

EFFECTS OF ADRENERGIC DRUGS AND MONOSODIUM
GLUTAMATE ON THE DEVELOPMENT OF SEX-
DEPENDENT CYTOCHROME P450 SYSTEM
IN MALE RAT LIVER

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CIDA ZHAO



EFFECTS OF ADRENERGIC DRUGS AND MONOSODIUM GLUTAMATE
ON THE DEVELOPMENT OF SEX-DEPENDENT CYTOCHROME P450 SYSTEM
IN MALE RAT LIVER

by

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A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements
for the degree of Master of Science

Interdisciplinary Programme in Toxicology
Memorial University of Newfoundland

June, 1994



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ISBN 0-612-01934-9

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In memory of my grandmother

ABSTRACT

The effects of adrenergic drugs on the development of male-specific cytochrome P450 in the male rat liver were studied. Pups, castrated on Day 0, were given (icv) α -antagonist or β -agonist with or without testosterone propionate (sc.), and assayed on Day 65. Other pups were administered with (icv) adrenergic agonists or antagonists on Day 0 & 3 of life, castrated on Day 55, and assayed on Day 70. Neither α -antagonist nor β -agonist defeminized the EMDM activity in neonatally castrated rats. In the rats castrated on Day 55, β -antagonist and α_1 -antagonist superdefeminized the EMDM activity. β -antagonist increased the total amount of P450 but α_1 -antagonist did not. In addition, the V_{max} of EMDM in β -antagonist-treated animals was increased; the K_m was unchanged. Further, β -antagonist stimulated the activities of 16 α -, 2 α -, and 6 β -testosterone hydroxylases in the superdefeminized rats. However, both β -antagonist and α_1 -antagonist, given simultaneously, suppressed the superdefeminization of EMDM activity.

The effect of monosodium glutamate (MSG) on the prepubertal development of male-specific cytochrome P450 in rat liver were also studied. Pups were treated on Day 1-9 with 2 or 4 mg/g MSG (sc), and on Day 35 the EMDM activities

in 2 and 4 mg/g MSG-treated groups were the same as control values. However, MSG stimulated, dose-dependently, the EMDM activity on Day 65. In addition, Vmax of EMDM was elevated in MSG-treated group; the Km was unchanged. Finally, 4 mg/g MSG stimulated the activity of 6 β - but not 16 α /2 α -testosterone hydroxylases.

ACKNOWLEDGMENTS

I wish to express my special thanks to Dr. Bruce B. Virgo, my supervisor, for his initial direction in selecting a research area, and his assistance and careful guidance throughout the research project and during preparation of the manuscript. I am also grateful to the members of my thesis committee, Drs. C. Loomis and D. Bieger, for their advice.

In addition, financial support from School of Pharmacy, Memorial University of Newfoundland is gratefully acknowledged.

Finally I am indebted to my parents who have given me my life, education, and more than I can say. I love them both dearly. My thanks are also due to my brother and sisters for their support and understanding.

ABBREVIATION

SHTP, 5-Hydroxytyptophan
ACTH, Adrenocorticotropic Hormone
AFP, α -Fetoprotein
AMN, Amygdaloid Nucleus
ARCN, Arcuate Nucleus
cAMP, Cyclic Adenosine 3',5'-Monophosphate
CLO, Clonidine
CNS, Central Nervous System
DAG, Diacylglycerol
EMDM, Ethylmorphine Demethylase
G-6-P, Glucose-6-Phosphate
G-6-P-DH, Glucose-6-Phosphate Dehydrogenase
GH, Growth Hormone
GHRH, Growth Hormone-Releasing Hormone
icv, Intracranial Injection
IP₃, Inositol-1,4,5-trisphosphate
ISO, Isoproterenolol
MPOA, Medial Preoptic Area
mRNA, Messenger Ribonucleic Acid
MSG, Sodium Glutamate
NADP, β -Nicotinamide Adenine Dinucleotide Phosphate
NADP, β -Nicotinamide Adenine Dinucleotide Phosphate
NADPH, Reduced Nicotinamide Adenine Dinucleotide Phosphate

p-NPH, p-Nitrophenol Hydroxylase
PAS, Permanent Anovulatory-Sterility
PCPA, p-Chlorophenylalanine
PH, Phentolamine
PHY, Phenylephrine
PRA, Prazosin
PRO, Propranolol
sc, Subcutaneous Injection
SDN-POA, Sexually Dimorphic Nucleus
SHS, Shaft Synapses
SS, Somatostatin
T₄, Thyroxone
TP, Testosterone Propionate
VMN, Ventromedial Nucleus
WY, WY 27127

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1.0 INTRODUCTION

Cytochromes P450 are a superfamily of heme proteins. These cytochromes are found mainly in the liver, but they also occur in other tissues, such as lung, kidney, intestine, skin, testis, placenta, and adrenal (Sipes and Gandollfi, 1991). These proteins are parts of a system that oxidizes steroids, vitamins, fatty acids and many xenobiotics such as drugs, pesticides. The cytochromes P450 are the terminal oxidase and substrate binding enzymes in some 30 different monooxygenase reactions that include aliphatic hydroxylation, aromatic hydroxylation, deamination, and N-hydroxylation, etc.(Sipes and Gandollfi, 1991).

The cytochrome P450 catalytic cycle is presented in Figure 1: the xenobiotic combines with the oxidized form of cytochrome P450 (Fe^{3+}) to form a substrate-cytochrome P450 complex. This complex accepts an electron from NADPH via NADPH-cytochrome c reductase, which reduces the iron in the cytochrome P450 heme to the Fe^{2+} state. The reduced complex then combines with one molecule of oxygen, which accepts another electron from NADP. This molecular oxygen radical is highly reactive and unstable and one atom of it is introduced into the substrate, while the other is reduced to water. Then the oxygenated substrate dissociates, regenerating the oxidized form of cytochrome P450.

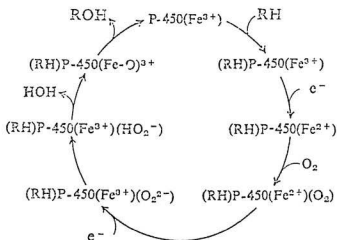


Figure 1. Cytochrome P450 Catalytic Cycle
 [modified from: Robert AN (1980). "Metabolism of toxic substances." In: Casarett and Doull's Toxicology, the Basic Science of Poisons, 2nd ed. edited by Doull J, Klaassen CD, and Amdur MO, Macmillan Publishing Co., Inc. New York]

The activity of cytochrome P450 mediated reactions can be influenced by factors such as diet (Anderson and Kappas, 1991), hormones (Lund et al., 1991), peptides (Urban et al., 1990), chemicals (Bock et al., 1990), sex (Kato, 1974), and age (Waxman et al., 1985). Alterations in the activity of cytochrome P450 will influence the normal physiological metabolism and the metabolism of drugs and other xenobiotics; which may interfere with the normal physiological functions in the body and induce pathogenesis. Therefore, studies on cytochrome P450 are important in toxicology. This thesis is an attempt to examine the role of neonatal adrenergic receptors in the central nervous system in the defeminization of hepatic male-specific cytochromes P450, and the role of growth hormone (GH) in the prepubertal development of hepatic cytochrome P450 in male rats.

1.1 HEPATIC CYTOCHROME AND BRAIN SEXUAL DIFFERENTIATION

1.1.1 Sexual Differences of Hepatic Cytochrome P450

Oxidations

Oxidation of a number of xenobiotics and steroids in the adult rat liver is sex specific. For example, female rats given hexobarbital sleep longer than males (Holck et al.,

1937) and similar results had also been shown by other authors (Quinn et al., 1958). Microsomes from the liver of adult male rats oxidize ethylmorphine and benzo(a)pyrene more rapidly than do those from the females (Chung et al., 1975; Davies et al., 1969; Gurtoo and Parker, 1976; Hietanen, 1974), and although microsomes from the livers of adult male rats have 10%-30% more cytochrome P450 than those from females (Chung, 1977; Colby, 1980), this difference does not account for the differences in the rates of metabolism of these xenobiotics (Kamataki et al., 1980; Kato, 1974). Moreover, other studies (Kato and Kamataki, 1982; Kamataki et al., 1982; MacGeoch et al., 1984; Morgan et al., 1985) showed that isoforms of hepatic cytochromes P450 were sexually differentiated in adult rats and that the existence of these sex-specific isoforms, such as cytochrome P450 2C11, 2C12, or 3A1/2, leads to the sexual differences in the substrate metabolism.

1.1.2 Sexual Differences of Cytochrome P450 and Neonatal Androgen

These sexually different activities of cytochrome P450-catalyzed oxidations are the result of neonatal and pubertal exposure of the rat to androgen. Castration of male rats in the neonatal period decreases ethylmorphine N-demethylation,

propoxycoumarin O-depropylation and benzo(a)pyrene hydroxylation to the female level (Shimada et al., 1987); neonatal administration of castrated male pups with testosterone increased the activities of the enzymes but not up to the levels of the normal adult males. However, others found that the activity of ethylmorphine demethylase (EMDM) in neonatal castrated male rats, treated neonatally and in adulthood with testosterone, was the same as that in intact males (Reyes and Virgo, 1988). The male isoforms of cytochromes P450 in adult male rats castrated neonatally are barely detectable while the female-specific isoforms appeared at a level comparable to those found in adult females (Kamataki et al., 1983, 1984; Kato and Kamataki., 1982; Waxman et al., 1985; Jansson et al., 1985a). Moreover, the administration to these animals of neonatal and postpubertal testosterone caused the appearance of male-type cytochromes P450 and the disappearance of the female types (Shimada et al., 1987). It appears that the sexually different cytochrome P450 enzymes are the results of neonatal and post-pubertal exposure of the rat to androgen.

Sexual differentiation of cytochrome P450 enzyme is believed to result from two distinct androgen-dependent processes: i) defeminization, which suppresses female

characteristics, permitting male characteristics to develop, and occurs during the neonatal period, and ii) masculinization, which enhances the male characteristics in adulthood (Reyes & Virgo, 1988). Furthermore, defeminization is thought to be a necessary prerequisite for the masculinization (MacLusky and Naftolin, 1981). Neonatal castration completely and irreversibly depresses the activities of ethylmorphine demethylase in adult males, but the enzyme activities can be permanently increased, albeit not to the levels in intact adult males, if these animals are given testosterone immediately after castration (Virgo, 1991). Adult castration, however, decreases the enzyme activities to the defeminized levels, which can be masculinized by testosterone. Thus, these data show that neonatal androgen permanently defeminizes the enzyme system, and pubertal androgen masculinizes the defeminized enzymes but not permanently. However, neonatal exposure of the rat to androgen is not an essential prerequisite for the sexual differentiation of drug metabolism. Both Shimada (1987) and Virgo (1991) have reported that masculinization can occur in the absence of defeminization. For example, neonatal castration completely suppressed the activities of the hepatic enzymes in adult male rats, but the levels of ethylmorphine N-demethylation (Shimada et al., 1987; Virgo, 1991),

propoxycoumarin O-depropylation and benzo(a)pyrene hydroxylation (Shimada et al., 1987) were increased to the levels of intact adult male rats by peripubertal testosterone treatment. These data suggest that neonatal androgen is not necessary for the effects of pubertal androgen though it can permanently defeminize the enzyme system. However, older adult male rats (Day 75-80) castrated neonatally do not respond to testosterone (Virgo, 1991), and female rats also do not respond to either neonatal or adult testosterone; the reasons for these phenomena are not yet known.

1.1.3 Sex Differences of Hepatic Cytochrome Oxidations and the Secretory Pattern of Growth Hormone

It was postulated originally that the female pituitary secretes a hormone, "feminotropin", that feminizes the female cytochrome P450 system (Gustafsson et al., 1977), and that "feminotropin" release is blocked in the male by a hypothalamic hormone, "feminostatin" (Gustafsson et al., 1976; Gustafsson et al., 1977). By investigating different pituitary hormones, it was found that only growth hormone has manifest effects on the sex-dependent cytochromes P450 in the rat liver (Gustafsson et al., 1983). Moreover, growth hormone was found to play the role of "feminotropin". For example,

exogenous GH feminizes the activities of cytochrome P450 system of intact, castrated, and hypophysectomized males (Kramer et al., 1975; Mode et al., 1981). Agents such as somatostatin (Virgo, 1985) that block GH-release will masculinize the cytochrome P450 system of castrated males while agents such as clonidine (Virgo, 1985) that stimulate GH release will feminize the cytochrome P450 system of intact males. All these observations suggest that GH plays the role of "feminotropin".

However, GH exists in both males and females. If it is the factor that feminizes the cytochrome P450 system, there should be some differences in GH between the males and the females. The current data indicates that the secretory patterns of GH are sexually different. Male rats exhibit very low nadir levels of plasma GH with marked peaks every 3-4 h. Female rats, in contrast, have a higher basal GH level and more irregular peaks of smaller amplitude (Jansson et al., 1985b). It appears that it is this sexual dimorphism that determines the activities of hepatic cytochrome P450 mediated oxidations. Cytochrome P450 2C11 is a male-specific isoform and exists in high levels in intact adult males but low levels in intact females. Cytochrome 2C12, however, is a female-specific isoform and appears in a high concentration in intact

female adults but low in intact adult males (Mode et al., 1989; Waxman et al., 1985). Hypophysectomy decreases the levels of both isoforms in adult rats. However, intermittent administration of GH, mimicking the male secretory pattern, caused complete masculinization of the male specific P450 2C11 in hypophysectomized males; when GH was administered continuously, mimicking the female secretory pattern, the female specific P450 2C12 was induced in the hypophysectomized females (Mode et al., 1989; Legraverend et al., 1992). These data suggest that some isoforms of sex-dependent P450 are related to the sexual dimorphism of GH secretory pattern.

This sexual dimorphism of GH secretion is the result of exposure of male rats to androgen during the neonatal period and adulthood (Jansson and Frohman, 1987). Neonatal castration causes a marked suppression in both the magnitude and the duration of GH secretion, and higher baseline GH levels than that in sham-operated controls, ie. feminine characteristics of GH secretion. Although neonatal testosterone replacement therapy restored high amplitude GH pulses, they occurred more frequently and the baseline GH levels were markedly higher than those in intact male rats. However, neonatally castrated rats given testosterone neonatally and also during adulthood displayed a normal GH

pattern comparable to that of intact males (Jansson and Frohman, 1987). Therefore, neonatal androgen can permanently defeminize the secretory pattern of GH, and androgen in adulthood can masculinize the defeminized GH secretion. However, a masculine GH pattern was also seen in the neonatally castrated male rats given testosterone only during adulthood (Jansson and Frohman, 1987). Thus, defeminization is not an essential prerequisite for masculinization. Concurrently, neonatal castration completely suppresses EMDM activities in adult males, and neonatal testosterone replacement partially restores the activity; but both neonatal and adult testosterone completely restores the enzyme activity (Virgo, 1991). Further, peripubertal testosterone can induce an intact adult level of enzyme activity in adult males castrated neonatally (Virgo, 1991). Thus, the sexual differentiation of this enzyme system is parallel to that of GH secretory pattern.

1.1.4 Hypothalamic Regulation of GH Secretion

GH secretion is under the control of the hypothalamus. The hypothalamus produces GH-releasing hormone (GHRH) which stimulates the synthesis and release of GH, and somatostatin (SS) which inhibits the synthesis and release of the hormone

(Jansson et al., 1985b). It has been reported (Painson et al., 1991) that the secretion of GHRH shows episodic pulses, and that androgen can increase the amplitude of the GHRH pulses. Male rats also display a periodic SS secretion with peaks at the time of the trough of GHRH secretion, and GH has a positive feedback on SS secretion (Painson et al., 1991); in contrast, female rats show a continuous secretory pattern of SS and the levels are higher. These data suggest that the GH secretory pattern is regulated by GHRH and SS. As for sexual differences of the release of both GHRH and SS, some evidence, which will be presented in the following section, show that they might be determined by the sexual differentiation of the brain.

1.1.5 Sexual Differentiation of the Brain

1.1.5.1 Defeminization and Masculinization

Sexual differentiation of the brain results from exposure to androgen during a short period of neural development when nervous tissue is still sufficiently plastic to respond permanently and irreversibly to gonadal hormones. In rats, it actually consists of two processes, the permanent defeminization and the reversible masculinization (MacLusky and Naftolin, 1981).

The short critical period when androgen irreversibly defeminizes the brain is marked by a suppression of female characteristics and an enhancement of the potential of the central nervous system (CNS) to respond to androgen in adulthood. This process is "defeminization" and only depends on the hormonal environment of the perinatal period. At puberty, the higher levels of androgen masculinize the defeminized brain. This process, termed "masculinization", activates masculine characteristics but is not permanent. Adult castration of male rats can inhibit the male type of drug metabolism, but the drug metabolism can resume to normal after injection of testosterone propionate (TP) to the castrated males (Shimada et al., 1987; Virgo, 1991).

The critical period for androgen to permanently organize the brain in rats is from just prior to birth to 10 days postpartum, ie, the perinatal period (MacLusky and Naftolin, 1981; McEwen, 1983). However, for the P450 system, the limit is 5 to 6 days postpartum. If exposure of the brain to androgen does not occur, the brain will innately develop female characteristics and the neural control of endocrine functions and sexual steroid-related cytochrome P450 system would be affected. The secretory pattern of gonadotropins is sexually different, with a cyclic pattern in female rats but

an acyclic or tonic pattern in males. This sexual dimorphism results from the sexual differentiation of the brain. If the brains of female rats are defeminized with neonatal testosterone treatment, the female rats will show an anovulatory syndrome due to the occurrence of an acyclic gonadotropin secretion. Adult male rats castrated between birth and 3 days of life and transplanted with ovarian and vaginal tissue can display vaginal cycles, ovulation (Harris, 1964) and cyclic gonadotropin secretion in adulthood (Gorski, 1966). Similarly, neonatal castration causes a completely suppression of male-specific cytochromes P450 in adult male rats but induces the expression of female-specific cytochromes P450 (Jansson et al., 1985a).

1.1.5.2 Morphological Changes of Brain and Neonatal Sex Hormones

It is well known that there are sexual differences in brain structure that are sex steroid-dependent. These differences include such aspects as size of nuclei in neuronal groups, number of neurons (Gorski et al., 1978; Breedlove and Arnold, 1980; Gurney and Konishi, 1980; Jordan et al., 1982), volume of neurons (Gorski et al., 1980), extent of dendritic extension and branching (Meyer et al., 1978; Ayoub et al.,

1982), number of dendritic spines (Greenough et al., 1977; Ayoub et al., 1982), and morphology (Güldner, 1982) and localization of synapses (Matsumoto and Arai, 1981).

In 1966, Pfaff first discovered the existence of sexual differences in the size of nerve cell nuclei in rat brains. Later, Raisman and Field (1973), Dyer (1984), Greenough et al. (1977), Nishizuka and Arai (1981), and Matsumoto and Arai (1981) found sexual dimorphisms in the pattern of neuronal connections in rat brains. All these differences proved to be dependent on exposure of the CNS to androgen during the perinatal period. For example, an injection of testosterone into female rats of day 5 caused a marked increase in the number of shaft synapses (SHS) in the medial amygdaloid nucleus (AMN). The incidence of SHS in androgenized females was almost the same as that in normal males. In contrast, neonatal orchidectomy resulted in a significant decrease in the number of SHS to a level comparable to that of normal females (Nishizuka and Arai, 1981). In another experiment, treatment with testosterone on day 5 to female rats increased the mean numbers of shaft and spine synapses in the hypothalamic arcuate nucleus (ARCN); moreover, the synaptic organization of the ARCN in the androgenized adult females was quite homologous to that observed in the normal adult males

(Matsumoto and Arai, 1981). These and other studies indicate that the presence of sex steroids during the perinatal period may permanently differentiate the developing process of the brain and irreversibly organize the brain structure.

