structural differences found between the SDN-POA and AVPV of males and females may be responsible for some physiological and behavioral differences exhibited by the sexes. Other areas of the brain, however, are also involved in sexual differentiation, and the information now known about the SDN-POA and AVPV may best serve as a model for understanding sexual differentiation of the brain in general.

As discussed above, sexual differentiation of the brain appears to be highly regulated

by the hormonal environment during the critical period. The mechanism by which steroids mediate these and many other actions involves intracellular steroid receptors. Therefore, sex differences seen in adults may be related to estrogen receptor (ER) concentrations during development. In fact, sexual differences in ER levels have been found in the medial preoptic area (MPOA) and the periventricular region of the preoptic area (PVP), where ER levels were higher in females than in males. These sex differences in ER levels have been detected as early as 24 hours after birth (Kuhnemann *et al.*, 1994). These data are further supported by sex differences found in ER mRNA levels in neonatal rats, with higher levels of ER mRNA found in females than males (DonCarlos *et al.*, 1995). Like the SDN-POA and AVPV, perinatal manipulation of hormones can also alter normal sexually differentiated ER mRNA levels. Perinatal treatment of female rats with estrogen reduced ER mRNA levels to levels comparable to males. Also, neonatal orchidectomy of male rats increased the level of ER mRNA to a level comparable to normal females (DonCarlos *et al.*, 1995).

The sexually differentiated structures of the brain which have just been discussed all appear to be influenced by the steroidal environment during the critical period for sexual differentiation of the brain. Raum *et al.* (1990) have shown that prenatal exposure to cocaine can alter the steroidal environment during the critical period. Therefore, perinatal exposure to cocaine may alter sexually differentiated structures involved in gonadotropin regulation.

Sexually differentiated behaviors:

The differences between male and female rats in the structures of certain regions of the brain (SDN-POA and AVPV) and in levels of ER may be responsible for behavioral

differences observed between males and females. For example, a female-typical mating behavior in rats is the lordosis reflex. The tactile stimulation of males' attempting to mount can induce females to arch their backs in a posture that permits intromission. Lordosis can be induced with steroids in adult females, but adult males do not exhibit this behavioral response when treated with the same steroids. Male-typical sexual behaviors in rats include mounting and intromission. Adult males treated with testosterone display these behaviors when placed with a receptive female. Normal adult females treated with testosterone cannot be induced to display the male-typical sexual behaviors.

Altering the steroidal environment during the critical period for sexual differentiation of the brain can, however, alter normal female and male behaviors. Transplantation of testes into females or treatment of females with testosterone or estrogen during the early postnatal period can disrupt the lordosis reflex in those rats when they are adults. These adult females also show the capacity to display mounting and intromission behaviors upon treatment with testosterone. Early postnatal treatment of males with an estrogen antagonist can interfere with adult male behaviors, and enable adult males to display the female lordosis reflex when treated with estrogen and progesterone (Dohler, 1991).

Hanousek *et al.* (1994, 1996) have shown that perinatal exposure to cocaine delayed development of sex differences in play behavior in juvenile rat pups without affecting most non-sexually differentiated aspects of open-field activity. Furthermore, Raum *et al.* (1990) demonstrated a decrease in mounting behavior in adult males perinatally exposed to cocaine. These results suggest that exposure to cocaine during the critical period for sexual differentiation of the brain may have an important impact on sexually differentiated function

in adult life.

Sexually differentiated physiology:

A highly sexually differentiated physiological function is the regulation of gonadotropins. The gonadotropins consist of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL). Gonadotropin regulation is a sex-dependent process that is programmed during the critical period for sexual differentiation for the brain. My research focused on the gonadotropin LH, a glycoprotein secreted by the anterior pituitary. In females, LH triggers ovulation and stimulates secretion of steroids by the ovaries, and in males, LH stimulates the testes to secrete testosterone.

In adult males LH release by the pituitary gland is tonic, whereas in females LH release is cyclic in nature (Gorski and Wagner, 1965; Dohler, 1991). Transplantation experiments have shown that pituitaries surgically removed from donors of one sex and implanted in recipients of the opposite sex perform in a normal manner with respect to the recipient (Gorski, 1985). Therefore, it appears that the difference in LH regulation between males and females is not due to the pituitary gland but rather to regions of the hypothalamus that regulate gonadotropin-releasing hormone (GnRH) or to regions innervating the hypothalamus.

LH release in males is regulated by a negative feedback loop involving the level of circulating steroids. Low steroid levels stimulate the hypothalamus to secrete GnRH which stimulates the pituitary gland to secrete LH. Increased levels of LH in circulation stimulate the gonads to secrete steroids. High steroid levels inhibit the hypothalamus, attenuating

release of GnRH. Attenuated GnRH secretion reduces release of LH by the pituitary which ultimately reduces steroid secretion by the gonads. This negative feedback loop is responsible for the relatively constant levels of LH characteristic of males (Gorski, 1985).

In females, LH release is under the influences of a negative feedback system similar to that of males, along with a positive feedback system unique to females. During the estrous cycle of the female rat, 4-5 days in length, levels of estrogen become increasingly high and peak on the day of proestrus. High levels of estrogen positively feedback at the level of the hypothalamus and the anterior pituitary, stimulating GnRH release which is followed by the preovulatory surge of LH during the afternoon of proestrus.

Like sexually differentiated structures and behaviors, regulation of gonadotropin secretion in adult rats also is influenced by the hormones present during the critical period. Transplantation of testes into newborn female rats has resulted in tonic LH release in adults, inducing permanent anovulatory syndrome. Similar results have been observed with early postnatal treatment of aromatizable androgen or estrogen (Gorski and Wagner, 1965; Dohler, 1991). Male rats that were orchidectomized *in utero* prior to birth or within 24 hours after birth showed the capacity to display steroid-induced LH surges (McPherson *et al.*, 1982; Wieland and Barraclough, 1984; Corbier, 1985). Therefore, these early orchidectomized rats appear to be feminized with respect to the regulation of LH. Perinatal exposure to cocaine, since it has been shown to reduce hypothalamic uptake of steroids, may alter LH regulation via a mechanism similar to that by which it alters sexually differentiated structures and behaviors.

The role of neurotransmitters in sexual differentiation of the brain:

The evidence presented thus far shows the strong influence hormones have on sexually differentiated structures, behaviors, and physiology during the critical period. Another important aspect of sexual differentiation which has not been discussed up to this point is the role of neurotransmitters (NTs). Lauder suggests that NTs involved in certain adult brain functions are probably the same NTs that direct fetal development of the structures that mediate those brain functions (Lauder, 1983). Therefore, manipulation of NTs during the critical period would be expected to alter normal sexual differentiation. Studies have shown that steroids can influence the NT content in brains, and NTs can modulate actions of steroids by influencing the number of steroid receptors (Dohler, 1991). Therefore, it appears that steroids and NTs are involved in a complex interaction.

Researchers have shown that manipulation of NTs during the critical period can alter normally sexually differentiated structures, behaviors, and functions. For example, pregnant rats treated with haloperidol, an antagonist of DA, gave birth to male offspring that exhibited reduced mounting behavior as adults (Dohler, 1991). Early postnatal treatment with L-tryptophan, a 5-HT precursor, inhibited expression of lordosis behavior in adult females (Dohler, 1991). Early postnatal stimulation of β_2 -adrenergic receptors with salbutomal caused the volume of the SDN-POA to increase in both females and males (Dohler, 1991). Early postnatal treatment with isoprenaline, an agonist of the β -adrenergic receptors, reduced the steroid-induced release of LH in females (Jarzab *et al.*, 1989). Therefore, alterations in the activity of NE, 5-HT, and DA can alter normal sexual differentiation of the brain. Since these are the same NTs affected by cocaine, perinatal exposure to cocaine may alter normal

sexual differentiation of the brain.

Effects of cocaine on sexual differentiation of the brain:

Studies have been conducted concerning the effects of cocaine on sexual differentiation of the brain. Dow-Edwards has shown that glucose utilization, a measure of metabolic activity, is sexually differentiated within certain areas of the brain of rats. For example, the MPOA in brains of male rats utilizes greater amounts of glucose than the MPOA in females. Sexual differentiation of this structure was altered by prenatal exposure to cocaine. In cocaine-treated males, many areas of the hypothalamus, including the MPOA and periventricular nucleus, had reduced amounts of glucose utilization compared to control males (Dow-Edwards *et al.*, 1990). Furthermore, when compared to a previous study done in females, glucose utilization within the MPOA in the cocaine-treated males was comparable to that in normal and cocaine-treated females (Dow-Edwards, 1988). This suggests that cocaine may demasculinize the brains of males with respect to glucose utilization.

Raum *et al.* have shown that prenatal exposure to cocaine reduced hypothalamic uptake of estradiol, the masculinizing hormone. They also have shown that perinatal exposure to cocaine reduced scent-marking behavior typical of males with no effect on this behavior in females (Raum *et al.*, 1990). Therefore, reduced availability of estradiol may be sufficient to prevent development of male-like patterns. Furthermore, Raum and his colleagues previously had shown that neonatal stimulation with β -adrenergic agonists also can reduce nuclear uptake of estradiol, suggesting cocaine's potential mechanism of action

(Raum *et al.*, 1984).

Studies have shown that prenatal exposure to cocaine also can reduce a female-typical behavior, lordosis. Vathy *et al.* (1993) observed reduced lordosis behavior in prenatally cocaine-treated females when compared to control females. Adult male rats that were prenatally cocaine-treated exhibited increased male sexual behavior (mounting and intromission) when compared to control males. This observation is difficult to interpret because increased male sexual behavior may imply increased development of male-like patterns, an interpretation inconsistent with the finding of Raum *et al.* (1990). It also may suggest that cocaine-treated males need greater stimulation to achieve ejaculation, therefore implying demasculinization. Vathy *et al.* also observed no differences with respect to estrous cyclicity between cocaine- and saline-treated females (Vathy *et al.*, 1993).

Another sex-dependent difference shown to be affected by prenatal exposure to cocaine is responsiveness to cocaine itself. In adult rats, there is an increase in certain locomotor activities after acute exposure to cocaine. Females have been shown to exhibit greater activation than males at any given dose. Miller and Seidler have shown that prenatal exposure to cocaine eliminates this sexually differentiated responsiveness. Cocaine-treated females exhibited reduced activation compared to that in control females. The activity level was comparable to that of control and cocaine-treated males, suggesting a defeminizing effect (Miller Seidler, 1994).

Exposure to cocaine during the critical period appears to alter normal sexual differentiation of the brain. Research suggests that the effect of cocaine is mediated through altered levels of NTs, which may, directly or indirectly, alter the action of steroids. Exposure

to cocaine during the critical period for sexual differentiation has altered sex-dependent behaviors and certain physiological functions. Therefore, it seems likely that perinatal exposure to cocaine would alter LH regulation, a highly sexually differentiated process. Although Vathy *et al.* (1993) have shown no effect of prenatal exposure to cocaine on estrous cyclicity, they did not test effects of cocaine on LH regulation in males or the degree to which LH-surges could be induced in females. Studies conducted by Raum *et al.* (1990) and Dow-Edwards (1988) suggest that cocaine may prevent the development of male-like patterns and possibly induce female-like patterns in males. Studies conducted by Vathy *et al.* (1993) and Miller and Seidler (1994) suggest that cocaine may prevent the development of female-like patterns and possibly induce male-like patterns in females. Therefore, the purpose of my experiments was to test the hypothesis that perinatal exposure to cocaine during the critical period of sexual differentiation of the brain will alter normal sexually differentiated gonadotropin regulation. Specifically, I hypothesized that males would exhibit steroid-induced LH release and females would exhibit either normal steroid-induced LH release or reduced levels of LH.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

The purpose of this experiment was to test the hypothesis that perinatal exposure to cocaine during the critical period for sexual differentiation of the rat brain interferes with regulation of LH, a highly sexually differentiated process. I hypothesized that LH-surges would be induced in males and LH-surges would either decrease in magnitude or remain the same in females. It has already been demonstrated that some behaviors that are male-specific in rats are reduced in frequency in males perinatally exposed to cocaine (Raum *et al.*, 1990). It has also been demonstrated that prenatal exposure to cocaine can inhibit sexual behavior in adult females (Vathy *et al.*, 1993). I proposed to study another trait that is sexually differentiated: regulation of the gonadotropin LH.

An important component in regulation of LH is the neurotransmitter system that governs secretion of GnRH from the hypothalamus. It has been suggested that NTs that are important for particular functions in adults are also important for correct embryonic development of the parts of the brain that regulate those functions (Lauder, 1983). Thus, the NTs important for gonadotropin regulation in adults may also be important for correct development of the brain areas that regulate gonadotropin release (Kalra and Kalra, 1983; Barraclough, 1994). Studies have shown that manipulating adrenergic and serotonergic

systems during the critical period for sexual differentiation of the brain can alter normal regulation of gonadotropins in adult rats (Jarzab *et al.*, 1989; Dohler *et al.*, 1991; Dohler, 1991). Cocaine is known to inhibit reuptake of NTs that regulate gonadotropin release in adults. I hypothesized that perinatal exposure to cocaine would alter development of the parts of the brain that control gonadotropin release.

The experimental approach was to treat some rats perinatally with cocaine, gonadectomize them after maturity, inject estradiol and progesterone, cannulate to allow repeated blood sample removal, and measure LH levels. Normal females demonstrate high-amplitude LH surges in response to sequential injections of estradiol and progesterone, and normal males do not. Since regulation of LH is highly sexually differentiated, this measurement of LH response to steroid treatment should distinguish clearly between male-like and female-like endocrine function of the rat brains perinatally exposed to cocaine, and should detect any differences from the responses of normal (untreated) controls.

If cocaine-treated males show an LH surge, I would interpret the result to mean that cocaine treatment prevented the normal differentiation of the parts of the brain responsible for the male-typical pattern of regulation. This result would be consistent with my hypothesis that perinatal treatment of male rats with cocaine will prevent normal LH regulation in their brains. Raum *et al.* have shown that neonatal stimulation with β -adrenergic agonists or prenatal exposure to cocaine can reduce nuclear content of estradiol (Raum *et al.*, 1984, 1990). NE is a β -receptor stimulator; therefore, increases in NE concentrations or hyperavailability within the synaptic cleft due to perinatal exposure to cocaine may be the mechanism by which cocaine mediates this action. It was previously

mentioned that estradiol is the masculinizing hormone. Thus, reduced availability of estradiol may be sufficient to prevent development of male-like patterns.

The magnitude of the LH surge may also depend on the magnitude of the reduction in estradiol content. The reduced availability of estradiol may be sufficient to prevent development of male-like patterns but may not be sufficient to feminize the brain fully. Thus, males perinatally exposed to cocaine may exhibit only small LH surges.

If cocaine-treated males show a normal response, that is, no generation of an LH-surge in response to estradiol treatment, I would interpret the result to mean that cocaine does not affect differentiation of the parts of the brain responsible for normal male regulation of the pattern of LH or it may be possible that I have missed the critical period. Gorski has recently shown that the SDN-POA of the rat brain can still be influenced by endogenous steroid up to postnatal day 29, suggesting a longer critical period that can extend beyond a few days postnatally (Davis *et al.*, 1995).

Since the majority of evidence suggests that perinatal exposure to cocaine appears to be blockage of masculinization, I expected no difference between treated and control females; both were predicted to show LH surges after injection of estradiol and progesterone. If cocaine-treated females exhibit no steroid-induced LH surge, I would interpret the result to mean that perinatal exposure to cocaine altered differentiation of the areas of the brain responsible for normal female regulation of LH. This could happen if perinatal exposure to cocaine depleted NTs, particularly NE. Jarzab *et al.* suggested that β -adrenergic stimulation by NE causes feminization (Jarzab *et al.*, 1987). Depletion of NE would reduce β -receptor stimulation, which would, according to this hypothesis, result in a lack of feminization.

Thus, cocaine-treated females would exhibit no steroid-induced LH surge. Evidence to support the defeminizing effect of cocaine has been given by Vathy *et al.* (1993), who showed that lordosis behavior was reduced in females prenatally exposed to cocaine.

If cocaine-treated females exhibit intensified LH surges compared to controls, I would interpret the result to mean that perinatal exposure to cocaine hyperfeminized female rats. This would be consistent with the hypothesized outcome for males, in which LH surges are induced in males, because it would suggest that sexual differentiation of the brain had been altered toward feminization in both males and females. Research has shown that manipulating NTs during the critical period can hyperfeminize females with respect to the LH surge. Neonatal inhibition of α -adrenergic neurotransmission with prazosin, an α_1 -antagonist, or yohimbine, an α_2 -antagonist, has been shown to potentiate LH surges in adult females (Jarzab *et al.*, 1987; Dohler *et al.*, 1991).

The hyperavailability and depletion of NTs appear to be contradictory mechanisms. The availability of NTs during treatment may depend on the amount of exposure to cocaine. It seems logical that at higher doses of cocaine one would expect the depletion of NTs to occur because homeostatic regulation would be unable to compensate for the ability of cocaine to block reuptake. Lower doses of cocaine may not be sufficient to out-compete the brain's homeostatic mechanisms for maintenance of normal NT levels. It is interesting to note that some effects of cocaine appear at low doses, and at higher doses the response disappears. For example, at low doses of cocaine scent-marking behavior was reduced in male rats, and at higher dose of cocaine scent-marking was normal (Raum *et al.*, 1990). My experimental design employed three different doses of cocaine. Therefore, I may have either

depletion or enhancement of NTs but only one at any given dose.

Materials and Reagents

Materials used during cannulation included: silicon tubing (#306-913; Baxter Scientific Products Division, McGraw Park, IL; i.d. 0.025in., o.d. 0.047 in.), Intramedic polyethylene tubing (#95452; Clay Adams, Parsippany, NJ; i.d. 0.58in., o.d. 0.965in.), and silk suture (#51-7706; Harvard Apparatus, S. Natick, MA). All animals were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL).

