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**Neonatal exposure to estradiol in female rats influences neuroactive  
steroid concentrations and behaviour in the adult rat**

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## Introduction

Sexual differentiation is the process through which individuals develop sexual characters and show masculine or feminine pattern of sexual behaviour. Most vertebrate species have two sexes, males and females which are defined by their ability to produce sperm or eggs, respectively. The sex chromosomes are the origin of all sex differences in a lot of animal species. In mammals, males have an X and a Y chromosome while females have two X chromosomes. The most important genetic difference occurs in a particular gene expressed only in the Y chromosome. This critical gene is SRY, which is necessary for differentiation of testes from the bipotential gonad (Koopman et al. 1991). The SRY gene encodes the transcription factor known as the testis-determining factor which upregulates the expression of other genes that induce testicular differentiation and development (Kashimada and Koopman 2010). In the absence of SRY, the gonad will develop into an ovary. In rodents, the development of testes in males and ovaries in females leads to sex differences in the perinatal secretion of testosterone, which induces differentiation of the genitalia and the brain. In fact, testosterone, released by fetal testes, enters the male brain, where it is converted to estradiol by the P450scc enzyme aromatase. Estradiol acts on estrogen receptors to cause masculine differentiation of several brain regions, inducing the formation of circuits that are required for masculine behaviours, including mating (mounting, intromission and ejaculation), aggression, territorial marking, and parental care (McCarthy 2008; Wu et al. 2009).

In particular, estradiol permanently alters the reproductive physiology by preventing its positive feedback effects on gonadotropin luteinizing hormone (LH) production and release from the pituitary in adulthood. By contrast, in females a lack of early exposure to high levels of estradiol is essential for the ovulatory surge of LH and for the development of sexual behaviour (Barraclough 1961). High levels of estradiol in maternal circulation could also access the fetal circulation, but perinatal fetuses and pups are protected by maternal estradiol because they carry the  $\alpha$ -fetoprotein (*Afp*), which binds estrogens. *Afp* does not bind testosterone, so this hormone can selectively enter into the neurons, where it is aromatized to estradiol. The importance of  $\alpha$ -fetoprotein in protecting female fetuses from behavioural masculinization and fertility has been established in *Afp* mutant

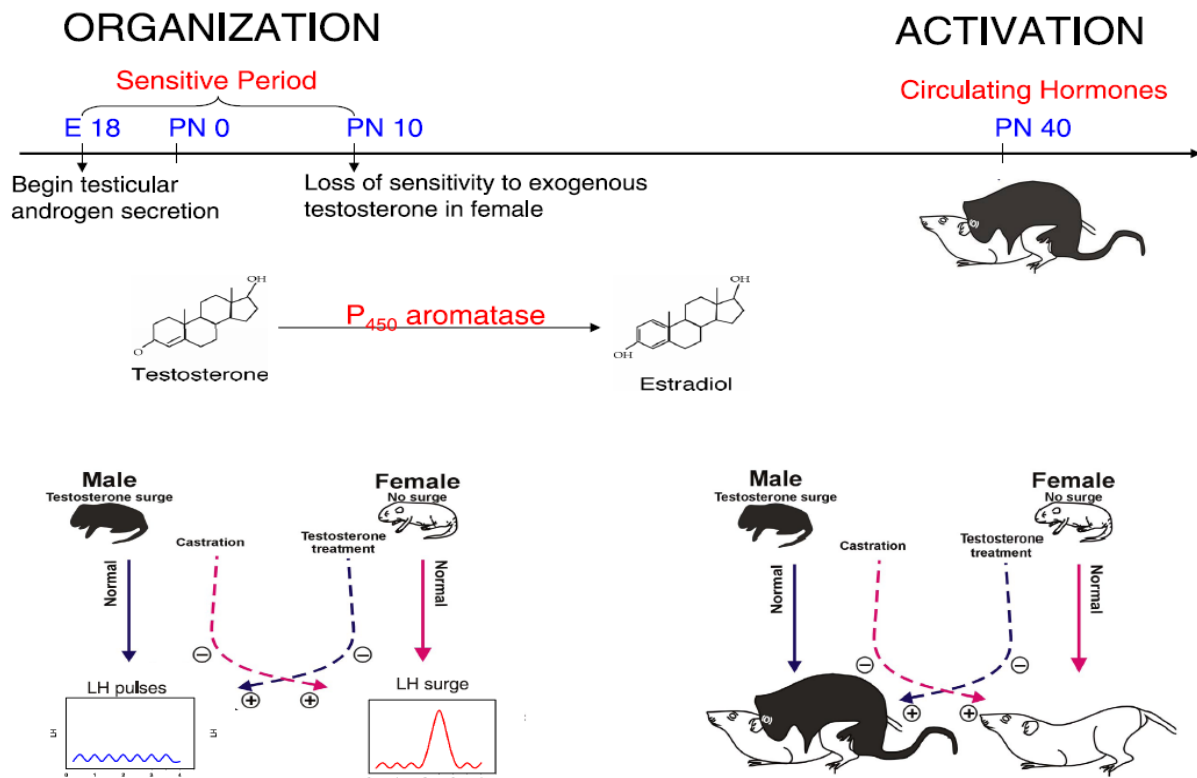
mice (*Afp*<sup>-/-</sup>). In fact, female offspring of *Afp*<sup>-/-</sup> dams were incapable of  $\alpha$ -fetoprotein production, and their brain was defeminized behaviourally and morphologically (Bakker et al. 2006).