The medial preoptic area (MPOA) of the hypothalamus is clearly important in the control of gonadotropin release and sexual behaviour (Preslock and McCann, 1987). Also, this area is generally accepted as a major site of neonatal hormone action during neural development. Within the MPOA is an area of darkly staining neurons named the sexually dimorphic nucleus (SDN-POA) (Gorski et al., 1980). The function of this nucleus is still not clear and most authors suppose that it is involved in masculine reproductive behaviour (Arendash and Gorski, 1982; Arendash and Gorski, 1983; Gorski, 1991).

The development of the SDN-POA starts during late fetal life and extends throughout the first ten days of postnatal life (Jacobson and Gorski, 1981; Jacobson et al., 1980). This period corresponds to that of brain sexual differentiation. The development of SDN-POA neurons is dependent upon androgen. Castration of the 1-day-old male rat significantly reduces SDN-POA volume in adulthood (Gorski et al., 1978; Jacobson et al., 1981), but immediate reimplantation of a testis or

injection of testosterone after neonatal castration restores SDN-POA volume to normal (Jacobson et al., 1981). Further, exogenous testosterone given postnatally to the genetic female significantly increases the volume of SDN-POA in adulthood (Gorski et al., 1978; Jacobson et al., 1981). Therefore, the SDN-POA, at least in terms of its volume, is clearly influenced by the perinatal hormone environment.

The structures of the ventromedial nucleus (VMN) and the arcuate nucleus (ARN) are also sexually different in synaptic pattern and nuclear volumes of neurons (McEwen et al., 1982; Toran-Allerand CD, 1980). The cell bodies of these nuclei contain GHRH; indeed, the ARC nucleus is the major site of GHRH in the brain. Furthermore, these morphologic differences in the rat are determined by the neonatal steroid environment.

Recently, Chowen and colleagues (1993) reported that neonatal and adult testosterone had effects on the organization and activation of GHRH and SS neurons in the hypothalamus. Neonatal testosterone increased the number of GHRH neurons in adulthood and adult testosterone stimulated GHRH mRNA levels; further, in female rats, adult testosterone stimulated GHRH mRNA levels, and the magnitude of the increase was higher in the animals exposed to neonatal testosterone

than those not exposed to neonatal sex steroids. In contrast, the number of SS neurons was not effected by testosterone, but both neonatal testosterone and adult testosterone significantly increased SS mRNA levels (Chowen et al., 1993).

1.1.5.3 Sex Steroids During the Neonatal Period

1.1.5.3.1 Androgen and Estrogen

It is known that the serum concentration of testosterone is significantly higher in male rats than in female ones during the perinatal period (Pang et al., 1979). Androgen can permanently defeminize the CNS during the critical period of neural development. It has been suggested that androgen is converted, via aromatization, to estradiol, which is commonly accepted as the precise molecular species of gonadal hormone that promotes the defeminization of the brain (Döhler, 1986; Toran-Allerand, 1986; Gorski, 1991). Administration with antiestrogens can block testosterone-induced (Doughty and McDonald, 1974) or estrogen-induced defeminization (Doughty et al., 1975). Furthermore, aromatizing enzyme inhibitors can also inhibit the defeminizing effects of both endogenous and exogenous testosterone (Booth, 1977). As for the brain-controlled hepatic cytochrome P450 system, neonatal

testosterone or estrogen defeminized sex-dependent drug metabolism, and aromatase inhibitor prevented the defeminization of hepatic drug metabolism induced by sex steroids (Reyes and Virgo, 1988). These data suggest that androgen is converted to estradiol to defeminize the brain. Further, the fetal and neonatal rat brain contains aromatizing enzymes which can convert aromatizable androgen to estradiol-17 β (George and Ojeda, 1982; MacLusky et al., 1985; Lephart et al., 1992), and estrogen receptor levels in the male and female rat brain increase markedly during the perinatal period (McEwen et al., 1982). These evidences support the possibility of androgen-aromatization and the involvement of estradiol in the defeminizing process. Therefore, it is assumed that androgen enters the cytosol of neuron and is converted to estradiol by aromatase; estradiol then interacts with specific receptors and ultimately the neuronal genome to alter mRNA activity which in turn stimulates or inhibits the production of some product, thereby modifying neuronal function (Döhler, 1986; Toran-Allerand, 1986; Gorski, 1991).

As for the effects of sex steroids on nerve cells, it has been demonstrated that estrogen or androgen can increase the rate and extent of neuritic outgrowth in neurons containing estrogen receptors (Toran-Allerand, 1980). The growth and

development of axons and dendrites in turn enhance synapse formation and neuronal survival, and increases neuron number. Since different circulating levels of sex steroids exist between sexes during the perinatal period, number of synapses, the spatial distribution of synapses, neuron number, neuronal volume and neural circuits will sexually differentiate. This hypothesis is currently involved in explaining the sexual morphogenetic mechanisms of the steroidal effects during the neural development (McEwen et al., 1982; Toran-Allerand, 1986; Gorski, 1991).

1.1.5.3.2 α -Fetoprotein (AFP)

Neonatal females have estrogen-rich plasma, and yet the brains of female rats do not become defeminized. This may be due to the presence of plasma α -fetoprotein.

AFP is a major fetal plasma protein which is present in most developing vertebrates. In rats, this protein occurs in high concentration during the first 3 weeks of postnatal life and decreases linearly from its very high concentration to trace levels at weaning (Raynaud et al., 1971). Although AFP can bind a variety of hormones and other biologically active ligands in other species, rat and mouse AFP alone binds

estrogens with a high affinity and protects the developing brain from excessive exposure to estrogens. Since AFP does not bind androgens, testosterone is free to enter the rodent brain. Thus, female brains do not become defeminized.

1.1.5.4 Hepatic Sex-Dependent Cytochrome P450

Based on the above, it can be concluded that estradiol binds estrogen receptors in the cytosol to modulate the expression of DNA and to organize the developmental course of the brains. Since the secretory pattern of growth hormone in adult rats results from the sexual differentiation of the brain, and the secretory pattern regulates the expression of sex-dependent cytochrome P450, we can infer that sexual differences in the activities of the cytochrome P450 system are the result of sexual differentiation of the brain. Reyes and Virgo (1988) showed that various sex-steroids which can defeminize gonadotropin secretion also defeminized drug metabolism, and that aromatase inhibitor which can block the defeminization of gonadotropin secretion prevented the defeminization of hepatic cytochrome P450. Therefore, it suggests that neonatal androgen defeminizes hepatic cytochrome P450 possibly via the sexual differentiation of the brain.

1.1.6 Catecholaminergic System in the Defeminization of the Brain

1.1.6.1 Neonatal catecholamines and behaviour

In addition to sex steroids, monoamine neurotransmitters are believed to be involved in the sexual differentiation of at least some aspects of CNS functions (Döhler, 1991). Thus, changing the amounts of these monoaminergic transmitters in CNS during the critical period of sexual differentiation of the brain would alter the steroid-dependent expression of sexually or nonsexually-related behaviours in adult rats. It was reported that reserpine, which depletes norepinephrine from nerve terminals by inhibiting uptake into storage vesicles, could prevent the defeminizing effects of neonatal testosterone in inducing anovulatory syndromes in the females (Gorski, 1973). Raum et al. (1990) reported that prenatal exposure of male rat fetuses to cocaine, which could increase synaptic concentrations of norepinephrine, inhibited sexually related behaviours in adult males. Reduction of serotonin by p-chlorophenylalanine (PCPA) enhanced masculine sexual behaviour in male rats and potentiated the defeminizing effect of exogenous testosterone in females; in contrast, increasing serotonin by 5-hydroxytryptophan (5HTP) inhibited masculine

behaviour induced by exogenous testosterone in female rats (Wilson et al., 1986). Neonatal administration of clonidine resulted in a significant reduction in the capacity to express female lordosis behaviour in female adulthood (Jarzab et al., 1987). Neonatal dopamine or noradrenaline was found to reduce the open field activity in female adult rats (González and Leret, 1992b). All the above evidence suggests that sexual differentiation of sexual behaviour is related to the neonatal catecholaminergic system in the brain.

1.1.6.2 Neonatal catecholamines and reproductive endocrine function

The secretory pattern of the gonadotropins is, as mentioned previously, sexually different, and this sexual dimorphism of gonadotropin secretion results from the sexual differentiation of the brain. If the female brain is defeminized with neonatal testosterone treatment, the female rat will become anovulatory due to the presence of acyclic gonadotropin secretion (Booth, 1979; Dörner, 1981; Goy and McEwen, 1980). Besides sex steroids, neurotransmitters during the neonatal period also have effects on the sexual differentiation of gonadotropin secretion. A series of experiments (Jarzab and Döhler, 1984; Jarzab et al., 1986,

1987, 1989a,b,c) demonstrated that postnatal treatment of female rats with the α -adrenergic antagonists prazosin or yohimbine, or with the nicotine receptor antagonist mecamylamine, significantly increased the release of LH by gonadal steroids in adulthood; and that postnatal treatment of female rats with the β -adrenergic agonist isoprenaline, the β_2 -adrenergic agonist salbutamol or β -adrenergic antagonist alprenolol reduced the release response of LH to gonadal steroids in adulthood. Undoubtedly, these data indicate neurotransmitters engage in the sexual differentiation of gonadotropin secretion.

1.1.6.3 Neonatal catecholamines and SDN-POA

SDN-POA is thought to be related to sexual behaviour (Arendash and Gorski, 1982; Arendash and Gorski, 1983; Gorski, 1991) and sexually differentiated (Gorski et al., 1980), the effect of neonatal catecholamines on the anatomy of SDN-POA have also been investigated. It was reported (Handa et al., 1986) that prenatal p-chlorophenylamine, an inhibitor of serotonin biosynthesis, increased the volume of the SDN-POA in female neonates compared to that of control males. Studies (Jarzab et al., 1989b, 1990a,b,c) show that salbutamol, a β_2 -receptor agonist, given neonatally, increased SDN-POA volume

in both female and male rats but the effect was particularly manifest in males; furthermore, the α_1 -receptor agonist clonidine augment the stimulatory effect of testosterone on SDN-POA differentiation in female rats. These data suggest that alteration of catecholamine levels in the neonatal brains would modify the sexual differentiation of the CNS.

1.1.6.4 Catecholamines and androgen during the neonatal period

There is evidence showing that catecholamines modify the defeminizing effect of neonatal testosterone. Postnatal serotonin delayed the permanent anovulatory-sterility (PAS)-inducing effect of postnatal testosterone (Shirama et al., 1975), and PCPA was found to prevent androgen-induced PAS in female rats (Reznikov et al., 1979). Tyramine, which releases noradrenaline from nerve terminals, as well as the α_1/α_2 receptor blockers phenoxybenzamine and phentolamine can inhibit the defeminizing action of testosterone on ovulatory cyclicity in female rats (Raum and Swerdloff, 1981). Although nuclear accumulation of estradiol derived from the aromatization of testosterone is inhibited by hypothalamic β -receptor stimulation in the neonatal female rat (Raum et al., 1984), the work of Canick et al. (1987) showed neonatal

administration of the β -agonist, isoproterenol, had no effect on aromatase activity in male or female pups (Canick et al., 1987).

In spite of that the mechanisms of neurotransmitter effects on defeminization is still not known; the fact of the involvement of these catecholamines in the defeminization of the brain cannot be denied. Therefore, due to the fact that sexual differences of hepatic cytochrome P450 result indirectly from the brain sexual differentiation and that sex differentiated characteristics can be influenced by adrenergic agonists/antagonists, we intend to study whether the neonatal adrenergic system is involved in the androgen-induced defeminization of hepatic cytochrome P450 in male rats.

1.2 GH and Development of Male-Specific Cytochromes P450

1.2.1 Developmental Expressions of Male-Specific Cytochromes P450

Sex-dependent cytochromes P450 of rat liver display developmental changes (Waxman et al., 1985; Imaoka et al., 1991). For example, during the neonatal period, the amount of cytochrome P450 2C11 is undetectable in both sexes; but in

male rats the amount of this isoform begins to rise at 4 weeks of age and reaches its highest level at puberty. The females show an almost undetectable levels of the isoenzyme from the neonatal period to puberty (Waxman et al., 1985; Imaoka et al., 1991). Another male-specific isoform of cytochrome P450, 3A1/2, also undergoes developmental changes, but it mainly shows a developmental decline in the females (Waxman et al., 1985; Imaoka et al., 1991). In the females, the amount of this isoenzyme is high during the neonatal period but decreases with age. The males, however, keep a high level of P450 3A1/2 from the neonatal period to puberty (Waxman et al., 1985; Imaoka et al., 1991). The activities of 2 α - and 16 α -testosterone hydroxylases are low in newborn rats, but rise at around 4 weeks of age and then reach an adult high level at puberty; since 2 α - and 16 α -testosterone hydroxylations are selective for cytochrome 2C11 which is barely detectable in female rats from the neonatal period to maturity, the females display low activities of 2 α - and 16 α -testosterone hydroxylases all the time. 6 β -Testosterone hydroxylation, which is catalyzed by cytochrome P450 3A1/2, shows a high activity in newborn female but decreases its activity with age. In males the activity of 6 β -hydroxylase rises at two weeks of age, reaches the highest at 4 weeks of age, then it decreases but arises again at 6 weeks of age (Waxman et al., 1985; Imaoka et al., 1991).

1.2.2 GH and Male-Specific Cytochromes P450

Ethylmorphine demethylase (EMDM), a sex-specific hepatic monooxygenase which exists in both sexes but shows a much higher activity in adult males, is neonatally defeminized by androgen (Reyes and Virgo, 1988; Virgo, 1991). The activity of this enzyme in male rats exhibits developmental changes (Gram et al., 1969). The activity is undetectable in newborn rats, begins to rise at 4 weeks of age and reach adult levels at puberty. However, the females display a low activity of the enzyme all the time.

The activities of EMDM (Kramer et al., 1975; Virgo, 1983; Vockentanz and Virgo, 1985) and male-specific cytochromes P450 such as 2C11 (Janeczko et al., 1990; Legraverend et al., 1992) and 3A1/2 (Waxman et al., 1990) in adult rats are regulated by GH. However, there are no reports regarding the factors responsible for the developmental expression of male specific isoforms during the prepubertal period. Plasma GH immediately after birth appears at very high levels, and then declines sharply to an almost undetectable level; it then begins to rise at 4 weeks of age, and reaches adult levels at puberty (Khorram et al., 1983; Rieutort, 1974; Strosser and Mialhe, 1975).

Although the male pattern of GH secretion is organized during the neonatal period (Jansson and Frohman, 1987), it becomes manifest only after puberty (Gabriel et al., 1992). During the prepubertal period, there is no apparently sexual difference in the secretory pattern of GH, the prepubertal expressions of male-specific P450 seems not related to the secretory pattern of the hormone. Therefore, we also intend to investigate whether GH plays a role in the prepubertal expression of the male-specific P450.

2.0 MATERIALS

AND

METHODS

2.1 GENERAL MATERIALS AND METHODS

2.1.1 Chemicals and Biochemicals

2.1.1.1 Chemicals for animal injection

DL-isoproterenol·HCl, DL-propranolol·HCl, clonidine·HCl, phenylephrine·HCl, phentolamine·HCl, prazosin·HCl, testosterone propionate, and propylene glycol were purchased from Sigma Chemical Company; WY27127 is the product of Wyeth Ltd., Philadelphia, PA, USA; sodium glutamate (MSG) is from G.E. Barbour Inc., Sussex, N.B.; and sodium chloride was purchased from Fisher Scientific Company, Nepean, Ont.

2.1.1.2 Chemicals for the enzyme assay

Glucose-6-phosphate dehydrogenase (G-6-P-DH), β -nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), oxidized cytochrome c and reduced β -nicotinamide adenine dinucleotide phosphate were purchased from Sigma Chemical Company; semicarbazide hydrochloride and 4-nitrophenol were the products of Aldrich Chemical Company, Inc., Milwaukee, WIS, USA; magnesium chloride, potassium cyanide, sucrose and sodium hydroxide were purchased from

Mallinckrodt Canada Inc, Pointe-Claire, Quebec; zinc sulfate, hydrochloric acid and sodium dithionite were from Fisher Scientific, Fair Lawn, NJ, USA; ethylmorphine chlorohydrate and perchloric acid were the products of BDH Chemicals, Canada; 16 α -hydroxytestosterone (4-androsten-16 α), 16 β -hydroxytestosterone (4-androsten-16 β), 11 α -hydroxytestosterone (4-androsten-11 α), 7 α -hydroxytestosterone (4-androsten-7 α), 2 α -hydroxytestosterone (4-androsten-2 α), 11 β -hydroxytestosterone (4-androsten-11 β), 6 β -hydroxytestosterone (4-androsten-6 β) and hydroxytestosterone (4-androsten-17 β -OL-3-ONE) were purchased from Steraloids, Inc., Wilton, N.H.; acetic acid glacial was from Baxter Corporation, Toronto, Ont; and L-ascorbic acid was purchased from United States Biochemical Corporation, Cleveland, Ohio, USA; glycerol, acetone, methanol and dichloromethane were the products of Fisher Scientific Company; BIO-RAD Protein Assay Dye Reagent Concentrate and bovine serum albumin are from BIO-RAD Laboratories, Richmond, CA, USA.

2.1.1.3 Chemicals for buffers

Buffers were prepared according to the methods described by Gomori (1955).

Trizma base (Tris[hydroxymethyl]amino-methane) is the product of Sigma Chemical Company; potassium phosphate monobasic, potassium phosphate dibasic and potassium chloride were purchased from Mallinckrodt Canada Inc.

2.1.2 Animals

Pregnant rats (Wistar), having successfully raised at least one previous litter, were purchased from Charles River Canada (St. Constant, Qué.) and were kept in solid-bottom plastic cages on chemical-free hardwood bedding. All rats were kept at 21°C under 12h of light per day and had free access to tap water and Agway PRO LAB chow (manufactured by Agway Inc., New York, USA). Newborn and adult rats were used in all experiments.

2.1.3 Intraventricular Injection

On Day 0 and 3 of birth, newborn male rats were anesthetized hypothermically. The pup was wrapped in a slightly damp piece of gauze bandage and placed in ice for 15 minutes to induce hypothermic anesthesia (Pfeiffer, 1936). The pup was then removed from ice, put in a holder, and the head placed in a horizontal position with the skull side up.

A midsagittal incision was made through the skin from a line joining the eyes to a line joining the ears, and the bregma was exposed. A 27-gauge needle was inserted vertically into the third ventricle (coordinates: 0.5 mm anterior to the bregma; 1.5 mm lateral to the midsagittal fissure) to a depth 2.5 mm ventral to the skull surface. The validity of these coordinates was confirmed by injecting dye (Methylene Blue-Certified, Sigma Chemical Company) and morphological examination of the brain after fixation in buffered 10% formalin. Ten μ l of vehicle or drug solution was injected into the ventricle over a period of 3 minutes and the needle was maintained in position for a further 5 minutes to prevent backflow from the puncture wound. The incision was closed by sealing the skin to the skull with cyanoacrylate glue.

After the injection, the pup was left undisturbed and warmed until its body returned to room temperature (ie. pink and active again). They were then warmed at 25°C for at least two hours before being returned to the dam.

2.1.4 Castration

2.1.4.1 Castration of the neonate

The procedure used was a modification of Pfeiffer's

method (Pfeiffer, 1936). Within 12 hours of birth, the pup was anesthetized hypothermically by placing it in ice for 15 min. The pup was then placed, ventral side up, on a surgical table. The area of incision was wiped with a piece of Kimwipe¹ soaked in 95% ethanol. An incision (2 mm) was made through the skin anterior to the urogenital papilla at a 45°-angle to the left (of the pup) by use of very fine iris scissors. The skin was then freed from subcutaneous tissues with a fine probe. A second incision was made horizontally through the musculature at the body wall of the initial cut. The right testis was then extruded by slightly pressing the abdomen and excised with a pair of scissors. The left testis was exposed by carefully probing through the viscera and also excised. The wound on the body wall was closed by aligning the cut edges of the musculature with small forceps and then sealing it with cyanoacrylate glue. The opening on the skin was then closed by overlapping the cut edges of the skin and sealing it with cyanoacrylate glue. After the surgery, the pup was left undisturbed, warmed and returned to the dam.

