Developmental Programming of Neuroendocrine and Behavioral Pathologies

Alexander Reznikov

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Table of Contents

Preface

This monograph addresses a critical domain of contemporary medical science—functional teratology, the study of predominantly functional disorders in the offspring of mothers exposed to adverse exogenous or endogenous influences during pregnancy or other vulnerable stages of early postnatal development. Such disruptions may remain latent at birth yet manifest later in life as chronic physiological or behavioral pathologies. Research in this field is inherently multidisciplinary, integrating physiology, pathophysiology, endocrinology, psychology, obstetrics and gynecology, as well as internal medicine.

It is now well recognized that the absence of overt teratogenic abnormalities in the newborn does not ensure a normal trajectory of subsequent development and health. This is especially pertinent for the neuroendocrine system, metabolic regulation, behavior, adaptive responses to homeostatic challenges, endocrine gland function, reproductive health, and immune competency. When such disorders become evident only in adulthood, reconstructing the maternal conditions during pregnancy and identifying the likely etiological factors is exceedingly difficult. Experimental studies in animals with relatively short life spans (rats, mice, hamsters) therefore provide a valuable opportunity to model early-life exposure to adverse influences and to assess their consequences for offspring health.

The first and second trimesters of human pregnancy are considered the most sensitive periods for intrauterine development. The first trimester is characterized by embryonic morphogenesis, whereas the second is marked by the maturation of the fetus's regulatory physiological systems, particularly the neuroendocrine system. One of the most critical processes occurring during this developmental window is the sexual differentiation of the brain, which programs sex-specific behavioral patterns, neuroendocrine regulation of gonadal function, and responses to stress.

Taking these considerations into account, the author and collaborators focused their research primarily on the final week of gestation in rats and, in some cases, on the first postnatal week. This strategy is justified by the fact that, compared with humans, early ontogenesis in rodents is developmentally delayed, with maturation of the neuroendocrine system occurring largely during this period.

I wish to express my deep respect and sincere gratitude to my colleagues for their professionalism and their dedicated contributions to the development of the

scientific field to which this monograph is devoted. Without their committed and productive efforts, the present work would not have been possible.

Alexander Reznikov

Chapter 1: The Concept of Early Epigenetic Programming of Functional Pathologies

1.1. Functional Teratology

Health may be conceptualized as an organism's adaptive capacity, reflected in the harmonious integration of physical and mental development and in the ability to mounting appropriate responses to internal and external stimuli. The genetically determined human phenotype provides the foundational framework for health, upon which numerous modifying influences accumulate over the lifespan, including lifestyle factors, social conditions, environmental exposures, and various exogenous and endogenous pathogenic agents.

As early as the latter half of the twentieth century, substantial experimental and clinical evidence demonstrated that certain hormonal agents (e.g., androgens, synthetic progestogens) and neurotropic drugs (e.g., reserpine, neuroleptics) administered during pregnancy could induce functional disturbances in the offspring, even in the absence of structural malformations at birth, with some effects persisting across subsequent generations. Later studies expanded this list of adverse influences to include maternal stress, nicotine, alcohol, undernutrition and overnutrition, narcotics and other psychotropic substances, as well as deficiencies in trace and macroelements or flavonoids. By crossing the placental barrier, the pathogenic factors act directly on the developing fetus, "programming" long-term alterations in physiological function and modifying the phenotype originally specified by the zygotic genome.

Hormonal imbalance during the critical window of development exerts particularly profound effects, modulating the maturation of neuroendocrine systems that regulate

behavior, reproductive function, and the activity of the hypothalamic–pituitary–adrenal (HPA) axis.

Current evidence demonstrates that prenatal disturbances of neuroendocrine development can lead to alterations in sexual, social, feeding, and aggressive behaviors. Such disruptions may also result in abnormalities of reproductive cycles, infertility, cognitive decline, reduced intellectual performance, impaired psycho-emotional stability, metabolic dysfunction, immune dysregulation, diminished endocrine adaptive reserves, and cardiovascular pathology. These outcomes arise through mechanisms of hormone–neurotransmitter imprinting—that is, long-term, and in some cases lifelong, programming mediated by disturbances in epigenetic regulation of genomic expression. As a consequence, persistent modifications occur in neuronal excitability, protein synthesis, hormonal and neurotransmitter metabolism, receptor sensitivity, and phosphorylation of brain proteins. Because these effects develop in the absence of overt anatomical malformations, this area of research was termed *functional teratology* (Dörner, 1976).

Functional teratology relies predominantly on experimental animal models, as most analogous abnormalities in humans, with few exceptions, manifest only years after birth. Therefore, efforts to establish causal links between adverse developmental exposures and subsequent functional pathology based solely on prospective or retrospective clinical studies face substantial methodological constraints.

A profound understanding of the pathways and mechanisms of epigenetic influences during early ontogenesis is crucial for preventing their deleterious consequences. Within this context, the neuroendocrine system represents the most vulnerable and thoroughly studied target. Perinatal exposures, however, frequently extend their effects beyond neuroendocrine regulation to the cardiovascular system and metabolic homeostasis, particularly in the pathogenesis of type 2 diabetes mellitus.

The British scientist David Barker, through his epidemiological studies, demonstrated that low birth weight relative to gestational age significantly increases the risk of type 2 diabetes (non-insulin-dependent), obesity, arterial hypertension, and mortality from myocardial infarction and stroke in later life (Barker, 2004). This relationship was documented, for instance, in individuals born during maternal starvation in Nazi-occupied countries of Western Europe in World War II. Retrospective analyses also revealed an association between type 2 diabetes and prenatal exposure to famine during the Holodomor of 1932–1933 in Ukraine (Lumey et al., 2015). It is now understood that insulin resistance in such individuals arises from aberrant methylation of the insulin receptor gene, reflecting epigenomic alterations.

Barker's hypothesis has since received extensive experimental and clinical confirmation (de Boo & Harding, 2006). For example, clinical observations indicate that individuals exposed to adverse prenatal conditions and born with low birth weight, exhibit altered hormonal and autonomic responses to psychogenic stress (Phillips & Jones, 2006). Prenatally stressed girls have been shown to display male-typical play behaviors (Barrett et al., 2014). Disruptions of prenatal programming of physiological functions may predispose not only the first generation of offspring but also subsequent generations to disease susceptibility (Messer et al., 2015).

Experimental research further indicates that prenatal stress substantially increases the risk of diabetes. In animal models, such exposures predispose offspring to type 1 (insulin-dependent) diabetes and elucidate the hormonal mechanisms involved (Abramov et al., 2004). Adult animals subjected to prenatal stress exhibit impaired glucose tolerance or severe hyperglycemia following administration of the diabetogenic agent streptozotocin, with these outcomes occurring 1.5 times more frequently than in intact controls. Pancreatic β-cells in prenatally stressed offspring demonstrate reduced insulin secretion in response to glucose stimulation (Krasova, 2004).

A considerable body of experimental evidence documents alterations in corticosteroid receptor density in the hippocampus and hypothalamus, modified stress reactivity of vasopressinergic and other peptidergic neuronal systems, as well as changes in regulatory peptides such as neuropeptide Y and leptin under conditions of prenatal stress. These neuroendocrine disturbances are implicated in the pathogenesis of impaired carbohydrate metabolism. Pronounced immune dysregulation has also been observed in prenatally stressed animals, associated with hippocampal and hypothalamic dysfunction (Tkachuk, 2005). The extent to which these findings translate to humans remains under investigation. Research conducted at the V.Ya. Danilevsky Institute of Endocrine Pathology Problems (Kharkiv, Ukraine) by Professor L.Yu. Serhienko and colleagues has demonstrated that chronic maternal stress throughout gestation in rats induces significant metabolic disturbances in offspring, including osteopenia and osteoporosis.

Maternal starvation may likewise be viewed as a form of metabolic stress. The principal mechanism underlying long-term disease risk in human offspring likely involves maternal and fetal hypercortisolemia triggered by metabolic stress. Through the catabolic actions of cortisol, fetal growth is restricted, the stress reactivity of the HPA axis becomes impaired, and nonspecific postnatal resistance is diminished. These alterations predispose offspring to autoimmune disorders, systemic connective tissue diseases, reduced glucose tolerance, and the development of insulin resistance (Barker, 2005).

The concept of the fetal origin of adult diseases was the central theme of the international congress *"Fetal Origin of Adult Diseases"* (Brighton, UK, 2003), which resulted in the establishment of a global scientific society of the same name—later renamed the *Developmental Origin of Health and Disease* (DOHaD). Various aspects of functional teratology have been explored in several monographs (Dörner, 1976; Reznikov, 1982, 1994, 2019; Reznikov et al., 2004b; Baraboy & Reznikov, 2013) and in numerous peer-reviewed publications.

In general, functional teratogenesis is characterized by the following features:
• Functional disorders arise in the absence of anatomical developmental anomalies.
• They manifest postnatally, most often after the onset of puberty.
• Functional pathology is long-term or lifelong.
• These disorders result from pathological programming of individual development *via* imprinting-like mechanisms.
• In some cases, functional abnormalities persist across several subsequent generations.

Functional teratology is directly relevant to the issue of drug safety for both mother and fetus. Even today, preclinical drug testing conducted within the field of medicinal toxicology remains largely focused on detecting visible structural defects in newborn offspring, while often neglecting the risk of latent functional disturbances. Yet many existing or potential pharmaceuticals possess hormonal properties, influence the synthesis, metabolism, or receptor activity of neurotransmitters, or block membrane ion channels. Some of these agents are capable of disrupting the programming of physiological functions, particularly the neuroendocrine regulation of reproduction and stress reactivity.

For example, the use of a progestin drug in pregnant women—one exhibiting weak androgenic activity—was associated with behavioral hypersexuality in male offspring and defeminization of behavior in female offspring (Reinisch & Sanders, 1984). Experimental studies in male mice have shown that prenatal exposure to paracetamol disrupts sexual differentiation of the brain, including behavioral endpoints (Hay-Schmidt et al., 2017). Results of a prospective study of individuals aged 31–33 years, born in Copenhagen in 1959–1961, revealed probable adverse long-term consequences associated with two major pregnancy-related factors: maternal smoking (2.7%) and the use of medications, especially analgesics (15.3%) and various psychotropic drugs.

Prevention of adverse developmental outcomes is possible only through a detailed understanding of the mechanisms underlying drug side effects. A notable example is the use of glucocorticoid medications in pregnant women with bronchial asthma or for accelerating fetal lung surfactant maturation in cases of threatened preterm birth. In the latter situation, up to 90% of pregnant women receive such treatment. Studies examining

the consequences of this hormonal therapy have demonstrated the necessity of avoiding multiple courses, recommending no more than two administrations.

Environmental pollutants, collectively termed *endocrine disruptors*, pose a substantial threat to fetal health. These substances may be of natural, industrial, or domestic origin. The hormone-like activity of dioxins, phthalates, bisphenols and their chlorinated derivatives renders them particularly harmful when exposure occurs prenatally through the maternal organism. The male reproductive system—especially its neuroendocrine and gonadal components—is especially vulnerable. Certain pesticides, herbicides, and nanoparticles of heavy metals also represent significant hazards.

Thus, the scope of functional teratology is exceptionally broad, encompassing a wide range of agents capable of distorting the perinatal programming of physiological functions and, consequently, health. Addressing the challenges inherent in this field requires the coordinated efforts of pathophysiologists, pharmacologists, clinicians, hygienists, ecologists, and, critically, the effective oversight of regulatory authorities.

1.2. Sexual Differentiation of the Brain and Its Disruption

The most thoroughly investigated domain of functional teratology concerns disturbances in the sexual differentiation of the brain, which historically marked the inception of research in this field. Sexual dimorphism in physiological functions is a firmly established phenomenon. It encompasses defensive, sexual, and parental behaviors; cognitive capacities; metabolic regulation; pituitary gonadotropin secretion; stress responsiveness; neurotransmission; and sensitivity of hormonal receptors, among other processes.

It is well documented that men typically exhibit superior spatial orientation, mathematical capability, and abstract reasoning, whereas women tend to demonstrate more advanced verbal abilities, artistic–creative skills, heightened emotional responsiveness, and a more concrete style of thinking. Men show a higher incidence of schizophrenia and Parkinson's disease, whereas depression and anorexia nervosa are more common in women. Behavioral dimorphism is also evident, with men displaying greater aggressiveness and accounting for approximately 80% of homicides and nearly all sexual offenses. At the neurobiological level, sexual dimorphism is reflected in differences in synaptic density, the distribution of steroid hormone receptors (estrogen and androgen), steroid metabolism within the brain, neurotransmitter levels and turnover, and the size of specific brain structures. Morphological studies have revealed pronounced dimorphism in the preoptic–hypothalamic region and in limbic formations related to hypothalamic neuroendocrine regulation, including the amygdala, hippocampus, and stria terminalis.

In mammals of reproductive age, the female reproductive system is regulated through reciprocal interactions between the hypothalamic–pituitary axis and the ovaries, and operates in a cyclical manner. The periodicity of ovarian–menstrual cycles in women, or estrous cycles in rodents, is determined by the capacity of the hypothalamic ovulatory center to respond to rising ovarian estrogen levels with increased secretion of gonadotropin-releasing hormone (GnRH). This, in turn, initiates the preovulatory surge of luteinizing hormone (LH) that induces follicular rupture and ovulation. By contrast, the male reproductive system functions in a relatively monotonic mode, governed by a negative feedback loop between the testes and the hypothalamic–pituitary axis, as testicular hormones do not exert positive stimulatory effects on this system. The male hypothalamus is largely unresponsive to the stimulatory action of estrogens, which occur only at low concentrations. Sexual functional dimorphism is therefore mediated by differences in the hormonal–receptor, neurotransmitter, peptidergic, and enzymatic systems of the hypothalamus and other brain regions (Cahill, 2006).

Regardless of genetic sex, the immature fetal brain is initially programmed toward a female, or possibly neutral, phenotype. In male fetuses, however, testicular testosterone directs the developing brain toward masculinization and defeminization. This androgen-dependent process—termed an organizational or programming effect, in contrast to the activation influences of androgens during puberty and adulthood—underlies sexual differentiation of the brain and occurs within a strictly defined critical period. Once this window closes, testosterone no longer affects brain differentiation. Consequently, testosterone deficiency during this period predisposes male fetuses to atypical sexual orientation or disturbances of gender identity. Conversely, androgen excess in female fetuses promotes defeminization and masculinization of neuroendocrine systems, which later manifests as menstrual irregularities, hyperandrogenic disorders such as polycystic ovary syndrome (PCOS), infertility, and alterations in sexual or social behavior.

Findings from animal studies have been supported by clinical evidence. For example, homosexual men exhibit a stimulatory effect of exogenous estrogens on luteinizing hormone (LH) secretion whereas this response is absent in heterosexual men (Rohde et al., 1978). Because a positive estrogen feedback response is a characteristic feature of the female hypothalamus, these results suggest that disturbances in the differentiation of male sexual behavior may be associated with altered hypothalamic control of gonadotropin secretion, despite normal circulating concentrations of total and free testosterone.

Postmortem neuropathological studies provide further support for this interpretation. In homosexual men who died suddenly from non-endocrine causes, the size of sexually dimorphic hypothalamic nuclei, including the suprachiasmatic nucleus (SCN),

resembled that of women, consistent with experimental neuroanatomical evidence of altered sexual differentiation of the brain.

Clinical evidence is also available for women. Reversal of sexual orientation and reduced fertility have been documented in daughters of mothers with congenital adrenal hyperplasia or PCOS—conditions characterized by chronic hyperandrogenism. In 80–90% of these cases, elevated levels of biologically active androgens were reported, supporting the hypothesis that maternal androgens crossing the placenta can disrupt fetal brain differentiation. This risk may be further exacerbated by assisted reproductive technologies applied in women with hyperandrogenic infertility.

Not only androgen deficiency but also imbalances in stress hormones and neurotransmitters can alter sexual differentiation of the brain. Maternal stress profoundly alters hormonal and neurotransmitter homeostasis in both mother and fetus, resulting in long-term developmental consequences. Prenatal stress has been associated with bisexual or homosexual behavior in men (Dörner et al., 1983) and in male animals (Ward, 1972), as well as with reduced fertility in female offspring.

In parallel with disturbances in the sexual differentiation of behavior and the hypothalamic–pituitary–gonadal (HPG) axis, prenatal stress also disrupts programming of the HPA axis. The adaptive function of the HPA axis during stress relies primarily on enhanced glucocorticoid secretion, which exhibits sex-specific characteristics shaped by perinatal influences of steroid hormones (testosterone, corticosteroids) and stress.

Animal research clearly indicates that male offspring exposed to prenatal stress carry a substantially higher risk of abnormal sexual differentiation of the brain than females. The translational relevance of these findings to humans is of particular importance. Long-term clinical follow-up studies (Dörner et al., 1980, 1983) demonstrated that maternal stressors such as family conflict or physical or sexual abuse during pregnancy markedly increased the risk of abnormal sexual differentiation in male offspring. In adulthood, these men exhibited a significantly higher prevalence of homosexual or bisexual behavior compared with controls. Similar conclusions were reached for men whose mothers experienced severe wartime stress, such as the Allied bombings of Berlin during World War II.

Based on current evidence regarding the pathogenesis of the prenatal stress syndrome, it is highly plausible that these disorders are driven by stress-induced hypersecretion of endogenous opioids, leading to testicular testosterone deficiency during the critical period of brain sexual differentiation (Barrett & Swan, 2015), along with increased maternal corticosteroid production and their transfer to the immature fetal brain.

1.3.Epigenetic Imprinting as a Mechanism of Developmental Programming of Physiological Functions and Their Disorders

The programming of physiological functions during early life represents a fundamental biological principle of individual development in both humans and animals. Among the various physiological systems, the neuroendocrine system has been the most thoroughly investigated in this context. In close interaction with the nervous and immune systems, it regulates essential life processes, including somatic and sexual development, growth, reproduction, behavior, homeostasis, and adaptation to changing environmental conditions. The imprinting mechanism has been demonstrated in the consequences of prenatal stress, hormonal imbalance, fetotoxic effects of certain pharmaceuticals, exposure to endocrine disruptors, and nutritional factors.

In general, two principal pathways can be identified through which hormones, neurotransmitters, cytokines, metabolites, calcium ions, and other regulatory factors exert programming effects on the developing brain during early ontogenesis. The first pathway involves their direct influence on neurogenesis—that is, on the formation of neural and neuroendocrine structures during the final stages of brain morphogenesis. This regulation is mediated through processes such as proliferation and apoptosis of neuroblasts and neurons, cellular migration, myelination of nerve fibers, synaptogenesis, and the growth of dendrites and axons. The role of the thyroid hormone thyroxine serves as a classical example of such regulation.

The second pathway encompasses long-term genomic and epigenetic effects, collectively referred to as imprinting. These effects extend to neurotransmission, receptor expression for endogenous bioactive molecules, DNA structure, protein synthesis, and metabolic regulation. The scientific concept of imprinting, widely applied in ethology, genetics, and developmental biology, refers to the stable encoding of information—a molecular “stamp” on the genetic program that irreversibly determines the trajectory of cell differentiation toward its definitive state.

A fertilized mammalian oocyte contains a diploid set of autosomal genes that are nonetheless subject to allele-specific expression, whereby only one allele is functionally active and the other remains silenced. This phenomenon, known as gametic imprinting, ensures the inheritance of certain phenotypic traits from a single parental line. Following fertilization, the sequential processes of histogenesis, morphogenesis, and organogenesis proceed, culminating in the completion of embryonic development. At this point, individual development enters the stage of fetogenesis, during which phenotypic characteristics begin to emerge (Fig. 1.1). However, the maturation of some cell populations and tissues specialized in distinct physiological functions continues after birth and, in the case of the reproductive system, extends until the completion of puberty.

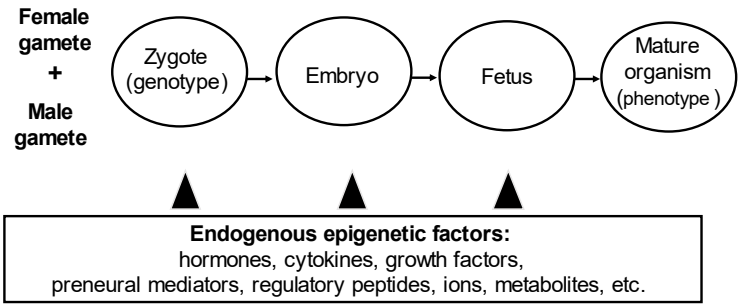

Fig. 1.1 Epigenetic factors in early mammalian ontogeny

Although imprinting is fundamentally a mechanism of normal ontogenesis, under certain conditions—particularly in response to factors within the cellular microenvironment—it may redirect development along an aberrant trajectory, thereby consolidating pathological changes for extended periods, in some cases throughout life. Thus, the pathogenesis of disease or pathological states is rooted in the same biological mechanisms that operate under physiological conditions. Clinical manifestations may emerge during early development, adulthood, or advanced age. Each physiological system possesses distinct critical periods during which interference with morphogenesis or functional differentiation can lead to developmental modification or pathological distortion.

In the context of sexual differentiation of the male rat brain, a pronounced surge in testosterone secretion on gestational days 16–18 plays a decisive role. In human male fetuses, testosterone production begins at 8–9 weeks after fertilization and reaches its peak around the 20th week of gestation. If the androgenic stimulus is absent during this critical window, masculinization of the brain does not occur.

As a result of many years of research, we have proposed the neurochemical concept of hormone–neurotransmitter imprinting of the developing brain (Reznikov A.G., 1982, 1994). According to its central principles, disturbances of imprinting give rise to a broad spectrum of dysregulations within physiological systems by altering cellular responsiveness to endogenous regulatory factors (Fig. 1.2). The underlying mechanisms involve changes in the density and ligand-binding capacity of steroid and other hormone

receptors, as well as alterations in the synthesis, metabolism, and receptor-mediated signaling of neurotransmitters and neuropeptides, including norepinephrine, serotonin, dopamine, acetylcholine, opioids, vasopressin, *etc*. These processes are further accompanied by modifications in gene expression governing protein synthesis and phosphorylation.

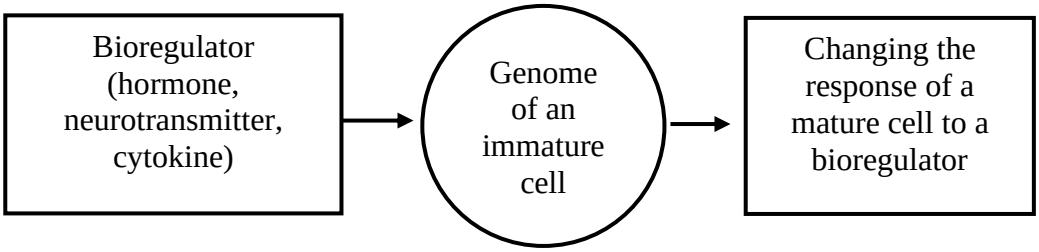

Fig. 1.2 Epigenetic imprinting-mediated automodification of cellular responsiveness

The fundamental mechanisms underlying the formation of sex differences in brain function in humans are largely conserved across numerous animal species, including rats, mice, monkeys, sheep, rabbits, and guinea pigs. Among the most extensively investigated manifestations of pathological imprinting are the syndromes of prenatal stress in male rats and neonatal androgenization in female rats. Prenatal stress in males is characterized by demasculinization and feminization of sexual behavior, along with altered stress reactivity of the HPA axis. In contrast, neonatal androgenization in females leads to defeminization and masculinization of these same functions. In both conditions, the disturbances originate from deviations in androgen-dependent sexual differentiation of the developing brain.

Animal studies conducted by the Department of Endocrinology of Reproduction and Adaptation at the V.P. Komisarenko Institute of Endocrinology and Metabolism (Kyiv, Ukraine), under the leadership of A. Reznikov, employing models of androgen-dependent sexual differentiation of the brain and prenatal stress pathology, identified the catecholestrogen 4-hydroxyestradiol-17β and norepinephrine as key inducers of sexual differentiation of the brain. A functional and neurochemical interaction between these agents was demonstrated (see Chapter 2 for details). Their effects are mediated through cooperative mechanisms consistent with the principle of co-induction.

Importantly, prenatal programming of neuroendocrine functions and behavior occurs before the maturation of both hypothalamic synaptic networks and the pituitary portal

vasculature. On the basis of these findings, we concluded that norepinephrine exerts its action outside the framework of conventional synaptic transmission, functioning instead as a "pre-neuronal" regulator of genes governing cellular differentiation.

In the course of this research, a biological mechanism of imprinting was conceptualized and initially termed "autorepression" (Reznikov, 1982), later refined as "automodification of cellular responsiveness" (Reznikov, 1994). A fundamental property of physiological systems is their capacity to respond to chemical, physical, and biological stimuli or inhibitory influences. Prenatal programming of the brain by microenvironmental factors acting on immature neural cells establishes a specific baseline level of their responsiveness (sensitivity) to the same physiologically active substances—regulators and modulators of physiological functions—including hormones, cytokines, neuropeptides, metabolites, neurotransmitters, and para- or autocrine agents.

When this programming process is disrupted, disorders of endocrine function and behavior emerge, because the ultimate consequence of epigenetic modification of gene expression is the development of hyperreactivity, hyporeactivity, or areactivity of neuronal and neuroendocrine cells. In the context of prenatal stress and disturbed sexual differentiation of the brain, these alterations manifest in adulthood as modified neuronal responses to stress hormones, estrogens, androgens, and norepinephrine, depending on the perinatally determined threshold of sensitivity established by these same factors. For example, elevated maternal corticosteroid levels during prenatal stress attenuate the HPA axis response to acute stress in male offspring. Likewise, administration of hydrocortisone to pregnant guinea pigs alters the expression of type II glucocorticoid receptors in the hippocampus of adult progeny.

Importantly, imprinting affects not only immature neurons but also glial cells. This is particularly significant for steroid hormones, whose effects on the brain are mediated in part through glia-dependent mechanisms of steroid metabolism, such as aromatization.
The concept of early self-modification of cellular reactivity as a biological principle of individual development encompasses not only hormone–neurotransmitter imprinting during neurogenesis but also the establishment of immunological tolerance. It was the analogy between these processes that prompted the author to propose such a generalization. Immunological tolerance is established in early ontogenesis through primary exposure of immunocompetent lymphocyte precursors to the organism's own antigens. As a result, clones of these cells fail to recognize endogenous antigens as foreign, despite retaining complementary receptors.

The phenomenon of early self-modification of cellular reactivity can also be reproduced *in vitro*. After undergoing such reorganization, neurons exhibit either increased or

decreased excitability in response to neurotransmitters. For example, the addition of norepinephrine to the culture medium of undifferentiated neural tissue from newborn rats modifies the threshold of noradrenergic excitation once the neurons have matured (Chubakov, 1984). Similar effects have been described for acetylcholine and serotonin.

Further support for the concept of self-modification of cellular reactivity comes from a 1991 study demonstrating that prenatal exposure to the adrenergic ligand propranolol alters quantitative binding parameters in the cerebral cortex of 7-day-old rat pups (Miller, 1991). Additional evidence is provided by observations that early exposure to sex steroids induces the formation of an electrical response in neurons of the rat preoptic area (POA) that is subsequently elicited by the same hormones (Babichev, 1981).

Based on our earlier *in vivo* animal research (Reznikov, 1982), subsequently supported by other researchers (Tretyak & Arkhipova, 1992; Whitaker-Azmitia et al., 1995; Buznikov et al., 1997), we proposed the existence of "pre-neuronal" forms of neurotransmitter activity during the early stages of ontogenesis. According to this concept, neurotransmitters initially act as intracellular regulators and local hormones—inducers of cellular differentiation—and only later acquire their function as synaptic transmitters. The aforementioned *in vitro* data demonstrating modification of the functional phenotype of immature neurons by neurotransmitters are consistent with the view of their pre-neuronal role in early development. As noted by Tretyak & Arkhipova (1992), our studies were among the first to initiate research into the inductive role of various neurotransmitters in embryogenesis and subsequent individual development in animals.

A substantial body of evidence supports the imprinting properties of insulin, glucocorticoids, sex steroids, catecholamines, opioids, vasopressin, oxytocin, thyrotropin-releasing hormone, and nicotine with respect to their target cells in neuroendocrine brain structures, reflecting the profound plasticity of the central nervous system.

Alterations resulting from pathological programming of physiological functions, which give rise to health disorders in the offspring, may also be transmitted to subsequent generations (Messer et al., 2015).

There is no doubt that early programming operates through effects on genes and their expression. For example, the programming action of testosterone on sexual differentiation of the brain can be abolished by inhibitors of transcription and translation. Although genetic defects—such as mutations and chromosomal aberrations—can produce not only anatomical malformations of fetal morphogenesis but also physiological dysfunctions manifesting postnatally at various stages of development,

these dysfunctions are predominantly the result of epigenetic modifications of gene expression (Fowden et al., 2006; Veiga-Lopez et al., 2013a; Messer et al., 2015; Vaiserman, 2015; Barouki et al., 2018).

The ultimate outcome of phenogenesis depends on the interaction between genes and stochastic influences during intrauterine life. Thus, the individual phenotype emerges from phenogenetic variability, which encompasses both physiological phenotypic variants and pathological deviations.

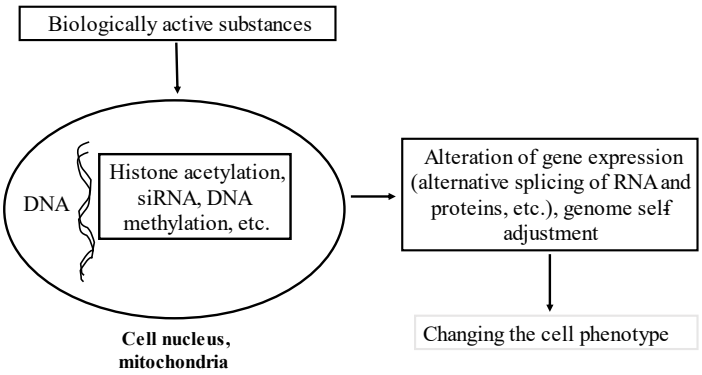

Fig. 1.3 Molecular mechanisms of epigenetic imprinting

Several pathways of epigenetic modification of gene expression by environmental factors have been identified. The principal mechanisms include DNA hypermethylation (gene silencing), DNA demethylation (gene activation), histone acetylation (gene activation), and microRNA-mediated interference with messenger RNA (20–30 nucleotides) (Fig. 1.3). For instance, perinatal administration of corticosteroids in rats induces DNA demethylation, which is associated with hyperexpression of the aminotransferase gene in the liver (Thomassin et al., 2001). Epigenetic histone modifications occur most frequently at the N-terminal tails and include, in addition to acetylation, phosphorylation, ubiquitination, sumoylation, and methylation. Epigenetic factors may also remodel chromatin by altering the density of DNA–protein (nucleosome) complexes.

We propose that another potential mechanism of epigenetic imprinting in early ontogenesis may hypothetically involve genome self-regulation mediated by transposons, mobile elements within the DNA molecule. The discovery of transposons

is credited to Barbara McClintock (USA), who received the Nobel Prize in 1983. The concept of genome self-regulation bridged the perspectives of neo-Lamarckians, who, following J.B. Lamarck, emphasized the inheritance of acquired traits, and those of classical geneticists, who considered mutagenesis the sole driver of evolutionary selection (genetic determinism).

The empirical material presented in the following chapters illustrates and further develops the fundamental principles of functional teratology outlined above.

Conclusions:

- The evidence underscores the central role of epigenetic mechanisms in programming physiological functions and establishing long-term phenotypic traits. Hormones, neurotransmitters, and environmental agents exert imprinting effects on neuroendocrine target cells, shaping both normal adaptive responses and maladaptive pathways that may lead to pathology. These influences may extend beyond the directly exposed generation, reflecting the transmissible nature of certain epigenetic modifications.

- Early-life programming operates primarily through changes in gene expression rather than alterations to the DNA sequence. Although genetic defects such as mutations or chromosomal abnormalities can produce major structural and functional disturbances, most deviations in postnatal neuroendocrine physiology arise from epigenetic modifications. DNA methylation, histone modifications, and RNA interference represent the principal molecular pathways through which endogenous and environmental signals interact with the genome.

- The concept of genome self-adjustment mediated by transposable elements offers an additional explanatory framework for how the genome responds to environmental challenges during early development. This perspective reconciles elements of Lamarckian theories of acquired trait inheritance with the deterministic views of classical genetics, supporting a more integrative understanding of developmental and evolutionary plasticity.

- Overall, phenogenesis emerges from the dynamic interplay among genetic, epigenetic, and stochastic factors during intrauterine development. Phenogenetic variability accounts for both normal physiological diversity and the emergence of pathological phenotypes. These principles constitute the theoretical foundation of functional teratology and will be elaborated in the chapters that follow.

Chapter 2: Programming of Neuroendocrine Disorders by Steroid Hormones in Early Life

2.1 Androgens

2.1.1 Androgen-dependent Sexual Differentiation of the Brain

Ovarian–Neuroendocrine Interactions. As noted in Chapter 1, the immature brain—regardless of genetic sex—is initially capable of developing along a female or, as some researchers propose, a neutral pathway. In adulthood, such a trajectory supports female sexual behavior, cyclic secretion of pituitary gonadotropins, and ovulation in response to endogenous or exogenous estrogens (Fig. 2.1). Under the influence of testicular androgens in males, or exogenous androgens in females, the neuroendocrine centers responsible for sexual behavior and gonadotropin secretion undergo epigenetic imprinting that redirects their development toward a male pattern. This process establishes the functional reproductive phenotype, which remains stable throughout life.

The consequences of impaired androgen-dependent differentiation of the brain have been analyzed in monographs and review articles (Dorner, 1976; Babichev, 1981; Reznikov, 1982, 1994, 2019; Reznikov et al., 2004a; Cahill, 2006; Negri-Cesi et al., 2008; Foecking et al., 2008; Baraboy & Reznikov, 2013; Abbott et al., 2020, 2025), as well as in numerous other studies. However, most authors have concentrated primarily on the descriptive aspects of reproductive dysfunction while giving comparatively limited attention to the neurochemical and epigenetic mechanisms underlying these disturbances.

The critical period of sexual differentiation of the brain—during which it remains sensitive to the organizing influence of testosterone and other androgens—occurs in humans during the mid-trimester of gestation. In rabbits, guinea pigs, sheep, and several other species, this period is likewise completed before birth. In contrast, in rodents (rats, mice, hamsters), the sensitive window extends through the final week of gestation (rat

gestation lasts 21–22 days) and continues into postnatal days (PND) 1–10. Consequently, research on alterations in neuroendocrine regulation of reproduction is conducted predominantly in rodent models.

Effects of Neonatal Androgenization in Females. A series of studies conducted in our department examined the immediate and long-term consequences of androgen imbalance during the early neonatal period in rats, with the aim of elucidating the pathogenesis of functional reproductive and HPA axis disturbances (Reznikov, 1978, 1986; Reznikov & Nosenko, 1983, 1987, 1995; Reznikov et al., 1990; Nosenko & Reznikov, 2001a, 2001b).

To model disruptions in the programming of sexual differentiation of the brain resulting from testosterone deficiency in males or excess in females, two approaches are used most commonly: castration of newborn male rats before PND 6, and administration of testosterone propionate to female rats either during gestation or within the first postnatal week. For instance, administration of testosterone to pregnant rats has been shown to delay the onset of puberty in female offspring. The likely mechanism involves reduced secretion of GnRH, potentially mediated by diminished expression of androgen, leptin, and melanocortin receptors (Ge et al., 2025).

In adult males castrated neonatally, signs of demasculinization and feminization of the brain are observed despite continuous androgen replacement therapy initiated on the tenth day of life. These manifestations include the exhibition of lordosis in response to a male's approach, as well as a positive gonadotropic response to estradiol—features typical of females rather than males. In our experiments, transplantation of ovarian fragments from immature rats into the anterior eye chamber of neonatally castrated adult males resulted in the formation of corpora lutea within the grafts. This outcome indicates that estrogens synthesized by the transplanted tissue induced a preovulatory surge of pituitary LH, reflecting preserved hypothalamic sensitivity to the stimulatory action of estrogens.

The consequences of neonatal androgenization in female rats model the effects of a hyperandrogenic state in pregnant women on the development of the female fetus, as observed in conditions such as PCOS, congenital adrenal hyperplasia, or during pharmacological treatment with agents possessing androgenic activity.

Ovulation, defined as follicular rupture and oocyte release, requires the sequential activation of hypothalamic GnRH secretion and pituitary LH release. The kisspeptin family plays a critical role in triggering the preovulatory surge of LH (Zhou et al., 2025). Following neonatal androgenization, the preovulatory increase in circulating GnRH and

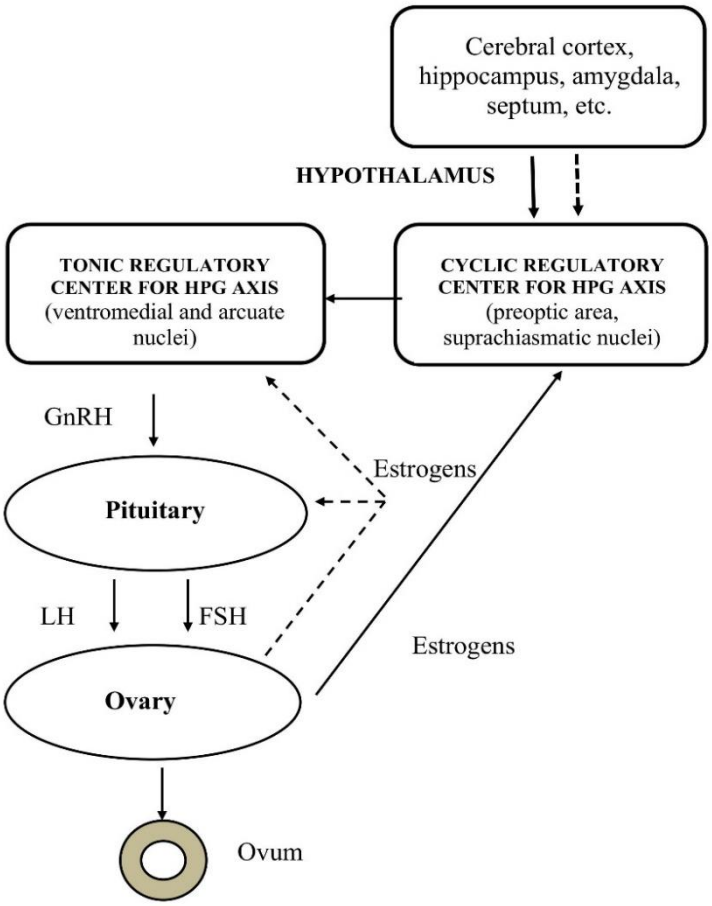

Fig. 2.1 Ovarian–neuroendocrine reciprocal interactions in the regulation of ovulation in rodents

Footnotes: HPG – hypothalamic–pituitary–gonadal. Solid arrows indicate stimulation; dashed arrows indicate inhibition.

LH fails to occur because the hypothalamus becomes refractory to the stimulatory action of estradiol. Consequently, anovulatory infertility develops, accompanied by polycystic ovarian morphology, absence of corpora lutea in rats, and a marked reduction in gonadal size (Fig. 2.2).

The degree of impairment in neonatally androgenized females depends on both the dose and the timing of testosterone administration. Under normal conditions, vaginal opening marks the completion of puberty, as the first ovulation typically occurs on the following day. In our studies on Wistar rats, this was observed on PND 41–48, with an average on PND 43 ± 0.5. In contrast, females that received a single injection of testosterone propionate between PND 3 and PND 5 (0.25 mg on day 3 or 4; 0.5 mg or 1.25 mg on day 5) exhibited vaginal opening 4–6 days earlier.

Androgenization by daily administration of testosterone propionate at a dose of 0.15 mg on PND 2–4 completely prevented vaginal opening. Furthermore, none of the subsequent hormonal stimulations in these animals (5α-androstanediol, estradiol, LH, FSH) produced any detectable effect. These findings indicate that the tissues of the external genitalia had lost sensitivity to hormonal stimuli as a result of local androgenic imprinting.

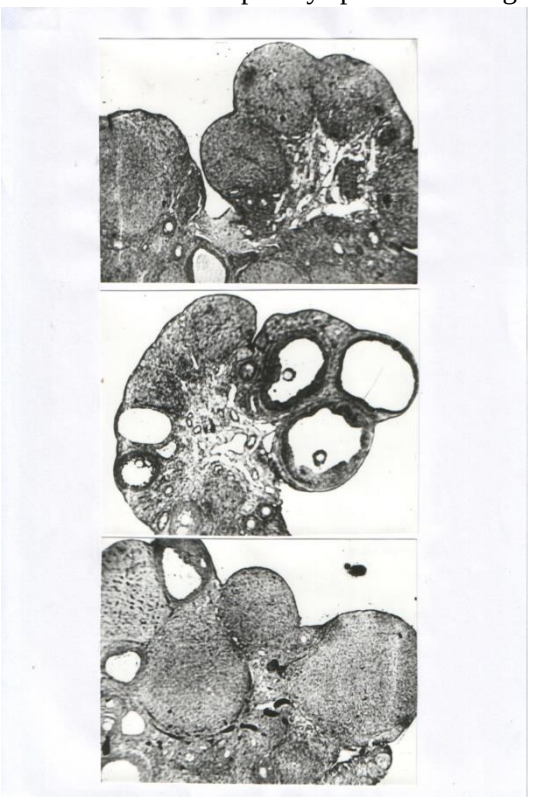

Fig. 2.2 Ovaries of sexually mature rats *Footnotes:* Top – normal, diestrous, postovulatory corpora lutea present; middle - neonatal androgenization, corpora lutea absent, follicular cysts present; bottom - restoration of normal structure after gonadotropic stimulation

In neonatally androgenized females aged 2–3 months, either persistent estrus or marked disturbances of estrous cyclicity were observed. Cycle length varied from 4 to 14 days (normal: 4–5 days). The cycles were irregular, characterized by a prolonged estrus phase, and frequently progressed to a state of permanent estrus.

Due to the reduced number or complete absence of corpora lutea in these females, the hormonal profile shifted toward a pattern consistent with polycystic ovarian

morphology. In normal rats, ovarian progesterone content ranged from 0.72 to 3.02 μg, whereas in neonatally androgenized animals the corresponding values did not exceed 0.60 μg.

Plasma concentrations of progesterone and 17β-estradiol were measured using radioimmunoassay. In females exhibiting persistent estrus, progesterone levels were markedly reduced. The concentration of 17β-estradiol also declined under conditions of moderate androgenization compared with control animals in estrus (Fig. 2.3). In contrast, administration of a higher dose of testosterone propionate (1.25 mg) induced more pronounced polycystic ovarian changes and was accompanied by elevated estradiol concentrations, which continued to increase with age. This elevation likely contributes to the development of endometrial and myometrial hypertrophy, as well as enhanced keratinization of the vaginal epithelium in neonatally androgenized females with a closed vaginal orifice.

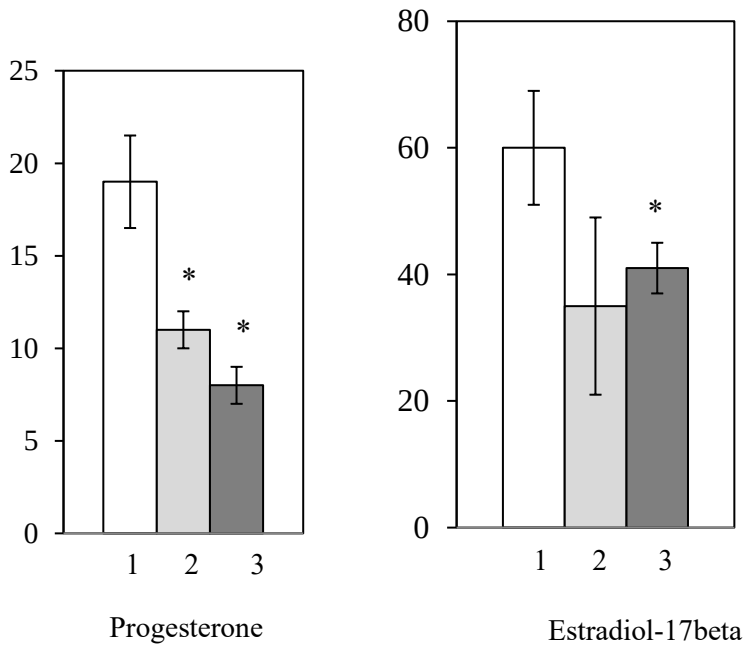

Fig. 2.3 Plasma concentrations of progesterone (ng/ml) and estradiol (pg/ml) in 3-month-old neonatally androgenized female rats

Footnotes: 1 – control, estrus; 2 – testosterone propionate, 0.25 mg on PND 4; 3 – testosterone propionate, 0.25 mg on PND 3.

* $p < 0.05$ compared with control.

Absence of the proestrous rise in estrogen levels, which normally triggers the preovulatory surge of pituitary LH, is typically attributable to impaired secretion of pituitary hormones, primarily FSH, LH, and prolactin. At 3.5 months of age, the prolactin content in the adenohypophysis, determined with electrophoresis, in females treated with testosterone propionate at a dose of 0.15 mg on PND 2–4 was nearly twice that of controls (control: 39.2 ± 2.1 μg/mg tissue; androgenization: 70.0 ± 3.0 μg/mg tissue; $p < 0.001$).

Plasma LH levels were assessed by radioimmunoassay using reagents kindly provided by Parlow, including purified rat pituitary LH, specific antibodies, and the NIAMD Rat LH-RP-1 calibration standard. Following radioiodination, the labeled LH was purified on a Sephadex column. No significant alterations in plasma LH concentrations were detected in neonatally androgenized females (0.25 mg testosterone propionate on PND 3): control values ranged from 5.7 to 8.3 ng/ml, whereas experimental values ranged from 3.0 to 8.1 ng/ml, with decreases observed only in some animals. In contrast, the contents of biologically active LH and FSH in the adenohypophysis, determined using NIH-LH-S-8 and NIAMD Rat FSH-B-1 reference standards, were markedly reduced. These changes corresponded to alterations in hypothalamic LH-releasing hormone (LHRH) levels, as assessed by the extent of LH depletion in the adenohypophysis of normal male rats following intravenous administration of hydrochloric acid extracts of hypothalamus from neonatally androgenized females.

As widely recognized, LHRH secretion depends, at least in part, on estrogen receptor-mediated signaling. Hypothalamic refractoriness to the stimulatory effects of estrogens within the "LHRH–gonadotropins" axis may arise from deficiencies at the receptor level. Neonatal androgenization of female rats suppresses synaptogenesis and simultaneously decreases estrogen receptor density in the hypothalamus and other target organs. Supporting this, our data on the uptake of tritium-labeled estradiol by target tissues of androgen-sterile rats demonstrate that the binding of high-specific-activity [6,7-^{3}H]estradiol-17β (10 Ci/mmol) decreased by 22% in the anterior hypothalamus, 25% in the medial basal hypothalamus (MBH), 44% in the adenohypophysis, and by approximately 50% in the uterus compared with controls.

Neurotransmitters. Regulation of the HPG axis involves multiple neurotransmitters, neuromodulators, neurosteroids, and neuropeptides. Regarding sex differences in brain neurotransmitter systems, it has been well established that such differences exist for norepinephrine, dopamine, and serotonin. In the POA and in hypothalamic neurosecretory nuclei, norepinephrine concentrations are higher in males than in females; dopamine levels are likewise higher in males in the arcuate nuclei, the median eminence, and other structures.

Concentrations of norepinephrine, dopamine, and serotonin were quantified in tissue samples from the POA and MBH of three-month-old females using spectrofluorometric analysis. Moderate androgenization with 0.25 mg testosterone propionate on PND 3 did not produce significant alterations in animals with persistent estrus compared with normal estrous females. In contrast, a more severe pathological state induced by administration of 0.15 mg testosterone propionate on PND 2–4 was characterized by a significant reduction in norepinephrine concentration to 1.53 ± 0.15 ng/mg tissue (control: 2.46 ± 0.13 ng/mg tissue) and dopamine concentration to 1.46 ± 0.37 ng/mg tissue (control: 3.35 ± 0.31 ng/mg tissue). Serotonin concentrations remained unchanged.

In addition to biogenic monoamines, the concentrations of histamine and acetylcholine were assayed, given the involvement of histamine in stimulating prolactin secretion and the role of acetylcholine in promoting the release of LH and FSH. Neonatal androgenization resulted in an increase in histamine content to 1.25 ± 0.10 ng/mg tissue compared with 0.91 ± 0.07 ng/mg in controls, along with a decrease in acetylcholine concentration to 0.68 ± 0.07 ng/mg tissue (control: 1.02 ± 0.09 ng/mg tissue).

A reduction in hypothalamic norepinephrine and acetylcholine concentrations correlates with decreased levels of gonadotropins and LHRH in androgenized females, whereas elevated histamine concentration is associated with increased prolactin content in the adenohypophysis. Notably, hyperprolactinemia is one of the characteristic endocrine features of PCOS in women.

Behaviors. Among the phenotypic manifestations of androgen-dependent disturbances in sexual differentiation of the brain are abnormalities in sexual and other behavioral patterns, a phenomenon also supported by clinical observations. For example, cases of reversed sexual orientation have been described in women whose mothers received hormonal medications during pregnancy, such as synthetic progestins exhibiting concomitant androgenic activity. Similar behavioral deviations have been reported in women born to mothers with hyperandrogenic conditions, including PCOS or congenital adrenal hyperplasia.

To investigate sexual behavior in neonatally androgenized females, an oil solution of testosterone propionate (0.25 mg) was administered subcutaneously on PND 3, whereas control animals received the vehicle. At 3 months of age, half of the experimental females exhibited persistent estrus, less commonly persistent diestrus, while the remaining animals demonstrated irregular estrous cycles.

Female-typical sexual behavior was assessed in 3-month-old ovariectomized females one week after surgery. Estrus was induced by administration of estradiol benzoate 48

hours prior to testing and progesterone 4–5 hours prior. Each female was paired with a male, and sexual behavior was recorded for 10 minutes or until 10 mountings occurred. Sexual motivation (approach, genital investigation, hopping, ear nibbling) and receptive behavior (number of lordotic responses) were documented. The lordosis quotient was calculated as the percentage of lordosis responses relative to the total number of male mountings.

Male-typical sexual behavior was evaluated by exposing the experimental female to a sexually receptive intact female in estrus for 10 minutes and recording the number of mountings.

All control females displayed active motivational and receptive sexual behavior. Normal lordotic responses occurred not only during male mountings but, on occasion, even in reaction to a male's mere approach, resulting in a lordosis quotient exceeding 100. In neonatally androgenized females, both motivational and receptive components of female sexual behavior were attenuated compared with intact animals: the proportion of females exhibiting Sexual motivation declined by one-third, and the number demonstrating lordotic responses decreased threefold. The lordosis quotient was markedly reduced, by an order of magnitude.

Male-typical sexual behavior in neonatally androgenized females was characterized by mounting activity observed in all experimental animals, whereas control females showed no such response toward a sexually receptive female. Thus, the behavioral alterations observed in androgenized females provide clear evidence of defeminization and masculinization induced by disrupted sexual differentiation of the brain.

Regarding other behavioral anomalies, it is notable that early androgenization in various animal species (monkeys, golden hamsters, mice) enhances aggressive behavior and alters feeding patterns. In free-choice paradigms, sexually mature female rats—unlike males—prefer saline to fresh water; however, this sex difference is abolished in neonatally androgenized females. Another noteworthy observation is the accelerated acquisition of maze-learning skills in female mice and rats subjected to early androgenization. The decisive role of androgens in the differentiation of parental behavior is supported by the finding that male rats castrated within hours of birth subsequently exhibit female-typical parental care despite testosterone replacement. These males attend to pups, groom them, and protect them from danger—behaviors never observed in intact males.

Neuromorphology. Neuromorphological features of sexual differentiation of the brain are also evident at the microstructural level. The MPN is larger in males than in females, a difference eliminated by neonatal castration in males or neonatal androgenization in

females. Similarly, sex differences in neuronal size within the SCN, ventromedial, and arcuate hypothalamic nuclei disappear under such hormonal manipulations. It has been demonstrated that sex hormones are essential for normal neurogenesis, as they stimulate the growth of neurocytes, dendrites, and axons and promote synaptogenesis. In the absence of hormonal influence, sexual dimorphism is lost, thereby creating a structural substrate for functional disturbances in neuroendocrine regulation later in life.

The Brain Proteins. Sexual differentiation of the brain is not confined to the hypothalamus but also extends to extrahypothalamic structures, including the amygdala and other regions. For example, electrical stimulation of the medial amygdala in neonatally androgenized female rats with anovulatory syndrome, unlike in normal animals, fails to enhance LH secretion. Several disturbances in sex-related differences in carbohydrate and protein metabolism, as well as in enzymatic activity across various brain regions, have been documented in neonatally androgenized females.

Disruptions in sexual differentiation of the brain induced by neonatal androgenization can be detected as early as the first PND. This applies particularly to alterations in the protein spectrum within neuroendocrine brain structures. Sexual dimorphism of the brain proteome is determined, at least in part, by the genome. For instance, two proteins with sex-specific expression levels have been identified in the POA. However, the extent to which such sex differences in protein composition are genetically predetermined, or alternatively programmed by hormonal influences during perinatal development, was remained unresolved. For this reason, we considered it appropriate to investigate the protein spectrum in discrete brain regions involved in the neuroendocrine regulation of sexual behavior and in the functions of the hypothalamic–pituitary–ovarian system and the HPA axis. Controls for neonatally androgenized animals with persistent estrus—induced by subcutaneous administration of 0.25 mg testosterone propionate on PND 3—were intact females in estrus, while males receiving the oil vehicle were used for comparative purposes.

The spectrum of soluble proteins in discrete brain structures, within the molecular mass (m.m.) range of 14.3–66.0 kDa, was analyzed by polyacrylamide gel electrophoresis in 5- and 10-day-old rat pups. A m.m. marker set (MW70LKB) served as reference. Gel plates were scanned using an Epson Perfection 1670 laser scanner. Densitometry and densitogram analysis were performed using the Scion Image software package.