Brain sexual differentiation is therefore largely controlled by steroids produced by the gonads that act on the brain during a perinatal sensitive period to organize a male or female phenotype. This sensitive period for steroid-induced sexual differentiation is defined by the onset of testicular androgen secretion in males (approximately embryonic day 18 in rodents) and by the loss of sensitivity of the female to exogenous androgen treatment (approximately postnatal day 10) (Figure 1).

Subsequent to this differentiation, the same gonadal hormones act on the male or female brain across the lifespan to produce sex-specific behaviours. This evidence leads to the concept of *organizational* and *activational* effects of gonadal steroids on the brain. Organizational effects are the permanent effects induced by gonadal steroids, during a critical perinatal period of fetal and neonatal life in mammals, which permanently alters the neural architecture of the brain. In contrast, activational effects are transient effects seen following steroids exposure in adulthood (Phoenix et al. 1959).

#### *Cellular mechanisms of Estradiol mediated sexual differentiation of the brain*

Sexual differentiation of the brain and sex behaviour involves three distinct processes: feminization, masculinization and defeminization. Masculinization and defeminization are distinct processes in the male brain, both of which rely on gonadal hormone action during the perinatal period. Masculinization is the process through which the brain becomes capable of producing male sexual behaviour in adulthood. Defeminization is the process whereby the ability to express female sexual behaviour or productive function is lost. Feminization is a default program that proceeds in the absence of organizing steroid hormone action and that organizes the brain regions controlling female sexual behaviour, so that the behaviours such as lordosis can be displayed under the proper hormonal conditions in adulthood (for a review see Lenz et al. 2012).



**Figure 1.** The organizational/activational hypothesis of estradiol action on the developing brain. Exposure of developing females to androgens during the period of sexual differentiation, which in rats begins around embryonic day 18 and extends up to 10 days postnatally, results in their aromatization to estradiol and masculinization of adult brain and behaviour. Estradiol induces a malfunction of the female neuroendocrine axis in adulthood, rendering females sterile and sexually unreceptive. In fact, females do not adopt the sexually receptive posture termed lordosis (from McCarthy 2008).