2.1.4.2 Castration of adult rats

The rat was lightly anesthetized with ether and placed, ventral side up, on a surgical table. The limbs were fixed to

the table. The hair on the scrotum was removed with a pair of surgical scissors and the skin of the scrotum was wiped with 75% ethanol. An incision was made through the midline of the scrotum followed by a second incision through the tunica of one testis. The testis was then exposed, the testicular artery and vein were ligated, and the testis was excised. The remaining testis was removed similarly. The scrotal incision was closed with 3 to 4 sutures.

2.1.5 Subcutaneous Injections During the Neonatal Period

On Day 0 to 10, chemicals were injected under the dorso-lateral skin of the pup immediately posterior to the front limb.

2.1.6 Preparation of Microsome

The method used to prepare hepatic microsome was based on that reported by Virgo (1991).

Food was removed from the rat twelve hours before it was killed in order to decrease glycogen in the liver. The rat was decapitated with a guillotine and exsanguinated. The abdomen was opened with a pair of surgical scissors and the

intact liver was excised as quickly as possible. The liver was then put into a beaker of ice-cold 50 mM Tris HCl buffer pH 7.4, containing 150 mM KCl. After the liver was rinsed, it was taken out, wiped dry with a piece of Kimwipe³ to remove blood and weighed. The liver was then put into a beaker containing 3 volumes of ice-cold 50 mM Tris HCl buffer pH 7.4 with 150mM KCl, homogenized with a Brinkmann Polytron at setting of 4 in a 4°C cold room. The homogenate was then centrifuged at 9000·g for 20 minutes at 4°C. The resultant supernatant was then centrifuged at 105,000·g at 4°C for 60 minutes. The supernatant was poured off and a similar volume of fresh 50 mM Tris HCl buffer pH 7.4 was added. The microsomes were resuspended with the homogenizer at setting of 3. The suspension was centrifuged once again at 105,000·g for 60 minutes at 4°C. The supernatant was discarded and the pellet was homogenized in a volume of 50 mM Tris HCl buffer pH 7.4 equal to half the volume of 9,000·g supernatant originally containing the microsomes. Glycerine (20% of the volume of the suspension) was added and the microsomes were stored at -80°C until use.

2.1.7 Measurement of Microsomal Protein

Concentration of microsomal protein was determined

according to the Bradford's method (1976) using the Bio-Rad Protein Assay kit:

Diluted microsomal protein (1:3000 and 1:6000) (0.8ml) and 0.8ml of standards (bovine serum albumin 2µg/ml, 3µg/ml, 4µg/ml, 6µg/ml and 8µg/ml) were placed in clean, dry test tubes, and 0.8ml distilled water was placed in "blank" test tube. Dye Reagent Concentrate 0.2ml was then added to the test tubes. All test tubes were shaken without causing excess foaming. After 5 minutes, the absorbance of the blank standards and samples were measured at 595 nm. The value of protein concentration was calculated from the standard curve.

2.1.8 Enzyme Assays

2.1.8.1 Measurement of ethylmorphine demethylase (EMDM) activity in hepatic microsome

The activity of EMDM was measured in vitro from the amount of formalin produced from the methyl group released from the substrate (Nash, 1953). The incubation medium contained the following in a total volume of 3 ml: 0.1 mM NADPH, 1 mM NADP, 3.3 mM glucose 6-phosphate, 2 Units glucose 6-phosphate dehydrogenase, 1.0 mM semicarbazide, 24 mM MgCl₂,

50 mM Tris HCl buffer (pH 7.4), 6 mg microsomal protein, and 6.7 mM ethylmorphine. After 5 min of preincubation of the mixture, the protein was added to the incubation mixture and the reaction was allowed to proceed for 20 min at 37°C under air. The reaction was then stopped after 20 min by adding 2 ml 20% ZnSO₄ and 2 ml saturated Ba(OH)₂ to the incubation mix. After the addition of 3 ml of distilled water, the resultant suspension was centrifuged at 3500 rpm for 15 minutes. Five ml of the supernatant was removed to another tube containing 2 ml double strength Nash Reagent (150 g ammonium acetate, 2 ml acetylacetone, 2 ml glacial acetic acid and diluted to 500 ml with distilled water), and then heated at 60°C for 30 minutes. The absorbance was then measured immediately at 413 nm. Concentration of formalin in the original incubation mix was calculated by: formalin (nmol/ml) = (ABS - 0.0069)/0.0006. This equation was obtained by using known concentrations of formalin in the Nash reaction (Nash, 1953).

The concentration of MgCl₂ used in the incubation was 24 mM, which was determined with preliminary experiments to select an appropriate MgCl₂ concentration. We used 1.2, 12, 24, 48 and 96 mM MgCl₂, and found that the amount of product was highest at 24 and 48 mM MgCl₂. Since there was no apparent difference in the amount of product between 24 and 48 mM MgCl₂ used, we decided to use 24 mM MgCl₂ for the reaction.

The incubation time was 20 min. Incubation of the mix for 5, 10, 20 and 30 minutes, respectively showed that the specific activity of ethylmorphine demethylase was the same at 5, 10 and 20 minute incubation and lower at 30 minutes (see Table 2-1); therefore, 20 min was chosen.

2.1.8.2 Measurement of p-nitrophenol hydroxylase (p-NPH) activity in hepatic microsome

The activity of p-nitrophenol hydroxylase was measured in vitro according to the method of Koop (1986). The incubation medium contained the following in a total volume of 1 ml: 0.1 mM p-nitrophenol solution, 0.5 mg microsomal protein, 1 mg NADPH and 100 mM phosphate buffer pH 6.8. After 5 min of preincubation, NADPH was added to start the reaction and water was added to the reference. The reaction was allowed to proceed for 6 min at 37°C under air and stopped by adding 0.5 ml 0.6 N perchloric acid. This mixture was then centrifuged for 15 min at 3500 rpm. At last, 0.1 ml of 10 N NaOH was added to 1 ml of the centrifuged supernatant, and the absorbance of this solution was measured at 546 nm. The concentration of the product, nitrocatechol, was calculated using a millimole extinction coefficient of $9.53 \text{ mM}^{-1}\text{cm}^{-1}$. The amount of the product was shown in preliminary experiments to

Table 2-1. Hepatic EMDM activity of intact and castrated male rats under different incubation time.

Incubation Time (minute)	[Formalin Product] (nmol/ml)	
	Int. ♂	g♂
5	65	12
10	133	22
20	268	46
30	327	66

Table 2-2. Hepatic p-NPH activity of male rats under different amounts of microsome protein & various incubation time.

[Microsome] (mg/per mix.)	[nitrocatechol] (nmol/mix.)		
	4 min.	6 min.	8 min.
0.1	0.44	1.04	2.20
0.25	1.35	2.03	3.13
0.5	2.28	2.68	4.37
1.0	3.33	4.73	5.82

be proportional to both the time of incubation and the concentration of microsomal protein (see Table 2-2).

2.1.8.3 Quantitation of cytochrome P450

The concentration was determined by the method of Omura & Sato (1964).

A few milligrams of sodium dithionite were added to the 2.5 ml microsomes suspended in 50 mM Tris HCl buffer (pH 7.4) (2 mg/ml) in a cuvet to reduce the cytochrome P450. The absorbance of the suspension was then measured from 400-500 nm yielding a baseline. The suspension was then bubbled with carbon monoxide for 30 seconds and its absorbance was measured from 400-500 nm based on the obtained baseline. Two different values of absorbance were selected to calculate, using a coefficient E as $91 \text{ mM}^{-1}\text{cm}^{-1}$, the concentration of cytochrome P450. The total P450 nmol/mg of the microsome is given by the formula:

$$(\text{ABS}_{450} - \text{ABS}_{490}) * 1000 / 91 / \text{mgMP}.$$

In the above formula, ' ABS_{450} ' was the absorbance at 450 nm and ' ABS_{490} ' the absorbance at 490 nm.

2.1.8.4 Measurement of NADPH cytochrome c reductase

The activity of this enzyme was determined by the procedure of Phillips and Langdon (1962) using $19.6 \text{ cm}^2 \text{ mM}^{-1}$ as the extinction coefficient for reduced cytochrome c and 100mM phosphate buffer for the reactions.

One ml of 50 μM cytochrome c reagent (1.86 mg oxidized cytochrome c in 1.0 ml of 100 mM phosphate buffer, pH 7.6) and 1 ml of 1 mM KCN (1.96mg in 10.0ml 100 mM phosphate buffer, pH 7.6), 90 μg microsome (100 μg microsomal protein in 1.0ml of 100 mM phosphate buffer, pH 7.6) were added to both reference and sample cuvetts. Then, 0.1 ml of 100 mM phosphate buffer pH 7.6 buffer was added to the reference cuvet, and the absorbance of the mixture was measured, with a spectrophotometer under the function of time drive mode at 550 nm, to obtain a baseline. The reference cuvet was then replaced with the sample cuvet. NADPH 40.9 μM dissolved in 100 μl of 100 mM phosphate buffer, pH 7.6, was added to the sample cuvet; the absorbance of the mixture was measured immediately. Two values of the absorbance were selected to calculate the activity based on the formula:

$$(\text{ABS}_2 - \text{ABS}_1) * 3 * 1000 / 19.6 / \text{time} / \text{mgMP}.$$

In the above formula, 'ABS₁' and 'ABS₂' were two values of the absorbance, '19.6' was the coefficient, '3' was the volume of the mixture, '1000' was used when millimole was converted to nanomole, and the 'time' was the period between the two absorbances selected.

2.1.8.5 Kinetics of ethylmorphine demethylase

The method to measure the kinetics of EMDM is identical to that for the measurement of EMDM activity except that the concentration of ethylmorphine varied, containing of 0.42, 0.84, 1.68, 3.35 or 6.7 mM ethylmorphine. The values of Km and Vmax are obtained based on the following calculation:

$$ABS = b[EM] + a$$

$$Km = b/a$$

$$Vmax = 1/a$$

In the above formulas, 'ABS' was the value of absorbance, '[EM]' was the concentration of ethylmorphine. Formula $ABS = b[EM] + a$ was obtained by linear regression analysis; therefore, 'b' was the slope of the curve, and 'a' was the intercept of the curve with the ordinate.

2.1.8.6 Measurement of 2 α -, 16 α -, and 6 β -testosterone hydroxylases

Cytochrome P450 2C11 is the major male-specific P450 and is responsible for the hydroxylation of testosterone in the 2 α and 16 α positions; cytochrome P450 3A is another male specific P450 and is responsible for testosterone hydroxylation in the 6 β position. Thus, we measured the activities of these hydroxylation reactions to observe the male-specific isoforms of hepatic P450 2C11 and 3A.

The incubation medium contained the following in a total volume of 1 ml (Wood et al., 1983): 1 mg microsome protein, 1 mM NADPH, 3 mM $Mg_2Cl \cdot 6H_2O$, 50 mM sucrose, 2.5 mM testosterone (4-androsten-17 β -ol-3-one) (dissolved and added in 20 μ l of methanol) and 50 mM phosphate buffer pH 7.4 buffer. After 5 min of preincubation, the testosterone was added and the reaction was allowed to proceed for 5 min at 37 $^{\circ}$ C under the air. The reaction was stopped by adding 6 ml dichloromethane. After mixing for 30 seconds, the liquid was centrifuged at 1000 rpm for 5 min. Then 5 ml of the dichloromethane was transferred to a 50 ml conical centrifuge tube and evaporated. After evaporation, the residue was dissolved in 100 μ l of solvent A (430 ml methanol + 11 ml acetone + distilled water to 1000 ml) and analyzed by HPLC.

The amounts of 2 α -, 16 α -, and 6 β -testosterone hydroxylases were determined with a Beckman System Gold high pressure liquid chromatography (HPLC) system by the method of Wood et al. (1983). The pumps were 11B Solvent Delivery Modules, the sample was placed on the column with a mold 507 Autosampler, coordinated with an Analog Interface Module Mold 406; products were identified with a Programmable Detector Module 166. The column used was a Supelcosil LC-18 from Supelco, Inc., Bellefonte, PA, USA. The particle size of packing was 5 μ m. The solvent systems consisted of solvent A (430 ml methanol + 11 ml acetone + distilled water to 1000ml) and solvent B (750 ml methanol + 19 ml acetone + distilled water to 1000 ml). The flow rate was 1.5 ml/min. The column was developed by a concave solvent gradient (Programme 4) beginning with 100% solvent A and progressing to 100% solvent B over 25 min. The column was washed for 8 min with 100% solvent A before each sample injection. Testosterone, 16 α -hydroxytestosterone, 16 β -hydroxytestosterone, 11 α -hydroxytestosterone, 7 α -hydroxytestosterone, 2 α -hydroxytestosterone, 11 β -hydroxy-testosterone and 6 β -hydroxytestosterone, dissolved in 100% solvent A, were analyzed at various concentrations to obtain retention times and to generate standard curves. The retention time of testosterone was 17.4 \pm 0.03 min (2 α), 12.4 \pm 0.05 min (16 α) and 9.05 \pm 0.07 min (6 β). The

concentrations of testosterone product were calculated according to the following formulas, which were obtained by using known concentrations of testosterone in the HPLC system:

for height of peak: (2a) $Y=15.30384X+0.148512$

(16a) $Y=13.81535X+0.138008$

(6B) $Y=17.57898X+0.134990$

for area of peak: (2a) $Y=0.053739X+0.153837$

(16a) $Y=0.037555X+0.157820$

(6B) $Y=0.051997X+0.155786$

In the above formulas, 'Y' was the concentration of product, and 'X' was the height of peak or area of peak depending what formula was used. Using either height of peak or area of peak would give same results.

2.2 ANIMAL PROCEDURES

2.2.1 Role of Adrenergic Receptors in the Neonatal

Defeminization of Hepatic Drug Metabolism in Male Rats

2.2.1.1 Drug metabolism in male rats castrated neonatally and given α - or β -adrenergic agonists or antagonists and testosterone propionate neonatally

To investigate the roles of adrenergic receptors and their relation with androgen in the defeminization of hepatic cytochrome P450 system, we castrated male pups within 12 hr after birth and administered (icv) isoproterenol (50 μ g dissolved in saline), phentolamine (25 μ g dissolved in saline), phenylephrine (50 μ g dissolved in saline) or WY27127 (50 μ g dissolved in saline) on Day 0 & 2. Some of these rats were given (sc) 1.45 μ mol testosterone propionate dissolved in propylene glycol on Day 0, 2 & 4, while the rest received the carrier. The rats were weaned on Day 28 and were killed on Day 65 for the enzymatic assays.

2.2.1.2 Drug metabolism in male rats given α - or β -adrenergic agonists or antagonists neonatally

To test whether α - or β -adrenergic receptors are involved

in the defeminization of hepatic cytochrome P450 enzymes, we treated (icv) male rat pups on Day 0 & 3 after birth with isoproterenol (50 µg dissolved in saline), clonidine (50 µg dissolved in saline), phenylephrine (50 µg dissolved in saline), propranolol (50 µg dissolved in saline), prazosin (50 µg dissolved in saline) or WY 27127 (50 µg dissolved in saline). The control group was given saline. Since pups from the same dam are more alike than those from different dam's due to genetics, dam's behaviour, etc., pups from different dams were mixed up together. Different litter sizes lead to different nutritional status for the pups, the litter size was reduced to 10 pups to minimize the bias of mean values. The rats were weaned on day 28 after birth, castrated on Day 55 to eliminate the masculinizing effect of androgen in adulthood, and killed on day 70 for the enzymatic assays.

2.2.1.3 Drug metabolism in male rats given propranolol together with other adrenergic agents neonatally

To study the exact role of α and β receptors in the defeminization of hepatic cytochrome P450 enzymes, we treated (icv) male rat pups on Day 0 & 3 after birth with WY27127 (50 µg in 25 µl saline), clonidine (50 µg in 25 µl saline), phenylephrine (50 µg in 25 µl saline), phentolamine (25 µg in 25 µl saline) or prazosin (50 µg in 25 µl saline),

respectively, together with propranolol (50 µg in 25 µl saline). The rats were weaned on day 28 after birth, castrated on Day 55 to eliminate the masculinizing effect of androgen in adulthood, and killed on day 70 for the enzymatic assays.

2.2.2 Monosodium Glutamate (MSG) and the Prepubertal and Pubertal Development of Hepatic Cytochrome P450 System in Male Rats

2.2.2.1 Drug metabolism in 35-day-old rats treated with MSG during the neonatal period

To study whether growth hormone (GH) plays any role in the pre-pubertal development of male hepatic cytochrome P450, we gave male newborn pups MSG subcutaneously to destroy growth hormone releasing hormone (GHRH) cells in the arcuate nucleus. The rats were then assayed on Day 35.

On Day 1, 3, 5, 7, & 9 after birth, male pups were given subcutaneously 0, 2, or 4 mg MSG dissolved in saline/g body weight. The litter size was 10 pups. The rats were weaned on Day 28 and killed on Day 35 for the enzymatic assays.

2.2.2.2 Drug metabolism in 65-day-old rats given MSG during the neonatal period

To investigate the role of GH in the expression of hepatic cytochrome P450 system, we gave male newborn pups MSG to observe the hepatic cytochrome P450 system.

On Day 1, 3, 5, 7 & 9 after birth, male pups were injected (sc) with 0, 2 or 4 mg MSG dissolved in saline/g body weight. The litter size was 10 pups. The rats were weaned on Day 28 and killed on Day 65 for the enzymatic assays.

2.3 STATISTICAL ANALYSES

Data were analyzed for overall significance by an one way analysis of variance. When a difference was found at $p < 0.05$, post-hoc tests (Bonferroni t tests) between selected pairs of groups were conducted.

Values of protein concentration were calculated by linear regression analysis using a CASIO scientific calculator programmed for linear regression analysis.

3.0 RESULTS

3.1 THE HEPATIC CYTOCHROME P450 SYSTEM: EFFECT OF ADRENERGIC DRUG ADMINISTERED NEONATALLY

3.1.1 Effect of Phentolamine and Isoproterenol on Defeminization in Neonatal Castrates Defeminized with Exogenous Androgen

To test whether, in the absence of testosterone, α -adrenergic receptors might stimulate defeminization or whether β -adrenergic receptors might inhibit the process, we used phentolamine (α_1/α_1 -adrenergic antagonist) and isoproterenol (β_1/β_1 -adrenergic agonist) in neonatally castrated males and observed whether defeminization was affected.

3.1.1.1 Hepatic ethylmorphine demethylase (EMDM) activity

The activity of hepatic ethylmorphine demethylase in intact adult males was 9.11 ± 1.12 nmol/min/mg, 6.6-fold that of the females ($p < 0.01$) (Table 3-1). Neonatal castration, as expected, prevented the defeminization of hepatic EMDM activity as the activity in the neonatally castrated males was 1.70 ± 0.14 (1.90 & 1.50) nmol/min/mg, which did not differ ($p > 0.05$) from that in the females.

Table 3-1. Effect of adrenergic agents on hepatic EMDM and p-NPH activities in neonatal castrated defeminized with exogenous androgen. The activities of hepatic ethylmorphine demethylase (EMDM) & p-nitrophenol hydroxylase (p-NPH) in male rats castrated within 12h after birth, treated with 25 µg phentolamine (PH) or 50 µg isoproterenol (ISO) (icv. on Day 0 & 2) together with or without 1.45 µmol testosterone propionate (TP) (sc. on Day 0, 2, & 4), & assayed on Day 70.

Treatment	n	EMDM Activity (nmol/min/mg)	p-NPH Activity (nmol/min/mg)
Intact ♂	4	9.11±1.12 ^a	1.37±0.17
Intact ♀	3	1.39±0.10 ^b	1.32±0.12
g♂+vehicle	2	1.70±0.14 ^b (1.90, 1.50)	1.70±0.25 (2.06, 1.34)
g♂+PH	2	0.97±0.36 ^b (1.48, 0.45)	1.62±0.14 (1.82, 1.42)
g♂+PH+TP	2	2.33±0.06 ^c (2.25, 2.41)	1.06±0.02 (1.02, 1.09)
g♂+ISO	3	1.81±0.61 ^{b,c}	1.35±0.12

Value is expressed as MEAN±SE; one replicate incubation was performed for each sample.