In intact 5-day-old males, the relative optical density of protein bands with m.m. of 66.0 kDa and 34.7 kDa in the POA was higher by 71% and 24%, respectively, whereas proteins of 24.0 kDa and 14.3 kDa were lower by 78% and 71% compared with females. No sex differences in the distribution of the analyzed proteins were detected in the MBH or hippocampus. At 10 days of age, sex differences in the preoptic hypothalamic region

persisted for proteins of 66.0 kDa, 34.7 kDa, and 24.0 kDa, with higher optical densities in males by 69%, 68%, and 31%, respectively. In the MBH, sex differences were identified in the distribution of proteins of 45.0 kDa, 24.0 kDa, 18.4 kDa, and 14.3 kDa, with male values exceeding those of females by 62%, 29%, 106%, and 51%, respectively.

These findings demonstrate pronounced sexual dimorphism in protein distribution across discrete brain structures involved in the regulation of reproductive function and sexual behavior during the critical period of sexual differentiation of the brain. Such differences likely reflect underlying structural and functional modifications arising from the developmental programming of sexual dimorphism.

Neonatal androgenization eliminated sex differences in the protein spectrum of the POA in 10-day-old pups by increasing the optical density of protein bands in females to levels comparable to those of intact males. At the same time, these females exhibited a 26% reduction in the 18.4 kDa protein relative to non-androgenized females. In the MBH of androgenized females, a significant 1.5-fold increase was observed in proteins of 66.0 kDa, 45.0 kDa, 24.0 kDa, 18.4 kDa, and 14.3 kDa (on average, a 67% increase compared with intact females), whereas the 34.7 kDa protein showed a 30% decrease. In the hippocampus, elevations in the 45.0 kDa and 14.3 kDa proteins and reductions in the 66.0 kDa and 18.4 kDa proteins were noted relative with the control groups.

The most striking observation concerns alterations in the 66.0 kDa protein in the POA of females treated with testosterone propionate. Sex differences in this protein may reflect variations in microtubulin content, a well-established marker of neuronal growth in the rodent brain, and are likely associated with normal circulating androgen levels.

The finding that testosterone administration to neonatal females abolished sex differences across a broad range of proteins indicates a shift in the neurochemical phenotype of the brain, resulting from developmental programming induced by early androgen exposure. The observed changes in protein distribution in androgenized females were associated with increased optical density of electrophoretic bands to values characteristic of intact males.

Testosterone Metabolism in the Brain. The most reliable early marker of disrupted sexual differentiation of the brain in neonatally androgenized females is altered steroid aromatase activity in the hypothalamus. As will be demonstrated later, the conversion of testosterone to estradiol represents the key neurochemical mechanism guiding the differentiation of the brain along the male neuroendocrine and behavioral trajectory. In addition, products of testosterone 5α-reduction (*e.g.*, 5α-dihydrotestosterone),

synthesized *via* the activity of steroid 5α-reductase, also contribute to the neuroendocrine regulation of reproductive function.

Hypothalamic aromatase activity differs between male and female rats, particularly in the POA, where it is higher in males than in females (MacLusky et al., 1985). In contrast, the highest aromatase activity in females has been detected in the paraventricular nuclei of the hypothalamus.

The activity of estrogen synthase and steroid 5α-reductase (3-oxo-5α-steroid:$NADP^+$-en-oxidoreductase) was assessed using a method developed by our group (Reznikov et al., 1990), which includes two-dimensional thin-layer chromatography on silica gel in organic solvent systems followed by radiometric analysis of the reaction products. These products were generated through a one-hour incubation of the supernatant fraction (after centrifugation at 1,000 rpm) of a 10% tissue homogenate with [1,2,6,7-^{3}H]-testosterone in the presence of an NADPH-generating system. Separated steroids were identified, extracted with diethyl ether, and quantified for radioactivity using a Mark III beta spectrometer (Beckman, USA).

Aromatase activity in the POA of 10-day-old intact males was nearly threefold higher than in intact females, whereas 5α-reductase activity in the POA was greater in females. No sex differences were detected in the activity of either enzyme within the MBH.

In 10-day-old neonatally androgenized females, aromatase activity exceeded normative values. This observation is consistent with the elevated testosterone levels characteristic of androgenized females, which function as an inducer of cerebral aromatase activity (Takahashi & Yamanaka, 1987). A trend toward increased 5α-reductase activity was also observed in the POA.

Subsequent experiments sought to clarify the neurochemical mechanisms underlying disorders of sexual differentiation of the brain. Research conducted in rats in the United States and Japan led to the paradoxical hypothesis that the masculinization and defeminization of the brain under perinatal exposure to testosterone or androstenedione are mediated not directly by androgens, but by their estrogenic metabolites—estradiol and estrone. This conclusion was supported by findings showing that non-aromatizable androgens, such as 5α-dihydrotestosterone, fail to induce male-typical brain development when administered systemically or intracerebrally to neonatal female rats.

The concept that brain masculinization is programmed not by male, but by female sex hormones was initially controversial. However, the effects of neonatal androgenization in female rats can be prevented by the concurrent administration of aromatase inhibitors, which block the conversion of androgens into estrogens. In male rats, the peak of

testicular testosterone secretion coincides with the critical period of behavioral and gonadotropin-related sexual differentiation. This developmental window is accompanied by elevated hypothalamic estradiol concentrations, likely resulting from increased aromatase activity.

We investigated the ability of the androgen receptor antagonists flutamide and its active metabolite hydroxyflutamide, as well as the steroidal aromatase inhibitors androsta-1,4,6-trien-3,17-dione and androsta-4-en-3,6,17-trione, to prevent the development of anovulatory syndrome in neonatally androgenized female rats. Testosterone propionate (0.05 mg) was administered subcutaneously on PND 5 together with the respective inhibitors (0.5 mg and 1 mg, respectively). At three months of age, vaginal cytology and ovarian morphology were examined. Disrupted estrous cycles and the presence of polycystic ovaries lacking corpora lutea were used as diagnostic indicators of anovulation.

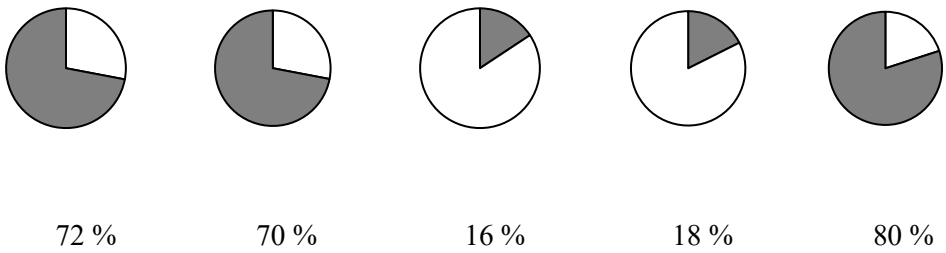

Figure 2.4 Effect of neonatal administration of testosterone propionate and its combinations with various compounds on the incidence of anovulation in adult female rats (dark gray color)
Footnotes: C – control, TP – testosterone propionate (50 μg on PND 5); Fl – flutamide; AT – androsta-4-en-3,16,17-trione; ATD – androsta-1,4,6-trien-3,17-dione; 4-OH-E_2 – 4-hydroxyestradiol-17β.

Pharmacological inhibition of steroid aromatase proved effective in preventing the development of anovulatory syndrome, thereby supporting the hypothesis that local estrogen biosynthesis is essential for the masculinization of fetal hypothalamic centers that regulate ovulation in adulthood (Fig. 2.4). In contrast, subcutaneous administration of the antiandrogen flutamide one hour prior to testosterone propionate injection failed to prevent its deleterious effects on the programming of neuroendocrine mechanisms governing ovulation. This finding can be attributed to the fact that the conversion of testosterone to estradiol does not require androgen receptor participation, meaning it proceeds independently of androgen receptor signaling. Nevertheless, androgen receptors remain critical for the organizational programming of male-typical mating behavior.

Catecholamine–Catecholestrogen Cooperation. The next step in advancing the neurochemical concept of sexual differentiation of the brain and its pathological disturbances was to investigate the potential role of catecholestrogens. These estrogenic metabolites, present in the hypothalamus and pituitary, are generated from monophenolic estrogens (estradiol, estrone) through hydroxylation at the 2- or 4-position of the A-ring of the steroid molecule. Despite their catechol structure, catecholestrogens retain the ability to bind to estrogen receptors and activate intracellular signaling cascades.

Although their estrogenic effects in peripheral target tissues are relatively weak, within the hypothalamus their potency may surpass that of natural estrogens, likely due to their capacity to modulate the membrane potential of neurosecretory neurons. For instance, they are more effective than estradiol in inducing preovulatory LHRH release and the subsequent pituitary LH surge. Notably, hypothalamic concentrations of catechol estrogens exceed those of estradiol and estrone by several-fold.

Interest in the role of catecholestrogens in the sexual differentiation of the brain emerged following reports that administration of 2-hydroxy- or 4-hydroxyestradiol-17β to neonatal female rats reproduced the effects of neonatal androgenization on the development of anovulation (MacLusky et al., 1983). We examined the ability of 2-hydroxyestrone and several catecholestrogen isomers (2- and 4-hydroxyestradiol-17β and 4-hydroxyestradiol-17α) to induce defeminization of the brain following systemic (subcutaneous) or intracerebral administration at doses of 5–25 μg from PND 1 to day 5. The estradiol metabolites 2-hydroxyestradiol-17β and 4-hydroxyestradiol-17α were ineffective in the early programming of anovulation in adulthood. In contrast, 4-hydroxyestradiol-17β produced effects equivalent to those of testosterone propionate. These differences in biological activity are likely attributable to the lower metabolic clearance and substantially higher affinity of 4-hydroxyestradiol-17β for cytosolic estrogen receptors relative to other catecholestrogens. By contrast, 4-hydroxyestradiol-17α exhibits weak estrogenic activity due to its low receptor affinity.

One intriguing finding associated with neonatal androgenization was the pronounced increase in norepinephrine content throughout the brain of newborn females. Its relevance to androgen-dependent brain differentiation, as opposed to other testosterone-mediated functions, remained uncertain. We demonstrated that a similar elevation occurs specifically in the hypothalamus. Following administration of 0.25 mg testosterone propionate to females on PND 3, hypothalamic norepinephrine content at seven days of age exceeded twice the normal level: experimental group – 7.3 ± 2.1 ng/mg tissue; control – 2.6 ± 0.1 ng/mg tissue. Dopamine concentrations remained unchanged, whereas serotonin content showed a slight decrease (experimental – 2.4 ± 0.2 ng/mg tissue; control – 3.1 ± 0.2 ng/mg tissue).

To determine the biological significance of hypothalamic monoamine alterations induced by neonatal androgenization in the pathogenesis of disrupted sexual differentiation of female brain, as well as their role in the normal programming of male-typical hypothalamic sexual dimorphism, we employed pharmacological agents with distinct mechanisms of action. Administration of the catecholamine synthesis inhibitor α-methyl-p-tyrosine to neonatal male rats prevented the programming of hypothalamic refractoriness to estrogenic stimulation of LH secretion in adulthood. This was evidenced by ovulation and the formation of corpora lutea in ovarian fragments transplanted into the anterior chamber of the eye of sexually immature males. Thus, hypothalamic norepinephrine proved to be critically important for normal male-typical differentiation of the hypothalamus, and its deficiency resulted in the retention of a genetically predetermined female-typical pattern of brain programming. Norepinephrine likely acts directly on the nuclear genome, similar to other biogenic monoamines (Tretiak & Arkhipova, 1992).

In experiments on neonatally androgenized female rats, α- and β-adrenergic blockers, presynaptic inhibitors of catecholamine and serotonin synthesis, catechol-O-methyltransferase (COMT) inhibitors, and additional neurotropic agents were employed. A single dose of 0.25 mg testosterone propionate was administered on PND 3 or 4 simultaneously with one of the neurotropic compounds, which was then continued for five consecutive days. At three months of age, vaginal smear cyclicity, ovarian morphology, and plasma concentrations of estradiol, progesterone, and LH were assessed.

The results demonstrated that blockade of adrenergic receptors did not prevent testosterone-induced disruption of sexual differentiation of the brain. The most effective protector against the development of anovulatory syndrome was *α*-methyl-*p*-tyrosine, which preserved the proestrous LH surge and prevented cystic transformation of the ovaries. Another effective protector was *p*-chlorophenylalanine, a selective inhibitor of serotonin synthesis. However, it would have been premature to conclude that serotonin participates in androgen-dependent defeminization of the developing brain without assessing the direct effects of both agents on hypothalamic neurotransmitter concentrations in 7-day-old neonatally androgenized females. It was found that both *α*-methyl-*p*-tyrosine and *p*-chlorophenylalanine prevented the testosterone-induced increase in hypothalamic norepinephrine, thereby raising doubts regarding the selectivity of the latter compound for serotonin.

These findings, among others, led to the conclusion that hypothalamic norepinephrine plays a primary role in the normal (male-typical) or disrupted (testosterone-exposed female) programming of sexual differentiation of the brain. Supporting this view, further elevation of hypothalamic norepinephrine in neonates—achieved through daily

administration of 0.1 mg tropolone, a COMT inhibitor, for PND 4-10 following a single injection of 0.25 mg testosterone propionate on PND 4—increased the proportion of animals exhibiting anovulatory syndrome from 70% to 100%.

The mechanism underlying the interaction between catecholestrogens, specifically 4-hydroxyestradiol-17β, and norepinephrine in these processes remained unclear. Our COMT activity in various brain regions (Breuer et al., 1974). COMT catalyzes the methylation of catecholamines using S-adenosylmethionine as the methyl donor. Catecholestrogens undergo the same methylation reaction, and COMT exhibits even higher affinity for these metabolites than for catecholamines. Competition between catecholestrogens and norepinephrine for COMT active sites inhibits the conversion of norepinephrine to normetanephrine, thereby increasing tissue norepinephrine concentrations. We hypothesized that 4-hydroxyestradiol-17β possesses this property and is responsible for the elevated hypothalamic norepinephrine induced by testosterone during sexual differentiation of the brain.

This hypothesis was confirmed experimentally: 24 hours after subcutaneous administration of 4-hydroxyestradiol-17β at 10 μg/day to females during the early postnatal period, hypothalamic norepinephrine levels increased from 3.04 ± 0.20 to 5.50 ± 0.62 nmol/g tissue, whereas dopamine concentrations remained unchanged. Thus, the elevation of hypothalamic norepinephrine induced by 4-hydroxyestradiol-17β is associated with its defeminizing effects in females during the critical window of sexual differentiation of the brain.

The pathogenetic role of COMT in neonatally androgenized females, as well as its physiological role in the programming of normal male-typical sexual differentiation of the brain, is further supported by evidence that enzyme activity is higher in males than in females during the first PNDs, and that hypothalamic norepinephrine levels in normal males are approximately twice those observed in females.

Since 4-hydroxyestradiol-17β is derived from estradiol formed *via* testosterone aromatization, it is understandable that administration of steroid aromatase inhibitors on PNDs 5 and 7 abolished the ability of a single 0.05 mg testosterone propionate injection on PND 5 to elevate hypothalamic norepinephrine in 10-day-old females and preserved regular estrous cyclicity in adult rats.

To establish a convincing neurochemical framework for androgen-dependent sexual differentiation of the brain, it was necessary to determine whether norepinephrine acts as a messenger of testosterone or its metabolites, or whether both “work” synergistically within the hypothalamus. Administration of tropolone to normal neonatal females increased hypothalamic norepinephrine levels but did not induce defeminization,

masculinization, or disturbances in sexual maturation and reproductive function. Thus, norepinephrine alone is insufficient to program the developing neuroendocrine system along a male-typical trajectory.

Conversely, the involvement of a hormonal—specifically estrogenic—component in this process is unequivocal, as aromatase blockade prevent the development of anovulatory syndrome in neonatally androgenized females. It is well established that estrogens stimulate synaptogenesis in developing neuroendocrine brain structures.

Therefore, androgen-dependent brain differentiation results from the cooperative action of catecholamines and neurosteroids—namely, estrogens and catecholestrogens synthesized within neural tissue. We termed this phenomenon *hormone–neurotransmitter co-induction*, which ensures the differentiation and functional maturation of immature neurons through epigenetic hormone–neurotransmitter imprinting. It should be emphasized that during the critical period of sexual differentiation of the brain, formation of the neuroendocrine system is still incomplete, and norepinephrine functions as a "presynaptic" inducer of cellular differentiation—an observation we first established in relation to mammalian development (see Chapter 1).

HPA Axis. Considering the involvement of brain catecholamines not only in the programming of the neuroendocrine reproductive system but also in the regulation of the HPA axis, it is reasonable to assume that, alongside reproductive neuroendocrine disturbances, the development of endocrine stress responses is likewise impaired.

The phenomenon of early hormonal programming of adaptive mechanisms, particularly HPA axis reactivity, has been widely discussed. It has been proposed that sex differences in HPA axis function, established prenatally or immediately postnatally in rodents, may result from sex hormone–dependent brain organization (Patchev et al., 1995; McCormick et al., 1998). In rats, there is some temporal overlap between the organizing influence of sex hormones on the brain and the maturation of the HPA axis (McEwen et al., 1991). Studies investigating the mechanisms underlying persistent HPA axis dysfunction in neonatally androgenized female rats have shown that these disturbances are associated with alterations in corticotropin-releasing hormone (CRH) mRNA expression and hypothalamic content, as well as increased cytoplasmic glucocorticoid receptor levels (Seale et al., 2005).

We examined HPA axis function in six-month-old females exhibiting persistent estrus that had received 0.25 mg testosterone propionate subcutaneously on PND 3. Plasma corticosterone (11-hydroxycorticosteroids) was quantified using a spectrofluorimetric method following extraction with methylene chloride. In control females, one hour of immobilization stress induced an average 2.2-fold increase in plasma corticosterone. In

neonatally androgenized females, the response to acute stress was either absent or markedly attenuated.

Noradrenergic reactivity of the HPA axis was examined in non-anesthetized, neonatally androgenized 8-month-old females that were allowed unrestricted movement within their cages. Ten days before the experiment, a metal cannula was stereotaxically implanted into the brain third ventricle, and a Silastic catheter was inserted into the external jugular vein the day prior to testing. Blood samples were collected at 30-min intervals, with the withdrawn volume replaced by isotonic NaCl containing heparin solution. Plasma corticosterone was measured 30, 60, and 90 minutes after application of norepinephrine bitartrate (10 μg in 2 μL of 0.9% pyrogen-free NaCl) into the third ventricle. Basal corticosterone concentrations were elevated compared with intact controls due to surgical manipulations.

Neonatal androgenization substantially impaired the noradrenergic reactivity of the HPA axis. In control animals, plasma corticosterone increased 1.6-fold 30 minutes after norepinephrine administration. In contrast, intraventricular application of norepinephrine failed to elevate corticosterone levels in neonatally androgenized females (Table 2.1).

Table 2.1 Plasma corticosterone concentrations (nmol/L) in neonatally androgenized female rats following application of norepinephrine bitartrate into the third ventricle of the brain (M ± SEM)

Animal group	**Time of blood sampling**			
	0 min	**30 min**	**60 min**	**90 min**
Control	774 ± 131	1213 ± 63*	1021 ± 56	956 ± 62
Testosterone propionate	862 ± 71	966 ± 62	935 ± 70	894 ± 100

Footnotes: * $p < 0.05$ compared with control. Each group contained five rats.

The data indicate impaired stress and noradrenergic reactivity of the HPA axis in adult female rats exposed to exogenous androgen during the neonatal period. This finding is consistent with report demonstrating reduced corticosteroid secretion in response to various stressors in neonatally androgenized female rats (Seale et al., 2005). The attenuated adrenocortical response to acute stress has been associated with reduced mRNA expression of arginine vasopressin (AVP) and CRH, as well as marked upregulation of glucocorticoid receptor mRNA in the paraventricular nuclei of the hypothalamus. It is likely that impaired adrenocortical responsiveness to stress or to central noradrenergic stimulation in androgenized females results from a direct action of

androgens on neural centers regulating HPA axis function, including those involved in hypothalamic negative feedback mechanisms.

Based on our findings, it may also be hypothesized that, in addition to disruptions in the negative feedback loop of the HPA axis, diminished hypothalamic sensitivity to norepinephrine plays a significant role in the altered stress reactivity observed in neonatally androgenized females.

When analyzing potential mechanisms underlying altered HPA axis reactivity in neonatally androgenized females, the influence of other hormonal factors—particularly estrogens—should also be considered. Estrogens are involved in regulating HPA axis activity. Typically, female rats exhibit greater HPA axis responsiveness to stress than males (Patchev et al., 1995; Reznikov & Nosenko, 2000; Seale et al., 2005), which is attributed to the stimulatory effects of estrogens on ACTH and corticosterone secretion (Burgess & Handa, 1992). Enhanced adrenal secretory activity may result from both the direct action of estrogens on CRH-producing neurons in the paraventricular hypothalamic nuclei and increased pituitary sensitivity to CRH (Vamvakopoulos & Chrousos, 1993).

In androgenized females, circulating estrogen levels are reduced due to inhibited folliculogenesis in the ovaries, thereby weakening their stimulatory influence on HPA axis stress reactivity. Neonatal administration of estradiol to ovariectomized, androgenized females normalizes mRNA expression of AVP and CRH, further supporting the activating role of estrogens in HPA axis function. However, this estrogenic effect is secondary to the organizational influence of androgens during the neonatal period on HPA axis reactivity in adulthood.

Role of Calcium Signaling in Androgen-Dependent Functional Disturbances. The cellular and molecular mechanisms underlying functional impairments in neonatally androgenized females require further elucidation. One potential mechanism involves calcium-dependent regulation of neurogenesis, including neuronal migration, proliferation, and apoptosis. Intracellular calcium plays a key role in neuronal development and migration in neonates (Mattson, 1992). Limited evidence suggests that calcium ions may contribute to hormone-dependent disruptions of neurogenesis. Specifically, androgens have been shown to modulate hypothalamic calcium-binding protein levels during perinatal development (Watson et al., 1998), indicating a possible role for calcium signaling in programming androgen-dependent neuroendocrine pathology.

Verapamil dissolved in isotonic NaCl was administered subcutaneously at 0.5 mg/kg b.w.t to neonatal female rats for five consecutive days starting on PND 3, concurrently

with testosterone propionate (0.25 mg on PND 3). Control animals received vehicle solutions, and all subjects were examined during estrus.

In verapamil-treated neonatally androgenized females, the distribution of 66.0, 34.7, and 24.0 kDa proteins in the POA remained comparable to that of intact females, showing respective reductions of 28%, 67%, and 29%. Verapamil administration also prevented testosterone-induced alterations in hippocampal protein distribution (66.0 and 45.0 kDa), characterized by an increase in the optical density of the 66.0 kDa protein and a decrease in that of the 45.0 kDa protein.

These findings indicate that pharmacological blockade of calcium channels during the critical period of sexual differentiation of the brain induces distinct modifications in the protein profile of neuroendocrine brain regions. This supports the hypothesis that intracellular calcium ions may mediate androgen-dependent mechanisms underlying sexual differentiation of the brain and that these mechanisms are susceptible to pharmacological disruption of calcium-regulated neurogenesis. Considering the masculinizing and/or defeminizing effects of androgens in female rats, as well as previous evidence of structural and neurochemical alterations in sexually dimorphic brain regions, the verapamil-induced changes in protein spectra likely reflect imprinting mechanisms that contribute to sexual differentiation of the brain.

Neonatal administration of verapamil together with testosterone propionate did not normalize aromatase activity, which remained elevated under testosterone exposure. Verapamil treatment exerted only a modest effect on the testosterone-induced disruption of female-typical sexual behavior, although the mean lordosis quotient increased 3.5-fold compared with neonatally androgenized animals.

Male-typical sexual behavior in androgenized females was characterized by mounting, which was observed in all subjects of the experimental group, whereas no control females displayed such behavior toward receptive females. Thus, androgenization in the presence of verapamil produced the same pathological outcomes as in females not treated with the calcium channel antagonist.

Regarding the potential role of calcium signaling in the pathogenesis of HPA axis dysfunction induced by neonatal androgenization, females subjected to combined neonatal exposure to testosterone and verapamil exhibited HPA axis responses to a one-hour immobilization stress that were comparable to those in intact animals. Verapamil-treated females showed a 1.9-fold increase in plasma corticosterone in response to immobilization stress.

Neonatal verapamil administration to androgenized females also largely preserved the normal pattern of noradrenergic HPA axis reactivity: the maximal rise in plasma corticosterone 30 minutes after intracerebroventricular application of norepinephrine bitartrate did not differ from that observed in control females.

These observations suggest that the impaired adrenocortical responses to stress or to central noradrenergic stimulation observed in androgenized females may arise from the direct action of androgens on the programming of neural centers regulating HPA axis function, particularly those involved in hypothalamic negative feedback mechanisms.

Based on the obtained data, it may also be hypothesized that, in addition to disruptions in negative feedback among HPA axis components, a major contributing factor to the reduced stress reactivity of neonatally androgenized females is a diminished hypothalamic sensitivity to norepinephrine.

The prevention of HPA axis functional impairments by verapamil in neonatally androgenized female rats indicates a potential involvement of calcium-dependent mechanisms in mediating the early organizational effects of androgens on stress responsiveness and noradrenergic reactivity of the HPA axis. This interpretation is supported by the ability of verapamil to cross the blood–brain barrier and block calcium channels in central neurons (Canchola-Martinez et al., 1997; Elsinga et al., 2004). Thus, the protective influence of verapamil on HPA axis reactivity following neonatal androgen exposure is likely mediated by the correction of testosterone-induced disturbances in neuronal calcium homeostasis within brain structures governing HPA axis regulation.

Overall, these findings underscore the critical role of calcium signaling in the perinatal programming of androgen-dependent alterations in HPA axis reactivity and in the sexual dimorphism of specific neurochemical brain parameters, as well as the ability of verapamil to exert a protective effect against these disruptions.

The culmination of these studies led to the formulation of a conceptual framework describing the neurochemical mechanisms underlying male-typical sexual differentiation of the brain, as illustrated in Figure 2.5.

2.2 Estrogens

Clinical observations have reported cases of sexual orientation reversal in women whose mothers were treated with the synthetic estrogen diethylstilbestrol during pregnancy

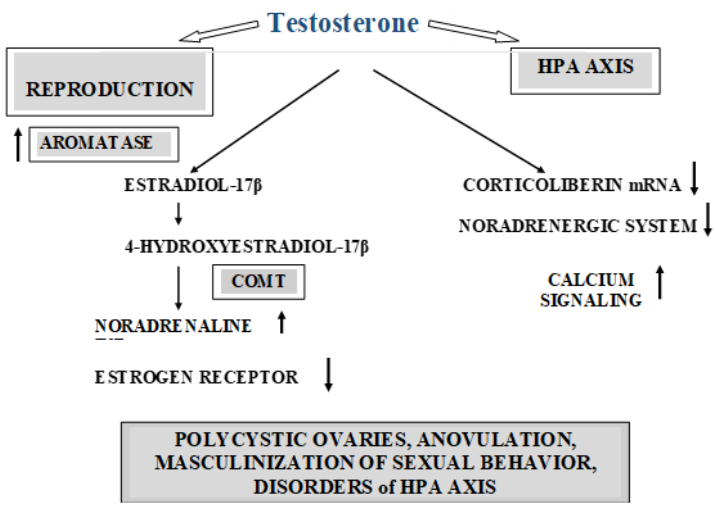

Fig. 2.5 Diagram of hormone–neurotransmitter imprinting during sexual differentiation of the brain in neonatally androgenized female rats

(Ehrhardt et al., 1985). This raises an important question: why do elevated maternal estrogen levels not normally disrupt sexual differentiation of the fetal brain? It is likely that estrogens synthesized locally within neural tissue play the primary role in governing sexual differentiation of the brain. With respect to prenatal exposure to diethylstilbestrol, its disruptive potential is explained by its low affinity for testosterone–estradiol binding globulin, which normally forms complexes with endogenous estrogens and thereby reduces their biological activity.

Administration of estrogens to neonatal female rats induces not only defeminization and/or masculinization of the reproductive system but may also partially impair neuroendocrine regulation of the HPA axis (McCormick et al., 1998). Conversely, a single injection of estradiol benzoate to male rats, administered no later than PND 5, results in testicular insufficiency, abnormalities of sexual development, and altered sexual behavior due to disrupted programming of the neuroendocrine system. Thus, dysregulation of central control over both the reproductive system and the HPA axis may occur under the influence of either androgens or estrogens, the latter producing such effects only at supraphysiological doses.

2.2 Hydrocortisone

Programming Effect of Glucocorticoids. The developing fetal brain is characterized by remarkable plasticity and heightened sensitivity to perturbations in hormonal

homeostasis. Maternal glucocorticoids readily cross the placental barrier and are critically involved in fetal neuronal differentiation and maturation, as well as in the regulation of genomic expression within neurons (Matthews, 2000; 2001; Welberg et al., 2001). Both endogenous and exogenous glucocorticoids can modify fetal HPA axis programming, which later manifests in adulthood as neuroendocrine and behavioral disturbances and, to some extent, influences the programming of reproductive function.

In clinical practice, several scenarios exist in which a pregnant woman may expose the fetus to an excessive glucocorticoid load, thereby increasing the risk of altered neuroendocrine development. Physiological pregnancy is accompanied by intrinsic activation of the HPA axis, reflected in elevated maternal plasma hydrocortisone (cortisol) concentrations. However, concomitant increases in corticosteroid-binding globulin—stimulated by estrogen-driven hepatic protein synthesis—and the presence of placental 11β-hydroxysteroid dehydrogenase, which partially inactivates cortisol by converting it into cortisone, collectively protect the fetus from glucocorticoid overexposure. Nevertheless, in cases of enzymatic insufficiency or impaired hepatic protein synthesis, these protective mechanisms may prove inadequate, resulting in elevated fetal cortisol levels and subsequent disruption of neuroendocrine programming.

A substantial body of animal research demonstrates that prenatal glucocorticoid exposure induces, in adulthood, a range of outcomes including altered sexual behavior, modifications in basal corticosteroid concentrations, altered HPA axis responses to stressors (Naumenko et al., 1990; Muneoka et al., 1997), and impaired reciprocal interactions between the adrenal glands and the hypothalamic–pituitary complex. These effects are associated with reduced hypothalamic CRH mRNA expression (McCabe et al., 2001) and altered density and distribution of corticosteroid receptors in the brain (Ordyan & Pivina, 1999; Dean et al., 2001), particularly within the hippocampus (Matthews, 2000; Liu et al., 2001; Welberg et al., 2001). Such alterations determine the sensitivity of neuroendocrine structures to corticosteroids under conditions of negative feedback and stress-induced HPA axis activation. These findings align with the concept of premature automodification of cellular responses (Reznikov, 1982; 1994).

Corticosteroids also contribute to neurogenesis, synaptogenesis, and the regulation of apoptosis in the embryonic and fetal nervous system, forming an essential component of HPA axis programming. According to Kalinina (2007), prenatal administration of hydrocortisone acetate inhibits neuronal apoptosis. Thus, disturbances in corticosteroid balance resulting from glucocorticoid treatment may lead to pathological consequences.

To clarify the role of glucocorticoids in early programming of the HPA axis and the reproductive neuroendocrine system, as well as their potential to modify the functional state of these hormonal systems, a series of studies was conducted in rats (Reznikov &

Nosenko, 2013; Reznikov et al., 1999a,b; 2004a,b; 2008a; Sinitsin et al., 2005; Reznikov, 2007). Particular attention was devoted to identifying sex-specific disturbances related to sexual differentiation of the brain, an aspect that remained insufficiently explored.

Hydrocortisone acetate suspension was administered subcutaneously to pregnant rats at a daily dose of 50 mg/kg b.w.t from gestational day (GD) 15 to 21, or on GD 16 and 18. The early and long-term neuroendocrine effects of this intervention were subsequently examined. Although corticosterone is the physiological glucocorticoid in rats, hydrocortisone acetate exhibits similar glucocorticoid properties and is prescribed during pregnancy for topical treatment of dermatological conditions. The clinical use of glucocorticoids during pregnancy for medical indications is a widely accepted practice.

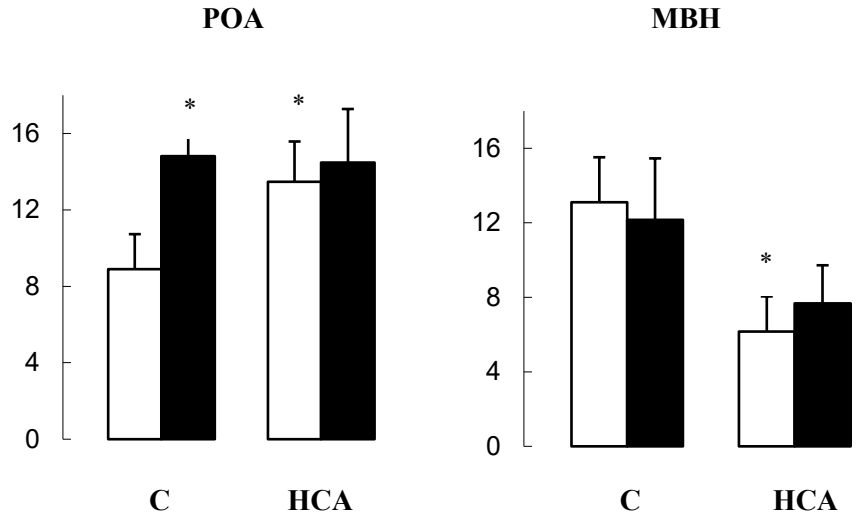

Fig. 2.6 Aromatase activity in the POA and MBH of ten-day-old rats following prenatal exposure to hydrocortisone acetate (HCA)
Footnotes: The ordinate axis represents pmol E_2/h/g protein. Light bars indicate females, black bars indicate males. C – control animals; HCA – HCA administration on GD 16 and 18. *p < 0.05 compared with control females.

Administration of the hormone throughout GD 15–21 resulted in a 53% increase in plasma 11-hydroxycorticosteroid levels on GD 21 compared to intact females.

Disruptions in the sexual differentiation of the brain caused by prenatal exposure to hydrocortisone acetate become evident in the early postnatal period, at a developmental stage when programming of the reproductive neuroendocrine systems is still incomplete.

Testosterone Metabolism in the Brain. In the assessment of hydrocortisone acetate effects on aromatase activity, ten-day-old control animals exhibited the same sex differences as described earlier; specifically, aromatase activity in the POA of males was significantly higher than in females. However, these sex differences were abolished following prenatal exposure to hydrocortisone acetate due to an increase in enzymatic activity in females (Fig. 2.6).

Prenatal exposure to hydrocortisone acetate also impaired the formation of 5α-reduced testosterone metabolites. Under normal conditions, steroid 5α-reductase activity in the POA of females was higher than in males (328.8 ± 63.9 pmol/h/g protein *vs.* 212.2 ± 62.9 pmol/h/g protein, $p < 0.05$, nonparametric *U* test). In the experimental animals, this difference disappeared (females: 236.7 ± 51.4 pmol/h/g protein; males: 190.0 ± 83.4 pmol/h/g protein, $p > 0.05$).

***Neurotransmitters*.** Given the essential role of norepinephrine as a neurochemical determinant of both brain sexual differentiation and HPA axis programming, it is noteworthy that prenatal hydrocortisone acetate abolished the sex difference in norepinephrine concentration in the POA of ten-day-old offspring by decreasing its level in females (control: females – 4.22 ± 0.48 nmol/g tissue, males – 2.74 ± 0.38 nmol/g tissue, $p < 0.05$; hydrocortisone acetate: females – 2.64 ± 0.37 nmol/g tissue, males – 2.16 ± 0.31 nmol/g tissue, $p > 0.05$). This effect is likely associated with the inhibitory prenatal influence of hydrocortisone acetate on tyrosine hydroxylase, the rate-limiting enzyme in norepinephrine biosynthesis in the fetal brain. Dopamine concentrations in the POA remained unchanged.

To assess catecholamine turnover in the aforementioned discrete hypothalamic structures of ten-day-old rats, the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine was administered subcutaneously at a dose of 0.25 mg/kg b.w.t, 1 or 2 hours prior to decapitation. Catecholamine turnover was calculated according to Brodie et al. (1966).

It is well established that changes in catecholamine turnover within the first hour following tyrosine hydroxylase inhibition reflect the utilization rate of the functional catecholaminergic pool. In control pups, no sex-dependent differences in catecholamine turnover were detected in any of the examined brain regions. Prenatal exposure to hydrocortisone acetate produced a significant reduction in norepinephrine turnover in the POA of males (0.33 ± 0.09 nmol/g·h *vs.* 1.89 ± 0.45 nmol/g·h in control males, $p < 0.05$), as well as an elevation in dopamine turnover in females (0.69 ± 0.12 nmol/g·h *vs.* 0.33 ± 0.11 nmol/g·h in control females, $p < 0.05$). No changes in these parameters were observed in the MBH of the experimental animals.

Serotonin concentrations and the terminal metabolite 5-hydroxyindoleacetic acid were quantified in the POA and MBH of ten-day-old rats using high-performance liquid chromatography. The serotonin metabolism index, which reflects turnover, was calculated as the ratio of 5-hydroxyindoleacetic acid to serotonin concentration.

No sex dimorphism was observed in any of the evaluated parameters. However, prenatal administration of hydrocortisone acetate resulted in the emergence of sex differences in 5-hydroxyindoleacetic acid concentrations in both brain regions and in serotonin

Table 2.2 Concentrations of serotonin and 5-hydroxyindoleacetic acid in the hypothalamus of 10-day-old female rats in the control group and following prenatal exposure to hydrocortisone acetate during GD 15–21 (M ± SEM)

Parameter	Control	Hydrocortisone acetate
POA		
Serotonin, ng/mg tissue	0.43 ± 0.03	0.43 ± 0.04
5-Hydroxyindoleacetic acid, ng/mg tissue	0.73 ± 0.14	1.71 ± 0.09 [a]
Serotonin metabolism index	1.80 ± 0.36	4.22 ± 0.58 [a]
MBH		
Serotonin, ng/mg tissue	0.44 ± 0.05	0.36 ± 0.02
5-Hydroxyindoleacetic acid, ng/mg tissue	0.74 ± 0.08	1.51 ± 0.14 [ab]
Serotonin metabolism index	1.85 ± 0.31	4.19 ± 0.39 [ab]

Footnotes: The table presents values of 6–11 analyses. Each group contained five-seven rats. [a] $p < 0.05$ compared with control females; [b] $p < 0.05$ compared with control males (refer to the Table 2.3). Statistical analysis performed using the Wilcoxon–Mann–Whitney *U* test.

metabolism in the POA, attributable to increased values in females. Serotonin concentrations in the POA were elevated in experimental males, whereas values in the MBH remained unchanged. In experimental females, the serotonin metabolism index increased in both examined brain regions (Tables 2.2, 2.3).

Early alterations in biogenic monoamine content and testosterone metabolism within neuroendocrine brain structures of animals exposed to prenatal hydrocortisone acetate were reflected in subsequent features of brain sexual differentiation and in the functional

Table 2.3 Concentrations of serotonin and 5-hydroxyindoleacetic acid in the hypothalamus of 10-day-old male rats in the control group and following prenatal exposure to hydrocortisone acetate during GD 15–21 (Mean ± SEM)

Parameter	Control	Hydrocortisone acetate
POA		
Serotonin, ng/mg tissue	0.35 ± 0.02	0.52 ± 0.03 [b]
5-Hydroxyindoleacetic acid, ng/mg tissue	0.87 ± 0.08	1.42 ± 0.07 [abc]
Serotonin metabolism index	2.49 ± 0.23	2.76 ± 0.15 [ac]
MBH		
Serotonin, ng/mg tissue	0.64 ± 0.15	0.33 ± 0.04
5-Hydroxyindoleacetic acid, ng/mg tissue	1.03 ± 0.14	1.11 ± 0.06 [ac]
Serotonin metabolism index	2.23 ± 0.57	3.57 ± 0.59 [a]

Footnotes: The table presents values of 6–11 analyses; [a] $p < 0.05$ compared with the control females (refer to the Table 2.2); [b] $p < 0.05$ compared with the control males; [c] $p < 0.05$ compared to females after prenatal exposure to hydrocortisone acetate. Statistical analysis performed using the Wilcoxon–Mann–Whitney *U* test.

state of the HPA axis in adulthood (6–8 months). The samples from experimental and control females were collected during diestrus.

Neuromorphology. A microstructural marker of hydrocortisone acetate–induced disturbances in sexual differentiation of the brain was the loss of sexual dimorphism in neuronal nuclear size within sex-dimorphic regions, specifically the MPN and SCN.

Morphometric analysis was conducted on frontal hypothalamic sections stained with Nissl. Maximal and transverse diameters of neuronal nuclei were measured, and nuclear volume was calculated using a standard geometric formula. Under physiological conditions, nuclear volume in males exceeds that of females by approximately 20–25%.

The mean nuclear volume of neurons in the SCN of males was 240 ± 11 μm³, whereas in females during diestrus it measured 202 ± 8 μm³ ($p < 0.05$). In the MPN, the corresponding values were 373 ± 14 μm³ and 286 ± 6.0 μm³ ($p < 0.05$). These sex differences were abolished in the SCN of experimental animals due to a reduction in male nuclear volume to 209 ± 8 μm³, a value comparable to that of control females. An

additional indicator of demasculinization in males was a reduced number of large neurons. No analogous changes were observed in experimental females.

In the MPN, sexual dimorphism likewise disappeared; however, in this region the effect was driven by an increase in nuclear volume in females to 354 ± 14 μm³ ($p < 0.05$). These neuroanatomical alterations are consistent with impairments in sexual behavior and fertility previously documented in male rats exposed to prenatal hydrocortisone acetate (Pereira et al., 2003).

HPA Axis. Particular attention was directed toward assessing the functional state of the HPA axis, particularly its responsiveness to acute stress. This included evaluation of plasma corticosterone levels under baseline conditions and following targeted stimulation of different HPA axis components, as well as analysis of hypothalamic noradrenergic mechanisms (which mediate HPA stress activation) and hippocampal GABAergic mechanisms (components of the stress-limiting system).

It was first established that the HPA axis of adult male rats born to mothers treated with hydrocortisone acetate during late gestation exhibited a markedly attenuated response to acute immobilization stress compared with controls, despite normal baseline corticosterone concentrations.

Baseline norepinephrine concentration in the whole hypothalamus of hydrocortisone-exposed males was reduced by approximately 20% compared with controls, whereas dopamine levels remained unchanged. In experimental males, acute stress elicited a decrease in hypothalamic dopamine concentration (baseline: 4.93 ± 0.37 nmol/g tissue; post-immobilization: 3.86 ± 0.23 nmol/g tissue, $p < 0.05$). However, these males failed to exhibit the typical stress-induced decline in hypothalamic norepinephrine concentration (normally 22%) or the characteristic increase in hippocampal glutamate decarboxylase activity (normally 40.3% in males and 38.6% in females, $p < 0.05$). Glutamate decarboxylase is the rate-limiting enzyme in GABA synthesis and constitutes a major regulatory component of the stress-limiting system.

In contrast to males, experimental females demonstrated a 30% increase in HPA axis stress reactivity (plasma corticosterone concentration) relative with the control groups, accompanied by a more pronounced decline in hypothalamic norepinephrine concentration (46% *vs.* 35% in controls, $p < 0.05$). Stress-induced elevation of hippocampal glutamate decarboxylase activity persisted in experimental females, although the magnitude of this increase was 21% lower than in controls. Importantly, baseline enzyme levels did not differ between sexes in either control or experimental groups, indicating that sex dimorphism becomes apparent only under conditions of adaptive activation of the HPA axis (Fig. 2.7).

The altered dynamics of hypothalamic norepinephrine and hippocampal glutamate decarboxylase activity were fully consistent with the observed modifications in HPA axis reactivity in males and females. These mechanisms may therefore serve as mediators of glucocorticoid-induced alterations in HPA axis stress responsiveness, complementing the previously described changes in the hippocampal corticosteroid receptor system.

It is well established that noradrenergic stimulation of hypothalamic structures responsible for the synthesis of CRH and AVP plays a critical role in activating the HPA axis in response to diverse stimuli, serving as a key mechanism of adaptation to disrupted homeostasis. Dysregulation of endogenous corticosteroid balance during the prenatal period may induce profound alterations in the noradrenergic mechanisms governing HPA axis activity in adult offspring. Notably, the HPA axis response to intracerebroventricular norepinephrine administration was significantly attenuated in adult offspring of females treated with hydrocortisone acetate on GD 16 and 18; however, these experiments were conducted exclusively in males (Naumenko & Dygalo, 1979).

In our study, we evaluated adrenal cortex responsiveness to corticotropic and central noradrenergic stimulation in 8-month-old male and female rats that had developed under conditions of artificial glucocorticoid excess induced by prenatal administration of hydrocortisone acetate during the final week of gestation. A major methodological advantage of the norepinephrine bitartrate application experiments was that they were performed in freely moving, non-anesthetized animals, a factor of fundamental importance when assessing HPA axis reactivity to stressogenic stimuli.

Control animals responded to central norepinephrine administration with a 20% increase in plasma corticosterone concentration at 30 min post-injection, which returned to baseline by 60 min. In contrast, in males prenatally exposed to hydrocortisone acetate, the adrenocortical response to norepinephrine was absent; moreover, corticosterone levels at 60 and 90 min were even lower than baseline.

In a comparable experimental group of females, the mean basal corticosterone concentration during diestrus was threefold lower than in control females at the same stage of the estrous cycle. Intracerebroventricular application of norepinephrine bitartrate in control females produced a 28% increase in plasma corticosterone at 30 min, followed by a slight decline below baseline at 60 min. In experimental females, the relative increase at 30 min was even greater (46% above baseline), and the response was more prolonged: plasma corticosterone remained elevated even 90 min after norepinephrine administration.

Because alterations in the adrenocortical response to intracerebroventricular norepinephrine might have reflected changes in adrenal sensitivity to pituitary corticotropin, this possibility was examined in a subsequent series of experiments. Plasma corticosterone levels were measured following exogenous corticotropic stimulation. However, neither males nor females prenatally exposed to hydrocortisone acetate exhibited altered adrenal responsiveness to β-1-24-corticotropin, administered intravenously at a dose of 200 μg/kg b.w. A twofold or greater increase in plasma corticosterone concentration occurred in all groups as early as 30 min after injection and persisted throughout the 2-h observation period. Thus, the HPA axis response to noradrenergic stimulation in these animals reflects the state of noradrenergic reactivity within the hypothalamic component of the system rather than altered adrenal sensitivity to ACTH.

The results obtained indicate that artificial elevation of glucocorticoid levels in the maternal and fetal circulation during pregnancy disrupts noradrenergic regulation of the HPA axis, with clear sex-specific differences in the programming effects of corticosteroids on the functional state of this system. The observed alterations in HPA axis function—particularly within its noradrenergic regulatory components—in rats prenatally exposed to hydrocortisone acetate may be attributable to glucocorticoid-induced disturbances in the imprinting of tyrosine hydroxylase gene expression and genes encoding noradrenergic receptors in the brain.

It is plausible that prenatal glucocorticoid exposure alters the functional state of the HPA axis in adult rats in a manner that reduces physical endurance (Nosenko & Reznikov, 2000). Impaired HPA axis activity is also considered to diminish nonspecific resistance of the organism and may contribute to increased susceptibility to autoimmune diseases and systemic connective tissue disorders.

The findings clearly demonstrate the modifying influence of prenatal glucocorticoid exposure on distinct neuromorphological and neurochemical features of discrete brain regions involved in the neuroendocrine regulation of reproduction and stress. These alterations exhibit all the characteristics of disruptions in the hormonal–neurotransmitter imprinting of the developing brain. Importantly, they were evident both in early postnatal life and in adulthood. Glucocorticoid “loading” coincided temporally with the period of sexual differentiation of the brain.

In 10-day-old rat pups prenatally exposed to hydrocortisone, sexual dimorphism of neurochemical and neuro-morphological parameters either disappeared (e.g., norepinephrine concentration, steroid aromatase activity in the POA) or emerged *de novo* (e.g., serotonin metabolism). Notably, these changes did not arise due to alterations in males but rather resulted from corresponding modifications in females.

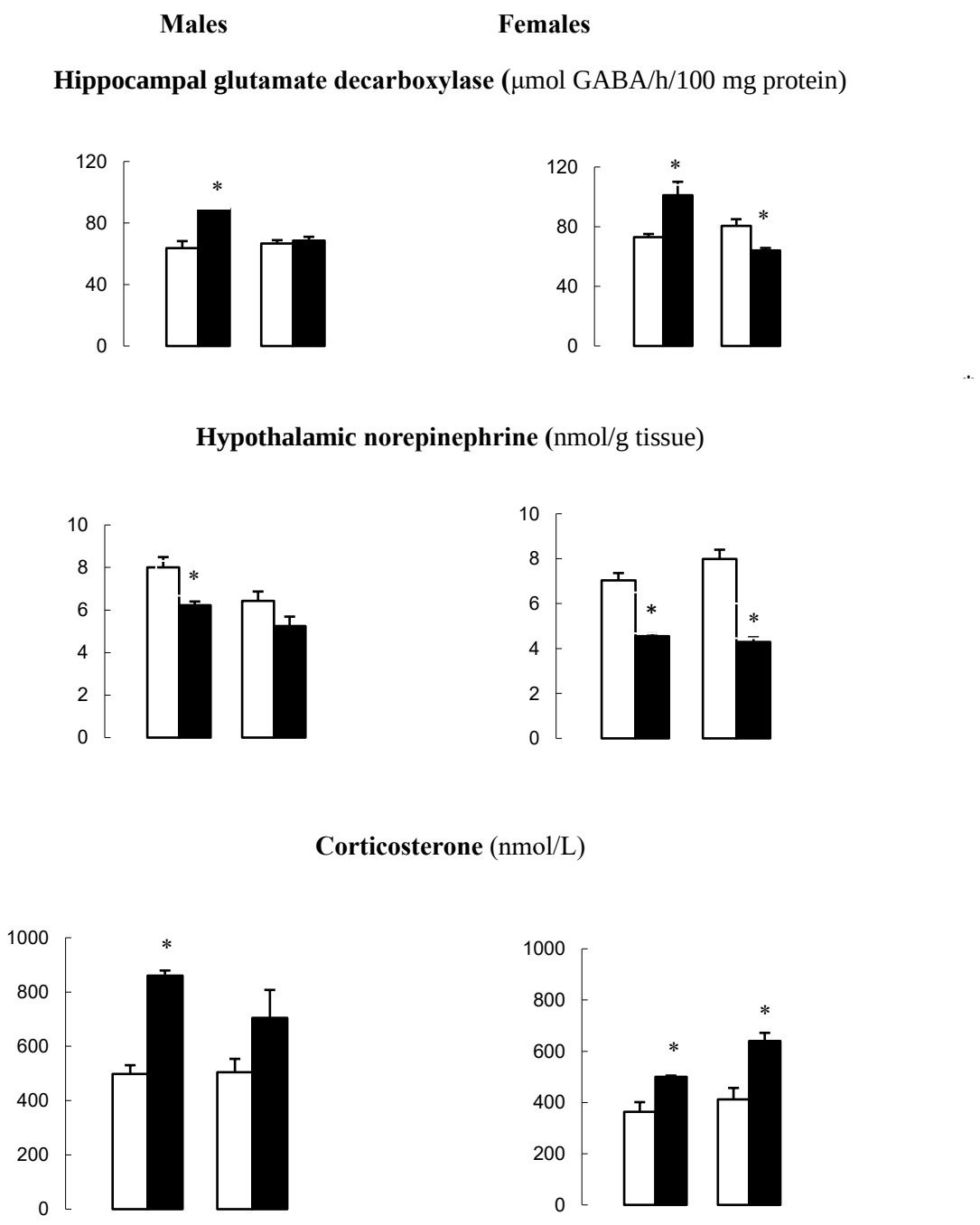

Fig. 2.7 Glutamate decarboxylase activity, norepinephrine concentrations, and plasma corticosterone levels in adult rats prenatally exposed to hydrocortisone acetate (right pairs of bars) or vehicle (left pairs of bars) under baseline conditions (open bars) and after immobilization (black bars). *$p < 0.05$ compared with baseline condition.

The findings from the hydrocortisone studies also provide important insights into the neurohormonal component of the first phase of the general adaptation syndrome, during which one-hour immobilization evokes corticosterone secretion by the adrenal cortex. These results confirm that glucocorticoid excess during the final week of gestation induces long-lasting alterations in both stress reactivity and noradrenergic responsiveness of the HPA axis in adult male offspring. Moreover, we demonstrated that these changes are distinctly sex-dependent: in males, HPA axis reactivity is attenuated, whereas in females it is moderately enhanced.

A key factor contributing to the diminished stress response of the HPA axis in experimental males appears to be increased corticosteroid receptor binding in the hippocampus—a brain region critically involved in regulating HPA axis activation in response to stress—combined with reduced stress-induced norepinephrine utilization in the hypothalamus. In contrast, the moderate enhancement of HPA axis stress activation observed in females may partly reflect a weakened inhibitory influence of the GABAergic system. This interpretation is supported by evidence showing reduced activity of glutamate decarboxylase, the rate-limiting enzyme in GABA synthesis, following acute stress compared with control females.

In summary, exogenous glucocorticoid "loading" of pregnant rats during the period of fetal sexual differentiation of the brain induces multiple programming disturbances in neuroendocrine systems governing reproductive function and HPA axis adaptation. The direction and magnitude of these alterations are strongly dependent on the sex of the offspring.

Conclusions:

- During intrauterine development of the male fetus, testosterone programs the brain toward a male-typical phenotype through epigenetic imprinting. In rats, the critical period of sexual differentiation of the brain spans the final week of gestation and the early neonatal period. Rat models have demonstrated that androgen excess in female fetuses disrupts neuroendocrine regulation of reproduction, leading to anovulation, PCOS–like manifestations, hyperprolactinemia, alterations in hormonal profiles, and behavioral masculinization. Conversely, insufficient testosterone exposure in male fetuses results in homo- or bisexual patterns of behavior later in life and reduced copulatory performance. These outcomes reflect underlying structural and functional changes in the hypothalamus, impaired gonadotropin secretion, alterations in hypothalamic biogenic amine content, modified aromatase and 5α-reductase activities, loss of sexual dimorphism in brain protein profiles, and demasculinization or feminization of neural and behavioral traits in males.

- Neonatal administration of androgens to female rats results in long-term disturbances of HPA axis function in adulthood, characterized by reduced stress reactivity and altered expression of neurohormones and their receptors. These effects likely reflect both the direct action of androgens on the developing brain and a concomitant decrease in estrogen levels. Calcium signaling also contributes to these alterations: treatment with verapamil partially normalized neurochemical parameters and the stress response, although it failed to restore sexually dimorphic behavioral patterns.