Neuronal sex differences occur on a variety of levels, including differences in the volume of certain brain nuclei, or difference in dendritic spine density, neurite branching, particular projection from one brain area to another or astrocyte complexity (Gorski et al. 1978; Mong et al. 2001). In the rodent, many of these sex differences are determined by steroid hormones, particularly estradiol, and are established by diverse downstream effects. Once aromatized from testosterone, estradiol acts in the brain on several different estrogen receptors (ERs): ER $\alpha$ , once thought to be the only estrogen receptor, the more recently characterized ER $\beta$ , and the G-protein-coupled receptors ER-X and GPR30. The

effects of ER $\alpha$  and ER $\beta$  can occur either in the nucleus in the ‘classical’ fashion or at the membrane (Mermelstein & Micevych 2008), ER-X appears to act also largely at the membrane and to activate intracellular signaling cascades (Toran-Allerand et al. 2002; Qiu et al. 2003), whereas GPR30 is most highly expressed on the endoplasmic reticulum and acts to alter intracellular calcium signaling (Revankar et al. 2005). Interestingly, recent knockout mouse models show that ER $\alpha$  is more closely coupled with masculinization and ER $\beta$  with defeminization (Kudwa et al. 2006).

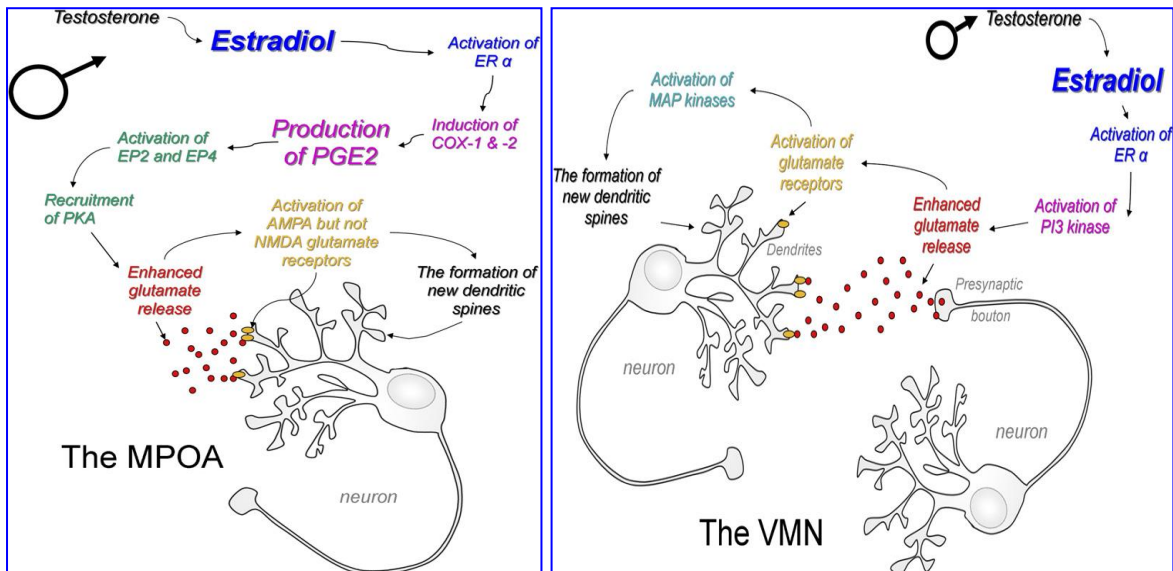
Many of sex differences are localized to regions that are necessary for adult sex behaviour or for determine the adult pattern of gonadotropin secretion. For instance, estradiol changes the preoptic area (POA), a crucial region involved in many sex behaviours, including copulatory and maternal behaviours. In this area, multiple cell types, including neurons, astrocytes, and microglia are masculinized by estradiol. Specifically, in the POA normal males have twice the density of dendritic spines than females, and the density of dendritic spines on these neurons correlates with measures of adult male sexual behaviour. Estradiol administration to a newborn female induces the male pattern of dendritic spines (Wright et al. 2008). Multiple downstream molecular mediators are involved in this effect of estradiol, including prostaglandins, glutamate receptors, protein kinases, and several immune signaling molecules (Figure 2).

Moreover, emerging evidence indicate epigenetic mechanism maintain sex differences in the POA that are organized perinatally and thereby produce permanent behavioural changes (Zhang & Ho 2011).