Radioimmunoassays (RIAs) for luteinizing hormone were performed according to standard procedures. RIA reagents were obtained from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. The current reference preparation for LH was used (NIH-rLH-RP-3). Iodination-grade LH was a gift from Dr. Leo E. Reichert, Jr., of Albany Medical College. LH was radioiodinated according to the chloramine T method. Intra-assay and inter-assay coefficients of variation for LH were 15.8% and 32.4% respectively. To eliminate inter-assay variability, all samples were evaluated in one assay. Upper and lower limits of detectability were 2.2ng/ml and 95ng/ml respectively. Protein A (IgG-sorb) was obtained from The Enzyme Center (Malden, MA).

Steroids used were estradiol benzoate (β -estradiol 3-benzoate; Sigma, St. Louis, MO) and progesterone (4-pregnene-3,20-dione; Sigma, St. Louis, MO) dissolved in peanut oil at concentrations of 0.5mg/ml and 25mg/ml, respectively. Cocaine (d-cocaine hydrochloride) was obtained from the National Institute on Drug Abuse and dissolved in 0.05M phosphate-

buffered saline (PBS) at a concentration of 15mg/ml.

Gonadectomy

Sexually mature animals were bilaterally ovariectomized or orchidectomized under methoxyflurane anesthesia. All surgeries and all other aspects of these protocols were performed according to procedures approved by the Loyola University Chicago Lakeside Campuses Institutional Animal Care and Use Committee (IACUC). The animal care facility is accredited by the Animal & Plant Health Inspection Services. The purpose of gonadectomizing the animals was to remove the endogenous source of steroid. After two weeks all animals received an injection of steroid or vehicle. The regimen of injected steroids served as a standard test of the animal's ability to respond to positive feedback with an LH surge. Exogenous steroid treatment also enabled me to collect blood samples at relatively convenient times. Thus, my ability to detect LH-surges was not dependent on the inherent estrous cycle of female rats, nor was it compromised by the presence of testosterone in the male rats.

Cannula and Bleeding Rig Preparation

In order to collect blood samples from the same animal at multiple time points, indwelling atrial cannulae were prepared. Silicon tubing 5cm in length was soaked in toluene for approximately 3 hours to expand its diameter. Next, one end of a 15cm length of Intramedic polyethylene (PE) tubing was held near a heat source, creating a flange approximately 1mm in diameter. The PE tubing was then inserted into the expanded Silicon

tubing until 0.5cm of Silicon tubing was overlapping the flange end the PE tubing. The cannula was then rinsed with deionized water and allowed to dry.

Cannula plugs were made from sewing pins that were cut to about 1cm in length using wire cutters. The cut ends of the plugs were filed down to provide a smooth tip that would not tear the PE tubing when inserted.

To facilitate blood sampling, a bleeding rig was attached to the cannula. The bleeding rig consisted of a 1cc syringe, PE tubing, a connector, and a 23 gauge needle with the bevel removed. The PE tubing was approximately 40cm in length. The connector was made from the cut end of a 23 gauge needle. The bevel and the cut end were filed, again to provide a smooth surface. The 23 gauge needle had its bevel cut and filed as well.

Prior to blood collection the bleeding rig was assembled. The syringe was connected to the cut 23 gauge needle. The needle was connected to one end of the 40cm PE tubing. The connector was inserted into the other end of the PE tubing. About 15 minutes prior to the first blood collection the cannula plug was removed, and the unattached end of the connector was inserted into the cannula. A separate 1cc syringe was filled with approximately 150 μ l heparinized saline. At the time of blood collection, approximately 600 μ l blood were collected. The syringe filled with blood was disconnected from the 23 gauge needle. The syringe filled with heparinized saline was connected, and the heparinized saline was injected. The bleeding rig was disconnected and the cannula plug was reinserted. For the first collection of blood in the afternoon, the bleeding rig and syringe were left attached until the next blood collection. After the last blood sample was collected the bleeding rig was disconnected and the cannula plug was reinserted. All blood samples were

collected with new syringes. Between all blood samples fresh heparinized saline in new syringes was always injected.

Cannulation

Before cannulating, the Silicon end of the cannula was beveled 2.8-2.9cm from the flange with a razor blade. The animal was then anesthetized with methoxyflurane. The rat was placed on its dorsal surface and its forelimbs secured with tape. Initially, the incision site was rinsed with ethanol. Using surgical scissors, a 2.0-2.5cm incision was made just right of the midline in the neck area. The underlying fat and connective tissue were teased away with fine forceps until the jugular vein was exposed. To separate the vein from the remaining tissue, a pair of fine forceps in the closed position was inserted underneath the vein and then released.

To control bleeding from the jugular vein when inserting the cannula, 7cm pieces of silk were loosely tied at the rostral and caudal ends of the vein. Each ligature had its ends clamped with a hemostat. To control bleeding, the hemostats were gently pulled until the sutures became taught, inhibiting blood flow.

Before inserting the cannula, the PE-end of the cannula was inserted into the unbevelled end of a metal 14 gauge cannula that served as a trochar. Blunt scissors were then gently pushed into the incision area just under the skin until they reached the dorsal portion of the neck. The scissors did not penetrate the skin layer at all. This procedure created a channel through the connective tissue under the skin for the metal cannula to follow. The blunt scissors were removed and the bevelled end of the metal cannula was

inserted, following the pathway laid by the scissor. Once the bevelled end reached the back of the neck, it was used to pierce the skin. The PE portion of the cannula was now exteriorized behind the neck of the animal, and the trocar drawn through. The cannula was attached to a 23 gauge needle and a 1cc syringe filled with approximately 500 μ l heparinized saline. The heparinized saline was injected until it dripped from the Silicon end of the cannula. The syringe remained connected to the end of the cannula throughout the surgery.

To insert the cannula into the right atrium, a small hole was cut into the jugular vein using very fine scissors. To ensure a hole was cut, the sutures were loosened at each end of the vein to allow blood flow through the vein and the hole. The bevelled end of the cannula was carefully inserted into the jugular until the flange was surrounded by the vein. The loosely tied ligature controlling the blood flow at the caudal end of the jugular vein was released from the hemostat and was used to tie the cannula to the vein. The ligature was tied just above the flange to ensure that the cannula did not slip from the vein. The ligature controlling the rostral blood flow was also released from the hemostat. The ligature was then closed at the rostral end. To anchor the cannula another piece of suture was threaded through muscle or connective tissue and around the cannula. Care was taken not to tie the suture so tightly that blood flow would be blocked.

A check was done to ensure blood could be drawn before closing the incision. The plunger on the syringe was pulled back until blood was drawn. The cannula was then flushed with approximately 150 μ l heparin. Next the incision was closed using a box stitch. Finally, the excess PE tubing was cut about 2cm above the skin and the cannula plug was inserted.

Preliminary Experimental Protocol

To study the effect of perinatal exposure to cocaine on gonadotropin secretion, particularly LH-surges, I first developed a protocol that would allow me to induce LH-surges in non-cocaine-treated, ovariectomized adult female rats. In previous literature, a single injection of estradiol benzoate (50 μ g/250g body weight [bwt]) given subcutaneously two weeks after ovariectomy was reported to be sufficient to induce LH-surges in adult female rats whose blood samples were collected in the afternoon 2 days later (Mann and Barraclough, 1973; Kalra *et al.*, 1973; Legan *et al.*, 1975). Accordingly, I performed the following preliminary tests.

In all my preliminary experiments, Sprague-Dawley rats were housed under a 12h:12h light:dark cycle with lights on at 0500h CST. Breeder animals for this experiment were obtained from Charles River (Wilmington, MA). Most animals from this experiment, however, were bred in the laboratory from the breeder animals. Temperature was maintained between 22-25°C. In preliminary experiment 1, 12 rats were bilaterally ovariectomized under methoxyflurane anesthesia between 1300h and 1500h (day 1). On day 15, 7 animals were subcutaneously injected with a single dose of estradiol benzoate (EB, 50 μ g/250gbwt) and 5 animals were injected with the vehicle, peanut oil (0.1ml/250gbwt). On day 16, an atrial cannula was inserted according to the procedure described above. On day 17, blood samples of approximately 600 μ l were collected in 1.5ml microtubes at 1000h, 1600h, 1700h, and 1800h. The LH surge normally occurs during the afternoon of proestrus in female rats. Therefore, the afternoon blood samples were collected every hour from 1600h-1800h. A morning blood sample was collected to observe the effect of steroid on

negative feedback. Blood samples were refrigerated until centrifugation on the following day. Samples were centrifuged (Biofuge A, American Scientific Products) at 10687g at room temperature. Serum was aspirated and stored in 2ml polypropylene screw top vials at -20°C until RIAs were performed. This procedure was unsuccessful in inducing significant LH surges in steroid-treated animals (see Results, Figure 1).

I speculated that stress induced during cannulation may have prevented me from successfully inducing significant LH-surges. Therefore, to obtain blood samples from non-stressed animals, I collected trunk blood by decapitation. In preliminary experiment 2, 32 animals were bilaterally ovariectomized under methoxyflurane anesthesia between 1300h and 1500h (day 1). On day 15, 16 animals received EB and 16 received oil, exactly as in preliminary experiment 1. On day 17, blood samples were collected by decapitation at 1000h, 1530h, 1600h, and 1630h. Each time point included 8 animals, 4 oil-treated and 4 EB-treated. In preliminary experiment 2, the afternoon time points for blood sampling were slightly different from preliminary experiment 1 because in preliminary experiment 1 and in a previous experiment conducted in the laboratory (Kefalas and Suter, unpublished), the steroid-induced LH-surges in animals housed in our facility appeared to occur around 1600h. Therefore, blood samples were collected at 1600h and one half-hour before and after (1530h and 1630h). Blood samples were collected in 15ml conical centrifuge tubes and refrigerated until centrifugation the following day. Samples were centrifuged (Sorvall SC) at 1302g at 4°C. Serum was collected and stored at -20°C until RIAs were performed. This procedure was also unsuccessful in inducing significant LH surges in the steroid-treated animals (see Results, Figure 2).

Since reduction of stress did not result in a successful induction of an LH surge in preliminary experiment 2, I decided that an injection of EB alone was not an adequate treatment regimen. Other steroid treatments have been successful in inducing LH surges in ovariectomized rats. Along with a single dose of EB, an injection of progesterone on the morning of blood sampling has proven successful in inducing LH surges in ovariectomized rats. Previous studies have used doses of progesterone ranging from 1mg/kgbw to 5mg per animal (Brown-Grant, 1973; Kalra *et al.*, 1973; Mann and Barraclough, 1973; Brann *et al.*, 1991; Bauer-Dantoin *et al.*, 1992). Preliminary experiment 3 combines data collected from 3 subset experiments which followed a procedure identical to that described in preliminary experiment 2, except for the additional injection of progesterone on the morning of blood sampling (day 17). Briefly, animals were gonadectomized on day 1, and 2 weeks later (day 15) the animals were treated with either oil or EB. On the day of blood sampling (day 17), EB-treated animals were injected with a single dose of progesterone (5mg/250gbwt), and oil-treated animals were given an additional oil injection (0.2ml/250gbwt).

The procedures for the subset experiments in preliminary experiment 3 had minor differences. First, in subset experiment 3a, due to unforeseen circumstances five of the EB-treated animals were not given an injection of progesterone on the morning of blood sampling (day 17). Blood collections from four of the animals not treated with progesterone were at the 1600h time point and one was at the 1630h time point. Second, in subset experiment 3c, I deleted the 1530h time point. In the previous experiments there had not been elevated LH levels at the 1530h time point, which indicated that the surge would be better observed during the later time points, 1600h and 1630h. Third, in subset experiment

3c, I also included male rats. Normal orchidectomized male rats treated with steroid do not exhibit an LH surge. I included males in this subset experiment to ensure that I would observe this lack of LH surges, and that male rats in our laboratory exhibit normal negative feedback in response to steroid treatment.

Experimental Protocol

Sprague-Dawley rats were housed as previously described. This experiment included 8-11 blocks of rats. Each block consisted of 10 pregnant females. Pregnant females were obtained by mating nulliparous adult females with adult males. Pregnancy was confirmed by the presence of sperm in vaginal lavages taken the morning after mating (gestational day 1 [D1]). Five pregnant females were assigned to 1 of 5 treatment groups: *ad libitum* control (ALC), pair-fed control (PFC), 30mg cocaine/kgbw dose, 15mg cocaine/kgbw dose, and 7.5mg cocaine/kgbw dose. The remaining 5 pregnant females were assigned as untreated surrogate mothers for the various treatment groups. Fostering all treated and control pups to surrogate mothers on the day of birth eliminated the possible confounding of effects of differences in maternal care or lactation, induced by exposing the mother to cocaine, with direct effects of cocaine on the pups. All surrogates delivered 1-6 days before their assigned treated mother. The first 2 pregnancies of each block always consisted of the 30mg/kgbw cocaine-treated mother and her assigned surrogate in order to permit pair-feeding, described below. The following pregnancies within the first block were randomly assigned and this selection order was rotated for the additional blocks. The order of the first block was

30mg/kgbw, 7.5mg/kgbw, 15mg/kgbw, PFC and ALC. The order of the second block was 30mg/kgbw, 15mg/kgbw, PFC, ALC and 7.5mg/kgbw, and the rotation of the order of treatments continued in this manner throughout the remaining blocks.

Treatment with cocaine has been shown to reduce nutritional intake in rats (Church *et al.*, 1990b), and the amount of nutritional intake appears to be inversely related to the dose of cocaine given. Therefore, to account for any confounding effects of undernutrition that might accompany treatment, animals in the PFC-, 7.5mg/kgbw-, and 15mg/kgbw-groups received exactly the amount of food and water the 30mg/kgbw-animal in that block ate and drank on that day of pregnancy. The first pregnant animal in each block was always assigned to the 30mg/kgbw-group to monitor its feeding habits, which determined the amount of food and water the PFC-animal and the two other cocaine-treated animals would receive in that particular block. The ALC-animals were allowed to eat and drink as much as they wanted. The purpose of the ALC-group was to observe effects that might be due to nutrition rather than cocaine, which would be evident in any differences between the ALC-group and the PFC-group.

The feeding habits of the ALC-animals were monitored and later compared to the feeding habits of the 30mg/kgbw-animals. Food and water consumption for the ALC- and 30mg/kgbw-groups were monitored from gestational day 15 (D15) up until delivery (D23). This time frame coincided with the time period of injection of cocaine or PBS into the pregnant animals. I also monitored gestational weight gain from D15 to delivery for all treatment groups.

A subcutaneous dose of 30mg/kgbw produces plasma levels of cocaine that mimic

plasma levels in human addicts, and that dose has been shown to alter neurobehavioral development (Seidler *et al.*, 1995). In previous literature my highest dose, 30mg/kgbw, has been shown not to cause obvious teratogenic effects or an increase in the mortality of rat pups. Animals injected with doses greater than 30mg/kgbw had a higher incidence of abruptio placentae, fetal edema, and cephalic hemorrhage than control animals (Church *et al.*, 1990a, b; Dow-Edwards, 1988). Observing morphological abnormalities such as these was not the purpose of this experiment. Cocaine is known to have opposite effects at different doses in some cases, so to ensure that the effective range was spanned I included two lower doses of cocaine (15- and 7.5mg/kgbw). My lowest dose is similar to the low dose used by Raum *et al.* (1990), who observed significant effects on male behavior. Including several dose levels was designed to allow me to observe possible effects of hyperavailability or depletion of NTs.

Daily subcutaneous injections of cocaine or vehicle (PBS) were given on D15 through D21 of gestation. Cocaine or PBS was also given daily to the pups on postnatal day 2 (pnd2, one day after birth) up until pnd10. The dose of cocaine per unit body weight given to each pup was the same as that given to its mother. The critical period for sexual differentiation of the brain in rats occurs between D15 of gestation and the first few days of postnatal life (Lauder, 1983; Jarzab *et al.*, 1987; Raum *et al.*, 1990). Therefore, by prenatally and postnatally exposing the pups to cocaine, I was confident that I was spanning the entire critical period for sexual differentiation of the brain.

On the day of birth the litters were culled to ten pups (five females and five males) and fostered to a surrogate mother. If there were not ten pups in the litter, I considered the

acceptable lower limit to be eight pups. I attempted to keep the number of males and females approximately equal in each litter, but this was not always possible. Litters were culled to 8-10 pups to reduce variability due to the differences in milk availability and nutritional intake by the pups. I monitored pup weight gain from pnd1 up to pnd10. All additional pups from the cocaine-treated groups were sacrificed. Some pups from the surrogate mothers and ALC-mothers were transferred to other experiments.

On pnd1 (day of birth), I recorded the size of the litter, total weight of the litter by sex, and sex ratio. These measurements were taken to observe possible gross effects of cocaine on the outcome of pregnancy. I also measured the anogenital distance (AGD) of each pup to monitor a simple sexually differentiated phenotype. The AGD was measured as the distance between the anus and the genital papilla and was measured using vernier calipers.

At pnd21, all pups were weaned from their mothers, and female and male siblings were housed in separate cages. I again recorded weight of the litter by sex and the anogenital distance of each pup. On pnd45, housing density was reduced to 2-3 animals per cage. For some groups, reducing the housing density at an earlier date would have been preferable due to the rapid growth of certain animals. In the interest of uniformity of the protocol among all experimental animals, however, housing density was always reduced on pnd45. Between pnd45 and pnd60, daily vaginal lavages were taken from female rats to ensure that any effect seen on LH levels in females was due to treatment of cocaine and not due to females with abnormal estrous cycles. To expose all rats to equal handling time, male rats were also handled in a fashion that simulated that involved in a vaginal lavage between pnd45 and

pnd60.