- Prenatal or early postnatal exposure to high doses of estrogens can disrupt sexual differentiation of the brain and impair neuroendocrine regulation, including HPA axis function. Estrogens—particularly synthetic compounds such as diethylstilbestrol—can interfere with the programming of reproductive behavior, overriding endogenous protective mechanisms.

- Prenatal administration of hydrocortisone acetate to pregnant rats disrupts the developmental programming of the HPA axis and the sexual differentiation of the offspring's brain, producing long-lasting, sex-dependent alterations in neurochemical, morphological, and hormonal parameters. In males, these effects manifest as demasculinization of brain structures, reduced stress reactivity, and impaired noradrenergic regulation of the HPA axis. In females, enhanced stress responsiveness and modifications within the GABAergic system are observed. Collectively, these findings underscore the critical role of glucocorticoids in both hormonal and neurotransmitter imprinting of the developing fetal brain.

Chapter 3: Pharmacological Analysis of Sexual Differentiation of the Rat Brain

3.1 Agonists and Antagonists of Neurotransmitters and Neuromodulators

The neonatal androgenization model in female rats has proven to be highly informative for elucidating how androgens reprogram the female brain toward masculinization and defeminization of the neuroendocrine reproductive system and the HPA axis. Equally important is the investigation of the mechanisms underlying normal sexual differentiation of the male brain, which unfolds under the influence of testicular androgens during intrauterine development, as well as the mechanisms operating in intact females. To address these questions, researchers have employed targeted pharmacological agents with well-characterized mechanisms of action.

Agents that Affect the Synthesis, Metabolism and Reception of Catecholamines. We investigated the prenatal effects of methyldopa on sexual behavior in rats (Reznikov & Limareva, 2017). Methyldopa is metabolized to α-methylnorepinephrine, which activates central α_2-adrenergic receptors and suppresses sympathetic outflow. Pregnant rats received methyldopa at a daily dose of 400 mg/kg b.w. on GD 15–21, and sexual behavior was assessed in their offspring at three months of age.

Administration of methyldopa to unstressed pregnant rats did not alter the timing of sexual maturation in either female or male offspring. However, it induced notable disturbances in sexual behavior. In males, during the first assessment of sexual behavior, the latencies to the first mounting and first intromission more than doubled. Despite this delay, copulatory activity increased: the number of intromissions rose by approximately 1.5-fold. During the second behavioral test, these differences were no longer evident—likely due to the acquisition of sexual experience—and the number of mountings without intromissions even decreased (Table 3.1).

Table 3.1 Parameters of male sexual behavior of three-month-old male rats following prenatal exposure to methyldopa (M ± SEM)

Parameter	Control	Methyldopa
	First testing	
Latency period (s):		
First mounting	37.2 ± 4.9	91.0 ± 18.7 [a]
First intromission	40.4 ± 5.3	93.0 ± 17.6 [a]
Number of:		
Mountings without intromissions	5.8 ± 0.9	9.0 ± 1.4
Intromissions	17.8 ± 3.9	28.2 ± 3.3 [a]
	Second testing	
Latency period (s):		
First mounting	11.2 ± 2.4 [b]	15.8 ± 2.1 [b]
First intromission	15.2 ± 2.6 [b]	16.8 ± 2.1 [b]
Number of:		
Mountings without intromissions	13.2 ± 1.9 [b]	5.0 ± 1.3 [ab]
Intromissions	22.6 ± 1.3	28.2 ± 3.2

Footnotes: [a] $p < 0.05$ compared with the control group; [b] $p < 0.05$ compared with the same group of animals in the first test. Each group contained five rats.

Testing of female-type sexual behavior in control males after orchidectomy followed by administration of estradiol benzoate and progesterone yielded negative results for all assessed parameters. In contrast, all experimental males exhibited lordosis responses during the 10-minute test when approached by a normal male. The mean number of lordosis responses was 6.8, and the lordosis quotient—defined as the percentage of lordosis responses relative to the number of approaches by a normal male—was 119.0. In addition, some of the males mounted the stimulus males (mean of 3.0 mountings), a behavior typically interpreted as homosexual. Thus, these animals expressed a bisexual pattern of sexual behavior.

In adult females, female-typical sexual behavior did not differ from controls. However, male-typical behavior emerged consistently, as all females exhibited mounting behavior toward receptive females (mean of 9.6 mountings), whereas such behavior was absent in control females.

Taken together, the administration of methyldopa to pregnant rats during the critical period of fetal sexual differentiation of the brain produced adverse effects on sexual behavior in both sexes, indicating the involvement of catecholaminergic mechanisms in the regulation of brain sexual differentiation.

Serotonin Reuptake Inhibitors. Because serotonin contributes to sexual differentiation of the brain and influences the development of the HPG axis during early ontogenesis, inhibitors of neuronal serotonin reuptake, including fluoxetine, interfere with these developmental processes. Experimental studies in rats have demonstrated that fluoxetine exposure during the perinatal period leads to alterations in sexual behavior in both males and females (Rayen et al., 2014). Administration of fluoxetine to female pups from PND 1 resulted, in adulthood, in enhanced motivational and receptive female sexual behavior during interaction with normal males, an increased lordosis quotient, and reduced avoidance behavior.

Clinical observations likewise indicate disturbances in psychological functions in children following prenatal pharmacological inhibition of serotonin reuptake (Hermansen & Melinder, 2015).

GABA Agonists and Antagonists. One of the most widely used γ-aminobutyric acid (GABA) agonists is phenibut, a β-phenyl derivative of GABA and an analogue of phenylethylamine. Phenibut exerts anxiolytic, tranquilizing, sedative, and antistress effects through stimulation of GABAergic receptors. This compound has been employed to examine the involvement of GABA receptors in the sexual differentiation of the rat brain (Reznikov & Lymareva, 2017).

Phenibut was administered intragastrically to pregnant rats at a daily dose of 100 mg/kg b.w. on GD 15–21. Sexual development of the offspring was monitored, and sexual behavior was assessed at three months of age. Phenibut accelerated testicular descent in males by an average of five days, occurring at 33.8 ± 0.1 days compared with 38.8 ± 0.3 days in controls ($p < 0.001$). In females, premature puberty was likewise observed: the age at vaginal opening decreased by an average of 4.5 days relative with the control groups (39.3 ± 1.0 *vs.* 43.8 ± 0.2 days, $p < 0.001$).

Prenatal exposure to phenibut had minimal effects on male copulatory behavior during the first test with receptive females. However, the number of mountings was reduced compared with intact controls, indicating impaired male sexual performance (Table 3.2).

Table 3.2 Parameters of male sexual behavior in three-month-old male rats following prenatal exposure to phenibut (M ± SEM)

Parameter	Control	Phenibut
	First testing	
Latency period (s):		
First mounting	39.4 ± 1.0	50.8 ± 12.3
First intromission	47.4 ± 11.4	54.8 ± 12.7
Number of:		
Mountings without intromissions	14.8 ± 2.5	15.4 ± 1.4
Intromissions	40.8 ± 2.4	27.8 ± 1.2 [a]
	Second testing	
Latency period (s):		
First mounting	4.0±1.1 [b]	7.0 ± 1.4 [b]
First intromission	4.8±1.2 [b]	8.0 ± 1.4 [b]
Number of:		
Mountings without intromissions	11.0±3.2	5.8 ± 1.1 [b]
Intromissions	43.0±5.9	31.2 ± 2.8 [a]

Footnotes: [a] $p < 0.05$ compared with the control group, [b] $p < 0.05$ compared with the same group of animals in the first test. Each group contained five rats.

Testing of female-type sexual behavior in prenatally phenibut-exposed males revealed a feminizing effect. The mean number of lordosis responses was 9.2 during the 10-minute test, and the lordosis quotient was 100.0.

In adult experimental females, indices of female-typical behavior did not differ from controls. However, male-typical behavior was consistently observed and was characterized by the presence of mountings directed toward receptive females (mean of 8.6 mountings), representing quasi-copulatory homosexual behavior, which was absent in control animals.

It is noteworthy that the $GABA_B$ receptor antagonist CGP 55845, administered to mice during embryonic days 7–11, was likewise capable of disrupting the development of hypothalamic neuroendocrine structures and programming abnormal behavior. The consequences of such interventions may include anxiety–depression-like disorders (Stratton et al., 2014).

3.2 Calcium Ion Channel Blockers

Intracellular calcium ions play a critical role in brain morphogenesis and functional maturation during early ontogenesis. Normal neurogenesis involves not only the formation of new neurons but also their programmed cell death. Accordingly, calcium is often described as both the “sculptor” and the “destroyer” of neuronal networks (Mattson, 1992).

One of the mechanisms implicated in the programming of neuroendocrine functions—and in their disruption—may involve the regulation of neurogenesis (including migration, proliferation, and apoptosis) by calcium ions. Calcium is recognized as one of the most essential ions required for interneuronal signaling, synaptogenesis, exocytosis, synaptic transmission, synaptic plasticity, and the processes of apoptosis and necrosis. Intracellular calcium also contributes to the regulation of neuronal development and migration during the neonatal period (Mattson, 1992). Evidence further suggests a role for calcium ions in apoptosis during the differentiation of sexually dimorphic nuclei of the hypothalamus (Davis et al., 1996). According to these authors, the increase in the volume of the sexually dimorphic nucleus of the medial POA in normal male rats is an androgen-dependent process that may be associated with reduced neuronal apoptosis relative to females.

Particular attention has been directed toward the limited reports addressing the involvement of calcium ions in hormone-dependent processes of perinatal neurogenesis. Notably, sex differences have been identified in the levels of calcium-binding proteins (calmodulin, calbindin, calretinin) within hypothalamic neurons of newborn rats

(Lephart, 1996). The calcium/calmodulin system has also been shown to participate in the hypothalamic regulation of female reproductive function (Rodriguez-Medina et al., 1993). Furthermore, glucocorticoids and androgens have been demonstrated to alter the expression of calcium-binding proteins in the hypothalamus during perinatal development (Lephart et al., 1997a; Rodriguez-Medina et al., 1998).

An important line of evidence supporting the role of calcium ions and intracellular calcium-binding proteins in the sexual differentiation of the brain is the colocalization of steroid aromatase with calbindin-D28k (Lephart et al., 1997b). In addition, maternal stress has been reported to influence the kinetic properties of L-type calcium channels in isolated pyramidal hippocampal neurons of adult offspring exposed to prenatal stress during the final week of gestation (Cai et al., 2007).

Taken together, the pivotal role of calcium signaling in neurogenesis suggests its potential involvement in the programming of neuroendocrine regulation and behavior.

To address this possibility, L-type calcium channel blockers such as nimodipine and verapamil have been employed as pharmacological tools. nimodipine readily crosses the blood–brain barrier (Biessels & Gispen, 1996) and selectively blocks L-type calcium channels in neurons of the central nervous system (Bar et al., 1990). Verapamil also penetrates the blood–brain barrier (Canchola-Martinez et al., 1997; Elsinga et al., 2004), although its actions extend to peripheral tissues. Accordingly, studies were conducted in Wistar rats in which these agents were administered during pre- and early postnatal development (Reznikov et al., 2012; Reznikov & Nosenko, 2013).

Nimodipine. Nimodipine was administered as a suspension prepared from tablet mass at a dose of 20 mg/kg b.w. daily *via* an intragastric feeding tube to pregnant Wistar rats during the final week of gestation. This dose is considered optimal for effective blockade of L-type calcium channels in brain cells (Biessels et al., 2005). In 5–10-day-old offspring, analyses included the profile of soluble cytosolic proteins and testosterone metabolism in hypothalamic structures involved in the programming of gonadotropin secretion and the regulation of sexual behavior, specifically the POA and MBH. In sexually mature rats, assessments encompassed sexual behavior, reproductive system function, and HPA axis responses to acute stress or intracerebroventricular administration of norepinephrine bitartrate.

On PND 10, experimental animals exhibited significant alterations in steroid 5α-reductase activity, specifically a pronounced increase in activity in both brain regions examined in females (by 50–100%) and in the POA of males (by 150%) (Table 3.3).

Table 3.3 Aromatase and 5α-reductase activities in discrete brain structures of 10-day-old rats following perinatal exposure to calcium channel blockers (M ± SEM; n = 5)

Animal group	Aromatase: pmol E_2/g tissue/h		5α-reductase: pmol 5α-reduced metabolites/g tissue/h	
	POA	MBH	POA	MBH
Control (males)	0.312 ± 0.079	0.081 ± 0.013	2.61 ± 0.67	5.28 ± 1.47
Nimodipine (males)	0.184 ± 0,095	0.127 ± 0,047	6.59 ± 1,58 [a]	7.34 ± 3,23
Control (females)	0.099 ± 0.017 [b]	0.057 ± 0.016	8.79 ± 0.88 [b]	7.88 ± 2.12
Nimodipine (females)	0.066 ± 0.008	0.105 ± 0.022	1.87 ± .,90 [ab]	15.60 ± 2.41[a]
Control (males)	0.241 ± 0.048	0.141 ± 0.058	5.99 ± 1.65	3.74 ± 0.71
Verapamil (males)	0.224 ± 0.036	0.124 ± 0.025	6.74 ± 1.09	3.24 ± 0.41
Control (females)	0.098 ± 0.010 [b]	0.096 ± 0.020	4.87 ± 0.94	5.06 ± 1.54
Verapamil (females)	0.128 ± 0.01 [ab]	0.186 ± 0.030[a]	4.46 ± 1.32	7.86 ± 0.70 [b]

Footnotes: [a] $p < 0.05$ compared with control; [b] $p < 0.05$ 05 compared with males of the same group; n - number of analyses in each group, each analysis was based on two-three animals. Prenatal exposure to nimodipine also produced a slight decrease in aromatase activity in the POA and an increase in the MBH, which represented only trends in both males and females ($0.05 < p < 0.1$).

Changes were also observed in the distribution of proteins within discrete brain structures (Fig. 3.1). Electrophoretic analysis in 5-day-old rats confirmed our earlier

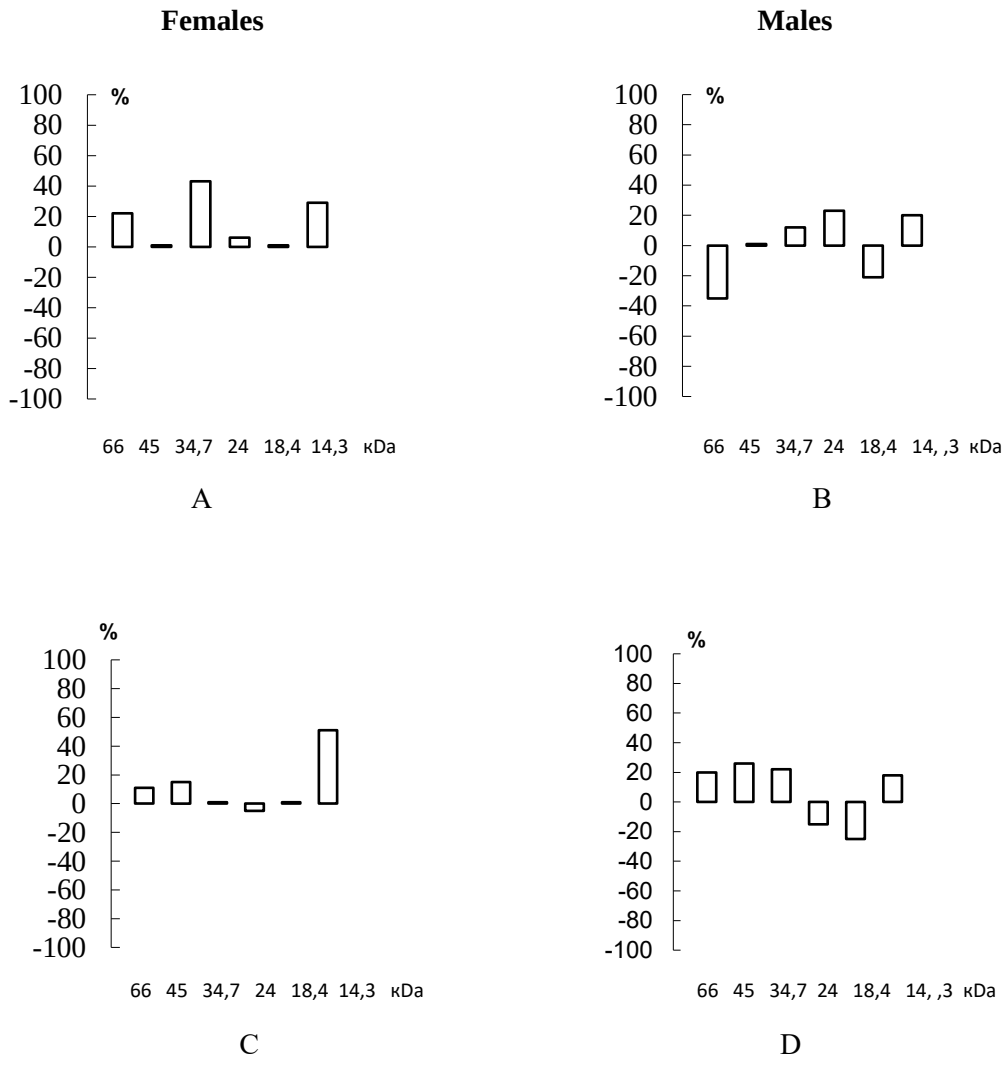

Fig. 3.1 Effect of prenatal nimodipine administration on the distribution of soluble proteins in the POA and MBH of 5-day-old rats

Footnotes: Mean values from five analyses per group are presented. Y-axis: percentage of the total area under the protein distribution curve in experimental females relative to intact females (A, C) and in experimental males relative to intact males (B, D); X-axis: m.m. (kDa). Upper panel – POA, bottom panel – MBH

findings (Reznikov et al., 2002) regarding sex-dependent spectra of soluble cytosolic proteins in the POA of intact animals at this age.

Prenatal exposure to nimodipine in females resulted in an increased relative abundance of proteins with m.m. of 66.0 kDa and 34.7 kDa (by 22% and 43%, respectively; $p <$

0.05) compared with intact females, bringing these values closer to those observed in intact males. Concurrently, optical density values associated with proteins of 24.0 kDa and 14.3 kDa also increased (by 6% and 29%, respectively; $p < 0.05$). In experimental males of the same group, the POA exhibited a significant decrease in proteins of 66.0 kDa and 18.4 kDa (by 35% and 21%, respectively) and an increase in proteins of 34.7 kDa, 24.0 kDa, and 14.3 kDa (by 12%, 23%, and 20%, respectively; $p < 0.05$) relative to intact males.

Although intact rats demonstrated no sex differences in protein distribution in the MBH, prenatal nimodipine produced similar alterations in both sexes. Specifically, there was a significant increase in the relative content of proteins with m.m. of 66.0 kDa, 45.0 kDa, and 14.3 kDa, and a decrease in 24.0 kDa proteins compared with respective controls. Additionally, experimental males showed an increase in optical density of the 34.7 kDa protein band and a decrease in the 18.4 kDa protein band relative to control males ($p < 0.05$)

In the hippocampus of males prenatally exposed to nimodipine, a significant increase in the optical density of protein bands with m.m. of 45.0 kDa, 34.7 kDa, and 14.3 kDa (by 23%, 12%, and 33%, respectively) was observed compared with intact animals. Conversely, the optical density of protein bands of 24.0 kDa and 18.4 kDa decreased (by 21% and 28%, respectively). The optical density of the 66.0 kDa protein band remained unchanged.

These findings indicate a pronounced modifying effect of nimodipine on the distribution of a broad spectrum of proteins within brain regions involved in the regulation of reproductive function and the neuroendocrine stress response.

Nimodipine also induced a marked delay in female puberty. Vaginal opening, which normally precedes the first estrus and ovulation by approximately one day, occurred on average 10 days later than in intact animals: on PND 62.3 ± 2.8 in the nimodipine group *vs.* 52.1 ± 0.9 in controls ($p < 0.01$). By three months of age, all control females exhibited regular estrous cycles, whereas 20% of experimental females showed irregular cycling. By six months of age, this proportion decreased to 6.7%.

Given the absence of data on the potential impact of prenatal nimodipine exposure on the programming of sexual behavior in offspring, an investigation was undertaken in sexually mature three-month-old Wistar rats.

Prenatal nimodipine exposure did not alter indices of female-typical sexual behavior in ovariectomized females with hormonally induced estrus when tested in contact with normal active males. However, the test revealed clear manifestations of male-typical

sexual behavior in these females. In contrast to hormonally primed ovariectomized controls, all females in the nimodipine group mounted receptive estrous females (7.0 ± 1.5 mountings per 10-min test, with some individuals performing up to 10 mountings; Fig. 3.2).

Moreover, these females exhibited markedly enhanced sexually motivational behavior: the number of approaches toward the receptive female increased significantly compared with controls (14.0 ± 1.5 *vs.* 5.8 ± 1.1, $p < 0.01$).

Thus, prenatal nimodipine administration induces masculinization of female sexual behavior without accompanying defeminization. This effect can be attributed to alterations in 5α-reductase activity within neuroendocrine brain structures during the critical period of sexual differentiation, particularly in the MBH, which in female rodents serves as a key regulatory center for sexual behavior.

Male sexual behavior of prenatally nimodipine-exposed males was evaluated during a 30-min session in the presence of an ovariectomized, hormonally primed estrous female. Latency to the first mounting, intromission, and ejaculation, as well as the number of mountings, intromissions, and ejaculations, were recorded. By all qualitative and quantitative indices, nimodipine did not affect male sexual behavior.

Female-typical sexual behavior was assessed in castrated males following estrogen–progesterone treatment during a 10-min test or until ten mountings were received from an active male.

Marked abnormalities were observed in the experimental group. Whereas none of the control males exhibited lordosis in response to mounting, four of five nimodipine-exposed males displayed typical female sexual behavior; the overall group lordosis quotient was 37.0 ± 14.6. Furthermore, four experimental males demonstrated homosexual behavior, and three of five exhibited bisexual behavioral patterns.

Collectively, the altered timing of female puberty onset and the abnormal sexual behavior in both sexes indicate the involvement of intracellular calcium ions in the programming of the developing fetal brain.

The effect of prenatal nimodipine exposure on the functional state of the HPA axis and its sex-specific features was also studied in 6-month-old animals. Plasma corticosterone levels were measured under basal conditions and following one-hour of immobilization stress.

In males from the nimodipine group, basal corticosterone levels were significantly reduced, by approximately 24% relative to controls. The stress-induced adrenocortical

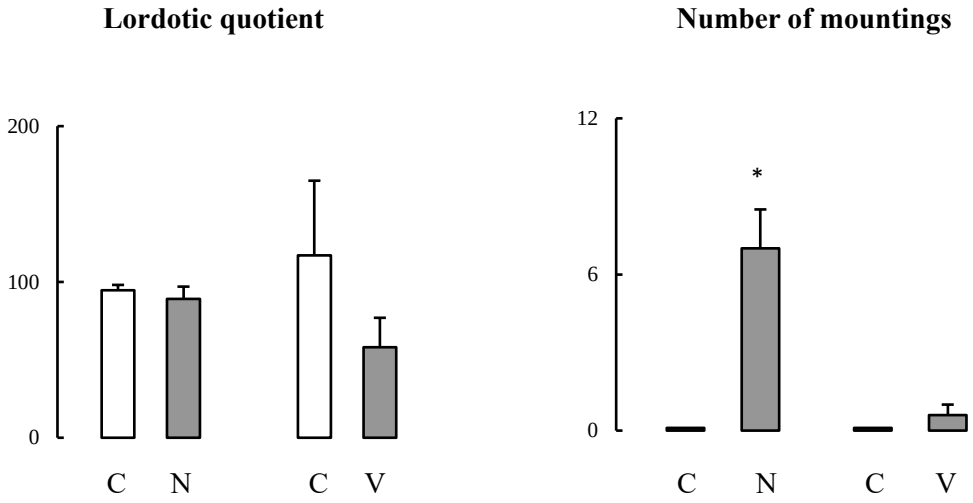

Figure 3.2. Prenatal effects of nimodipine (N) and verapamil (V) on sexual behavior in adult female rats (M ± SEM)
Footnotes: * $p < 0.05$ 05 compared with control (C); each group contained five rats.

Table 3.4 Effect of prenatal nimodipine administration on plasma corticosterone levels (nmol/L) in 6-month-old male and female rats under basal conditions and following one-hour immobilization (acute stress) (M ± SEM)

Animal group	Baseline level	Acute stress
	Males	
Control	567 ± 14	770 ± 36 [b]
Nimodipine	456 ± 41[a]	822 ± 36 [b]
	Females	
Control	607 ± 62	928 ± 42 [b]
Nimodipine	747 ± 26	1063 ± 23 [ab]

Footnotes: [a] $p < 0.05$ compared with control; [b] $p < 0.05$ compared with basal level. Each group contained five animals.

response in these animals was moderately enhanced: plasma corticosterone concentrations increased by 80% following acute immobilization stress compared with a 36% increase in controls. In contrast, females prenatally exposed to Nimodipine did not exhibit significant alterations in basal corticosterone levels; however, their corticosterone response to immobilization stress was markedly amplified (Table 3.4).

Thus, Nimodipine administration during pregnancy exerts a modulatory influence on the development of HPA axis stress reactivity in adult rats. These alterations may result from antenatal disturbances in calcium homeostasis within brain structures—particularly the hippocampus—which plays a central role in regulating HPA axis activity. Moreover, given the observed reduction in basal corticosterone secretion following prenatal Nimodipine exposure, the possibility cannot be excluded that Nimodipine interacts with

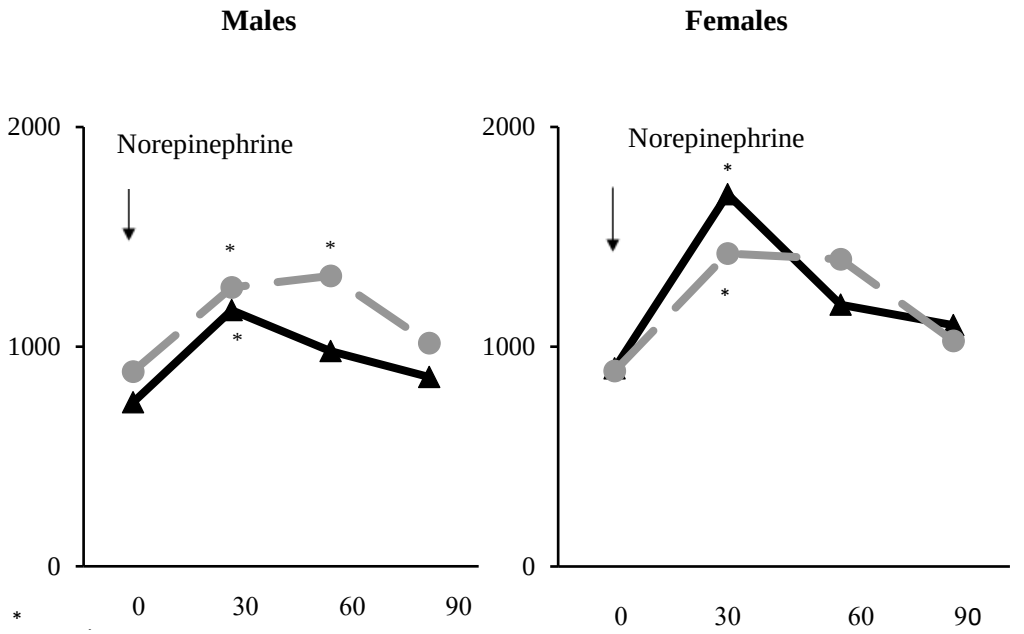

Figure 3.3 Effect of nimodipine on noradrenergic reactivity of the HPA axis in 8-month-old rats

Footnotes: Solid line – control, dashed line – nimodipine. Y-axis: plasma corticosterone concentration (nmol/L) following administration of norepinephrine bitartrate (10 μg/2 μl of 0.9% pyrogen-free NaCl solution) into the third ventricle of the brain; X-axis: min. Arrow indicates start of norepinephrine bitartrate administration. * $p < 0.05$ compared with baseline level.

hippocampal mineralocorticoid receptors (Dietz et al., 2008), known to participate in the regulation of tonic glucocorticoid secretion (Dallman et al., 1994; Matthews, 2001)

The prenatal effect of nimodipine on the HPA axis response to central noradrenergic stimulation was also examined (Fig. 3.3). Control males and females responded to

administration of norepinephrine bitartrate into the third ventricle of the brain with a significant rise in plasma corticosterone at 30 min, followed by a return to baseline by 60 min. The HPA axis of male rats treated prenatally with nimodipine, responded to administration of norepinephrine bitartrate with protracted rise of corticosterone level, meanwhile females did not.

Verapamil. Verapamil blocks the transmembrane flow of calcium ions primarily in cardiomyocytes and smooth muscle cells of peripheral blood vessels. However, it has been demonstrated that verapamil is capable of crossing the blood–brain barrier and inhibiting calcium channels in neurons of the central nervous system (Canchola-Martinez et al., 1997; Elsinga et al., 2004). These properties justified its use in the present study.

Verapamil was administered subcutaneously to newborn rats in an isotonic NaCl solution at a daily dose of 0.5 mg/kg b.w. on PND 3–5. Control animals received the vehicle only.

At five days of age, the spectrum of soluble cytosolic proteins was analyzed, and on PND 10, sex-specific characteristics of testosterone metabolism were examined in discrete hypothalamic structures. In rats aged 3, 6, or 8 months, assessments included sexual behavior, functional status of the reproductive system, and the HPA axis response to acute stress or to intracerebroventricular administration of norepinephrine bitartrate.

In adult females, stages of the estrous cycle were determined by microscopic examination of vaginal smears for two weeks prior to decapitation. Both control and experimental females were included in the experiment during estrus. For evaluation of sexual behavior, females in both groups were ovariectomized one week before testing and treated with ovarian hormones as described above.

In control rats at 5 days of age, sex-specific differences in the electrophoretic spectrum of soluble brain proteins were detected in the POA. Following verapamil administration, females exhibited a substantial increase in the relative content of proteins with m.m. of 66.0 kDa, 45.0 kDa, 34.7 kDa, and 24.0 kDa (by 86%, 66%, 72%, and 64%, respectively) compared with controls ($p < 0.01$). In the MBH, the relative content of all analyzed protein fractions increased, except for the 34.7 kDa protein, which showed a decrease (Fig. 3.4).

Administration of verapamil to male rats during the neonatal period produced a modest increase in the relative content of proteins with m.m. of 66.0 kDa and 45.0 kDa in the

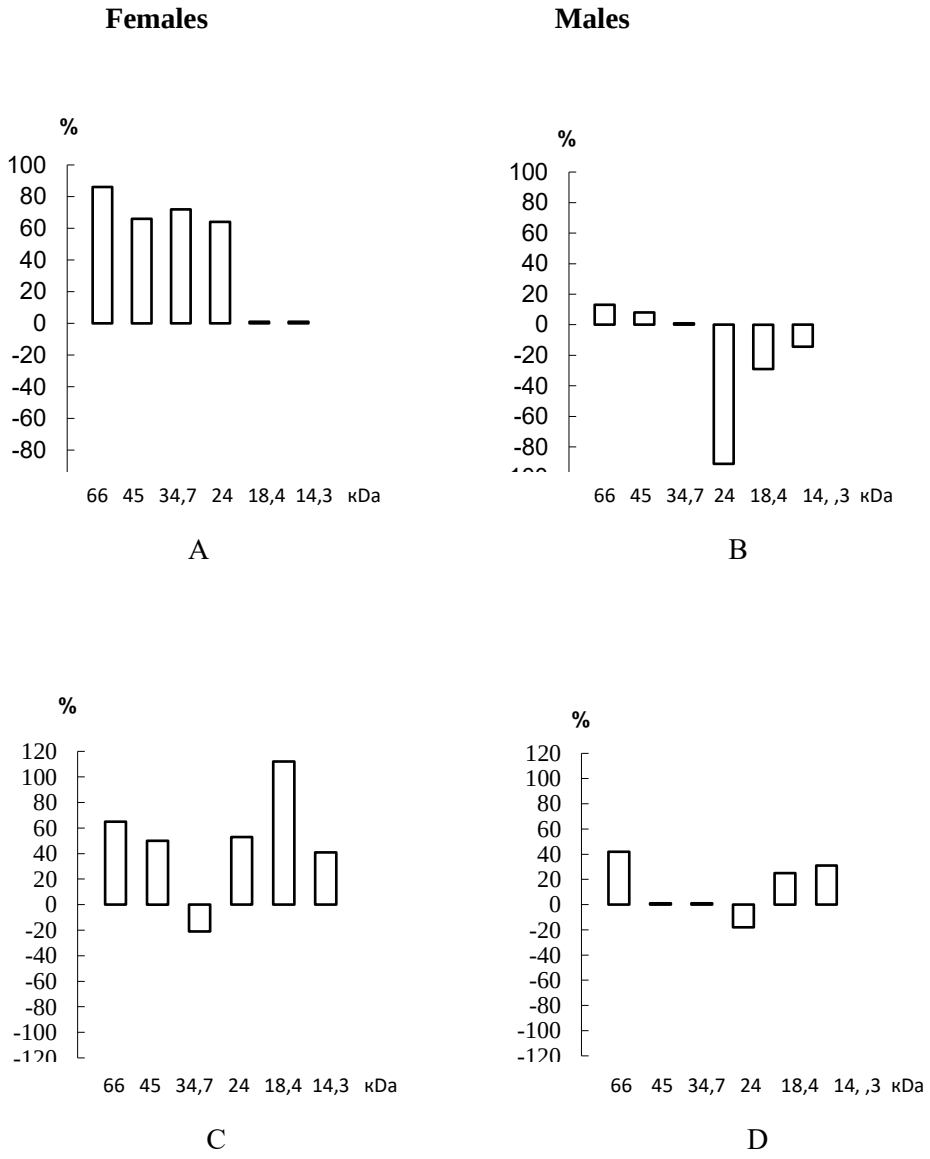

Fig. 3.4 Effect of prenatal verapamil administration on the distribution of soluble proteins in the POA and MBH of 5-day-old rats

Footnotes: Mean values from five analyses per group are presented. Y-axis: percentage of the total area under the protein distribution curve in experimental females relative to intact females (A, C) and in experimental males relative to intact males (B, D); X-axis: m.m. (kDa). Upper panel – POA, bottom panel – MBH.

POA, along with a decrease in proteins of 24.0 kDa, 18.4 kDa, and 14.3 kDa (by 90%, 29%, and 11%, respectively) compared with control males ($p < 0.01$). In the MBH, verapamil administration resulted in increased relative optical density of the 66.0 kDa,

18.4 kDa, and 14.3 kDa protein bands (by 42%, 25%, and 31%, respectively), and an 18% decrease in the 24.0 kDa protein compared with controls ($p < 0.01$).

In the hippocampus of newborn females exposed to verapamil, a 26.5% increase in the content of the 34.7 kDa protein was observed relative to control females ($p < 0.01$). The optical density of proteins with m.m. of 66.0 kDa and 45.0 kDa significantly decreased, whereas proteins of 24.0 kDa, 18.4 kDa, and 14.3 kDa remained unchanged. In the hippocampus of verapamil-exposed males, increases in the 24.0 kDa and 18.4 kDa proteins and a decrease in the 45.0 kDa protein were detected compared with controls ($p < 0.05$).

On PND 10, verapamil significantly increased aromatase activity in the MBH of females, without affecting metabolic parameters in the POA or the activity of aromatase and steroid 5α-reductase in either hypothalamic structure in males (Table 3.3). Verapamil did not significantly influence the timing of sexual maturation in females; however, upon reaching sexual maturity it induced disturbances in estrous cyclicity, manifested as irregular cycles and altered sexual behavior. In particular, a twofold reduction in the lordosis quotient during interactions between hormone-primed females and normal males indicated suppression of female-typical sexual behavior. The proportion of females exhibiting sexual motivational and receptive behaviors was likewise reduced. By contrast, evaluation of male-typical behavior in females revealed no significant effects of verapamil, except for the appearance of several females displaying male-like pseudocopulatory behavior toward another female.

Thus, both nimodipine and verapamil exerted a modifying influence on the distribution of a broad range of proteins and on testosterone metabolism in the POA during the early postnatal period, indicating disruption of sexual differentiation of the brain (defeminization in females).

Noteworthy are the changes in aromatase activity induced by neonatal exposure to calcium channel blockers, particularly within the MBH, which contains the key regulatory center of female-typical sexual behavior in rats. Such alterations are likely manifestations of a direct pharmacological impact on the calcium/calmodulin signal transduction system in the hypothalamus during early postnatal development. In adulthood, these early disturbances are expressed, in part, as impairments of female-typical sexual behavior (Rodriguez-Medina et al., 1993; Canchola et al., 1997).

Neonatal administration of verapamil did not alter the HPA axis response to a one-hour immobilization stress in 8-month-old females tested during estrus. However, these females exhibited a prolonged adrenocortical response following injection of norepinephrine bitartrate into the third ventricle of the brain.

In 6-month-old males treated neonatally with verapamil, the hormonal response to acute immobilization stress was markedly enhanced compared with controls: plasma corticosterone levels increased 2.6-fold, whereas in control males the increase was 1.7-fold. Administration of dexamethasone (0.1 mg/kg b.w. intramuscularly, 30 min before immobilization) to evaluate the integrity of HPA axis negative feedback attenuated the corticosterone response in both groups; however, the reduction in plasma corticosterone was smaller in verapamil-treated males than in controls (18% *vs.* 25%, respectively) (Table 3.5).

Table 3.5 Effect of neonatal verapamil administration on plasma corticosterone levels (nmol/L) in 6-month-old male rats in the physiological resting stage, following one-hour immobilization (acute stress) and pre-stress dexamethasone administration (M ± SEM)

Sr. No.	Animal group	Control	Verapamil	*p*
1	Basal level	387.5 ± 27.1	270.8 ± 46.1	< 0.1
2	Acute stress	672.4 ± 25.1	700.6 ± 16.2	> 0.05
	$p_{1,2}$	< 0.001	< 0.001	
3	Acute stress + dexamethasone	502.9 ± 21.6	574.21 ± 34.8	> 0.05
	$p_{1,3}$	< 0.001	< 0.001	
	$p_{2,3}$	< 0.001	< 0.02	

Footnote: Each group contained five rats.

The HPA axis response to intracerebroventricular administration of norepinephrine bitartrate into the third ventricle of the brain was examined in 8-month-old male rats. The relatively high basal plasma corticosterone levels observed in control operated males are noteworthy. This is most likely attributable to a state of chronic stress resulting from the previous neurosurgical intervention. In verapamil-treated males, the response to central noradrenergic stimulation was both enhanced and prolonged in duration (Table 3.6).

Thus, neonatal administration of the calcium channel blocker verapamil to male rats

Table 3.6 Effect of administration of norepinephrine bitartrate into the third ventricle of the brain on plasma corticosterone levels in 8-month-old male rats given verapamil in the neonatal period (M ± SEM)

Animal group	Time of blood sampling			
	0 min	30 min	60 min	90min
Control	966 ± 71	1532 ± 85 *	1174 ± 107	931 ± 58
Verapamil	1539 ± 86	2355 ± 49 *	1989 ± 54 *	1608 ± 67

Footnotes: * $p < 0.05$ compared with the basal level; each group contained five rats.

exerts a distinct modulatory influence on the development of stress-related and noradrenergic reactivity of the HPA axis, shifting it toward enhanced responsiveness in adulthood. These findings highlight the critical role of calcium ions in the early programming of HPA axis function.

3.3 Prostaglandin Biosynthesis Inhibitor

Ibuprofen, a prostaglandin biosynthesis inhibitor, was suspended in Dorfman's gel and administered to pregnant rats intragastrically *via* a feeding tube at a dose of 30 mg/kg b.w. twice daily on GD 15–21. Control females received an equivalent volume of Dorfman's gel. Male offspring from experimental and control groups were evaluated at 5 months of age for plasma testosterone concentrations and for the HPA axis response to one-hour restraint stress. At 8 months of age, males were tested for male-typical sexual behavior, and at 8.5 months for female-typical sexual behavior.

Prenatal ibuprofen exposure resulted in incomplete masculinization of copulatory behavior in adult offspring. Significant prolongation of the latencies to the first mounting and first intromission, as well as a reduction in the number of intromissions during interaction with receptive females, was observed. In the initial test, the latency to the first mounting increased approximately fourfold, and the latency to the first intromission increased more than threefold; meanwhile, the number of mountings with intromission was reduced by half. None of the ibuprofen-exposed males achieved ejaculation. One week later, impairments became even more pronounced: the latency to the first mounting increased by a factor of 23, the latency to the first intromission by 14, and the number of mountings with intromission decreased 6.5-fold (Table 3.7). Taken together, these findings indicate marked disruption of both central and peripheral components of male sexual behavior.

Table 3.7 Effect of prenatal ibuprofen exposure on male-type sexual behavior in male rats of 8-month age

Parameter	Control	Ibuprofen
	First testing	
Latency period (s):		
First mounting	64.0 (9-201)	184.0 (114-590) [a]
First intromission	73.0 (11-390)	324.0 (243-695) [a]
Number of:		
Mountings without intromission	4.0 (2-6)	7.0 (3-8)
Mountings with intromission	6.0 (5-16)	2.0 (2-7) [a]
Ejaculations	0	0
	Second testing	
Latency period (s):		
First mounting	7.0 (0-21)	60.0 (24-577) [a]
First intromission	19.0 (2-57) [b]	303.0 (73-601) [a]
First ejaculation	543.0 (193-717) [b]	-
Number of:		
Mountings without intromission	4.0 (3-11)	5.0 (2-7)
Mountings with intromission	15.0 (14-20) [b]	2.0 (1-5) [a]
Ejaculations	1.0 (1-1) [b]	0 [a]

Footnotes: The test lasted 15 min. The data are presented as medians with minimum and maximum values in parentheses. Each group contained five rats. Statistical analysis by Wilcoxon-Mann-Whitney *U* test; [a] $p \leq 0.05$ compared with the control groups; [b] $p \leq 0.05$ compared with the first test.

To assess female-type sexual behavior, male offspring prenatally exposed to ibuprofen were orchiectomized one week prior to testing and treated with steroid hormones as described above for preparing receptive females. The males were then introduced to sexually experienced males. Testing continued for 10 minutes or until the active male achieved 10 mountings. The number of lordosis responses to approaches and mountings by the stimulus male was recorded. However, none of the ibuprofen-exposed males exhibited lordosis, in contrast to the findings of Balin et al. (2020).

Under basal conditions, testosterone, estradiol, and corticosterone levels in blood plasma of 5-month-old males did not differ between experimental and control groups. Likewise, no statistical differences were observed in the HPA axis response to one-hour immobilization stress. Ibuprofen exposure also did not affect sperm content in the epididymis.

There are at least two plausible mechanisms underlying the incomplete masculinization of the male fetal brain following prenatal ibuprofen exposure. One involves reduced testosterone production by the fetal testes, resulting from ibuprofen-induced inhibition of steroidogenic enzymes (Ben Maamar et al., 2017; Kristensen et al., 2018). The second mechanism relates to ibuprofen-induced suppression of prostaglandin synthesis. Sexual differentiation of the embryonic brain in male songbirds—but not quail—has been shown to be disrupted by cyclooxygenase inhibition (Delage et al., 2021). Furthermore, prostaglandin E_2, which is induced by estradiol in neonatal POA microglia, acts cooperatively with estrogen to masculinize neuroanatomical architecture in the male brain (Lenz et al., 2018; Amateau et al., 2004). Therefore, inhibition of prostaglandin E_2 synthesis by ibuprofen likely contributes to incomplete sexual differentiation of the male fetal brain. It is probable that both mechanisms operate in parallel.

Considering that prenatal ibuprofen did not alter either basal corticosterone levels or corticosterone responses to acute stress in adult males, we suggest that this drug exerts a selective influence on the neuroendocrine reproductive system.

Conclusions:

- Drugs that modulate catecholaminergic, serotonergic, and GABAergic activity are capable of altering sexual differentiation of the rat brain in both males and females. Prenatal administration of methyldopa, fluoxetine, or phenibut induced marked changes in sexual behavior, including feminization, masculinization, or homosexual responses. These findings underscore the essential role of neurotransmitter systems in the physiological programming of sexual behavior.

- Prenatal or neonatal administration of the calcium channel blockers alters sexual behavior, enzyme activity, brain protein composition, and HPA axis stress reactivity in rats. Nimodipine induces masculinization of the female brain and promotes bisexual behavioral patterns in males. Nimodipine and verapamil disrupt normal sexual differentiation of the brain, indicating a pivotal role of calcium signaling in neuroendocrine programming.

- Administration of ibuprofen to female Wistar rats during the final week of gestation partially disrupts the neuroendocrine programming of male-typical copulatory behavior in male offspring, without affecting female-typical sexual behavior or HPA axis function. The incomplete masculinization of the developing fetal brain is hypothesized to result from two cooperative mechanisms: inhibition of testosterone synthesis in the fetal gonads and suppression of prostaglandin E_2 production in the POA of the hypothalamus.

Chapter 4: Prenatal Stress and Its Consequences

4.1. General Concepts of Stress

The concept of stress, originally introduced by the Canadian pathologist Hans Selye, has been progressively elaborated over the past decades by researchers across virtually all medical disciplines and has simultaneously acquired broad biological significance. Today, it stands as one of the foundational constructs of pathophysiology and has retained its relevance over time.

Stress represents a state of tension within physiological systems that emerges in response to diverse external or internal stimuli and is expressed through the general adaptation syndrome. This adaptive response functions to preserve homeostasis and enhance the organism's nonspecific resistance to potentially pathogenic influences. Numerous such factors exist, and various aspects of this subject have been examined in our monograph (Baraboy & Reznikov, 2013), one of many published worldwide.

The doctrine of stress is rooted in the foundational work of Claude Bernard on the principles of homeostasis and the conceptualization of disease as a deviation from stable internal conditions. Modern interpretations of stress are inseparable from the pioneering studies of Walter Cannon, who between 1911 and 1929 formulated the theory describing the role of the sympathetic nervous system and the adrenal medulla in mediating emotional responses to threat. Cannon emphasized that the organism's reaction to threat can be characterized by the classic "fight-or-flight" response, which entails rapid activation of the sympathetic–adrenomedullary system. He defined this reaction as a "nonspecific response of the organism to certain demands upon it," mediated through

autonomic neural pathways and endocrine mechanisms. In this respect, Cannon may be regarded as a precursor to Hans Selye in the development of stress theory.

Between 1948 and 1952, Ulf von Euler demonstrated that norepinephrine is secreted predominantly by sympathetic nerve terminals, whereas epinephrine is produced by the chromaffin cells of the adrenal medulla. Under conditions of psycho-emotional strain, secretion of both catecholamines increases sharply. Today, activation of the sympathetic–adrenomedullary system is regarded as an integral and obligatory component of the stress response.

In experiments on animals subjected to trauma, pain, emotional stimuli, infection, intoxication, hyper- or hypothermia, muscular overload, and other stressors, Selye observed a stereotypical triad of changes: hypertrophy of the adrenal cortex, involution of thymic and lymphoid tissues, and the occurrence of hemorrhages and peptic ulcers in the stomach.

Research conducted by followers of the stress theory has enriched it with a multitude of experimental and clinical findings concerning alterations at the molecular level (e.g., *c-fos* and other "immediate" genes, heat shock proteins, or chaperones, interleukins), at the cellular level (mitochondrial stress, endoplasmic reticulum stress), at the biochemical level (oxidative stress), and at the systemic level (immune, nervous, endocrine, and other systems).

Concepts regarding the nature and mechanisms of stress have evolved significantly over time, both through the broadening of the definition and the deepening of mechanistic understanding down to the cellular, subcellular, and molecular levels. At the early stages of stress theory development, stress was primarily regarded as part of the pathophysiological scenario of catastrophic events (fractures, hemorrhage, burns, explosions, etc.). Initially, Selye underestimated the role of psycho-emotional and social factors in the genesis of stress (a point he himself acknowledged in 1983). Thanks to the works of Lazarus (1957–1966), the crucial—indeed, paramount for humans—role of psychological, emotional, and psychosocial factors (conflicts, bereavement, divorce, etc.) as causes and concomitants of stress became evident.

It has gradually become evident that stress is not limited to humans and higher animals, but represents a truly universal syndrome of adaptation. Fish, insects, plants, and even unicellular organisms encounter a wide range of extreme environmental conditions throughout their life cycle and actively resist them through both inherited and acquired stereotypical responses. In terms of the biological essence of its functional role in vital activity, stress is similar across all levels of living organization, although the underlying

protective mechanisms, driving forces, and components of the stress response may differ considerably, though not fundamentally.

In recent decades, the genetic and evolutionary aspects of stress, as well as its role in both species and individual selection, have been successfully investigated. The biological basis of this phenomenon is thought to include, in particular, the imprinting effect of corticosteroids on inducible metabolic enzymes, gene expression (owing to the ability of steroids to activate or repress genes), the development of the neuroendocrine system, and the establishment of specific behavioral patterns, as exemplified by prenatal (maternal) stress.

It should be noted that repeated stressors of moderate intensity induce a state of eustress, which "trains" the organism's defenses and maintains it in an active state. Distress, by contrast, emerges as a response to excessively strong stimuli (infectious, psycho-emotional, physical, etc.). Its course exhibits certain staged patterns, with outcomes ranging from recovery or progression to chronic conditions, and in cases of irreversible changes, death.

The deterioration of ecological conditions, social upheavals, wars, technogenic disasters, fear of unemployment, physical violence, and family conflicts can all serve as potent causes of distress.

One of the negative consequences of stress is the limitation of reproductive potential, a phenomenon with an evident biological rationale. In adverse times, food becomes scarce, pregnancy maintenance and offspring care are at risk. In males, distress reduces pituitary gonadotropin and testosterone secretion, impairs the fertilizing capacity of seminal fluid, and induces pathological alterations in spermatozoa. In females, distress manifests as disturbances of cyclic processes within the hypothalamic–pituitary–ovarian axis, inhibition or complete blockade of ovulation, and menstrual irregularities.

The key role in these disturbances belongs to the neuroendocrine system of reproduction, primarily the hypothalamus and the structures of the amygdaloid complex. As demonstrated in animal experiments (mainly in rodents), norepinephrine, neuropeptide Y, and serotonin—originating from brainstem structures—along with glutamate in the medial preoptic area and neuropeptide Y in the arcuate nuclei of the hypothalamus, excite the neurosecretory cells of female rats that synthesize GnRH, thereby stimulating the secretion of LH. In male rats, in contrast to females, intracerebroventricular administration of neuropeptide Y aimed at hypothalamic structures inhibits LH secretion (Reznikov & McCann, 1993).

Conversely, the secretion of GnRH and, consequently, gonadotropins in both sexes is inhibited by GABA in the medial preoptic area and by opioids in the arcuate nuclei. As already mentioned, in rodents the medial preoptic area of the hypothalamus is identified as the neuroendocrine center regulating ovulation in females and male sexual behavior in males. The arcuate nuclei, belonging to the MBH, are involved in regulating female sexual behavior in females and tonic gonadotropin secretion *via* the feedback loop between the gonads and the hypothalamic–pituitary complex. In humans and primates, however, the regulation of sexual cyclicity is likely carried out within the MBH.

According to numerous experimental studies, stress disrupts the normal balance of neurotransmitters and regulatory neuropeptides. In particular, neuropeptide Y-ergic neurons of the arcuate nuclei are activated, and norepinephrine release from the synaptic terminals of brainstem neurons, whose axons project to hypothalamic structures, increases. These include the paraventricular nuclei, where CRH is synthesized, and the supraoptic nuclei, the site of vasopressin synthesis. Axons of these nuclei form synaptic contacts with GnRH-producing neurons in the medial preoptic area. Excitation of CRH neurons activates opioid and GABA secretion in the medial preoptic area. Moreover, CRH and vasopressin stimulate the secretion of pituitary ACTH, which during stress is accompanied by the release of large amount of β-endorphin into the bloodstream, thereby inhibiting gonadotropin secretion. Following intracerebroventricular administration of arginine–vasopressin, a marked reduction in testosterone secretion has been observed against the background of elevated plasma ACTH and corticosterone in male rats (Sinitsin et al., 2007).

Most neuroendocrine structures are involved in the hormonal response to stress, *i.e.*, in alterations of the secretory activity of the adrenal cortex, thyroid gland, pituitary, and gonads. The hippocampus, particularly its corticosteroid receptors, plays an important role in the regulation of the HPA axis during stress. The amygdala and the *bed nucleus* of the *stria terminalis* also participate in these processes, as they, like the paraventricular nuclei of the hypothalamus, contain CRH-producing neurosecretory cells. At the same time, the stress-limiting GABAergic system of the hippocampus becomes activated. As a result of complex interactions among neuropeptides and neurotransmitters, primarily GABA and endorphins, sexual activity and testicular testosterone secretion are reduced in males, while in females the pulsatile secretion of LH is inhibited and its amplitude decreases, the preovulatory surge of pituitary LH and FSH is suppressed, leading to anovulation.

Under resting conditions, the normal level of circulating glucocorticoids is maintained through negative feedback within the functional circuitry of the HPA axis. Glucocorticoid hormones (cortisol in humans, primates, dogs, and guinea pigs; corticosterone in rabbits, mice, and rats) inhibit the secretion of CRH and ACTH,

whereas at low blood levels of these hormones, the release of CRH and ACTH is disinhibited. During stress, the sensitivity threshold of neuroendocrine centers to corticosteroids increases, resulting in elevated circulating levels. A key role in these hormonal interactions is played by corticosteroid receptors and their signaling.

Two types of corticosteroid receptors are distinguished: mineralocorticoid (type I) and glucocorticoid (type II). Type I receptors display high affinity primarily for mineralocorticoids (aldosterone, deoxycorticosterone), lower affinity for cortisol and corticosterone, and the lowest affinity for synthetic glucocorticoids (*e.g.*, dexamethasone). In the central nervous system, they are localized mainly in the hippocampus and are absent in the hypothalamus. Type I receptors are involved in the physiological regulation of basal as well as stress-induced CRH and arginine–vasopressin secretion, and thus ACTH release. The response of the HPA axis to psychogenic and possibly other stressogenic stimuli depends, to some extent, on mineralocorticoid receptor polymorphism.