#### *Effect of estradiol administration during development*

Classical and recent studies have used different experimental paradigms to study sexual differentiation of the brain, including the prenatal or neonatal exposure of female rats to gonadal steroids. The first evidence of sexual differentiation of the brain was discovered in 1959, when Phoenix and colleagues reported that female guinea pigs, prenatally exposed to testosterone, showed little or no female sex behaviour as adults. One of the surprises was that treatment of neonatal females with exogenous estradiol, originally

administered as a control for testosterone, also induced complete masculinization of brain and behaviour (McEwen et al. 1977).



**Figure 2.** Representation two different mechanisms of masculinization of the POA and the VMN induced by estradiol during the development period (from McCarthy et al. 2009).

In the following years, numerous studies have examined the brain differentiation induced by estradiol, using different protocols. For example, neonatal administration of 100 µg of estradiol to female rats for five consecutive days after birth induces a premature vaginal opening and an altered estrous cycle with persistent estrus or prolonged estrus (Kouki et al. 2003). Similarly, a single injection of estradiol (100 µg) on the day of birth (Pinilla et al. 2002) or at day 5 after birth (Kanaya & Yamanouchi 2012) to female rats, causes alterations like acyclicity, anovulation, ovarian atrophy and loss of negative and positive feedback between estradiol and LH. Furthermore, treatment of newborn female rats with a low dose (10 µg) of estradiol benzoate for five consecutive days after birth, inhibits the growth and differentiation of the ovary (Ikeda et al. 2001). Similarly, the injection of 100 µg of estradiol benzoate to female rats, 4 days after birth, abolished sexual receptivity in adulthood, even with estrogen and progesterone replacement after ovariectomy (Levine and Mullins 1964), while neonatal administration of a low doses of the 17 $\alpha$ -ethinylestradiol, the main estrogen component in the contraceptive pill, alters female



sexual behaviour and specifically the appetitive components of sexual behaviour that influence the rate of copulation (Della Seta et al. 2008) Postnatal estradiol exposure during development also affected partner preference. In fact, female rats that received exogenous estradiol on postnatal day 0 and up to 3 weeks, spent more time with an estrous female and less time with a sexually active male (Henley et al. 2011).

In addition, there are also numerous studies that have looked at the effects of androgens, aromatase inhibitors or environmental pollutants that show estrogenic action, also called xenoestrogens. If a developing female is exposed to testosterone propionate, she will be sterile, losing the capacity to ovulate and she will lack sexual receptivity as an adult (Barraclough 1961; Barraclough & Gorski 1961). Moreover, if as an adult she is treated with testosterone, she will exhibit male behaviour when presented with a sexually receptive female (Baum 1979; Whalen 1964). Neonatal exposure to genistein, a phytoestrogen found in soy-based foods, altered vaginal opening, induced an aberrant cycle with a persistent or prolonged estrus and caused the formation of small ovaries lacking corpora lutea (Kouki et al. 2003). Moreover, exposure to bisphenol A, a common environmental endocrine disruptor with estrogenic properties present in the manufacture of plastics used for containers, bottles, dental composites, and other plastics items induces lower levels of proceptive behaviour and a down-regulation of the estrogen receptor alpha expression in specific hypothalamic regions necessary for sexual receptivity and the lordosis posture (Monje et al. 2009). Overall, exposure to estrogen or estrogen-like compounds during development induces a broad spectrum of effects, which vary depending on dose and duration of exposure, age at exposure and sex of the individual.

The neural mechanism underlying the effects of a short exposure of developing females to estradiol at the level of genitalia and sexual behaviour have been extensively studied. Treatment of female neonates with testosterone or estrogen changes the responsiveness of the hypothalamus and pituitary gland to the feedback action of ovarian steroids, leading to dysregulation of the secretion of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH), in adulthood (Robinson 2006). However, it is not clear whether these long-lasting effects that “program” the female neuroendocrine axis to

malfunction in adulthood have consequence in the brain hormonal milieu and/or in the behaviour influenced by gonadal hormones.