At pnd 60, the sexually mature rats were bilaterally gonadectomized. At pnd74, 3 males and 3 females were injected with EB (50 μ g/250gbwt) between 0900h and 1100h. The remaining rats (2 males and 2 females) were injected with peanut oil (0.1ml/250gbwt). At pnd76, the day blood samples were taken, EB-treated rats were injected with progesterone (5mg/250gbwt) between 0800h and 0900h. Oil-treated rats received another injection of peanut oil (0.2ml/250gbwt) between 0800h and 0900h. If there were not 10 pups in a litter, I attempted to have at least 2 males and 2 females injected with steroids and at least 2 males and 2 females injected with oil. If this was not possible, an additional litter of the same treatment was later created to fill in the missing animals.

Blood samples were collected by decapitation at two time points, 1000h and 1630h. Each time point included at least one male and one female steroid-treated animal and also one male and one female oil-treated animal. If any time point had sibling animals of the same sex and treatment, I averaged the results of the duplicates to avoid pseudoreplication. Samples were collected and assayed for LH exactly as in preliminary experiment 3. A time line of the experimental protocol is included (Diagram 2).

Statistical Analysis

Hormone data from preliminary experiment 1 and maternal and pup weight gain, and daily food and water consumption from the main experiment were analyzed using two-way analysis of variance (ANOVA) with repeated measures. The main effects were treatment and time. AGDs on pnd1 and pnd21 were analyzed using two-way ANOVA with treatment and

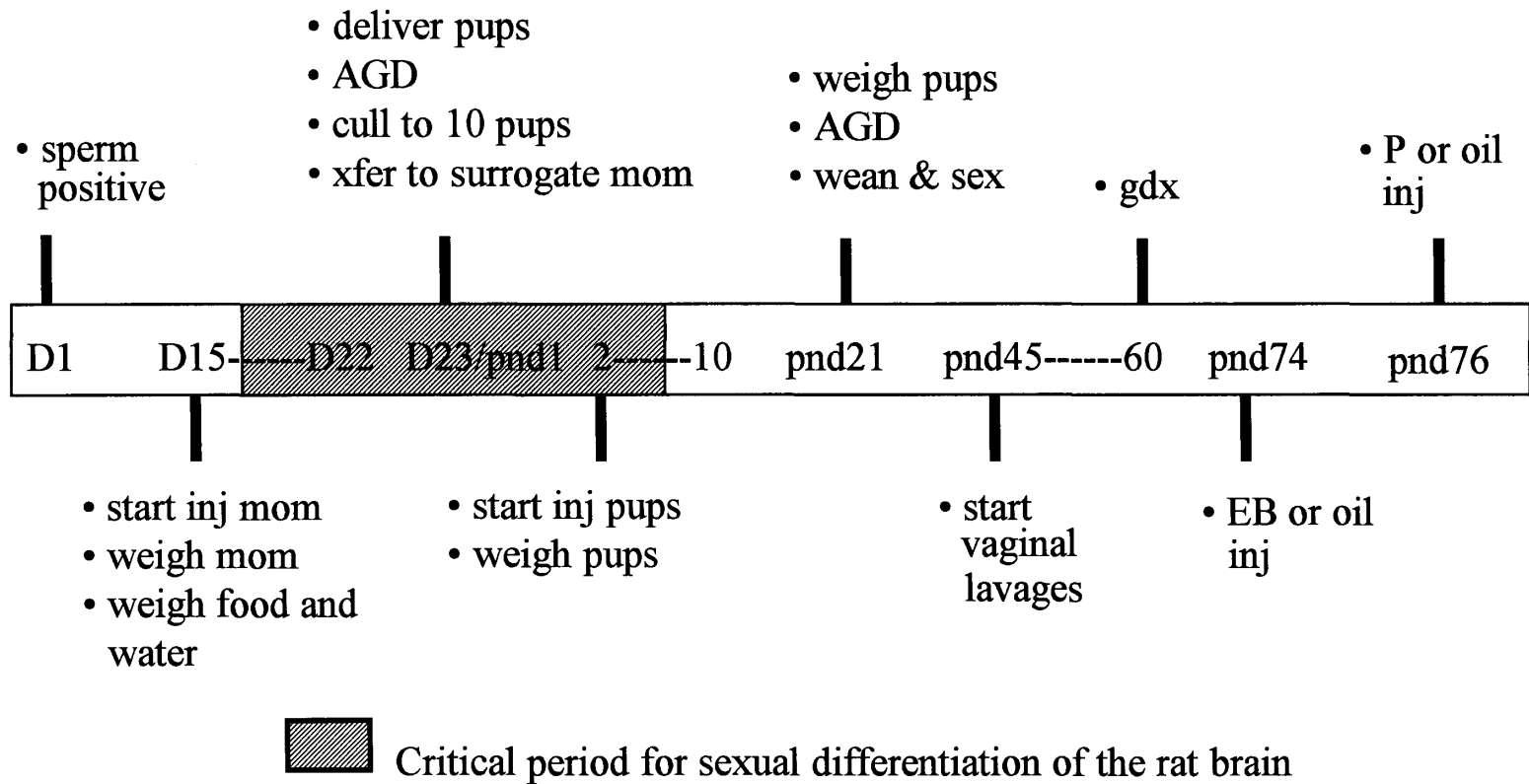


Diagram 2. Timeline of the experimental protocol.

sex as the main effects. Preliminary experiments 2 and 3, and hormone data from ALC-, PFC-, 30mg/kg-, 15mg/kg-, and 7.5mg/kg-groups were analyzed using two-way ANOVA with treatment and time as the main effects. Total food and water consumption, litter size, birth weight, and sex ratio presented as percent female were analyzed using one-way ANOVA with treatment as the main effect. When a significant F-value was obtained for a main effect, Tukey's post-hoc tests determined significant differences between specific groups within the ANOVA. Data that did not meet the assumptions of ANOVA were transformed accordingly. If the data still did not meet the assumptions of ANOVA, the data were analyzed using the appropriate non-parametric tests. Differences with p-values < 0.05 were considered statistically significant. Sex ratios of the offspring from the various treatment groups were analyzed using a chi-square test. Cyclicity data were analyzed using a Kruskal-Wallis Test.

To determine a minimal sample size that would enable me to detect a possible LH-surge in males or to detect possible differences in LH-surges between different females, I performed a power analysis. The following equation was used:

$$\frac{d}{\sqrt{2\sigma^2/n}} = t_{\alpha} + \Phi^{-1}(p)$$

- d =detectable difference between [LH] values in different treatment groups (ng/ml)
n =sample size
 σ^2 =variance from a sample population

- $\Phi^{-1}(p)$ =under a normal distribution at a power of 0.83 for a significance level, α , of 0.05
Table 3, Column A (Rosner, 1990)
- t_{α} =under a t-distribution at α level of 0.05; degrees of freedom $df=N-k$
Table 5 (Rosner, 1990)
N=total number of rats
k=number of treatment groups

Before I determined my sample size I had already collected blood samples from 199 animals: 99 males and 100 females. I measured LH levels from these samples via RIA, and used the variance of these measurements as an estimate of σ^2 .

To determine the minimal sample size that would enable me to detect a significant LH surge in cocaine-treated males, I first chose a value of 10ng/ml for d . This meant I wanted to be able to declare as significant a difference of 10ng/ml between treatment groups. The value of σ^2 is the mean square error term (M.S.E.) from the statistical analysis of my sample population ($\sigma^2=43$). The value of $\Phi^{-1}(p)$ with a p value of 0.83 under a normal distribution equaled 0.96. The t_{α} -value at $t_{0.05}$ under a t-distribution with $df=79$ equaled 1.671. My degrees of freedom were equal to 79 because I had 99 blood samples from males ($N=99$), and I had 5 treatment groups, 2 steroid-treatments per cocaine-treatment group, and 2 time points within each steroid-treatment group ($k=5 \times 2 \times 2$). Since I expected the LH value in the afternoon for some of the treatment groups to surge I needed only to concern myself with a one-sided distribution in this case. Solving for n , I calculated a sample size equal to 6. Therefore, to be able to declare a minimal difference of 10ng/ml between my treatment groups as significant, I would need a sample size of 6 males for each treatment group.

To determine a sample size that would allow me to detect differences in LH surge values for the females between treatment groups, I used the same equation as before with a

slightly different approach. I decided to calculate the value of d given a certain sample size that I predetermined. I chose my sample size to equal 8. From the data collected on 100 females I obtained a $\sigma^2=0.0571$. The M.S.E. was much lower than the male M.S.E. because the female data were log-transformed. The variance of the raw data from the females was not homogenous, which is an assumption that needs to be fulfilled when using ANOVA. Therefore, I log-transformed the data. Since I could not anticipate whether the female LH surge value would increase or decrease, I obtained a two-sided t_{α} -value at $t_{0.025}$ with $df=80$ ($N=100, k=20$) ($t_{0.025}=2$). Solving for d I got a value equal to 0.354 which is the log value. Therefore, I needed to take the antilog of this value which equaled 2.25. This value does not mean I can detect differences as small as 2.25ng/ml. It meant that if I had a mean equal to 50ng/ml, I would be able to detect a difference that was approximately lower than half the value of 50ng/ml or greater than twice the value of 50ng/ml, 25ng/ml and 100ng/ml respectively. Satisfied with my ability to detect significant differences at these levels I determined a sample size of 8 to be sufficiently large. Although I determined a sample size of 6 from my male data, the power analysis for the female data determined a larger minimal sample size of 8.

CHAPTER III

RESULTS

Preliminary Experiment 1

To study the effect of perinatal exposure to cocaine on LH secretion, I first developed a protocol that would allow me to induce LH surges in untreated, ovariectomized adult female rats. To collect serial blood samples from single animals in preliminary experiment 1, I cannulated my animals one day after injection of EB and one day prior to blood collection. LH values for control (vehicle-injected) animals ranged from 12-21 ng/ml (Figure 1. The y-axes in all figures related to the preliminary experiments were set to the same value in order to facilitate comparisons between subsets of data.). The data were not normally distributed ($p < 0.05$) and demonstrated heterogeneous variances ($p < 0.05$). The normal probability plot displayed a logarithmic pattern and therefore the data were log-transformed. The transformed data were normally distributed and had homogeneous variance. LH levels in EB-treated animals were not significantly different from those in control animals ($p = 0.19$). There was a significant effect of time ($p = 0.001$). Tukey's post hoc test indicated that the 1600h time point was significantly different from the 1000h and 1700h time points ($p < 0.05$). The significant difference among time points, however, is not indicative of an LH-surge. Significant LH-surges would be evident as a significant treatment x time interaction, with a.m. values from EB-treated animals lower than a.m. values from oil-treated animals, and

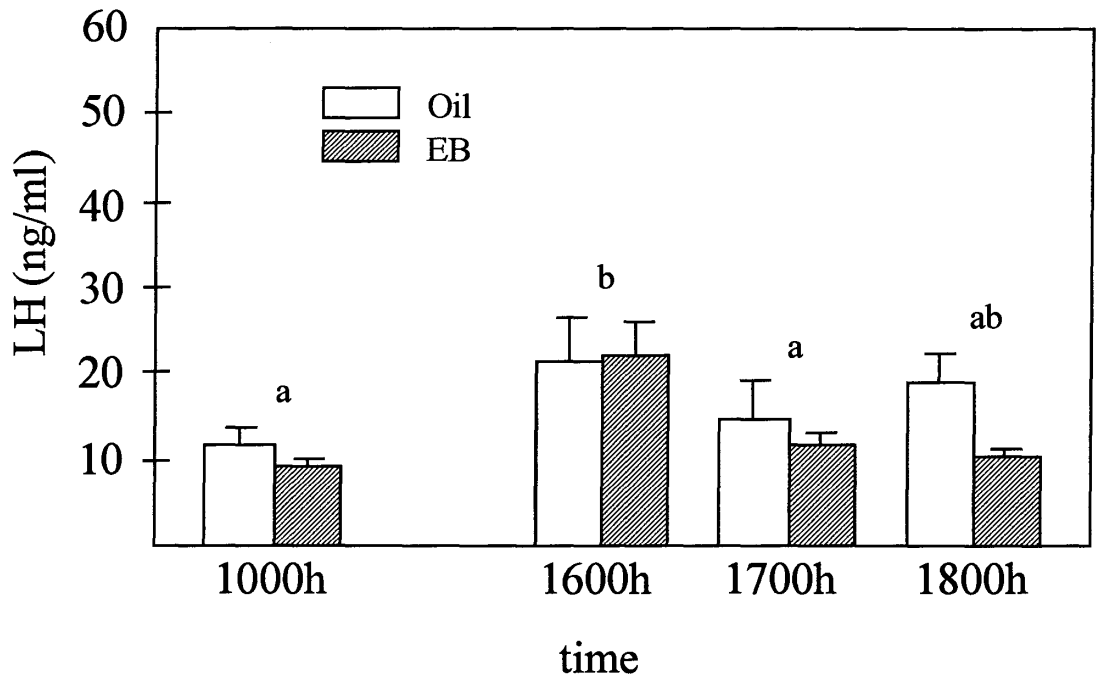


Figure 1. Preliminary Experiment 1. Effect of EB on LH in ovariectomized adult female rats. Serial blood samples were collected via cannulation of the right jugular vein. There was no significant main effect of treatment ($F_{1,7}=1.10$, $p=0.19$). There was a significant main effect of time ($F_{3,21}=8.81$, $p=0.001$). Times labeled with different letters are significantly different from each other. There was no significant treatment x time interaction ($F_{3,21}=1.53$, $p=0.24$). Each bar represents the mean \pm S.E.M. of 4 to 6 animals.

with p.m. values from EB-treated animals higher than p.m. values from oil-treated animals. There was no significant treatment x time interaction ($p=0.24$). Therefore, a single injection of EB (50ug/250gbwt) two weeks after ovariectomy was unsuccessful in inducing significant LH surges in EB-treated animals.

Preliminary Experiment 2

I speculated that stress during the cannulation procedure prevented me from successfully inducing significant LH surges in preliminary experiment 1. To obtain blood samples from non-stressed animals, I collected trunk blood by decapitation in preliminary experiment 2. The disadvantage of this procedure as compared to blood collection via a cannula was the increased number of animals used. The advantages of this procedure were the reduced stress on the animals prior to the collection of blood and the increased volume of serum collected. From preliminary experiment 1 and previous experiments performed in this laboratory (Kefalas and Suter, unpublished), I knew that LH surges would most likely occur around 1600h. Therefore, I collected p.m. blood samples at 1530h, 1600h, and 1630h. The data satisfied the assumptions of ANOVA and therefore were not transformed. LH values for control animals ranged from 8-13ng/ml (Figure 2). LH levels in EB-treated animals were not significantly different from those in control animals ($p=0.73$). There was also no significant effect of time ($p=0.11$) nor a significant treatment x time interaction ($p=0.39$). Therefore, this procedure was also unsuccessful in inducing significant LH surges in EB-treated animals.

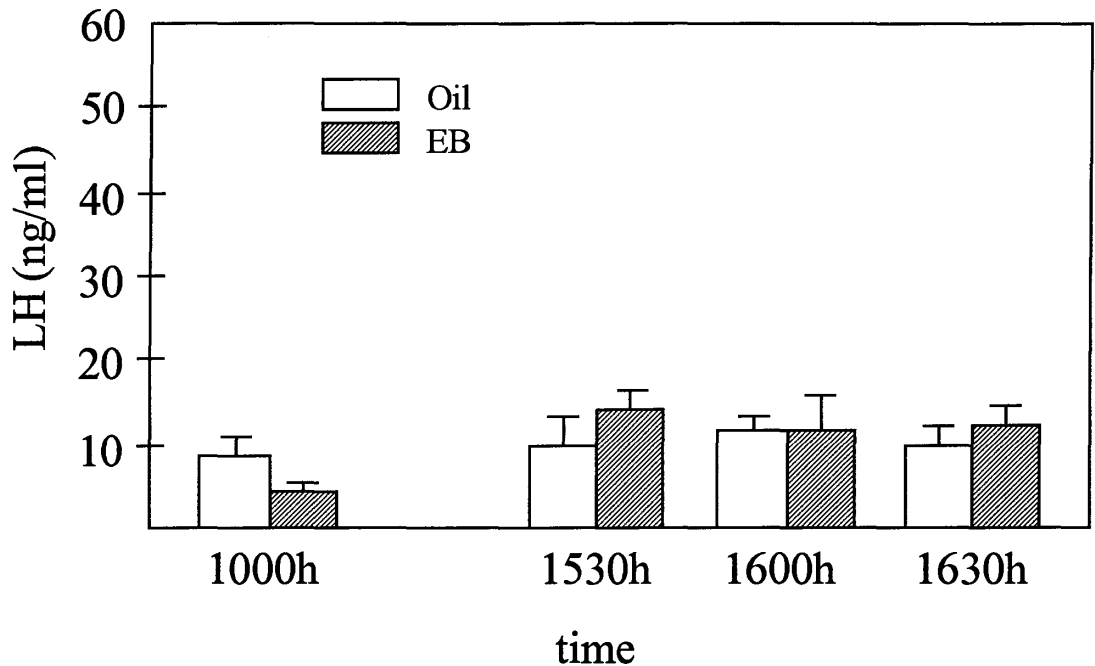


Figure 2. Preliminary Experiment 2. Effect of EB on LH in ovariectomized adult female rats. Trunk blood samples were collected via decapitation. There was no significant main effect of treatment ($F_{1,6}=0.14$, $p=0.73$). There was no significant main effect of time ($F_{3,18}=2.33$, $p=0.11$). There was also no significant treatment x time interaction ($F_{3,18}=1.06$, $p=0.39$). Each bar represents the mean \pm S.E.M. of 4 animals.