Type II receptors are more widely distributed in the brain: they are present not only in the hippocampus but also in the hypothalamus, amygdala, and the perikarya of catecholaminergic brainstem neurons. These receptors bind dexamethasone and natural glucocorticoids more readily than mineralocorticoid hormones; however, their affinity for glucocorticoids is tenfold lower than that of mineralocorticoid receptors. It is primarily these receptors that are engaged in the regulation of ACTH and corticosteroid secretion during stress. Because their saturation requires large concentrations of circulating glucocorticoids, the inhibitory effect of glucocorticoids on the HPA axis becomes attenuated, allowing the system to function at a higher secretory level under stress conditions.

The limitation of excessive HPA axis activation is mediated by corticosteroid receptor interactions in the hippocampus, including the enhancement of GABAergic inhibition and the reduction of catecholaminergic activity. Ultimately, modulation of HPA axis responses to stress depends on the balance between type I and type II receptors.

4.2. The Impact of Stress During Early Gestation

Contemporary life imposes a substantial stress load on humans. The proportion of so-called socio-emotional stress is rapidly increasing, often resulting in psychosomatic pathology.

Stress poses significant risks for the pregnant woman and the fetus she carries. These include not only the possibility of miscarriage and preterm or complicated delivery but also stillbirth, as well as risks of somatic and neuropsychiatric disorders in the offspring.

According to the concept of epigenetic modification in the programming of the neuroendocrine system, behavior, reproductive, and other physiological functions, such consequences may manifest at later stages of life. Evidence suggests that maternal stress, for example in rats, is accompanied by elevated ACTH and corticosteroid levels in the blood of the fetus, *i.e*., by the development of a stress *in utero*. For this reason, the concept of "maternal stress" with respect to the fetus is equated with that of "prenatal stress."

There is a considerable body of clinical evidence regarding the adverse consequences of prenatal stress (Barrett & Swan, 2015). In particular, prenatal stress increases the risk of schizophrenia, depression, autism spectrum disorder, and attention-deficit/hyperactivity disorder. It also disrupts the functioning of the HPA axis. In female offspring, the risks of bronchial asthma, genital masculinization, and masculinized patterns of play behavior are elevated.

The adverse effects of prenatal stress demonstrate sex-specific differences. Deviations in sexual behavior are observed predominantly in male offspring, while depressive states and asthma are more frequently reported in female offspring.

Although the consequences of chronic stress during gestation have been extensively studied in animal models with respect to offspring health, less is known about the effects of stress in the early stages of gestation. This gap has been largely addressed through the comprehensive animal research conducted by Professor L.Yu. Sergienko and colleagues at the V.Ya. Danilevsky Institute of Endocrine Pathology (Kharkiv, Ukraine), who reported numerous hormonal, metabolic, and other alterations in the offspring.

Socio-emotional stress was experimentally induced in rats from the 2nd to the 8th day of pregnancy by housing them among non-pregnant females and repeatedly altering the composition of this group for six hours per day. Following this period, the animals were housed individually until the end of gestation. The stressed state of the experimental females was confirmed by elevated plasma corticosterone levels.

Birth weights of prenatally stressed male and female offspring were slightly reduced, likely reflecting the catabolic effects of elevated corticosterone. At three months of age, plasma corticosterone concentrations in both sexes were significantly increased. However, the response of this parameter to stress-inducing conditions (daily one-hour restraint in tubes for a week) was markedly attenuated. Morphological changes in the adrenal cortex, indicative of activation of its hormone-producing function and even functional overstrain, corresponded with this diminished responsiveness. Presumably, this underlies the blunted reaction to immobilization.

Significant alterations were observed in the reproductive system, generally reflecting deterioration. At 12 months of age, the mass of the testes was below normal, and fertility, including the ability to impregnate and achieve conception, was substantially reduced. These changes were associated with quantitative and qualitative impairments in sperm composition in seminal fluid. Mean plasma testosterone concentration was more than halved, whereas estradiol levels were significantly elevated compared to age-matched intact animals. Histological examination of the gonads revealed destructive processes. Hormonal and morphological indicators worsened further under additional stress in prenatally stressed males.

Equally dramatic pathological changes were observed in the reproductive organs of prenatally stressed females. Sexual maturation, as indicated by vaginal opening, was delayed by several days. The number of primordial follicles in the ovaries during the first days after birth was halved. After one month, disruptions in folliculogenesis were evident, including a reduced number of developing follicles, degenerative changes in follicular cells and oocytes, and formation of follicular cysts. The histopathological picture of the ovaries in 12-month-old rats was characterized by numerous involuted corpora lutea, atretic follicles, expansion of the medullary area, and fibrotic degeneration of the gonads. Plasma estradiol levels were sharply decreased. Surprisingly, estrous cyclicity remained normal. Nevertheless, the overall conclusion regarding the reproductive effects of early gestational stress in females is robust: by 12 months of age, oogenesis is nearly completely arrested.

At 90 days of age, insulin levels in prenatally stressed males and females were above normal, and the pancreatic microstructure exhibited an increased number of islets, some formed *de novo*, particularly following stress exposure. Endocrinocytes had signs of heightened functional activity.

Prenatally stressed rats also exhibited metabolic and structural changes in bone tissue, creating a predisposition for osteopenia and osteoporosis. Stress exposure exacerbated these destructive changes in the skeleton.

A significant finding of studies on early gestational stress is the identification of neurological disturbances, manifested as increased alcohol-seeking behavior, alterations in behavior under alcohol deprivation, elevated hypothalamic catecholamine and serotonin content, and modified neurotransmitter responses to stress.

Maternal stress during early pregnancy potentiates gestational diabetes-induced disturbances in glucose and lipid homeostasis and oxidative stress in first-generation rat offspring. These alterations are mediated by the development of insulin resistance.

4.3. Prenatal Stress Syndrome

The concept of the consequences of maternal stress for offspring health emerged after the publication in *Science* in 1972 of a study reporting an increased incidence of bi- and homosexual behavior in adult male rats whose mothers were subjected to daily stress (immobilization combined with bright illumination) during the final week of gestation (Ward, 1972). Prenatally stressed males exhibited reduced copulatory activity (21% of animals copulated and ejaculated compared with 64% in the control group) and a high frequency of lordotic responses, *i.e.*, female-type sexual behavior. Postnatal stress did not affect the parameters studied. The author attributed the observed alterations to stress-induced shifts in the ratio of adrenal to gonadal androgens during the critical stage of sexual differentiation of the brain. Specifically, it was suggested that stress enhances secretion of the weak androgen androstenedione by the maternal or fetal adrenal cortex, or by both sources, while competitively decreasing secretion of the active testicular androgen testosterone. Due to the disturbances in sexual behavior, this pathology became known as the "prenatal stress syndrome."

Subsequent studies conducted in various laboratories worldwide demonstrated that even a single one-hour immobilization of pregnant rats during the specified gestational period, without the illumination factor, was sufficient to reproduce in male offspring disturbances in sexual behavior consistent with altered sexual differentiation of the brain. Similar impairments were observed in other animal species, as well as under alternative stress inducers (pain, cold, fasting, emotional stressors).

The prenatal stress syndrome is characteristic not only of animals but also of humans (Dorner et al., 1980; Dorner et al., 1983b). The risk of giving birth to boys who later in life display alterations in sexual orientation (manifesting as homo- or bisexuality) increases when the mother experienced intense and prolonged stress during pregnancy, for instance, related to military actions or family conflicts. Evidence of the negative impact of gestational stress on offspring continues to accumulate across different countries, including Ukraine and Israel, where populations are exposed to rocket attacks, and Canada, where natural disasters have occurred.

The scope of the concept of the "prenatal stress syndrome" has significantly expanded after it was established that this condition induces not only alterations in sexual behavior but also a wide range of other abnormalities. These include disturbances in lipid and carbohydrate metabolism, changes in HPA axis stress responses, impairments of fertility, alterations in hepatic microsomal oxidation, imbalances in pro- and antioxidant processes, and others, occurring in both sexes. Stress exposure of rats during the early postnatal period produces similar consequences, which can be explained by the fact that

maturation of the neuroendocrine system is not yet complete during this developmental stage.

One of the most significant pathogenetic mechanisms of the prenatal stress syndrome is disruption of androgen-dependent sexual differentiation of the brain, manifesting as feminization/demasculinization. This provides an explanation for why males are considerably more vulnerable to prenatal stress. For this reason, we proposed that the mechanisms underlying this phenomenon should be considered from the perspective of imprinting-related self-modification of cellular response, which underlies disturbances of sexual differentiation of the brain observed in neonatally castrated males and neonatally androgenized females (Reznikov, 1990).

In our department, the pathogenesis of the prenatal stress syndrome has been investigated for over 25 years. The main experimental model involved daily one-hour immobilization of pregnant female rats by firm fixation of their limbs in the supine position during GD 15–21. The consequences of prenatal stress were studied in rats at 3, 6–8, and 19–28 months of age, *i.e.,* in sexually mature young and aging animals. A number of priority findings were obtained and published in leading national and international journals (Reznikov & Nosenko, 1996; Reznikov et al., 1997, 1999; Reznikov et al., 1999a,b; Nosenko & Reznikov, 2001a,b; Reznikov et al., 2001; Reznikov et al., 2005; Reznikov & Tarasenko, 2007; Reznikov et al., 2008b; Limareva & Reznikov, 2012). Below is a list of phenotypic features of the prenatal stress syndrome. Findings from our laboratory are marked with an asterisk.

Prenatal Stress Syndrome in Males

- Homo- or bisexual behavior, reduced number of mountings, intromissions, ejaculations, and increased latency of these behaviors *
- Intensification of aggressive behavior
- Microstructural manifestations of brain “feminization”: reduced size of the sexually dimorphic nucleus in the preoptic-anterior hypothalamic region; reduced volume of neuronal nuclei in the preoptic-medial and suprachiasmatic hypothalamic nuclei *
- Early changes in the content and metabolic turnover of catecholamines in the brain *
- Early impairment of testosterone conversion to estradiol-17β *
- Early impairment of testosterone conversion to 5α-dihydrotestosterone and other 5α-reduced metabolites in the MBH *
- Suppression of pituitary LH response to LHRH *
- Suppression of pituitary ACTH response to AVP*
- Acceleration of age-related involution of spermatogenesis *

- Attenuation of adrenal cortex responses to acute stress *
- Decrease in type II glucocorticoid receptors in the hippocampus
- Delayed release of ACTH granules from pituitary corticotrophs during acute stress *
- Insufficient release and utilization of catecholamines in the hypothalamus during acute stress *
- Impaired serotonergic regulation of HPA axis responses to acute stress *
- Noradrenergic hypersensitivity of the HPA axis *
- Activation of brain stress-limiting systems (GABA, atrial natriuretic peptide, melatonin, β-endorphin, prostaglandin PG2, prostacyclin, and others)
- Elimination of early sex differences in the protein spectrum across different brain regions *
- Disturbances of lipid and protein peroxidation in the brain
- Decreased activity of microsomal monooxygenases in the liver *

Prenatal Stress Syndrome in Females

- Delay of sexual maturation *
- Alterations in estrous cycle structure *
- Reduction of fertility potential and fecundity
- Moderate enhancement of adrenal cortex responses to acute stress *
- Decrease in physical endurance *
- Attenuation of noradrenergic sensitivity of the HPA axis *
- Increased activity of hepatic microsomal monooxygenases *

Thus, our findings, consistent with those of many other researchers, confirm that the immature neuroendocrine system of male fetuses is substantially more sensitive to stressogenic factors than that of females. As shown below, in male fetuses, masculinization/defeminization of the neuroendocrine centers of reproduction is caused by a transient suppression of testicular testosterone secretion. In females, reproductive disturbances are likely due to the entry of maternal adrenal androgens into the fetal circulation, the secretion of which (primarily androstenedione) increases under stress in parallel with elevated glucocorticoid secretion.

4.4 Early Neurochemical and Microstructural Markers of Brain Demasculinization/Feminization in Prenatally Stressed Males

Studies of testosterone metabolism, the status of biogenic monoamines, protein spectra, and microstructural features of the whole hypothalamus and discrete brain structures in prenatally stressed males revealed alterations in the neurochemical determinants of sexual differentiation of the brain. These changes primarily affect the preoptic–anterior

hypothalamic zone, which is the critical locus of androgen-dependent sexual differentiation of the brain.

Testosterone Metabolism in the Brain. The activity of steroid aromatase and 5α-reductase was assessed in 10-day-old rats by measuring the quantity of testosterone metabolites in the incubation medium following incubation of the supernatant fraction of a 10% tissue homogenate with tritiated testosterone. In intact 10-day-old rats, hypothalamic aromatase activity in males was significantly higher than in females, reflecting the process of brain masculinization. Examination of the whole hypothalamus revealed the disappearance of sex differences in aromatase activity as a consequence of prenatal stress exposure due to a twofold reduction of this parameter in males (Table 4.1).

Table 4.1 Effects of prenatal stress on steroid aromatase activity in the whole hypothalamus of 10-day-old rat pups (M ± SEM)

Animal group	pmol E_2/h/g tissue	pmol E_2/h/g protein
Intact females (control)	0.66 ± 0.18 (5)	23.8 ± 8.4 (5)
Intact males (control)	3.53 ± 1.09 (5) *	125.0 ± 20.5 (5) *
Prenatally stressed females	2.96 ± 0.29 (5) *	102.8 ± 0.5 (6) *
Prenatally stressed males	1.71 ± 0.49 (5)	71.3 ± 24.2 (5)

Footnotes: Values are presented as M ± SEM. Numbers in parentheses indicate the number of assays, each including 3–5 animals; * $p < 0.05$ compared with intact females.

Significant changes were also observed in the formation of 5α-reduced testosterone metabolites in the brain. In the whole hypothalamus of intact pups, no statistically significant sex differences in the biosynthesis of 5α-dihydrotestosterone, 3α-diol, or overall 5α-reductase activity were detected, although a clear trend toward higher formation of 3α-diol and total 5α-reductase activity was noted in females. In contrast, maternal stress exposure induced the emergence of sex differences in all parameters due to a significant reduction in females (Table 4.2).

Considering the crucial role of the preoptic region in sexual differentiation, results from the studies in this neural structure, as well as in the MBH, are particularly informative. Under normal conditions, aromatase activity in the POA of newborn males was approximately 1.5 times higher than in females, whereas 5α-reductase activity was about twofold lower. Prenatal stress completely eliminated sex differences in aromatase activity in the POA by reducing it to the level observed in normal females. At the same

time, a complete inversion of sex differences in 5α-reductase activity occurred in this structure, accompanied by an increase in the MBH of males (Table 4.2).

Table 4.2 Effect of prenatal stress on the formation of 5α-reduced testosterone metabolites and total 5α-reductase activity (pmol/h/mg protein) in the whole hypothalamus of 10-day-old rat pups (M ± SEM)

Animal group	DHT		3α-diol		5α-reductase (DHT + 3α-diol)	
	pmol/g tissue/h	pmol/g protein/h	pmol/g tissue/h	pmol/g protein/h	pmol/g tissue/h	pmol/g protein/h
Control females	12.7 ± 2.4	502 ± 116	50.1± 15.1	1930 ± 632	60.6 ± 17.4	2339 ± 746
Control males	16.7 ± 4.2	724 ± 220	22.0 ± 7.7	943 ± 374	32.6 ± 12.6	1397 ± 608
Prenatally stressed females	8.5 ± 2.6	280 ± 83	10.3 ±2.3 [a]	332 ± 69 [a]	17.1 ± 3.5 [a]	560 ± 166 [a]
Prenatally stressed males	21.1 ± 1.4[ab]	883 ± 83[a b]	29.0 ±5.9[ab]	1170 ±279[b]	51.1 ± 5.8 [b]	2053 ± 296 [b]

Footnotes: DHT – 5α-dihydrotestosterone. 4-6 assays per group, each including 3–5 animals; [a] $p < 0.05$ compared with. control females; [b] $p < 0.05$ compared with prenatally stressed females.

It is evident that a deficiency of local estrogen formation is the key pathophysiological mechanism determining disrupted normal androgen-dependent brain differentiation, since estradiol serves as a substrate for the formation of its 4-hydroxylated derivative, one of the main determinants of this process. Our data indicate that insufficient androgenic induction of hypothalamic steroid aromatase occurs not only prenatally, on GD 18–20, against the background of reduced LH and testosterone levels in the blood of male fetuses (Ward & Weisz, 1984), but also continues in early postnatal life until the end of the critical period of sexual differentiation of the brain.

Neurotransmitters. Given that, according to the neurochemical concept of sexual differentiation of the brain, this process is mediated by the cooperative action of

Table 4.3 Effect of prenatal stress on sexual dimorphism of biogenic monoamine contents and testosterone metabolism in discrete brain regions of 10-day-old rats

Parameter	Intact rats (control)		Prenatally stressed rats	
	Males	Females	Males	Females
POA				
Norepinephrine, nmol/g tissue	2.9 ± 0.2	4.4 ± 0.6 [a]	4.7 ± 0.6 [a]	3.5 ± 0.4
Dopamine, nmol/g tissue	3.8 ± 0.4	4.0 ± 0.2	5.9 ± 1.6	3.0 ± 0.3
Serotonin, nmol/g tissue	1.6 ± 0.3	1.4 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
5- Hydroxyindoleacetic acid, nmol/g tissue	5.6 ± 1.5	2.7 ± 0.05 [a]	4.1 ± 0.6	4.8 ± 0.2
Aromatase, pmol E_2/h/·g tissue	0.62 ± 0.07	0.40 ± 0.05 [a]	0.38 ± 0.05 [a]	0.42 ± 0.04
5α-Reductase, pmol 5α-reduced metabolites/ h/ g tissue	6.2 ± 1.9	14.3 ± 2.2 [a]	18.6 ± 2.0 [ab]	12.1 ± 1.8 [b]
MBH				
Norepinephrine, nmol/g tissue	2.6 ± 0.3	1.9 ± 0.1 [a]	2.1 ± 0.1	1.7 ± 0.04
Dopamine, nmol/g tissue	4.7 ± 0,7	2.4 ± 0.1 [a]	3.8 ± 0.5	3.3 ± 0.3
Serotonin, nmol/g tissue	3.3 ± 0.2	2.8 ± 0.6	3.0 ± 0.1	2.0 ± 0.1 [b]
5- Hydroxyindoleacetic acid, nmol/g tissue	8.0 ± 0.9	6.2 ± 1.0	11,6 ± 1.7	5.5 ± 0.5 [b]
Aromatase, pmol E_2/h/·g tissue	0.25 ± 0.04	0.28 ± 0.04	0.17 ± 0.05	0.31 ± 0.05 [b]
5α-Reductase, pmol 5α-reduced metabolites/ h/ g tissue	8.2 ± 2.2	8.8 ± 2.2	22.4 ± 3.5	9.9 ± 1.4 [b]

Footnotes: [a] $p < 0,05$ compared with intact males; [b] $p < 0.05$ compared with prenatally stressed animals of the opposite sex.

catecholamines and estrogenic testosterone metabolites *via* an imprinting mechanism (refer to Chapter 2), it was reasonable to investigate the status of brain neurotransmitter systems in prenatally stressed animals.

Sex differences in hypothalamic norepinephrine content in rats emerge during maturation of the brain catecholaminergic system on PND 7 and reach statistical significance by day 10. Accordingly, we conducted relevant studies in 10-day-old rats. In normal animals, these differences are characterized by lower norepinephrine concentrations in the POA and higher levels in the MBH of males. In the preoptic region, this corresponds to a 90% higher metabolic turnover of the functional norepinephrine pool, assessed using the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine, compared with females. Dopamine concentration in the MBH was higher in males, while serotonin levels were similar in both hypothalamic structures. Notably, the turnover rate of serotonin in the male preoptic region was higher, as indicated by a markedly elevated concentration of 5-hydroxyindoleacetic acid compared with females. In the male MBH, the functional dopamine pool exhibited a twofold faster turnover (Table 4.3).

Prenatal stress caused a complete elimination of sex differences in catecholamine concentration and turnover in both examined hypothalamic regions, while it induced the appearance of sex differences in dopamine turnover in the POA due to an increase in females. Additionally, serotonin metabolism was accelerated in the preoptic region of females and in the MBH of males.

The final week of gestation represents the critical period during which the catecholaminergic system of the rat fetal brain is being established. This explains the high sensitivity of the developing system to stressogenic factors. One such factor is likely the increased corticosterone levels in maternal and fetal blood, given the ability of glucocorticoids to induce tyrosine hydroxylase synthesis. Thus, early stress-induced alterations of the catecholaminergic, and possibly serotonergic, systems of the hypothalamus can be considered determinative mechanisms that, together with hormonal factors, underlie future disturbances of reproductive function and, as shown below, adaptive HPA axis responses.

The Brain Proteins. A reflection of disrupted sexual differentiation of the brain is also evident in changes in the spectrum of soluble proteins. Analyses were conducted on tissue samples from the POA, MBH, and hippocampus of 5-day-old rats. Prenatal stress eliminated sex differences in the relative content of the 66.0 kDa protein, while inducing sex differences in the 18.4 kDa protein in the POA. Although under normal conditions no sex differences were observed in the examined protein spectrum in the MBH or hippocampus, such differences appeared as a consequence of prenatal stress. In the MBH, optical density of electrophoretic protein bands at 14.3 kDa and 24.0 kDa was higher in females, whereas the 34.7 kDa protein was more abundant in males. In the hippocampus, the relative content of 24.0 kDa and 66.0 kDa proteins was higher in females, while that of 34.7 kDa and 45.0 kDa proteins was higher in males.

Neuromorphology. Stress-induced disruptions of the maternal neuroendocrine system also affect neuromorphological features of hypothalamic structures. For histological analysis, the hypothalamic regions of 10-day-old males and females and were fixed in Bouin's solution, and frontal paraffin sections were stained with cresyl violet using Nissl staining. The intensity of staining, degree of cytoplasmic vacuolization in neurocytes, and the size and location of nucleoli were evaluated.

Sexually dimorphic brain structures, which are targets of early sexual differentiation, include the SCN of the hypothalamus. Specifically, the mean neuronal nuclear volume in males averaged 233 ± 7 μm³ *vs.* 193 ± 10 μm³ in females ($p < 0.05$). This sex difference disappeared in prenatally stressed neonates due to a reduction in males to 189 ± 7 μm³, while remaining at 189 ± 21 μm³ in females. The distribution of neuronal nuclear volumes in prenatally stressed males and females was nearly identical. Therefore, the SCN of prenatally stressed male neonates exhibit altered sexual differentiation toward demasculinization, reflecting disturbances in male sexual behavior in adulthood and likely resulting from prenatal stress-induced reductions in circulating testosterone levels in intra-uterine fetuses.

Of particular interest was the study of the arcuate nuclei, which are involved in regulating reproductive function in females. In the rostral portion of the arcuate nuclei, neurons form a relatively compact cluster between the ventral surface of the third ventricle and the structures of the median eminence. In intact males and females, the neuronal cytoplasm appeared light, mostly vacuolated, and finely granular. The granules were intensely stained with cresyl violet, especially along the periphery of the perikaryon, resembling Nissl substance. Neuronal nuclei were light, round to slightly elongated, and varied in size, containing predominantly a single nucleolus, occasionally two.

In prenatally stressed females, perikarya were smaller, the cytoplasm appeared dense, and staining with cresyl violet was more intense. Only in a few cells were large perikarya with vacuolated cytoplasm observed, indicative of high functional activity. The histological appearance of the arcuate nuclei in prenatally stressed males remained largely unchanged, resembling that of intact animals.

Neuron karyometry also revealed a more pronounced effect of prenatal stress on female rats. The mean nuclear volume decreased by 29%, reaching 288 ± 13 μm³ compared with 406 ± 47 μm³ in intact animals ($p < 0.05$). The variability in nuclear size was substantially reduced, with cells possessing nuclei larger than 550 μm³ nearly disappearing; their proportion was 1.8% of the total, whereas in intact females, such cells constituted 20.5%.

In prenatally stressed males, the overall distribution of neuronal nuclei in the arcuate nuclei remained unchanged compared with intact animals. Nuclear size varied within a

relatively wide range, averaging 381 ± 48 μm³ *vs.* 366 ± 44 μm³ in controls. However, the proportion of neurons with small nuclei decreased: cells with nuclei around 200 μm³ comprised 8.6% compared with 15.2% in intact animals.

Thus, there is a correlation between neuromorphological alterations of the arcuate nuclei in females and the prenatal stress-induced disturbances in estrous cycles and fertility in adulthood. The absence of significant changes in males is not surprising, as the arcuate nuclei do not belong to sexually dimorphic regions of the hypothalamus, which is evident, for example, from the lack of statistically significant sex differences in neuronal nuclear sizes in intact males and females.

4.5. Long-Term Neuroendocrine Manifestations of Prenatal Stress Syndrome

Sexual Maturation and Reproductive Function. Maternal stress affected the timing of sexual maturation in offspring. In males, the age at testicular descent into the scrotum was recorded, whereas in females, the age at vaginal opening was noted. These parameters varied slightly across experiments and were influenced by housing conditions, season, lighting, and other environmental factors. Therefore, special attention was paid to these indicators in control animals. In one study, the mean age of sexual maturation in normal males was 38.8 ± 0.3 days, whereas prenatally stressed males matured at 48.0 ± 0.2 days, indicating a significant delay (p <0.001). In intact females, vaginal opening occurred at 52.1 ± 0.9 days postnatally, while in prenatally stressed females, it was delayed to 65.1 ± 2.2 days (p <0.001). Approximately one-third of the prenatally stressed females subsequently exhibited irregular estrous cycles and reduced fertility. Other researchers have similarly reported delayed sexual maturation, attributing it to suppression of hypothalamic kisspeptin gene expression (Medeiros et al., 2023).

Regulation of LH Secretion. The physiological "hypothalamus–anterior pituitary–testes" axis operates via negative feedback, in which circulating testosterone inhibits hypothalamic GnRH synthesis and secretion, thereby regulating pituitary LH output. A decrease in circulating testosterone leads to disinhibition of LH secretion. Interestingly, GnRH neurons are influenced not only by testosterone but also—likely predominantly—by estradiol-17β, locally synthesized in the mediobasal hypothalamus from testosterone. Numerous experiments have long supported this conclusion. However, assessing LH regulatory status requires information on hypothalamic steroid aromatase activity.

At 20 days of age, hypothalamic aromatase activity is well expressed in rats, with higher activity in males than in females. In prenatally stressed rats, these sex differences were abolished due to reduced enzymatic activity in males.

In 3-month-old normal rats, hypothalamic aromatase activity is markedly reduced relative to 20-day-old animals, and sex differences are absent, as in prenatally stressed animals. In contrast, under normal conditions, 5α-reductase activity is significantly higher in females, whereas prenatal stress reduces it to male levels. This likely contributes to ovarian cycle disturbances induced by prenatal stress.

To assess the sensitivity of neuroendocrine centers to inhibitory effects of estrogens and androgens, we employed the steroid aromatase inhibitor 1,4,6-androstatrien-3,17-dione and the androgen receptor antagonist flutamide. Additionally, GnRH administration was used to evaluate LH reserves and pituitary sensitivity in 3-month-old rats. These functional tests allow quantitative assessment of hypothalamic–pituitary gonadotropic reserves. Biologically active LH in plasma was determined based on its ability to stimulate testosterone synthesis in cultured mouse testicular interstitial cells. Testosterone levels were measured by radioimmunoassay, using human LH calibrated against the 2nd International Standard 69/45 (Sigma, USA).

Intact male rats responded to administration of 1,4,6-androstatrien-3,17-dione and flutamide with a twofold increase in plasma levels of bioactive LH. In contrast, these responses were completely absent in prenatally stressed males. Thus, prenatal stress significantly disrupts the programming of pituitary gonadotropic regulatory mechanisms.

Furthermore, intravenous application of 25 ng porcine LHRH hormone in non-anesthetized animals—which under normal conditions elicits more than a twofold increase in plasma bioactive LH within 15 min (from 5.44 ± 0.35 IU/L to 12.12 ± 1.16 IU/L, $p < 0.001$)—in prenatally stressed males produced only a 37% increase (from 5.61 ± 0.26 IU/L to 7.66 ± 0.51 IU/L, $p < 0.001$). The preservation, albeit attenuated, of pituitary gonadotroph responsiveness to GnRH suggests that the lack of gonadotropic response to the aromatase inhibitor and androgen receptor antagonist results from prenatal stress–induced functional insufficiency of the hypothalamic component of the HPG axis.

Neuromorphology. On frontal sections of the hypothalamus, the sexually dimorphic nucleus SDN of the medial POA, stained with cresyl violet by the Nissl method, was located laterally to the supraoptic recess of the third ventricle. Its neurons were clearly delineated from the markedly smaller neurons of the anterior periventricular nucleus by a narrow band of neuropil. On the dorsal and ventrolateral sides, neurons were arranged more loosely and lacked distinct boundaries of cellular aggregation. They were intensely stained with cresyl violet, and their perikarya contained finely dispersed Nissl substance. Many neurons exhibited 2–3 processes. They possessed large round or oval nuclei,

sometimes of slightly irregular shape reflecting the contours of the perikaryon. The nuclei contained a prominent, clearly visualized nucleolus.

In three-month-old intact males, the sexually dimorphic nucleus occupied a much larger area than in females in the metestrus stage and consisted of a significantly greater number of neurons. Moreover, it was observed on a larger number of sections, indicating larger rostro caudal dimensions. Overall, the neurons of males and females did not differ significantly in their general morphology.

The difference in the area of sexually dimorphic nuclei between males and females was absent in cases of demasculinization/feminization of the brain, induced by androgen deficiency at early stages of functional formation of the neuroendocrine system.

In three-month-old prenatally stressed females, neurons in the sexually dimorphic nucleus were loosely distributed and lacked distinct borders of cellular aggregation. Morphologically, the sexually dimorphic nuclei of prenatally stressed males and females showed no differences. However, in comparison with intact males, experimental animals exhibited significant differences in the volumes of neuronal nuclei.

Our laboratory has demonstrated that the microstructural equivalent of altered brain programming in prenatally stressed males is a reduction in the volume of neuronal nuclei in the suprachiasmatic nuclei (SCN) of the hypothalamus to levels characteristic of normal females (Reznikov, Pyshak & Nosenko et al., 2004b). These nuclei are located at the angle between the surface of the optic chiasm and the third ventricle. In three-month-old rats, the SCN consists of small, densely packed neurons. In intact males, neurons are larger than in intact females (315 ± 20 μm³ *vs*. 257 ± 15 μm³). The proportion of neurons with a nuclear volume more than 400 μm³ was 29% in males and only 8% in females. Large, pale nucleoli, typical of actively synthesizing cells, were markedly more numerous in male neurons than in female neurons.

Prenatal stress reduced the average nuclear volume in males to 193 ± 15 μm³ ($p < 0.001$), whereas in females it remained unchanged at 211 ± 11 μm³ ($p > 0.05$). In males, the number of neurons with vacuolated cytoplasm, i.e., functionally active cells, also decreased. Most neuronal nuclei were approximately 200 μm³, clearly contoured, exhibited heterochromatin, and appeared darker than in controls. A few cells were twice the mean size; however, overall, the morpho functional state of SCN neurons in prenatally stressed males was less active compared with controls. Morphometric analysis supported these observations: in control males, 73% of neurons had nuclei more than 300 μm³, whereas in prenatally stressed males, only 12% reached this size.

Similar alterations were observed in the MPN of rats. In intact males, the MPN were larger than in intact females in terms of area, neuron number, cell size, and staining intensity. The proportion of neurons with nuclear volumes more than 500 μm³ was 80% in males and 39% in females. Under normal conditions, the average nuclear volume in three-month-old male neurons is approximately 30% higher than in females.

In prenatally stressed males, a reduction in the area of the MPN occupied by large neurons was observed. Neuronal nuclear volumes decreased by 37%, resulting in the disappearance of sexual dimorphism both in mean values and in the distribution of nuclear volumes, as shown in the histogram (Fig. 4.1). Neurons with nuclear volumes more than 500 μm³ constituted only 27%. Wrinkled, hyperchromatic neurons, typically absent in normal animals, appeared. Glial cell nuclei were more prominent, and some exhibited hyperchromatism.

Females did not exhibit significant changes in neuronal nuclear volumes in response to prenatal stress. The proportion of neurons with nuclear volumes more than 500 μm³ increased to 44%. Thus, prenatal stress induces demasculinization of the sexually dimorphic hypothalamic zone in males at the microstructural level, without substantially affecting

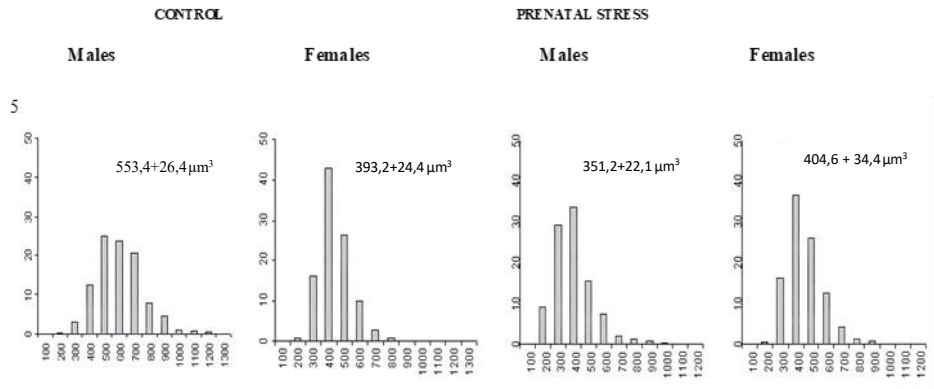

Fig. 4.1 Effect of prenatal stress on the distribution of neuronal nuclear sizes in the MPN of 3-month-old rats
Footnotes: Y-axis – percent of total; X-axis - nuclear volumes (X-axis)

microstructural parameters in females. Therefore, there is a complete correspondence between the microstructural alterations as characteristic of prenatal stress syndrome and

the anomalies of sexual behavior observed in males. Although the arcuate nuclei of male and female rats do not exhibit sexual dimorphism in terms of karyometric parameters, their involvement in reproductive regulation makes it relevant to investigate neuromorphological features in prenatally stressed and intact animals. In three-month-old intact females, the arcuate nucleus consisted of relatively large neurons at the periphery and smaller neurons in the central cluster. The cytoplasm of neurons in intact animals displayed varying degrees of vacuolization, was finely granular, and weakly stained with cresyl violet, except for peripheral regions where intensely stained areas were observed. Neuronal nuclei were round, with an average volume of 455 ± 19 μm³, showing a wide range of variability. Glial cell nuclei were light, large, perineuronal, and inconspicuous.

In prenatally stressed females, histological signs indicated a marked increase in neuronal functional activity of the arcuate nuclei. The intensity of cytoplasmic staining in neuronal perikarya slightly increased, with vacuolization observed only in a few cells. The average nuclear volume increased by 19% to 541 ± 51 μm³. Hypertrophic neurons with nuclear volumes more than 650 μm³ accounted for 29.6%, compared with 13.2% in intact females. Changes were also observed in glial cells: glial nuclei were well-defined, slightly smaller, and overall exhibited signs of functional overstrain.

In intact males, morphological signs of neuronal activity were similar to that of intact females in the diestrus stage. However, perikarya were slightly larger and the cytoplasm stained more intensely with cresyl violet. The mean nuclear volume was 452 ± 37 μm³, not significantly different from females.

In prenatally stressed males, the mean nuclear volume of neurons in the arcuate nuclei was 486 ± 57 μm³, showing no significant change. The proportion of neurons with nuclei more than 650 μm³ increased from 13% to 22%. Glial cell alterations were similar to those observed in prenatally stressed females.

Thus, prenatal stress determines in adult rats an increase in the functional activity of a specific population of neurons in the rostral arcuate nucleus, with changes more pronounced in females than in males. The medial part of the rostral arcuate nucleus contains the terminals of neurosecretory cells that produce LHRH, specifically in the median portion of the preoptic-infundibular tract. The majority of arcuate nucleus neurons belong to the tubero-infundibular dopaminergic system, which is involved in ovarian regulation. Increased neuronal functional activity in the arcuate nucleus of sexually mature prenatally stressed females correlates with reproductive disorders, specifically reduced fertility and disrupted estrous cyclicity.

Sexual Behavior. The assessment of male sexual behavior lasted for 15 min. Parameters of copulatory behavior in prenatally stressed males are presented in the Table 4.4.

Table 4.4 Effect of prenatal stress under nimodipine treatment on male-typical sexual behavior parameters in male rats (M ± SEM)

Parameter	Control	Prenatal stress	Prenatal stress + nimodipine
	First testing		
Latency period (s):			
First mounting	29.0 ± 3.6	55.2 ± 17.1	18.0 ± 2.9 *
First intromission	63.4 ± 14.1	115.8 ± 40.6	42.6 ± 24.0
First ejaculation	723.6 ± 74.6	671.6 ± 147.7	686.6 ± 289.0
Number of:			
Mountings without intromissions	3.8 ± 1.6	1.6 ± 0.7	1.0 ± 0.6
Intromissions	15.2 ± 1.9	12.0 ± 0.9	13.4 ± 1.8
Ejaculations	2.2 ± 0.2	1.4 ± 0.2 *	1.8 ± 0.6
	Second testing		
Latency period (s):			
First mounting	7.6 ± 1.3	33.0 ± 7.0 *	10.2 ± 3.9 [a]
First intromission	13.2 ± 4.1	38.4 ± 10.0*	12.2 ± 6.0
First ejaculation	402.6 ± 52.1	638.6 ± 77.9*	393.4 ± 145.1
Number of:			
Mountings without intromissions	0.6 ± 0.6	0.4 ± 0.4	0
Intromissions	15.4 ± 1.1	16.8 ± 1.8	16.6 ± 3.4
Ejaculations	3.0 ± 0.3	1.6 ± 0.2*	2.0 ± 0.6

Footnotes: [a] $p < 0.05$ compared with the control group; [b] $p < 0.05$ compared with the prenatally stressed group. Each group contained five rats.

The results confirm and further specify the abnormalities of sexual behavior in males, which manifest as both feminization and demasculinization. All prenatally stressed males exhibited elements of female-type sexual behavior (lordosis responses), which are normally absent in intact rats. At the same time, these animals demonstrated significant alterations in male-type sexual behavior: prolonged latency periods to the first mounting, first intromission, and first ejaculation. During the second testing, these alterations were less pronounced (the values of the studied parameters decreased on average by 1.4–2 times) compared to the first testing, which is attributable to the acquisition of sexual experience. However, due to a more marked decline in control indices during the second

testing (on average by 2.0–3.3 times), the impairments of male sexual behavior parameters induced by prenatal stress became more apparent. The number of ejaculations was reduced twofold during the second testing, whereas the number of mountings without intromissions and the total number of intromissions did not significantly differ from control values.

HPA Axis. Since prenatal stress has a substantial impact on the development of the catecholaminergic system of the brain during early ontogenesis, the expected consequence of this disruption in the normal maturation of the neuroendocrine system would be a modification of HPA axis stress reactivity. This assumption logically follows from the key role of norepinephrine in mediating stress-induced stimulation of ACTH and corticosteroid secretion. Nevertheless, one cannot exclude the pathogenetic contribution of other neurotransmitters and neuromodulators such as opioids, vasopressin, neuropeptide Y, and others.

The adaptive role and functional reserves of the HPA axis are best revealed under conditions of stimulation by different factors. In our experiments, such stimuli included one-hour immobilization stress, intracerebroventricular administration of norepinephrine and arginine–vasopressin to target circumventricular organs (hypothalamic nuclei and the median eminence of the hypothalamus), and functional ACTH challenge tests both *in vivo* and *in vitro*.

The first step was to investigate the HPA axis response in normal and prenatally stressed 3-month-old rats subjected to one-hour or thirty-min immobilization. At the time of testing, all control and experimental females were in the diestrus stage, determined by vaginal smear cytology. Such standardization is necessary given the dependence of HPA axis fluctuations on the estrous cycle. Immediately after stress exposure, animals were sacrificed by decapitation, and the trunk blood samples were collected for assay of plasma corticosterone concentration.

Basal corticosterone levels in intact females were significantly higher than those in males. Although prenatal stress did not affect corticosterone levels in males under resting conditions, the post-stress elevation following a one-hour immobilization (an average increase of 54%) was threefold lower compared to the response of intact males (an increase of 162%). This was consistent with a delayed release of adrenocorticotropic hormone (ACTH) secretory granules from pituitary corticotrophs, as demonstrated by ultrastructural examination of the adenohypophysis. The dynamics of the adrenocortical stress response were also altered: immediately after 30 min of immobilization, corticosterone levels in control males increased by 44%, and by 77% relative to baseline after an additional 30 min. In contrast, the rise in corticosterone in prenatally stressed

males reached 68% but returned to baseline within the following 30 min, indicating a rapid depletion of the hormonal reserve of the HPA axis.

The cause of the attenuated stress reactivity of the HPA axis in males became clear after measurements of hypothalamic norepinephrine concentrations. Intact males responded to one-hour acute stress with a typical stress-associated decrease in hypothalamic catecholamine concentration from 8.4 ± 0.4 nmol/g tissue to 6.3 ± 0.2 nmol/g tissue $p < 0.05$), reflecting its utilization as a neurotransmitter within the stress-implementing neural system. In contrast, prenatally stressed males showed no such response (baseline 8.0 ± 0.2 nmol/g tissue; after stress 7.2 ± 0.6 nmol/g tissue, $p > 0.05$).

Unlike males, adult prenatally stressed females of the same age responded to acute stress similarly to intact animals, with a marked increase in plasma corticosterone concentrations of 73% (intact – 79%) and a significant reduction in hypothalamic norepinephrine levels from 8.4 ± 0.4 nmol/g tissue to 6.0 ± 0.3 nmol/g tissue ($p < 0.05$) (intact – from 8.1 ± 1.0 to 6.0 ± 0.3 nmol/g tissue, $p < 0.05$). These findings highlight clear sex differences in the effects of prenatal stress on the development of HPA axis stress responsiveness.

The prenatally stress-modified HPA axis reactivity in males persisted into aging: one-hour immobilization almost failed to elevate corticosterone levels in the blood. In contrast, aged prenatally stressed females exhibited a pronounced adrenocortical response to acute stress, although the magnitude of corticosterone increase was lower than in 3-month-old females (69% *vs*. 91% in intact animals of the same age). A similar age-dependent decline in adrenocortical responsiveness was also observed in intact females.

Of note, the adrenocortical response to acute stress in both intact and prenatally stressed aging females was not accompanied by a reduction in hypothalamic norepinephrine levels: intact animals – baseline 4.3 ± 0.7 nmol/g tissue, post-stress 3.0 ± 0.1 nmol/g tissue ($p > 0.05$); prenatally stressed – baseline 3.5 ± 0.2 nmol/g tissue, post-stress 3.3 ± 0.3 nmol/g tissue ($p > 0.05$). It is therefore likely that adrenocortical responses to acute stress in these animals are mediated by other neurotransmitter or neuropeptide factors. Thus, sex-specific differences in the effects of prenatal stress on the neuroendocrine system are also evident across the lifespan.

Importantly, maternal stress did not affect hypothalamic dopamine concentrations under physiological conditions or during acute stress in any of the studied groups.

Although prenatal stress programmed an abnormal hypothalamic norepinephrine response to acute stress, norepinephrine sensitivity of the hypothalamus as a trigger of

HPA axis activation appeared not only preserved but even moderately enhanced in 5-month-old prenatally stressed males. Intracerebral application of norepinephrine bitartrate in control males aged 5 months caused a mean corticosterone increase of 31.6% above baseline at 30 min, with recovery to basal levels by 60 min. Prenatally stressed males of the same age displayed a more pronounced and prolonged HPA axis response: corticosterone levels were elevated by 45.5% at 60 min. In another similar experiment, corticosterone returned to baseline by 90 min in both control and experimental groups. In aged control rats (21 months), the response to noradrenergic stimulation remained substantial, whereas in prenatally stressed males it was indistinguishable from controls (Fig. 4.2).

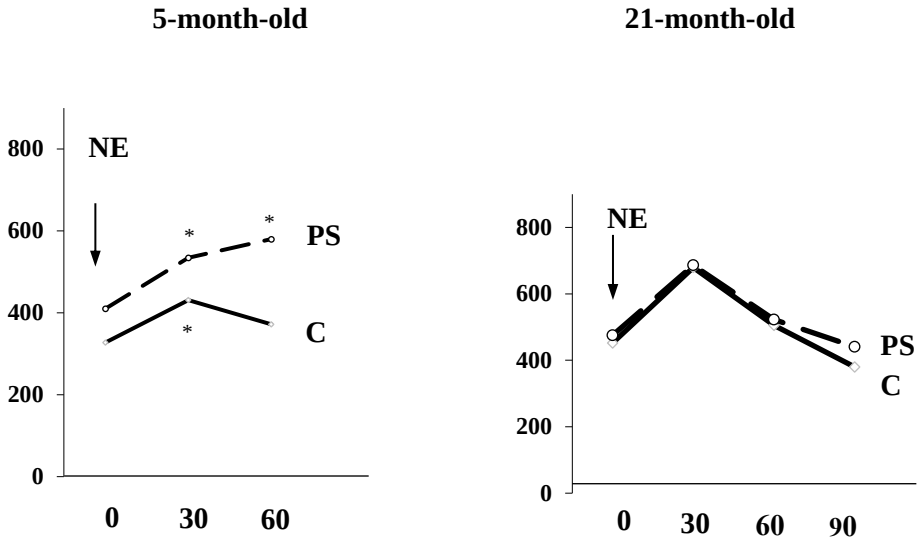

Fig. 4.2 Age-related characteristics of noradrenergic reactivity of the HPA axis in 5-month-old prenatally stressed male rats

Footnotes: The ordinate axis represents the plasma corticosterone level (nmol/L), while the abscissa axis indicates the time (min) after administration of norepinephrine bitartrate (NE) into the third ventricle of the brain. C – control, PS – prenatal stress. $^{*}p < 0.05$ compared with the baseline level.

Control sham-operated 5-month-old females responded to norepinephrine with a 64.1% increase in plasma corticosterone concentration 30 min after application, from 468 ± 37 nmol/Lto 768 ± 93 nmol/L($P < 0.05$), which was twice as strong as the response observed in control males. This finding is consistent with the well-documented higher stress reactivity of the female HPA axis, which is known to result from elevated estrogen levels that directly stimulate the expression of the CRH gene in neurons of the paraventricular

hypothalamic nuclei. In control females, plasma corticosterone concentrations returned to baseline 60 min after norepinephrine application and remained stable at 90 min.

In contrast to both males and control females, prenatally stressed females exhibited no increase in corticosterone secretion in response to central noradrenergic stimulation. Their baseline corticosterone level was 513 ± 118 nmol/L, declining to 434 ± 54 nmol/Lat 30 min, and remained unchanged thereafter. A repeated experiment confirmed these findings. However, this effect of prenatal stress was not detected with aging. In 21- month-old control females, the amplitude of the HPA axis response was approximately threefold lower than in young females, most likely due to age-related alterations inhormonal status, specifically the decline in estrogen production following cessation of ovulatory cycles. Prenatally stressed aged females did not differ from control females in the dynamics of plasma corticosterone concentrations (Fig. 4.3)**.**

Documenting the markedly impaired HPA axis response of prenatally stressed sexually mature females to norepinephrine, it must be acknowledged that the underlying mechanisms of this pathology remain unresolved. It is conceivable that elevated corticosterone levels observed during maternal stress in pregnant rats exert an impact on the HPA axis of the fetuses. Given the disturbances in estrous cycle structure in prenatally stressed adult females, characterized by prolongation of diestrus, and considering the dependence of stress responses on the phase of the cycle, it may be

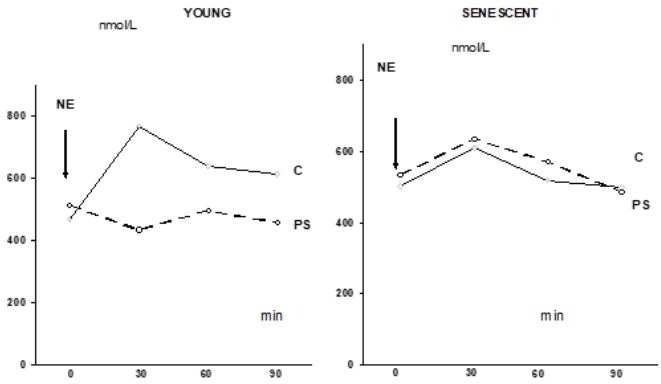

Fig. 4.3 Age-related characteristics of noradrenergic reactivity of the HPA axis in 5-month-old prenatally stressed female rats
Footnotes: Refer to Fig. 4.2.

postulated that the sensitivity threshold of CRH–producing neurons in the paraventricular nuclei of the hypothalamus to the stimulatory action of estrogens increases. As a consequence, norepinephrinergic reactivity of the HPA axis becomes attenuated.

With regard to prenatally stressed sexually mature males, the enhancement of the norepinephrinergic response of the HPA axis proved unexpected, since their adrenocortical response to acute stress was diminished, primarily due to insufficient release and utilization of hypothalamic norepinephrine. It may be assumed that this enhanced responsiveness to central norepinephrinergic stimulation represents a compensatory mechanism counteracting the inadequate release of norepinephrine from the terminals of norepinephrinergic neurons.

Table 4.5 Plasma ACTH levels (M ± SEM, pg/mL) in control and prenatally stressed (PS) adult male and female rats before and following application of AVP into the third ventricle of the brain

Sr. No.	Animal group	Basal level	20 minutes following AVP application
1	Control males	98.9 ± 13.8	408.1 ± 85.5 *
2	PS males	176.2 ± 19.6	622.6 ± 65.9 *
	$p_{1,2}$	< 0.01	> 0.05
3	Control females	180.7 ± 19.7	492.7 ± 52.6 *
	$p_{1,3}$	< 0.01	> 0.05
4	PS females	197.4 ± 42.7	458.1 ± 33.6 *
	$p_{2,4}$	< 0.05	> 0.1
	$p_{3,4}$	< 0.05	> 0.05

Footnotes: * $p < 0.05$ compared with basal level; each group contained 6-8 rats.

Considering that the natural stimulator of glucocorticoid secretion is pituitary ACTH, experiments were conducted to determine its plasma levels in normal and prenatally stressed rats. The concentration of ACTH in the plasma of sexually mature female and male rats was measured by radioimmunoassay using IRMA-ACTH reagents (Immunotech, France). Blood samples from females were collected during the diestrus stage.

The basal ACTH level in non-stressed female offspring was significantly higher than in males. This finding can be explained by the well-documented stimulatory effect of endogenous estrogens on the neurosecretory cells of the hypothalamic paraventricular nuclei and on CRH gene expression, and consequently on corticotropin secretion. Males responded to prenatal stress with an increase in basal ACTH level in adulthood, and by this parameter they did not differ from either prenatally stressed or control females (Table 4.5). The absence of sexual dimorphism in basal ACTH levels thus reflects the modifying influence of maternal stress on the functional state of the HPA axis.

One of the neurosecretory products of the hypothalamus is AVP (antidiuretic hormone), which, in cooperation with CRH, stimulates the release of ACTH from corticotrophs *via* interaction with V1b receptor subtypes located in the plasma membrane of pituitary corticotrophs. Under these conditions, the stimulatory effect of CRH on ACTH secretion is potentiated. In normal rats, approximately 50% of parvocellular neurons of the hypothalamic paraventricular nucleus that produce CRH also co-express arginine-vasopressin, which is released into the hypophyseal portal circulation from nerve terminals located in the external zone of the median eminence. The biosynthesis of these neuropeptides is regulated by multiple mechanisms, including hormonal ones: the biosynthesis of AVP is testosterone-dependent (in addition to its regulation by plasma osmolality and extracellular fluid volume), whereas CRH production is modulated by corticosterone levels through negative feedback mechanisms. Male rats exhibit higher expression levels of AVP mRNA in the bed nucleus of the stria terminalis compared to females, with these sex-related differences primarily determined by variations in testosterone levels and its metabolites during the neonatal period. The number of paraventricular hypothalamic neurons containing AVP positively correlates with plasma testosterone concentrations.

The activity of the parvocellular vasopressinergic system is enhanced following adrenalectomy or under conditions of chronic stress. In contrast, the magnocellular vasopressinergic system of the hypothalamic supraoptic nuclei remains uninvolved in the stress response, since, according to immunohistochemical studies and *in situ* hybridization data, the activation of this system is primarily driven by osmotic or chemoreceptive stimuli.

Despite the large body of research on the role of the vasopressinergic system in mediating the effects of chronic stress, no data have been available in the literature regarding the specific features of the HPA axis response to central vasopressinergic stimulation in prenatally stressed animals. In this context, it was of particular interest to investigate the effects of central vasopressinergic stimulation of the brain's circumventricular structures on pituitary and adrenocortical responses of the HPA axis

in prenatally stressed male and female rats, as well as the specific features of the HPG axis response under these conditions.

The experiments were conducted on unanesthetized male and female rats aged 8 months (n = 6 per group). Blood samples were collected at 20 and 40 min following intracerebroventricular administration of 0.5 ng (Arg^8)-vasopressin acetate dissolved in 2 μL of isotonic, pyrogen-free sodium chloride solution into the third cerebral ventricle. Plasma levels of ACTH, corticosterone, and testosterone were measured.

An increase in plasma ACTH concentration at 20 min following AVP administration was nearly identical in prenatally stressed and control (sham-operated) female rats. In contrast, prenatally stressed males demonstrated a pronounced enhancement of the hormonal response compared with the sham-operated control group (Table 4.5).

At 20 min after AVP application, the adrenocortical response in prenatally stressed males was twice as low as that observed in normal animals. By 40 min, the response continued to develop but remained attenuated. The dynamics of plasma corticosterone concentrations were as follows: in control rats, corticosterone levels increased from 887 ± 134 to 2298 ± 161 nmol/Lat 20 min ($p < 0.001$); in prenatally stressed males, levels rose from 1086 ± 138 to 1641 ± 133 nmol/L($p < 0.02$). At 40 min, plasma corticosterone levels were significantly elevated in both normal (3.5-fold increase, $p < 0.001$) and prenatally stressed (2.6-fold increase, $p < 0.001$) males; however, the intergroup difference remained significant.

In control females, the adrenal cortical response was slower: no changes were detected at 20 min, whereas at 40 min plasma corticosterone levels increased by 64%. In prenatally stressed females, corticosterone concentrations increased comparably at 20 and 40 min after AVP application (by 26% and 35%, respectively). Thus, the HPA axis response to central AVP stimulation in prenatally stressed males and females was markedly attenuated, but the degree of alteration differed substantially between sexes.