^a Significantly different from ^b (p<0.01) & ^c (p<0.05);

^b Significantly different from ^c (p<0.05).

g♂: castrated male

The activity of hepatic EMDM in neonatally castrated (feminized) male rats given phentolamine or isoproterenol on Day 0 & 3 did not differ ($p>0.05$) from that in females (Table 3-1). This shows that phentolamine (PH) or isoproterenol (ISO) do not cause defeminization in the absence of testosterone. When testosterone was given concurrently with phentolamine, the EMDM activity significantly increased, by 67% ($p<0.05$), over that of the intact females. Thus, although the defeminization was not as great as one would expect (normally 75% of adult values, see Virgo, 1991), the drug did not prevent it. The sample size, however, is small.

3.1.1.2 Hepatic p-nitrophenol hydroxylase (p-NPH) activity

The activity of p-nitrophenol hydroxylase, as expected, did not display sexual differences ($p>0.05$) (Table 3-1). Neonatal castration did not effect p-NPH activity. Given phentolamine, phentolamine plus testosterone or isoproterenol, the castrated rats did not show any change in p-NPH activity ($p>0.05$) (Table 3-1). The data indicate that castration, testosterone, phentolamine, or isoproterenol has no influence on the non-sexually differentiated p-nitrophenol hydroxylase.

3.1.1.3 Hepatic cytochrome c reductase activity and cytochrome P450 amount

The activity of cytochrome c reductase and the total P450 did not show a significant difference ($p>0.05$) between intact males and females, and neonatal castration had no effects on these parameters (Table 3-2). The administration of PH, ISO, or PH plus testosterone did not alter significantly ($p>0.05$) the total amount of P450 (Table 3-2) or the reductase activity (Figure 2) in the adult rats castrated neonatally. These latter data suggest that the increase of EMDM activity in testosterone and phentolamine-treated rats does not result from an increase of either the amount of P450 or the reductase.

Due to the length of time required to conduct these types of experiments and the high mortality we experienced in the neonatal castrates (up to 70%), we decided to proceed to our main investigation on the roles of adrenergic receptors in the defeminization process.

Table 3-2. Effect of adrenergic agents on hepatic cytochrome c reductase activity and cytochrome P450 amount in neonatal castrated defeminized with exogenous androgen. The total amount of hepatic cytochrome P450 & the activity of hepatic cytochrome c reductase in male rats castrated within 12 h after birth, treated with 25 µg phentolamine (PH) or 50 µg isoproterenol (ISO) (icv) on Day 0 & 2 together with or without 1.45 µmol testosterone propionate (TP) (sc) on Day 0, 2, & 4, & assayed on Day 70.

Treatment	n	Cyt. P450 (nmol/mgMP)	Cyt. c Reductase (nmol/min/mg)
Intact ♂	4	0.97±0.10	96±7
Intact ♀	3	0.64±0.02	95±5
g♂+vehicle	2	0.72±0.04 (0.77, 0.66)	109±2 (105, 112)
g♂+PH	2	0.72±0.05 (0.79, 0.65)	113±4 (119, 108)
g♂+PH+TP	2	0.51±0.01 (0.52, 0.50)	71±1 (69, 73)
g♂+ISO	3	0.67±0.02	101±10

Values are expressed as MEAN±SE; Values are not significantly different ($p>0.05$) within columns. One replicate incubation was performed for each sample.

g♂: castrated male

3.1.2 Effect of Neonatal Adrenergic Agents on Defeminized Cytochrome P450 System

3.1.2.1 Hepatic ethylmorphine demethylase activity

It is well known that neonatal testosterone defeminizes EMDM activity which by definition, refers to a permanent increase in enzyme activity coupled with the capacity for a further reversible increase in activity upon exposure to androgen in adulthood (ie. masculinization). Defeminization is best measured by the permanent increase in activity and this can be unmasked by simply castrating the adult. We chose to castrate on Day 55 as this is five days after puberty which begins on Day 35 and extends for 14 Days (Ojeda et al., 1980). The assay was conducted on Day 70 when any androgen effects had dissipated.

The activities of hepatic ethylmorphine demethylase in intact adult males, males castrated on Day 55 (postpubertal) and intact adult female rats were 6.64 ± 0.18 , 2.79 ± 0.26 and 0.90 ± 0.06 nmol/min/mg (Table 3-3). These results show that the hepatic activity of EMDM in adult male rats is 7-fold higher than that of the females ($p < 0.01$). Castration of postpubertal rats decreased the EMDM activity, although the activity was

Table 3-3. Effect of neonatal adrenergic agents on hepatic EMDM and p-NPH activities in defeminized male rats. The activities of hepatic ethylmorphine demethylase (EMDM) & p-nitrophenol hydroxylase (p-NPH) in male rats treated neonatally with 50 µg isoproterenol (ISO), 50 µg propranolol (PRO), 50 µg phenylephrine (PHY), 50 µg clonidine (CLO), 50 µg WY27127 (WY) or 50 µg prazosin (PRA) on Day 0 & 3, castrated on Day 55, and assayed on Day 70.

Treatment	n	EMDM activity (nmol/min/mg)	p-NPH activity (nmol/min/mg)
Intact ♂	7	6.64±0.18 ^a	1.25±0.08
g♂	8	2.79±0.26 ^b	0.94±0.10
g♂+vehicle	5	2.46±0.28 ^b	1.25±0.12
Intact ♀	3	0.90±0.06 ^c	0.98±0.24
g♂+ISO	5	3.00±0.99 ^b	0.94±0.03
g♂+PRO	4	5.56±0.60 ^a	1.20±0.05
g♂+PHY	4	3.14±0.79 ^b	0.86±0.18
g♂+PRA	3	6.06±1.36 ^a	0.82±0.06
g♂+CLO	4	2.74±0.73 ^b	1.18±0.10
g♂+WY	4	2.65±0.16 ^b	1.05±0.08

Values are presented as MEAN±SE; One replicate incubation was performed for each sample.

^a Significantly different from ^b (p<0.05) & ^c (p<0.01) within column;

^b Significantly different from ^a (p<0.05) & ^c (p<0.05) within column;

^c Significantly different from ^a (p<0.01) & ^b (p<0.05) within column.

g♂: castrated male

not completely depressed ($p < 0.05$) and was higher than that in the females ($p < 0.05$) (Table 3-3). Thus, these data show that the neonatal defeminization in the males increases the hepatic EMDM activity over the levels in the females, and that masculinization at puberty elevates the defeminized EMDM activity to a much higher level.

Since there was no significant difference in the EMDM activities between castrated male rats and castrated male rats given the vehicle only (Table 3-3), the injection procedure did not affect the results and the change of EMDM activity in the animals injected with adrenergic drugs only reflects the effects of the drugs.

The EMDM activity at Day 70 of male rats castrated at Day 55 and given, neonatally, isoproterenol (ISO), propranolol (PRO), phenylephrine (PHY), clonidine (CLO), WY27127 (WY) or prazosin (PRA), were 3.00 ± 0.99 , 5.56 ± 0.60 , 3.14 ± 0.79 , 2.74 ± 0.73 , 2.65 ± 0.16 or 6.06 ± 1.36 nmol/min/mg, respectively (Table 3-3). The activities of EMDM in both the propranolol- and the prazosin-treated groups were increased approximately 2.5 fold ($p < 0.05$) compared to that of the defeminized males given only vehicle neonatally, and did not differ ($p > 0.05$) from the levels in intact males. This result shows that the

neonatal inhibition of one or both β -adrenergic receptors or prazosin inhibition of the α_1 -adrenergic receptor leads to a "super-defeminization" of hepatic EMDM in the males. This "super-defeminization" does not differ in magnitude from that in normal rats where the total activity represents defeminization and masculinization (Table 3-3). Therefore, these results suggest that in the normal animal the α_1 -adrenergic agonist complex inhibits defeminization. Alternatively, blockade of the α_1 -adrenergic receptor may permit more of the norepinephrine in the synapse to bind to a stimulatory receptor. Similarly, these data suggest that at least one of the β -adrenergic receptor is inhibitory to defeminization (or that β blockade increases norepinephrine for binding to a stimulatory receptor).

The animals, treated with ISO, PHY, CLO or WY showed no significantly different ($p > 0.05$) activity of EMDM, compared with the vehicle control under the conditions used (Table 3-3). As neither clonidine nor WY27127 influenced the defeminization process one might speculate that the α_2 -adrenergic receptor is not involved in the process of defeminization. However, only one dose was used and other doses may have demonstrated effects. Similarly, the apparent lack of effects from either PHY or ISO may reflect inadequate

doses rather than a lack of agonist effect (eg. maximal defeminization from endogenous agonists).

3.1.2.2 Hepatic p-nitrophenol hydroxylase activity

The hepatic p-NPH activities in intact males, males castrated at Day 55 and intact female rats were 1.25 ± 0.08 , 0.94 ± 0.10 and 0.98 ± 0.24 nmol/min/mg (Table 3-3). These data show that there is no sexual difference ($p > 0.05$) in hepatic p-NPH activity, and that castration neither stimulates nor inhibits p-NPH activity ($p > 0.05$) (Table 3-3). The animals, given ISO, PRO, PHY, CLO, WY or PRA, displayed neither an increase nor decrease in hepatic p-NPH activity. The above data, therefore, imply that the neonatal stimulation or inhibition of α - or β -adrenergic receptors do not affect the hepatic p-NPH activity. This is what was expected from this enzyme, which is not sexually differentiated, and served as a control in our experiments.

3.1.2.3 Hepatic cytochrome c reductase activity

In this experiment, there were no significant differences in the hepatic cytochrome c reductase activities between intact males, males castrated at Day 55 and intact female

rats; nor were there any significant differences in the reductase activities between animals receiving different drugs and intact males, males castrated at Day 55 or intact females. Intact male rats had a value of 111 ± 8 nmol/min/mg, and intact females 87 ± 10 nmol/min/mg (Table 3-4). The value of the reductase activity in defeminized male rats was 98 ± 9 nmol/min/mg, which did not differ ($p > 0.05$) from those of either intact males or females. Defeminized animals given adrenergic agonists or antagonists exhibited values of ranging from 102 ± 13 to 87 ± 7 nmol/min/mg. However, none of these agents influenced the reductase activity ($p > 0.05$) (Table 3-4). Thus, the alteration of hepatic EMDM activity seen with PRO and with PRA does not arise from a change in the reductase activity.

3.1.2.4 Hepatic cytochrome P450 amount

The level of cytochrome P450 in intact male rats (0.79 nmoml/mg protein) was 31% higher ($p < 0.05$) than that in intact females (0.60 nmol/mg protein) (Table 3-4). Postpubertal castration significantly reduced ($p < 0.05$) the level in males to that of females. However, these small differences in the total amount of cytochrome P450 seem unlikely to correspond to the 6-fold differences in EMDM activity (Table 3-3) between these animals. Propranolol, however, increased the amount of

Table 3-4. Effect of neonatal adrenergic agents on hepatic cytochrome c reductase activity and cytochrome P450 amount in defeminized male rats. The total amount of hepatic cytochrome P450 & the activity of hepatic cytochrome c reductase of male rats treated (icv) neonatally with 50 µg isoproterenol (ISO), 50 µg propranolol (PRO), 50 µg phenylephrine (PHY), 50 µg clonidine (CLO), 50 µg WY27127 (WY) or 50 µg prazosin (PRA) on Day 0 & 3, castrated on Day 55, and assayed on Day 70.

Treatment	n	Cyt. P450 (nmol/mgMP)	Cyt. c Reductase (nmol/min/mg)
Intact ♂	7	0.79±0.04 ^a	111±8
g♂	8	0.62±0.03 ^b	98±9
g♂+vehicle	5	0.66±0.04 ^{b,c}	101±8
Intact ♀	3	0.60±0.04 ^{b,c}	87±10
g♂+ISO	5	0.62±0.04 ^{b,c}	100±4
g♂+PRO	4	0.82±0.03 ^a	100±6
g♂+PHY	4	0.65±0.03 ^{b,c}	102±13
g♂+PRA	3	0.67±0.02 ^{a,b,c}	89±3
g♂+CLO	4	0.75±0.08 ^{b,c}	93±8
g♂+WY	4	0.63±0.03 ^{b,c}	87±7

Value is expressed as MEAN±SE; one replicate incubation was performed for each sample.

^a Significantly different from the groups without ^a within column;

^b Significantly different from the groups without ^b within column;

^c Significantly different from the groups without ^c within column.

g♂: castrated male

P450 in "super-defeminized" male rats up to the levels found in intact adult males just as it increased the EMDM activity up to that of intact males (Table 3-3). In contrast, PRA which also caused "super-defeminization" did not significantly increase the P450 levels, however the sample size is small and perhaps not representative. Propranolol (PRO) is a competitive β -adrenergic antagonist and has equal affinity for β_1 and β_2 receptors; thus it inhibits both β_1 and β_2 receptors (Hoffman and Lefkowitz, 1990). This drug has been reported to have effects on sexual differentiation of the brain, which was attributed to its β -antagonism (McGivern et. al., 1986). Since PRO (β -adrenergic antagonist) increases the total amount of P450 but PRA (α -adrenergic antagonist) does not, the "super-defeminizing" mechanisms of PRO and PRA may be different. The other groups displayed a 20% decline in the P450 amount (Table 3-4); however, they did not significantly differ ($p>0.05$) from the castrated control groups, so that no drug effects can be attributed.

3.1.2.5 Kinetics of hepatic EMDM in PRO-induced "super-defeminization"

Since propranolol caused "super-defeminization" of EMDM, we investigated the kinetics of EMDM in the propranolol-treated rats. However, since PRA induced high mortality and

we had to repeat the experiment on the effect of PRA; by the time when we obtained the data, we did have enough time to further investigate the kinetics of EMDM and the activities of testosterone hydroxylases under the influence of PRA.

The values of the apparent K_m in intact males, castrated (defeminized) males and castrated (defeminized) males treated with neonatal propranolol were 0.55 ± 0.06 , 0.64 ± 0.04 and 0.73 ± 0.10 mM (Table 3-5). There was no significant difference ($p > 0.05$) between these values among the three groups, which suggests that the "super-defeminization" of the EMDM activity does not result from a change of K_m . These results suggest that since the K_m of an enzyme is a constant, propranolol did not significantly change the relative proportions of those isoforms that demethylate ethylmorphine in the microsome.

The values of V_{max} were 6.96 ± 1.04 , 7.15 ± 0.40 and 3.13 ± 0.36 nmol/min/mg (Table 3-5) for the defeminized rats given PRO, intact males, and defeminized rats. The V_{max} of the rats treated neonatally with PRO was not significantly different from that of intact controls ($p > 0.05$) but was 2.22 times greater than the defeminized controls ($p < 0.01$) (Table 3-5). Thus, the PRO induced "super-defeminization" is associated with an increase in the V_{max} of the isoform(s) of P450 that demethylate ethylmorphine. However, it is not possible to

Table 3-5. Kinetics of hepatic EMDM in propranolol-super-defeminized rats. The apparent Vmax (nmol/min/mg) and Km (mM) of the hepatic ethylmorphine demethylase (EMDM) in male rats treated with 50 µg propranolol (PRO) (icv) on Day 0 & 3, castrated on Day 55, & analyzed on Day 70.

Treatment	n	Km (mM)	Vmax (nmol/min/mg)
PRO+castrate	4	0.73±0.10	6.96±1.04 [†]
Intact	5	0.55±0.06	7.15±0.40 [†]
Castrate	5	0.64±0.04	3.13±0.36

Values are expressed as MEA±SE; one replicate incubation was performed for each sample.

[†]Significantly different from castrate (p<0.01).

infer whether the increased V_{max} results from more enzyme(s) or from a more active enzyme(s).

3.1.2.6 Hepatic 6 β -, 16 α -, and 2 α -testosterone hydroxylases in PRO-induced "super-defeminization"

In order to determine whether any of the major male specific isoforms of cytochrome P450, namely 2C11 and 3A1/2 might be affected by propranolol, we measured the activities of 6 β -, 16 α - and 2 α -testosterone hydroxylases.

The values of the enzyme activities in defeminized male rats with propranolol treatment, intact males and defeminized males were: (6 β) 0.84 ± 0.08 , 0.80 ± 0.12 and 0.19 ± 0.10 nmol/min/mg; (16 α) 1.41 ± 0.13 , 1.41 ± 0.10 , 0.58 ± 0.10 nmol/min/mg; (2 α) 1.25 ± 0.14 , 1.32 ± 0.09 , 0.56 ± 0.10 nmol/min/mg (Table 3-6). The activities of 6 β -, 16 α -, and 2 α -hydroxylases in PRO-treated group were increased 342%, 143%, and 123% over the defeminized controls ($p < 0.01$). Since the 6 β -hydroxylated product is the regio- and stereo-selective product of cytochrome P450 3A1/2 while the 2 α - and 16 α -products, which are usually present in 1:1 ratio (approximately 1:1 in our results), are selectively produced by the P450 2C11 isoform, PRO-induced "super-defeminization" is associated with increased activities of the isoforms, 3A1/2 and 2C11.

Table 3-6. Hepatic 6 β -, 16 α -, & 2 α -testosterone hydroxylases in propranolol-induced superdefeminization. Hepatic 6 β -, 16 α -, & 2 α -testosterone hydroxylase activities in male rats treated with 50 μ g **propranolol (PRO)** (icv) on Day 0 & 3, castrated on Day 55, & analyzed on Day 70.

Treatment	n	6 β (nmol/min/mg)	16 α (nmol/min/mg)	2 α (nmol/min/mg)
PRO+castrate	4	0.84 \pm 0.08	1.41 \pm 0.13	1.25 \pm 0.14
Intact	5	0.80 \pm 0.12	1.41 \pm 0.10	1.32 \pm 0.09
Castrate	5	0.19 \pm 0.04 [†]	0.58 \pm 0.10 [†]	0.56 \pm 0.10 [†]

Values are expressed as MEAN \pm SE; one replicate incubation was performed for each sample.

[†]Significantly different from PRO+castrate and Intact within each column (p<0.01).

3.1.3 Effects of Neonatal Adrenergic Drugs in PRO-Treated Neonates

In this experiment, neonatal males were injected with PRO neonatally to produce "super-defeminization". However, other adrenergic agonists and antagonists were given simultaneously to determine their influence on the "super-defeminization". Although PRA also increased defeminization, the effects of other adrenergic agents on PRA-induced "super-defeminization" was not tested due to the high mortality caused by PRA.

3.1.3.1 Hepatic ethylmorphine demethylase activity

Administration of CLO or WY to the propranolol-treated defeminized males did not cause significant differences ($p > 0.05$) in EMDM activity, compared to the PRO-treated castrated males (Table 3-7), which had been demonstrated to be "super-defeminized" in the previous experiment (Table 3-3). Thus, these data suggest that the α_2 -adrenergic receptor is not involved in the defeminization. PH decreased the enzyme activity by 35% (Table 3-7), but the decline was not significant ($p > 0.05$); therefore, a mixed α_1/α_2 antagonism does not appear to significantly alter PRO-induced "super-defeminization". PHY increased the enzyme activity by 56%

Table 3-7. Effect of neonatal adrenergic agents on hepatic EMDM and p-NPH activities in propranolol-superdefeminized rats. The activities of hepatic ethylmorphine demethylase (EMDM) & p-nitrophenol hydroxylase (p-NPH) in male rats treated neonatally (icv) with 50 µg propranolol (PRO) plus 50 µg WY27127 (WY), 50 µg clonidine (CLO), 50 µg phenylephrine (PHY), 25 µg phentolamine (PH) or 50 µg prazosin (PRA) on Day 0 & 3, castrated on Day 55, and assayed on Day 70.