The purpose of this thesis is to describe an animal model of defeminization induced by a single treatment with a low dose (10  $\mu$ g) of  $\beta$ -estradiol-3-benzoate to female rats on the day of birth. We used  $\beta$ -estradiol-3-benzoate because it is the most commonly used ester of the naturally occurring estrogen estradiol in endocrine and neuroendocrine research involving estrogen replacement paradigms. In fact, this is a hormone that is rapidly hydrolyzed in vivo to the physiologically active  $17\beta$ -estradiol following its systemic administration in oil. Firstly, we evaluated whether neonatal administration of  $\beta$ -estradiol-3-benzoate changes the brain and plasma concentrations of gonadal hormones and their neuroactive steroid metabolites in the adult brain and alters the development of the female reproductive tract and the sexual behaviours (Chapter 1). Subsequently, we assessed whether this treatment may results in an alteration of behaviour through a series of behavioural tests that evaluate the emotional state, the cognitive function and the social behaviour in adulthood (Chapter 2). Moreover, given that we have recently demonstrated that neonatal administration of  $\beta$ -estradiol-3-benzoate alters the expression of specific GABA<sub>A</sub> receptor subunits (Calza et al. 2010), that may alter the behavioural sensitivity to GABA<sub>A</sub> receptor-targeting-drugs, such as benzodiazepines (Pritchett et al. 1989), we investigated whether sensitivity to diazepam in adulthood might be affected by neonatal administration of  $\beta$ -estradiol-3-benzoate (Chapter 3).

## Chapter 1

### **Characterization of an animal model of defeminization induced by a single treatment with $\beta$ -estradiol-3-benzoate on the day of birth**

#### **1.1 Introduction**

Abnormal levels of sex hormones in the perinatal period can induce profound alterations in neuroendocrine function, which have repercussions in adulthood for both reproductive and hormonal levels. These changes might involve alterations at every level of the hypothalamic-pituitary-gonadal axis. For example, estrogens may act on the hypothalamus by changing the release of gonadotropin-releasing hormone (GnRH), and thereby affecting gonadotropin and steroid hormone secretion; they may act on pituitary responsiveness to GnRH and thereby alter gonadotropin secretion and ovarian physiology; they may directly disrupt ovarian function, by inducing changes in ovarian cycle and steroid output. Moreover, it has been shown that treatment of female neonates with testosterone or estrogen changes the responsiveness of the hypothalamus and pituitary gland to the feedback action of ovarian steroids, leading to dysregulation of GnRH and the luteinizing hormone (LH) secretion in adulthood (Robinson 2006). This phenomenon results in adult females that exhibit persistent vaginal cornification, acyclicity and ovulatory failure associated with an altered production of steroids by the gonads (Handa et al. 1985; Rodriguez et al. 1993).

Given that gonadal steroids, besides their role in the control of the reproductive function, affect the nervous system in many different ways, we evaluated whether the changes in the hormonal milieu induced by  $\beta$ -estradiol-3-benzoate administration to neonatal female rats might influence the brain and plasma concentrations of gonadal steroid and their neuroactive steroid metabolite, allopregnanolone, in the adult rat. Allopregnanolone has been proposed to be involved in neuroendocrine functions such as GnRH release (Vicems et al. 1994), gonadotropin release (Brann et al. 1990), inhibition of ovulation (Genazzani et al. 1995), modulation of female sexual behaviour (McCarthy et al. 1995), and the response to stress (Biggio et al. 2007). We also examined the effect of administration of a

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single dose of  $\beta$ -estradiol-3-benzoate to neonatal female rats on estrus cycle and ovarian morphology in adulthood.