Plasma testosterone levels in both control and prenatally stressed males exhibited a sharp decline at 20 min following intracerebroventricular AVP administration (5.9-fold and 3.7-fold decreases, respectively). By 40 min, testosterone concentrations showed partial recovery but remained significantly below baseline values in both groups. Such a decline appears to be consistent with the role of AVP as one of the mediators of stress, with intracerebral administration of this hormone mimicking stress-related effects. It is well established that in both humans and rats, acute stress and exogenous glucocorticoids cause a pronounced reduction in circulating androgen levels without affecting LH secretion (Cumming et al., 1983; Srivastava et al., 1993).

A plausible mechanism underlying the testosterone-lowering effect of AVP administration may involve inhibition of testicular steroidogenic enzymes, specifically 3β- and 11β-hydroxysteroid dehydrogenases (Cumming et al., 1983; Nwe et al., 2000). Additional evidence supporting this assumption comes from studies on Brattleboro rats, which have a genetic deficiency of AVP and demonstrate no testicular response to a 2-hour immobilization stress (Collu et al., 1984). These findings suggest that the inhibitory effect of stress on testosterone biosynthesis during the initial two hours of immobilization in normal animals is at least partially mediated by arginine-vasopressin; according to the cited authors, this inhibition occurs at a post-cAMP level.

It is well established that the regulation of AVP and CRH synthesis in parvocellular neurons of the hypothalamic paraventricular nucleus is mediated by different mechanisms. As shown above, analysis of noradrenergic reactivity of the HPA axis revealed an enhancement and prolongation of corticosterone elevation in prenatally stressed male rats in response to central stimulation of hypothalamic structures with

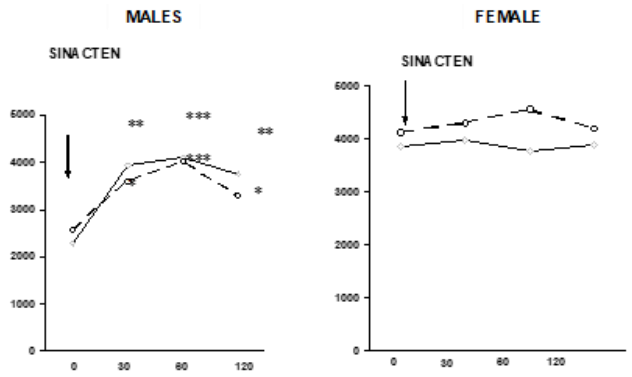

Fig. 4.4 Adrenocortical response of prenatally stressed adult rats to β-1-24-corticotropin (Sinacthen) administration

Footnote: The Y-axis represents the plasma corticosterone level (nmol/L), while the X-axis indicates the time after administration of Sinacthen (min)

*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs.* baseline.

norepinephrine, compared with the response observed in normal animals. In contrast, the HPA axis response to intracerebroventricular administration of AVP was diametrically opposite. Considering the presence of vasopressinergic neurons in the hypothalamus and the ability of AVP to stimulate ACTH secretion by directly acting on pituitary corticotrophs through increasing their sensitivity to CRH, it is currently not possible to distinguish the contribution of each mechanism at this stage of research. Nevertheless, there is evidence that the number of vasopressin-producing neurons in the hypothalamic paraventricular nucleus is reduced during the early postnatal period in prenatally stressed male rats, accompanied by suppressed vasopressin synthesis in this structure (Kolesnyk et al., 2006). The results of our study suggest a likely pathogenetic role of diminished vasopressinergic reactivity of the HPA axis in the attenuation of adrenocortical responses in prenatally stressed males and females compared with control rats.

Taken together with relevant literature data, these findings indicate that vasopressinergic hypothalamic regulation is substantially involved in the modification of HPA axis reactivity in prenatally stressed rats. However, drawing a definitive conclusion regarding the pathogenetic role of impaired noradrenergic and vasopressinergic regulation of the HPA axis induced by prenatal stress would be premature without assessing the adrenal cortical response to the stimulatory effect of ACTH

Experiments were carried out on 3-month-old intact and prenatally stressed rats of both sexes. To stimulate adrenal cortical activity, a synthetic ACTH analog, β-1-24-corticotropin, was employed. Blood samples for the determination of plasma corticosterone concentration before and after Synacthen administration were collected via a Silastic catheter implanted into the external jugular vein 24 hours prior to the experiment. The animals were unanesthetized and allowed free movement in the cage. β-1-24-Corticotropin was administered at a dose of 60 μg/kg b.w. through the catheter, and subsequent blood sampling was performed at 30, 60, and 120 min. During the experiments, all females were in diestrus.

As expected, β-1-24-corticotropin significantly increased corticosterone secretion in control males, and a similar response was observed in prenatally stressed males. Somewhat unexpectedly, no response was detected in either experimental or control females (Fig. 4.4). A convincing explanation for this finding lies in the well-documented dependence of both basal and stress-induced glucocorticoid secretion on the level of estrogen saturation, which is minimal during diestrus. Nevertheless, the absence of a hormonal response in intact females does not contradict their more pronounced response, compared to intact males, to central noradrenergic stimulation of the HPA axis, where massive ACTH release into the circulation substantially exceeds the threshold sensitivity of the adrenal cortex.

The *in vivo* experiments were complemented by *in vitro* studies. Adrenal slices from intact and prenatally stressed males and females aged 6 months were incubated for one hour in Krebs–Ringer bicarbonate buffer containing glucose, with or without 0.8 IU of synthetic porcine ACTH. Corticosterone concentrations were measured in the incubation medium. Basal corticosterone levels (unstimulated by ACTH) were higher in intact and prenatally stressed females compared with males. This finding does not contradict the *in vivo* results, as the applied ACTH dose was sufficient to elicit stimulation. The increase in corticosterone concentration following ACTH addition was almost identical in males and females (1.5–1.6-fold), and prenatal stress did not influence adrenal cortical sensitivity to ACTH.

With respect to prenatally stressed males, the results of β-1-24-corticotropin experiments suggest that alterations in stress reactivity, arginine-vasopressinergic, and noradrenergic sensitivity of the HPA axis reflect disturbances in central regulatory mechanisms as a consequence of deviations in early-life neuroendocrine programming. Another important conclusion is that these consequences are sex-dependent, manifesting differently in males and females, which is likely attributable to differences in the hormonal status of the fetus during maternal stress exposure.

The attenuation of the HPA axis response to acute stress in prenatally stressed males may be associated with an increased number of type 2 glucocorticoid receptors in the frontal cortex and hippocampus, which enhances the inhibitory influence of circulating corticosterone on its own secretion. The inducer of *de novo* receptor synthesis is evidently serotonin, the utilization of which in the hypophysiotropic region of the hypothalamus during acute stress is elevated in prenatally stressed 10-day-old males compared with intact animals (see above). This interpretation is supported by evidence indicating a serotonergic mechanism underlying the increase in glucocorticoid receptor density in the brain of adult male rats exposed to early-life stress through daily 20-min maternal separation (“handling”) for three weeks after birth (Medeiros et al., 1991).

The involvement of serotonin in limiting the HPA axis response to acute stress in prenatally stressed adult animals is further supported by data showing the absence of plasma corticosterone elevation in male rats after stimulation of central serotonin receptors *via* intracisternal administration at a dose of 50 μg, in contrast to the stimulatory stress-effect observed in vehicle-treated controls (Tkachuk, 1998). According to the author, this finding indicates long-term modification of the feedback interactions between the adrenal cortex and serotonergic systems of the brain.

Glucocorticoid receptors are not the only component of the brain’s stress-limiting system, whose primary function is to restrain excessive HPA axis activation in response to stressors. An equally important mechanism involves the GABAergic system, which

mediates the inhibitory effects of GABA within the central nervous system. To analyze the impact of prenatal stress on the GABAergic system, baclofen, a GABA-B receptor agonist, was used. Baclofen was administered subcutaneously at a dose of 10 mg/kg b.w.t to 6-month-old rats 30 min prior to a one-hour immobilization session. Immediately after acute stress, animals were sacrificed by decapitation to collect blood samples.

Activation of GABA-B receptors suppressed the stress-induced increase in plasma corticosterone in control males and females but failed to prevent this response in prenatally stressed animals of both sexes. Thus, prenatal stress appears to program a reduction in sensitivity, or even refractoriness, of GABA-B receptors under conditions of acute stress. Similar results were obtained by S. Tkachuk, V. Pishak & V. Myslytsky from the Bukovinian State Medical Academy (Chernivtsi, Ukraine), who applied intracisternal administration of baclofen at a dose of 2.5 μg prior to one-hour immobilization in prenatally stressed or control adult rats (Reznikov et al., 2004b). Furthermore, using muscimol, a GABA-A receptor agonist, at a dose of 15 μg in analogous experiments, the same authors demonstrated the involvement of this receptor subtype in the long-term modification of HPA axis function in prenatally stressed males.

Comprehensive studies of the brain's stress-limiting system in prenatally stressed rats, conducted by S. Tkachuk, have also demonstrated disturbances in the interactions of melatonin, β-endorphin, and other peptides in modulating the HPA axis response to acute stress. In particular, administration of melatonin prior to one-hour immobilization in prenatally stressed adult males failed to increase β-endorphin levels in the POA and MBH, a response characteristic of intact animals.

Furthermore, extensive experimental evidence has been obtained regarding the status of cyclic nucleotide systems, prostaglandins, lipid peroxidation (LPO), protein free-radical oxidation, and other neurochemical parameters of the brain, all of which support the pathogenic role of prenatal stress in programming brain function. More detailed information on these findings is presented in our joint monograph (Reznikov et al., 2004b).

Summarizing the results concerning the functional state of the HPA axis in prenatally stressed animals, the following conclusions can be drawn. The prenatal stress syndrome is characterized by an imbalance between the brain's stress-activating and stress-limiting systems. In prenatally stressed males, the hypothalamus does not respond to acute stress with a reduction in norepinephrine concentration, whereas in females the normal response is preserved, which corresponds to the maintenance and even enhancement of the HPA axis response to acute stress.

Against the background of a weakened HPA axis response to acute stress, prenatally stressed males exhibit a paradoxical increase in the sensitivity of the hypothalamic component of the HPA axis to norepinephrine and a refractoriness of GABAergic receptors to agonists. These modifications are likely compensatory mechanisms for the insufficiency of the catecholaminergic component of the brain's stress-activating system.

Taken together, the results of studies on the mechanisms underlying HPA axis dysfunction in prenatally stressed animals lead to the conclusion that the primary cause of the attenuated HPA axis stress reactivity is the inadequacy of the hypothalamic norepinephrine response to stressors.

4.4. Pharmacological Analysis of Neurohormonal Pathways and Mechanisms of Stress-Induced Programming Disorders of the Neuroendocrine System and Behavior

To elucidate the pathways and mechanisms underlying the pathogenic impact of maternal stress on the programming of neuroendocrine functions during early ontogenesis, various pharmacological agents were employed in the laboratory. These included compounds with intrinsic hormonal activity, inhibitors of biosynthesis, receptor agonists and antagonists, as well as ion channel blockers.

First and foremost, it was essential to determine whether activation of the HPA axis indeed plays a decisive role in the pathogenesis of the prenatal stress syndrome. Experiments on adrenalectomized pregnant rats and their offspring demonstrated the absence of increased anxiety in both males and females, as well as the lack of spatial learning deficits in males (effects typically observed in prenatally stressed animals) (Zagron & Weinstock, 2006). However, adrenalectomy eliminates not only the secretion of corticosterone but also that of the adrenal medulla, which produces adrenaline. Secondly, the authors' conclusion that corticosterone mediates the maladaptive programming of behavior does not account for abnormalities in sexual behavior, which represent a hallmark feature of the prenatal stress syndrome. Finally, doubts regarding the mediatory role of corticosteroids in the pathogenesis of prenatal stress syndrome are reinforced by reports showing no significant differences in plasma corticosterone concentrations between intact and stressed rats on GD 21 (Ward & Weisz, 1984). We confirmed these findings in our own experiments performed on days 15 and 21 of gestation. Immediately following a one-hour immobilization stress on day 15, plasma corticosterone levels were 747 ± 28 nmol /L compared with a basal level of 711 ± 84 nmol /L. On day 21, values were 848 ± 112 nmol/Land 1110 ± 72 nmol /L, respectively ($p > 0.05$).

Although the involvement of glucocorticoids in the pathogenesis of the prenatal stress syndrome is supported by the similarity of some of its manifestations to the effects of exogenous glucocorticoids (refer to Chapters 2 and 3), important differences also exist. For example, according to our data, administration of hydrocortisone acetate to rats during the final week of pregnancy did not reproduce the early prenatal stress-induced changes in hypothalamic norepinephrine content or in the HPA axis response to intracerebral administration of norepinephrine bitartrate in adult offspring. Furthermore, it has been reported that ACTH and corticosterone are not implicated in the abnormalities of sexual behavior observed in prenatally stressed rats. Since corticosteroids exert their biological effects via receptor-mediated signaling, the role of glucocorticoid receptors in mediating the programming effects of prenatal stress on behavioral sexual dimorphism, the balance between stress-activating and stress-limiting systems of the brain, and other neuroendocrine mechanisms remained unresolved.

Preventive Effect of Dexamethasone. To address these questions, we employed the synthetic glucocorticoid dexamethasone, which is commonly used in experimental studies to suppress the stress response of the HPA axis. This feature of dexamethasone

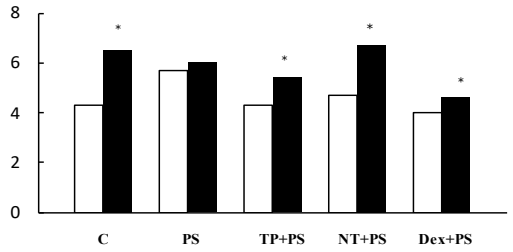

Fig. 4.5. Comparative preventive effects of pharmacological agents with different mechanisms of action on sex differences in the content of the 66 kDa protein in the POA of the brain of 5-day-old prenatally stressed rats.

Footnotes: The ordinate shows the percentage of the total area under the curve of the electrophoretic densitogram. Open bars – females; black bars – males. C – control, PS – prenatal stress, TP – testosterone propionate, NT – naltrexone, Dex – dexamethasone. *P<0.05 compared with females, Wilcoxon–Mann–Whitney *U* test.

is attributed to its high glucocorticoid activity, resulting from strong affinity for glucocorticoid receptors and weak binding to plasma proteins. Pregnant female rats were subjected to a standard procedure of one-hour daily immobilization stress from GD 15 to 21. A subset of these animals received intramuscular injections of dexamethasone at a dose of 100 μg/kg b.w., administered 30 min. before immobilization. Control animals were injected with isotonic sodium chloride solution. Both early and delayed effects of these interventions were examined.

In the POA, MBH, and hippocampus of 5-day-old offspring, the electrophoretic spectrum of soluble cytosolic proteins was analyzed according to m.m. For protein extraction, pooled tissue samples from 5–7 animals were used for each measurement.

Similar to our previous studies, the POA of the brain in normal animals displayed sexual dimorphism in specific proteins within the m.m. range of 14.3–66.0 kDa. The relative abundance of proteins with m.m. of 14.3 and 24.0 kDa was higher in females, whereas proteins of 34.7 and 66.0 kDa predominated in males. No sex differences were detected in the protein spectrum of the MBH.

Prenatal stress abolished the sex differences in the relative abundance of the 66.0 kDa protein in the POA due to its elevation in females. Dexamethasone blockade restored the sexual dimorphism of this parameter, although the effect was somewhat attenuated (Fig. 4.5).

Aromatase activity in the early postnatal period clearly reflects both the direction and neurochemical mechanism of sexual differentiation of the brain. Therefore, it is essential to trace the causal relationship between disturbances in testosterone metabolism within brain structures involved in the regulation of sexual behavior during early ontogenesis and the characteristics of male sexual behavior in adulthood in prenatally stressed animals exposed to dexamethasone.

As demonstrated above, stress during pregnancy leads to a reduction in enzyme activity in the POA of the male brain, thereby abolishing sex differences in the offspring. On PND 10, the offspring of females subjected to stress against the background of dexamethasone administration showed no changes in aromatase activity in the POA, indicating preservation of sexual dimorphism. In the MBH, no significant alterations in aromatase activity were detected in any of the experimental groups (Fig. 4.6).

Prenatal stress induced an almost twofold increase in the activity of steroid 5α-reductase in the MBH of males, whereas dexamethasone blockade prevented these alterations. In the examined discrete hypothalamic structures of control females, 5α-reductase activity was nearly twice as high as in males. Following prenatal dexamethasone blockade, the

HPA axis response to immobilization was accompanied by enhanced 5α-reduction of testosterone in the POA of males, whereas in females it decreased in the MBH. This resulted in the disappearance of sex differences in enzymatic activity within the MBH and their inversion in the POA.

In full accordance with the protective effect of dexamethasone on aromatase activity in the preoptic region of the male brain, the results of investigations into male sexual behavior at the age of three months were obtained (Table 4.6). As is well established, male sexual behavior comprises two major components: sexual motivation (libido) and the copulatory act itself (sexual capacity, or potency). These two components are regulated by distinct mechanisms: the "appetitive" phase (number of mountings, latency of intromissions, and post-ejaculatory refractory period) is governed by central mechanisms, whereas the copulatory phase (number of intromissions and ejaculations) is regulated by the peripheral nervous system. Prenatal stress disrupted both of these components. Prenatal stress significantly prolonged the latency to the first mounting and reduced the number of ejaculations during both the first and the second testing sessions.

Furthermore, in the second testing session, males exposed to prenatal stress demonstrated an increased latency to the first intromission. Prenatal dexamethasone administration largely normalized male sexual behavior, with the exception of the number of ejaculations during the second testing session: this parameter remained significantly lower compared with the control groups, although still higher than in prenatally stressed rats. At the same time, in comparison to the results of the first testing, this parameter remained practically unchanged.

At first glance, there appears to be a contradiction between the preventive effect of dexamethasone on stress-induced alterations in aromatase activity within the POA and on male sexual behavior, and its detrimental influence on reproductive system parameters when administered to non-stressed pregnant rats (refer to Chapter 3). However, it is well established that dexamethasone administration to females during the last third of pregnancy produces a fourfold reduction in plasma corticosterone levels on GD 18, and in their adult male offspring induces demasculinization and feminization of sexual behavior (Holson et al., 1995). Nevertheless, glucocorticoid deficiency in pregnant rats is relative, since it can be assumed that, under dexamethasone treatment, the total biological activity of maternal glucocorticoids during the critical period of sexual differentiation of the offspring brain differs little from the physiological norm.

Thus, the results of the present study suggest that dexamethasone, when administered prior to maternal stress exposure, interrupts the cascade of physiological processes in

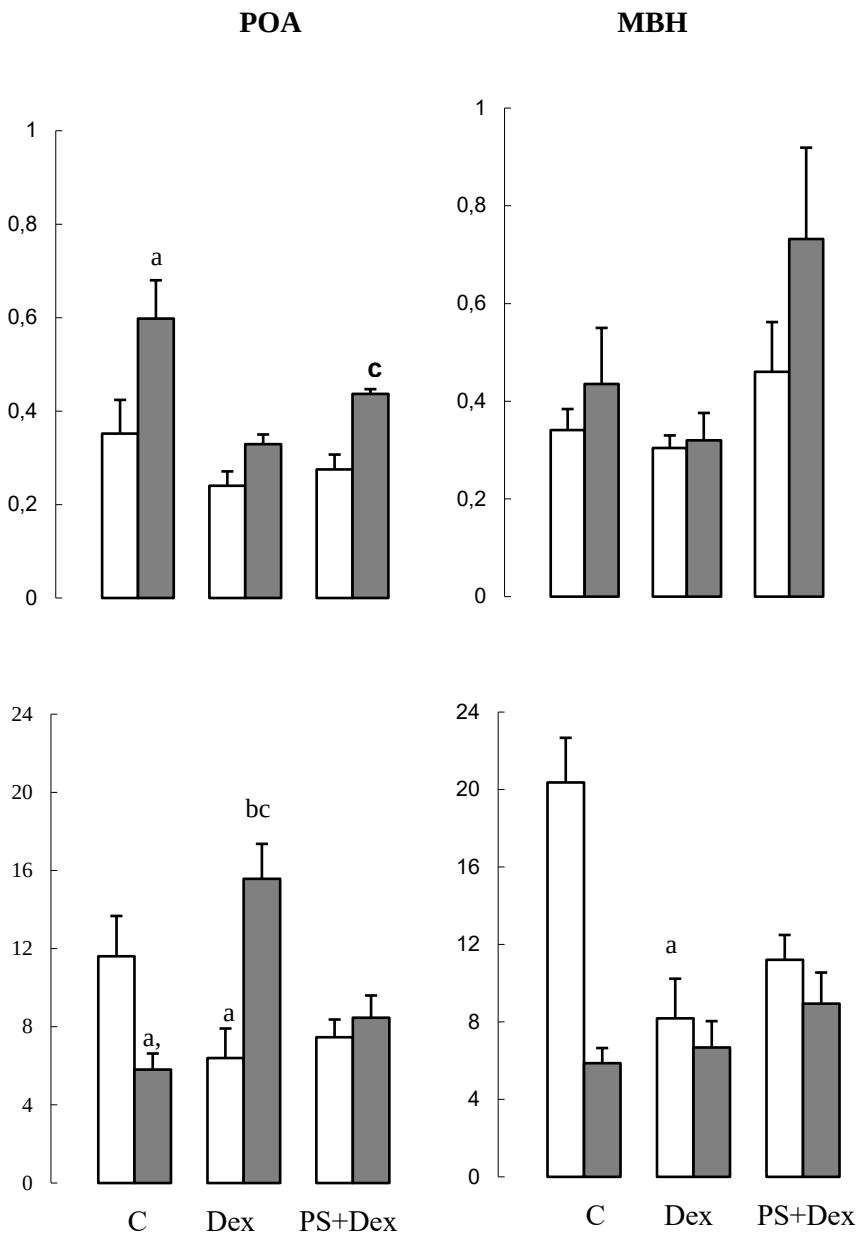

Fig. 4.6. Aromatase (upper panel) and 5α-reductase (bottom panel) activities in the POA and MBH of 10-day-old rats prenatally treated with dexamethasone (Dex) or prenatally stressed (PS) under Dex administration

Footnotes: The ordinate axis represents aromatase (upper panel, pmol E_2/h/g tissue) and 5α-reductase (bottom panel, 5α-reduced metabolites /h/g tissue) activity. Open bars – females, gray bars – males. [a] $p < 0.05$ compared with the control females; [b] $p < 0.05$ compared with the control males; [c] $p < 0.05$ for difference between females and males within the same group.

Table 4.6. Effect of prenatal stress under dexamethasone treatment on parameters of male sexual behavior in male rats (M ± SEM)

Parameter	Control	Prenatal stress	Prenatal stress + dexamethasone
		First testing	
Latency period (s):			
First mounting	10.8 ± 2.9	33.6 ± 7.8 *	28.2 ± 8.2
First intromission	15.8 ± 4.3	38.1 ± 10.6	32.2 ± 9.8
First ejaculation	617.5 ± 87.1	727.8 ± 44.3	657.2 ± 59.2
Number of:			
Mountings without intromissions	0.2 ± 0.2	0.8 ± 0.3	0.7 ± 0.2
Intromissions	11.5 ± 1.0	13.9 ± 1.4	9.5 ± 1.2
Ejaculations	2.2 ± 0.2	1.1 ± 0.2	2.0 ± 0.3
		Second testing	
Latency period (s):			
First mounting	3.5 ± 0.5	12.3 ± 0.9 *	5.8 ± 1.9
First intromission	4.0 ± 0.6	25.0 ± 12.0 *	9.5 ± 4.1
First ejaculation	368.8 ± 56.7	470.8 ± 81.4	421.5 ± 57.1
Number of:			
Mountings without intromissions	0.5 ± 0.5	1.2 ± 0.6	1.0 ± 0.4
Intromissions	23.5 ± 3.4	15.8 ± 4.4	22.5 ± 2.5
Ejaculations	3.5 ± 0.3	1.5 ± 0.2 *	2.3 ± 0.2 *

Footnotes: * $p < 0.05$ compared with control males; each group contained six rats.

both the mother and fetus that contribute to disturbances in sexual differentiation of the brain, in particular testosterone aromatization. In adult males, such dexamethasone pretreatment leads to near-complete normalization of both the motivational and copulatory components of sexual behavior. Overall, the preventive effect of dexamethasone on the development of the prenatal stress syndrome can be attributed to pharmacological blockade of the HPA axis response and, indirectly, to suppression of the hypothalamic-pituitary-testicular system's response to stress.

Table 4.7. Effect of one-hour immobilization (acute stress) on norepinephrine (NE) and dopamine (DA) content in the hypothalamus of control and prenatally stressed (PS) 6-month-old male rats exposed to dexamethasone (Dex) during the prenatal period (M ± SEM)

Parameter	NE, nmol/g/tissue	DA, nmol/g/tissue
	Experiment 1	
Control:		
Basal level	8.36 ± 0.36	4.64 ± 0.20
Acute stress	6.34 ± 0.17 *	4.82 ± 0.15
PS + Dex:		
Basal level	8.00 ± 0.17	5.37 ± 0.21
Acute stress	7.21 ± 0.59	5.29 ± 0.49
	Experiment 2	
Control:		
Basal level	7,2 ± 0,3	4,8 ± 0,4
Acute stress	5,4 ± 0,4 *	4,4 ± 0,4
PS + Dex:		
Basal level	6,8 ± 0,5	4,7 ± 0,3
Acute stress	5,4 ± 0,3 *	4,9 ± 0,2

Footnotes: * $p < 0.05$ compared with basal level; each group contained 5-6 rats.

Another compelling line of evidence for the protective role of dexamethasone administered prenatally comes from investigations of brain catecholamine status and HPA axis stress response to noradrenergic stimulation. These experiments were performed on adult offspring (males and females in diestrus) at the age of 6 months. For comparison, certain parameters were also assessed in prenatally stressed animals that did not receive dexamethasone.

A similar pattern was observed for hypothalamic norepinephrine content. In prenatally stressed male and female rats exposed to dexamethasone during the prenatal period, the stress-induced norepinephrine response to one hour of immobilization was preserved, resembling that of intact controls, *i.e.* indicating a protective effect of this synthetic corticosteroid on the catecholaminergic component of the stress-activating system (Table 4.7, 4.8). No hypothalamic dopamine response to stress was observed in any of the experimental groups.

The state of GABA receptor systems was evaluated using the GABA-B receptor agonist baclofen and the GABA-A receptor agonist muscimol, which were administered intraperitoneally at doses of 10 mg/kg and 0.1 mg/kg, respectively, 30 min before the immobilization stress.

Table 4.8 Effect of one-hour immobilization (acute stress) on hypothalamic norepinephrine (NE) and dopamine (DA) content in control and prenatally stressed (PS) 6-month-old female rats, exposed to dexamethasone (Dex) during the prenatal period (M ± SEM)

Parameter	NE, nmol/g/tissue	DA, nmol/g/tissue
Control:		
Basal level	7.9 ± 0.3	5.6 ± 0.3
Acute stress	4.9 ± 0,2 *	5.0 ± 0.1
PS + Dex:		
Basal level	7.8 ± 0.3	4.8 ± 0.3
Acute stress	5.5 ± 0.2 *	4.6 ± 0.2

Footnotes: * $p < 0.001$ compared with basal level; each group contained 5-6 rats.

Similar to 3-month-old males, prenatally stressed 6-month-old males exhibited a reduced corticosterone response in plasma following one hour of immobilization stress. However, when pregnant mothers had received dexamethasone prior to stress exposure,

their 6-month-old male offspring displayed an adrenocortical response to acute stress that was indistinguishable from that of intact animals, although their basal corticosterone levels were reduced by 24% (p <0.05).

It has been previously reported that in prenatally stressed adult male rats, both GABA-A and GABA-B receptors exhibit refractoriness to their respective agonists, muscimol and baclofen, when assessed through the adrenocortical response to acute stress. Similar experiments were conducted in males born to dams that had been exposed to stress during the final week of gestation under conditions of prior administration of these agonists.

The basal plasma corticosterone level in adult males stressed under dexamethasone administration was significantly lower compared to intact control animals (Table 4.9). However, the adrenocortical response to a one-hour immobilization was markedly elevated: the post-stressor increase in plasma corticosterone in experimental rats averaged 170%, in contrast to 90% in controls. The relative adrenal weight in experimental males under resting physiological conditions was also significantly increased (intact males: 8.2 ± 0.5 mg/100 g b.w.; experimental males: 11.3 ± 0.6 mg/100 g b.w., $p < 0.05$).

In prenatally stressed females, prenatal dexamethasone administration did not affect basal corticosterone secretion; however, it induced a moderate enhancement of corticosterone release under conditions of acute stress (Table 4.9). Immobilization-induced corticosterone levels increased by 120% in experimental females versus 70% in controls. A similar moderate potentiation of the stress-induced adrenocortical response had previously been observed in prenatally stressed female rats.

In control male and female rats, baclofen administration 30 min prior to one-hour immobilization resulted in a reduction of the adrenocortical response to acute stress by 60% and 30%, respectively. In males, plasma corticosterone levels returned to baseline values, whereas in females they remained elevated during experiment. In prenatally stressed rats treated with dexamethasone, C administration also attenuated the stress-induced adrenocortical response, although to a lesser extent compared with the control animals.

In experiments employing the GABA-A receptor agonist muscimol, both control and experimental males demonstrated a comparable and statistically significant reduction of plasma corticosterone levels after stress (by 15.3% and 17.4%, respectively). However, in both groups, corticosterone concentrations remained elevated relative to basal levels. Notably, the amplitude of the stress-induced adrenocortical response under muscimol

Table 4.9 Plasma corticosterone levels (M ± SEM nmol/L) after one-hour immobilization (acute stress) and pre-stressor administration of the GABA-B receptor agonist baclofen in sexually mature intact (control) and prenatally stressed (PS) rats exposed to dexamethasone (Dex) during the prenatal period

Sr. No.	Animal group	Control	Dex + PS
		Males	
1	Basal level	668.4 ± 27.7 (16)	457.2 ± 72.6 (5) *
2	Acute stress	1276.3 ± 45.4 (11)	1245.6 ± 78.3 (5)
	$p_{1,2}$	< 0.001	< 0.001
3	Baclofen + acute stress	819.4 ± 95.1 (6)	998.3 ± 28.9 (6)
	$p_{1,3}$	> 0.5	< 0.001
	$p_{2,3}$	< 0.001	< 0.02
		Females	
4	Basal level	550.5 ± 62.3 (5)	581.9 ± 103.4 (5)
	$p_{1,4}$		> 0.5
5	Acute stress	981.5 ± 17.2 (5)	1297.9 ± 38.8 (5) *
	$p_{4,5}$	< 0.001	< 0.001
6	Baclofen + acute stress	763.6 ± 49.6 (6)	1078.9 ± 40.3 (6) *
	$p_{4,6}$	< 0.02	< 0.001
	$p_{5,6}$	< 0.02	< 0.01

Footnote: * $p < 0.05$ compared with basal level; number of rats is presented in parentheses.

administration in males prenatally exposed to dexamethasone (2.5-fold increase) exceeded that observed in control males (1.6-fold increase) (Table 4.10).

Thus, the obtained data demonstrated that maternal stress during pregnancy under dexamethasone administration did not exert a damaging effect on the formation of the stress response of the HPA axis in adult male offspring. In adult males, the characteristic

Table 4.10 Plasma corticosterone levels (M ± SEM, nmol/L) after one-hour immobilization (acute stress) and pre-stressor administration of the GABA-A receptor agonist muscimol in sexually mature prenatally stressed (PS) male rats exposed to dexamethasone (Dex) during the prenatal period

Sr. No.	Animal group	Control	Dex + PS
1	Basal level	587.4 ± 73.3	409.7 ± 30.9
2	Acute stress	1126.9 ± 22.4	1243.8 ± 27.4 *
	$p_{1,2}$	< 0.001	< 0.001
3	Muscimol + acute stress	955.2 ± 60.7	1027.9 ± 36.0
	$p_{1,3}$	< 0.05	< 0.001
	$p_{2,3}$	< 0.01	< 0.001

Footnote: * $p < 0.05$ compared with control; each group contained 5-6 rats.

post-stress changes typical of normal animals were preserved, including alterations in hypothalamic norepinephrine content and plasma corticosterone levels, alongside the retained ability of GABA-A and GABA-B receptors to participate in stress-induced activation. This effect may be attributed to dexamethasone-mediated blockade of HPA axis stress activation, while the combined action of synthetic and endogenous glucocorticoids appeared insufficient to impair fetal HPA axis under maternal stress.

In contrast to males, no preventive effect of dexamethasone on HPA axis stress reactivity was observed in females. In experimental females, prenatal dexamethasone exposure, similar to prenatal stress alone, resulted in a moderate enhancement of the adrenocortical response to a stressor, while GABA-B receptor activity remained preserved. It is likely that under pharmacological blockade of the HPA axis, other regulatory systems involved in HPA axis dysfunction, such as opioid mechanisms, may remain active.

Norepinephrine reactivity of the HPA axis under conditions of pre-stressor prenatal dexamethasone administration was investigated in sexually mature (8-month-old) non-anesthetized males and females (in diestrus) implanted with a cannula into the third cerebral ventricle and a Silastic catheter in the jugular vein. In all experimental groups, the adrenal response to central noradrenergic stimulation was unidirectional, manifesting as an increase in plasma corticosterone 30 min after norepinephrine administration, which remained significantly elevated compared to baseline even after 60 min. The only difference observed between offspring of intact and stressed dams treated with dexamethasone concerned the amplitude of the adrenocortical response at the 30-min

time point: in males, it was more pronounced in control animals (three-fold increase) compared to prenatally dexamethasone-exposed males (2.4-fold increase). In females, the opposite trend was observed, with the adrenocortical response being more pronounced in the experimental group than in controls (2.1-fold *vs.* 1.8-fold) (Table 4.11).

Thus, the similar direction and magnitude of corticosterone elevation in both male and female animals of control and experimental groups in response to central noradrenergic stimulation indicate the protective properties of prenatal dexamethasone administration in prenatally stressed animals with respect to HPA axis responsiveness.

The preventive effect of dexamethasone on the development of the prenatal stress syndrome can be explained by pharmacological blockade of HPA axis activity and, indirectly, by preventing the inhibitory response of the hypothalamic–pituitary–testicular system to stress. Based on the dualistic concept of the HPA axis response to stress (defined by two opposing mechanisms, stress-executing and stress-limiting) it is reasonable to assume that programming of the prenatal stress syndrome could be prevented not only through blockade of corticosteroid receptors, but also by other

Table 4.11 Effect of norepinephrine bitartrate application into the third ventricle of the brain on plasma corticosterone concentrations (M ± SEM, nmol/L) in rats prenatally stressed (PS against the background of dexamethasone (Dex) treatment

Animal group	Time of blood sampling		
	0 min	30 min	60 min
Males:			
Control	666 ± 36	2000 ± 141 [a]	1320 ± 92 [a]
PS + Dex	626 ± 34	1545 ± 74 [ab]	1309 ± 85 [a]
Females:			
Control	1152 ± 144	2060 ± 86 [a]	1832 ± 98 [a]
PS + Dex	1044 ± 114	2187 ± 284 [a]	2084 ± 12 [a]

Footnotes: [a] $p < 0.05$ compared with the basal level; [b] $p < 0.05$ compared with the corresponding time point in control animals. Each group contained six rats.

approaches, such as enhancement of GABAergic signaling by GABA agonists, inhibition of catecholamine synthesis in the hypothalamus, elimination of androgen deficiency during sexual differentiation of the brain, or modulation of opioid pathways affecting the HPG axis. These possibilities were systematically tested using various pharmacological agents.

Preventive Effects of GABA Agonist. One pharmacological approach to blocking HPA axis stress responses in pregnant rats involves the use of phenibut, a well-known tranquilizer and nootropic agent with agonistic properties at GABA receptors, the key component of the stress-limiting system of the central nervous system. The study investigated the prenatal effects of immobilization stress during GD 15–21 under phenibut treatment on the timing of puberty, sexual behavior, and HPA axis in the offspring. The drug was suspended in Dorfman's gel and administered intragastrically *via* a metal probe at a daily dose of 100 mg/kg b.w. 30 min before immobilization. Control pregnant females received Dorfman's gel only. The objective of the study was to clarify the role of GABA in the disruption of neuroendocrine development during early life under prolonged stress exposure and to evaluate the possibility of preventing pathological manifestations of prenatal stress through activation of stress-limiting GABA-dependent mechanisms.

Maternal stress during pregnancy caused a significant reduction in aromatase activity in the POA but did not affect enzyme activity in the MBH of male offspring. In contrast to aromatase activity, 5α-reductase activity (*i.e.*, the sum of 5α-dihydrotestosterone and 5α-androstan-3α,17β-diol formation) remained unchanged in both brain regions of prenatally stressed males.

Phenibut administration prior to maternal stress prevented the prenatal stress-induced early postnatal alterations in aromatase activity in the POA of the offspring's brain, which may indicate the protective action of the drug with respect to the programming of male sexual behavior. At the same time, 5α-reductase activity remained unchanged in both examined hypothalamic regions (Table 4.12).

Prenatal stress caused a significant delay in male sexual maturation: testicular descent into the scrotum occurred at 38.8 ± 0.29 days postpartum in the control group, whereas in the experimental group this process was delayed until 48.0 ± 0.21 days ($P<0.001$). In contrast, under phenibut administration, the adverse effect of prenatal stress was attenuated: the delay in puberty was reduced by half, with testicular descent observed at 43.5 ± 0.17 days ($P<0.001$). Thus, phenibut exerted a partial protective effect on the disturbances in the timing of sexual maturation in prenatally stressed males.

The results of the study of male-type sexual behavior in 3-month-old male rats are presented in Table 4.13. Temporal and quantitative parameters of male-type sexual behavior were analyzed during a 30-min period of cohabitation with a hormonally primed receptive female, conducted twice with a one-week interval.

Prenatal stress in young 3-month-old male rats led to a threefold prolongation of the latency to the first mounting and a delay in the first intromission. These disturbances

represent manifestations of the demasculinizing effect of prenatal stress on the nervous mechanisms regulating male copulatory behavior in rats. The degree of these alterations

Table 4.12 Effect of prenatal phenibut administration on aromatase activity (pmol estradiol/h/g tissue) and 5α-reductase activity (pmol 5α-reduced metabolites/h/g tissue) in the POA and MBH of prenatally stressed male rats at PND 10

Animal group	POA	MBH
	Aromatase	
Control	0.540 ± 0.003	0.438 ± 0.015
Prenatal stress	0.423 ± 0.031*	0.409 ± 0.180
Prenatal stress + phenibut	0.538 ± 0.035	0.491 ± 0.053
	5α-reductase	
Control	3.19 ± 1.01	6.01 ± 2.06
Prenatal stress	5.86 ± 2.59	9.78 ± 0.87
Prenatal stress + phenibut	6.00 ± 2.53*	9.35 ± 1.83

Footnotes: * $p < 0.05$ compared with the control animals. Each group contained ten rats; pooled tissue samples from two animals were used in each assay.

was reduced during the second testing, which was expected and associated with the acquisition of sexual experience. The number of mountings on receptive females without intromissions, as well as the number of intromissions, did not significantly differ from control values. However, in both tests, the number of mountings with intromissions was reduced by 1.5–2-fold.

Phenibut administration prior to maternal stress completely prevented the prolongation of the latency to the first mounting and the first intromission. The number of mountings with intromissions increased 1.5-fold in both tests and did not statistically differ from control values, while the difference compared to prenatally stressed animals in the first test approached statistical significance.

All males from the experimental groups demonstrated lordosis responses when in contact with active males, whereas no lordotic behavior was observed in control animals. Phenibut attenuated the feminizing effect of prenatal stress on female-type sexual behavior in male offspring. This was evidenced by an almost one-third reduction in the

Table 4.13 Parameters of male-type sexual behavior in 3-month-old rats prenatally stressed (PS) against the background of phenibut treatment (M ± SEM)

Parameter	Control (n = 5)	PS (n = 4)	PS + phenibut (n = 5)
	First testing		
Latency period (s):			
First mounting	39.4 ± 1.0	126.0 ± 32.1	27.0 ± 12.0 [b]
First intromission	47.4 ± 11.4	163.6 ± 44.0	28.8 ± 12.4 [b]
Number of:			
Mountings without intromission	14.8 ± 2.5	13.6 ± 0.7	14.4 ± 1.9
Intromissions	40.8 ± 2.4	22.4 ± 2.4 [a]	33.4 ± 5.8 [a]
	Second testing		
Latency period (s):			
First mounting	4.0 ± 1.1	12.2 ± 2.5 [a]	4.0 ± 0.6 [b]
First intromission	4.8 ± 1.2	13.0 ± 2.5 [a]	5.0 ± 0.6 [b]
Number of:			
Mountings without intromission	11.0 ± 3.2	12.2 ± 1.4	7.0 ± 0.6 [b]
Intromissions	43.0 ± 5.9	29.4 ± 1.6 [a]	34.2 ± 3.2

Footnotes: [a] $p < 0.05$ compared with the control animals; [b] $p < 0.05$ compared to prenatally stressed animals; n –numbers of rats.

number of lordosis responses during testing of female-type sexual behavior in males: on average, from 10.0 in prenatally stressed animals to 7.2 under phenibut treatment (testing duration was 10 min or until 10 mountings by the active male).

Based on the obtained data, it can be concluded that the GABAergic stress-limiting system is involved in the pathogenesis of disturbances in sexual maturation and sexual behavior in male rats induced by prenatal stress. The protective action of phenibut on behavioral disorders is likely mediated by activation of the brain's stress-limiting system, which counteracts disturbances of hormonal and neurotransmitter balance. Considering that prenatal stress disrupts the formation of social behavior not only in animals (see above) but also in humans (Barrett et al., 2014), the use of phenibut and

possibly other GABA receptor agonists during maternal and fetal stress may be of potential benefit. At the same time, the risk–benefit ratio of their administration for maternal and fetal health must be carefully evaluated.

The features of the HPA axis hormonal response to acute stress (one-hour immobilization) in sexually mature male rats, whose mothers received phenibut prior to immobilization stress during the final week of pregnancy, were studied at the age of 6 months.

In prenatally stressed animals, the response to one-hour immobilization was markedly attenuated: the hormone level increased by only 35%, compared to 78% in the control group, which is consistent with the findings of our previous studies. Phenibut prevented the suppression of HPA axis stress reactivity in prenatally stressed males: the post-stress increase in corticosterone level was even higher than in controls, amounting to 123% compared to 78% in the control group and 35% in prenatally stressed animals. In all three groups, the basal plasma corticosterone concentration did not differ significantly (Table 4.14).

Table 4.14 Effect of combined prenatal stress against the background of phenibut treatment on plasma corticosterone levels (M ± SEM, nmol/L) in 6-month-old male rats before and following one-hour immobilization (acute stress)

Sr. No.	Animal group	Basal level	Acute stress	*p*
1	Control	544 ± 83	971 ± 42	< 0.001
2	Prenatal stress	479 ± 27	646 ± 19	< 0/001
	$P_{1,2}$	> 0.5	<0.001	
3	Prenatal stress + phenibut	571 ± 85	1272 ± 78	< 0.001
	$p_{1,3}$	> 0.5	< 0.01	
	$p_{2,3}$	> 0.1	< 0.001	

Footnotes: p - compared with the basal hormone level within the corresponding animal group. Each group contained ten rats.

Prenatal stress induced a prolongation of the adrenocortical response to intracerebroventricular administration of norepinephrine bitartrate into the third cerebral ventricle. Prenatal administration of phenibut did not prevent the effect of prenatal stress on alterations of the adrenocortical response to norepinephrine stimulation (Table 4.15).

The role of GABA in the regulation of the stress response is determined by its ability to influence various components of the HPA axis as well as extrahypothalamic structures *via* GABA receptors. An important mechanism of GABA involvement in the implementation of stress responses is its colocalization with the factors of the stress-executing system. Hypothalamic neurons that synthesize CRH and proopiomelanocortin are GABAergic (Blasquez et al., 1994), and the majority of GABA-immunopositive neurons in the *locus coeruleus* of rats are simultaneously noradrenergic (Jijima et al., 1992). In the mechanisms of GABAergic regulation of ACTH secretion through corticosteroid feedback, GABA receptors of the adenohypophysis are involved. Moreover, GABA can directly affect the adrenal hormonal response to stress. Conversely, changes in corticosterone and ACTH levels in the blood influence the content, metabolism, transport, and receptor activity of GABA in the hypothalamus and hippocampus, which are the central structures of neuroendocrine regulation of stress responses (Myshunina & Kononenko, 1997).

Table 4.15 Effect of administration of norepinephrine bitartrate into the third ventricle of the brain on plasma corticosterone levels (M ± SEM, nmol/L) in 8-month-old male rats prenatally stressed against the background of phenibut treatment

Animal group	Time of blood sampling			
	0 min	30 min	60 min	90 min
Control	715 ± 68	961 ± 69 [a]	767 ± 60	602 ± 55
Prenatal stress	854 ± 60	1152 ± 58 [a]	1259 ± 47 [ab]	936 ± 57 [b]
Prenatal stress + phenibut	952 ± 62 [b]	1228 ± 47 [ab]	1301 ± 45 [ab]	1052 ± 51 [b]

Footnotes: [a] $p < 0.05$ compared with the basal hormone level within the corresponding animal group; [b] $p < 0.05$ compared with the corresponding value in the control group. Each group contained five rats.

Phenibut acts as an indirect GABA mimetic. It is capable of crossing the blood-brain barrier and, by interacting with presynaptic GABA receptors, facilitates presynaptic release of GABA and its accumulation in synapses, thereby enhancing GABAergic inhibition of neuronal activity. These properties of phenibut most likely determine its protective effects against the consequences of prenatal stress.

GABA appears in the embryonic brain on day 15 (Vandenpol et al., 1995). In our experiments, this was precisely the stage when pregnant females were subjected to stress in the presence of phenibut administration. Considering the colocalization of GABA with the factors of the stress-executing system, as well as the possibility of its direct influence on extrahypothalamic structures and on both central and peripheral

components of the HPA axis involved in the regulation of stress responses, it can be hypothesized that the administration of the GABA agonist phenibut to pregnant females prior to stress exposure enhances the stress-limiting effect of the GABAergic system of the brain in relation to the implementation of prenatal stress.

Thus, stimulation of GABAergic mechanisms in the brain prevents the development of stress induced by immobilization of pregnant animals and thereby mitigates its adverse neuroendocrine and behavioral consequences in male offspring.

Preventive Effects of Methyldopa. According to current views on the role of the HPA axis in stress, noradrenergic mechanisms of the sympathetic nervous system occupy a central position in its activation. According to the literature, disturbances of sexual behavior in adult male rats subjected to prenatal stress can be prevented by administering tyrosine, an amino acid precursor of catecholamine synthesis, to their pregnant mothers (Rohde et al., 1989). The preventive action of tyrosine is associated with the restoration of hypothalamic norepinephrine levels, which are reduced as a consequence of stress.

One of the approaches to elucidating the involvement of norepinephrine in the cascade of physiological processes occurring in the pregnant mother and fetus during stress, which determine programming disturbances of the neuroendocrine system, was the use of methyldopa. Following its conversion into α-methylnorepinephrine, methyldopa exerts an inhibitory effect on catecholamine synthesis in the brain.

Using methyldopa, the role of sympathetic mediation was investigated in prenatal stress-induced modifications of postnatal changes in testosterone metabolism in the brain and in sexual behavior of male rats. The experiments were conducted on 10-day-old and 3-month-old Wistar male rats born to mothers assigned to three groups: (1) control females; (2) females exposed to chronic stress during the final week of pregnancy (immobilization for 1 h daily from day 15 to day 21); and (3) females that, during the same gestational period, received methyldopa at a dose of 400 mg/kg 30 min before immobilization. The drug was administered orally through a metallic gastric probe in the form of a suspension prepared from ground tablets using Dorfman's's gel as a vehicle.

The analysis of sexual behavior confirmed the presence of disturbances in prenatally stressed males, manifested as feminization and demasculinization. All males displayed lordosis behavior in response to mounting by an active male. In prenatally stressed males treated with methyldopa, female-type receptive behavior was weaker: the number of lordosis reactions decreased by one-third (from 7.5 ± 1.0 to 5.2 ± 0.9); however, this difference did not reach statistical significance compared to prenatally stressed animals. At the same time, it should be noted that homosexual behavior was observed in 4 of 5 examined rats of this group, expressed as mounting of an active male by castrated males.

Furthermore, 4 of 5 animals exhibited bisexual behavior, i.e., they simultaneously demonstrated both lordosis reactions and mounting attempts.

The assessment of male-type sexual behavior demonstrated that prenatal stress disrupted the central regulatory mechanism of male copulatory behavior (prolongation of the latency period to the first mounting and first intromission). Methyldopa not only failed to improve indices of copulatory activity but instead exacerbated them, particularly by

Table 4.16 Parameters of male sexual behavior in 3-month-old male rats following prenatal exposure to methyldopa (MD) and stress (M ± SEM)

Parameter	Control (n = 5)	Prenatal stress (n = 4)	Prenatal stress + MD (n = 5)
	First testing		
Latency period (s):			
First mounting	37.2 ± 4.9	51.0 ± 0.4 [a]	77.0 ± 13.3 [a]
First intromission	40.4 ± 5.3	53.7 ± 0.7	75.0 ± 11.1 [a]
Number of:			
Mountings without intromissions	5.8 ± 0.9	5.7 ± 1.5	4.8 ± 1.4
Intromissions	17.8 ± 3.9	13.5 ± 0.9	24.0 ± 4.7 [b]
	Second testing		
Latency period (s):			
First mounting	11.2 ± 2.4	22.2 ± 1.6 [a]	19.2 ± 1.9 [a]
First intromission	15.2 ± 2.6	31.7 ± 0.6 [a]	20.4 ± 2.0 [b]
Number of:			
Mountings without intromissions	13.2 ± 1.9	13.5 ± 0.9	6.2 ± 1.2 [ab]
Intromissions	22.6 ± 1.3	18.5 ± 2.3	23.2 ± 1.3

Footnotes: [a] $p < 0.05$ compared with the control males; [b] $p < 0.05$ compared to prenatally stressed males. Number of rats is presented in parentheses.

further prolonging the latency periods of the first mounting and first intromission and reducing the number of mountings without intromissions (Table 4.16).

It is well known that stress-induced activation of the HPA axis exerts an inhibitory effect on the reproductive system of animals. It is assumed that the reduction in hypothalamic LHRH production, which occurs as a result of stress-related increases in opioids and glucocorticoid hormones, leads to decreased pituitary LH secretion and, consequently, to reduced testosterone levels in the blood. In our studies, pharmacological inhibition of the sympathetic pathway of HPA axis activation by prestress administration of methyldopa prevented the stimulating effect of norepinephrine on HPA axis activity, which may reduce the inhibitory pressure of the HPA axis on the male reproductive system and restore androgen balance. Since testosterone is a substrate inducer of aromatase in neuroendocrine brain structures, the normalization of testosterone aromatization processes in the POA of male rats during the critical period of sexual differentiation of the brain becomes understandable.

Although prenatal administration of methyldopa exerted a pronounced protective effect against the demasculinizing influence of prenatal stress on aromatase activity in the POA, it was insufficient in mitigating its feminizing effect. Furthermore, prenatally stressed animals treated with methyldopa exhibited signs of homo- and bisexual behavior that were absent in normal males. These findings can be explained by the attenuation of norepinephrine action, which, as demonstrated in Chapter 2, plays a role as an inducer of androgen-dependent sexual differentiation of the brain.

The features of the HPA axis hormonal response to acute stress in adult prenatally stressed males treated with methyldopa were studied in 6-month-old rats.

In adult prenatally stressed males, plasma corticosterone level after acute stress increased on average by only 24%, compared to 73% in the control group (Table 4.17). In prenatally stressed males treated with methyldopa, the post-stress increase in plasma corticosterone levels amounted to 49%, which differed significantly from prenatally stressed males but did not differ statistically from controls.

At the age of 8 months, males were examined to assess the noradrenergic reactivity of the HPA axis. As in previous studies, prenatally stressed males responded to norepinephrine bitartrate administration into the third cerebral ventricle with a prolonged adrenocortical reaction. A similar pattern of adrenocortical response to central noradrenergic stimulation was observed in males prenatally stressed under methyldopa treatment. At the same time, it should be noted that the basal corticosterone level in these animals was significantly higher than in controls (Table 4.17).

Thus, prenatal administration of phenibut and methyldopa did not affect the changes in

Table 4.17 Effect of prenatal administration of methyldopa on plasma corticosterone levels (M ± SEM, nmol/L) in adult prenatally stressed male rats under basal conditions and following one-hour immobilization (acute stress)

Sr. No.	Animal group	Basal level	Acute stress	*p*
1	Control	565 ± 23	980 ± 39	< 0.001
2	Prenatal stress	489 ± 20,0	608 ± 12	< 0.001
	$p_{1,2}$	< 0.05	< 0.001	
3	Prenatal stress + methyldopha	542 ± 24	810 ± 78	< 0.002
	$p_{1,3}$	> 0.5	> 0.05	
	$p_{2,3}$	> 0.1	< 0.05	

Footnotes: p – significance of difference with the basal level within the corresponding animal group; each group contained ten rats.

noradrenergic reactivity of the HPA axis induced by prenatal stress in adult male rats. It is likely that mechanisms initiated by corticosteroids play a leading role in the

Table 4.18 Effect of norepinephrine bitartrate administration into the third ventricle of the brain on plasma corticosterone levels (M ± SEM, nmol/L) in male rats prenatally stressed against the background of methyldopa treatment

Animal group	Time of blood sampling			
	0 min	30 min	60 min	90 min
Control	670 ± 63	926 ± 67 [a]	728±57,5	553± 54
Prenatal stress	809 ± 70	1110 ± 57 [a]	1229 ± 52,8 [ab]	982 ± 54 [b]
Prenatal stress + methyldopa	918 ± 59 [b]	1180 ± 48 [b]	1261 ± 45 [ab]	1012 ± 50 [b]

Footnotes: [a] $p < 0.05$ compared to baseline level; [b] $p < 0.05$ compared with the corresponding value of the control group. Each group contained five rats.

pathogenesis of the prenatal stress syndrome, as evidenced by the prevention of its development by dexamethasone.