Treatment	n	EMDM activity (nmol/min/mg)	p-NPH activity (nmol/min/mg)
gσ+PRO	5	5.47±1.16 ^a	1.03±0.09
gσ+PRO +WY	4	4.27±1.13 ^c	1.03±0.08
gσ+PRO +CLO	2	6.42±1.74 ^a (4.68, 8.16)	1.02±0.06 (0.96, 1.07)
gσ+PRO +PH	2	3.57±0.24 ^c (3.81, 3.33)	0.94±0.07 (1.00, 0.87)
gσ+PRO +PHY	2	8.54±1.90 ^a (10.43, 6.64)	0.81±0.05 (0.85, 0.76)
gσ+PRO +PRA	5	2.90±0.54 ^b	1.19±0.16

Values are expressed as MEAN±SE; one replicate incubation was performed for each sample.

^a Not significantly different (p>0.05) from gσ+PRO within column;

^b Significantly different (p<0.05) from gσ+PRO within column;

^c Not significantly different (p>0.05) from gσ+PRO within column.

gσ: castrated male

though again this effect was not significant ($p>0.05$). Thus, stimulating the α_1 -adrenergic receptor had no significant effect on the "super-defeminization", and this may be due to the full occupancy of α_1 -adrenergic receptors by norepinephrine. Unexpectedly, PRA depressed the "super-defeminized" enzyme activity by 47% (Table 3-7); the value of the depressed activity was 2.90 ± 0.54 nmol/min/mg, which was significantly different ($p<0.05$) from that in propranolol-treated male rats. Therefore, α_1 -adrenergic agonist complexes inhibit the process of defeminization.

Based on the above data, stimulation of the α_1 -adrenergic receptor inhibits defeminization and, under physiological conditions, does so maximally; at least one of β -adrenergic receptors inhibits defeminization, and does so maximally under physiological conditions. However, the results suggest that there may be a stimulatory receptor, which might be β_1 or β_2 , or another unknown receptor, for the defeminization. Thus, blockade of either α_1 - or β -adrenergic receptors would permit more norepinephrine to bind with the stimulatory receptor and induce "super-defeminization". Similarly, the α_2 -adrenergic receptor may inhibit defeminization since blockade of both α_1 and β_1/β_2 -adrenergic receptors, in which the α_2 -adrenergic receptor is still open to norepinephrine, suppresses "super-defeminization".

3.1.3.2 Hepatic cytochrome c reductase activity and the amount of cytochrome P450

The activities of hepatic cytochrome c reductase in the animals treated neonatally with PRO plus WY, CLO, PHY, PH or PRA were 89 ± 8 , 106 ± 10 , 83 ± 2 , 74 ± 12 and 70 ± 4 nmol/min/mg (Table 3-8), and were not significantly different ($p > 0.05$) from those of the propranolol-treated, castrated males (81 ± 17 nmol/min/mg). The amounts of hepatic cytochrome P450 in the animals given adrenergic agents were similarly not altered (Table 3-8), compared to the propranolol-treated, castrated males ($p > 0.05$). The inhibition of PRO-induced "super-defeminization" by PRA is therefore not mediated by a decrease in either total cytochrome P450 or cytochrome P450 reductase, and suggests that PRO-effect on P450 3A1/2 and 2C11 may not be affected by PRA.

3.1.3.3 Hepatic p-nitrophenol hydroxylase activity

The values of hepatic p-NPH activity of the male rats treated neonatally with PRO plus WY, CLO, PHY, PH or PRA were 1.03 ± 0.08 , 1.02 ± 0.06 , 0.94 ± 0.07 , 0.81 ± 0.05 or 1.19 ± 0.16 nmol/min/mg, respectively (Table 3-7). None were significantly different ($p > 0.05$) from those of the propranolol-treated,

Table 3-8. Effect of neonatal adrenergic agents on hepatic cytochrome c reductase activity and cytochrome P450 amount in propranolol-superdefeminized rats. Total amount of hepatic cytochrome P450 and the activity of cytochrome c reductase in male rats treated (icv) neonatally with 50 µg **propranolol (PRO)** plus 50 µg **WY27127 (WY)**, 50 µg **clonidine (CLO)**, 50 µg **phenylephrine (PHY)**, 25 µg **phentolamine (PH)** or 50 µg **prazosin (PRA)** on Day 0 & 3, castrated on Day 55, and assayed on Day 70.

Treatment	n	Cyt. P450 (nmol/mgMP)	Cyt. c Reductase (nmol/min/mg)
gσ+PRO	5	0.62±0.02	81±17
gσ+PRO +WY	4	0.64±0.01	89±8
gσ+PRO +CLO	2	0.65±0.04 (0.61, 0.68)	106±10 (96, 116)
gσ+PRO +PH	2	0.58±0.02 (0.60, 0.55)	83±2 (81, 85)
gσ+PRO +PHY	2	0.69±0.02 (0.71, 0.67)	74±12 (86, 62)
gσ+PRO +PRA	5	0.64±0.02	70±4

Value is expressed as MEAN±SE; Values are not significantly different ($p>0.05$) within columns. One replicate incubation was performed for each sample.

gσ: castrated male

castrated males. Therefore, neonatal stimulation or inhibition of adrenergic receptors does not influence the non-sexually differentiated p-NPH activity in adulthood.

3.2 THE HEPATIC CYTOCHROME P450 SYSTEM: EFFECTS OF MONOSODIUM GLUTAMATE (MSG) ADMINISTERED NEONATALLY

3.2.1 MSG Effects in Males Assayed on Day 35

It has been reported (Bloch et al., 1984) that MSG damages GHRH cells in the neonatal arcuate nucleus and thus abolishes the release of growth hormone (GH) in the adults. The role of GH in maintaining the sex differences of the hepatic cytochrome P450 enzyme system is well understood in adult rats, but there is no information for its role in prepubertal rats. Thus, we treated neonatal male rats with various amounts of MSG and observed the hepatic cytochrome P450 system in prepubertal and adult rats.

Male rat pups were given 0, 2 or 4 mg/g MSG on Day 1, 3, 5, 7, & 9 of life, respectively, and analyzed on Day 35 and 65. In this experiment, we have obtained data which show that neonatal MSG had no influence on the prepubertal hepatic EMDM (Table 3-9). Since neonatal MSG can decrease serum GH

Table 3-9. Effect of neonatal MSG on hepatic EMDM and p-NPH activities at Day 35. The activities of hepatic ethylmorphine demethylase (EMDM) & p-nitrophenol hydroxylase (p-NPH) in male rats treated with monosodium glutamate (MSG) (0, 2 or 4mg/g, sc) on Day 1, 3, 5, 7 & 9 of life, and assayed on Day 35.

Dosage (mg/g)	n	EMDM Activity (nmol/min/mg)	p-NPH Activity (nmol/min/mg)
0	5	3.41±0.44	1.51±0.23
2	5	3.43±0.18	1.40±0.20
4	5	3.63±0.37	1.38±0.21

Value is expressed as MEAN±SE; The values are not significantly different within each column ($p>0.05$). One replicate incubation was performed for each sample.

(Bloch et al., 1984) and the activities of EMDM at Day 35 were not decreased by neonatal MSG, prepubertal development of EMDM seems to be independent upon plasma GH. The activities of EMDM at Day 35 were approximately 25% higher than that of defeminized rats (Table 3-3); thus, defeminization still occurred in MSG-treated rats and the increase of EMDM activity at Day 35 probably reflects the increase of other plasma hormones.

The reductase activities and the total amounts of cytochrome P450 in the 0 mg/g, 2 mg/g and 4 mg/g MSG groups of 35-day-old rats were not significantly different ($p>0.05$) (Table 3-10). The data suggest that GH does not influence the prepubertal reductase activity or the total amount of P450.

The prepubertal p-NPH activities in MSG-treated rats were not significantly different comparing with the controls ($p>0.05$) (Table 3-9) and they all reached adult levels. Therefore, these data indicate, as expected, that GH is also unrelated to the prepubertal development of p-nitrophenol hydroxylase and p-NPH activity seems to reach adult levels early before puberty.

Table 3-10. Effect of neonatal MSG on hepatic cytochrome c reductase activity and cytochrome P450 amount at Day 35. The total amount of hepatic cytochrome P450 & the activity of hepatic cytochrome c reductase in male rats treated with monosodium glutamate (MSG) (0, 2 or 4mg/g, sc) on Day 1, 3, 5, 7 & 9 of life, assayed on Day 35.

Dosage (mg/g)	n	Cyt. P450 (nmol/mgMP)	Cyt. c Reductase (nmol/min/mg)
0	5	0.85±0.12	40±3
2	5	0.76±0.05	40±1
4	5	0.71±0.03	42±2

Value is expressed as MEAN±SE; The values are not significantly different within each column ($p>0.05$). One replicate incubation was performed for each sample.

3.2.2 MSG Effects in Males Assayed on Day 65

3.2.2.1 Hepatic EMDM activity and p-NPH activity

In 65-day-old rats, 2 mg/g and 4 mg/g MSG caused a 50% and 43% increase ($p < 0.01$) in EMDM activity respectively (Table 3-11). These data imply that EMDM activity is inversely related to GH after puberty. However, neither 2mg/g nor 4mg/g MSG altered the activity of p-nitrophenol hydroxylase (Table 3-11) and these data imply that GH is not related to adult p-NPH activity.

3.2.2.2 Cytochrome c reductase activity and total amount of cytochrome P450

At 4 mg/g MSG, the total amount of cytochrome P450 was significantly increased ($p < 0.01$) by 26% but it was unaffected by 2mg/g (Table 3-12). The reductase was unaffected by either dose of MSG (Table 3-12).

3.2.2.3 Kinetics of hepatic EMDM

Since the increase in EMDM activity that MSG caused might have been related to changes in cytochrome P450, we examined the kinetics of the EMDM in MSG treated rats.

Table 3-11. Effect of neonatal MSG on hepatic EMDM and p-NPH activities at Day 65. The activities of hepatic ethylmorphine demethylase (EMDM) & p-nitrophenol hydroxylase (p-NPH) in male rats treated with monosodium glutamate (MSG) (0, 2 or 4mg/g, sc) on Day 1, 3, 5, 7 & 9 of life, & assayed on Day 65.

Dosage (mg/g)	n	EMDM Activity (nmol/min/mg)	p-NPH Activity (nmol/min/mg)
0	4	6.68±0.51	0.86±0.10
2	4	10.04±1.06 ^{††}	0.84±0.07
4	4	9.52±0.78 ^{††}	0.89±0.13

Value is expressed as MEAN±SE; one replicate incubation was performed for each sample.

^{††}Significantly different from controls (p<0.01).

Table 3-12. Effect of neonatal MSG on hepatic cytochrome c reductase activity and cytochrome P450 amount at Day 35. The total amount of hepatic cytochrome P-450 & the activity of hepatic cytochrome c reductase in male rats treated with monosodium glutamate (MSG) (0, 2 or 4mg/g, sc) on Day 1, 3, 5, 7 & 9 of life, & assayed on Day 65.

Dosage (mg/g)	n	Cyt. P450 (nmol/mgMP)	Cyt. c Reductase (nmol/min/mg)
0	4	0.54±0.03	110±8
2	4	0.53±0.01	102±6
4	4	0.67±0.05 ^{**}	105±5

Value is expressed as MEAN±SE; one replicate incubation was performed for each sample.

^{**}Significantly different ($p < 0.01$) from other doses.

The apparent K_m of EMDM in 65 day old rats treated neonatally with 0mg/g, 2mg/g, or 4mg/g MSG are presented in Table 3-13 and are not significantly different ($p>0.05$) from each other. Thus, these data suggest that the increase of EMDM activity does not result from an change in K_m and suggest that there is no change in the isoform composition of the enzymes.

In contrast, the apparent V_{max} of EMDM in MSG-treated male rats was increased dose-dependently (Table 3-13) with that of the group treated with 4 mg/g MSG being significantly increased ($p<0.05$) by 38%. Therefore, the increase of EMDM activity in the neonatal MSG-treated animals can be related to an elevation of the V_{max} , which implies an increase in the amount of the isoforms responsible for ethylmorphine demethylation or an increase in their activity.

3.2.2.4 Hepatic 6 β -, 16 α -, and 2 α -testosterone hydroxylases

To investigate the activities of sexually differentiated isoforms of hepatic P450s in MSG-treated male rats, we measured the activities of 6 β -, 16 α - and 2 α -testosterone hydroxylases. The 6 β -hydroxylated product is regio- and stereo-selectively produced by cytochrome P450 3A1/2 while the

Table 3-13. Kinetics of EMDM in 65-Day-old male rats treated neonatally with MSG. The apparent Km (mM) and Vmax (nmol/min/mg) of the hepatic ethylmorphine demethylase (EMDM) in male rats treated with monosodium glutamate (MSG) (0, 2 or 4mg/g, sc) on Day 1, 3, 5, 7, & 9 of life, and assayed on Day 65.

Dosage (mg/g)	n	Km (mM)	Vmax (nmol/min/mg)
0	4	0.50±0.03	8±0.8
2	4	0.46±0.03	9±0.7
4	4	0.52±0.03	11±0.3 [†]

Values are expressed as MEAN±SE; one replicate incubation was performed for each sample.

[†]Significantly different (p<0.05) from other doses.

2 α - and 16 α -products are indicative of the 2C11 isoform. It is clear that MSG increased the 6 β -activity but not that of the 2 α /16 α activities (Table 3-14). Thus, it appears that the MSG increased P450 3A1/2 but not 2C11.

Table 3-14. Hepatic 6 β -, 16 α -, & 2 α -testosterone hydroxylases in 65-Day-old male rats treated neonatally with MSG. Hepatic 6 β -, 16 α -, & 2 α -testosterone hydroxylase activities in male rats treated neonatally with monosodium glutamate (MSG) (0, 2 or 4mg/g, sc), on Day 1, 3, 5, 7, & 9 of life, & analyzed on Day 65.

Dosage (mg/g)	n	6 β (nmol/min/mg)	16 α (nmol/min/mg)	2 α (nmol/min/mg)
0	4	0.36 \pm 0.06	0.53 \pm 0.13	0.52 \pm 0.10
2	4	0.52 \pm 0.04 [†]	0.66 \pm 0.04	0.60 \pm 0.04
4	4	0.53 \pm 0.04 [†]	0.68 \pm 0.09	0.63 \pm 0.03

Values are expressed as MEAN \pm SE; one replicate incubation was performed for each sample.

[†]Significantly different (p<0.05) from controls.

4.0 DISCUSSION

4.1 ADRENERGIC RECEPTORS AND THE DEFEMINIZATION OF EMDM

From our studies, we conclude that mixed α_1/α_2 -adrenergic agonist (phentolamine), mixed β -adrenergic agonist (isoproterenol), α_1 -adrenergic agonist (phenylephrine), α_2 -adrenergic agonist (clonidine) & antagonist (WY27127), at the dose of 50 μg (phentolamine given at 25 μg), do not inhibit defeminization of EMDM, cause significant changes in the total amount of P450, activity of p-NPH or cytochrome c reductase; that propranolol, a mixed β_1/β_2 -adrenergic antagonist, and prazosin, an α_1 -adrenergic antagonist, both given at the dose of 50 μg , increased defeminization of EMDM, and the super-defeminization induced by propranolol is not via increasing the activity of cytochrome c reductase, the total amount of P450, or the apparent K_m , but due to the increase of the apparent V_{max} of EMDM. Further, propranolol superdefeminizes the activities of 2 α -, 16 α -, & 6 β -testosterone hydroxylases; but both prazosin and propranolol, given simultaneously at equal doses of 50 μg , prevent super-defeminization. However, prior to our studies it was not known whether adrenergic receptors are involved in the defeminization of hepatic cytochrome P450 system, and the mechanism of the action of adrenergic receptors in defeminization is still not clear.

4.1.1 Adrenergic Receptors and CNS Defeminization

A large amount of evidence has shown that adrenergic receptors are involved in the defeminization of the brain in rats. Administration of adrenergic drugs to newborn rats altered their sexual function, behaviour and the structures of the brain (Jarzab et al., 1989a,b, 1990a,b,c). Due to the fact that sexual differences in hepatic cytochromes P450 probably result from the brain's sexual differentiation, we hypothesised that intervention on the adrenergic system during the neonatal period would alter the sexually dependent cytochrome P450 in the liver. Our data show that neonatal propranolol (β -adrenergic antagonist) or prazosin (α_1 -adrenergic antagonist), given intraventricularly, caused "super-defeminization" of EMDM activity in postpubertal male rats. These results suggest that α_1 - and at least one β -adrenergic receptor are involved in inhibiting defeminization of the hepatic cytochromes P450 in the normal rats.

In our study, stimulation of the β -adrenergic receptor by isoproterenol (Table 3-3) did not decrease defeminization. This may reflect the full occupancy of inhibitory β -adrenergic receptors by norepinephrine in the physiological condition. On the other hand, inhibiting β_1/β_2 receptors by propranolol

might simply allow more norepinephrine to bind to an unknown receptor that stimulates defeminization. Our result is in agreement with a previous conclusion regarding the role of β -adrenergic receptors (Raum and Swerdloff, 1981; McGivern et al., 1986).

Similarly, the increase of defeminization by PRA-induced inhibition of the α_1 -adrenergic receptor (Table 3-3) indicates that the α_1 -adrenergic receptor is involved in the defeminization and that α_1 -adrenergic agonist (norepinephrine) complex under physiological condition inhibits the defeminization of hepatic cytochrome P450. Although phenylephrine (a specific α_1 -adrenergic agonist) (Table 3-3) given neonatally did not decrease the defeminization of EMDM, it does not exclude the possibility that an α_1 -adrenergic receptor-complex may cause the defeminizing process of hepatic cytochrome P450. The lack of a response to the agonist may result from fully occupied α_1 -adrenergic receptors under normal physiological conditions. Also only one dose of agonist was used. Our data that propranolol and prazosin together suppressed superdefeminization suggest that both two drugs inhibit each other.

Previous reports showed that perinatal stimulation or inhibition of CNS adrenergic activity affected the control of

gonadotropic secretion and sexual behaviour. It appears that the roles of the different types of adrenergic receptors in the modulation of these defeminizations are different.

Stimulation of the β -adrenergic receptor during the neonatal period prevents the defeminizing effect of neonatal testosterone in female rats (Raum and Swerdloff, 1981), which prevents the occurrence of a cyclic gonadotropin secretion and leads to anovulation, interrupts the vaginal cyclicity and suppresses the lordosis reflex, a behaviour of adult female rats responding to the mounting attempts of a sexually active male by an arching of the back. Their data suggested that activation of the β -adrenergic receptor inhibits defeminization. Other evidence also supports this hypothesis. Prenatal stress has been demonstrated to cause a small rise in catecholamine levels in the dam, but a 3- to 4-fold rise in the fetus (Rohde et al., 1983). Thus, the development of female patterns of saccharin preference and maze learning behaviour is thought to result from the prenatal stress-induced increase in norepinephrine. It was reported (McGivern et al., 1986) that propranolol prevented this development of female patterns of saccharin preference and maze learning behaviour in adult male rats. Furthermore, since androgen is converted to estradiol, which acts to defeminize the brain,

via aromatization and β -adrenergic antagonist prevents the inhibition of nuclear estradiol accumulation produced by β -adrenergic agonist (Raum et al., 1984), it is hypothesized that the inhibition of defeminizing effects may be mediated via stimulating the β -adrenergic receptor (Raum et al., 1984).

In contrast, the α_1 -adrenergic receptor seems to play another role in the defeminization of gonadotropin secretion. It was found that prazosin, an α_1 -adrenergic antagonist, prevented the reduction of lordosis behaviour induced by neonatal testosterone in female rats (Jarzab et al., 1987), indicating that activation of the α_1 -adrenergic receptor stimulates defeminization. They suggested that blockade of the α_1 -adrenergic receptor allows more norepinephrine to bind to the β -adrenergic receptor and inhibits defeminization. Besides, Raum and his colleagues (1984) reported that phenoxybenzamine, an α -adrenergic antagonist, inhibited the nuclear accumulation of estradiol converted from androgen (Raum et al., 1984); thus, blockade of the α -adrenergic receptor allowed less estradiol to defeminize the brain.