## 1.2 Materials and methods

### Animals

Female Sprague-Dawley rats (Charles River, Calco, Italy) were bred in our colony and maintained on an artificial 12-h-light, 12-h-dark cycle (light on from 08:00 to 20:00 hours) at a constant temperature of  $22^{\circ} \pm 2^{\circ}\text{C}$  and a relative humidity of 65%. Food and water were available ad libitum. Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethics committee. Females were mated with males at regular intervals. On the day of birth (day 0), the male pups were removed from the litter while the female pups were injected subcutaneously (s.c.) with 10  $\mu\text{g}$  of  $\beta$ -estradiol 3-benzoate in 50  $\mu\text{l}$  of sesame oil or with 50  $\mu\text{l}$  of sesame oil (controls) (Rodriguez et al. 1993; Solum and Handa 2002). In order to avoid leakage of  $\beta$ -estradiol 3-benzoate pups were injected under hypothermia anaesthesia. After injection, neonatal  $\beta$ -estradiol-3-benzoate-treated females were randomly distributed among litters of the same age so that each mother had five to eight pups. All female pups within a litter received the same treatment. After weaning, female rats were housed in groups (control and neonatal  $\beta$ -estradiol-3-benzoate-treated) of six to eight per cage. They were sacrificed on day 60 for measurement of plasma and brain steroid levels. To avoid effects of estrous cycle status and the stress of vaginal smears on steroid concentrations control animals were sacrificed in randomized phases of the estrous cycle. The female rats used for induced sexual behaviour were bilaterally ovariectomized (OVX) following anaesthesia with Equithesin (a mixture of Pentobarbital, ethanol 96% and chloral hydrate; 3ml/kg), three weeks prior to the behavioural tests. The vaginal cytology of the OVX rats confirmed that they were not cycling.

### Steroid extraction and assay

Animals were sacrificed by decapitation, the brain was rapidly (<1 min) removed, and the cerebral cortex was dissected and frozen at  $-80^{\circ}\text{C}$  until steroid extraction. Blood was collected from the trunk into heparinized tubes and centrifuged at  $1000 \times g$  for 15 min,

after which the plasma supernatant was frozen until assayed for steroids. All steroids were assayed in the same tissue sample. Steroids present in cerebral cortical homogenates (400 mg of tissue in 4 ml of phosphate-buffered saline) were extracted four times with an equal volume of ethyl acetate. The combined organic phases were dried under vacuum, the resulting residue was dissolved in 4 ml of *n*-hexane and applied to a Seppak silica cartridge (Waters, Milan, Italy), and residue components were eluted with a mixture of *n*-hexane and 1-propanol (7:3, v/v). Steroids were further purified by HPLC on a 5- $\mu$ m Lichrosorb-diol column (250 x 4 mm; Phenomenex, Castel Maggiore, BO, Italy) with a gradient of 1-propanol in *n*-hexane. Given that cholesterol, which coelutes from the Lichrosorb-diol column with progesterone, was found to reduce the sensitivity of the radioimmunoassay for progesterone, this latter steroid was separated from cholesterol by washing the corresponding dried column fractions twice with 200  $\mu$ l of dimethylsulfoxide and once with 400  $\mu$ l of water. Progesterone was extracted from the aqueous phase twice with 1.5 ml of *n*-hexane. The recovery of each steroid through the extraction-purification procedures (70 to 80%) was monitored by the addition of trace amounts (4000 to 6000 cpm) of  $^3$ H-labeled standards to the brain tissue homogenate. Steroids were quantified by radioimmunoassay as described (Porcu et al. 2003). Plasma steroid concentrations, were measured in 1 ml after extraction with 1.5 ml of ethyl acetate for four times.

### **Measurement of anogenital distance, vaginal opening and vaginal smear checks**

The anogenital distance in rats treated neonatally with  $\beta$ -estradiol-3-benzoate or with vehicle was measured on days 7, 21 and 60 after birth. All female pups were checked daily for vaginal opening. The differences in the anogenital distance and in the day of vaginal opening among the groups were detected by t-test. Vaginal smears were checked from vaginal opening until day 60. Every morning between 8:30 and 9:30 a.m. vaginal secretion from each animal was collected by carefully inserting the tip of a cotton swab (pre-wet in normal saline, NaCl 0.9%) into the vaginal orifice. It is important that the cotton swab is not inserted too deeply (more than 1 cm), in order to avoid excessive cervical stimulation, which may induce a pseudopregnancy state, characterized by a persistent diestrus (Goldman et al. 2007). Vaginal fluid was placed on glass slides and was observed under a light microscope. Three types of cells could be recognized: round

and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. The proportion among them was used for the determination of the estrous cycle phases (Long & Evans 1922; Mandl 1951). A female rat which showed a constant 4- or 5-day vaginal estrous cycle was regarded as an animal with a regular estrous cycle. When a vaginal smear contained cornified cells through the examination term, it was determined as persistent estrous, even if a few leukocytes were seen occasionally. If the diestrous condition appeared in some intervals in persistent estrous, the condition was regarded as a prolonged estrous.