At the same time, it should be noted that the preventive effect of methyldopa on prenatal stress-induced alterations in HPA axis stress reactivity was rather moderate: the HPA axis response to stress stimulation approached the parameters typical of normal males. When analyzing the cause of such an effect of methyldopa, it is important to take into

account the multifactorial nature of the stress response, which involves, in addition to norepinephrine, other stress mediators such as corticoliberin, vasopressin, and opioid peptides. Apparently, pharmacological blockade of norepinephrine synthesis by methyldopa prior to stress exposure in pregnant dams does not prevent the release of other stress mediators.

In 6-month-old female offspring, the adrenocortical response to one-hour immobilization was examined. Prenatally stressed females responded to acute stress with a moderate increase in the hormonal activity of the adrenal cortex compared with the control animals. Prenatal administration of methyldopa produced a protective effect against the action of prenatal stress: the plasma corticosterone level after one-hour immobilization increased by 72%, compared to 97% in prenatally stressed animals (Table 4.19).

Table 4.19 Plasma corticosterone levels (M ± SEM, nmol/L) in 6-month-old female rats prenatally stressed under methyldopa treatment under basal conditions and following one-hour immobilization (acute stress)

Sr. No.	Animal group	Basal level	Acute stress	*p*
1	Control	619 ± 60	1126 ± 39	< 0.001
2	Prenatal stress	707 ± 42	1395 ± 36	< 0.001
	$p_{1,2}$	> 0.5	< 0.001	
3	Prenatal stress + methyldopa	634 ± 72	1091 ± 90	< 0.01
	$p_{1,3}$	> 0.05	> 0.05	
	$p_{2,3}$	> 0.05	< 0.001	

Footnote: Each group contained ten rats.

Thus, based on the obtained results, it can be postulated that the brain noradrenergic system is involved in the development of prenatal stress-induced disturbances of HPA axis stress reactivity in adult female offspring. The results of the present study also indicate a significant role of the central noradrenergic system in the mechanisms underlying the development of endocrine and behavioral disorders in male rats caused by prenatal stress.

The Role of Opioids in the Pathogenesis of the Prenatal Stress Syndrome. It is evident that pharmacological blockade of norepinephrine synthesis by methyldopa prior to stress exposure in pregnant animals does not prevent the release of other stress mediators, particularly β-endorphin. The enhanced production of endogenous opioids, especially β-

endorphin, is known to be associated with stress-induced activation of ACTH secretion. Chronic stress exposure in pregnant rats is accompanied by an increased content of opioids, including β-endorphin, in the hypothalamus and adenohypophysis of both the mother and the fetus (Rohde et al., 1990).

Autoradiographic studies of kappa-, delta-, and mu-opioid receptors in the offspring of immobilization-stressed female rats demonstrated a selective down-regulation of mu-opioid receptors (Sanchez & Milanes, 1996). These findings are consistent with data indicating that during the prenatal period of brain development in rats, only mu-opioid receptors are functionally active and mediate the effects of endogenous opioids (Volterra et al., 1986).

Administration of β-endorphin (33 μg, three times daily) to pregnant female rats during the final week of gestation, *i.e.* the critical period of sexual differentiation of the brain, was shown to program abnormalities of sexual behavior in adult male offspring (Kashon et al., 1992). These males approached females in estrus less frequently and more often exhibited female-type lordosis responses when exposed to sexually active males.

In our department, the state of neurochemical determinants of the prenatal stress syndrome, namely, hypothalamic aromatase and catecholamines, was investigated using a model of prenatal β-endorphin exposure. The study was based on the experimental model proposed by Kashon et al. (1992) for investigating sexual behavior, with slight modifications: β-endorphin was administered subcutaneously to pregnant females (33 μg three times daily) only on gestation days (GD) 16–18, covering the developmental period during which the male fetus is most sensitive to testosterone deficiency.

The effect of prenatal β-endorphin exposure on testosterone metabolism in discrete brain structures was studied in neonatal rats. It was found that the neurochemical basis of β-endorphin–induced disturbances in male brain sexual differentiation lies in the suppression of steroid aromatase activity in the POA. In normal control rats, sex differences in aromatase activity within the POA were pronounced: enzyme activity in males was four times higher than in females. The difference in the MBH did not reach statistical significance. On PND 10, the sex differences in aromatase activity in the POA of β-endorphin–exposed males were absent, due to a reduction in activity to the level observed in normal females (Fig. 4.7).

These findings indicate that prenatal β-endorphin exposure reproduces the effects of prenatal stress, specifically the elimination of sex differences in aromatase activity in the POA of neonatal rat brains.

In the POA, the sex differences in steroid 5α-reductase activity, which are normally higher in females, also disappeared due to a slight decrease in females and an increase

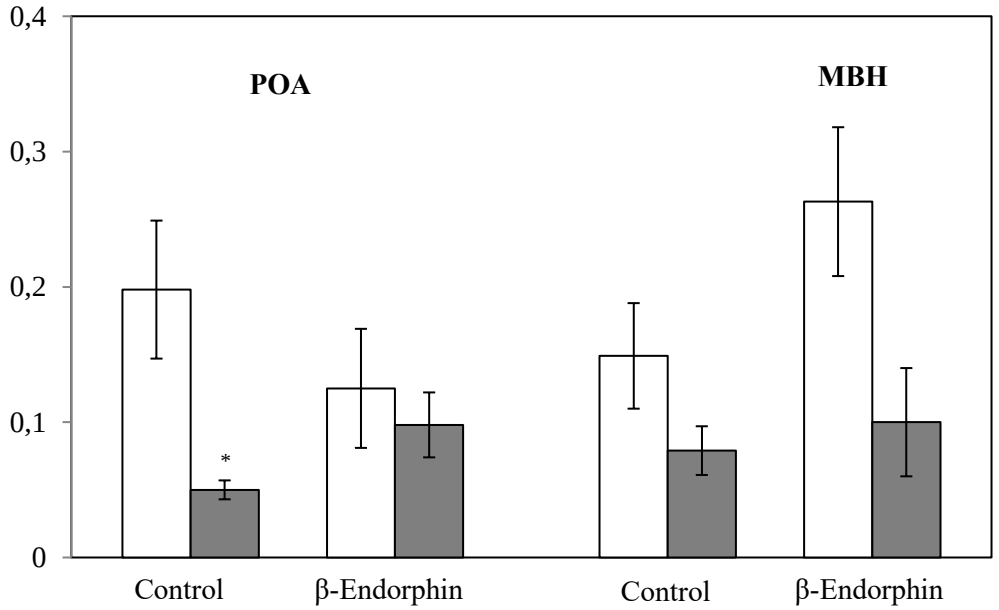

Fig. 4.7 Aromatase activity (pmol E_2/h/g tissue) in discrete brain regions of 10-day-old rat pups whose mothers received β-endorphin during GD 16–18
Footnotes: Open bars – males, gray bars – females; * $p < 0.05$ compared with males.

in males. In the MBH, prenatal exposure to β-endorphin did not affect 5α-reductase activity in offspring of either sex.

Normal levels of opioids in the brain during early ontogeny are essential for its proper development. Opioids can alter neurogenesis processes through a direct activating effect on neuronal systems involved in the maturation of the HPA axis. Chronic administration of the opioid receptor blocker naltrexone disrupts proliferation processes in certain brain regions depending on the dose (Zagon & McLaughlin, 1986). At high doses, naltrexone accelerates the maturation of cortical and hippocampal neurons and stimulates dendritic growth, while at low doses, it inhibits these processes (Hauser et al., 1987).

As a logical continuation of studies on the role of opioids in the pathogenesis of the prenatal stress syndrome, experiments were conducted involving blockade of endogenous opioids released into the bloodstream during stress. To achieve this, naltrexone, a competitive opioid receptor blocker, was used. Previous studies showed that administration of naltrexone during maternal stress prevented disturbances in the differentiation of sexual behavior in male offspring (Ward et al., 1986). However, the

neurochemical basis of this effect remained unknown, which motivated the present study conducted on offspring during early postnatal development. In addition, the components of male copulatory behavior were examined in greater detail, which had not been done in the cited work.

Experiments were conducted on male and female rats born to mothers that, 30 minutes before a one-hour stress exposure during the final week of pregnancy, received subcutaneous injections of naltrexone at a dose of 10 mg/kg b.w. The control group of pregnant rats received the vehicle — an isotonic sodium chloride solution.

Prenatal administration of naltrexone completely preserved sexual differences in aromatase activity in the POA of the hypothalamus in 10-day-old rats, which disappeared as a result of maternal stress.

The same applies to the norepinephrine content in the POA. In normal females, it was twice as high as in males. In the present study, sexual dimorphism was observed in the dopamine content of the MBH: 2.33 ± 0.11 nmol/g in females versus 3.82 ± 0.54 nmol/g tissue in males ($p < 0.05$). No sex differences were found in the norepinephrine content in the MBH or in the dopamine content in the POA. As a result of prenatal stress, sex differences in norepinephrine levels in the POA disappeared due to an increase in males, and sex differences in dopamine levels in the MBH disappeared due to an increase in females. However, administration of naltrexone exerted a preventive effect on these changes: the norepinephrine content in the POA retained its sexual difference, amounting to 4.03 ± 0.47 nmol/g tissue in females and 2.33 ± 0.17 nmol/g tissue in males ($p < 0.05$). In contrast, naltrexone did not affect the sexual dimorphism in dopamine content in the MBH.

The obtained experimental data are consistent with the protective effect of naltrexone on brain proteins that serve as markers of sexual differentiation and its disturbances. The experiments were conducted on 5-day-old prenatally stressed rats. The comparison group consisted of control rats of the same age. Soluble proteins of the cytosolic fraction from tissue homogenates of the POA of the hypothalamus, the MBH, and the hippocampus were separated by electrophoresis, stained, and analyzed using a densitometer.

Densitogram analysis showed that in the POA of newborn offspring of females that received naltrexone prior to stress exposure, the sex-specific differences characteristic of intact animals were preserved in the protein spectrum at m.m. of 14.3 kDa, 24.0 kDa, 34.7 kDa, and 66.0 kDa. The relative content of proteins with m.m. of 14.3 kDa and 24.0 kDa, expressed as a percentage of the total area under the densitometric curve, was

higher in females compared to males (by 21% and 22%, respectively, $p < 0.01$). In the range of proteins with m.m. of 34.7 kDa and 66.0 kDa, the protein bands were denser in

Table 4.20 Effect of prenatal stress against the background of naltrexone treatment on parameters of male sexual behavior in male rats (M ± SEM)

Parameter	Control	Prenatal stress	Prenatal stress + naltrexone
		First testing	
Latency period (s):			
First mounting	26.7 ± 3.8	57.1 ± 16.3	22.5 ± 7.3
First intromission	46.7 ± 8.7	116 ± 21 [a]	34.2 ± 12.6 [b]
First ejaculation	653 ± 86	756 ± 53	538 ± 90
Number of:			
Mountings without intromissions	2.3 ± 0.8	2.4 ± 0.8	1.8 ± 0.8
Intromissions	26.8 ± 2.1	15.6 ± 1.6 [a]	31.0 ± 4.7 [b]
Ejaculations	2.7 ± 0.3	1.4 ± 0.3 [a]	2.7 ± 0.3 [b]
		Second testing	
Latency period (s):			
First mounting	4.3 ± 0.7	6.9 ± 0.7 [a]	6.0 ± 0.9
First intromission	4.3 ± 0.7	9.0 ± 1.4 [a]	6.0 ± 0.9
First ejaculation	378 ± 76	648 ± 96 [a]	393 ± 56 [b]
Number of:			
Mountings without intromissions	0	0.6 ± 0.4	0
Intromissions	22.7 ± 2.7	18.9 ± 0.7	25.5 ± 2.3 [b]
Ejaculations	3.2 ± 0.3	2.1 ± 0.3 [a]	2.7 ± 0.2

Footnotes: [a] $p < 0.05$ *vs.* control; [b] $p < 0.05$ *vs*. prenatally stressed males; each group contained 5-6 rats.

males than in females (by 8% and 43%, respectively, $p < 0.05$).

In the MBH and hippocampus, the prenatal administration of naltrexone eliminated the sex differences in the protein spectrum at 14.3 kDa and 66.0 kDa that had been induced by prenatal stress.

Administration of naltrexone proved to be effective in normalizing disturbances of male sexual behavior induced by prenatal stress in male offspring, indicating the involvement of endogenous opioids in its demasculinization (Table 4.20).

It is known that a functional relationship exists between the HPA axis and the HPG axis, and the presence of sexual dimorphism in the stress reactivity of the HPA axis supports this notion. Considering the lack of literature data regarding the involvement of opioids in the prenatal stress–induced modification of HPA axis function, a corresponding study was conducted using naltrexone in adult offspring (males and females in the diestrus stage), aged 6 months, whose mothers received naltrexone injections according to the above-mentioned protocol.

The HPA axis response to acute stress was evaluated by measuring plasma corticosterone levels and catecholamine content in the hypothalamus. Analysis of GABA receptor mechanisms was carried out using the GABA-B receptor agonist baclofen, administered intraperitoneally at a dose of 10 mg/kg, 30 min before a one-hour immobilization session.

As in the previous experiment, administration of naltrexone before maternal stress preserved in the offspring of both sexes the characteristic post-stressor changes in plasma corticosterone levels observed in normal animals. Both basal and stress-induced corticosterone levels in the plasma did not differ from those of the corresponding control groups.

In control pubertal male and female rats, administration of baclofen 30 minutes before a one-hour immobilization stress caused a reduction in corticosterone response to acute stress by 2.0-fold and 1.5-fold, respectively. As shown earlier, in prenatally stressed males, the inhibitory effect of baclofen on the acute stress–induced adrenocortical response was absent, which is consistent with reports from other researchers indicating impaired feedback mechanisms between GABAergic receptors and corticosteroids (Reznikov et al., 2004b).

Administration of naltrexone before maternal stress resulted in complete preservation of the ability of GABA-B receptors in sexually mature male offspring to respond to acute stress stimulation: prestress administration of baclofen limited corticosterone response to one-hour immobilization similarly to that observed in control animals (Table 4.21).

Table 4.21 Plasma corticosterone levels (nmol/L) following one-hour immobilization (acute stress) and prestress administration of the GABA-B receptor agonist baclofen in intact and prenatally stressed (PS) under naltrexone treatment rats aged 6 months (Mean ± SEM)

Sr. No.	Animal group	Control	Naltrexone + PS
		Males	
1.	Basal level	586 ± 71	520 ± 39
2.	Acute stress + baclofen	1152 ± 41	1044 ± 22
	$p_{1,2}$	< 0.001	< 0.001
3.	PS + acute stress + baclofen	891 ± 19	853 ± 37
	$p_{1,3}$	< 0.001	< 0.001
	$p_{2,3}$	< 0.001	< 0.001
		Females	
4.	Basal level	738 ± 45	624 ± 103
	$p_{1,4}$		> 0.5
5.	Acute stress + baclofen	1127 ± 42	1104 ± 93
	$p_{4,5}$	< 0.001	< 0.01
6.	PS + acute stress + baclofen	847 ± 30	840 ± 51
	$p_{4,6}$	> 0.1	> 0.1
	$p_{5,6}$	< 0.001	< 0.05

Footnote: Each group contained 5-6 rats.

In both males and females of the control and experimental groups, baclofen significantly reduced the plasma corticosterone levels induced by acute stress, with the values in females approaching their baseline levels.

In our previous studies, we found that the attenuated HPA axis response to acute stress in prenatally stressed adult males was associated with the absence of post-stress changes in hypothalamic norepinephrine content. The results of subsequent experiments demonstrated the preventive effect of prenatal naltrexone administration on disturbances in the norepinephrine response to acute stress. The acute stress–induced decrease in hypothalamic norepinephrine concentration in adult experimental males averaged 37%, which did not differ from the control group value (a 39% decrease). The dopamine

content under acute stress conditions remained unchanged. Basal levels of both catecholamines in the control and experimental groups were identical (Table 4.22).

Table 4.22 Effect of one-hour immobilization (acute stress) on hypothalamic catecholamine levels in adult male rats prenatally stressed (PS) against the background of naltrexone treatment (Mean ± SEM)

Sr. No.	Animal group	Norepinephrine, nmol/g tissue	Dopamine, nmol/g tissue
	Control:		
1	Basal level	7.1 ± 0.2	5.1 ± 0.2
2	Acute stress	5.2 ± 0.1	4.9 ± 0.1
	$p_{1,2}$	< 0.001	> 0.05
	PS + naltrexone:		
3	Basal level	6.8 ± 0.3	5.0 ± 0.1
	$p_{1,3}$	> 0.05	> 0.05
4	Acute stress	4.9 ± 0.1	4.9 ± 0.1
	$p_{3,4}$	< 0.001	> 0.05

Footnote: Each group contained 5-6 rats.

The hypothalamic catecholamine response to acute stress in females in similar experiments also demonstrated an identical effect of naltrexone in both experimental and control groups: the norepinephrine concentration decreased by 49% and 58%, respectively (Table 4.23). The basal norepinephrine content in the hypothalamus of females remained unchanged. The dopamine concentration under physiological resting conditions or during acute stress in experimental female rats did not differ from control values.

The effects of prenatal naltrexone administration on the hormonal response of the HPA axis to norepinephrine bitartrate injection into the third cerebral ventricle were studied

Table 4.23 Effect of one-hour immobilization (acute stress) on hypothalamic catecholamine levels in adult female rats prenatally stressed (PS) against the background of naltrexone treatment (Mean ± SEM)

Sr. No.	Animal group	Norepinephrine, nmol/g tissue	Dopamine, nmol/g tissue
1	**Control:**		
	Basal level	7.6 ± 0.3	5.5 ± 0.1
2	Acute stress	4.8 ± 0.1	5.3 ± 0.2
	$p_{1,2}$	< 0.001	> 0.05
	PS + naltrexone:		
3	Basal level	7.9 ± 0.2	4.9 ± 0.2
	$p_{1,3}$	> 0.5	> 0,05
4	Acute stress	5.3 ± 0.3	4.8 ± 0.2
	$p_{3,4}$	< 0.001	> 0.05

Footnote: Each group contained 5-6 rats.

in male and female rats aged 8 months. With this method of administration, the catecholamine diffuses into the circumventricular structures of the hypothalamus. As in previous experiments, the studies were performed on non-anesthetized animals equipped with a pre-implanted intracerebral cannula and a venous catheter. All experiments on females were carried out during the diestrus phase, which was determined by microscopic examination of vaginal smears.

Application of norepinephrine bitartrate into the third ventricle of the brain in both control and prenatally stressed male rats whose mothers received naltrexone prior to stress exposure caused a significant increase in plasma ACTH levels 20 min after administration compared to baseline values (Table 4.24). In males of the experimental group, the response to noradrenergic stimulation was somewhat weaker than in control animals (a 3.5-fold versus 5.5-fold increase, respectively) due to the fact that the basal ACTH level in the “prenatal stress + naltrexone” group was significantly higher than in

Table 4.24 Effect of norepinephrine bitartrate administration to the third ventricle of the brain on plasma ACTH and corticosterone levels in rats prenatally stressed (PS) under naltrexone treatment (Mean ± SEM)

Sr. No.	Animal group	ACTH, pg/ml		Corticosterone, nmol/L	
		0 min	20 min	0 min	20 min
		Males			
1	Control	94 ± 10	515 ± 24 *	618 ± 20	1678 ± 48 *
2	PS + naltrexone	154 ± 17	545 ± 68 *	1098 ± 134	1853 ± 140 *
	$p_{1,2}$	< 0.01	> 0.05	< 0.01	> 0.05
		Females			
3	Control	169 ± 15	541 ± 23 *	1840 ± 162	2720 ± 142 *
	$p_{1,3}$	< 0.01	> 0.05	< 0.001	< 0.001
4	PS + naltrexone	191 ± 17	620 ± 32 *	1311 ± 164	2599 ± 138 *
	$p_{2,4}$	> 0.05	> 0.05	> 0.05	< 0.01
	$P_{3,4}$	> 0.05	> 0.05	< 0.05	> 0.05

Footnotes: * $p < 0.05$ relative to baseline level; each group contained 5-6 rats.

controls. Females of both studied groups also showed higher basal ACTH levels compared with the control males. However, the rise in ACTH levels following intracerebroventricular norepinephrine bitartrate application was somewhat less pronounced than in males: a 3.2-fold increase in both the control and experimental groups.

The observed differences in basal plasma ACTH levels in intact rats, with females showing 1.8 times higher values than males, can likely be explained by the stimulatory effect of endogenous estrogens on the neurosecretory cells of the paraventricular nuclei of the hypothalamus and on the expression of the CRH gene, thereby enhancing ACTH secretion (Burgess & Handa, 1992; Atkinson & Waddell, 1997). According to our data (Table 4.5), in prenatally stressed rats the ACTH level not stimulated by AVP application into the third cerebral ventricle did not differ between males and females, unlike in

normal animals, which reflects the modifying influence of maternal stress on the hypothalamic–pituitary component of the HPA axis.

The similar direction and magnitude of ACTH elevation in both sexes of control and experimental groups in response to central noradrenergic stimulation indicate the protective properties of prenatal naltrexone administration with respect to the HPA axis response in prenatally stressed animals.

Plasma corticosterone levels measured 20 mins after norepinephrine bitartrate application into the third cerebral ventricle were significantly increased in both control (2.7-fold) and experimental males (1.7-fold). A pronounced adrenocortical response was also observed in females of both control and experimental groups (1.5-fold and 2.0-fold increases, respectively). Corticosterone levels after norepinephrine administration did not differ between prenatally stressed rats treated with naltrexone and non-stressed animals of either sex.

The absence of significant differences in corticosterone elevation patterns between intact and prenatally stressed rats exposed to naltrexone indicates that the opioid receptor blocker prevents the development of characteristic alterations in noradrenergic reactivity of the HPA axis induced by prenatal stress. The protective properties of naltrexone confirm the pathogenic role of excessive endogenous opioid production, induced by prenatal stress, in the development of functional disorders of one of the organism's key adaptive systems. Thus, naltrexone may be considered a potential pharmacological agent capable of preventing long-term effects of prenatal stress on reproductive and stress-response systems by correcting disturbances in neuronal opioid homeostasis. Given the interactions between the opioid system and noradrenergic (Douglas, 2005) as well as vasopressinergic systems (Watson. et al., 1982), the involvement of additional factors in this process cannot be excluded.

Preventive Effect of Exogenous Testosterone. The ability of testosterone administration prior to maternal stress exposure to prevent abnormalities of male sexual behavior in rats was first demonstrated by Dörner et al., (1983a) and provided evidence that a deficiency of fetal testicular testosterone before birth is a cause of brain demasculinization and feminization. Since the critical period of sexual differentiation of the brain in rats encompasses not only the final prenatal days but also the PND 7–10, abnormalities in the behavior of prenatally stressed males can also be prevented by testosterone administration during this period (Pereira et al., 2006).

However, the neuroendocrine mechanisms underlying the preventive effect of testosterone in the prenatal stress syndrome remained unclear. To address this gap, the spectrum of soluble cytosolic proteins and the activity of testosterone-metabolizing

enzymes were analyzed in discrete neuroendocrine brain regions. In our view, the preventive action of androgen on aromatase activity and other parameters could provide further convincing evidence for the determining role of this enzyme in the pathogenesis of the prenatal stress syndrome.

During the final week of pregnancy, rats were subjected to daily one-hour immobilization stress. Testosterone propionate was administered subcutaneously on GD 17, 19, and 21 at a dose of 5 mg/kg b.w., 30 min before immobilization. Control animals received the oil vehicle of testosterone propionate.

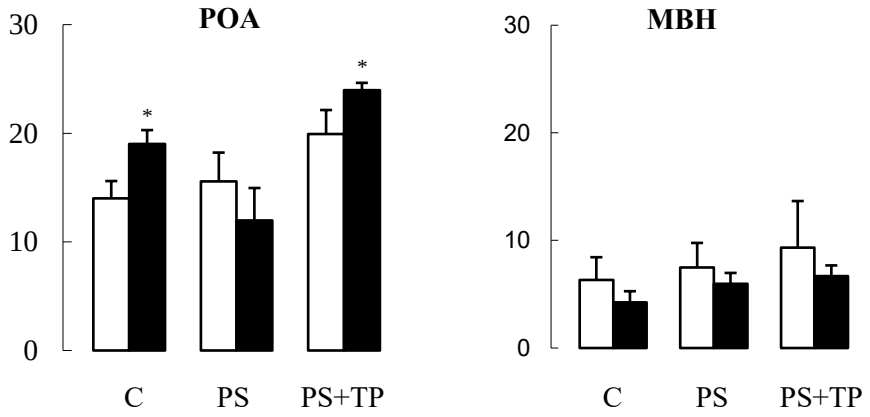

Fig. 4.8 Preventive effect of testosterone propionate on changes in aromatase activity in the POA and MBH of prenatally stressed 10-day-old rats (M ± SEM).
Footnotes: C – control; PS – prenatal stress; TP – testosterone propionate. Open bars – females, black bars – males. Ordinate axis – pmol E_2/hour/g protein (M ± SEM). * $p < 0.05$ compared with females.

In the present study, prenatal stress caused a significant reduction of aromatase activity in the POA of 10-day-old male rats by an average of 38%, resulting in the disappearance of sexual differences in this parameter. These differences were restored in the group of animals exposed to prenatal stress in the presence of testosterone, due to an increase in enzyme activity in males, which even exceeded the control value by 20%. No sex dimorphism in enzyme activity was observed in the MBH in any of the experimental groups (Fig. 4.8).

Prenatal stress affected the activity of steroid 5α-reductase in a different way. Sexual differences in this parameter were absent in both examined hypothalamic regions; however, in prenatally stressed animals, a sex difference emerged in the POA due to a 68% decrease in enzyme activity in females ($p < 0.05$). Prenatal administration of

testosterone propionate not only restored 5α-reductase activity but also induced its more than two-fold increase compared with control females, thus producing a significant sex difference. The only change in 5α-reductase activity in the MBH induced by prenatal stress was its 46% reduction in females compared with controls. These changes were fully normalized by prenatal testosterone propionate administration (Table 4.25).

Table 4.25 Effect of prenatal testosterone propionate (TP) administration on steroid 5α-reductase activity (pmol of 5α-reduced metabolites/hour/g protein) in the POA and MBH of prenatally stressed (PS) 10-day-old rats (Mean ± SEM)

Animal group	POA	MBH
	Control	
Females	1185 ± 140 (5)	967 ± 146 (5)
Males	972 ± 192 (5)	869 ± 225 (5)
	PS	
Females	377 ± 22 [a] (4)	519 ± 128 [a] (4)
Males	941 ± 160 [a] (4)	686 ± 96 (4)
	PS + testosterone	
Females	2461 ± 343 [abc] (5)	886 ± 129 (5)
Males	1222 ± 218 (6)	946 ± 296 (6)

Footnotes: Values in parentheses indicate the number of analyses (2–3 animals per analysis). [a] $p < 0.05$ compared with control females; [b] $p < 0.05$ compared with PS females; [c] $p < 0.05$ compared with the "PS + testosterone" group.

Changes in 5α-reductase activity in the MBH of females are consistent with the pathogenic impact of prenatal stress on their reproductive function in adulthood, manifested by reduced fertility, disrupted estrous cycles, and related alterations. However, this effect cannot be explained by testosterone deficiency in the fetus, since female fetuses lack testes. The preventive effect of testosterone on this enzyme is therefore particularly intriguing and likely involves secondary mechanisms influencing the programming of the developing neuroendocrine reproductive system.

In males, the preventive effect of testosterone on prenatal stress–induced changes in aromatase activity is most evident in the POA, which regulates male sexual behavior and

represents a *locus minoris resistentiae* to the damaging influence of prenatal stress. The role of aromatase in the programming of neuroendocrine functions extends beyond sexual differentiation to other behavioral forms as well. For example, administration of an aromatase inhibitor to pregnant rats disrupts the sexual dimorphism of anxiety levels and other behavioral responses in offspring (Pivina et al., 2007).

In normal adult rats, testosterone induces an increase in hypothalamic aromatase activity (Roselli & Resko J.A., 1984), and this phenomenon is also observed in the brains of fetuses and newborns (Takahashi et al., 1987). Our findings on the preventive effects of naltrexone and testosterone in relation to the prenatal stress syndrome support the point of view that the stress-induced decrease in fetal plasma testosterone levels is mediated by opioids and underlies the reduction of hypothalamic aromatase activity, which in turn leads to impaired programming of the neuroendocrine reproductive system in males. Moreover, prenatal stress indeed reduces plasma testosterone levels in male rat fetuses during the critical period of sexual differentiation of the brain. Animal studies from our laboratory showed that plasma testosterone levels in 5-day-old prenatally stressed males were on average 40% lower than in control males of the same age (0.35 nmol/L*vs*. 0.58 nmol/L in controls). Administration of testosterone propionate to pregnant rats prior to stress exposure prevented these hormonal alterations. Under these conditions, plasma testosterone levels averaged 3.8 nmol/L, not only fully restoring but even exceeding control values.

Certain brain proteins appear to be important targets and potential participants in the disturbances of sexual differentiation of the brain. Therefore, the study of the preventive effects of testosterone on its metabolism in the hypothalamus was complemented by an analysis of sex-dependent cytosolic protein distribution in the POA, MBH, and hippocampus of 5-day-old offspring. For analysis, tissue samples from 3–5 animals (MBH or hippocampus) and from 7 animals (POA) were pooled, with five analyses performed for each brain region.

Stress exposure of pregnant rats in the presence of testosterone propionate did not impair the formation of sexual dimorphism in the distribution of the 66.0 kDa protein band in the POA. Typical sex differences, characterized by a higher optical density of this band in males ($p < 0.05$), were maintained in 5-day-old offspring. At the same time, androgen administration to stressed pregnant rats eliminated the sex difference in the 18.4 kDa protein band in the POA due to a 12% increase in the optical density of this protein in males compared with prenatally stressed females ($p = 0.05$).

In the MBH and hippocampus, testosterone treatment abolished the prenatal stress–induced sex differences in the 14.3 kDa protein spectrum, and in the hippocampus, in the 66.0 kDa band as well.

The obtained data on the protective properties of testosterone with respect to prenatal stress–induced early postnatal alterations of sex dimorphism in protein distribution in neuroendocrine brain structures of male rats confirm the pathogenic role of androgen deficiency in the development of sexual differentiation disturbances.

The Role of Calcium Ions in Prenatal Stress Syndrome. We performed a pharmacological analysis of the involvement of intracellular calcium ions in the pathogenesis of disturbances in steroid metabolism and protein profiles within neuroendocrine brain structures, as well as in the disorders of reproductive function, sexual behavior, and HPA axis reactivity to stress and noradrenergic stimulation in rats exposed to prenatal stress. For this purpose, we used nimodipine, a blocker of L-type voltage-gated calcium channels that is capable of crossing the blood–brain barrier and directly influencing neuronal activity. This compound is widely used in clinical practice for the treatment of neurological and cardiovascular disorders. Its use during pregnancy is not contraindicated, when necessary, which makes it particularly interesting to investigate the early and long-term consequences of perinatal calcium channel blockade by nimodipine on the development of neuroendocrine regulatory mechanisms of reproduction and adaptation. These issues have not been previously studied.

Elucidating the mechanisms of interaction between intracellular calcium ions and hormonal factors of the internal milieu may be of primary importance for understanding the nature of congenital neuroendocrine pathologies of epigenetic origin and for identifying effective strategies to prevent them.

Pregnant rats subjected to stress during the final week of gestation, received daily oral administration of nimodipine during the same period, in the form of a suspension of crushed tablets in Dorfman's gel, at a dose of 20 mg/kg b.w. This dose of nimodipine is optimal for blocking L-type calcium channels in brain cells (Biessels et al., 2005). Testosterone metabolism in discrete brain structures was examined in 10-day-old offspring**.**

The sexual dimorphism of aromatase activity in the POA, typical for non-stressed animals, disappeared in prenatally stressed rats due to a threefold reduction in enzyme activity in males. Administration of nimodipine prior to stress exposure exerted a preventive effect against the demasculinization of testosterone metabolism in the POA of male offspring. In contrast, in the MBH, a significant increase in aromatase activity was observed compared with control animals (Table 4.26). No significant changes in aromatase activity relative with the control group values were observed in females of any group in either of the studied brain structures.

Table 4.26 Prenatal effect of nimodipine on steroid aromatase activity (pmol estradiol/h/g tissue) in the POA and MBH of 10-day-old prenatally stressed and non-stressed under nimodipine male rats (Mean ± SEM)

Sr. No.	Animal group	POA	MBH
1	Control	0.518 ± 0.131* (4)	0.134 ± 0.022 (4)
2	Prenatal stress	0.171 ± 0.027 (3)	0.139 ± 0.065 (3)
	$p_{1,2}$	< 0.05	> 0.05
3	Nimodipine	0.305 ± 0.158 (4)	0.210 ± 0.078 (4)
	$p_{1,3}$	> 0.05	> 0.05
	$p_{2,3}$	> 0.05	> 0.05
4	Prenatal stress + nimodipine	0.307 ± 0.106 (4)	0.215 ± 0.083 (4)
	$p_{1,4}$	> 0.05	< 0.05
	$p_{2,4}$	> 0.05	> 0.05
	$p_{3,4}$	> 0.05	> 0.05

Footnotes: Values in parentheses indicate the number of analyses; * $p < 0.05$ compared with females of the same group (refer to Table 4.27).

Control 10-day-old rats exhibited sexual dimorphism in 5α-reductase activity in the POA, with higher enzyme activity in females compared to males. Prenatal stress did not affect 5α-reductase activity in the POA of females but significantly increased it in males, thereby eliminating the sexual dimorphism in enzymatic activity (Tables 4.27). No preventive effect of nimodipine on the prenatal stress-induced alterations in 5α-reductase activity in the POA of male brains was detected. Moreover, in prenatally stressed females treated with nimodipine, enzyme activity in the POA was significantly reduced, whereas in males it was increased compared with the control animals. As a result, a complete inversion of the sexual dimorphism of enzyme activity occurred in this brain region relative with the control group animals.

Our findings on the modulatory effect of nimodipine on androgen metabolism suggest that Ca^{2+}-dependent mechanisms are involved in mediating the effects of prenatal stress through the products of androgen aromatization and 5α-reduction in males.

The effect of prenatal nimodipine administration on the brain protein spectrum of

Table 4.27 Prenatal effects of nimodipine on steroid 5α-reductase activity (pmol of 5α-reduced metabolites/hour/g tissue) in the POA and MBH of 10-day-old prenatally stressed and non-stressed male rats (Mean ± SEM)

Sr. No.	Animal group	POA	MBH
1	Control	4.29 ± 1.10 (5)	8.71 ± 2.43 (4)
2	Prenatal stress	11.86 ± 2.59 (5)	9.78 ± 0.87 (5)
	$p_{1,2}$	< 0.05	> 0.5
3	Prenatal stress + nimodipine	14.40 ± 3.66 (5)	7.54 ± 1.30 (5)
	$p_{1,3}$	< 0.05	> 0.5
	$p_{2,3}$	> 0.5	> 0.5

Footnotes: Values in parentheses indicate the number of analyses.

prenatally stressed rats was examined in 5-day-old offspring. As expected, prenatal stress resulted in the disappearance of sex differences in the relative content of a 66.0 kDa protein in the cytosolic fraction of the POA, due to a decrease in its expression in males. At the same time, a new sex difference emerged in the relative abundance of an 18.4 kDa protein, which was more pronounced in males (p <0.01).

Prenatal administration of nimodipine partially prevented the prenatal stress–induced alterations in the distribution of the 66.0 kDa protein. In addition, the sex difference in the relative content of the 24.0 kDa cytosolic protein disappeared, primarily due to an increase in the optical density of this protein band on the densitogram in males.

These findings suggest that nimodipine exerts a modulatory and potentially neuroprotective effect on the developing brain under prenatal stress conditions. By stabilizing calcium homeostasis and preventing aberrant protein expression patterns, nimodipine may contribute to the preservation of sex-specific neurochemical differentiation in the POA during early postnatal development.

Prenatal administration of nimodipine to prenatally stressed rats resulted in a reversal of sex differences in the distribution of the 14.3 kDa protein: unlike in intact animals, the optical density of this protein band was higher in males than in females. Moreover, in prenatally stressed rats exposed to nimodipine, sex differences were observed in the relative content of the 18.4 kDa protein, with a higher level detected in females.

In the MBH and hippocampus of intact rats, no sex dimorphism was found in any of the proteins examined. Prenatal stress led to the appearance of sex differences in the MBH for the 14.3 kDa and 34.7 kDa proteins. In this region, the content of the 14.3 kDa and 24.0 kDa proteins was higher in prenatally stressed females, while the 34.7 kDa protein level was substantially greater in prenatally stressed males. The optical density of the 45.0 kDa protein band also increased.

In the hippocampus, prenatal stress induced significant sex-related differences in the proteins ranging from 24.0 to 66.0 kDa, with higher levels in females, whereas proteins in the 34.7–45.0 kDa range were more abundant in males.

Evidence of the protective effect of nimodipine against the adverse influence of maternal stress included alterations in the relative content of the 34.7 kDa protein in the MBH and the 45.0 kDa protein in the hippocampus. In the MBH of this group, sex differences appeared in the 18.4 kDa and 66.0 kDa proteins, due to increased levels in males ($p < 0.01$). In females, a significant increase was observed in the 14.3 kDa and 24.0 kDa proteins. In the hippocampus, sexual dimorphism was manifested in the content of the 14.3 kDa and 24.0 kDa proteins (higher in females, $p < 0.01$), as well as in the 18.4 kDa and 34.7 kDa proteins (higher in males, $p < 0.01$).

Thus, disorders of sexual differentiation in the protein composition of neuroendocrine hypothalamic structures induced by prenatal stress in rats can be mitigated or modified by concurrent administration of nimodipine. These data provide grounds to assume that calcium ion signaling is involved in the pathogenesis of the prenatal stress syndrome. This is particularly evident in the analysis of the sex-dimorphic 66.0 kDa protein (presumably tubulin) in the POA of the hypothalamus. Tubulin is known to serve as a marker of neuronal growth in the rodent brain, and its synthesis is stimulated by androgens. It is therefore plausible that an early testosterone deficiency in male fetuses is associated with suppressed neuronal growth in the sex-dimorphic brain region.

Oral administration of nimodipine to stressed pregnant rats did not affect the prenatal stress–induced disturbances in reproductive function of female offspring. In prenatally stressed females, vaginal opening occurred on average 13 days later than in controls, and in those prenatally exposed to nimodipine, 12 days later. In 26.7% of 3-month-old prenatally stressed females, irregular estrous cycles were observed, characterized by a

prolonged period of quiescence (metaestrus and diestrus stages), while in the group stressed in the presence of nimodipine, irregular cycles were found in 33.3% of animals.

By the end of the 6-month observation period, disturbances in estrous cyclicity were less pronounced. The number of females exhibiting regular estrous cycles increased significantly, while the proportion of animals with disrupted cycle structure decreased on average 2–3-fold, reaching 14.3% in prenatally stressed females and 13.3% in those stressed in the presence of nimodipine. In the vast majority of animals, the phase structure of the estrous cycle normalized.

Behavioral testing of sexual receptivity in 3-month-old females that underwent ovariectomy followed by estradiol and progesterone administration revealed no significant changes among the three groups (intact, prenatally stressed, and prenatally stressed with nimodipine treatment). However, prenatally stressed females, both with and without nimodipine exposure, exhibited distinct elements of male sexual behavior during a 10-minute interaction with receptive females in estrus. In the control group, no mountings or male-type receptive behaviors were observed. In prenatally stressed females, the mean number of mountings and incidence of male-type behavior reached 1.6 and 80%, respectively, whereas in the nimodipine-treated stressed group, these values were reduced to 0.6 and 20%, respectively.

Taken together, these findings support the involvement of Ca^{2+}-dependent mechanisms in the disturbances of early programming of heterotypic sexual behavior in female rats, presumably mediated by adrenal androgen activity.

The study of male sexual behavior under conditions of combined prenatal stress and nimodipine administration was conducted in 3-month-old rats. Parameters of male sexual behavior were recorded during a 30-minute period of cohabitation between the test male and a receptive female in the estrus stage. Behavioral testing was performed twice, with a one-week interval between sessions.

Immobilization of pregnant dams induced alterations in both central and peripheral mechanisms regulating male copulatory behavior in their male offspring (Table 4.28). Administration of nimodipine to pregnant rats prior to stress exposure restored the examined parameters of male-type sexual behavior in their male progeny. In particular, the calcium channel blocker prevented the prolongation of the latency to the first mounting and even shortened it during the first testing session, indicating activation of the motivational component of sexual behavior.

The subsequent stage of the study aimed to investigate the sex-specific characteristics of HPA axis functioning in male and female rats (examined during the diestrus stage),

whose mothers had been subjected to immobilization stress during the final week of pregnancy under conditions of concurrent nimodipine administration or nimodipine exposure alone.

Table 4.28 Parameters of male sexual behavior in 3-month-old male rats prenatally stressed under nimodipine treatment (Mean ± SEM)

Parameter	Control	Prenatal stress	Nimodipine	Prenatal stress + nimodipine
		First testing		
Latency period (s):				
First mounting	29.0 ± 3.6	55.2 ± 17.1	32.6 ± 9.2	18.0 ± 2.9*
First intromission	63.4 ± 14.1	115.8 ± 42	34.0 ± 9.8	42.6 ± 24.0
First ejaculation	724 ± 74	672 ± 148	715 ± 137	686 ± 289
Number of:				
Mountings without intromissions	3.8 ± 1.6	1.6 ± 0.7	0.6 ± 0.4	1.0 ± 0.6
Intromissions	15.2 ± 1.9	12.0 ± 0.9	13.8 ± 1.6	13.4 ± 1.8
Ejaculations	2.2 ± 0.2	1.4 ± 0.2*	2.0 ± 0.3	1.8 ± 0.6
		Second testing		
Latency period (s):				
First mounting	7.6 ± 1.3	33.0 ± 7.0*	8.2 ± 1.2	10.2 ± 3.9
First intromission	13.2 ± 4.1	38.4 ± 10*	36.4 ± 26.5	12.2 ± 6.0
First ejaculation	403 ± 52	639 ± 78*	775 ± 233	393 ± 145
Number of:				
Mountings without intromissions	0.6 ± 0.6	0.4 ± 0.4	0.6 ± 0.5	0
Intromissions	15.4 ± 1.1	16.8 ± 1.8	1 4.0 ± 3.8	16.6 ± 3.4
Ejaculations	3.0 ± 0.3	1.6 ± 0.2*	2.0 ± 0.3	2.0 ± 0.6

Footnotes: * $p < 0.05$ compared with the controls. Each group contained 5-6 rats.

Administration of nimodipine prior to stress exposure in pregnant females prevented the development of typical alterations in HPA axis stress reactivity in their adult offspring (both males and females): the response to acute stress was comparable to that of sex-matched control animals (Fig. 4.9). Although no statistically significant difference was observed between the post-stress corticosterone levels in prenatally stressed females and

those prenatally stressed under nimodipine administration, the difference in the mean amplitude of corticosterone elevation (83% and 36%, respectively) was statistically significant ($p < 0.05$).

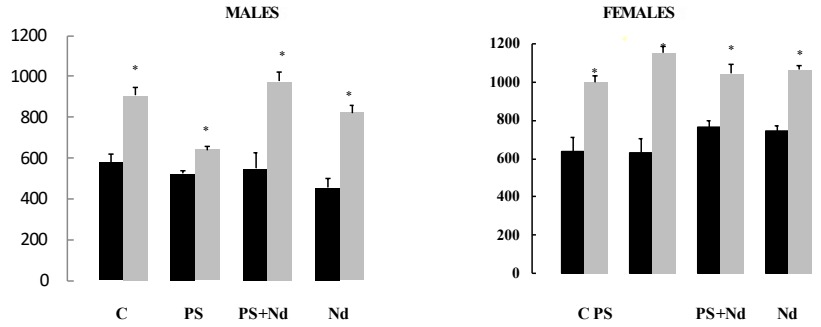

Fig. 4.9 Adrenocortical response to one-hour immobilization in rats prenatally stressed under nimodipine treatment (Mean ± SEM)

Footnotes: Ordinate axis – plasma corticosterone level (Mean ± SEM, nmol/L). C – control; PS – prenatal stress; Nd – nimodipine. Black bars – baseline level; gray bars – post-immobilization values. * $p < 0.05$ compared with baseline level.

Noradrenergic reactivity of the HPA axis was examined in 8-month-old rats. Consistent with previous experiments, prenatal stress in males enhanced and prolonged the corticosterone response: at 60 min after norepinephrine bitartrate application, corticosterone levels remained significantly elevated compared to baseline, in contrast with the control group animals (Table 4.29). In prenatally stressed females, the dynamics of the hormonal response were opposite—more precisely, absent (Table 4.30). Conversely, in prenatally stressed females, nimodipine demonstrated a protective effect, which was not observed in males.

The establishment of a protective effect of nimodipine administered during the prenatal period against stress-induced alterations in HPA axis reactivity in adult prenatally stressed rats suggests that prenatal stress–induced changes in HPA axis activity are most likely associated with enhanced calcium signaling within brain regions involved in HPA axis regulation. Considering the pivotal role of glucocorticoid hormones in mediating the effects of prenatal stress, it may be inferred that calcium signaling plays an essential role in the prenatal programming of glucocorticoid-dependent disturbances in HPA axis

reactivity in adult rats, and that nimodipine is capable of exerting a protective effect against these impairments.

On the other hand, when analyzing the potential pathways and mechanisms underlying

Table 4.29. Effect of norepinephrine bitartrate administration to the third ventricle of the brain on plasma corticosterone levels (nmol/L) in 8-month-old male rats prenatally treated with nimodipine or prenatally stressed under nimodipine treatment (Mean ± SEM)

Animal group	Time of blood sampling			
	0 min	30 min	60 min	90 min
Control	746 ± 129	1166 ± 82*	980 ± 64	862 ± 52
Prenatal stress	852 ± 69,7	1189 ± 82*	1232 ± 93*	984 ± 68
Prenatal stress + nimodipine	843 ± 85	1319 ± 94*	1296 ± 63*	933 ± 77
Nimodipine	889 ± 67	1269 ± 57*	1322 ± 75*	1015 ± 79

Footnotes: PS – prenatal stress; * $p < 0.05$ relative to baseline level. Each group contained seven rats.

the protective action of nimodipine, it is also important to consider the possible interaction of intracellular calcium ions with other stress mediators, particularly opioids,

Table 4.30. Effect of norepinephrine bitartrate administration to the third ventricle of the brain on plasma corticosterone levels (nmol/L) in 8-month-old female rats prenatally treated with nimodipine or prenatally stressed (PS) under nimodipine treatment (Mean ± SEM)

Animal group	Time of blood sampling			
	0 min	30 min	60 min	90 min
Control	901 ± 108	1699 ± 73*	1191 ± 91	1098 ± 84
PS	939 ± 103	1033 ± 48	1089 ± 47	869 ± 66
PS + nimodipine	918 ± 66	1349 ± 70*	1258 ± 58*	1001 ± 57
Nimodipine	889 ± 84	1424 ± 68*	1398 ± 75*	1026 ± 68

Footnotes: Refer to the Table 28.

which are involved in mediating the pathogenic effects of prenatal stress on the neuroendocrine system. Given that opioid receptors can interact with calcium channels and exert activating effects on these channels (Lorentz, 1988; Chen et al., 2000), the involvement of intracellular calcium ions in mediating opioid effects on the maturation of HPA axis function is not unexpected. Therefore, blockade of calcium channels by nimodipine may interfere with the realization of endogenous opioid–mediated effects induced by prenatal stress on the development of HPA axis stress reactivity in adult animals. This assumption is supported by other authors (Esmaelli-Mahani et al., 2007), who demonstrated an inhibitory effect of another calcium channel blocker, nifedipine, on morphine-induced corticosteroid secretion.

It should once again be emphasized that the term "prenatal stress syndrome," which was originally introduced into the scientific literature to describe abnormalities in male sexual behavior, has significantly broadened its scope and now encompasses a wide range of physiological and metabolic dysfunctions. The most pronounced long-term consequences of maternal stress during the period of sexual differentiation of the brain include disturbances in reproductive function and alterations of the HPA axis in the offspring, predominantly in males. These effects are determined by neurotransmitter and hormonal factors, the analysis of which has been presented in this chapter.

Undoubtedly, the noradrenergic system of the brain represents the first link in the neurohormonal response to a stressor. Acting in concert with other hypothalamic neurotransmitters and neuropeptides, it initiates a cascade of hormonal changes that disrupt maternal and fetal hormonal homeostasis. Excessive secretion of glucocorticoids and opioids inhibits the release of LHRH, followed by suppression of pituitary LH secretion and fetal testicular testosterone production. As a consequence of reduced testosterone levels in the male fetal circulation, along with catecholaminergic stimulation of hypothalamic adrenoceptors, aromatase activity in the fetal brain decreases. Simultaneously, norepinephrine content in the hypothalamus is reduced, while its level in the POA selectively increases, partly due to opioid involvement. Insufficient local estradiol synthesis in the hypothalamus and disruption of the interaction among the key neurochemical determinants of androgen-dependent differentiation of the neuroendocrine center controlling male sexual behavior hinder its normal masculinization.

As for the attenuated stress reactivity of the HPA axis observed in prenatally stressed adult male rats, it is most likely attributable to enhanced corticosterone secretion of both maternal and fetal origin induced by prenatal stress. This interpretation is supported by the reproduction of the same phenomenon following prenatal administration of a glucocorticoid (hydrocortisone).

The signs of behavioral masculinization in prenatally stressed adult females are most likely determined by hypersecretion of androstenedione by the adrenal cortex of both the mother and the fetus. This excessive androgen exposure may program the developing brain along a masculinized trajectory, resulting in aberrant sexual behavior patterns.

Attention should be drawn to the dual nature of the effects produced by pharmacological agents used to analyze the pathogenetic mechanisms underlying the detrimental impact of prenatal stress on the programming of reproductive functions and the HPA axis. Their protective properties against prenatal stress may be counterbalanced by potential adverse pathogenic effects on the fetus when administered to non-stressed pregnant females.

4.4. Remote Reproductive Consequences of Adolescent Stress

The second critical period in the development of neuroendocrine systems regulating reproduction and stress response is puberty. Adolescence represents one of the most dynamic stages of brain and body development after birth, second only to early childhood. However, unlike childhood, adolescents are considerably more sensitive to a wide range of stressors. During puberty, both the hormonal and psychoemotional states undergo profound changes, characterized by pronounced lability and increased reactivity of the nervous and endocrine systems.

The adverse effects of adolescent stress may manifest later in life as cognitive, behavioral, hormonal, and reproductive disorders (Hueston et al., 2017; Mancha-Gutiérrez et al., 2021; Harris et al., 2022). In adolescent females, stress-induced elevation of CRH suppresses the pulsatile secretion of GnRH, thereby reducing the overall levels of FSH and LH. This, in turn, leads to functional hypothalamic amenorrhea (Gibson et al., 2020). Approximately half of these patients develop PCOS (Hager et al., 2023).

Stress-induced hyperprolactinemia in adolescent girls may result in menstrual irregularities and hirsutism, while in boys it can cause gynecomastia, insufficient development of secondary sexual characteristics, and other endocrine imbalances (Pańubska et al., 2017; Levine et al., 2018; Abaturov et al., 2022).

Early-life stress may contribute to disorders of somatosexual development, including delayed puberty in boys and accelerated puberty in girls, impaired growth, immune dysfunction, and various neuropsychiatric and mental health disturbances. Chronic stress during adolescence is frequently associated with elevated anxiety and depressive symptoms resulting from stress-induced hormonal and neurotransmitter imbalances within the central nervous system, as demonstrated in both clinical and experimental studies (Mancha-Gutiérrez et al., 2021; Harris et al., 2022).

In males, common manifestations of post-traumatic stress disorder include reduced libido, erectile dysfunction, and impaired sperm quality and fertilizing capacity. Early-life stress is also recognized as one of the contributing factors to decreased male fertility. Numerous studies have documented the emergence of risky sexual behaviors in young men following early-life stress, particularly when associated with sexual abuse. However, the long-term effects of chronic stress during adolescence on sexual function in adult men remain insufficiently characterized in the scientific medical literature.

Given that puberty represents the critical period of sexual maturation, disturbances in hormonal balance caused by various stressors may adversely affect reproductive function. This effect is further amplified by the fact that, at the onset of puberty, the responsiveness of the HPA axis to stress stimuli in both humans and animals is significantly higher than in adulthood (Romeo et al., 2016; Romeo & Sciortino, 2021; Kann & Romeo, 2022).

One of the earliest experimental demonstrations of stress-induced alterations in hormonal reactivity before and after puberty was provided by Goldman and colleagues (Goldman et al., 1973). Other researchers also reported higher reactivity and slower recovery of plasma corticosterone levels in 30-day-old rats following restraint stress compared to adult animals (Foilb et al., 2011). Experimental data indicate that chronic exposure to exogenous corticosterone throughout adolescent development induces profound, sex-dependent somatic and neuroendocrine alterations (Kaplowitz et al., 2016).

Studies in rats have demonstrated a disruption of pituitary–testicular hormonal balance in adolescent males subjected to four weeks of stress. Specifically, a marked decrease in FSH, LH, and testosterone levels was accompanied by a significant elevation in prolactin and estradiol concentrations (Liu et al., 2019). Similarly, male golden hamsters exposed to chronic stress during puberty exhibited delayed sexual maturation and reduced testosterone levels compared with controls, although no changes in gonadal mass were observed (Wommack et al., 2004).

LPO and protein oxidation play an essential role in the pathogenesis of various diseases. The damaging effects of LPO products constitute an integral component of distress — a condition that arises when the organism fails to adapt to excessive disturbances in homeostasis. This mechanism is also relevant to the adverse consequences of stress on the reproductive system (Reznikov, 2023). However, the state of LPO processes in the gonads of humans and animals exposed to chronic pubertal stress has remained largely unexplored.

A review of major scientific databases revealed a limited number of studies addressing the long-term reproductive consequences of chronic adolescent stress, particularly those concerning alterations in sexual behavior. Therefore, we conducted several investigations in this area (Reznikov et al., 2024; Sachynska et al., 2024 a,b).

During PND 30–45, male offspring were subjected to daily restraint stress by placement in plastic tubes for 1 hour per day. Considering the circadian rhythm of corticosterone fluctuations, the stress procedure was performed between 9:00 and 12:00 a.m. Non-stressed animals served as controls.