The α_2 -adrenergic agonist, clonidine, also attenuated the inhibitory effect of neonatal testosterone on the differentiation of lordosis behaviour in female rats (Jarzab

et al., 1987). Jarzab and his colleagues hypothesized that presynaptic stimulation of the α_1 -adrenergic receptor reduces the release of norepinephrine into the synaptic cleft and leads to less stimulation of postsynaptic α_1 - and β -adrenergic receptors, and attenuates the defeminizing effect of neonatal testosterone.

Briefly, the authors above hypothesized that the defeminizing effects of postnatally acting steroids may be mediated via stimulating the α_1 -adrenergic (postsynaptic) receptor, and that blockade of defeminization may be mediated via stimulating the β -adrenergic receptor, and activation of the α_1 -adrenergic receptor may indirectly inhibit defeminization activity via the β -adrenergic receptor.

However, this model cannot fully explain all results available, for example, activation or inhibition of β -adrenergic receptors during postnatal development permanently impaired the responsiveness of the centre for cyclic gonadotropin release to gonadal steroids in female rats (Jarzab et al., 1989a), and might not be true for the sexually differentiated hepatic cytochrome P450 system. Reznikov et al. (1990) showed that tropolone, which inhibits catechol-o-methyltransferase so that norepinephrine increases in the

hypothalamic synaptic clefts augmented the sterilizing effect of neonatal testosterone on female rats. However, this study tells nothing of receptor type involved but only that increase of norepinephrine stimulates defeminization. In our study (Table 3-3), both neonatal propranolol (β -adrenergic antagonist) and neonatal prazosin (α_1 -adrenergic antagonist) stimulated the defeminization of hepatic EMDM; these data suggest that both α_1 - and β -adrenergic receptors are inhibitory to the defeminization of hepatic cytochrome P450 under physiological condition.

Our conclusion about the role of β -adrenergic receptors in defeminization is agreement with Jarzab et al.'s hypothesis. However, our conclusion concerning the role of α_1 -adrenergic receptors in defeminization is contradictory to that of the above researchers (eg. Jarzab et al., 1986, 1987). That stimulation of the α_1 -adrenergic receptor inhibits defeminization is concluded from our data; but an opposing role of α_1 -adrenergic receptor is deducted from their results.

One explanation for this discrepancy might be that different parameters were used to measure defeminization (eg. reproductive endocrine status and behaviour vs. cytochrome P450). It has been reported (Jarzab et al., 1989a) that

stimulation of the β -adrenergic receptor during the perinatal period intensified differentiation of feminine reproductive behaviour but impaired differentiation of feminine gonadotropin secretion. Thus, prazosin, an α_1 -adrenergic antagonist, attenuated the inhibitory effect of neonatal testosterone on the differentiation of lordosis behaviour in female rats but failed to prevent the anovulatory syndrome induced by neonatal testosterone (Jarzab et al., 1987). These studies indicate that differentiation of different sexual functions may not overlay identical adrenergic mechanisms, and imply the possibility that different systems might be differently effected.

It was reported that stimulation of the α_2 -adrenergic receptor by clonidine attenuated the reduction of lordosis capacity, induced by postnatal treatment of female rats with testosterone (Jarzab et al., 1986). The authors hypothesized that presynaptic stimulation of the α_2 -adrenergic receptor decreases the release of norepinephrine into the synaptic cleft and leads to less stimulation of postsynaptic α_1 - and β -adrenergic receptors. However, this speculation contradicts our clonidine data in which clonidine did not change defeminization. In our study, the prevention of propranolol-induced "super-defeminization" by prazosin, a very potent and selective α_1 -adrenergic antagonist, suggests that more

norepinephrine might bind to the α_2 -adrenergic receptor, when both α_1 - and β -adrenergic receptors are blocked, so that the α_2 -adrenergic-agonist (norepinephrine) complex prevents "super-defeminization". Our data on phentolamine are consistent with this. Phentolamine blocks both α_1 and α_2 -adrenergic receptors, and the "super-defeminization" caused by propranolol is not affected. That neither clonidine (α_2 -adrenergic agonist) nor WY27127 (α_2 -adrenergic antagonist) influenced PRO-induced "super-defeminization" implies that the α_2 -adrenergic receptor under physiological condition might be of minor importance in the defeminization of hepatic cytochrome P450, but it might replace the inhibitory role of both α_1 - and β -adrenergic receptors under some circumstance.

4.1.2 Mechanism of the Action of Adrenergic Receptors in Defeminization

Defeminization is induced by neonatal sex steroids. Under the influence of neonatal and adult sexual steroids, the morphology and the neurotransmission of the brain develop in sexually different ways; consequently, reproductive hormone and behaviour, and other brain-controlled physiological functions such as the secretory pattern of GH are sexually differentiated.

During the neonatal period androgen in the brain is converted to estradiol via aromatization. Estradiol then binds to estrogen receptors in cytosol and the complex enters the nucleus and presumably combines with the genes which regulate defeminization. The essential role of estradiol in defeminization has been confirmed by McCarthy et al. (1993). They infused a 15-mer antisense oligodeoxynucleotide to the translation start codon of estrogen receptor messenger RNA into the hypothalamus of rat pups, and found protection against many of the defeminizing effects of testosterone.

Although it is known that adrenergic receptors modulate the defeminizing effect of neonatal testosterone, the mechanism of the action of adrenergic receptors on the defeminization is not known. That tropolone alone did not defeminize female rats but augmented the sterilizing effect of neonatal testosterone on female rats (Reznikov et al. 1990) and that neither phentolamine nor isoproterenol (Table 3-1) increased the activity of EMDM in male rats castrated neonatally suggest adrenergic receptors modulate but do not mediate the defeminizing effect of neonatal androgen.

Raum et al. (1984) showed that the nuclear estradiol in hypothalamus was decreased in rats treated with β -adrenergic

agonists and that both exogenous testosterone and β -adrenergic antagonists prevented the decrease. They suggested, therefore, that stimulation of β -adrenergic receptors decreases the activity of aromatase and reduces the aromatization of Δ^4 -androgens. However, evidence indicates that the aromatase is activated by phosphorylation (eg. β -adrenergic activation). It has been demonstrated that the activity of aromatase was decreased when alkaline phosphatase was added to the buffer and was stabilized by phosphate buffer or phosphatase inhibitors (Bellino and Holben, 1989). Further, it is known (Lefkowitz et al., 1990) that stimulation of the β -adrenergic receptor activates, under the influence of a stimulatory guanine nucleotide-binding regulatory protein (G_s), adenylyl cyclase, which promotes the accumulation of cAMP, at the cytoplasmic face of the plasma membrane. cAMP then activates cAMP-dependent protein kinase in cytosol. The activated cAMP-dependent protein kinase (protein kinase A) catalyzes the phosphorylation of phosphorylase kinase, thereby activating it. Phosphorylase kinase then phosphorylates and activates phosphorylase which phosphorylates a variety of cellular proteins and regulates their activities. Therefore, Raum's theory is inconsistent with these facts, and the prevention of defeminization by stimulating the β -adrenergic receptor does probably not occur via the aromatase. On the

other hand, it has been reported that increasing cellular cAMP markedly decreased estrogen binding to the nuclear receptor (Pedersen et al., 1991). Thus, it seems that the prevention of defeminization by β -adrenergic agonists might occur via a decrease in estrogen binding to the nuclear receptor or the genome. However, although there were reports that phosphorylation participates in the transcription regulation by estrogen receptor complex (Ishibe, 1993; Ali et al., 1993) and that phosphorylation of estrogen receptor increased the affinity of estrogen receptor for specific DNA sequences (Denton et al., 1992), it seems that estrogen receptor has autophosphorylation activity, ie. the estrogen receptor can be phosphorylated with ATP without any protein kinase (Ishibe, 1993). Thus, whether the increase of cAMP induced by the activation of adrenergic receptors is necessary for the phosphorylation of estrogen receptors is not known. Further, it was suggested that an increase in overall estrogen receptor phosphorylation does not necessarily result in the increase of transcriptional activity (Aronica and Katzenellenbogen, 1993); that the modulation of defeminization by adrenergic receptors is via regulating the estrogen behaviour is still discussable. Therefore, as functions of receptors that are regulated by phosphorylation are only beginning to be investigated, more studies are needed to solve the problems concerning the

relationship between estrogen receptor and phosphorylation, and also the mystery regarding the mechanism of adrenergic receptor action in defeminization.

Stimulation of the α_1 -adrenergic receptor leads to the activation of a membrane-bound phospholipase C which hydrolyses a membrane phospholipid to form diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 then causes the release of Ca^{2+} from intracellular stores, which initiates a variety of cellular responses; and DAG stimulates the activity of a Ca^{2+} -sensitive enzyme, protein kinase C, which phosphorylates a distinct set of substrates (Lefkowitz et al., 1990) including some substrates also for protein kinase A. It has been found (Petitti and Etgen, 1991) that stimulation of the α_1 -adrenergic receptor enhanced β -adrenoceptor-stimulated cAMP formation in rat hypothalamus, and protein kinase C appeared to mediate this process. Thus, stimulation of EMDM defeminization by prazosin does not seem to be related to the aromatase. Since stimulation of the α_1 -adrenergic receptor appears to intensify the β -adrenoceptor-induced cAMP increase which will decrease estrogen binding to nuclear receptors, the inhibition of defeminization by the α_1 -adrenergic agonist complex might occur via cAMP increase. However, since some substrates phosphorylated by protein kinase C are different

from the substrates phosphorylated by both protein kinases C and A, it implies that the α_1 -adrenergic receptor act differently from the β -adrenergic receptor. In our data, the total amount of cytochrome P450 was increased by propranolol but not by prazosin in the superdefeminized animals and this suggests that the α_1 -adrenergic receptor in defeminization might act in a way completely independent of the β -adrenergic receptor.

There is evidence showing that alteration of catecholamine transmission during the brain development causes long-term changes in neuronal organization. For example, prenatal exposure of rats to the dopamine (DA) antagonist haloperidol induces a long-term decrease in DA receptor number in the brain (Rosengarten and Friedhoff, 1979). Neonatal clonidine treatment causes long-term changes in noradrenaline sensitivity (Gorter et al., 1989).

Adrenergic receptors might generate the formation of cellular cAMP, stimulate phosphorylation, and activate protein kinases and similarly stimulate the neuronal development. Stimulation of the α_2 -adrenergic receptor inhibits adenyl cyclase; as a consequence, intracellular concentration of cAMP are lowered and the activity of cAMP-dependent protein kinase

is decrease (Lefkowitz et al., 1990). Therefore, blockade of both α_1 - and β -adrenergic receptors allows more norepinephrine bind to the α_1 -adrenergic receptor. The activation of α_1 -adrenergic receptors decreases intracellular concentrations of cAMP and leads to the dephosphorylation of cellular protein. Thus, the activity of aromatase might be lowered and the defeminization might be inhibited. In another way, activation of the presynaptic α_2 -adrenergic receptor inhibit the release of norepinephrine and leads to less stimulation of the stimulatory receptor for the defeminization of hepatic cytochrome P450.

However, the role of the β -adrenergic receptor in these processes is probably different from that of the α_1 -adrenergic receptor. Thus, activation of the β -adrenergic receptor stimulates mainly adenylate cyclase, but the α_1 -adrenergic receptor is mainly responsible for the regulation of protein kinase C and Ca^{2+} /calmodulin-sensitive protein kinases. Furthermore, the β -adrenergic receptor is able to regulate the activity of ornithine decarboxylase, which is involved in the processes of axogenesis and synaptogenesis (Morris and Slotkin, 1985; Slotkin and Bartholome, 1986; Slotkin et al., 1988); However, no data indicate that the α_1 -adrenergic receptor is related to the regulation of this enzyme.

Therefore, the β -adrenergic receptor might have different approach(es) to modulate the defeminization from that of the α_1 -adrenergic receptor.

Although alteration of some catecholamine transmission with dopamine (DA) antagonist or clonidine during the brain development caused long-term changes in neural organization such as changes in DA receptor number and noradrenaline sensitivity of the brain (Rosengarten and Friedhoff, 1979; Gorter et al., 1989), changes of β -adrenergic activity do not seem to have long-term effect on noradrenergic system of the rat brain (Erdsieck-Ernste et al., 1993). Postnatal blockade of the β -adrenergic receptor with labetalol, an α_1 - and β -adrenergic antagonist, increased α_1 -adrenergic receptor binding; however, neither propranolol nor labetalol had long-term effect on the β -adrenoceptor number in the mature rat brain. Although postnatal propranolol caused an immediate increase of noradrenaline (NA) metabolism, the long-term effect of this drug on NA metabolism is still questionable. Therefore, the β -adrenergic receptor might have immediate effect on the action of neonatal androgen, which defeminizes the brain structure and some neurotransmitter systems which are related to the secretion of GH. However, since the data concerning the long-term effect of β -adrenoceptor agents on β -

adrenoceptor number and NA metabolism were obtained based on the usage of non-selective β -adrenergic agents, further studies are still needed to confirm the conclusion. Furthermore, the evidence above does not exclude the long-term effect of α_1 - and α_2 -adrenergic receptors on adrenoceptor number and NA metabolism.

4.1.3 Kinetics of EMDM and 16 α -, 2 α -, and 6 β -Testosterone Hydroxylases in PRO-induced Superdefeminization

Since adrenergic drugs do not result in significant changes in the total amount of P450 and the activities of cytochrome c reductase and p-nitrophenol hydroxylase, the final effect induced by adrenergic agents on the defeminization of EMDM impacts on the isoforms of sex-dependent cytochrome P450. Although propranolol increased the total amount of P450, the increase was not significant and is unexplainable to the marked elevation of EMDM activity. Propranolol did not change the apparent K_m of EMDM but did increase the apparent V_{max} in the superdefeminized group; therefore, the enhancement of EMDM activity by propranolol is due to the increase of V_{max} . Since the alteration of activity or amount of the isoform(s) demethylating ethylmorphine can

result in changes in the V_{max} of EMDM, increase of EMDM activity by propranolol might be related to a rise in the activity or amount of isoform(s) responsible for ethylmorphine demethylation.

Cytochrome P450 2C11, a major male-specific P450, is responsible for 16 α - and 2 α -testosterone hydroxylations (Kato et al., 1986) and cytochrome P450 3A1/2, another male-specific isoform, catalyses 6 β -testosterone hydroxylation (Imaoka et al., 1988) and is also a major isoform for ethylmorphine demethylation (Wrighton et al., 1985). In our study, propranolol increased the activities of 6 β -, 16 α -, and 2 α -testosterone hydroxylases; therefore, propranolol stimulated the defeminization of hepatic cytochrome P450 by increasing the expression of at least two isoforms 2C11 and 3A1/2.

It has been reported neonatal defeminization induced high amplitude GH pulses (Jansson and Frohman, 1987), which is parallel to the defeminization of EMDM activity (Virgo, 1991), in adulthood. Furthermore, both neonatal defeminization and adult masculinization together lead to typical masculine pattern of GH secretion (Jansson and Frohman, 1987), which is parallel to the pubertal activity of EMDM (Virgo, 1991). Besides, it was found that decline in the amplitude of GH

pulses with intact ultradian rhythm caused 16 α -, 2 α -, and 6 β -testosterone hydroxylases, and isoforms of P450 2C11 and 3A2 to exceed normal levels (Shapiro et al., 1989; Pampori et al., 1991). These data suggest that the secretory pattern of GH determines the expression of some sex-dependent cytochromes P450, and that alteration of GH secretion might change the activities of these isoforms. Therefore, the superdefeminizing effect of propranolol on the activities of 16 α -, 2 α -, and 6 β -testosterone hydroxylases might be via altering the secretory pattern of GH, which in turn stimulates the expression of sex-dependent P450 isoforms, in adulthood.

4.2 NEONATAL MONOSODIUM GLUTAMATE AND PREPUBERTAL DEVELOPMENT OF MALE SPECIFIC CYTOCHROMES P450

From our data, we conclude that neonatally administered monosodium glutamate (MSG) does not affect the activities of hepatic EMDM, p-NPH or cytochrome c reductase, or the total amount of cytochrome P450 in 35 day old (pre-pubertal) rats. Furthermore, p-NPH activity reaches adult levels before puberty. However, neonatal MSG increased EMDM activity between day 35 and 65 (post-puberty) while p-NPH and cytochrome c reductase activity were not altered. The total amount of cytochrome P450 was increased by 26% in the

postpubertal rats given neonatally 4 mg/g MSG. Neonatal 2 mg/g or 4 mg/g MSG did not change the apparent K_m of EMDM activity in the postpubertal rat liver, but 4 mg/g MSG increased the apparent V_{max} by 38%. Further, neonatal MSG increased the activity of 6 β - but not 2 α /16 α -testosterone hydroxylases in the rat liver at Day 65.

4.2.1 Prepubertal Development

Neonatal administration of monosodium glutamate (MSG) to rats produces almost complete disappearance of GH releasing hormone (GHRH) immunoreactive cell bodies in the arcuate nucleus and GHRH-immunoreactive fibres in the median eminence (Olney, 1981; Lemkey-Johnston and Reynolds, 1974), while apparently having no effect on adjacent areas in the hypothalamus. Consequently, serum concentrations of GH (Terry et al., 1981; Nemeroff et al., 1977) are profoundly reduced; whereas serum levels of ACTH (Krieger et al., 1979), PRL (Terry et al., 1981), TSH (Nemeroff et al., 1977), FSH, and LH (Dada and Blake, 1985) are normal. Therefore, we administered MSG to male pups to eliminate plasma GH and observed the relationship between GH and the development of hepatic cytochrome P450.

It is well known that the activity of sex-dependent cytochrome P450 in rat liver displays developmental changes. The activity of hepatic EMDM in male rats is low during the neonatal period, but begins to rise gradually at 4th week and reaches adult levels at or after puberty (Gram et al., 1969). Cytochrome P450 2C11, a male specific isoform, is undetectable in male pups; it then expresses a gradual increase of activity to the 4th week, and shows high adult activity at puberty (Waxman et al., 1985; Imaoka et al., 1991). Cytochrome 3A1/2, other male specific isoforms, also displays developmental expression. In contrast to the increase of 2C11 isoform in male rats, it is mainly suppressed in female rats (Waxman et al., 1985; Imaoka et al., 1991). All the activities of EMDM (Colby, 1980), 2C11 (Mode et al., 1989; Legraverend et al., 1992) and 3A1/2 (Waxman et al., 1988) are regulated by GH at adulthood. The secretory pattern of GH was demonstrated to determine the expression of male specific isoforms 2C11 and 2C12 (Mode et al., 1989; Legraverend et al., 1992). Male-type of GH secretion stimulates the expression of the male isoform 2C11, but suppresses the appearance of the female isoform 2C12; while female pattern of GH secretion suppresses the expression of 2C11 isoform, but promotes the 2C12 expression. Although the expression of isoform 3A1/2 is not dependent on pulsatile plasma GH (Waxman et al., 1988), its expression is suppressed by GH (Waxman et al., 1988).

Our data (Table 3-9) show that neonatal administration of 2 or 4mg/g MSG did not change the activity of hepatic EMDM in 35-day old rats. Since it was reported (Shapiro et al., 1989; Waxman et al., 1990) that 2mg/g MSG caused an 80-90% reduction in the pulse amplitudes of plasma GH and 4mg/g MSG resulted in the total loss of all measurable levels of circulating GH in adulthood, and further, that plasma GH in prepubertal rats is undetectable (Khorram et al., 1983; Strosser and Mialhe, 1975); therefore, in this study, there should be no plasma GH during the prepubertal period. Thus the data suggest that the prepubertal development of the male type P450 in the rat liver is not related to plasma GH. Further, since there is no significant difference in the total amount of cytochrome P450, the activities of cytochrome c reductase and p-nitrophenol hydroxylase, it seems that GH is not related to the prepubertal development of hepatic cytochrome P450 system.