Preliminary Experiment 3 (females)

Other researchers have been able to induce LH surges in ovariectomized adult female rats by giving not only an injection of EB but also an injection of progesterone. To test the efficacy of the addition of an injection of progesterone to my treatment regimen, I followed the protocol of preliminary experiment 2 exactly, except that steroid-treated animals received an injection of progesterone (5mg/250gbwt) on the morning of blood sampling. Control animals received vehicle injections. LH values for the female oil-treated control animals for blood samples collected in the morning and afternoon ranged from 15-20ng/ml (Figure 3). The data did not satisfy the assumptions of normality ($p < 0.001$) or homogeneity of variance ($p < 0.001$). Plotted residuals displayed a sigmoidal pattern; therefore the data were square-root transformed. Although the transformation resulted in data that were normally distributed, the transformation was unsuccessful in producing variances that were homogeneous ($p < 0.001$); therefore the appropriate non-parametric test was used, therefore the data were analyzed using Mann-Whitney U tests. In the morning, steroid-treated LH levels were significantly lower than oil-treated LH levels ($p = 0.013$). In the afternoon, LH levels in steroid-treated females were significantly higher than in the oil-treated females ($p = 0.004$). The data from this experiment demonstrate that I was successful in producing steroid-induced LH surges in ovariectomized adult female rats.

Although there were highly significant treatment effects in the morning and in the afternoon samples for preliminary experiment 3, each subset of experiments analyzed individually did not yield significant differences, teaching me the importance of a sufficiently large sample size. Confident I could induce an LH surge in steroid-treated, non-

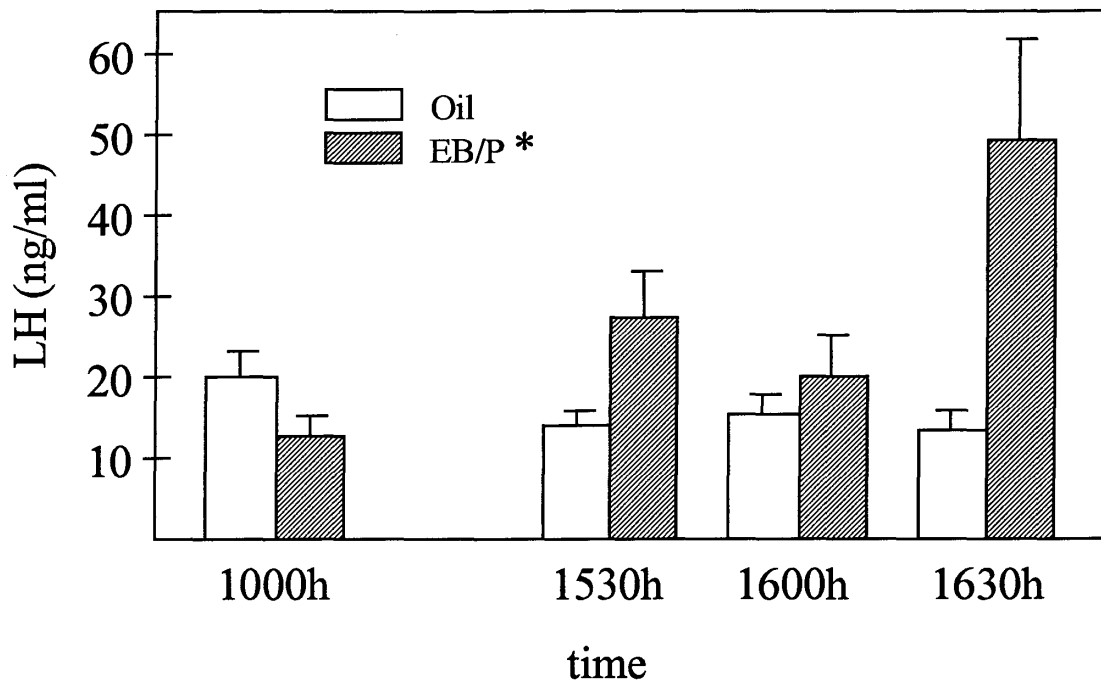


Figure 3. Preliminary Experiment 3 (female). Effect of combined treatment with EB and P on LH in ovariectomized adult female rats. Trunk blood samples were collected via decapitation. *There was a significant main effect of treatment at the 1000h time point ($p=0.013$). There was also a highly significant main effect of treatment for the combined afternoon time points ($p=0.004$). Each bar represents the mean \pm S.E.M. of 8 to 12 animals.

cocaine-treated females, I needed to calculate the minimum sample size that would enable me to detect an LH surge in males or to detect a difference in LH surges of females from different treatment groups. To do this I performed a power analysis that produced a sample size of 8 (see statistical analysis section of the Materials and Methods)

Preliminary Experiment 3 (males)

To ensure that orchidectomized adult male rats in our laboratory exhibit normal negative feedback responses to treatment with steroid, I orchidectomized adult rats and steroid-treated them two weeks later. LH values for oil-treated males ranged from 17-25ng/ml (Figure 4). The data did not satisfy the assumptions of normality ($p < 0.001$) or homogeneity of variance ($p = 0.001$). The normal probability plot displayed a logarithmic pattern, and therefore the data were log-transformed. The transformation resulted in normally distributed data and variances that were homogeneous. There was a highly significant main effect of treatment ($p < 0.001$). Oil-treated animals had LH values that were approximately four-fold higher than those of steroid-treated animals at all time points. There was no significant effect of time ($p = 0.75$). There also was no significant treatment x time interaction ($p = 0.40$). These data allowed me to conclude that the male rats did exhibit normal negative feedback in response to the steroid treatment.

Since I was successful in producing significant LH surges in steroid-treated females and my control males exhibited normal negative feedback, I proceeded with my main experiment. The following results are from the main experiment. It should be noted that the sample sizes within groups were not always 8. The reason for this was the loss of pups

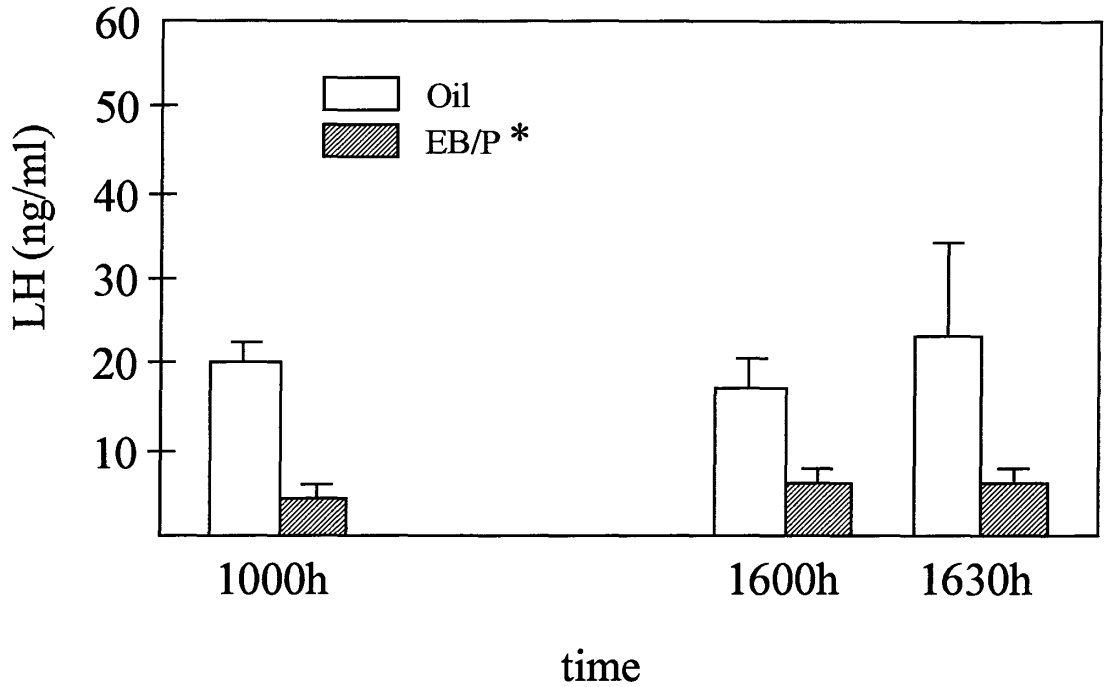


Figure 4. Preliminary Experiment 3 (male). Effect of combined treatment with EB and P on LH in orchidectomized adult males. Trunk blood samples were collected via decapitation. *There was a highly significant main effect of treatment ($F_{1,18}=46.05$, $p<0.001$). There was no significant main effect of time ($F_{2,18}=0.36$, $p=0.75$). There was no significant treatment x time interaction ($F_{2,18}=0.97$, $p=0.40$). Each bar represents the mean \pm S.E.M. of 4 animals.

during the experiment. In order to have a sample size of at least eight, I needed to have make-up litters. The loss of pups occurred in a number of the treatment groups, but seemed to be more problematic within my 30mg/kgbw group. Therefore, the additional make-up litters caused my sample size to exceed 8 in some cases.

Food and water consumption

Treatment with cocaine has been shown to reduce nutritional intake (Church *et al.*, 1990b). I compared the amounts of food and water consumed by the ALC-group and the 30mg/kgbw-group. There was a highly statistical significant main effect of treatment ($p < 0.001$) for the total amount of food consumption as a percent of body weight from D15 to D22 (Figure 5). The 30mg/kgbw group ate approximately 10% less than the ALC group. There was no significant difference in water consumption between the 30mg/kgbw- and ALC-groups ($p = 0.68$).

I also wanted to see if there was a difference between the two groups in the *pattern* of food and water consumption. I therefore analyzed daily food and water consumption from D15 to D22. In Figure 6, the daily food intake as a percent of body weight from D15 to D22 is shown. All percent values were below 10%; therefore the data were arcsin square-root transformed. The transformed data did satisfy the assumption of normality but not homogeneity of variance ($p < 0.05$). Specifically, D16, D20 and D21 demonstrated heterogeneous variance, whereas data from the other five days had homogenous variances. The 2-way ANOVA indicated a highly significant treatment x time interaction ($p = 0.001$), indicating that the ALC-group and the 30mg/kgbw-group did have different patterns of food

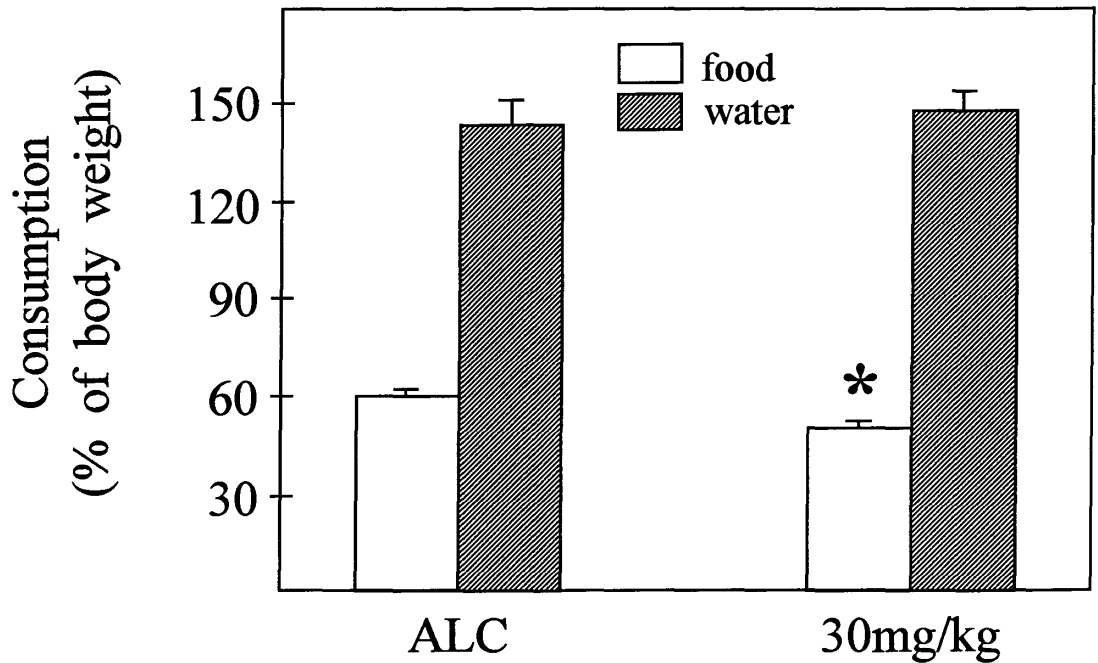


Figure 5. Total food and water intake from D15 to D22 measured as a percent of weight. *The 30mg/kgbw group consumed less food than the ALC group, therefore, there was a significant main effect of treatment for the total amount of food consumed ($F_{1,25}=18.93$, $p<0.001$). There was no significant main effect of treatment for the total amount of water consumed ($F_{1,25}=0.18$, $p=0.68$). Each bar represents the mean \pm S.E.M. of 10 to 17 animals.

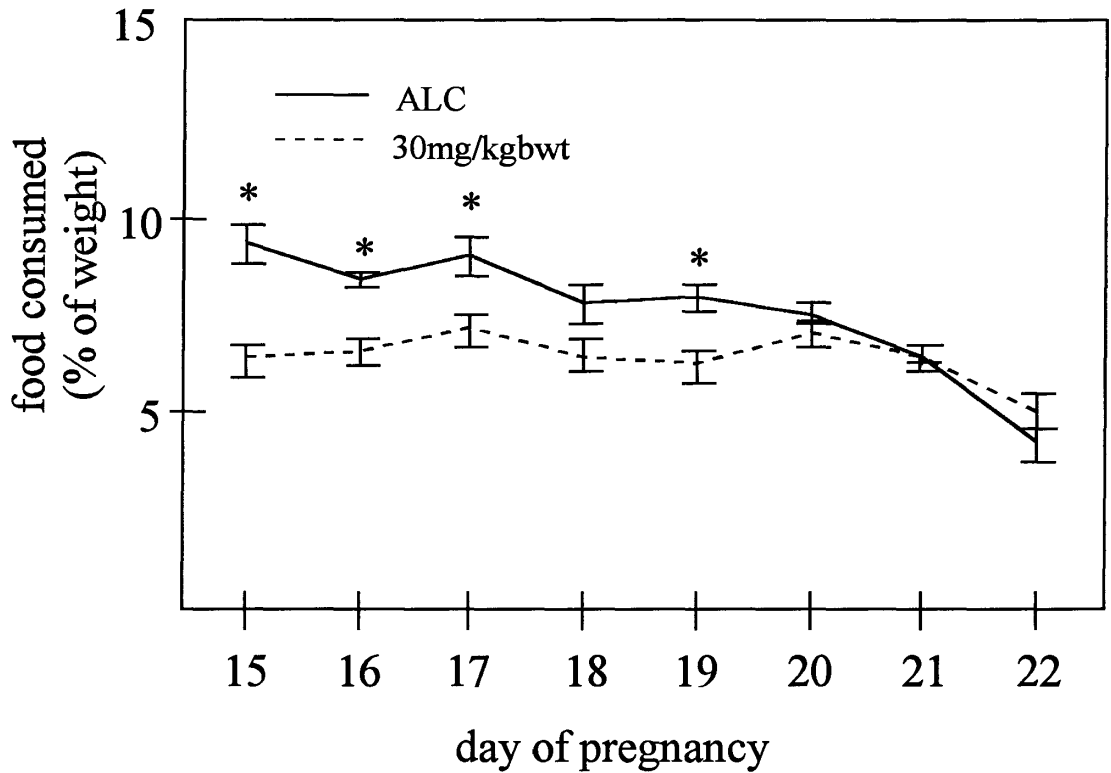


Figure 6. Daily amount of food intake from D15 to D22 measured as a percent of weight. There was a significant time x treatment interaction ($F_{7,140}=3.90$, $p=0.001$). *Subsequent t-tests and Mann-Whitney U tests indicated significant differences from D15 through D17 and on D19. Each point represents the mean \pm S.E.M. of 9 to 17 animals.

consumption. Post hoc tests were performed on each day with treatment as the main effect. Food consumption on those days with heterogeneous variances were analyzed using the Mann-Whitney U test; on those days with homogenous variances, post-hoc t-tests were performed. All p-values from t-tests and Mann-Whitney U tests were adjusted with a Bonferroni correction factor. The ALC-group consumed significantly more food than the 30mg/kgbw group from D15 through D17 and on D19 ($p < 0.05$). On D18 and D20 though D22, both groups consumed approximately the same amount of food ($p > 0.05$).

I attempted to analyze differences in the pattern of water consumption, but the data did not satisfy the assumptions of parametric analysis even after transformation. Furthermore, available non-parametric tests were not appropriate. By inspection of Figure 7, it does not appear there are any differences in the pattern of daily water consumption for the days measured.