A subset of males was decapitated at 6 months of age, and the testes were collected to assess the levels of LPO products. At 7 months of age, the males were evaluated for the expression of male-type sexual behavior, and at 8 months, for female-type sexual behavior.

On PND 22, rat pups were weaned and separated by sex into individual cages, ensuring that animals from different litters were represented in both the control and experimental groups. The animals were exposed to daily restraint stress between PND 30 and 45 by placing them for 1 hour into plastic tubes equipped with ventilation holes. During the stress period and after its completion, the onset of puberty was monitored in both control (non-stressed) and experimental animals by testicular descent into the scrotum in males and vaginal opening in females. Subsequent experiments were conducted on sexually mature rats aged 6 months.

Timing of Puberty and Testosterone Levels. Chronic stress during puberty did not affect the timing of puberty onset in males but significantly delayed sexual maturation in females (46.0 ± 1.1 days *vs*. 42.8 ± 1.0 days in controls, $p < 0.05$), which is consistent with previous findings (Kinsey-Jones et al., 2010; Li et al., 2019). Serum testosterone levels in 6-month-old rats exposed to stress during puberty (n = 5) did not differ significantly from those of the controls (n = 7): 16,98±2,66 nmol/L*vs.* 15,08±2,03 nmol/L, respectively ($p > 0.05$).

Gonads. The spermatogenesis index, reflecting the ratio of different cell types within the seminiferous epithelium, was significantly lower in stressed animals than in controls due to a reduced number of late spermatids (3.41 ± 0.02 *vs*. 3.51 ± 0.02, $p < 0.05$). Similar reductions in the number of mature spermatids have been reported by other authors using a slightly different stress model (Almeida et al., 2000). Ribeiro et al. (2018) also observed irreversible testicular alterations in pubertal rats subjected to a comparable stress protocol, including an increase in the density of the intertubular compartment and a reduction in the density of the tubular compartment of the testes.

In animals exposed to pubertal stress, a significant reduction in sperm count from epididymal washes and a 2.4-fold decrease in redox activity (lower respiratory index) in spermatozoa were recorded (Table 4.31). However, sperm motility remained unchanged.

Table 4.31 Sperm counts and respiratory indexes in rats (Mean ± SEM)

Animal group	n	Sperm count ($\times 10^6$/mL)	Respiratory index
Control	7	59.50 ± 2.49	0.79 ± 0.12
Stress	6	44.08 ± 3.44*	0.33 ± 0.05*

Footnote: $p < 0.05$ compared with the control group.

Examination of sperm morphology revealed that males from the experimental group had a higher proportion of spermatozoa with "soft" head morphology and more twisted

Table 4.32 Percentages of sperm morphologies in rats (Mean ± SEM)

Animal group	n	Normal	"Soft" head	Curved	Head deformity	Tail deformity
Control	7	44.93 ± 2.23	30.21 ± 3.07	12.07 ± 1.49	3.57 ± 0.77	9.21 ± 1.91
Prenatal stress	6	40.00 ± 2.21	42.75 ± 1.49*	10.25 ± 1.76	1.17 ± 0.48*	5.75 ± 1.17

Footnote: $p < 0.05$ compared with the control group; n – number of rats.

midpieces compared with normal sperm, whereas control animals exhibited a greater number of spermatozoa with abnormalities in the neck region (Table 4.32). Such deviations in sperm quality may contribute to a reduction in the fertilizing capacity of males exposed to pubertal stress.

The morphological structure of the ovaries and the number of follicles, as well as the duration and phase composition of the estrous cycle, did not differ between females in the control and experimental groups.

LPO. The results of malone dialdehyde (MDA) measurements in the testes and ovaries of control and pubertally stressed rats are presented in Figure 4.13. Regardless of sex, the MDA content increased in stressed animals. In addition, the level of diene conjugates in the testes of stressed rats rose from 0.73 ± 0.01 to 1.27 ± 0.04 nmol/mg protein. These findings indicate an intensification of LPO processes within the gonads, which is a

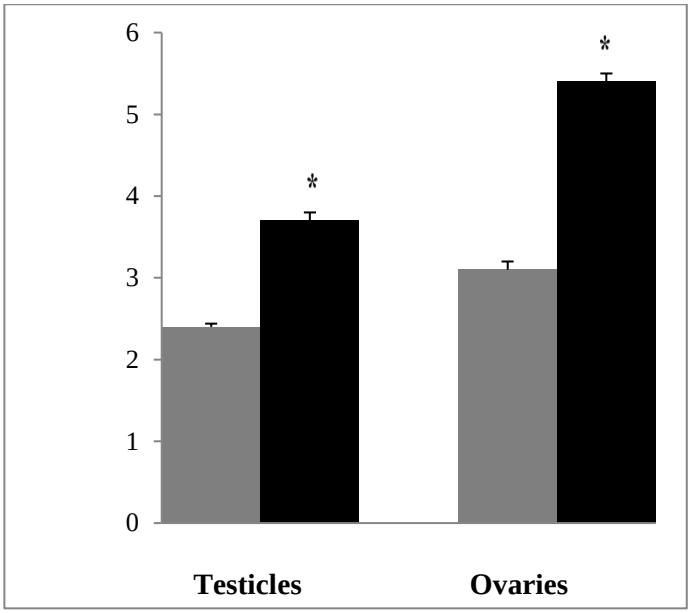

Fig. 4.10 MDA content in the gonads of control and stressed rats (Mean ± SEM).
Footnotes: Ordinate axis — MDA content (nmol/mg protein). Grey bars represent the control group; black bars represent rats exposed to pubertal stress. * $p < 0.05$ compared with the control group.

hallmark of oxidative stress. One possible consequence of impaired antioxidant defense in the gonads may be an acceleration of the age-related decline in both generative and steroidogenic functions (Wang et al., 2017).

Sexual Behavior. The results of the assessment of male-type sexual behavior in 7-month-old male rats are presented in Table 4.33. Since the distribution of quantitative parameters in both the control and experimental groups was non-parametric, the data are expressed as medians with range values for each group.

In the present study, the analysis of sexual behavior demonstrated a marked and statistically significant decrease in the latency to the first mounting: in adult stressed males, the median value during the first testing session was 8.7-fold lower compared with intact control males. The latency parameters for the first and second intromissions during the first testing showed a clear downward trend, and during the second testing, the latency to the second intromission was reduced by more than half. When assessing female-type sexual behavior, no lordosis responses were observed in males of either group. However, in the group of stressed males, homosexual behavior was recorded in one animal, as Mounting attempts were directed toward an active male.

Table 4.33 Effect of pubertal stress on parameters of sexual behavior in 7-month-old male rats

Parameter	First testing		Second testing	
	Control	Stressed	Control	Stressed
Latency period (s):				
First mounting	74 (8-279)	8.5 (2-15) *	2 (1-355)	1 (1-3)
First intromission	89 (28-519)	27 (9-548)	10 (2-464)	9 (3-10)
Second intromission	161 (45-611)	37 (11-675)	29 (4-576)	13 (7-22) *
First ejaculation	–	–	–	550
Number of:				
Mountings without intromission	4.5 (4-9)	3,5 (1-6)	3 (2-6)	3 (1-5)
Mountings with intromission	8 (2-17)	22(2-22)	12 (4-26)	16 (14-21)
Ejaculations	0	0	0	1

Footnotes: The test lasted 15 min. The data are presented as medians with minimum and maximum values (in the parentheses). Each group contained 6 rats. Statistical analysis was performed using Wilcoxon-Mann-Whitney *U* test; * $p \leq 0.05$ compared with the control.

Previous reports have described a weakening of sexual motivation in three-month-old males exposed to stress between PND 25 and 50 (Hernández-Arteaga et al., 2020). In contrast, the data obtained in our study indicate a significant increase in sexual motivation, accompanied by normal serum testosterone levels, in adult males examined five and a half months after prolonged pubertal stress. This discrepancy may be due not so much to the duration of stress exposure as to the different stress paradigms used. In the study cited above, rats were subjected to social isolation by housing singly in cages, which likely induced psychoemotional stress.

In another investigation (Almeida et al., 2000 a), daily immobilization of male rats for 6 hours over a period of 15 or 60 days, starting from PND 40, resulted in an increased latency to the first mounting. However, the authors concluded that stress exerted a stimulating effect on copulatory behavior, as the frequency of intromissions increased 2.5-fold. It is worth noting that sexual behavior in that study was assessed immediately after the stress period, which precludes direct comparison with our results.

Based on the findings of the present study, it can be concluded that prolonged immobilization stress during puberty exerts a stimulating effect on male sexual behavior in adulthood. This effect was independent of circulating testosterone levels and is therefore likely associated with stress-induced modulation of the neuroendocrine regulation of sexual behavior.

As noted above, testosterone levels in the experimental males remained within the normal range. Interestingly, other researchers reported an increase in testosterone concentrations in male rats exposed to daily 6-hour immobilization from PND 40 to 55 (Almeida et al., 2000 b), while prolonged exposure up to day 100 resulted in a decrease (Almeida et al., 1998). However, in both studies, hormone assays were performed immediately after the stress period, suggesting that the timing of the experiment may play a crucial role in these discrepant findings.

There are also reports of delayed testicular maturation and decreased spermatogenic and androgenic activity in adult rats subjected to immobilization-induced prepubertal stress (Almeida et al., 1998; 2000 a,b). It is plausible that oxidative stress contributes to these alterations, leading to impairments in the generative and steroidogenic functions of the testes.

Conclusions

- Prenatal stress activates the maternal HPA axis, inducing hormonal and neurotransmitter alterations that influence fetal development. The early gestational period is the most vulnerable. The *prenatal stress syndrome* represents a complex of neuroendocrine, behavioral, and metabolic alterations in the offspring resulting from severe maternal stress during pregnancy, particularly in rats, during its final week. In male rats, these effects manifest as demasculinization and feminization of sexual behavior, changes in aromatase and 5α-reductase activity, microstructural alterations in the hypothalamus, modifications in the protein spectrum of neuroendocrine brain structures, and disruptions of neurotransmitter systems (catecholamines, serotonin). In females, reproductive and metabolic disorders are observed. Similar effects have been reported in humans. The main underlying mechanism is believed to involve disruption of sexual differentiation of the brain through hormonal imbalance, affecting androgen-dependent imprinting of the fetal neuroendocrine system. These changes exhibit clear regional and sex-specific patterns, reflecting the profound influence of prenatal stress on neuroendocrine system programming.

- Prenatal stress attenuates HPA axis responses to acute stress in adult male rats due to impaired noradrenergic and vasopressinergic regulation, whereas in

females, HPA reactivity is slightly enhanced. Dexamethasone-induced blockade of HPA axis activation in stressed pregnant females prevents the long-term effects of prenatal stress: it preserves sexual dimorphism, normalizes enzymatic activity, HPA axis stress reactivity, and male sexual behavior in adulthood by inhibiting maternal HPA axis.

- The GABA agonist phenibut, administered to pregnant rats prior to stress exposure, mitigates the adverse effects of prenatal stress in male offspring: it normalizes sexual maturation, behavior, and HPA axis reactivity. Its action is associated with activation of the GABAergic stress-limiting system. Methyldopa, as a catecholamine synthesis inhibitor, partially prevents the effects of prenatal stress by attenuating sympathetic nervous system activation.

- Prenatal stress increases opioid levels in the brains of both the mother and the fetus. Administration of β-endorphin to pregnant females mimics the negative effects of prenatal stress on the reproductive system and HPA axis in rat offspring, whereas the opioid receptor antagonist, naltrexone, prevents these effects. Naltrexone normalizes sex-related differences in neurochemical and behavioral parameters of the offspring and preserves normal HPA axis responses to stress and noradrenergic stimulation. Thus, endogenous opioids are key mediators of the effects of prenatal stress on the offspring.

- Administration of testosterone to pregnant rats prior to stress prevents prenatal stress–induced disturbances in sexual behavior, enzymatic activity in the brain, and brain protein composition in the offspring. This finding underscores the critical role of androgens, in programming sexual differentiation of the male brain.

- The calcium channel blocker, Nimodipine, administered to pregnant rats prior to stress, partially prevented the adverse consequences of prenatal stress: it normalized androgen metabolism, preserved sexual dimorphism in aromatase activity and brain protein spectra, reduced behavioral masculinization in females and demasculinization in males, and maintained HPA axis functionality. Hence, calcium ions are involved in the programming of the developing fetal neuroendocrine system.

- In adult males exposed to pubertal stress, a deterioration in the quantitative and qualitative parameters of the spermogram was observed, despite maintaining normal plasma testosterone levels.

Chapter 5: Developmental Reprogramming of the Neuroendocrine System by Endocrine Disruptors

5.1. General Background

Population decline in many countries is largely attributed to the negative impact of environmental pollutants on fertility, primarily due to their interference with hormonal regulatory systems. Of particular concern is the ability of chemical endocrine disruptors to induce disorders of embryonic and fetal development, characterized not only by morphological abnormalities but also by epigenetic disturbances affecting the formation of physiological systems.

The term *"endocrine disruptors"* is currently used to a broad group of anthropogenic or naturally occurring environmental pollutants that, upon entering the organism, disrupt hormonal homeostasis. This disruption occurs through their ability to interact with hormone receptors or directly affect the activity of endocrine glands (Diamanti-Kandarakis et al., 2009; Zoeller et al., 2012; Taxvig et al., 2013; Reznikov, 2014; Kabir et al., 2015; Darbre, 2018). These substances exhibit hormone-like or anti-hormonal biological activity.

Chemical endocrine disruptors are ubiquitous in the environment and can enter the body via food, drinking water, air, or through dermal absorption. Their sources include pharmaceuticals, cosmetics, clothing materials, and industrial emissions; as a result, these compounds or their metabolites are almost invariably detected in human and animal biological fluids such as urine and blood. A notable example is acetaminophen (paracetamol), commonly detected in urine samples from the general population. It is a metabolite of aniline, a compound used in veterinary medicine, pharmaceuticals, and in the manufacture of pesticides, rubber, food dyes, textiles, and cosmetic products. Paracetamol has been shown to possess anti-androgenic properties, and its pathogenic effects on the fetus during the first two trimesters of pregnancy are associated with the

abnormal development of the male reproductive system, including cryptorchidism, hypospadias, and hypofertility (Thiele et al., 2013; Modick et al., 2014; Arendrup et al., 2018).

Numerous diseases and pathological conditions have been linked to chemical pollutants, including hypofertility, diabetes mellitus, obesity, immune disorders, and cardiovascular diseases. Some researchers suggest that the pathogenic influence of these substances on the developing offspring may underlie the increasing incidence of neurodegenerative and neurodevelopmental disorders such as Parkinson's disease, cognitive dysfunction, attention deficit disorder, and autism — the latter having increased nearly fiftyfold over the past 40 years.

Disorganization of endocrine system activity is most often caused by the entry of by-products of fuel and lubricants, acrylamides, heavy metals, plant growth stimulators and inhibitors, pesticides, fertilizers, phytoestrogens, and plastic hardeners or plasticizers into the body. Endocrine disruptors such as polychlorinated hydrocarbons and DDT possess strong lipophilic and cumulative properties, allowing them to accumulate in adipose tissue and the liver. These compounds are highly resistant to degradation and may persist in the human body for decades.

The adverse effects of endocrine disruptors can extend across several generations due to epigenetic modifications in the genome of germ cells. For instance, the grandsons of women who had been prescribed the synthetic estrogen diethylstilbestrol to prevent miscarriage were found to have a significantly higher incidence of hypospadias compared to the general population (Kalfa et al., 2011).

In 2009, the Endocrine Society of the United States issued a scientific statement highlighting the harmful effects of endocrine disruptors, including impaired reproductive function in both men and women, increased risks of breast and prostate cancer, disturbances of neuroendocrine regulation, metabolic syndrome, thyroid dysfunction, and cardiovascular diseases (Diamanti-Kandarakis et al., 2009).

A distinctive feature of the pathogenic effects of endocrine disruptors is the absence of a straightforward "dose–response" relationship, which complicates the determination of toxic thresholds and the predictability of biological responses. The exposure of the maternal organism to such chemicals triggers a cascade of neurohormonal alterations in both the mother and fetus. Through the involvement of imprinting mechanisms, these changes may program long-term disturbances of neuroendocrine regulation, affecting numerous physiological functions—including behavior, reproductive processes, and adaptive responses—in adult offspring.

Below are examples of the most prevalent endocrine-disrupting chemicals found in the environment:

- **BPA (BPA):** present in the white inner coating film of metal food cans and bottle caps, plastic packaging materials, and certain dental compounds.
- **Polycarbonates:** used in rigid plastic products, including hard contact lenses.
- **Epoxy resins and phthalates:** found in food packaging made of foil or cardboard, emulsion paints, floor coverings produced from polyvinyl chloride, and in printing inks.
- **Alkylphenols:** contained in industrial detergents, shampoos, shaving creams, spermaceti-based cosmetics, gasoline, and antioxidants that prevent yellowing of transparent synthetic materials.
- **Dioxins and chlorine-containing compounds:** detected in lipid-rich food products.
- **Pesticides and their degradation products** (e.g., vinclozolin and others): found in vegetables, fruits, and dairy products.
- **Insecticides:** including DDT, methoxychlor, and permethrin.
- **Pentachlorophenol:** used in wood preservatives and leather treatment products.
- **Xenohormones** (e.g., phytoestrogens): naturally occurring in soybeans, clover, alfalfa, hops, parsley, and celery.
- **Nanomaterials:** such as nanometals, nanocarbons, and dendrimers.
- **Heavy metals:** including cadmium, lead, arsenic, mercury, and others.

5.2. Mechanisms of Action of Endocrine Disruptors

The endocrine system of humans and other mammals possesses a highly complex structural and functional organization. Endocrine regulation operates through several hierarchical levels that are interconnected by direct or reciprocal functional relationships. These include neuroimmunoendocrine, hormonal, and metabolic mechanisms controlling the synthesis, secretion, and metabolism of hormones; their binding to transport proteins in the bloodstream; as well as systems of hormone receptor interaction and signal transduction. In essence, endocrine signaling translates into electrophysiological, biochemical, and genetic responses within target cells.

Endocrine disruptors interfere with one or, more commonly, multiple biological processes involved in hormonal regulation (Pinto & Carvalho, 2013; Reznikov, 2014; Sidorkiewicz et al., 2017; Yilmaz et al., 2020; Ahn & Jeung, 2023; Michelangeli et al., 2024; Soyer-Gobillard et al., 2025).

In the pathogenesis of disorders induced by endocrine disruptors, their hormone-like or antihormonal activity is accompanied by disturbances in fundamental biochemical

pathways that sustain essential cellular functions. One of the most universal mechanisms of such damage involves disruption of the physiological balance between oxidative and antioxidative processes, a phenomenon referred to in modern pathophysiology as oxidative stress**.** This condition is characterized by excessive generation of free radicals and reactive oxygen species, leading to impaired function and structural integrity of cellular membranes and organelles. Environmental endocrine disruptors, in particular, have been shown to alter both the quantity and quality of sperm through metabolic disturbances in testicular tissue.

A distinctive feature of endocrine disruptor toxicity lies in their ability to interact with hormone receptors. A vast body of experimental evidence indicates that their agonistic or antagonistic effects toward natural hormonal ligands occur *via* binding to membrane, nuclear, or cytoplasmic receptors for estrogens, androgens, and progesterone, thereby disrupting intracellular signaling mechanisms and genomic function (Stavros et al., 2025). For example, BPA acts as a direct estrogen receptor agonist (Fig. 5.1).

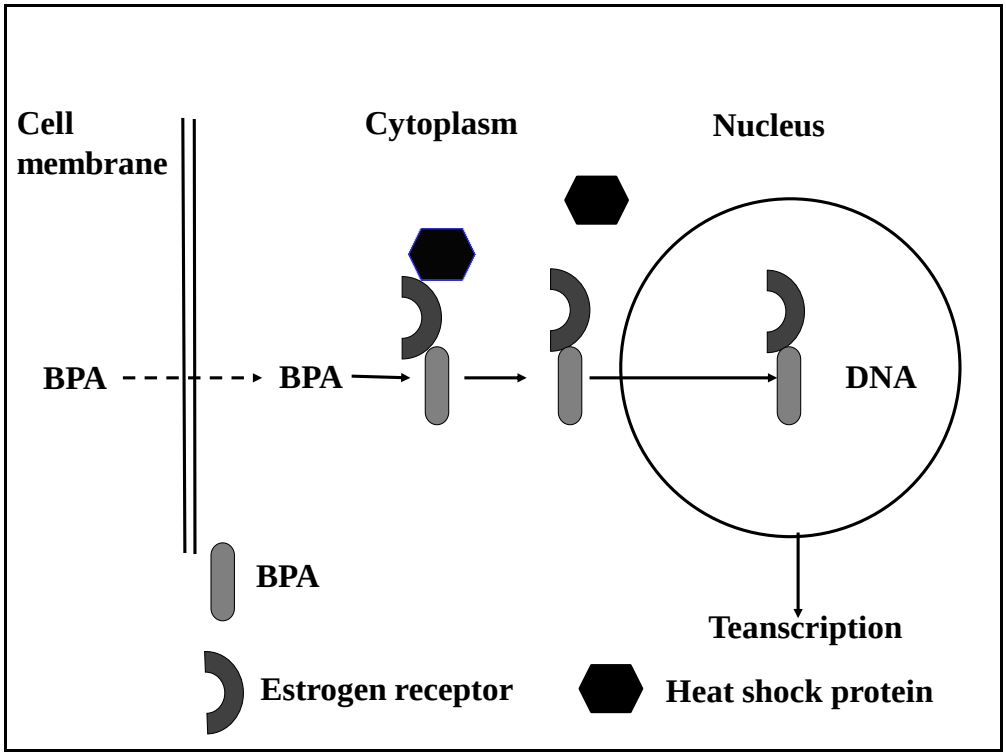

Figure 5.1 Interaction of BPA with estrogen receptor and the genome

Some endocrine disruptors interfere with the synthesis and metabolism of hormones either directly or indirectly by suppressing the secretion of pituitary hormones. The

formation of estrogens may be reduced through inhibition of aromatase, an enzyme that plays a key role in the conversion of testosterone to estradiol and androstenedione to estrone.

Endocrine disruptors are also capable of inducing epigenetic reprogramming *via* altered transcriptional mechanisms, primarily through DNA methylation at cytosine–guanine sequences and through post-translational histone modifications such as methylation, phosphorylation, acetylation, and ubiquitination.

Another critical pathogenic mechanism of endocrine disruptor action on the developing fetus is the impairment of blood flow within the feto-placental system and the weakening of histohematous barrier functions. Damage to the blood–testis barrier caused by certain disruptors has been demonstrated experimentally; it may contribute to the development of autoimmune orchitis**.**

In real-life conditions, humans are usually exposed not to a single disruptor but to complex mixtures of such compounds. The outcomes of combined exposure are difficult to predict, as the interactions between individual chemicals may result in synergistic potentiation in some cases and mutual antagonism in others (Germaine & Buck, 2014).

The major experimentally confirmed mechanisms underlying the harmful effects of endocrine disruptors are summarized below:

- **Genomic effects *via* aryl hydrocarbon receptors:** binding to dioxin-responsive elements in nuclear DNA.
- **Genomic effects *via* estrogen and androgen receptors:** interaction with hormone-responsive elements in nuclear DNA.
- **Epigenomic effects:** involving DNA hypo- or hypermethylation; histone acetylation, methylation, phosphorylation, or ubiquitination; and interference with non-coding microRNAs.
- **Non-genomic effects *via* membrane estrogen receptor:** modulation of intracellular kinase signaling cascades.
- **Oxidative stress:** damage to cellular membranes and organelles induced by reactive oxygen species.
- **Hormonal imbalance:** disruption of steroidogenesis.

5.2. Reproductive Targets of Endocrine Disruptors

The reproductive system appears to be one of the most vulnerable targets of endocrine disruptors. Numerous studies have documented a progressive decline in the fertility potential of the global population over recent decades. According to a meta-analysis of

ten prospective and retrospective investigations evaluating the effects of endocrine disruptors—including heavy metal salts, dioxins, polychlorinated and polybrominated bisphenols, DDT, DDE, and related compounds—together with the characterization of individual exposure doses, a significant association was established between environmental exposure to these pollutants and reduced fertility rates among married couples (Pinto & Carvalho, 2013).

Exposure to BPA and di-(2-ethylhexyl) phthalate (DEHP) has been implicated in the disruption of reproductive function, particularly through alterations in folliculogenesis and steroidogenesis. Experimental data consistently support these effects; however, clinical evidence remains inconclusive. A recent meta-analysis reported an inverse association between BPA exposure and *in vitro* fertilization outcomes (Yuan et al., 2025), whereas other human studies have not confirmed significant changes in ovarian reserve or fertilization success (Incognito et al., 2025).

Adverse Effects of Endocrine Disruptors on the Male Fetus and Offspring

Fetus:

- Cryptorchidism
- Hypospadias
- Impaired spermatogenesis
- Decreased testosterone secretion
- Micropenis

Adult male:

- Hypogonadism and infertility
- Autoimmune orchitis
- Increased risk of testicular cancer

Adverse Effects of Endocrine Disruptors on the Female Fetus and Offspring

Fetus:

- Fetal hypoxia
- Impaired intrauterine development

Adult female:

- Disorders of sexual development

- Premature thelarche
- Endometrial hypoplasia
- Carcinoma of the uterus, vagina, or mammary glands
- Endometriosis
- Menstrual irregularities and PCOS (PCOS)

Studies examining the structure and function of ovaries damaged by endocrine disruptors have revealed the presence of ovarian dysgenesis syndrome, disturbances in folliculogenesis, premature follicular atresia, meiotic arrest of oocytes, and inhibition of steroidogenesis in granulosa and theca cells.

Given the complexity of these processes, the condition of reproductive targets affected by endocrine disruptors warrants a more detailed consideration.

5.3. Effects of Endocrine Disruptors on Fetal and Neonatal Development

Prolonged exposure of pregnant women to endocrine disruptors poses a serious threat to fetal development, particularly to the differentiation and maturation of the reproductive system. For example, a higher incidence of male infants born with micropenis, hypospadias, and cryptorchidism has been associated with maternal and fetal exposure to pesticides in Northeastern Brazil, where their agricultural use is extensive (Gaspari et al., 2012). Similar findings regarding the impact of endocrine disruptors have been reported by Egyptian researchers (El Kholy et al., 2013).

Polychlorinated bisphenols are known to exhibit estrogenic properties (Lee et al., 2013). Accordingly, administration of these compounds to pregnant rats resulted in prolonged estrous cycles and morphological alterations in the POA and MBH of adult female offspring—regions critically involved in the neuroendocrine regulation of ovarian function and reproductive cyclicity (Walker et al., 2013). In neonates of both sexes, prenatal exposure to BPA caused aberrant expression of estrogen receptor subtypes α and β within the amygdala and MBH (Cao et al., 2013).

Because BPA, methoxychlor, and vinclozolin are capable of crossing the fetal blood–brain barrier, they can induce epigenetic modifications in genes responsible for the sexual differentiation of the brain. These changes may later manifest as abnormalities in sexual behavior and other hormone-dependent neural functions. Moreover, endocrine disruptors impair the synthesis and secretion of GnRH and kisspeptins—key stimulators of the HPG axis. As a consequence of such neuroendocrine alterations, delayed puberty and various reproductive pathologies are often observed in the offspring.

As mentioned above, acetaminophen (paracetamol) poses a considerable risk to both the mother and the developing fetus, particularly during the second trimester of pregnancy. The compound readily crosses the placental barrier and enters the fetal systemic circulation. Its sources are not limited to paracetamol itself but also include pork and poultry meat (as the use of paracetamol is permitted in animal production technologies), as well as colored textiles, rubber, and cosmetic products manufactured using aniline-based processes.

Studies on organotypic testicular cultures and on Leydig cells isolated from adult men and neonates have elucidated the mechanisms underlying reduced testosterone synthesis and the increased risk of cryptorchidism following paracetamol exposure. These effects appear to result from the suppression of insulin-like growth factor 3 (IGF-3) and prostaglandin production due to the inhibitory action of paracetamol on cyclooxygenase activity (Modick et al., 2014).

Experimental studies in rats demonstrated decreased sperm production and disruption of the histological architecture of the testes in neonates and at 10 months of age following maternal administration of paracetamol, endocrine-disrupting chemicals with estrogenic or antiandrogenic activity, or their mixtures. The exposure began on GD 7 and continued through the end of pregnancy and lactation (until PND 21) (Axelstad et al., 2018). Notably, the paracetamol dose used corresponded to therapeutic levels in humans, while the concentrations of other substances approximated real-life environmental exposure. Even the lowest tested doses caused significant impairment of spermatogenesis, leading the authors to conclude that current permissible environmental concentrations of these compounds should be reconsidered.

One of the key mechanisms underlying impaired spermatogenesis following prenatal exposure to endocrine disruptors involves their direct action on Leydig cells, Sertoli cells, and testicular stem and germ cells. The process of spermatogenesis critically depends on Sertoli cells, which are localized within the seminiferous epithelium of the testicular tubules. Germ cell precursors of spermatogonia form tight junctions with Sertoli cells through actin filament connections, ensuring structural and functional integrity of the spermatogenic epithelium. Endocrine disruptors interfere with this interaction and induce apoptosis of Sertoli cells, leading to disorganization of spermatogenesis and impaired sperm maturation.

Prenatal exposure to paracetamol has also been shown to adversely affect the female reproductive system, particularly folliculogenesis, by reducing the number of primordial follicles in the ovaries (Arendrup et al., 2018). In adulthood, these females exhibit delayed follicular development, premature follicular atresia, irregular estrous cycles, and

reduced or absent fertility. Overall, this condition resembles the clinical manifestations of premature ovarian insufficiency in women.

Among external factors contributing to endocrine disruption, phytoestrogens occupy a prominent place (Boberg et al., 2013). Depending on their chemical structure, dose, and duration of exposure, these compounds exert agonistic, antagonistic, or modulatory effects on estrogen receptor in target tissues. Two major groups of phytoestrogens are distinguished: isoflavones (heterocyclic phenols) and lignans (bisphenolic compounds). They are found in a wide variety of plants consumed by humans and animals, including soybeans, red clover leaves, lentils, dates, black grapes, and sunflower seeds, among others (Akingbemi et al., 2007).

Feeding pregnant rats and their offspring a mixture of twelve xenoestrogens, primarily the lignan resinol and the isoflavones genistein and daidzein, resulted in a reduction of AGD in male neonates, hypertrophy of the mammary glands in adulthood, and elevated serum estradiol concentrations. However, no significant changes were observed in the weights of the gonads, accessory sex glands, or in circulating testosterone levels (Bonde et al., 2017).

In female mice whose mothers consumed a low-phytoestrogen diet during gestation, premature sexual maturation was observed, accompanied by elevated blood estradiol concentrations and increased uterine sensitivity to estrogen. Male offspring, by contrast, exhibited reduced testicular, epididymal, and seminal vesicle weights, along with an enlarged prostate gland. This constellation of symptoms is recognized as the *fetal estrogenization syndrome* (Ruhlen et al., 2008). Administration of genistein to pregnant laboratory animals resulted in endometrial hypoplasia and an increased incidence of uterine and mammary gland tumors in the offspring. The phytoestrogen coumestrol demonstrated embryotoxic effects in mice, and the surviving progeny showed markedly reduced fertility.

Among the most widespread endocrine disruptors are phthalate esters**,** commonly used as plasticizers. The most extensively applied compounds include DEHP and di(n-butyl) phthalate (DBP**)**, the latter being comparatively less studied. Phthalates are primarily employed in the manufacture of polyvinyl chloride products such as floor and roof coverings, packaging materials for cosmetics and medical devices (e.g., household and surgical gloves), beverage containers, coatings for orally administered pharmaceuticals, and even children's toys.

Phthalates are non-covalently bound to polymer matrices and can easily leach into the environment, leading to widespread contamination. Their metabolites are frequently detected in the urine and blood of pregnant women, in amniotic fluid, in cord blood, and

in both children and adults. Notably, monobutyl phthalate, the primary metabolite of DBP, has been detected at elevated concentrations in the urine of pregnant women and in individuals taking medications coated with DBP-containing materials (Hernandez-Diaz et al., 2009).

Extensive epidemiological and experimental studies have demonstrated a clear association between prenatal and postnatal phthalate exposure and disorders of the male reproductive system (Barrett and Swan, 2015; Bonde et al., 2017; Lara et al., 2017; Axelstad et al., 2018). Owing to their antiandrogenic activity, phthalates interfere with the normal development of the male reproductive tract during fetal life. Compounds such as diethyl phthalate and other phthalate esters directly target the testicular genome and disrupt steroid biosynthesis, resulting in decreased testosterone production (Gray et al., 2025).

By contrast, the female reproductive system appears to be relatively resistant to the adverse effects of phthalates (Ema & Miyawaki, 2001; Guerra et al., 2010; Xie et al., 2016). However, some rodent studies report opposing outcomes, including polycystic ovarian morphology and altered hormonal profiles in adult offspring following prenatal exposure to DBP and DEHP, resembling the PCOS phenotype in women (Hewlett et al., 2017). As will be discussed below, phthalate-induced alterations in the female reproductive system are now supported by a growing body of evidence.

The feminizing effect of phthalates in males is primarily attributed to inhibition of testosterone synthesis in fetal testes and disorganization of Leydig cell histoarchitecture, the main testosterone-producing cell population. At high doses, DBP suppresses cholesterol side-chain cleavage within the mitochondria of steroidogenic cells (Hallmark et al., 2007), a rate-limiting step in steroidogenesis.

However, both human and animal studies indicate that the testicular dysgenesis syndrome induced by phthalates—manifesting as impaired spermatogenesis, cryptorchidism, hypospadias, and an increased risk of testicular cancer—does not necessarily result from androgen deficiency alone. Instead, elevated fetal estradiol levels and/or alterations in the testicular proteome have been implicated (Veeramachaneni & Klinefelter, 2014). According to these authors, phthalates activate gonadal aromatase, leading to excessive estradiol production. This hormonal imbalance disrupts the synthesis of key proteins responsible for normal testicular differentiation and development.

As a result, vascular injury and interstitial biochemical disturbances occur, followed by local hemorrhage and inflammation. These pathological processes lead to abnormal clustering and functional impairment of Leydig cells, thereby reducing testosterone

synthesis. The subsequent hormonal insufficiency and structural disorganization contribute to abnormal differentiation and morphogenesis of the seminiferous tubules and accessory sex glands.

Along with the inhibition of steroidogenesis in the testes of rat fetuses, DBP decreases the expression of the insulin-like factor 3 (Insl-3), which led the authors of the study to confirm that the male fetal gonads represent a primary target of this endocrine disruptor (Ge & Chen, 2007; Howdeshell & Rider, 2008). The deficiency of this peptide factor, produced by Leydig cells and critical for the first phase of testicular descent into the scrotum, explains the occurrence of cryptorchidism following prenatal exposure to DBP (Anand-Ivell & Ivell, 2014). DBP disrupts the maturation of both Leydig and Sertoli cells, resulting in a reduction of the germinal epithelium in pathologically altered seminiferous tubules of adult individuals, as well as the appearance of multinucleated germ cells. The clustering of Leydig cells becomes disorganized, leading to their hypoplasia, which persists into adulthood.

Because the structure of androgen and Insl-3 receptors is highly conserved among mammals, there is a strong likelihood that phthalates present in the biological fluids of pregnant women and pose a threat to the reproductive health of offspring. A large, controlled clinical study demonstrated that the concentrations of Insl-3 and phthalate metabolites in the amniotic fluid of women at 13–16 weeks of gestation, i.e., during the critical "window" of male reproductive organogenesis, correlate with the incidence of cryptorchidism and hypospadias (Anand-Ivell et al., 2018).

Administration of DBP to pregnant rats at doses of 50, 250, or 500 mg/kg b.w. daily from GD 12.5 to 21.5 induced enhanced apoptosis in the fetal testes through the disruption of key proteins regulating apoptosis and cell proliferation (Ma et al., 2017).

Abnormal development of the male reproductive tract in rats has also been observed following maternal exposure to dipentyl phthalate (Gray et al., 2015) and DEHP. Along with the absence of effects on sexual maturation and ovulation in females prenatally exposed to DEHP, this finding supports the antiandrogenic nature of the disruptor. However, certain distinctions have been noted compared to the effects of di(n-butyl) phthalate, including the occurrence of prostatic agenesis.

Testing of the estrogenic potential of DBP and butyl benzyl phthalate in female rats demonstrated the absence of any estrogen-like effects (Ahmad & Verma, 2015).

The pathogenic impact of endocrine disruptors on fetal testes leads to impaired sexual differentiation of the brain, resulting from decreased testosterone production during the critical period of brain masculinization.

5.4. Phthalates as Etiological Factors within the Concept of Functional Teratology

The long-term effects of pre- and perinatal exposure to phthalates, particularly dibutyl phthalate (DBP), on the fetus have been scarcely investigated within the framework of functional teratology. Such experiments are best conducted on animals whose offspring exhibit no overt anatomical malformations or only minimal morphological abnormalities. A sensitive indicator of the antiandrogenic activity of the studied compound is the AGD.

The distance between the anus and the external genitalia in male mammals is greater than that in females and serves as one of the morphological markers of sex. Fusion of the perineal seam occurs prenatally and requires the presence of the male sex hormone—testosterone. Therefore, a reduction in AGD in newborn male rodents indicates testosterone deficiency or an antiandrogenic influence of the tested compound on the developing fetus. This mechanism underlies the observed decrease in AGD in male neonates born to mothers exposed to DBP (Swan et al., 2005; Van den Driesche et al., 2017).

Among the functional disturbances induced by perinatal exposure to dibutyl phthalate, behavioral effects occupy a prominent place. Modification of various behavioral patterns as a result of perinatal phthalate exposure is now considered an established scientific fact. For example, oral administration of a phthalate mixture to pregnant rats maintained on a high-fat diet during gestation and the first ten days *post partum* led to reduced social and play behavior in male offspring, whereas female offspring exhibited enhanced play activity (Kougias et al., 2018).

5.5. Effects of Low-Dose DBP Prenatal Exposure in Males

It is believed that the harmful effects of DBP on the developing fetus are mediated by its active metabolite, monobutyl phthalate (Ema & Miyawaki, 2001). Administration of what the authors considered to be relatively low doses of monobutyl phthalate (250–750 mg/kg b.w.t per day during GD 15–17) to rats resulted in reduced AGD in male offspring and the occurrence of cryptorchidism. However, the presence of cryptorchidism indicates that the doses used induced a pronounced teratogenic effect.

Researchers studying the prenatal effects of DBP predominantly report severe anatomical malformations in male offspring (teratogenic effects), as well as hypogonadism and other signs of impaired sexual function when pregnant rats were administered DBP at doses exceeding 250 mg/kg b.w. (Zhang et al., 2004). Nevertheless, the possibility of functional disturbances in the absence of gross teratogenic effects cannot be excluded, and this aspect should serve as a key criterion for defining threshold

exposure levels. Therefore, it is reasonable to consider the results of experiments involving low-dose DBP administration during pregnancy, particularly during the critical periods of reproductive organ formation and sexual differentiation of the brain.

Within the framework of the U.S. National Toxicology Program, studies were conducted on male offspring of rats exposed to DBP through dietary intake during GD 12–20. The minimal daily dose that produced no observable adverse reproductive effects (NOAEL) was established at approximately 66 mg/kg/day (Foster & Cattley, 2000). A corresponding dose determined in experiments where DBP was administered throughout the entire gestation period was 50 mg/kg/day (Zhang et al., 2004). Extrapolation of this exposure level to humans corresponds to an estimated oral daily intake of about 500 mg/kg/day. In experimental studies on rats, a dose of 100 mg/kg/day is considered to be near the NOAEL threshold and, therefore, classified as low.

In this context, particular interest is drawn to a study that examined the state of hormonal receptors in Leydig cells of rats whose mothers received DBP at a dose of 100 mg/kg/day since GD 12 until PND 21 of lactation (Wakui et al., 2014). The authors based their investigation on prior experimental data in rodents showing that prenatal exposure to DBP induces atypical Leydig cell hyperplasia in the testes of adult animals. Their findings indeed confirmed atypical Leydig cell hyperplasia accompanied by degenerative changes in the seminiferous tubules. Using Western blotting, polymerase chain reaction, and immunohistochemistry, they detected a marked upregulation of estrogen receptor alpha (ERα) expression and a downregulation of both estrogen receptor, ERα and beta (ERβ), and androgen receptor expression in the testes and Leydig cells, along with corresponding alterations in receptor mRNA levels.

A considerable number of male rats perinatally exposed to DEHP were sexually inactive in the presence of receptive females, a phenomenon not correlated with any morphological abnormalities, suggesting an isolated disruption of sexual differentiation in the fetal brain (Moore et al., 2001).

In contrast to most published studies investigating the effects of endocrine disruptors administered during embryonic and fetal development, our research selectively examined the epigenetic reprogramming of neuroendocrine development and behavior that occurs after the completion of embryogenesis, specifically, during the critical period of sexual differentiation of the brain. Therefore, we investigated the consequences of prenatal exposure of rats, *via* the maternal organism, to relatively low doses of DBP during the period corresponding to androgen-dependent programming of the fetal rat brain, *i.e.*, during the final week of gestation (Reznikov et al., 2017; Reznikov et al., 2020a).

Study Design. Pregnant female rats were administered a 10% oil solution of DBP intragastrically via a metal catheter at a daily dose of 100 mg/kg b. w. since GD 15 to 21. Control animals received the vehicle only, in the same manner. Male sexual behavior was evaluated from birth to senescence—at 6, 10, and 18 months of age. Lordosis responses were tested in orchiectomized males activated by estradiol and progesterone administration in the presence of a normal male.

On PND 2, 7, and 10, the AGD was measured. Subsequently, the timing of testicular descent into the scrotum was recorded. For behavioral testing, five males born to different mothers in each group (experimental and control) were selected. Male sexual behavior was examined at 6 months of age (in December) and in another cohort at 10 months (in April), whereas female sexual behavior was assessed at 10.5 months. A subset of males from each group (n=6 per group) was euthanized by decapitation at 6 months of age, and plasma testosterone concentrations were measured.

Offspring Characteristics. Ten pregnant rats exposed to DBP gave birth to a total of 87 pups, averaging 8.7 per litter. Four control females produced 45 pups, averaging 11.2 per litter. A reduction in litter size under prenatal DBP exposure, in the absence of overt adverse effects on the dams, was also noted previously (Zhang et al., 2004).

AGD. Prenatal exposure to DBP resulted in a statistically significant reduction of AGD in male offspring, indicating antiandrogenic activity of the compound. When normalized to b.w.t (mm/g), AGD was 0.51 ± 0.01 mm *vs*. 0.56 ± 0.01 mm in controls on PND 2; 0.52 ± 0.01 mm *vs.* 0.59 ± 0.02 mm on PND 7; and 0.47 ± 0.01 mm *vs.* 0.51 ± 0.01 mm on PND 10.

Sexual Maturation. In rats, testicular descent into the scrotum is an indicator of pubertal onset; spermatozoa typically appear in the epididymides 5–7 days thereafter. In control males (n = 28), testicular descent occurred at 38.54 ± 0.14 days postnatally, whereas in DBP-exposed males (n = 47), it occurred at 33.18 ± 0.12 days, *i.e.,* 5.4 days earlier ($P < 0.001$). Similar results were obtained in mice, where maternal DBP exposure induced early puberty in male offspring (Ma et al., 2017).

Testosterone Levels. The plasma testosterone concentration in sexually mature control males (n = 6) was 13.03 ± 2.96 nmol/L, whereas in DBP-exposed males (n = 6) it was 26.13 ± 5.04 nmol/L, *i.e.,* approximately twofold higher ($P < 0.001$), indicating marked hyperandrogenization.

Testicular and Accessory Sex Gland Morphology. Prenatal exposure to DBP did not alter the structure of the spermatogenic layer in the testes compared with controls. All stages of spermatogenesis were observed without visible abnormalities. Clusters of

Leydig cells were present in the interstitial tissue. Although the number of Leydig cells appeared lower than in controls, most were larger and exhibited features of active steroidogenesis, explaining the elevated plasma testosterone levels. Sperm counts determined in epididymal flushes (2 mL per sample) using a Goryaev's chamber showed a tendency toward increase: 38.2 ± 1.6 million/mL in the DBP-exposed group *vs.* 31.9 ± 2.9 million/mL in controls ($0.05 < p < 0.1$).

Sexual Behavior. Male sexual behavior was assessed for 15 minutes in the presence of a receptive female that had been previously ovariectomized and hormonally stimulated with sex steroids.

The sexual behavior of control males at 6 and 10 months of age exhibited certain differences that could be attributed to seasonal variations. The low sexual activity of 6-month-old males coincided with the winter period (December), during which sexual activity, testosterone secretion, and fertility in rats are known to decline. During the first 15-minute behavioral test, no ejaculations were recorded in these males (Table 5.1). By 10 months of age, i.e., in late February, approaching the spring season, ejaculations were observed and the number of intromissions slightly increased (Table 5.2). One of the five males in this age group was excluded from further testing because no signs of sexual motivation toward the receptive female were detected during the 15-minute session.

During the second testing session, both age groups of control males exhibited reduced latency periods to the first mounting and first intromission, shorter post-ejaculatory refractory periods, and an increased number of intromissions compared with the first test. In 6-month-old males, ejaculations were recorded during the repeated testing. Overall, these results indicate the acquisition of sexual experience by the animals.

Against the background of seasonally reduced sexual activity observed in 6-month-old control males, the DBP-exposed group demonstrated a pronounced enhancement of male sexual behavior across nearly all parameters in both testing sessions. The observed activation involved both central (motivational) and peripheral (copulatory and ejaculatory) components of male sexual behavior (Table 5.1).

A significant difference in several parameters of male sexual behavior between the experimental and control groups persisted in 10-month-old males (Table 5.2). This difference was characterized by the appearance of ejaculations during the first testing session, an increase in the number of ejaculations, and a shortening of the latency to the first intromission during the second testing session. Changes in other behavioral parameters were also pronounced and occurred in the same direction as those observed in 6-month-old animals. These differences approached statistical significance, although they did not reach the accepted level of reliability.

Table 5.1 Male-type sexual behavior in male rats aged 6 months after prenatal action of DBP

Parameter	Control	DBP
	First testing	
Latency period (s):		
First Mounting	109.4 (10-219)	15.2 (3-40) [a]
First intromission	125.2 (15-259)	21.2 (8-51) [a]
First ejaculation	-	490.6 (315-743) [a]
Number of:		
Mountings without intromission	3.8 (1-6)	2.4 (2-3)
Intromissions	9.8 (1-19)	18.8 (17-25) [a]
Ejaculations	0 (0)	1.2 (1-2) [a]
	Second testing	
Latency period (s):		
First Mounting	3.8 (2-9)	20.4 (2-73) [a]
First intromission	28.0 (3-113) [b]	30.8 (9-88)
First ejaculation	361.4 (244-602) [b]	451.0 (273-658)
Number of:		
Mountings without intromission	4.0 (3-5)	2.8 (2-4) [ab]
Intromissions	17.4 (11-35)	26.6 (20-32) [b]
Ejaculations	1.2 (1-2) [b]	1.8 (1-2) [ab]

Footnotes: Here and in Table 5.2, data are presented as medians (in parentheses – the range of individual variations). Each group contained 5 rats. Statistical analysis of differences was performed using the Wilcoxon–Mann–Whitney *U* test.

[a] $p < 0.05$ compared with control males; [b] $p < 0.05$ compared with the same group of animals during the first testing.

Along with the activation of male sexual behavior, testing of female-type sexual behavior in 10-month-old males of the experimental group revealed a significant increase in the number of lordosis reactions. At the same time, these males exhibited Mounting attempts toward normal active males, indicating the emergence of homosexual behavior.

Table 5.2 Parameters of male-type sexual behavior in 10-month-old male rats after prenatal exposure to DBP

Parameter	Control	DBP
	First testing	
Latency period (s):		
First Mounting	67.7 (6-217)	94.2 (1-312)
First intromission	124.7 (40-310)	121.0 (5-380)
First ejaculation	393.7 (150-555)	384.2 (146-859)
Number of:		
Mountings without intromissions	7.5 (4-14)	9.8 (8-12)
Intromissions	14.0 (10-22)	20.8 (18-23)
Ejaculations	1.0 (1)	1.8 (1-2) [a]
	Second testing	
Latency period (s):		
First Mounting	41.0 (1-159)	1.8 (1-3)
First intromission	81.5 (5-295)	6.8 (2-10) [ab]
First ejaculation	282.5 (150-380)	376.2 (184-580)
Number of:		
Mountings without intromissions	3.7 (2-5)	6.0 (2-12) [b]
Intromissions	20.2 (13-34)	28.8 (24-42) [b]
Ejaculations	1.2 (1-2) [b]	1.6 (1-2) [ab]

Refer to the Table 5.1.

Thus, administration of DBP to pregnant rats at a daily dose of 100 mg/kg b.w. during the period of sexual differentiation of the fetal brain resulted in the programming of hypersexual behavior and a hyperandrogenization syndrome in male offspring of the first generation. Although elevated plasma testosterone levels were detected in adult experimental males, the earlier descent of the testes into the scrotum strongly suggests that a hyperandrogenic state had already been established before the onset of puberty.

The results of this study demonstrate a certain discrepancy between the shortening of the AGD, which is typically associated with androgen deficiency, and the acceleration of sexual maturation, which is stimulated by testosterone. Reduction of the AGD is a characteristic feature of the so-called "phthalate syndrome," which, at high doses (≥ 250 mg/kg b.w.), also induces testicular dysgenesis, reduced testosterone synthesis, cryptorchidism, hypospadias, hypo- and pathospermia, and other malformations of the urogenital system. However, apart from a slight delay in perineal fusion, no other signs of urogenital morphogenetic abnormalities were observed in our study following

prenatal exposure to a lower dose of DBP (100 mg/kg b.w.). Therefore, the most probable cause of the detected deviation in sexual dimorphism is a hormonal imbalance during gestation.

The hypersexual behavior of males exposed prenatally to DBP can be attributed to excessive testosterone saturation. Testosterone activates hypothalamic centers and the cerebral cortex, stimulating sexual motivation toward females, whereas its active metabolite, 5α-dihydrotestosterone, primarily acts at the level of spinal sympathetic and parasympathetic centers, mediating sexual reflexes of erection, copulation, and ejaculation. The elevated testosterone level explains the reduced latency to the first mounting and first intromission, the doubling of the number of intromissions, and the appearance of ejaculations in the DBP-exposed group, unlike in controls, during the first testing of male sexual behavior in 6-month-old animals. In contrast, during the second testing, *i.e.* after acquisition of sexual experience, the differences in the latency periods to the first intromission and ejaculation between control and DBP-exposed males disappeared. Changes in other behavioral parameters remained significant and followed the same direction as during the first testing.

Neuromorphology. Given the physiological role of the POA of the male rodent brain, it was considered appropriate to conduct a detailed neuromorphological examination of this region. It is well known that the MPN of the rodent brain is directly involved in the regulation of male sexual behavior.

Histological analysis of this structure in 6-month-old control male rats revealed that the majority of its neurons were polygonal in shape and contained oval nuclei. The size of the neurons, as well as the size of their nuclei, varied considerably (Fig. 5.2). Larger neurons were predominantly located in the peripheral portion of the MPN, where they were arranged less densely than in the central region. Their perikarya exhibited a well-defined Nissl substance with intensive staining. Neurons with vacuoles in the perikaryon were encountered only occasionally. In the central portion of the nucleus, clusters of tightly packed small neurons were observed, each possessing a thin rim of cytoplasm surrounding small nuclei. A minor proportion of neurons demonstrated hyperchromatic staining, and shrunken (pyknotic) neurons were also detected in two of the six examined animals.

In the MPN of male rats whose mothers received DBP, most neurons were characterized by larger nuclei and perikarya compared with those of control animals (Fig. 5.3). The cytoplasm of the perikarya in many neurons appeared lighter and exhibited signs of vacuolization. The perikarya were often oval in shape, and the majority of neurons contained large, spherical nuclei. The number of hyperchromatic neurons within the nucleus was markedly greater than in controls, and such cells were observed in all

animals of the experimental group. The neuronal density within the nucleus was lower than that in the control rats.

Overall, the histological pattern was interpreted as indicative of enhanced functional activity of the MPN, consistent with the hypersexual behavior observed in the experimental males. Hypersexual male-type behavior and the hyperandrogenic state

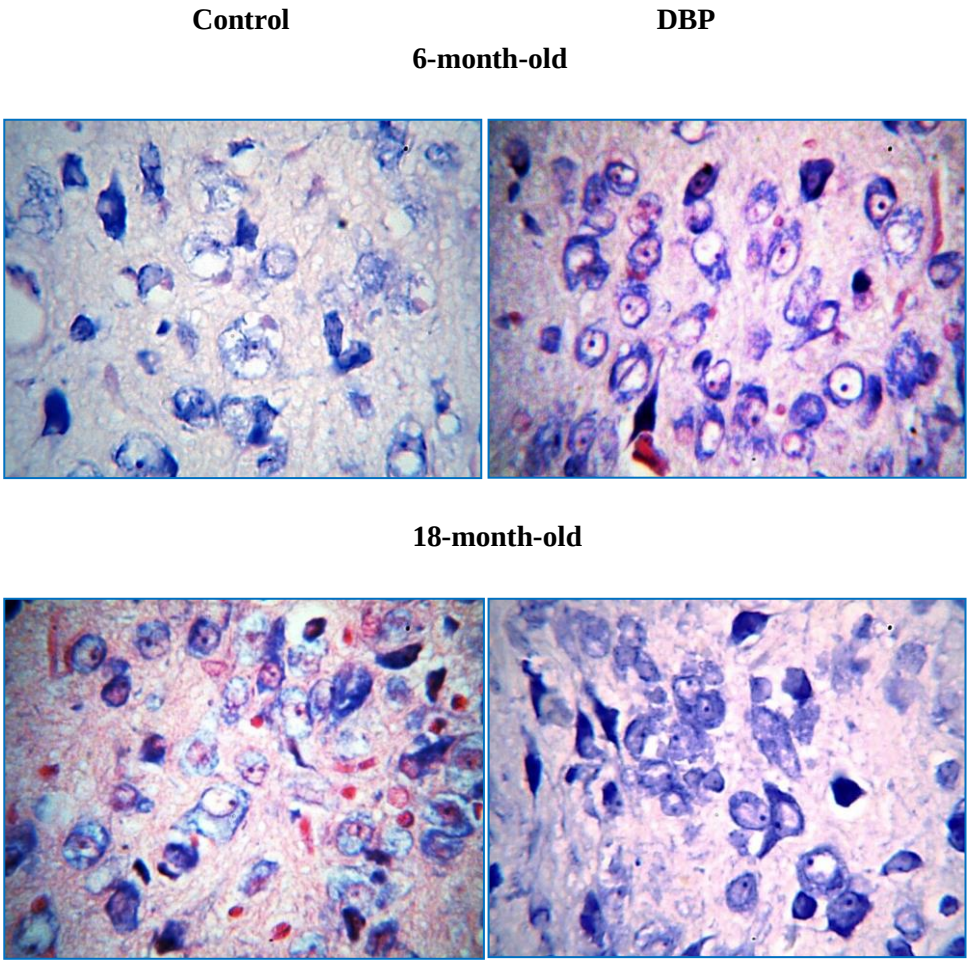

Fig. 5.2 Microphotographs of the MPN in male rats of different ages whose pregnant mothers were exposed to DBP.
Azure–eosin staining. x400

observed in sexually mature offspring exposed to DBP are most likely the result of excessive masculinization of the fetal brain during its sexual differentiation.