Since the hepatic EMDM activity in male rats at Day 35 is significantly higher than that of defeminized rats, the EMDM activity does express a developmental increase. As GH does not seem to be involved in the prepubertal development of EMDM activity, there may be other hormones responsible for this EMDM development.

Serum corticosterone in rats shows developmental changes (Lu et al., 1987; Henning, 1978). During the neonatal period, the concentration of plasma corticosterone is quite low. It increases gradually and reaches the highest point at the 24th Day but then declines. It was reported that this hormone increased the activity of hepatic ethylmorphine demethylase in adult rats (Colby, 1980), and that dexamethasone elevated the amount of hepatic cytochrome P450 3A (Gonzalez et al., 1986; Wrighton et al., 1985; Nagata et al., 1990), which is associated with ethylmorphine demethylation (Wrighton et al., 1985). The concentration of plasma FSH in male rats also displays developmental changes (Ketelslegers et al., 1978). At birth, plasma FSH shows high levels but declines to its lowest levels in the second week; it then climbs gradually and reaches the highest concentration at the 40th Day. This hormone was reported to be able to increase the rate of EMDM in castrated rats of either sex (Krammer et al., 1977), suggesting that the increase of hepatic EMDM activity induced by FSH may not via the FSH-stimulated release of testosterone. Thus the prepubertal development of EMDM activity might reflect the combined effect of these hormones. However, plasma testosterone begins to rise at the 4th week of life in male rats; further, androgen receptors have been found in the rat liver (Eagon et al., 1989; Konoplya and Popoff, 1992), the

possibility that prepubertal development of hepatic EMDM activity result from the gradual increase of plasma androgen before puberty (Corpéchet et al., 1981) should not be excluded.

4.2.2 Pubertal Development

Since the secretory pattern of GH is important to the activities of sex-dependent P450 isoforms in adulthood, neonatal MSG which can eliminate plasma GH will change the activity of sex-dependent cytochrome P450 in adulthood. Our data (Table 3-11) show that neonatal administration of 2 or 4mg/g MSG increased the EMDM activity in adulthood. Since hypophysectomy increased the EMDM activity (Colby, 1980) and GH together with decreased the EMDM activity in the thyroxine (T_4)-, ACTH-, or T_4 - and ACTH-treated hypophysectomized male rats (Colby, 1980); and further, clonidine which increases GH release inhibited EMDM activity, but somatostatin which lowers the trough of GH secretion pattern decreases GH release stimulated EMDM activity (Virgo, 1985), GH is thought to be inhibitory to the EMDM activity (Virgo, 1985). Therefore, the increase of EMDM activity in MSG-treated groups suggest that neonatal MSG reduces plasma GH and lessens the suppression by GH on EMDM activity.

In our study, MSG at dose of 4mg/g enhanced the apparent V_{max} of hepatic EMDM but did not affect the apparent K_m . The increase of the V_{max} of EMDM might result from an elevation in the activity or the amount of isoform(s) for ethylmorphine demethylation, suggesting that the increase of EMDM activity induced by neonatal MSG is due to an increase of the activity or amount of EMDM. MSG at dose of 4mg/g increased the total amount of cytochrome P450 by 24%, which might result from an increase in the amount of isoform(s) and may partly explain the changes in EMDM activity.

Microsomal activities of 6 β -testosterone hydroxylase, in 2 or 4 mg/g MSG-treated rats, rose by 44%-47% (Table 3-14); however, MSG did not effect on the activities of 16 α - and 2 α -testosterone hydroxylases. Because cytochrome P450 3A1/2 is responsible for 6 β -testosterone hydroxylation (Imaoka et al., 1988), and cytochrome P450 2C11 for 16 α - and 2 α -testosterone hydroxylations, the data imply that neonatal MSG influences the expression of cytochrome P450 3A1/2 but not cytochrome P450 2C11. EMDM is mainly associated with 3A1/2 isoform (Wrighton et al., 1985); therefore, the increase of EMDM activity in MSG-treated groups is due to the elevation of 3A1/2 amount.

However, our suggestion differs from the observation of Pampori and his colleagues (1991). They showed that the neonatal administration of 2mg/g MSG did not change the ultradian pattern of circulating GH, although it reduced in the pulse amplitudes of plasma GH by 80%, while it increased the levels of cytochrome P450 2C11 and P450 3A1/2 in male rats. At 4mg/g the levels of these isoforms were depressed. Concurrently, the activities of 16 β -, 2 α -, and 6 β -testosterone hydroxylases increased or decreased in parallel with the changing levels of their substrate specific isoforms of P450 (Pampori et al., 1991). The expression of cytochrome P450 2C11 is dependent on the secretory pattern of GH (Mode et al., 1989; Legraverend et al., 1992), and low peaks of GH with male secretory pattern are still sufficient to stimulate the expression of 2C11 isoform (Legraverend et al., 1992). The above researchers' studies suggest that decreasing plasma GH without changing the male secretory pattern of GH may intensify the expression of 2C11 isoform and the activities of 16 α - and 2 α -testosterone hydroxylases. Thus, the data in our study that 2 mg/g MSG did not change the activities of 16 α - and 2 α -testosterone hydroxylases might that the dosage of MSG used did not cause a low enough reduction in plasma GH, and that 4mg/g MSG did not influence the activities of the 16 α - and 2 α -hydroxylases in our study might be reflect that the plasma GH was not totally absent.

In Pampori et al.'s study (1991), 2mg/g MSG increased the activity of 6 β -testosterone hydroxylase, but 4mg/g MSG depressed the activity. The expression of cytochrome P450 3A1/2 which catalyses 6 β -hydroxylase was demonstrated to be not dependent on the secretory pattern of GH, but depressed by continuous GH (Waxman et al., 1988). Hypophysectomy increased the levels of cytochrome P450 3A1/2, but injection of GH to these rats depressed the levels of the enzyme (Waxman et al., 1990). All these data imply that continuous GH is inhibitory to the expression of 3A1/2 isoform and the activity of 6 β -testosterone hydroxylase.

Our data show that both 2 and 4mg/g MSG enhanced the activity of 6 β -testosterone hydroxylase (Table 3-14). The increase of the activity of 6 β -testosterone hydroxylase in MSG-treated rats, therefore, might be due to MSG-induced loss of plasma GH; that 4 mg/g MSG did not suppress the activity of 6 β -testosterone hydroxylase might be due to the failure of the dosage in this study to completely eliminate plasma GH.

REFERENCES

- Ali S, Metzger D, Bornert JM, and Chambon P (1993). "Modulation of transcription activation by ligand-dependent phosphorylation of the human receptor A/B region." *EMBO. J.* 12(3): 1153-1160
- Anderson KE and Kappas A (1991). "Dietary regulating of cytochrome P450." *Annu. Rev. Nutr.* 11: 141-167
- Arendash GW and Gorski RA (1982). "Enhancement of sexual behavior in female rats by neonatal transplantation of brain tissue from males." *Science* 217: 1276-1278
- Arendash GW and Gorski RA (1983). "Effects of discrete lesions of the sexually dimorphic nucleus of the preoptic area or other medial preoptic regions on the sexual behavior of male rats." *Brain Res. Bull.* 10: 147-154
- Aronica SM and Katzenellenbogen BS (1993). "Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I." *Mol. Endocrinol.* 7(6): 743-752
- Ayoub DM, Greenough WT, and Juraska JM (1982). "Sex differences in dendritic structure in the preoptic area of the juvenile macaque monkey brain." *Science* 219: 197-198
- Bellino F and Holben L (1989). "Placental estrogen synthetase (aromatase): phosphatase-dependent inactivation." *Biochem. Biophys. Res. Commun.* 162(1): 498-504
- Bloch B, Ling N, Benoit R, Wehrenberg WB, and Guillemin R (1984). "Specific depletion of immunoreactive growth hormone-releasing factor by monosodium glutamate in rat median eminence." *Nature (London)* 307: 272-273
- Bock KW, Lipp HP, and Bock-Henning BS (1990). "Induction of drug-metabolizing enzymes by xenobiotics." *Xenobiotica* 20(11): 1101-1111

- Booth JE (1977). "Effects of the aromatization inhibitor, androst-4-ene-3,6,17-trione on sexual differentiation induced by testosterone in the neonatally castrated rat." *J. Endocrinol.* 72: 53-54
- Booth JE (1979). "Sexual differentiation of the brain." In: *Oxford Reviews of Reproductive Biology*, ed. Finn CCA, 1: 58-158
- Bradford M (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." *Anal. Biochem.* 72: 248-254
- Breedlove SM and Arnold AP (1980). "Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord." *Science* 210: 564-566
- Canick JA, Tobet SA, Baum MJ, Vaccario DE, Ryan KJ, Leeman SE, and Fox TO (1987). "Studies on the role of catecholamines in the regulation of the developmental pattern of hypothalamic aromatase." *Steroids* 50(4-6): 509-521
- Chowen JA, Argente J, Gonzalez-Parra S, and Garcia-Segura LM (1993). "Differential effects of the neonatal and adult sex steroid environments on the organization and activation of hypothalamic growth hormone-releasing hormone and somatostatin neurons." *Endocrinology* 133(6): 2792-2802
- Chung LWK (1977). "Characteristics of neonatal androgen-induced imprinting of rat hepatic microsomal monooxygenases." *Biochem. Pharmacol.* 26: 1979-1984
- Chung LWK, Raymond J, and Fox S (1975). "Role of neonatal androgen in the development of hepatic microsomal drug-metabolizing enzymes." *J. Pharmacol. Exp. Ther.* 193: 621-630
- Colby HD (1980). "Regulation of hepatic drug and steroid metabolism by androgens and estrogens." *Adv. Sex Horm. Res.* 4: 27-71
- Corpéchet C, Baulieu EE, and Robel P (1981). "Testosterone, dihydrotestosterone and androstenediols in plasma, testes and prostates of rats during development." *Acta Endocrinol.* 96: 127-135

- Dada MO and Blake CA (1985). "Monosodium L-glutamate administration: effects on gonadotropin secretion, gonadotrophs and mammatrophs in prepubertal female rats." *J. Endocrinol.* 104: 185-192
- Davies DS, Gigon PL, and Gillette JR (1969). "Species and sex differences in electron transport system in liver microsomes and their relationship to ethylmorphine demethylation." *Life Sci.* 8: 85-91
- Döhler KD (1981). "Sexual differentiation of the brain." *Vitam. Horm.* 38: 325-381
- Döhler KD (1986). "The special case of hormonal imprinting, the neonatal influence of sex." *Experientia* 42: 759-769
- Döhler KD (1991). "The pre- and postnatal influence of hormones and neurotransmitters on sexual differentiation of the mammalian hypothalamus." *Int. Rev. Cytol.* 131: 1-57
- Doughty C, Booth JE, McDonald PG, and Parrott RF (1975). "Inhibition, by the anti-oestrogen mer-25, of differentiation induced by the synthetic oestrogen RU 2858." *J. Endocrinol.* 67: 459-460
- Doughty C and McDonald PG (1974). "Hormonal control of sexual differentiation of the hypothalamus in the neonatal female rat." *Differentiation* 2: 275-285
- Dyer RG (1984). "Sexual differentiation of the forebrain-relationship to gonadotropin secretion." *Prog. Brain Res.* 61: 223-236.
- Eagon PK, Seguiti SM, Rogerson BJ, McGuire TF, Porter LE, and Seeley DH (1989). "Androgen recepto: in rat liver: characterization and separation from a male-specific estrogen-binding protein." *Arch. Biochem. Biophys.* 268(1): 161-175
- Erdtsieck-Ernste EBHW, Feenstra MGP, and Boer GJ (1993). "Perinatal influence of beta-adrenergic drugs on the noradrenergic system of the rat brain." *Gen. Pharmacol.* 24(5): 1069-1078

- Evans CT, Jo Corbin C, Saunders CT, Merrill JC, Simpson ER, and Mendelson CR (1987). "Regulation of estrogen biosynthesis in human adipose stromal cells: effects of dibutyryl cyclic AMP, epidermal growth factor, and phorbol esters on the synthesis of aromatase cytochrome P-450." *J. Biol. Chem.* 262: 6914-6920
- Gabriel SM, Roncancio JR, and Ruiz NS (1992). "Growth hormone pulsatility and the endocrine milieu during sexual maturation in male and female rats." *Neuroendocrinology* 56: 619-628
- George FW and Ojeda SR (1982). "Changes in aromatase activity in the rat brain during embryonic, neonatal, and infantile development." *Endocrinology* 111: 523-529
- Gomori G (1955). "Preparation of buffer for use in enzyme studies." *Meth. Enzymol.* 1: 138-146
- González MI and Leret ML (1992). "Neonatal catecholaminergic influence on behaviour and sexual hormones." *Physiol. and Behav.* 51: 527-531
- Gonzalez FJ, Song B-J, and Hardwick JP (1986). "Pregnenolone-16 α -carbonitrite-inducible P-450 gene family: gene conversion and differential regulation." *Mol. Cell Biol.* 6: 2969-2976
- Gorski RA (1966). "Localization and sexual differentiation of the nervous structure which regulate ovulation." *J. Reprod. Fertil. Suppl.* 1: 67
- Gorski RA (1973). "Perinatal effects of sex steroids on brain development and function." *Prog. Brain Res.* 39: 149-163
- Gorski RA (1991). "Sexual differentiation of the endocrine brain and its control." *Brain Endocrinology*, second edition, Raven Press, Ltd., New York: 71-104
- Gorski RA, Gordon J, Shryne JE, and Southam AM (1978). "Evidence for a morphological sex difference within the medial preoptic area of the rat brain." *Brain Res.* 148: 333-346
- Gorski RA, Harlan RE, Jacobson CD, Shryne JE, and Southam AM (1980). "Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat." *J. Comp. Neurol.* 193: 529-539

- Gorter JA, Mirmiran M, Bos NPA, and Van Der Werf D (1989). "Hippocampal neuronal responsiveness to different transmitters in the adult rat after neonatal interference with noradrenaline transmission." *Brain Res. Bull.* 23: 293-297
- Goy RW and McEwen ES (1980). eds. *Sexual differentiation of the brain*, MIT Press, Cambridge, Massachusetts, pp 211
- Gram TE, Guarino AM, Schroeder DH, and Gillette JR (1969). "Changes in certain kinetic properties of hepatic microsomal aniline hydroxylase and ethylmorphine demethylase associated with postnatal development and maturation in male rats." *Biochem. J.* 113: 681-685
- Greenough WT, Carter CS, Steerman C, and DeVoogd TJ (1977). "Sex differences in dendritic patterns in hamster preoptic area." *Brain Res.* 126: 63-72
- Güldner FH (1982). "Sexual dimorphisms of axo-spine synapses and postsynaptic density material in the suprachiasmatic nucleus of the rat." *Neurosci. Lett.* 28: 145-150
- Gurney ME and Konishi M (1980). "Hormone-induced sexual differentiation of brain and behavior in zebra finches." *Science* 208: 1380-1383
- Gurtoo HL and Parker NB (1976). "Organ specificity of the sex dependent regulation of aryl hydrocarbon hydroxylase (AHH) in rat." *Biochem. Biophys. Res. Commun.* 72: 216-222
- Gustafsson J-Å, Ingelman-Sundberg M, Stenberg Å, and Hökfelt T (1976). "Feminization of hepatic steroid metabolism in male rats following electrothermic lesion of the hypothalamus." *Endocrinology* 98: 922-926
- Gustafsson J-Å, Eneroth P, Pousette Å, Skett P, Sonnenschein C, Stenberg Å, and Åhlén A (1977). "Programming and differentiation of rat liver enzymes." *J. Steroid Biochem.* 8: 429-437
- Gustafsson J-Å, Edén S, Eneroth P, Hökfelt T, Isaksson D, Jansson J-O, Mode A, and Norstedt G (1983). "Regulation of sexually-dimorphic hepatic steroid metabolism by the somatostatin-growth hormone axis." *J. Steroid Biochem.* 19: 691-698

- Handa RJ, Hines M, Schoonmaker JN, Shryne JE, and Gorski RA (1986). "Evidence that serotonin is involved in the sexually dimorphic development of the preoptic area in the rat brain." *Dev. Brain Res.* 30: 278-282
- Harris GW (1964). "Sex hormones, brain development and brain function." *Endocrinology* 75: 627-648
- Henning SJ (1978). "Plasma concentrations of total and free corticosterone during development in the rat." *Am. J. Physiol.* 235: E451-E456
- Hielanen E (1974). "Effect of sex and castration on hepatic and intestinal activity of drug-metabolizing enzymes." *Pharmacology* 12: 84-89
- Hoffman BB and Lefkowitz RJ (1990). "Adrenergic receptor antagonists" In: Goodman and Gilman's *The Pharmacological Basis of Therapeutics* 8th ed. edited by Gilman AG, Rall TW, Nies AS and Taylor P, Pergamon Press
- Holck HGO, Munir AK, Mills LM, and Smith EL (1937). "Studies upon the sex differences in rats in tolerance to certain barbiturates and to nicotine." *J. Pharmacol. Exptl. Ther.* 60: 323-335
- Imaoka S, Terano Y, and Funae Y (1988). "Constitutive testosterone 6 β -hydroxylase in rat liver." *J. Biochem.* 104: 481-487
- Imaoka S, Fujita S, and Funae Y (1991). "Age-dependent expression of cytochrome P-450s in rat liver." *Biochim. Biophys. Acta* 1097: 187-192
- Ishibe Y (1993). "Studies on phosphorylation of estrogen receptor from porcine uterus." (Abstract) *Hokkaido-Igaku-Zasshi* 68(5): 728-735
- Jacobson CD and Gorski RA (1981). "Neurogenesis of the sexually dimorphic nucleus of the preoptic area of the rat." *J. Comp. Neurol.* 196: 519-529
- Jacobson CD, Shryne JE, Shapiro F, and Gorski RA (1980). "Ontogeny of the sexually dimorphic nucleus of the preoptic area." *J. Comp. Neurol.* 193: 541-548
- Jacobson CD, Csernus VJ, Shryne JE, and Gorski RA (1981). "The influence of gonadectomy, androgen exposure, or a gonadal graft in the neonatal rat on the volume of the

- sexually dimorphic nucleus of the preoptic area." J. Neurosci. 1: 1142-1147
- Janeczko R, Waxman DJ, Le Blanc GA, Morville A, and Adesnik M (1990). "Hormonal regulation of levels of the messenger RNA encoding hepatic P450 2c (IIC11), a constitutive male-specific form of cytochrome P450." Mol. Endocrinol. 4: 295-303
- Jansson J-O and Frohman LA (1987). "Differential effects of neonatal and adult androgen exposure on the growth hormone secretory pattern in male rats." Endocrinology 120(4): 1551-1557
- Jansson J-O, Ekberg S, Isaksson O, Mode A, and Gustafsson J-Å (1985a). "Imprinting of growth hormone secretion, body growth, and hepatic steroid metabolism by neonatal testosterone." Endocrinology 117(5): 1881-1889
- Jansson J-O, Edén S, and Isaksson O (1985b). "Sexual dimorphism in the control of growth hormone secretion." Endocr. Rev. 6(2): 128-150
- Jarzbab B and Döhler KD (1984). "Serotonergic influences on sexual differentiation of the rat brain." Prog. Brain Res. 61: 119-126
- Jarzbab B, Lindner G, Lindner T, Sickmüller PM, Geerlings H, Döhler KD (1986). "Adrenergic influences on sexual differentiation of the rat brain." Monogr. Neural Sci. 12: 191-196
- Jarzbab B, Sickmüller PM, Geerlings H, and Döhler KD (1987). "Postnatal treatment of rats with adrenergic receptor agonists or antagonists influences differentiation of sexual behavior." Horm. and Behav. 21: 478-492
- Jarzbab B, Gubala E, Achtelik W, Lindner G, Pogorzelska E, and Döhler KD (1989a). "Postnatal treatment of rats with beta-adrenergic agonists or antagonists influences differentiation of sexual brain functions." Exp. Clin. Endocrinol. 94(1/2): 61-72
- Jarzbab B, Gubala E, Achtelik W, and Döhler KD (1989b). "Postnatal treatment with the β -adrenergic agonist isoprenaline influences steroid-induced LH release in adult female rats." Acta Endocrinol. (Copenhagen) 120 (Suppl.1): 227-228