Maternal weight gain

Maternal weight gain, expressed as the percent difference from weight measured on the morning of D15, is presented in Figure 8. The data satisfied the assumptions of ANOVA and therefore were not transformed. There was a significant treatment x time interaction due to different patterns in weight gain among treatment groups ($p < 0.001$). The ALC-group gained weight linearly from D15-22 of gestation. The PFC-group, however, gained no weight for the first 2 days of treatment, and then gained weight throughout the rest of pregnancy at a rate parallel to the ALC-group. Subsequent 1-way ANOVAs were performed on each day to better describe the differences observed in Figure 8. At all days measured

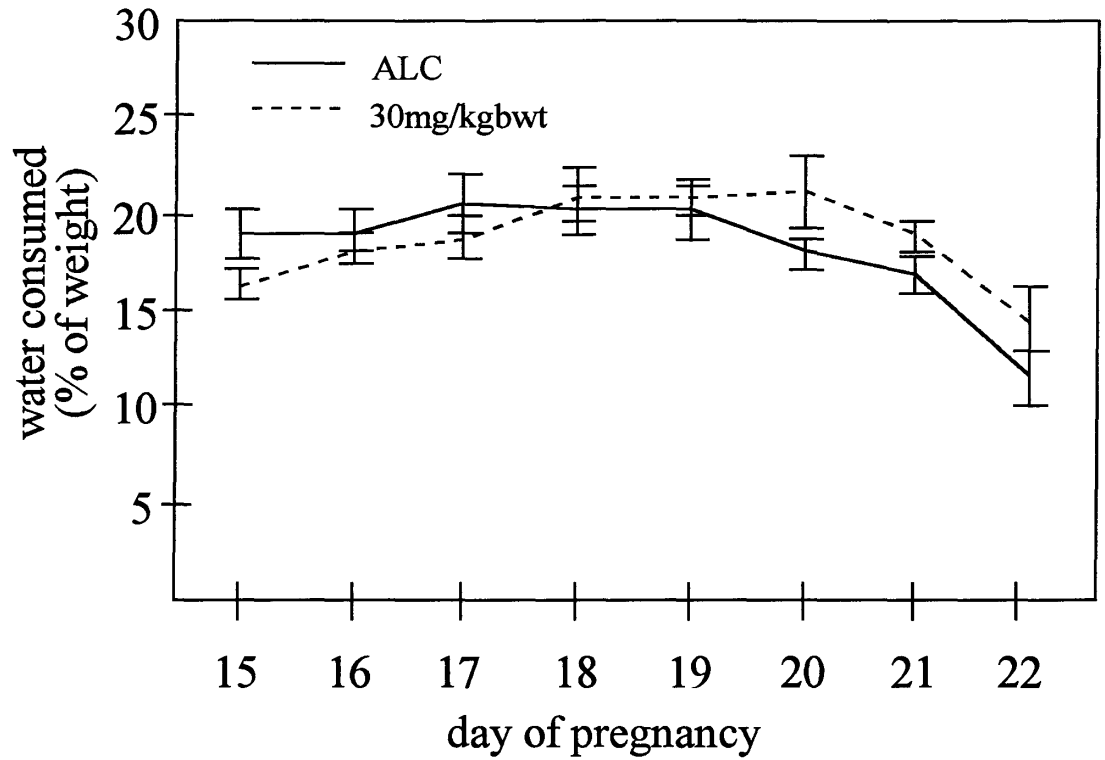


Figure 7. Daily amount of water intake from D15 to D22 measured as a percent of weight.

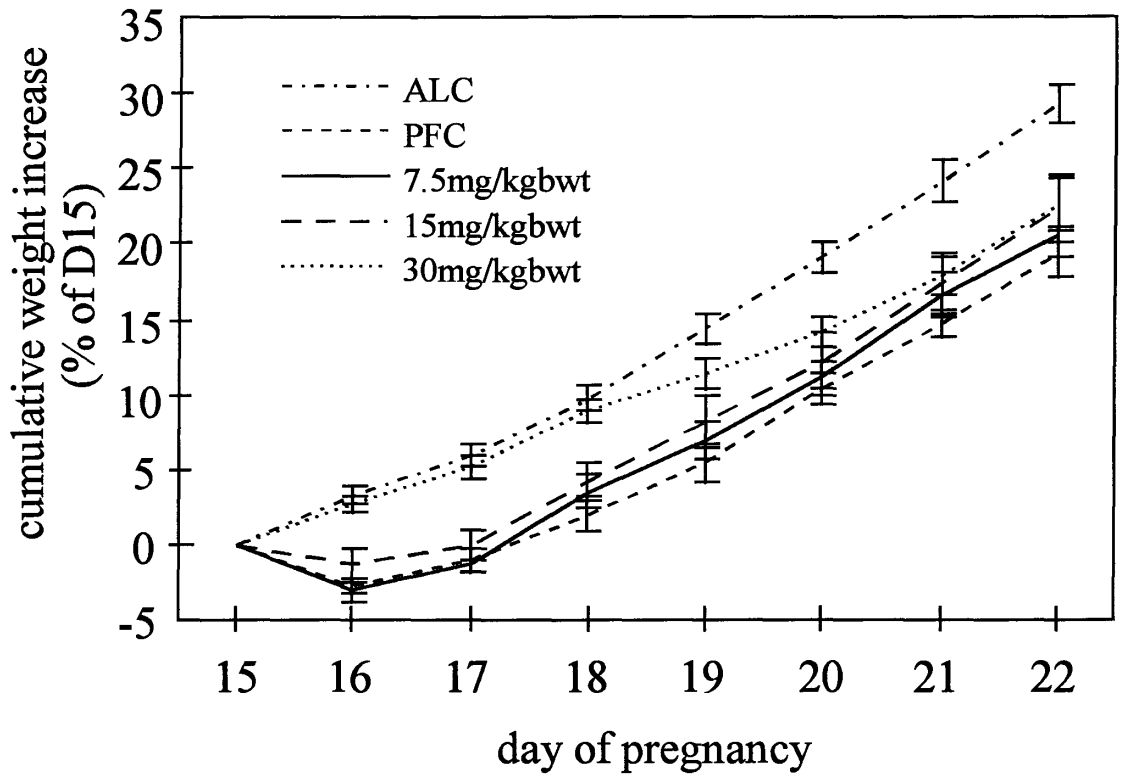


Figure 8. Daily percent increase in maternal weight from D15 to D22. There was a significant treatment x time interaction ($F_{24,282}=2.48$, $p<0.001$). Separate 1-way ANOVAs were performed on each day. There was a significant main effect of treatment for every day measured ($p<0.05$). Post hoc Tukey's tests indicated the PFC-, 7.5mg/kgbw-, and 15-mg/kgbw-group were significantly different from the ALC-group throughout all days measured ($p<0.05$). The 30mg/kgbw group was not significantly different from the ALC-group from D15 up to and including D19 ($p>0.05$). From D20 up to and including D22, the 30mg/kgbw group was significantly different from the ALC-group. Each point represents the mean \pm S.E.M. of 9 to 17 animals.

there was a significant main effect of treatment after adjusting p-values with a Bonferonni correction factor ($p < 0.05$). Post hoc Tukey's tests indicated that the animals in the PFC-group gained significantly less weight than the ALC-group throughout all of the measured days ($p < 0.05$). The 7.5- and 15-mg/kgbw group gained weight in a pattern identical to the PFC-group ($p > 0.05$). The 30mg/kgbw group exhibited a different pattern, gaining almost as much weight as the ALC-group for the first 4 days of treatment ($p > 0.05$). In fact, subsequent analysis indicated the ALC-group was not significantly different from the 30mg/kgbw group up to and including D19. After D19 the 30mg/kgbw group gained weight more slowly and was not significantly different from the PFC-group, 7.5- or 15-mg/kgbw groups ($p > 0.05$).

Data were also analyzed without adjusting for starting weights when measurements began. Again, the data satisfied the assumptions of ANOVA and therefore were not transformed. As for the adjusted data, there was a highly significant treatment x time interaction ($p < 0.001$). Subsequent 1-way ANOVAs were performed for each day measured. Unlike the case of the adjusted data, there was no significant main effect of treatment for maternal weights for any of the days measured ($p > 0.05$). There was a highly significant main effect of time due to the overall increase in weight for all groups throughout gestation ($p < 0.001$).

Total maternal weight gain also was analyzed using a one-way ANOVA. There was a highly significant main effect of treatment ($p = 0.001$). Tukey's test showed that the ALC-group gained more weight than the PFC-group and the cocaine-treated groups, approximately 25g more, but that there was no difference in total weight gain among the PFC-group and the

cocaine-treated groups. To ensure that differences in total weight gain were not due to differences in maternal weights at the start of treatment (D15), maternal weights on D15 were analyzed using a one-way ANOVA with treatment as the main effect. The data satisfied the assumptions of normality and homogeneity of variance and therefore were not transformed. No significant differences were found between treatment groups ($p=0.59$). To determine if the lack of significance was biologically real or due to a lack of statistical power, a power analysis was performed. A power analysis determines the probability of making a type II error, accepting the null hypothesis when it should be rejected. The P value calculated from the power analysis was 0.72 which translates into a 72% probability of correctly rejecting the null hypothesis. Therefore, differences in total weight gain between the ALC-group and the PFC- and cocaine-treated groups appear to be real and not due to differences in starting maternal weights on D15.

Total birth weight, litter size and sex ratio

Figure 9 represents the average weights of the litters at pnd1 for each treatment group. The weights ranged between 80-90g. The data satisfied the assumptions of ANOVA and therefore were not transformed. There was no significant difference between treatment groups ($p=0.60$).

The average litter size for each treatment group is shown in Figure 10. The average size of the litters for all groups was approximately 13 pups. There was no significant main effect of treatment ($p=0.74$) The variance was homogeneous, but the data were not normally distributed ($p=0.01$). The non-transformed data were analyzed due to the robust nature of

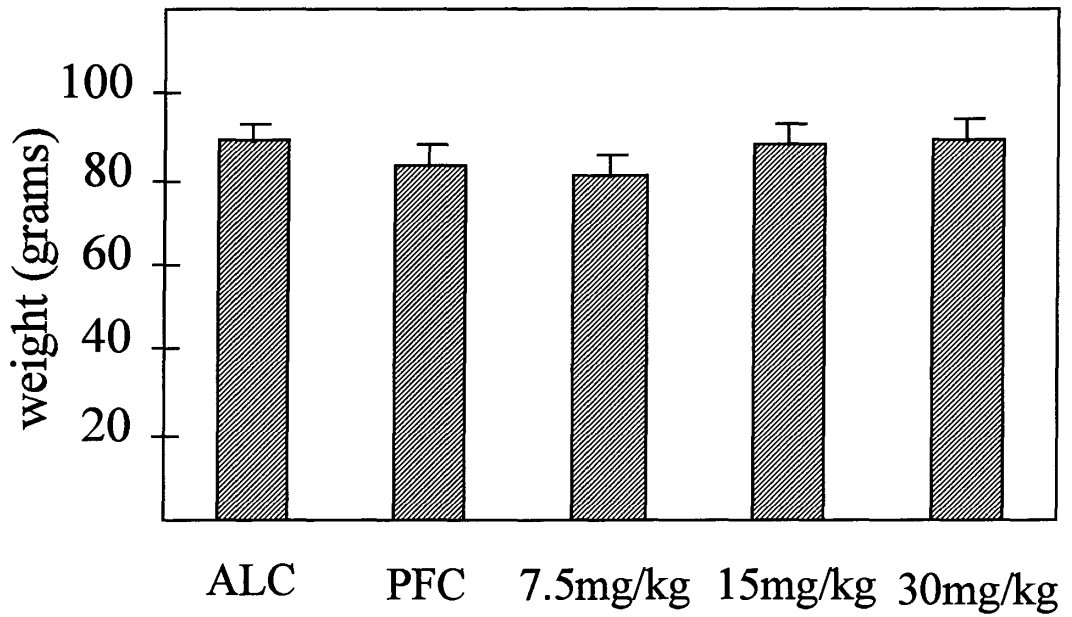


Figure 9. Average total weight of pups delivered at birth (pnd1). There was no significant main effect of treatment ($F_{4,48}=0.69$, $p=0.60$). Each bar represents the mean \pm S.E.M. of 9 to 16 litters.

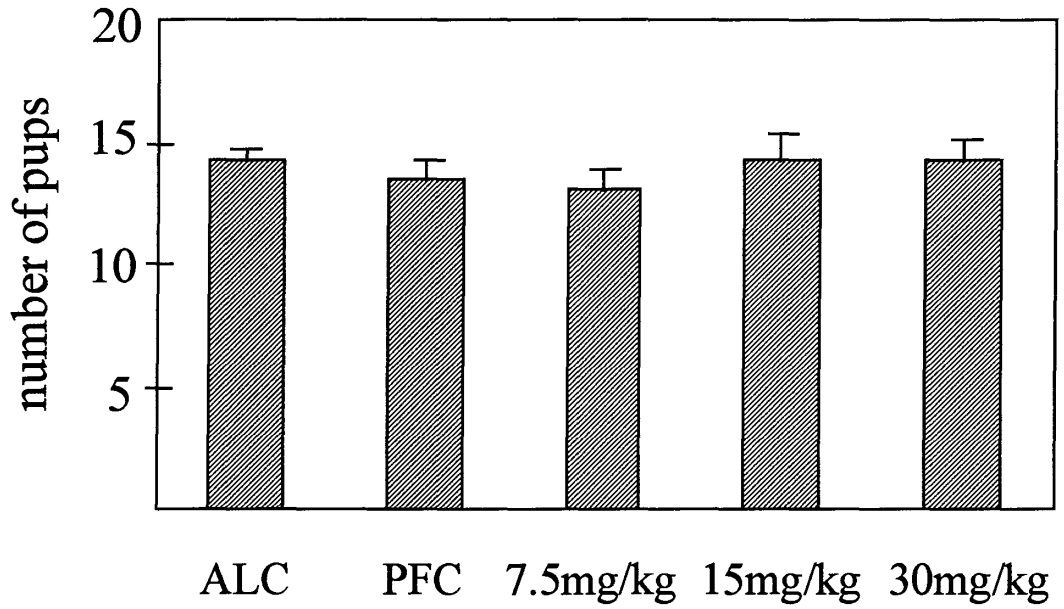


Figure 10. Litter size at birth (pnd1). There was no significant main effect of treatment ($F_{4,49}=0.49$, $p=0.74$). Each bar represents the mean \pm S.E.M. of 9 to 16 litters.

the ANOVA procedure to departures from normality.

To determine if the sex ratio were significantly different from a 1:1 ratio for each of the treatment groups, a χ^2 test was performed. The data satisfied the assumptions of parametric tests and therefore were not transformed. Sex ratios were not significantly different from a 1:1 ratio for any of the treatment groups ($p>0.05$). To determine if any of the treatment groups were significantly different from each other, sex ratios expressed as % female were analyzed using a one-way ANOVA. There was no significant main effect of treatment ($p=0.94$) (Figure 11).

Pup weight gain

The body weight of each pup was measured daily from pnd1-pnd10 and also at pnd21. The average body weights per pup for pnd1-pnd10 and pnd21 were analyzed using 3-way ANOVA with repeated measures with sex, treatment, and time as the main effects. The data did not satisfy the assumptions of normality ($p<0.05$) or homogeneity of variance ($p<0.05$). Plotted residuals displayed a logarithmic pattern, and therefore the data were log-transformed. The data still did not satisfy assumptions of normality or homogeneity. Therefore, pup weights were analyzed separately for males and females and only for pnd1, pnd10 and pnd21. These data did satisfy assumptions of parametric testing. I analyzed the data using a 2-way ANOVA with repeated measures with time and treatment as the main effects. Figure 12 represents the average body weights per pup for each treatment group. On pnd1 the average weight of a pup was approximately 6.5g. By pnd10 the average pup weight increased to approximately 20g. Pup weight gain from pnd1 to pnd21 was not

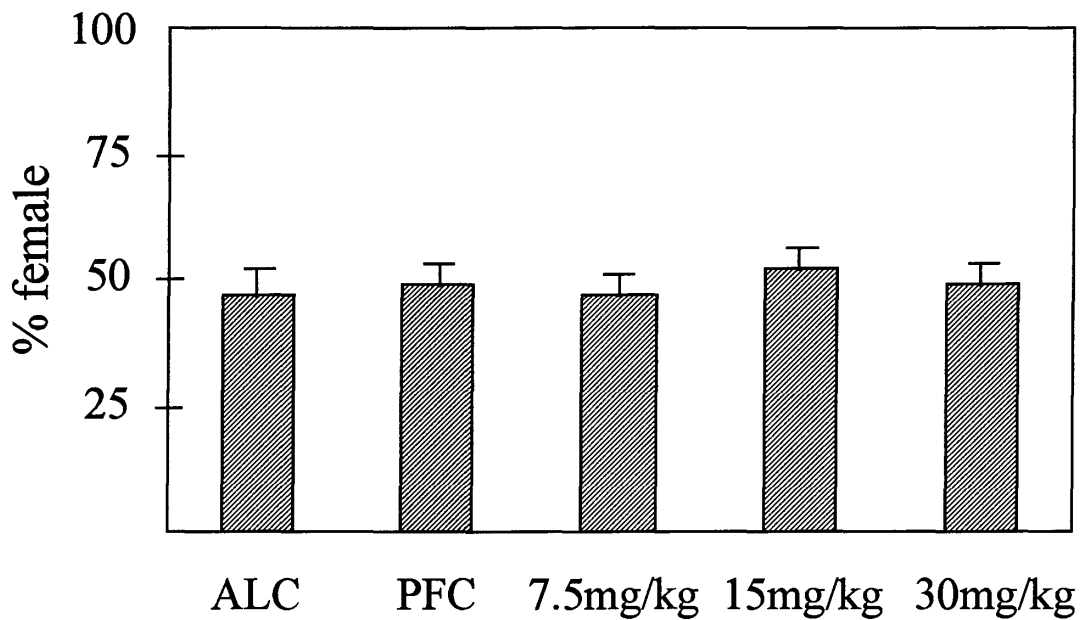


Figure 11. Sex ratio expressed as % female. There was no significant main effect of treatment ($F_{4,47}=0.20$, $p=0.94$). Each bar represents the mean \pm S.E.M. of 9 to 14 litters.