In male rats, this process occurs during the final week of gestation and may extend into the first few PND under the influence of testosterone secreted by the fetal testes. It is well established that the pathogenic effects of endocrine disruptors on the reproductive system are mediated through the signaling pathways of estrogen and androgen receptors, disruption of steroidogenesis, or *via* oxidative stress (Pinto & Carvalho, 2013; Aly et al., 2016; Sidorkiewicz et al., 2017).

Among these potential mechanisms underlying the behavioral alterations observed, the most plausible appears to be an increased synthesis of testosterone in the fetal testes. This hypothesis is supported by data demonstrating the direct stimulatory effect of DBP and its metabolite, monobutyl phthalate, at low concentrations on testosterone synthesis in cultured murine Leydig cells (MLTC-1) (Wang et al., 2006; Chen et al., 2013). At higher concentrations, however, both compounds inhibited steroidogenesis at the enzymatic level—specifically, cytochrome P450scc (responsible for cholesterol side-chain cleavage) and 17β- and 3β-hydroxysteroid dehydrogenases (cytochromes P450c17 and 3β-HSD, respectively). The authors demonstrated that the enhancement of steroidogenesis was associated with stimulation of the synthesis of the steroidogenic acute regulatory protein (StAR), which facilitates the transport of the steroidogenic substrate—cholesterol—from the cytosol to the inner mitochondrial membrane.

The unexpected activation of female-type sexual behavior in the DBP -exposed males may appear contradictory to the hypothesis of excessive brain masculinization during early neuroendocrine programming. However, this phenomenon may result from impaired defeminization pathways within neuroendocrine structures, possibly due to oxidative stress.

Thus, the functional alterations in the reproductive system identified in the present study are consistent with the general concept of functional teratology. Although DBP produced activation of androgenic function and male sexual behavior—contrary to the expected suppressive effects—against the background of an almost complete absence of teratogenic manifestations, this paradoxical effect should not be regarded as beneficial. If similar mechanisms occur in humans, such alterations may underlie the development of so-called criminal hypersexuality, which is often associated with abnormally elevated blood testosterone levels (Cooper, 1986). Moreover, the emergence of homosexual-type behavior and female-pattern brain differentiation in DBP -exposed males may indicate a potential risk of bisexual behavioral tendencies.

The initial stimulation of male sexual behavior and increased hormone-synthesizing activity in young males do not remain without long-term consequences. As demonstrated in 18-month-old males prenatally exposed to DBP under the aforementioned protocol, these early effects manifest as accelerated reproductive aging. Specifically, a

prolongation of the latency to the first ejaculation compared with the control groups was observed, and the difference in latency to the first mounting between experimental and control groups disappeared (Table 5.3, Fig. 5.3). The most pronounced changes were noted in the number of intromissions and ejaculations, which were drastically reduced—or even completely absent—in the experimental group.

Table 5.3 Parameters of male-type sexual behavior in 18-month-old male rats after prenatal exposure to DBP

Parameter	Control	DBP
	First testing	
Latency period (s):		
First mounting	53.7 (7-100)	3.5 (11-147)
First intromission	299.7 (76-900)	101.0 (35-240)
First ejaculation	751.7 (307-900)	735.5 (513-836)
Number of:		
Mountings without intromission	5.5 (4-8)	5.0 (4-7)
Intromissions	7.5 (0-19)	5.5 (1-9)
Ejaculations	0.2 (0-1)	0 [a]
	Second testing	
Latency period (s):	27.0 (1-60)	
First mounting		27.0 (18-39)
First intromission	51.4 (18-138) [b]	478.8 (42-878) [a]
First ejaculation	223.4 (125-349) [b]	744.8 (433-878) [a]
Number of:		
Mountings without intromission	6.60 (5-7)	2.8 (1-5) [a]
Intromissions	24.8 (20-32) [b]	2.6 (0-5) [a]
Ejaculations	1.8 (1-3) [b]	0 [a]

Footnote: Refer to the Table 5.1.

When tested for female-type sexual behavior, aging males from both the control and experimental groups (five rats per group) did not display any lordosis responses when in contact with sexually active males. However, three out of five animals in the DBP-exposed group exhibited homosexual behavior, performing an average of 9.4 mountings toward an active male within a 10-minute testing period, compared with an average of 0.8 mounting in the control group.

Remarkable alterations in male-type sexual behavior in aging males can be explained by a more than twofold decrease in plasma testosterone levels compared with age-matched control males. This decline may also account for the accelerated atrophy of accessory

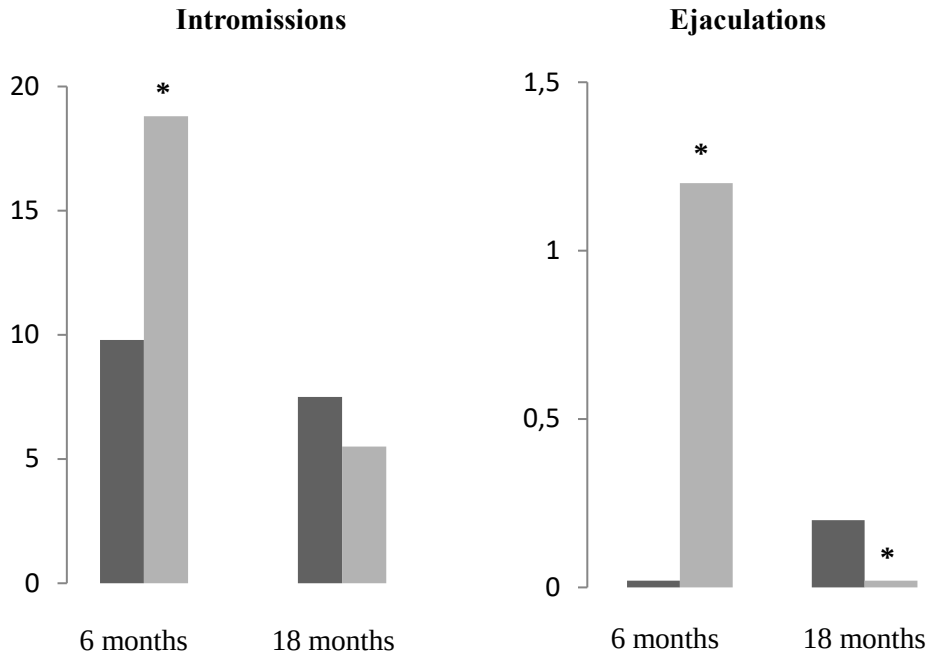

Fig. 5.3 Effect of prenatal exposure to DBP on male sexual behavior parameters in 6- and 18-month-old male rats.
Footnote: Dark gray bars represent control group; light gray bars represent DBP-exposed group. * $p < 0.05$ compared with the control group.

sex glands — the ventral prostate, coagulating gland, and seminal vesicles. The mass of the ventral prostate and coagulating gland decreased by approximately 50%, and that of the seminal vesicles by more than one-third compared with control animals of the same age.

Histological examination. More pronounced involutional changes in the ventral lobe of the prostate of DBP-exposed males were revealed in comparison with controls. The sperm concentration in the epididymal fluid of DBP-exposed males significantly differed from the control group, averaging $38.3 \pm 4.6 \times 10^6$/ml *vs.* $53.2 \pm 2.9 \times 10^6$/ml in controls ($p < 0.05$).

Histological analysis of the MPN demonstrated, unlike in young males, reduced functional activity of neurons compared with controls.

It was shown that maternal DBP exposure of male mouse fetuses led to accelerated brain aging in postnatal life, and that monobutyl phthalate, the principal DBP metabolite, promotes *in vitro* senescence in both hippocampal and GnRH-producing hypothalamic neuronal cells (Ma et al., 2025). These findings are consistent with our data, indicating that prenatal exposure to DBP results in accelerated reproductive system aging in male rats.

5.6. Prenatal Effects of Low-Dose DBP in Females

Functional reproductive disorders have been reported in female mice as a result of prenatal exposure to mono(2-ethylhexyl) phthalate on GD 17–19 at doses of 100–1000 mg/kg b.w. (Moyer & Hixon, 2012). These effects were manifested as delayed puberty onset, elevated plasma FSH and estradiol levels in adulthood, disrupted estrous cyclicity, and prolonged estrus stage. Decreased ovarian mRNA expression of the steroidogenic acute regulatory protein (*StAR*) and the aromatase enzyme (*Cyp19a1*) indicated impaired steroidogenesis, specifically reduced conversion of androgenic precursors into ovarian estrogens. Similarly, decreased mRNA expression of *StAR*, *Cyp19a1*, and *Cyp17a1* in mouse ovaries was observed following perinatal exposure to di(2-ethylhexyl) phthalate at doses of 0.05–5.0 mg/kg b.w. throughout gestation and lactation (Pocar et al., 2012). Thus, an apparent discrepancy exists between reduced aromatase activity and elevated estrogen levels, which warrants further investigation.

In contrast, intragastric administration of DBP to pregnant rats from GD 7 to 21 at a daily dose of 600 mg/kg b.w. resulted in increased ovarian *Cyp19a1* mRNA expression in prepubertal offspring and in a longer AGD in newborn pups (Boberg et al., 2008). However, changes in AGD cannot be considered definitively established, since another study (Ivell et al., 2013) found no differences after intragastric DBP exposure from GD 14.5 to PND 6. Likewise, no reproductive alterations were observed in female offspring of rats exposed to DBP at 100 mg/kg b.w. from GD 12 until term or until PND 21 (Guerra et al., 2010).

Puberty onset, ovarian and uterine weights, and estrous cycle regularity remained unchanged even after increasing the disruptor dose to 600 mg/kg b.w. and administering it during the same gestational period. However, at a lower dose of 10 mg/kg b.w., serum estradiol and progesterone concentrations increased, which may potentially affect female fertility (Xie et al., 2016).

We used a conditionally low dose of DBP (100 mg/kg b.w. daily) administered intragastrically to Wistar rats on GD 15–21 to examine possible adverse functional outcomes related to reproductive health, with a particular focus on pubertal development

and sexual behavior as reliable indicators of disturbed sexual differentiation of the brain. Female (lordosis) and male-type sexual behaviors were tested at 10 months of age in a 10-minute interaction with a receptive female (ovariectomized and hormonally primed).

AGD. On the PND 2, the AGD in female offspring exposed to DBP was reduced compared to the control group: 1.52 ± 0.04 mm *vs*. 1.84 ± 0.08 mm ($p < 0.05$). On the PND 7, no significant difference from the control was observed, whereas on the 10th day, the AGD was again reduced: 4.12 ± 0.11 mm *vs*. 4.67 ± 0.17 mm ($p < 0.05$). When normalized to b.w.t, a minor yet statistically significant difference was detected only on the PND 2: 0.24 ± 0.01 mm/g b.w. *vs*. 0.28 ± 0.01 mm/g b.w. in the control group ($p < 0.05$).

Pubertal Development. The onset of puberty was determined by the day of vaginal opening, which typically precedes the first ovulation by one day. In the control females, vaginal opening occurred on average on PND 39.00 ± 0.21, whereas in the DBP-exposed group it was observed on PND 36.93 ± 0.12, indicating an acceleration of approximately two days ($p < 0.001$).

Testosterone Levels. The plasma testosterone concentration in sexually mature control females aged six months was 1.25 ± 0.10 nmol/L, compared to 1.01 ± 0.14 nmol/L in the DBP-exposed group; this difference was not statistically significant.

Sexual Behavior. Prenatal exposure to DBP did not alter typical female sexual behavior. In the presence of a male, the exposed females exhibited normal sexual behavior, as well as lordosis responses, with a 100% lordosis index. However, all experimental females also displayed male-type sexual behavior, performing mountings toward receptive females. This finding clearly indicates the emergence of homosexual behavioral orientation, suggesting disruption of sexual differentiation of the brain.

Thus, the prenatal adverse impact of DBP on the reproductive system appears to be more pronounced in males, yet it also affects females.

5.7. Effects of BPA in the Context of Functional Teratology

A series of studies provides unequivocal evidence implicating BPA as an etiological factor underlying functional health disturbances associated with its impact on the developing intrauterine fetus. These findings substantiate a central postulate of functional teratology, namely that one of the most vulnerable physiological systems to environmental disruption is the neuroendocrine regulatory network governing behavior, reproduction, and the HPA axis**.**

Such effects are presumed to originate from BPA-induced alterations in the microstructural organization of the brain and in the status of hormonal receptors within its neuroendocrine regions. Due to their limited metabolic capacity to biotransform BPA, immature fetal systems are considerably more susceptible to its harmful effects than adult tissues (Nahar et al., 2013).

Investigations of estrogen receptor subtypes ERα and ERβ in the hypothalamus and amygdala of offspring born to dams orally exposed to BPA (2.5 µg/kg b.w. or 25.0 µg/kg b.w. daily during GD 6–21 demonstrated significant changes in receptor expression within the MBH and amygdala of both sexes. Following perinatal exposure to low doses of this endocrine disruptor, female rats exhibited reduced expression of estrogen receptors in the sexually dimorphic medial preoptic area and periventricular nuclei of the hypothalamus (Cao & Rebuli, 2013; Rebuli et al., 2014; Arambula et al., 2016). In males, a reduction in the size of the MPN was observed, consistent with feminization of the brain as a manifestation of disrupted sexual differentiation (McCaffrey et al., 2013).

Furthermore, male offspring exposed *in utero* to low doses of BPA exhibited a fourfold increase in mRNA expression of ERβ within the POA of the brain (Ramos et al., 2003). It is essential to emphasize that these findings pertain to very low BPA doses, comparable to—or even below—the estimated daily exposure levels in the general human population.

In contrast, other studies have reported enlargement of the same nuclei (He, Paule & Ferguson, 2012), whereas earlier research detected no significant **c**hanges in their size (Takagi et al., 2004), nor did later investigations on sexually dimorphic brain regions of rats reveal any alterations (Arambula et al., 2017).

Evidence of disrupted programming of neuroendocrine functions also includes decreased secretion of estradiol and testosterone in female rat offspring (Cao et al., 2025), as well as reduced hypothalamic GnRH, altered GABA production, and changes in glutamate receptor expression in the hypothalamus of male rats. Therefore, the claim of some researchers (Kobayashi et al., 2012) regarding the harmlessness of BPA under conditions of low exposure appears questionable. Nonetheless, the issue remains open for future investigation.

Among neuroendocrine structures, the arcuate nucleus of the hypothalamus occupies a central position, as it integrates the regulation of reproduction and energy balance. In this regard, particular attention has been drawn to findings indicating a reduction in the expression of muscarinic receptor type 3 and adiponectin receptor type 1, as well as altered expression of serotonin and cholecystokinin receptors in the arcuate nucleus of

female rats exposed prenatally to BPA during the last four days of gestation and the first postnatal week (Roepke et al., 2016).

With respect to other effects of prenatal BPA exposure, a number of studies suggest its influence on various behavioral domains, including sexual differentiation and cognitive performance (Richter et al., 2007). Interactions between environmental endocrine disruptors and the fetal genome are believed to contribute to the increasing prevalence of attention-deficit/hyperactivity disorder in children. Regarding BPA, this association has been confirmed by both original studies and meta-analyses (Harley et al., 2013; Rochester et al., 2018). The authors emphasize that these conclusions are consistent with extensive experimental data obtained from laboratory animal models. Symptoms of depression and anxiety have been observed predominantly in boys, but not in girls, and correlate with maternal urinary BPA concentrations during pregnancy.

In the absence of anatomical or histological abnormalities of the reproductive organs, Wistar rats exposed prenatally to BPA from GD 7 to 22 exhibited a reduction in sperm count in males and impaired spatial memory in females, as demonstrated in maze performance tests (Hass et al., 2016). Manifestations of sexual dysfunction in male mice and rats following perinatal BPA exposure included prolonged latency to initiate contact with a receptive female, disruptions of copulatory behavior, and reduced numbers of intromissions and ejaculations (Farabollini et al., 2002; Jones et al., 2011; De Catanzaro et al., 2013).

Even extremely low doses of BPA, lower than the acceptable daily intake for humans, administered perinatally to rats, were shown to disrupt sexual differentiation of the brain. In the absence of anatomical anomalies of the reproductive system and AGD, BPA exposure caused an inversion in the size of the locus coeruleus, which is normally larger in females, as well as alterations in open-field behavior (Kubo et al., 2003). Perinatal exposure to low BPA doses in mice similarly resulted in the elimination of sex-specific behavioral patterns in the open-field test and abolished sexual dimorphism in the number of tyrosine hydroxylase-positive neurons in the preoptic region of the brain due to a reduction in females (Rubin et al., 2006). In male rats, prenatal exposure to the disruptor manifested as depressive-like behavior and impaired sexual differentiation of behavior in open-field and forced-swim tests (Fujimoto et al., 2006).

With regard to the hormonal mechanisms underlying the demasculinizing prenatal effects of BPA on brain sexual differentiation and male sexual behavior, attention should be drawn to the reduced serum testosterone levels observed in fetuses at the end of gestation and two hours after birth (Tanaka et al., 2006), as this period corresponds to the androgen-dependent programming of male sexual behavior in rats.

It is well established that dysfunction of the HPA axis is associated with various age-related neuropsychiatric, cognitive, and neurodegenerative disorders (De Kloet et al., 2005). Therefore, the state of the HPA axis in individuals prenatally exposed to BPA is of considerable interest. A study involving low-dose BPA exposure in pregnant rats (40 µg/kg b.w. daily throughout gestation and lactation) demonstrated in female offspring an elevation in basal plasma corticosterone levels, a reduction in glucocorticoid receptor expression in the hypothalamus, impaired spatial memory, anxiety-like behavior, and an attenuated corticosterone response to stress (swimming). Unlike typical stress-induced downregulation, no reduction in glucocorticoid receptor expression was observed under stress conditions. In contrast, male offspring displayed a markedly stronger HPA axis response to stress than females, higher mRNA expression of proopiomelanocortin, and decreased expression of CRH receptors in the pituitary (Poimenova et al., 2010; Panagiotidou et al., 2014). The authors suggest that BPA-induced alterations in perinatal HPA axis programming may compromise stress resilience later in life.

Since the hypothalamic CRH acts as the primary trigger of the HPA axis, it is notable that perinatal BPA exposure eliminates normal sex differences in the number of CRH-immunoreactive neurons within the bed nucleus of the stria terminalis in rats. This occurs through an increase in males and a decrease in females (Funabashi et al., 2004).

One could assume that the observed alterations in the offspring's HPA axis result from BPA-induced activation of the maternal HPA axis, leading to elevated cortisol levels and its transplacental transfer to the fetal circulation. However, in pregnant women exposed to BPA, cortisol concentrations are in fact reduced (Giesbrecht et al., 2016). Therefore, a more plausible explanation is the direct action of BPA on the fetal neuroendocrine system, causing premature activation of the HPA axis and subsequent reprogramming of its development through glucocorticoid hormone excess.

There is also medical and psychological evidence indicating alterations in sexually dimorphic behavior in children resulting from prenatal exposure to BPA. These changes may stem from sex-dependent modifications of HPA axis function. Elevated concentrations of the disruptor in maternal urine were found to correlate with increased salivary cortisol levels in three-month-old female infants and decreased levels in males. Conversely, HPA axis reactivity was reduced in females and enhanced in males (Giesbrecht et al., 2017).

Prenatally determined impairments of spermatogenesis, partially discussed above, were observed in 21-day-old rats whose mothers received BPA during the final week of gestation. These effects were attributed to oxidative stress products and activation of apoptosis in testicular tissue involving mitochondrial pathways and the Akt/mTOR signaling cascade (Quan et al., 2017). Functional outcomes of prenatal and early

postnatal BPA exposure in male rodents include decreased sperm count and motility, enhanced apoptosis, and DNA damage in gametes. These manifestations are likely associated with BPA-induced endocrine dysfunctions in the testes, particularly weakened steroidogenesis and testosterone production due to decreased expression of the steroidogenic acute regulatory protein (StAR), as well as adverse effects on gonadal hormone receptors (Peretz et al., 2014). Notably, such effects are induced not only by high, but also by low exposure doses of the disruptor.

Further development of this research was presented in a study in which BPA was administered to pregnant rats during the critical period of sexual differentiation (GD 12–22) (Abdel-Maksoud et al., 2015). In the testes of prepubertal offspring, BPA induced variable changes in the expression of StAR-1 and GATA-binding protein, and increased the expression of the sex-determining region Y-box 9 (SOX9) and anti-Müllerian hormone compared with the control group animals. At the same time, the expression of β-subunits of LH and FSH was elevated in the pituitary of prepubertal males but reduced in adults. Importantly, the authors identified epigenetic alterations in the genome, specifically a decrease in global DNA hydroxymethylation in the gonads accompanied by suppression of DNA methyltransferase activity. Thus, prenatal BPA exposure disrupted gonadal developmental programming through DNA methylation changes.

The direct impact of BPA on the development of the fetal ovarian follicular apparatus is well established, having been documented in numerous studies (Peretz et al., 2014). In macaques exposed prenatally to low BPA doses, an increased number of secondary and antral follicles was observed at birth. Under similar conditions in mice, the number of primordial follicles was found to decrease in a dose-dependent manner. Prenatal BPA effects in sheep have been shown to include reduced expression of steroidogenic genes and microRNAs involved in gonadal differentiation and folliculogenesis. Collectively, the available data indicate that gestational exposure to BPA results in the formation of multinucleated oocytes within fetal follicles due to disruption of germ cell clone integrity.

In one of the comprehensive studies on the effects of BPA conducted in rats, administration of the disruptor during pregnancy and lactation was shown to reduce the number of primordial follicles in the ovaries of the offspring, while the number of corpora lutea increased. This was accompanied by elevated plasma progesterone levels, enhanced gonadal 3β-hydroxysteroid dehydrogenase activity, an imbalance of androgen receptors among different follicular types, and increased FSH receptor expression (Santamaría et al., 2016).

It is believed that some inconsistencies in the results of similar studies are due to species-specific differences. In sheep fetuses on the 65th day of intrauterine development—

whose mothers received BPA at a dose of 500 μg/kg b.w. (a dose relevant to real human population exposure) from GD 30 to 90 (with the total gestation period being 145–147 days)—no significant effects on the transcriptomes of steroidogenic enzymes or signal transduction factors in the ovaries were observed, except for increased mRNA expression of Cyp19 (aromatase) and 5α-reductase genes, as well as altered microRNA expression. However, by day 90, the enzyme gene expression changes previously observed were no longer detectable (Veiga-Lopez et al., 2013b).

For BPA and other chemical endocrine disruptors, non-monotonic dose–response relationships are well established. Investigating the long-term physiological effects of perinatal exposure to BPA at doses below the currently acceptable daily intake is therefore of particular importance. The US Food and Drug Administration has defined the no-observed-adverse-effect level (NOAEL) for BPA in rodents as 5 mg/kg/day. Nevertheless, alterations in physiological functions resulting from perinatal exposure may occur at significantly lower doses. Evidence concerning these low-dose BPA effects remains contradictory (Beronius et al., 2010).

Several authors have reported sex-dependent alterations in HPA axis function in rats treated perinatally with low doses of BPA (Poimenova et al., 2010; Chen et al., 2014; Panagiotidou et al., 2014). This issue, as well as many others related to BPA developmental neurotoxicity, warrants further investigation. Of particular interest are the changes in sexual behavior in male rats exposed to low-dose BPA during the final week of gestation—a critical period for sexual differentiation of the brain.

A depotentiation effect of BPA administered at low doses (40–50 μg/kg b.w. daily) throughout pregnancy and lactation on the sexual behavior of adult male offspring was confirmed by Jones et al. (2011). Paradoxically, administration of BPA at doses 100 times higher did not influence male sexual development or behavior in that study.

To clarify these controversial findings, we examined the morphology of the sexually dimorphic regions of the hypothalamus, assessed the hormonal profile, and male and female sexual behaviors in the male offspring subjected *in utero* to low-dose BPA exposure (Reznikov et al., 2020b, 2023). The objective of this investigation was to evaluate developmental, behavioral, and neuroendocrine alterations in the male progeny of rats exposed to low doses of BPA during the critical time window of sexual differentiation of the brain.

BPA was administered by oral gavage during the final week of gestation at a daily dose of 25 μg/kg b.w. Estradiol-17β diacetate (E_2D) was injected subcutaneously at a dose of 10 μg/kg b.w. and served as an estrogenic reference compound.

The mean litter size in control and BPA-treated pregnant females was similar (10.6 and 10.8 pups, respectively), indicating that low-dose exposure to the disruptor during the last trimester of gestation did not exert a toxic effect on fecundity.

5.8. Prenatal Effects of Low-Dose BPA in Male Rats

AGD. Small increases in average relative anogenital distances in the BPA and E_2D groups comparing to the control on PND 2 were found: control 0.56 ± 0.01 mm/g b.w., BPA 0.60 ± 0.01 mm/g b.w. ($p < 0.05$), and E_2D 0.62 ± 0.02 mm/g b.w. ($p < 0.05$). These alterations almost disappeared on PND 10: control 0.40 ± 0.01 mm/g, BPA 0.42 ± 0.01 mm/g ($p \leq 0.05$), and E_2D 0.45 ± 0.01 mm/g ($p < 0.05$) due to a further concrescence of the perineal suture.

Pubertal Development. Neither prenatal BPA nor E_2D did not affect the timing of puberty detected by the descent of the testes into the scrotum.

Hormone Levels. At a 6-month age, blood plasma steroid hormone basal levels were as follows: testosterone 10.8 ± 1.9 nmol/L in the BPA group ($p > 0.05$) and 28.0 ± 7.1 nmol/L in the E_2D group ($p < 0.05$) against 10.1 ± 1.4 nmol/L in the control; estradiol: 0.15 ± 0.02 nmol/L in the BPA group ($p > 0.05$) and 0.14 ± 0.02 02 nmol/L in the E_2D group ($p > 0.05$) against 0.15 ± 0.01 nmol/L in the control. Testosterone/estradiol-17β ratios were 75.5 ± 15.2, 257.8 ± 89.9, and 71.9 ± 14.2 correspondingly ($p > 0.05$).

Basal levels of corticosterone showed rather mild changes and were as follows: 878 ± 105 nmol/L in the BPA group against 1035 ± 69 nmol/L in the control ($P > 0.05$). The corticosterone response to one-h-long immobilization in both treatment groups also did not change compared to the control.

Sexual Behavior. At 10 months of age, control males in the first testing of male sexual behavior that lasted 15 min demonstrated typical male motivated behavior, mountings, and intromissions, while ejaculations were not observed. In the BPA group, male sexual behavior was characterized by almost complete lack of the sexual potency, in particular by the absence of intromissions and ejaculations. The rats developed only a few mountings without intromission. One out of five males was absolutely sexually inactive and, thus, was excluded from statistical processing.

In the second behavioral assessment, control males exhibited a marked increase in sexual motivation compared to the first test. They performed ejaculations and demonstrated a higher frequency of intromissions. The latencies to mounting and intromission were substantially shorter than those recorded in the initial trial.

Table 5.4. Parameters of male-type sexual behavior in 10-month-old male rat offspring prenatally exposed to E_2D or BPA.

Parameter	Control (*n*=5)	E_2D (*n*=5)	BPA (*n*=4)
	First testing		
Latency period (sec):			
First mounting	37.8 (14–100)	35.6 (10–79)	87.0 (23–175)
First intromission	104.2 (49-185)	64.0 (20-126) *	–
First ejaculation	–	667 (525–829)	–
Numbers of:			
Mountings without intromission	4.8 (2–10)	1.0 (1–1) *	1.8 (1–3) *
Intromissions	5.4 (2–9)	10.4 (8–14) *	0 *
Ejaculations	0	1.0 (1–1) *	0 *
	Second testing		
Latency period (sec):			
First mounting	7.2 (2–15)	3. 0 (1–8) *	149 (68–213) *
First intromission	17.0 (6–33)	8.4 (3–15) *	380 #
First ejaculation	285 (179–371)	164 (33–312) *	–
Numbers of:			
Mountings without intromission	5.8 (4–7)	1.2 (1–2) *	2.0 (0–3) *
Intromissions	18.4 (14–27)	19.4 (16–26)	0.5 (0–2) *
Ejaculations	1.2 (1–2)	2.2 (2–3) *	0 *

Footnotes: Mediana values are presented; ranges of the individual parameters are shown in parentheses. The test lasted 15 min. $^{*}p \leq 0.05$ in comparison with the control group using the Wilcoxon–Mann–Whitney nonparametric *U*-criterion.

In contrast, all males prenatally exposed to BPA displayed a pronounced suppression of sexual motivation toward receptive females. In four out of five animals, copulatory elements of sexual behavior were completely absent. The remaining male also failed to ejaculate, and the numbers of mountings—both with and without intromission—were significantly reduced relative to the control.

Conversely, males exposed to E_2D exhibited a complete repertoire of male-typical sexual behaviors, including normal frequencies of intromissions and ejaculations (Table 5.4). In the BPA group, all male offspring pre-treated with sex hormones exhibited pronounced lordosis behavior in the presence of a sexually active male, indicating a feminization of behavioral responses.

***Neuromorphology*.** At the age of six months, rat brains were dissected for microscopic examination of the MPN. Tissue fragments were fixed in Bouin's solution, and serial frontal sections (7 μm thick) were stained with azure–eosin according to the Pappenheim method. Two mutually perpendicular diameters (D) of neuronal nuclei were measured, and the nuclear volume (V) was calculated using the formula $V = D \cdot 2d \cdot \pi/6$ μm³. For each animal (n = 5 per group), at least 100 neuronal nuclei were analyzed.

Control rats demonstrated pronounced heterogeneity in the neuronal population of the MPN. Neurons with large perikarya and a high degree of cytoplasmic vacuolization predominated, which is interpreted as a morphological correlate of elevated functional activity (Fig. 5.4). The mean nuclear volume was 361.96 ± 20.27 μm³. Larger neurons were predominantly localized in the peripheral portion of the nucleus, where they were distributed less densely than in the central region. The Nissl substance within their perikarya was intensely stained. A substantial proportion of neurons displayed a rounded morphology with a weakly stained, "foamy" cytoplasm. A minor proportion of cells were hyperchromatic, and occasional shrunken pyknotic neurons were also observed.

In contrast, the MPN of the offspring born to BPA-exposed mothers contained markedly fewer activated neurons (Fig. 5.4). The nuclei were composed predominantly of small and medium-sized neurons with moderately vacuolated cytoplasm. Neurons with large perikaryal vacuoles were rare. The perikaryal volume was significantly smaller than that observed in control animals, indicating reduced functional activity of these cells. Hyperchromatic neurons with homogeneous cytoplasm and thin dendrites were less frequent compared with the control group. The mean volume of neuronal nuclei was

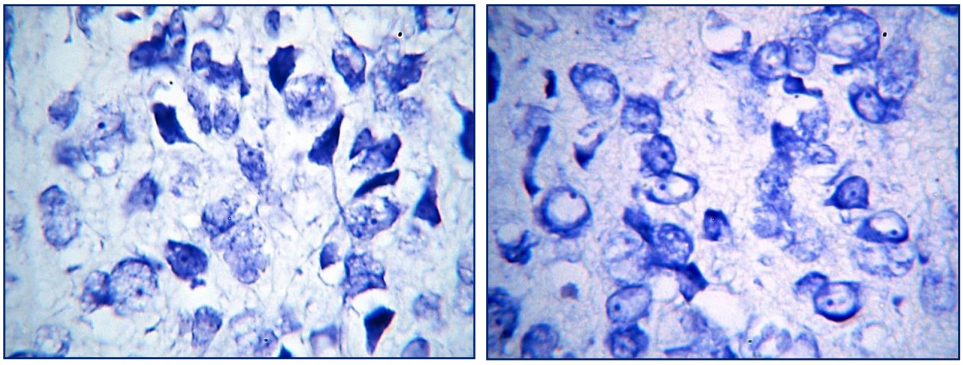

Fig. 5.4 Micrographs of representative samples from the central portions of the MPN in control (left) 6-month-old male rats and those prenatally exposed to BPA (right) Pappenheim staining. x 400

statistically significantly lower than that in the control group (287.10 ± 7.95 μm³; $p < 0.05$). The distribution pattern of neuronal nuclei volumes also differed substantially from that in the control group (Fig. 5.5). No signs of neuronal degeneration or necrosis

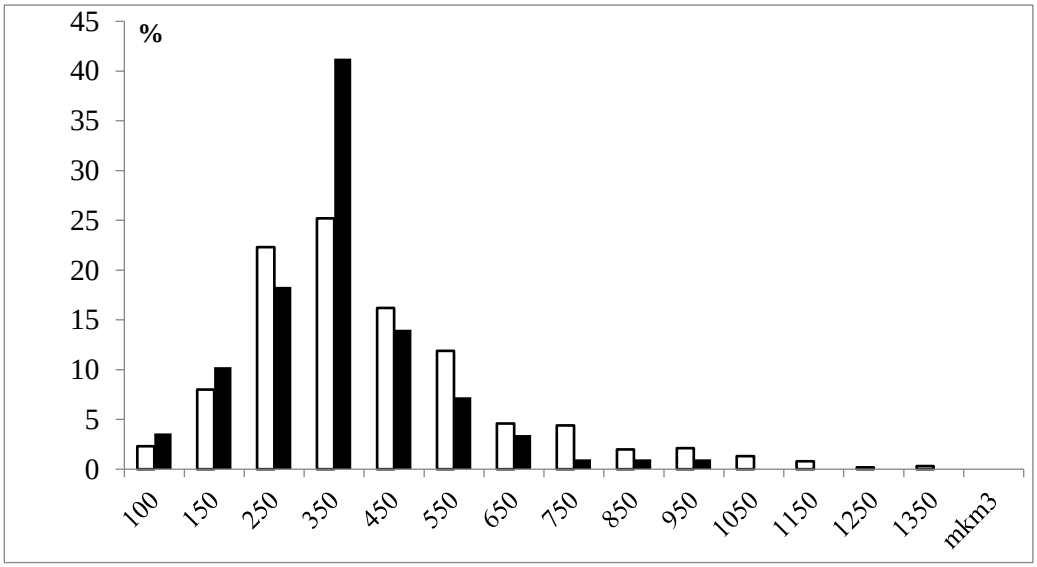

Fig. 5.5 Normalized distribution of neuronal nuclear volumes in the MPN of control and BPA-exposed male rats (open and filled bars, respectively).
Footnote: The total number of neurons subjected to karyometric analysis is taken as 100%.

were observed, suggesting the absence of direct neurotoxic effects. The morphological alterations appear to be associated with the influence of BPA on the sexual differentiation of the brain during critical developmental periods.

In E_2D -exposed animals, most neurons exhibited signs of functional activation. The perikaryal volumes were significantly larger than those in the control group. The neuronal density within the MPN was also higher. Compared with the control groups, these animals exhibited fewer neurons with large cytoplasmic vacuoles or lightly stained, foamy cytoplasm. The number of hyperchromatic, shrunken neurons with homogeneous cytoplasm was also lower. Neurons with large perikarya and intensely stained Nissl substance predominated, indicating an overall enhancement of neuronal metabolic and synthetic activity.

The BPA dose selected in the present study corresponds to a low level of human environmental exposure (Fujimoto et al., 2006). The experimental design was based on the assumption that E_2D and BPA would exert similar developmental effects. The observed alterations in the AGD-to-body-mass ratio are consistent with the ability of both compounds to activate estrogen receptor–mediated signaling pathways in developing tissues.

Our data differ from those reported by Ferguson et al. (2011), who administered BPA to rats at a daily dose two orders of magnitude higher, from GD 6 to PND 21, and observed no changes in the AGD-to-body-mass ratio. Their findings were interpreted as evidence for the absence of a direct dose-dependent disruptive effect, a conclusion echoed in several other studies.

The results of our study demonstrated that BPA, administered during GD 15–21 at a dose far below the established no-observed-adverse-effect level (NOAEL) for rodents, almost completely suppressed the motivational and copulatory components of male sexual behavior in adult offspring prenatally exposed to this compound. BPA appears to disrupt sexual differentiation of the brain by altering the sensitivity of neuroendocrine centers to estrogenic stimulation, which becomes evident through the occurrence of female-typical lordosis responses after priming with E_2D combined with progesterone. This phenomenon most likely reflects the programming effect of prenatal low-dose BPA exposure on the expression of estrogen receptors in the anterior hypothalamus (Rebuli et al., 2014).

It should be emphasized that prenatal BPA exposure induced behavioral abnormalities in adult animals despite normal plasma concentrations of testosterone and estradiol. Consistent with these findings, Kobayashi et al. (2012) also reported no significant alterations in plasma levels of reproductive steroid hormones in rat offspring exposed to low doses of BPA during intrauterine and lactational development.

Prenatal exposure to E_2D markedly enhanced sexual activity in adult offspring and elevated plasma testosterone levels. These findings are consistent with current understanding that the organizational, or programming, effect of fetal testosterone on the developing brain is mediated through its estrogenic metabolites (Reznikov, 1986).

Morphological sexual dimorphism within the medial preoptic area of the rat brain was first identified by Gorski et al. (1978). Two regions are primarily associated with sexually dimorphic morphology in rodents: the MPN and the anteroventral periventricular nucleus located in the preoptic bay of the third ventricle. In both regions, morphometric sex differences are linked to estrogen-mediated masculinization of the brain, where estrogens are synthesized locally from fetal testicular testosterone. However, the direction of apoptotic regulation in these nuclei differs between sexes—resulting in an enlarged sexually dimorphic nucleus in males (Davis et al., 1996) and a more prominent anteroventral periventricular nucleus in females (Walker & Gore, 2017). The mechanisms underlying sexual differentiation of both the sexually dimorphic and the anteroventral periventricular nuclei are well established and depend on the activation of estrogen receptor alpha (ERα).

The MPN represents the principal structure of the sexually dimorphic region in the rodent brain. Histological data obtained in the present study align, in several respects, with the developmental and behavioral alterations induced by BPA and E_2D exposure. Morphological and karyometric analyses revealed that prenatal administration of low-dose BPA during the critical period of sexual differentiation of the brain in males resulted in a pronounced reduction in the functional activity of neurons within the MPN. These structural changes correspond to the behavioral findings, namely, the suppression of male sexual behavior, which is regulated by neuroendocrine circuits localized within the preoptic area of the hypothalamus.

We hypothesize that the disruption of androgen-dependent sexual differentiation of the brain observed in our study results from the antagonistic action of BPA toward fetal testosterone. This antagonism may stem from the ability of BPA to bind to estrogen receptor and to compete with testosterone-derived estrogenic metabolites—estradiol-17β and 4-hydroxyestradiol—which exert essential organizational effects on the programming of male sexual behavior.

Prenatal administration of E_2D, an ether of natural estrogen, produced the opposite outcome, manifested by functional activation of neurons within the MPN, enhanced sexual activity, and increased testosterone secretion in adult males. In other words, hypermasculinization of the developing brain appears to be a consequence of the pronounced estrogenic activity of E_2D through activation of ERα.

No morphological indications of direct neurotoxic effects of BPA on neurons of the MPN were detected. The observed deviations in neuronal morphology most likely reflect indirect influences of BPA on neuroendocrine regulatory pathways governing sexual differentiation of the brain.

BPA has been shown to induce developmental epigenetic alterations in rat brain DNA (Walker & Gore, 2017; Cheong et al., 2018). In the embryonic mouse forebrain, BPA exposure resulted in changes in DNA methylation patterns that may underlie disturbances in sexual differentiation of the brain (Yaoi et al., 2008). Beyond its effects on reproductive behavior, BPA also interferes with the development of other sexually dimorphic behavioral domains in rodents, including spatial learning and memory performance (Johnson et al., 2016).

Considering the normal levels of circulating steroid hormones and the unaltered responsiveness of the HPA axis to acute stress in adult BPA-exposed offspring, it can be concluded that the neuroendocrine hypothalamus exhibits relative resistance to alterations induced by prenatal low-dose BPA exposure. In contrast, the structures of the preoptic area appear to be the most vulnerable to BPA impact, which may be attributed to the high local concentration of estrogen receptor capable of binding this compound.

No significant differences were observed in either basal or stress-induced plasma corticosterone levels between the control and experimental groups. Similar findings regarding the absence of BPA effects on basal corticosterone concentrations have been reported by other researchers (Poimenova et al., 2010; Panagiotidou et al., 2010; Silva et al., 2019), although some studies demonstrated an increased stress response under comparable conditions (Poimenova et al., 2010; Panagiotidou et al., 2010). These inconsistencies may stem from methodological variations across experimental designs.

In summary, transplacental exposure of male rat fetuses to low doses of BPA during the final week of intrauterine development results in pronounced suppression of male sexual behavior and preservation of the sensitivity of sexually dimorphic neuroendocrine pathways to stimulation by female sex hormones in adulthood. These findings indicate a strong demasculinizing and feminizing effect of BPA on sexual differentiation of the brain. Notably, behavioral abnormalities in BPA-exposed males occur against the background of normal plasma testosterone and estradiol-17β concentrations. In contrast, E_2D exerts a masculinizing organizational influence on male-type behavior and testosterone secretion. The morphology and karyometric parameters of MPN neurons strongly correlate with the observed behavioral alterations in both BPA- and E_2D-exposed male offspring.

5.9. Prenatal Effects of Low-Dose BPA in Female Rats

Hormonal Parameters. The plasma testosterone concentrations in adult female offspring did not differ significantly among the three groups. In contrast, plasma estradiol levels were significantly lower in females prenatally exposed to E_2D (0.17 ± 0.02 nmol/L) or BPA (0.20 ± 0.01 nmol/L) compared with controls (0.25 ± 0.01 nmol/L). No statistically significant differences were observed in basal plasma corticosterone concentrations between control (349± 46 nmol/L) and experimental groups (BPA, 402 ± 48 nmol/L; E_2D, 344 ± 27 nmol/L). A pronounced adrenocortical response to acute stress was detected in all groups of animals.

Sexual behavior. Testing of female-type sexual behavior in 10-month-old females whose mothers were treated with BPA or E_2D revealed no differences compared with control animals. All experimental females displayed normal motivational (hopping, darting, approaching, and other solicitations) and receptive (lordosis) behaviors. The number of lordosis responses and the lordosis quotient were similar across experimental and control groups.

Despite the absence of changes in female sexual behavior, experimental females demonstrated elements of male-type sexual behavior in the presence of a receptive female, including mounting and pseudocopulatory movements. Such reactions were not observed in the control group. Moreover, females exposed prenatally to BPA or E_2D exhibited a significant increase in the motivational component of male-type sexual behavior, with the number of approaches toward receptive females more than doubled compared with controls (Table 5.5).

Table 5.5. Parameters of male-type sexual behavior in 10-month-old female rats prenatally subjected to BPA or E_2D (Mean ± SEM)

Animal group	Number of approaches to the female	Number of mountings	Females displaying sex motivational behavior	Females displaying pseudocopulatory behavior
Control	7.0 ± 0.7	0	5/5	0/5
BPA	15.8 ± 2.2*	6.2 ± 1.3*	5/5	5/5
E_2D	15.2 ± 0.4*	3.0 ± 0.8*	5/5	4/5

Footnotes: * $p < 0.01$ compared with the control; each group contained five rats.

Based on the obtained results, it can be concluded that administration of BPA or E_2D to rats during the final week of pregnancy induces masculinization of sexual behavior in female offspring; however, this effect is not accompanied by defeminization.

Neuromorphology. In 6-month-old control females, neurons within the MPN were relatively sparsely distributed. Small neurons predominated; they possessed modest-sized perikarya with intensely stained or moderately vacuolated cytoplasm and small nucleoli. A considerable proportion of the neuronal population consisted of hyperchromic neurons. Neurons with a high degree of cytoplasmic vacuolization—an indicator of increased functional activity—were relatively few. In all animals, clusters of small cells were observed in the caudal portion of the MPN.

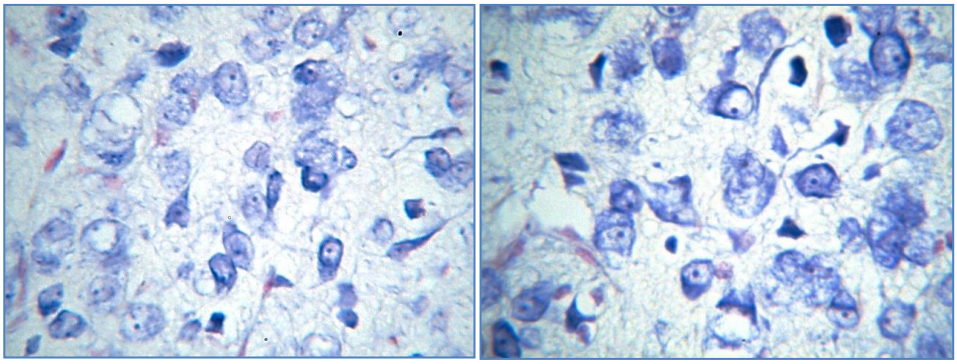

Fig. 5.6 Micrographs of representative samples from the central portions of the MPN in control (left) 6-month-old female rats and those prenatally exposed to BPA (right) Pappenheim staining. ×400

In the BPA-exposed progeny group, the MPN also consisted predominantly of small and medium-sized neurons. However, these nuclei contained a somewhat larger number of activated neurons with moderately vacuolated cytoplasm, as well as neurons with large vacuoles within the perikarya and enlarged nucleoli (Fig. 5.6). Compared with control females, the proportion of hyperchromic neurons with homogeneous cytoplasm and thin dendrites was slightly lower. Overall, the perikaryal volume was greater than in control animals, suggesting enhanced functional activity.

The results of the morphological examination are consistent with the observed masculinization of sexual behavior in females prenatally exposed to a low dose of BPA, while simultaneously preserving the typical female behavioral pattern.

In control females, the mean nuclear volume of the MPN neurons was 323 ± 21 μm³, whereas in rats prenatally exposed to BPA it was 307 ± 28 μm³ ($P > 0.05$). Histogram

analysis revealed no substantial differences in the distribution of neuronal nuclear sizes between the two groups.

Thus, even brief exposure of pregnant rats to an ultra-low dose of BPA is capable of disrupting the genetically determined programming of female fetus neuroendocrine system responsible for sexual behavior and ovarian function.

The ovaries are the primary source of sex hormones in females. It is likely that the reduced estradiol levels observed in the BPA-treated group (Mahalingam et al., 2017; Reznikov et al., 2023) result from a decreased number of follicles and their dysfunction (Santamaría et al., 2016; Patel et al., 2017), as well as diminished activity of ovarian steroidogenic enzymes.

Studies evaluating the effects of low-dose BPA exposure in pregnant rats (40 μg/kg b.w. daily throughout gestation and lactation) demonstrated that female offspring at puberty exhibited elevated basal corticosterone, reduced glucocorticoid receptor expression in the hypothalamus, impaired spatial memory, anxiety-like behavior, a blunted corticosterone response to stressor (swimming), and the absence of stress-induced downregulation of glucocorticoid receptor expression (Panagiotidou et al., 2014). The divergence between those findings and our results is most likely attributable to differences in exposure duration. According to our data, short-term BPA administration does not alter either basal corticosterone levels or the stress response. Similarly, another study reported no changes in basal corticosterone in sexually mature offspring exposed to BPA from GD 10 to PND 7 (Chen et al., 2014).

Our investigation revealed pronounced alterations in female sexual behavior. Although female-typical behavior remained unchanged, BPA-exposed females exhibited sexual motivation toward receptive females and demonstrated pseudocopulatory behavior. These findings indicate partial masculinization of the fetal brain, *i.e.*, disruption of sexual differentiation of the brain.

A characteristic feature of endocrine disruptor-induced effects is the absence of a linear dose–response relationship, which also applies to BPA. According to Farabollini et al. (1999), exposure to high doses of BPA from GD 14 to PND 6 did not induce signs of brain masculinization in female rat offspring. Similar results were obtained with daily doses of 50 μg/kg b.w. or 5 mg/kg b.w. administered during GD 6-21, followed by exposure of newborn females during lactation (Jones et al., 2011), as well as with maternal ingestion of 2–200 μg/kg b.w. throughout pregnancy and lactation (Ryan et al., 2010). Farabollini et al. (1999) reasonably suggested that prolonged BPA exposure may activate compensatory mechanisms counteracting potential disturbances of sexual differentiation of the brain caused by the disruptor's estrogenic activity. It is likely that

under short-term exposure such compensatory mechanisms do not have sufficient time to develop.

Feeding rats BPA across a wide dose range (10–10,000 μg/kg b.w.) during the period corresponding to the critical window of sexual differentiation of the brain did not reveal changes in the volume of the so-called sexually dimorphic nucleus of the POA, which is typically smaller in females than in males (Kwon et al., 2000; McCaffrey et al., 2013; Arambula et al., 2017). However, in our experiments, the histological characteristics of the MPN were altered and correlated with behavioral abnormalities observed in the experimental females. These findings are consistent with reports of defeminization of sociosexual behavior in juvenile females born to mothers exposed to low-dose BPA during pregnancy and lactation (Porrini et al., 2005).

The unidirectional nature of functional disturbances induced by prenatal exposure to BPA and the reference estrogenic drug E_2D in both male and female offspring indicates that these effects are mediated by the estrogenic properties of both compounds. The present results point to epigenetic disturbances in brain sexual programming caused by prenatal exposure to BPA at a dose 200 times lower than the NOAEL (no-observed-adverse-effect level) established for rodents. These findings should be taken into account when assessing the potential health risks posed by BPA to humans.

Conclusions

- Endocrine disruptors are widespread chemical pollutants that, through hormone-like or antihormonal activity, induce reproductive, metabolic, immune, and neurodegenerative disorders in the offspring. Their effects may be transmitted across several generations via epigenetic mechanisms and do not correlate with exposure dose. Pathogenesis involves receptor-mediated, epigenomic, oxidative, and hormonal effects, as well as damage to barrier structures. The combined action of several compounds may be synergistic or antagonistic.

- Endocrine disruptors (pesticides, bisphenols, phthalates, phytoestrogens, paracetamol, etc.) administered during pregnancy induce persistent developmental and functional impairments of the reproductive system in offspring of both sexes, ranging from cryptorchidism, hypospadias, and infertility to hormonal and behavioral disorders, endometriosis, and cancers of the reproductive organs. In particular, phthalates exert antiandrogenic effects under pre- and perinatal exposure and alter behavioral outcomes in the progeny.

- Prenatal exposure to DBP at a moderate dose (100 mg/kg b.w., GD 15–21) induces a hyperandrogenic state and hypersexual behavior in young male rats

due to excessive masculinization of the brain. With aging, however, this results in accelerated reproductive senescence and reduced functionality of hypothalamic structures. In females, DBP accelerates puberty and induces male-type sexual behavior in adulthood while preserving normal female behavior.

- BPA, even at very low doses, disrupts the programming of neuroendocrine systems, sexual differentiation of the brain, behavior, and reproductive functions in male offspring during prenatal development, leading to long-lasting functional impairments without significant anatomical abnormalities. In females, BPA and E_2D induce masculinization of sexual behavior without defeminization, accompanied by reduced estradiol levels and morphological alterations of the medial preoptic nuclei, consistent with estrogen-mediated epigenetic disturbances of brain sexual differentiation programming.

Overall Conclusions

- The results summarized in this monograph demonstrate that the intrauterine environment plays a decisive role in programming neuroendocrine development, sexual differentiation of the brain, and long-term physiological and behavioral outcomes. A wide range of prenatal influences—hormonal, neurotransmitter-mediated, pharmacological, stress-related, and environmental—act primarily through epigenetic mechanisms that modify gene expression without altering DNA structure. These epigenetic imprints shape the maturation of neuroendocrine circuits in a sex-specific and region-specific manner and may persist into adulthood or even across generations.

- Disruption of androgen- or estrogen-dependent programming during critical developmental windows leads to predictable alterations in sexual differentiation of the brain, reproductive functions, and stress reactivity. Pharmacological agents and endocrine disruptors, even at very low doses, can disturb these processes through estrogenic, antiandrogenic, neurotransmitter-modulating, or calcium-dependent pathways. Prenatal stress further amplifies these effects by activating maternal HPA mechanisms, resulting in long-lasting neurochemical and behavioral abnormalities in the offspring.

- Collectively, the evidence supports a unified concept: phenogenesis reflects the dynamic interaction of genetic, epigenetic, hormonal, and environmental factors during early development. These findings underscore the sensitivity of the fetal

neuroendocrine system to external influences and highlight the need for careful evaluation of environmental chemicals, medications, and stressors during pregnancy because of their potential to induce persistent and sometimes transgenerational effects on health.

Abbreviations

AGD - anogenital distance

AVP – arginine vasopressin

COMT - catechol-O-methyltransferase

CRH – corticotrophin-releasing hormone

E_2D - estradiol-17β diacetate

GABA - γ-aminobutyric acid

GD - gestation day

GnRH - gonadotropin-releasing hormone

HPA - hypothalamic-pituitary-adrenal

HPG - hypothalamic–pituitary–gonadal

LH - luteinizing hormone

LHRH - LH-releasing hormone

MBH - medial basal hypothalamus

MDA - malone dialdehyde

m.m. - molecular mass

MPN - medial preoptic nucleus

PCOS – polycystic ovary syndrome

PND – postnatal day (s)

SCN - suprachiasmatic nucleus

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