- Jarzab B, Kaminski M, Gubala E, Achteulik W, Wagiel J, and Döhler KD (1990a). "Postnatal treatment of rats with the β_2 -adrenergic agonist salbutamol influences the volume of the sexually dimorphic nucleus in the preoptic area." *Brain Res.* 516: 257-262
- Jarzab B, Kokocinńska D, Kaminski M, Gubala E, Achteulik W, Wagiel J, and Döhler KD (1990b). In: *Hormones, Brain and Behavior in Vertebrates. I: Sexual Differentiation, Neuroanatomical Aspects, Neurotransmitters and Neuropeptides. Comparative Physiology*, edited by Balthazart J : 41-50
- Jarzab B, Sickmüller PM, Kokocinńska D, Kaminski M, Gubala E, Achteulik W, Wagiel J, and Döhler KD (1990c). In: *Neuroendocrinology: New Frontiers*, edited by Gupta D, Wollmann HA, and Ranke MB : 87-96. *Brain Res. Promotion, Tübingen, Germany.*
- Jordan CL, Breedlove SM, and Arnold AP (1982). "Sexual dimorphism and the influence of neonatal androgen in the dorsolateral motor nucleus of the rat lumbar spinal cord." *Brain Res.* 249: 309-314.
- Kamataki T, Ando M, Yamazoe Y, Ishii K, and Kato R (1980). "Sex difference in the O-dealkylation activity of 7-hydroxycoumarin O-alkyl derivatives in liver microsomes of rats." *Biochem. Pharmacol.* 29: 1015-1022
- Kamataki T, Maeda K, Yamazoe Y, Nagai T, and Kato R (1982). "Evidence for the involvement of multiple forms of cytochrome P-450 in the occurrence of sex-related differences of drug metabolism in the rat." *Life Sci.* 31: 2603-2610
- Kamataki T, Maeda K, Yamazoe Y, Nagai T, and Kato R (1983). "Sex difference of cytochrome P-450 in the rat: purification, characterization, and quantitation of constitutive forms of cytochrome P-450 from liver microsomes of male and female rats." *Arch. Biochem. Biophys.* 225: 758-770
- Kamataki T, Maeda K, Shimada M, Nagai T, and Kato R (1984). "Neonatal testosterone imprinting of hepatic microsomal drug metabolism and a male-specific form of cytochrome P-450 in the rat." *J. Biochem. (Tokyo)* 96: 1939-1942
- Kato R (1974). "Sex-related differences in drug metabolism." *Drug Metab. Rev.* 3: 1-32

- Kato R and Kamataki T (1982). "Cytochrome P-450 as a determinant of sex differences of drug metabolism in the rat." *Xenobiotica* 12: 787-800
- Kato R, Yamazoe Y, Shimada M, Murayama N, and Kamataki T (1986). "Effects of growth hormone and ectopic transplantation of pituitary gland on sex-specific forms of cytochrome P-450 and testosterone and drug oxidations in rat liver." *J. of Biochem.* 100: 895-902
- Ketelslegers JM, Hetzel WD, Sherins RJ, and Catt KJ (1978). "Developmental changes in testicular gonadotropin receptors: plasma gonadotropins and plasma testosterone in the rat." *Endocrinology* 103: 212-222
- Khorram O, DePalatis LR and McCann SM (1983). "Development of hypothalamic control of growth hormone secretion in the rat." *Endocrinology* 113: 720-728
- Konoplya EF and Popoff EH (1992). "Identification of the classical androgen receptor in male rat liver and prostate cell plasma membranes." *Int. J. Biochem.* 24(12): 1979-1983
- Koop DR (1986). "Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a." *Mol. Pharmacol.* 29: 399-404
- Kramer RE, Greiner JW and Colby HD (1975). "Divergence of growth hormone actions on hepatic drug metabolism in the presence and absence of the pituitary gland." *Life Sci.* 17: 779-786
- Kramer RE, Greiner JW, Rumbaugh RC, Sweeney TD, and Colby HD (1977). "Relation of the gonadal hormones to growth hormone actions on hepatic drug metabolism in rats." *J. Pharmacol. Exptl. Ther.* 204(2): 247-254
- Krieger DT, Liotta AS, Nichol森 G, and Kizer JS (1979). "Brain ACTH and endorphin reduced in rats with monosodium glutamate-induced arcuate nuclear lesions." *Nature* 273: 562-563
- Lefkowitz RJ, Hoffman BB, and Taylor P (1990). "Neurohumoral transmission: the autonomic and somatic motor nervous system." In: Goodman and Gilman's *The Pharmacological Basis of Therapeutics* 8th ed. edited by Gilman AG, Rall TW, Nies AS, and Taylor P, Pergamon Press.

- Legraverend C, Mode A, Westin S, Ström A, Eguchi H, Zaphiropoulos PG, and Gustafsson J (1992). "Transcriptional regulation of rat P-450 2C gene subfamily members by the sexually dimorphic pattern of growth hormone secretion." *Mol. Endocrinol.* 6: 259-266
- Lemkey-Johnston N and Reynolds WA (1974). "Nature and extent of brain lesions in mice related to ingestion of monosodium glutamate. A light and electron microscopic study." *J. Neuropathol. Exp. Neurol.* 33: 74-97
- Lephart ED, Simpson ER, McPhaul MJ, Kilgore MW, Wilson JD, and Ojeda SR (1992). "Brain aromatase cytochrome P-450 messenger RNA levels and enzyme activity during prenatal development in the rat." *Mol. Brain Res.* 16: 187-192
- Lu R-B, Lebenthal E and Lee P-C (1987). "Developmental changes of glucocorticoid receptors in the rat pancreas." *J. Steroid Biochem.* 26(2): 213-218
- MacGeoch C, Morgan ET, Halpert J, and Gustafsson J-A (1984). "Purification, characterization and pituitary regulation of the sex-specific cytochrome P-450 15 α -hydroxylase from liver microsomes of untreated female rats." *J. Biol. Chem.* 259: 15433-15439
- MacLusky NJ and Naftolin F (1981). "Sexual differentiation of the central nervous system." *Science* 211: 1294-1303
- MacLusky NJ, Philip A, Hurburt C, and Naftolin F (1985). "Estrogen formation in the developing rat brain: sex differences in aromatase activity during early post-natal life." *Psychoneuroendocrinology* 10: 355-361
- Matsumoto A and Arai Y (1981). "Effect of androgen on sexual differentiation of synaptic organization in the hypothalamic arcuate nucleus: an ontogenetic study." *Neuroendocrinology* 33: 166-169
- McCarthy MM, Schlenker EH, and Pfaff DW (1993). "Enduring consequences of neonatal treatment with antisense oligodeoxynucleotides to estrogen receptor messenger ribonucleic acid on sexual differentiation of rat brain." *Endocrinology* 133(2): 433-439
- McEwen BS (1983). "Gonadal steroid influences on brain development and sexual differentiation." *Int. Rev. Physiol.* 27: 99-145

- McEwen BS, Biegon A, Davis PG, Krey LC, Luine VN, McGinnis MY, Paden CM, Parsons B, and Rainbow TC (1982). "Steroid hormones: humoral signals which alter brain cell properties and functions." *Rec. Prog. Horm. Res.* 38: 41-92
- McGivern RF, Poland RE, Taylor AN, Branch BJ, and Raum WJ (1986). "Prenatal stress feminizes adult male saccharin preference and maze learning: antagonism by propranolol." *Monog. Neural Sci.* 12: 172-178
- Meyer G, Ferres-Torres R, and Mas M (1978). "The effects of puberty and castration on hippocampal dendritic spines of mice. A Golgi study." *Brain Res.* 155: 108-112
- Mode A, Norstedt G, Simic B, Eneroth P, and Gustafsson J-Å (1981). "Continuous infusion of growth hormone feminizes hepatic steroid metabolism in the rat" *Endocrinology* 108 (6): 2103-2108
- Mode A, Wiersma-Larsson E, Ström A, Zaphiropoulos PG, and Gustafsson J-Å (1989). "A dual role of growth hormone as a feminizing and masculinizing factor in the control of sex-specific cytochrome P-450 isozymes in the liver." *J. Endocrinol.* 120: 311-317
- Morgan ET, MacGeoch C, and Gustafsson J-Å (1985). "Sexual differentiation of cytochrome P-450 in rat liver: evidence for a constitutive isozyme as the male-specific 16 α -hydroxylase." *Mol. Pharmacol.* 27: 471-479
- Morris G and Slotkin TA (1985). "Beta-2 adrenergic control of ornithine decarboxylase activity in brain regions of the developing rat." *J. Pharmacol. Exp. Ther.* 233: 141-147
- Magata K, Gonzalez FJ, Yamazoe Y, and Kato R (1990). "Purification and characterization of four catalytically active testosterone 6 β -hydroxylase P-450s from rat liver microsomes: comparison of a novel form with three structurally and functionally related forms." *J. Biochem. (Tokyo)* 107: 718-725
- Nash T (1953). "The colorimetric estimation of formaldehyde by means of the Hantzsch reaction." *Biochem. J.* 55: 416-421

- Nemeroff CB, Konkol RJ, Bissette G, Youngblood W, Martin JB, Brazeau P, Rone MS, Prange AJ Jr, Breese GR, and Kizer JS (1977). "Analysis of the disruption in hypothalamic-pituitary regulation in rats treated neonatally with monosodium L-glutamate (MSG): evidence for the involvement of tuberoinfundibular cholinergic and dopaminergic systems in neuroendocrine regulation." *Endocrinology* 101: 613-622
- Nishizuka M and Arai Y (1981). "Sexual dimorphism in synaptic organization in the amygdala and its dependence on neonatal hormone environment." *Brain Res.* 212: 31-38
- Ojeda SR, Andrews WW, Advis JP, and White SS (1980). "Recent advances in the endocrinology of puberty." *Endocr. Rev.* 1(3): 228-257
- Olney JW (1981). "Excitatory neurotoxins as food additives: an evaluation of risk." *Neurotoxicology* 2: 163-192
- Omura T and Sato RS (1964). "The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature." *J. Biol. Chem.* 230: 2370-2378
- Painson J-C and Tannenbaum GS (1991). "Sexual dimorphism of somatostatin and growth hormone-releasing factor signaling in the control of pulsatile growth hormone secretion in the rat." *Endocrinology* 128(6): 2858-2866
- Pampori NA, Agrawal AK, Waxman DJ, and Shapiro BH (1991). "Differential effects of neonatally administered glutamate on the ultradian pattern of circulating growth hormone regulating expression of sex-dependent forms of cytochrome P450." *Biochem. Pharmacol.* 41(9): 1299-1309
- Pang SF, Caggiula AR, Gay VL, Goodman RL, and Pang CSF (1979). "Serum concentrations of testosterone, oestrogens, luteinizing hormone and follicle-stimulating hormone in male and female rats during the critical period of neural sexual differentiation." *J. Endocrinol.* 80: 103-110
- Pedersen SB, Børghlum JD, Eriksen EF, and Richelsen B (1991). "Nuclear estradiol binding in rat adipocytes: regional variations and regulatory influences of hormones." *Biochim. Biophys. Acta* 1093: 80-86

- Petitti N and Etgen AM (1991). "Protein kinase C and phospholipase C mediate alpha 1- and beta-adrenoceptor intercommunication in rat hypothalamic slices." *J. Neurochem.* 56(2): 628-635
- Pfaff DW (1966). "Morphological changes in the brains of adult male rats after neonatal castration." *J. Endocrinol.* 36: 415-416
- Pfeiffer CA (1936). "Sexual differences of the hypophysis and their determinization by the gonads." *Am. J. Anat.* 58: 195-226
- Philip AH and Langdon RG (1962). "Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization and kinetics." *J. Biol. Chem.* 237: 2652-2660
- Preslock JD and McCann SM (1987). "Lesions of the sexually dimorphic nucleus of the preoptic area: effects upon LH, FSH and prolactin in rats." *Brain Res. Bull.* 18: 127-134
- Quinn JP, Axelrod G, and Brodie BB (1958). "Species, strain and sex differences in metabolism of hexobarbitone, amidopyrine, antipyrine and aniline." *Biochem. Pharmacol.* 1: 152-159
- Raisman G and Fields PM (1973). "Sexual dimorphism in the neuropil of the preoptic area of the rat and its dependence on neonatal androgen." *Brain Res.* 54: 1-29
- Raum WJ and Swerdloff RS (1981). "The role of hypothalamic adrenergic receptors in preventing testosterone-induced androgenization of the female rat brain." *Endocrinology* 109: 273-287
- Raum WJ, Marciano M, and Swerdloff RS (1984). "Nuclear accumulation of estradiol derived from the aromatization of testosterone is inhibited by hypothalamic beta-receptor stimulation in the neonatal female rat." *Biology of Reprod.* 30: 388-396
- Raum WJ, McGivern RF, Peterson MA, Shryne JH, and Gorski RA (1990). "Prenatal inhibition of hypothalamic sex steroid uptake by cocaine: effects on neurobehavioral sexual differentiation in male rats." *Dev. Brain Res.* 53: 230-236

- Raynaud JP, Mercier-Bodard C, and Baulieu EE (1971). "Rat estradiol binding plasma protein (EBP)." *Steroids* 18: 767-788
- Redding TW, Schally AV, Arimura A, and Wakabayashi I (1971). "Effect of monosodium glutamate on some endocrine functions." *Neuroendocrinology* 8: 245-255
- Reyes EF and Virgo BB (1988). "Neonatal programming of ethylmorphine demethylase and corticosteroid 5 α -reductase by testosterone, dihydrotestosterone, and estradiol: effects of an anti-estrogen, an anti-androgen, and an inhibitor of estrogen synthetase." *Drug Metab. Dispos.* 16(1): 93-97
- Reznikov AG, Nosenko ND, and Demkiv LP (1979). "New evidences for participation of biogenic monoamines in androgen-dependent sexual differentiation of hypothalamic control of gonadotropin secretion in rats." *Endokrinologie* 73(1): 11-19
- Reznikov AG, Nosenko ND, and Tarasenko LV (1990). "Augmentation of the effect of neonatal androgenization with tropolone, a catechol-o-methyltransferase inhibitor, in female rats." *Neuroendocrinology* 52: 455-459
- Rieutort M (1974). "Pituitary content and plasma levels of growth hormone in feotal and weaning rats" *J. Endocrinol.* 60: 261-268
- Robert AN (1980). "Metabolism of toxic substances." In: Casarett and Doull's Toxicology, the Basic Science of Poisons, 2nd ed. edited by Doull J, Klaassen CD, and Amdur MO, Macmillan Publishing Co., Inc. New York
- Rohde W, Ohkawa T, Dobashi K, Arai K, Okinaga S, and Dörner G (1983). "Acute effects of maternal stress on fetal blood catecholamines and hypothalamic LH-RH contents." *Exp. Clin. Endocrin.* 82: 268-274
- Rosengarten H and Friedhoff AJ (1979). "Enduring changes in dopamine receptor cells of pups from drug administration to pregnant and nursing rats." *Science* 203: 1133-1135
- Shapiro BH, MacLeod JN, Pampori NA, Morrissey JJ, Lapenson DP, and Waxman DJ (1989). "Signalling elements in the ultradian rhythm of circulating growth hormone regulating expression of sex-dependent forms of hepatic cytochrome P450." *Endocrinology* 125(6): 2935-2944

- Shimada M, Murayama N, Yamazoe Y, Kamataki T, and Kato R (1987). "Further studies on the persistence of neonatal androgen imprinting on sex-specific cytochrome P-450, testosterone and drug metabolism." Japan J. Pharmacol. 45: 467-478
- Shirama K, Takeo Y, Shimizu K, and Maekawa K (1975). "Inhibitory effect of 5-hydroxytryptophane on the induction of persistent estrus by androgen in the rat." Endocrinol. Jpn. 22: 575-579
- Sipes IG and Gandolfi AJ (1991). "Biotransformation of toxicants." In: Casarett and Doull's Toxicology, 4th ed. Pergamon Press, Inc. USA : 88-126
- Slotkin TA and Bartolome J (1986). "Role of ornithine decarboxylase and the polyamines in nervous system development: a review." Brain Res. Bull. 17: 307-320
- Slotkin TA, Windh R, Whitmore WL, and Seidler FJ (1988). "Adrenergic control of DNA synthesis in developing rat brain regions: effects of intracisternal administration of isoproterenol." Brain Res. Bull. 21: 737-740
- Strosser MT and Mialhe P (1975). "Growth hormone secretion in the rat as a function of age." Horm. Metab. Res. 7: 275-278
- Terry LC, Epelbaum J, and Martin JB (1981). "Monosodium glutamate: acute and chronic effects on rhythmic growth hormone and prolactin secretion, and somatostatin in the undisturbed male rat." Brain Res. 217: 129-142
- Toran-Allerand CD (1980). "Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro: implications for sexual differentiation." Brain Res. 106: 407-412
- Toran-Allerand CD (1986). "Sexual differentiation of the brain." Developmental NeuroPsychology, Academic Press, Inc.
- Urban RJ, Garmey JC, Shupnik MA, and Veldhuis JD (1990). "Insulin-like growth factor type I increases concentrations of messenger ribonucleic acid encoding cytochrome P450 cholesterol side-chain cleavage enzyme in primary cultures of porcine granulosa cells." Endocrinology 127(5): 2481-2488

- Virgo BB (1983). "Endogenous growth hormone and the regulation of hepatic drug oxidation in the male rat." *Developmental Pharmacology* : 379-382 (Alan R. Liss, Inc., New York)
- Virgo BB (1985). "Effects of somatostatin and testosterone on the hepatic monooxygenase system in castrated male rats." *Drug Metab. Dispos.* 13(1): 9-13
- Virgo BB (1991). "The effects of peripubertal testosterone on ethylmorphine demethylase activity in adult rats testectomized neonatally." *Canadian J. Physiol. Pharmacol.* 69: 459-463
- Vockentanz BM and Virgo BB (1985). "Feminization of the hepatic monooxygenases by growth hormone is mimicked by puromycin and correlates with a decrease in male-type cytochrome P450." *Biochem. Biophys. Res. Commun.* 128(2): 683-688
- Waxman DJ, Dannan GA, and Guengerich FP (1985). "Regulation of rat hepatic cytochrome P-450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isozymes." *Biochemistry* 24: 4409-4417
- Waxman DJ, LeBlanc GA, Morrissey JJ, Staunton J, and Lapenson DP (1988). "Adult male-specific and neonatally programmed rat hepatic P-450 forms RLM2 and 2a are not dependent on pulsatile plasma growth hormone for expression." *J. Biol. Chem.* 263(23): 11396-11406
- Waxman DJ, Ram PA, Notani G, LeBlanc GA, Alberta JA, Morrissey JJ, and Sundseth SS (1990). "Pituitary regulation of the male-specific steroid 6 β -hydroxylase P-450 2a (gene product IIIA2 in adult rat liver. Suppressive influence of growth hormone and thyroxine activating at a pretranslational level." *Mol. Endocrinol.* 4: 447-454
- Weniger JP and Zeis A (1988). "Stimulation of aromatase activity in the fetal rat gonads by cAMP and FSH." *Acta Endocrinol-Copenh.* 119(3): 381-385
- Wilson CA, Pearson JR, Hunter AJ, Tuohy PA, and Payne AP (1986). "The effect of neonatal manipulation of hypothalamic serotonin levels on sexual activity in the adult rat." *Pharmacol. Biochem. Behav.* 24: 1175-1183

- Wood AW, Ryan DE, Thomas PE, and Levin W (1983). "Regio- and stereoselective metabolism of two C₁₉ steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes." J. Biol. Chem. 258(14): 8839-8847
- Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B, and Guzelian PS (1985). "Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450_p." Biochemistry 24: 2171-2178