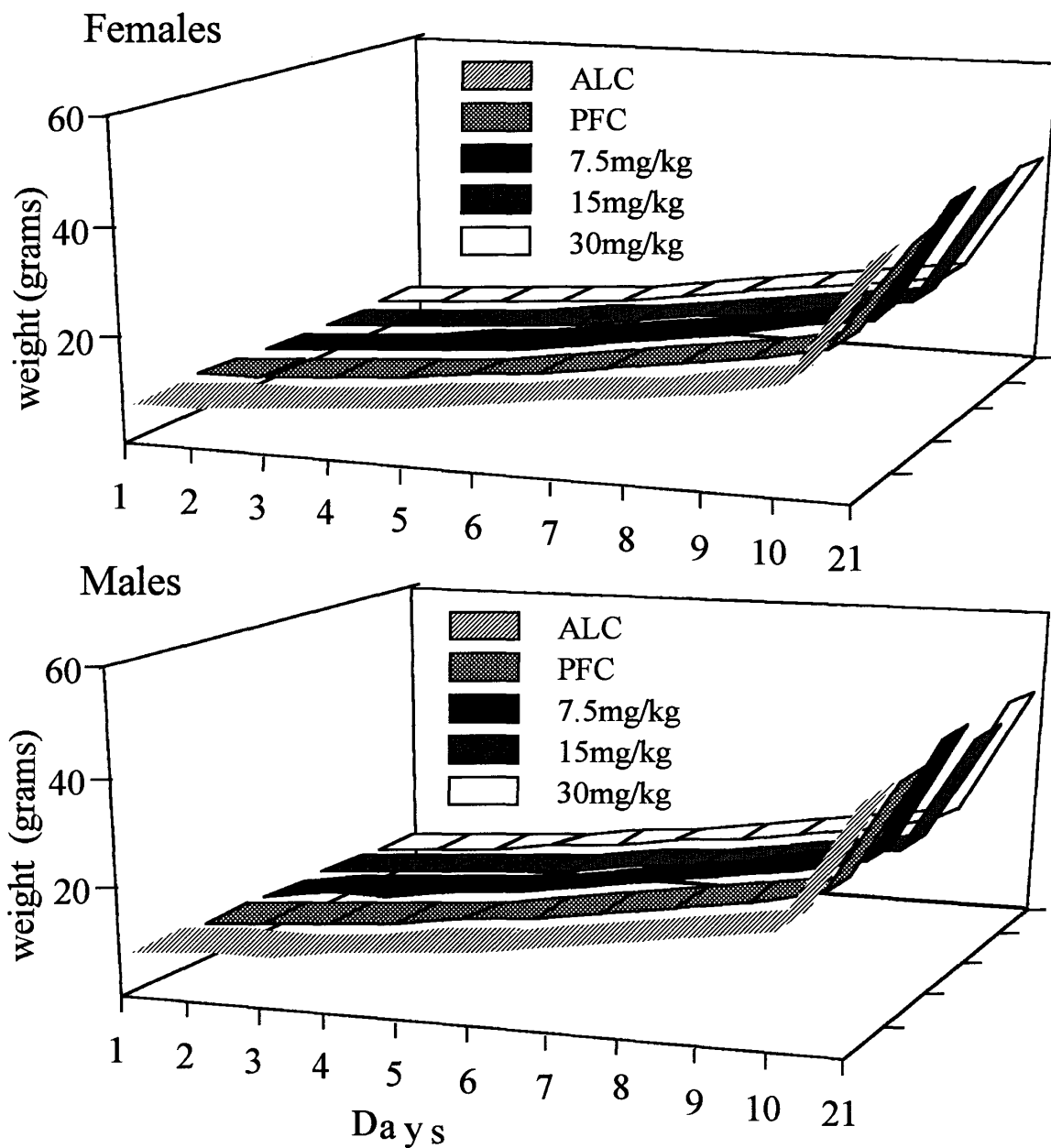


Figure 12. Body weights of male and female offspring, pnd1-10 and pnd21. Separate statistical analyses for each sex were performed on data from pnd1, pnd10, and pnd21. Pup weight gain did not significantly differ among treatment groups for either sex (males, $F_{4,38}=0.34$, $p=0.85$; females, $F_{4,38}=0.86$, $p=0.50$). There was also no significant treatment x time interaction for either sex (males, $F_{8,76}=0.75$, $p=0.65$; females, $F_{8,76}=0.88$, $p=0.54$). There was a significant time effect for both sexes (males, $F_{2,76}=1830$, $p<0.001$; females, $F_{2,76}=1518$, $p<0.001$). Each point represents the mean \pm S.E.M. of 8 to 11 litters.

significantly different among treatment groups for either sex (males, $p=0.85$; females, $p=0.50$). There also was no significant treatment x time interaction (males, $p=0.65$; females, $p=0.54$). There was a significant main effect of time ($p<0.001$) for both sexes due to the overall increase in weight throughout the days measured.

Anogenital Distance

To observe effects of cocaine on a sexually differentiated morphological attribute, I measured the AGDs at pnd1 and pnd21. A significant correlation has been found between weight and AGD, so I adjusted AGD by dividing by the pup weight. At pnd1 and pnd21, these adjusted data satisfied the assumptions of ANOVA and therefore were not transformed.

Figure 13 displays adjusted AGDs that were measured at pnd1 and pnd21. AGDs at pnd1 and pnd21 were analyzed separately, using 2-way ANOVA with sex and treatment as the main effects. At pnd1 there was a highly significant main effect of sex ($p<0.001$) because the males had longer AGDs than the females. There was no significant main effect of treatment ($p=0.68$) or treatment x sex interaction ($p=0.51$) for adjusted AGDs at pnd1.

At pnd21, there was a significant treatment x sex interaction ($p=0.04$) indicating that the sex difference in AGD varied among treatment groups. The AGD in males was 11% greater than in females in the PFC-group, but only 7% greater in the 30mg/kgbw group. Separate 1-way ANOVAs were performed on each treatment group with sex as the main effect. For every group, males had significantly longer AGDs than females ($p<0.05$) except for the 30mg/kgbw group ($p=0.06$). Separate ANOVAs also were performed on each sex with treatment as the main effect. Within males and within females, there was no significant

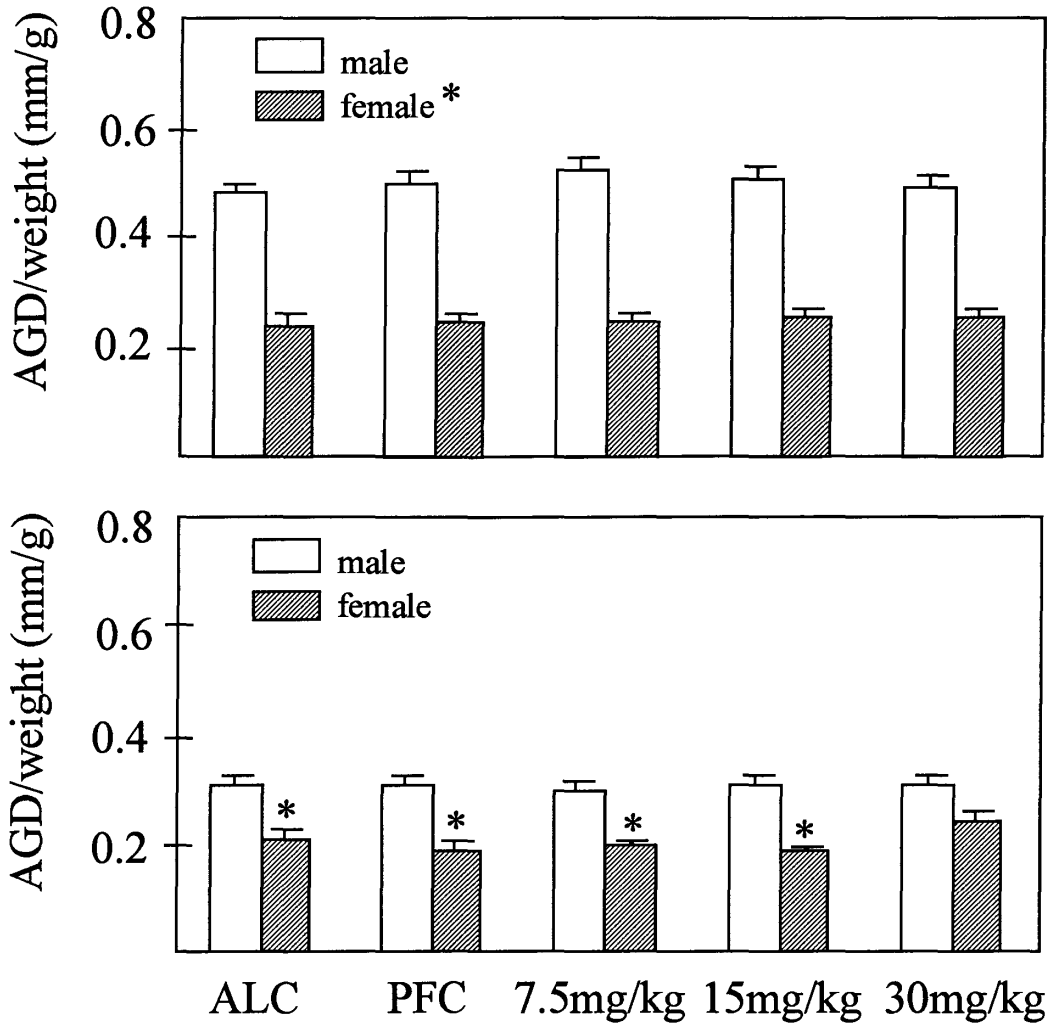


Figure 13. Anogenital distance adjusted for weights on pnd1 and pnd21 for male and female offspring. *For pnd1 there was no significant main effect of treatment ($F_{4,96}=0.58$, $p=0.68$). There was a significant main effect of sex ($F_{1,96}=1007.34$, $p<0.001$). There was no significant treatment x sex interaction ($F_{4,96}=0.83$, $p=0.51$). For pnd21 there was a significant treatment x sex interaction ($F_{4,74}=2.69$, $p=0.04$). *Separate 1-way ANOVAs for each treatment indicated significant differences in AGDs between males and females ($p<0.05$) except for the 30mg/kgbw group ($p=0.06$). Separate 1-way ANOVAs for each sex indicated no significant differences between treatment groups ($p>0.05$). Each bar represents the mean \pm S.E.M of 8 to 15 litters.

difference among treatment groups ($p > 0.05$). All p-values for separate 1-way ANOVAs were adjusted with a Bonferonni correction factor.

Estrous Cyclicity

To ensure that any effect seen on LH levels in females was due to treatment of cocaine and not due to females with abnormal estrous cycles, I performed vaginal lavages from pnd45-60. I determined the vaginal cytology from the lavages, and the data were recorded. Cell types were designated 'l' for leukocytic, 'n' for nucleated, and 'c' for cornified. Females were cycling normally if they exhibited at least 3 consecutive 4- or 5-day estrous cycles. Estrous cycles were indicated by the presence of an overnight conversion from 'c' cells to 'l' cells. There were no significant differences among treatment groups ($p = 0.77$; data not shown).

LH levels in oil-treated females

After perinatal exposure to cocaine or vehicle, pups were allowed to mature. To observe effects of perinatal exposure to cocaine on regulation of LH secretion, the adult female and male rats were gonadectomized and two weeks later treated with either oil or steroid. LH levels of oil-treated females are graphed in Figure 14. The y-axes in all figures related to LH data were set to the same scale in order to facilitate comparisons between the different subsets of data.

Although I had two control groups, ALC and PFC, my graphs display only one control group, the PFC-group. I compared LH levels between the ALC- and PFC-groups

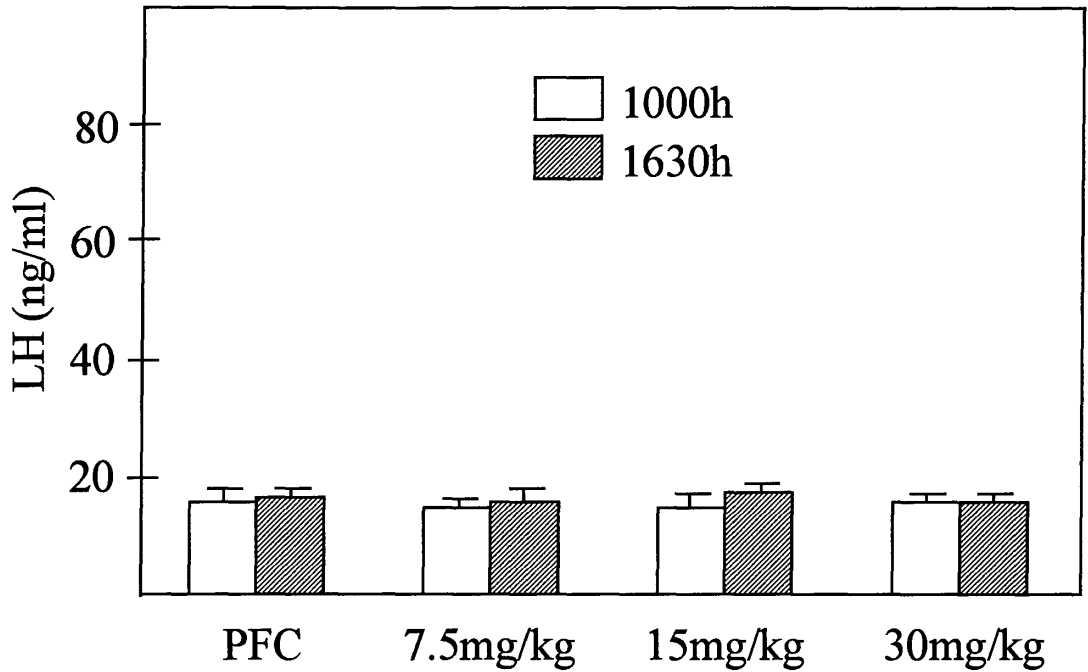


Figure 14. Effect of perinatal exposure to cocaine on LH secretion in oil-treated ovariectomized adult female rats. There was no significant main effect of time ($F_{1,61}=0.77$, $p=0.38$) or treatment ($F_{3,61}=0.21$, $p=0.89$). There was also no significant treatment x time interaction ($F_{3,61}=0.22$, $p=0.88$). Each bar represents the mean \pm S.E.M. of 8 to 10 animals.

using a two-way ANOVA with treatment and time as the main effects, and found no significant difference between the two ($p=0.22$). Therefore, nutrition did not appear to affect LH values for the oil-treated females. All subsequent comparisons of LH levels between the ALC- and PFC-groups used the same statistical analysis.

When analyzing the effect of cocaine on LH regulation I used the appropriate control, the PFC-group. The PFC-group received treatment identical to the cocaine-treated groups except for the injection of cocaine. The PFC-group received injections of vehicle (PBS).

The LH values in the morning were approximately 15ng/ml for all groups. The data satisfied the assumptions of normality and homogeneity of variance and therefore were not transformed. The LH values in the afternoon were not significantly different from the values in the morning ($p=0.38$). There was also no significant treatment effect ($p=0.89$) or treatment x time interaction ($p=0.88$).

LH levels in steroid-treated females

Female steroid-treated animals all exhibited significant LH surges, indicated by a significant main effect of time ($p<0.001$) (Figure 15). The LH values in the morning were approximately 8ng/ml for all treatment groups. The LH values in the afternoon were approximately 8-fold higher than in the morning. The data did satisfy the assumption of homogeneity of variances but were not normally distributed ($p<0.001$). The non-transformed data were analyzed due to the robust nature of the ANOVA procedure. There was no significant treatment effect ($p=0.36$). There was also no significant treatment x time interaction ($p=0.45$), indicating that the pattern of low a.m values and high p.m. values had

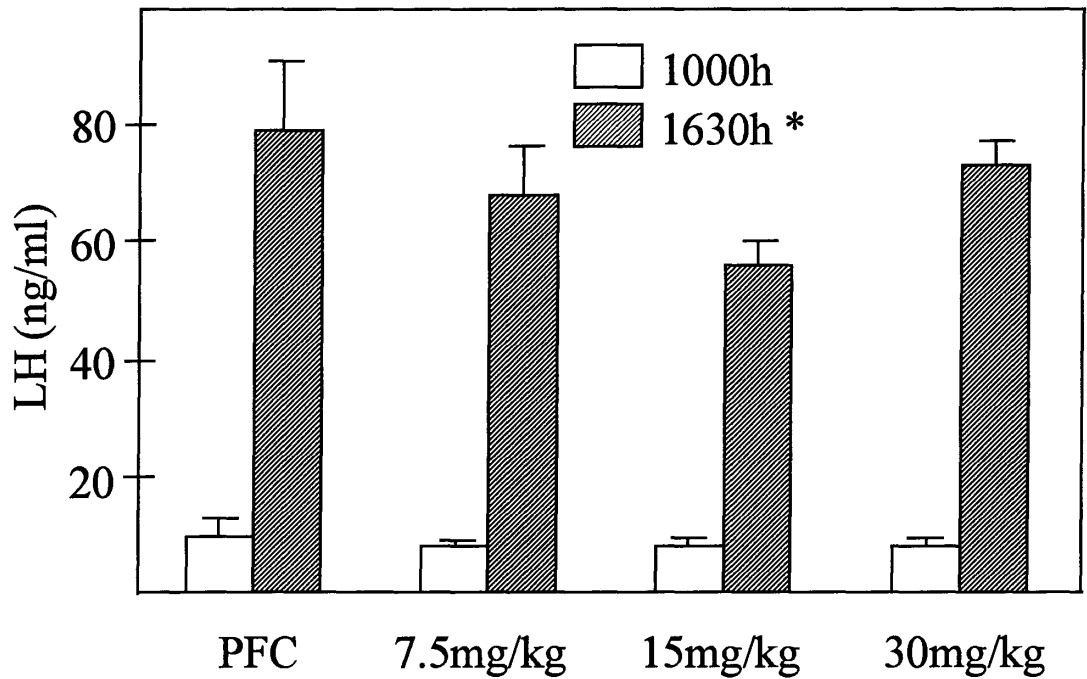


Figure 15. Effect of perinatal exposure to cocaine on LH secretion in steroid-treated ovariectomized adult female rats. *There was a significant main effect of time ($F_{1,63}=248.7$, $p=0.0001$). There was no significant main effect of treatment ($F_{3,63}=1.70$, $p=0.18$) or treatment x time interaction ($F_{3,63}=1.27$ $p=0.29$). Each bar represents the mean \pm S.E.M. of 8 to 11 animals.

a similar appearance in each treatment group. As for the oil-treated females, the ALC-group was not statistically different from the PFC-group ($p=0.42$).

LH levels in oil-treated males

LH levels in oil-treated males are shown in Figure 16. The data did satisfy the assumption of normality but not homogeneity of variance ($p=0.003$). Plotted residuals displayed a logarithmic pattern, therefore the data were log-transformed. After the transformation, the distribution was now not normal ($p=0.02$), but the variances were homogenous. Although the data did not satisfy the assumption of normality, transformed data were analyzed due to the robust nature of the ANOVA procedure. There was a significant time effect ($p<0.001$). The significance can be attributed to the lower LH values in the morning than in the afternoon for all treatment groups. The mean LH values in the morning and afternoon were approximately 14ng/ml and 18ng/ml, respectively. There was no significant treatment effect ($p=0.86$) or treatment x time interaction ($p=0.22$). The ALC-group for the oil-treated males also was not statistically different from the PFC-group ($p=0.90$).

LH levels in steroid-treated males

The data did not satisfy the assumptions of normality ($p=0.006$) or homogeneity of variance ($p<0.001$). Plotted residuals displayed a logarithmic pattern, and therefore the data were log-transformed. Transformed data were normally distributed, and the variance was homogeneous. For steroid-treated males there was also a highly significant time effect

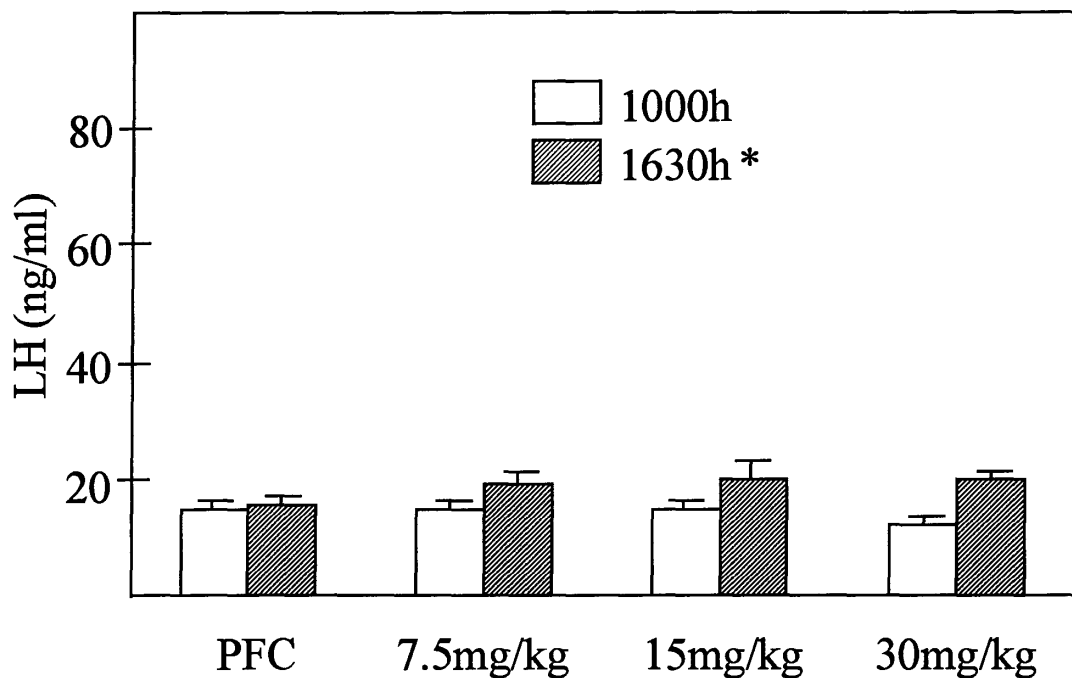


Figure 16. Effect of perinatal exposure to cocaine on LH secretion in oil-treated orchidectomized adult male rats. *There was a significant main effect of time ($F_{1,60}=13.30$, $p=0.0006$). There was no significant main effect of treatment ($F_{3,60}=0.52$, $p=0.67$) or treatment x time interaction ($F_{3,60}=1.14$, $p=0.34$). Each bar represents the mean \pm S.E.M. of 8 to 10 animals.

($p < 0.001$) (Figure 17). The significance can be attributed to the higher LH values in the morning than in the afternoon for all treatment groups. There was no significant treatment effect ($p = 0.50$) or treatment x time interaction ($p = 0.50$). The ALC-group and the PFC-group also were compared in steroid-treated males, and were not significantly different from one another ($p = 0.13$).

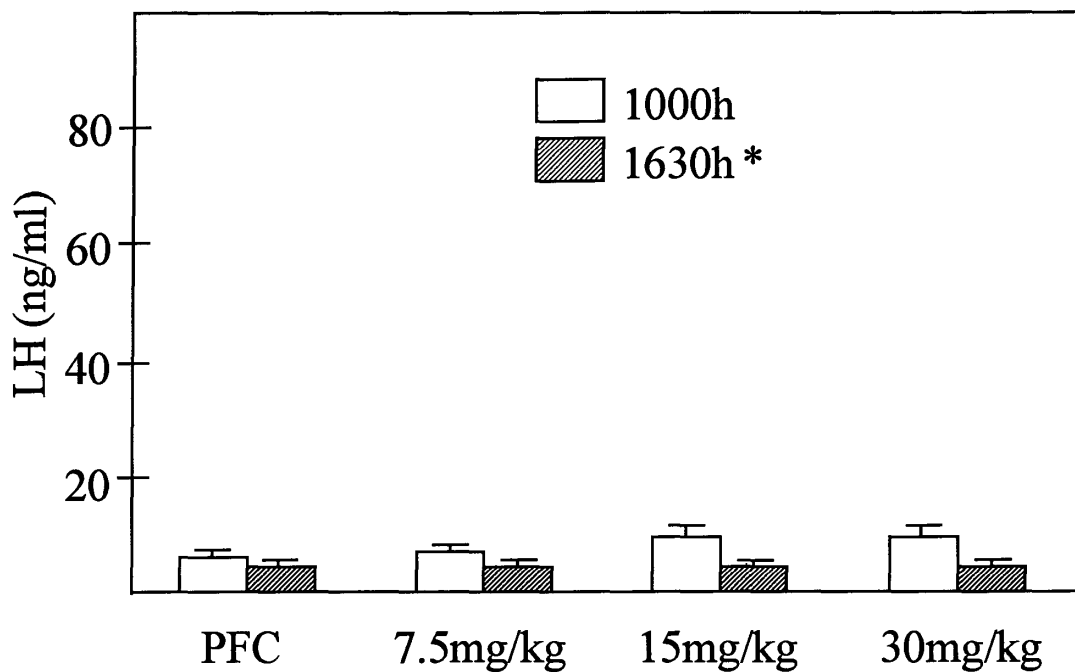


Figure 17. Effect of perinatal exposure to cocaine on LH secretion in steroid-treated orchidectomized adult male rats. *There was a significant main effect of time ($F_{1,61}=32.83$, $p<0.001$). There was no significant main effect of treatment ($F_{3,61}=0.80$, $p=0.50$) or treatment x time interaction ($F_{3,61}=0.79$, $p=0.50$). Each bar represents the mean \pm S.E.M of 8 to 11 animals.

CHAPTER 4

DISCUSSION

The purpose of this experiment was to study the effects of exposure to cocaine during the critical period for sexual differentiation of the brain on regulation of LH, a highly sexually differentiated physiological function. I also was interested in cocaine's effects on maternal weight gain and nutritional intake, as well as, perinatal effects of cocaine on offspring litter size, sex ratio, ano-genital distance, and birth weight and offspring weight gain. Researchers have shown that treatment with cocaine during the critical period for sexual differentiation of the brain can alter sexually differentiated behaviors, such as lordosis (Vathy *et al.*, 1993) and scent-marking (Raum *et al.*, 1990). Researchers have also shown that prenatal exposure to cocaine can alter sexually differentiated glucose utilization in certain areas of the brain (Dow-Edwards, 1988; Dow-Edwards *et al.*, 1990). The ability of cocaine to affect these sexually differentiated processes prompted me to test the hypothesis that perinatal exposure to cocaine during the critical period for sexual differentiation of the brain would alter normal sexually differentiated LH regulation. To test my hypothesis I perinatally exposed rats to cocaine during the critical period for sexual differentiation of the brain. Male and female cocaine-exposed pups were allowed to grow to sexual maturity and as adults were then gonadectomized. Animals were then injected with steroids known to be successful in inducing LH surges in non-cocaine treated ovariectomized adult females.

Morphological Measurements

The subcutaneous dose of 30mg/kgbw, the highest I used, produces plasma levels of cocaine that mimic those in human addicts (Seidler *et al.*, 1995) and has been shown not to cause teratogenic effects or an increase in the mortality of the pups (Church *et al.*, 1990a, b; Dow-Edwards, 1988). To ensure that this dose of cocaine did not cause serious teratogenic effects in my hands, I measured litter size, total birth weight, and individual pup weight. Significant differences in these measurements among treatment groups were not found, nor did I anecdotally observe any morphological abnormalities. Therefore, my results are in agreement with those of other researchers (Church *et al.*, 1990b; Henderson and McMillen, 1990).

Researchers have shown that treatment with cocaine reduces nutritional intake. Therefore, I gave animals in the PFC-, 7.5mg/kgbw-, and 15mg/kgbw-groups exactly the amount of food and water that animals in the 30mg/kgbw-group ate and drank to account for any confounding effects of undernutrition. My data clearly indicate that the treatment of cocaine I administered reduced nutritional intake. Mothers from the ALC-group ate significantly more food than mothers from the 30mg/kgbw-group. I mentioned in the results that the ALC-groups ate approximately 10% more food than the 30mg/kgbw-group. Although, this does not seem like a large amount of nutritional loss, small changes in food consumption do result in altered physiology. Furthermore, reduced nutritional intake translated into reduced maternal weight gain during treatment. This reduced maternal weight gain, however, did not affect litter size or total birth weight. Therefore, the maternal physiology successfully transferred adequate nutrition to the pups, reducing the weight of

the mothers. My results are in agreement with those of other researchers who have administered doses similar to mine to rats (Church *et al.*, 1990b; Henderson and McMillen, 1990). Low birth weight in human babies prenatally exposed to cocaine is a consistent finding (Petitti and Coleman, 1990; Lutiger *et al.*, 1991). I was unable, however, to observe similar results in my rat pups prenatally exposed to cocaine, suggesting that low birth weight in human babies maybe due to some factor or combination of factors other than cocaine, such as multiple drug abuse or a reduced level of prenatal care, or that rats and humans respond differently to cocaine.

In addition to birth weight, I monitored pup weight gain from pnd1-pnd10 and once again at pnd21. I did not observe any effect of cocaine on pup weight gain at any of the days measured. Therefore, cocaine does not appear to delay or impair physical development as neonates or juveniles. Again, my results coincide with those of other researchers administering similar doses of cocaine for similar lengths of time (Church *et al.*, 1990b).

I also measured AGDs of pups and the sex ratio (as determined by AGDs) of the litters. Researchers have shown that the steroidal environment influences sexually differentiated characteristics. The AGD can be altered by altering the hormonal environment during gestation. For example, the AGD in females can be increased by hormones secreted by adjacent male siblings within the uterus (Clemens *et al.*, 1978). Therefore, as a simple (although not necessarily conclusive) indicator of the prenatal hormonal environment, I measured the sex ratio and AGDs of the offspring. The AGD has been shown to be longer in males than females at all ages even after correction for differences in body size. Not surprisingly, at pnd1 I observed males to have longer AGDs than females. At pnd21,

however, I did observe a significant treatment x sex interaction. For all treatment groups males had significantly longer AGDs than females except for the 30mg/kgbw group, in which the AGDs were statistically equal between sexes ($p=0.06$). Although individual ANOVAs indicated no main effect of treatment within a sex, it appeared that the female animals had AGDs that were as long as the male AGDs within the 30mg/kgbw group, suggesting masculinization of this morphological characteristic. Although the p-value of 0.06 approached significance, a power analysis indicated a power >0.99 , indicating the unlikely possibility of not finding a real significance. Furthermore, a recent study by Cutler *et al.* (1996) supports my finding. In that study, at pnd1 AGDs of females prenatally exposed to cocaine were greater than AGDs of those not exposed. These observations were evident on pnd1 whereas mine were not apparent until pnd21. The discrepancy between my results and those of Cutler *et al.* in the time of appearance of cocaine's effect on genital development may partially be explained by the different exposure lengths between the two studies. Cutler *et al.* exposed pregnant females from gestational day 8 to 20. I exposed females later in the gestational period, days 15-22, and further exposed the neonates, from pnd2 to pnd10. Early exposure to cocaine may have a greater impact on the morphological development of the ano-genital region. Therefore, cocaine's effect on AGD may have been more dramatic for pups on pnd1 for Cutler *et al.* than for the pups in my experiment. Since I began cocaine injection on D15 and continued until pnd10, cocaine's effect on AGD in my study may have been subtle on pnd1 with no apparent effect until pnd21. The exact mechanism by which cocaine affects genital development is not known. Cocaine may alter the neuroendocrine systems which may indirectly interfere with or alter genital development.

Cocaine does cause increased levels of DA, which can result in prolactin levels to fall since DA is inhibitory to prolactin release. And since prolactin does inhibit LH secretion, lower levels of prolactin may result in higher levels of LH which would stimulate steroid production (Kalra and Kalra, 1983). Higher levels of testosterone secretion by male fetal gonads may be sufficient to masculinize the AGD of females.

LH regulation

Oil-treated females had LH levels that did not differ between morning and afternoon. These results were not surprising since I did not expect to observe an oil-induced LH surge. The oil-treated control females did not differ from the cocaine-treated females. I also did not observe LH surges in oil-treated males and the control group did not differ from the cocaine-treated groups. I did, however, observe a significant time effect in oil-treated males. LH levels were greater in the afternoon than in the morning for all treatment groups, suggesting a diurnal pattern. Others have observed diurnal variations in LH secretion in non-orchidectomized males (Dunn *et al.*, 1972; Taya and Igarashi, 1974; Huhtaniemi *et al.*, 1982). Peak levels of LH were observed at various times but the ranges of time were generally between late evening and early morning (2300-0300h). Others have also reported observing an absence of diurnal secretion of LH in non-orchidectomized (Shin and Kraicer, 1974; McLean *et al.*, 1977; Kalra and Kalra, 1977) and orchidectomized males (Yamamoto *et al.*, 1970; Henderson *et al.*, 1977). So, it is still not well established whether LH in males is secreted in a diurnal pattern. I collected blood samples at limited time points, and further experiments would need to be performed in order to make a more conclusive argument. My

data do suggest, however, that a diurnal rhythm of LH exists in males.

I observed normal male-typical regulation of LH in the steroid-treated PFC control group. Normal sexual differentiation of the male brain renders it unable to respond in a positive feedback manner to high levels of estrogen with enhanced secretion of LH. Upon steroid treatment, males in the PFC-group exhibited normal negative feedback. Control males treated with steroids had lower LH levels in the afternoon compared to the morning, which can be attributed to the injection of progesterone on the morning of blood sampling. The control males did not differ from the cocaine-treated males. Therefore, perinatal exposure to cocaine does not appear to alter regulation of LH in adult male offspring.

Normal females in the PFC-group did exhibit LH surges upon steroid stimulation. Normal sexual differentiation of the female brain renders it able to respond positively to the steroid treatment with enhanced secretion of LH late in the afternoon. The cocaine-treated females responded to the steroid treatment with LH surges that were not significantly different from the control females. Therefore, perinatal exposure to cocaine does not appear to alter gonadotropin regulation in adult female rats.

I had hypothesized that masculinization would be blocked in cocaine-treated males, and thus that steroid-induced LH surges would be demonstrated in orchidectomized cocaine-treated males. My reasoning followed two lines of evidence. First, Raum *et al.* (1990) have shown that prenatal exposure to cocaine demasculinized at least one male behavior, scent-marking; and Dow-Edwards has shown that prenatal exposure to cocaine demasculinized glucose utilization in the brains of adult male rats (Dow-Edwards, 1988; Dow-Edwards *et al.*, 1990). Second, NE, a NT strongly affected by cocaine, is also the same NT involved in

LH regulation in adults. Lauder and Krebs (1986) have suggested that NTs direct embryological development of systems they will control in adulthood.

Adrenergic receptors located on postsynaptic neurons are thought to be involved in development and sexual differentiation of the brain. Raum *et al.* (1984) showed that neonatal stimulation with a β -adrenergic agonist reduced hypothalamic nuclear content of estradiol, the active hormone involved in masculinization of the brain. Dohler *et al.* (1991) have also shown that neonatal treatment with a β -agonist, isoprenaline, in males and females increased steroid-induced adult lordosis behavior compared to controls. Therefore, stimulation of β -receptors with exogenous agonists appears to hyperfeminize sexual behaviors in females, and to feminize the male brain, enabling males to perform female-typical lordosis behavior in response to steroid treatment. NE is a β -receptor stimulator; therefore, increases in NE concentrations or hyperavailability within the synaptic cleft due to perinatal cocaine exposure may also result in reduced estradiol content and at least partial feminization of the brain. Further studies by Raum *et al.* (1990) have suggested that prenatal exposure to cocaine demasculinized at least one male behavior, scent-marking, possibly due to reduced nuclear uptake of steroids, stimulated by β -receptor activation during the critical period.

As my results show, LH surges were not steroid-inducible in adult male rats perinatally exposed to cocaine. Seidler *et al.* (1995) have recently shown that adrenergic activity is actually inhibited in rats neonatally treated with the same dose of cocaine used in my study. Compared to untreated rats, cocaine-treated rats had reduced NE turnover, expressed as the fraction of NE lost after treatment with a catecholamine synthesis inhibitor.

If less NE were lost in cocaine-treated animals compared to controls, this would suggest that adrenergic activity is reduced in the cocaine-treated animals. Therefore, β -receptors do not appear to be hyperstimulated but just the opposite. This finding would suggest that less feminization, not more, should occur in cocaine-exposed males.

Although the findings by Seidler *et al.* appear to contradict the findings by Raum *et al.*, a closer look at the variation in their methods needs to be taken. The length of exposure to cocaine differed between these two experiments. Both administered cocaine subcutaneously, but Raum *et al.* exposed animals prenatally (D15-D20) as well as postnatally (pnd1-5), and Seidler *et al.* administered cocaine only postnatally (pnd1, pnd7, pnd14, and pnd21). Another discrepancy is the specificity of the area of the brain in which measurements were taken. Raum *et al.* measured nuclear uptake of steroids specifically in the hypothalamus. Seidler *et al.* measured fractional turnover of neurotransmitters in the entire forebrain, which includes the hypothalamus along with other regions of the brain. In terms of the length of exposure to cocaine, my experimental protocol more closely followed that of Raum and his colleagues. In addition, I focused on the effect of cocaine on LH secretion, which is regulated by the hypothalamus. Therefore, conclusions drawn from Raum *et al.* seem to be more pertinent to my experiment than those from Seidler *et al.*

A possible explanation as to why I did not observe steroid-induced LH surges in males perinatally treated with cocaine has to do with the nature of the surge mechanism. LH surges can be steroid-induced in normal ovariectomized adult female rats. Crowley demonstrated that, prior to the LH surge, a sequential accumulation followed by a decline in luteinizing hormone-releasing hormone (LHRH) occurs. He also demonstrated an

increase in turnover rates of NE concomitant with the accumulation of LHRH (Crowley, 1988; Crowley and Kalra, 1994). LH surges cannot be steroid-induced in normal orchidectomized adult male rats, and the changes in LHRH and NE triggered by steroid treatment in females were not observed in similarly treated male rats. Adult ovariectomized female rats that were neonatally treated with estradiol did not respond to the steroid treatment as adults; thus, no LH surges were observed. Nevertheless, all the female-typical changes in LHRH and NE prior to an LH surge were observed in these “masculinized” females. Therefore, estradiol may be responsible for the uncoupling of excitatory influences of NE and LHRH from secretion of LH, but some other factor may be responsible for the lack of adrenergic activity in response to steroidal treatment in males (Crowley and Kalra, 1994). Crowley suggests these other factors may include testosterone itself or some other non-estrogenic metabolite. The capacity to have LH surges seems to require a number of necessary aspects and to compromise any one of them disables the entire surge mechanism. Crowley and Kalra (1994) have shown that although the females neonatally exposed to estradiol exhibited many aspects of an LH surge in non-treated females, they were unable to have an LH surge. Therefore, it is possible I was able to feminize some aspects of my cocaine-treated males, but not all the necessary aspects needed for LH surges. This may explain why I was unable to induce LH surges in my male rats that were perinatally exposed to cocaine.

Crowley *et al.* also bring up an interesting point regarding the exact roles of estrogen and testosterone in masculinization. If testosterone is responsible for the lack of adrenergic stimulation upon steroid treatment in males, then estradiol may not be responsible for *all*

masculinizing effects during the critical period. I did not gonadectomize my male rats during the critical period for sexual differentiation of the brain. Therefore, the presence of testosterone may have been sufficient to cause a lack of adrenergic stimulation upon steroidal treatment in adult males. LH surges can be steroid-induced in adult male rats that have been orchidectomized at birth or shortly after birth (McPherson *et al.*, 1982, Weiland and Barraclough, 1984, Corbier, 1985). So it is possible to induce LH surges in male rats but it requires the removal of androgens during the sensitive period of masculinization, providing further evidence that testosterone itself may play a role in masculinization of the brain. I should mention that these were the *only* references that demonstrated LH surges in males compared to the large number of papers on induced anovulatory syndrome in females. Therefore, the induction of LH surges in males appears to be more difficult, requiring orchidectomy prenatally or early neonatally. This interpretation of the literature is consistent with the theory suggested above, that feminization of LH regulation is a more intricate process than feminization of many behaviors.

Evidence does exist on the differential roles of androgen and estrogen in sexual differentiation of the brain. Studies have been conducted using 5 α -dihydrotestosterone (DHT), a non-aromatizable androgen. Valencia *et al.* (1992) have demonstrated that postnatal administration of DHT to male pups reduced the volume of the rat accessory olfactory bulb, a sexually dimorphic structure, to a volume not different from normal females. Therefore, at least for this characteristic, DHT appeared to demasculinize the male brain of rats. Tonjes and Dorner (1987) have shown postnatal administration of DHT to female pups increased sexually differentiated prepubertal play behavior to levels not different

from normal juvenile males. Administration of EB to female pups, however, did not alter prepubertal play behavior. Therefore, estrogen and androgens may be responsible for different aspects of sex-specific brain differentiation.

The ability of cocaine to alter certain sexually differentiated behaviors and not certain sexually differentiated physiological phenomena, in my case LH regulation, suggests a theory of the process of sexual differentiation. Various sexually differentiated behaviors are likely to be influenced by different areas of the brain. A particular female-typical behavior may be controlled by certain areas of the brain, while a particular male-typical behavior may be controlled by others. In the brain of a female, areas regulating female-typical behaviors would develop, while the areas regulating male-typical behaviors would degenerate in some sense, resulting in a genetic female with female-typical behaviors. The opposite would also be true for males.

An important example of such a process of development can be found in other areas of the body. Within the abdominal cavity of the undifferentiated fetus lie the Wolffian and Mullerian ducts, which differentiate into the male and female internal genitalia. In the presence of the testosterone, the Wolffian ducts develop into male internal genitalia and secrete mullerian-inhibiting hormone (MIH), which causes regression of the Mullerian ducts. In the absence of testosterone, the Wolffian ducts regress and MIH is not secreted. Therefore, the Mullerian ducts develop (Diagram 3). In the internal genitalia model, it is possible for both sets of organs to exist simultaneously. For example, in a genetic male with normal testes, the Wolffian ducts will develop. If, however, the gene for MIH is non-functional, MIH would not be secreted, and therefore the Mullerian ducts would develop

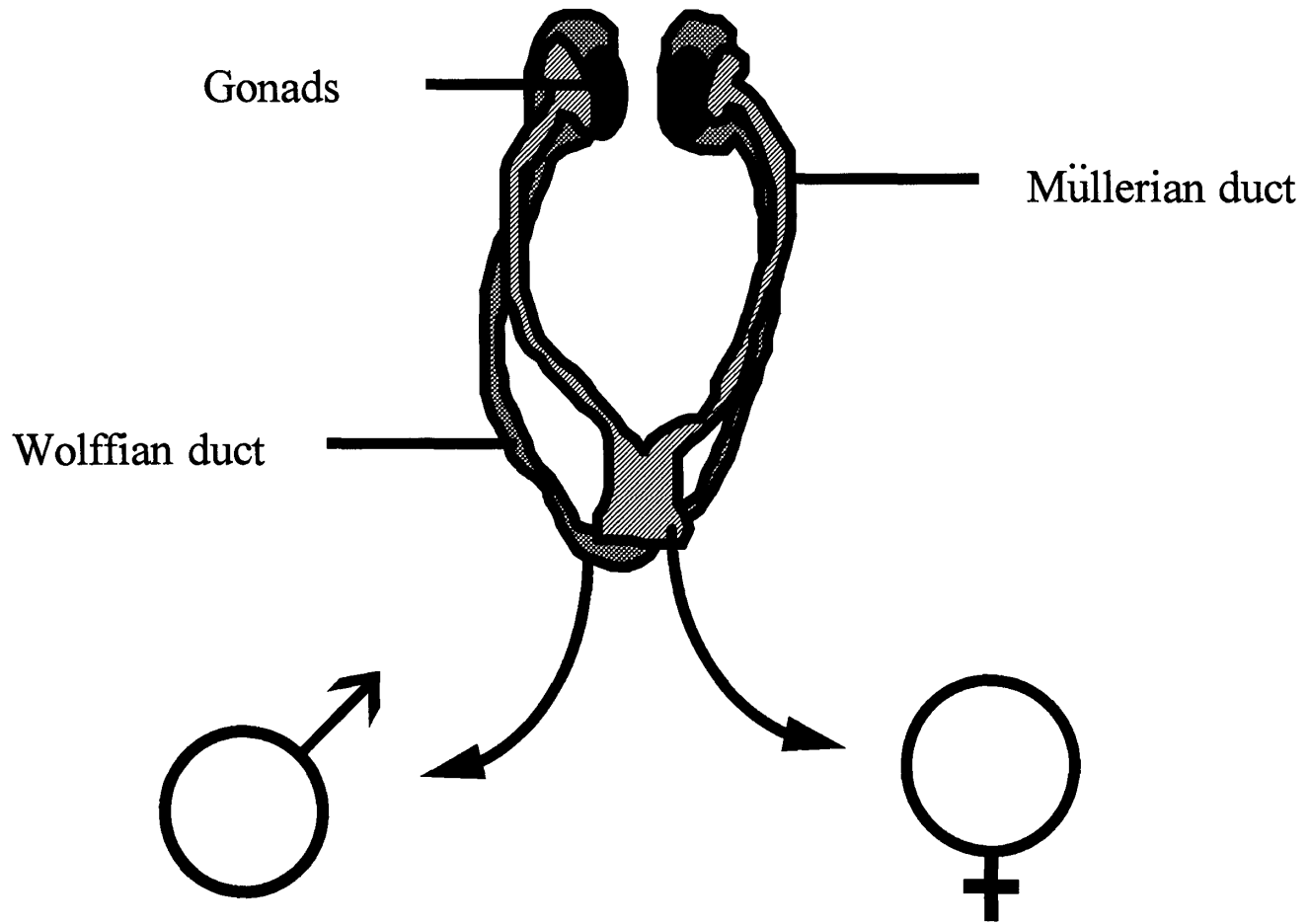


Diagram 3. Differentiation of the internal genitalia.

along with the Wolffian ducts. In development of the internal genitalia, two separate structures are present and in the presence of the appropriate signals either the female or the male structures develop while the others regress. If this is also the case for development of brain structures controlling sexually differentiated behaviors as I have suggested, then the capacity to exhibit both male- and female-typical neural events in a single animal would be possible.

The process of development is quite different, however, in the external genitalia. The external genitalia of males and females develop from identical sexually indifferent embryonic structures. In the presence of testosterone the urogenital fold, genital tubercle, and labioscrotal swelling differentiate into the shaft of the penis, glans penis, and the scrotum. In the absence of testosterone the undifferentiated external genitalia become the labia minora, clitoral glans, and labia majora of females (Diagram 4). Unlike the internal genitalia, the existence of both male and female external genitalia is not possible since both differentiate from the same structures. There are cases where the external genitalia do not fully develop into the male or female structures. These structures have an ambiguous appearance that is somewhere between fully differentiated male and female structures. As in the external genitalia model, some areas of the brain controlling LH regulation may develop by a similar process. LH regulation may be controlled by one area of the brain that is the same for both males and females. In the presence of testosterone, LH release displays a tonic pattern typical of males. In the absence of testosterone, LH release displays a cyclic pattern typical of females. The external genitalia model suggests that a certain structure is basically neutral until a signal triggers which path of differentiation will be taken. If this is

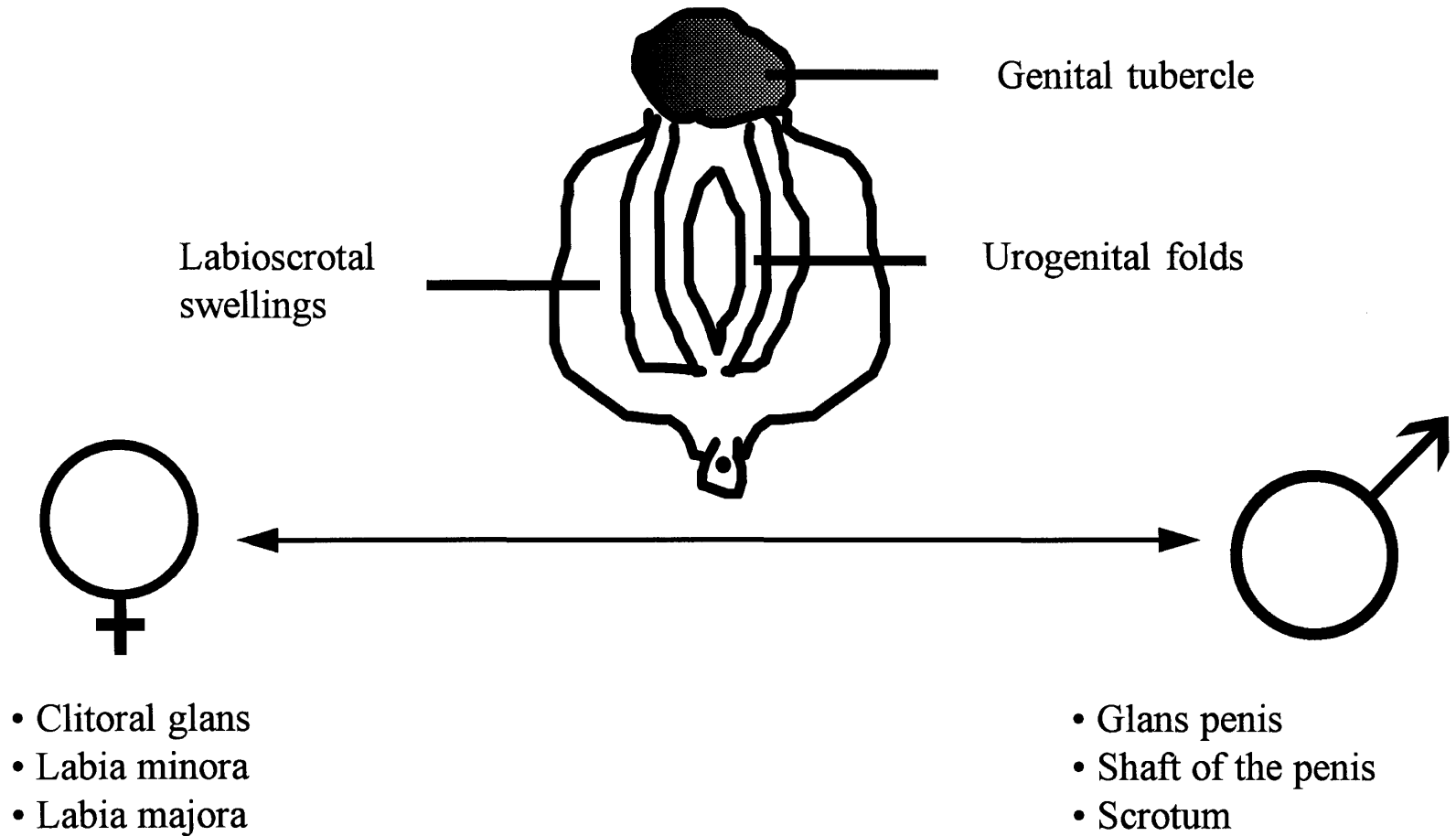


Diagram 4. Differentiation of the external genitalia.

the case for the development of structures of the brain controlling LH regulation, the capacity to generate both male and female patterns of LH release in a single individual is not possible. Like the occasionally ambiguous structures of the external genitalia, however, a kind of LH regulation might exist somewhere on a spectrum between the extremes of a fully differentiated male brain on one end and a fully differentiated female brain on the opposite end.

These two models suggest a more complex model for development of sexually differentiated behaviors and physiological processes, both contributing to a common endpoint. I propose that separate sexually differentiated behaviors occur because separate neural structures develop in the brains of males and females. Sexually differentiated LH regulation, however, occurs because a single set of structures develops differently in males and females. Since the basic model of development for sexually differentiated characteristics may be different, different stimuli may have different effects on behavior and endocrinology. This theory could explain the ability of cocaine to affect behavior but not LH regulation. Vathy *et al.* (1993) have shown that prenatal exposure to cocaine (20mg/kgbw) did reduce female sexual behavior, but did not alter estrous cyclicity in female rats. Both cocaine-treated and control females had similar 4-5 day estrous cycles. I also observed no alterations in estrous cyclicity and further demonstrated that the capacity of females to respond to steroidal positive-feedback stimulation was not different between control and cocaine-treated rats, because the intensity of the LH surge was not different among treatment groups. Dohler *et al.* (1991) have shown that neonatal treatment with L-tryptophan, a 5-HT precursor, reduced the capacity for lordosis behavior but had no effect on LH regulation, demonstrating

again the ability of a given stimulus to have different effects on various aspects of sexual differentiation. Therefore, the developmental events that cause sex differences in behavior and LH regulation may share some common elements, but may not be identical. In summary, my experiment suggests that sexual differentiation of LH regulation may occur by a different mechanism from sexual differentiation of behaviors. This model would explain why some behaviors are affected by treatment with cocaine but LH regulation is not.

The purpose of my experiment was to study the effects of exposure to cocaine on sexual differentiation of the brain. The increased use of cocaine by pregnant women emphasizes the need to understand better the effects of cocaine on the developing fetus. I have shown that cocaine does not appear to affect sexually differentiated LH regulation in the developing rat. This result suggests that, although sexual differentiation of some behaviors may be affected by prenatal exposure to cocaine, sex differences in basic physiology are not compromised. I mentioned earlier that SDN-POA of the rat brain appears to be influenced by endogenous steroid up until postnatal day 29, suggesting I may have not exposed my pups long enough to cocaine's effects. In terms of LH regulation, inducing LH-surges in males is only possible if the gonads are removed neonatally. Therefore, this suggests that LH regulation is established during the time I exposed my pups to cocaine. Understanding the complexities of sexual differentiation of the brain in humans or in rats is far from complete and further research needs to be done. Sexual differentiation seems not to be as simple as past models have suggested. Many questions remain unanswered, including the exact roles of estrogen and testosterone, the interactions between NTs and steroids, the possibility of different critical periods for different areas of the brain, and which

specific areas of the brain control specific behaviors and physiological functions. Future experiments regarding cocaine's effect on sexual differentiation may provide more insight into sexual differentiation of the brain as a whole as well as possible options to protect developing fetuses exposed to cocaine.

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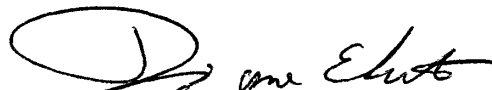
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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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