### **Hematoxylin & eosin staining of ovarian sections**

Rats (treated with  $\beta$ -estradiol-3-benzoate or vehicle) were ovariectomized at 60-70 days after birth during the estrous or diestrous phase of their cycle. The ovaries were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) through three consecutive steps: the first two steps were performed at room temperature for 1 hour each, the last step was performed at 4°C overnight. The tissue was then rinsed in PBS, dehydrated and embedded in paraffin. Ovarian tissue sections of 10  $\mu$ m were prepared for hematoxylin & eosin (H&E) staining. Tissue sections of ovaries were deparaffinised in xylene, dehydrated through an ethanol series of 100%, 90%, 80%, 70%, and 50%, and stained with H&E. After dehydration and clearing with fresh xylene, sections were mounted with Canada balsam and observed on an inverted microscope in brightfield (ZEISS, Axio Observer Z.1). The images were acquired with a high resolution camera (ZEISS AxioCam MR-m) and were analyzed by Axiovision software to confirm the existence of corpora lutea (CL) and Graafian follicles (GF).

### **Spontaneous sexual behaviour**

In order to evaluate the effect of neonatal treatment with  $\beta$ -estradiol-3-benzoate on sexual behaviour, each experimental animal was placed with a “vigorous” male in a cage, with clear plexiglass lids to allow videotaping with an 8 mm Sony Handycam (in nightshot) for subsequent analysis of female sexual behaviour. The experiment lasted overnight, for 4 consecutive nights in order to capture the estrous cycle stage when females usually mate. The day before and the day after each mating, experimental animals were subjected

to vaginal smears in order to assess the stage of the cycle, given that females are receptive, and thus available to mate, only during the estrus phase. Each night, the male was placed in a cage with a different female, to promote the "Coolidge effect", whereby a male tends to mate more easily if it interacts with a different female.

Behaviours were video recorded for analysis. Each female, was monitored for both receptive and proceptive behaviours. The receptivity is the "consummatory" component of sexual interaction, characterized by the lordosis posture, a postural reflex with a dorsiflexion of the vertebral column. The receptive behaviour is expressed through a lordosis quotient (LQ) ( $\text{lordosis} / \text{mounts} + \text{intromissions} \times 100$ ). Proceptive behaviours represent the "appetitive", motivational component of sexual interaction and include those behaviours shown by a receptive female (in estrus) in order to attract and facilitate the male mounts such as "ear wiggling" (rapid oscillatory movement of the ears due to fast head movements subsequent to a high degree of tension in the axial muscles) and hops and darts (jumping and scattering in front of the male).

### **Induced sexual behaviour**

Beginning at 90 days after birth, and 30 days after ovariectomy, estrus was induced in all females by administering estradiol (200  $\mu\text{g}/\text{rat}$ , s.c.) and progesterone (500  $\mu\text{g}/\text{rat}$ , s.c.), 48 hours and 6 hours before the test, respectively. Lordosis and proceptive behaviours were examined for 60 minutes, under a dim red light, in a rectangular plexiglass mating arena (40 x 20 x 17 cm). A sexually vigorous male was allowed to acclimate to the testing chambers for 5 minutes before introducing the female. All tests were videotaped with a Sony Handicam (in nightshot) for subsequent analysis of female sexual behaviour as described above (LQ and proceptive behaviour).

### **Reagents**

$\beta$ -Estradiol 3-benzoate and Progesterone were obtained from Sigma-Aldrich (Milan, Italy). Antiserum to allopregnanolone was generated and characterized as described (Purdy *et al.* 1990). Antisera to progesterone and to  $17\beta$ -estradiol were obtained from MP Biomedicals (Solon, OH, USA). All other chemicals were of the best available quality from commercial sources.



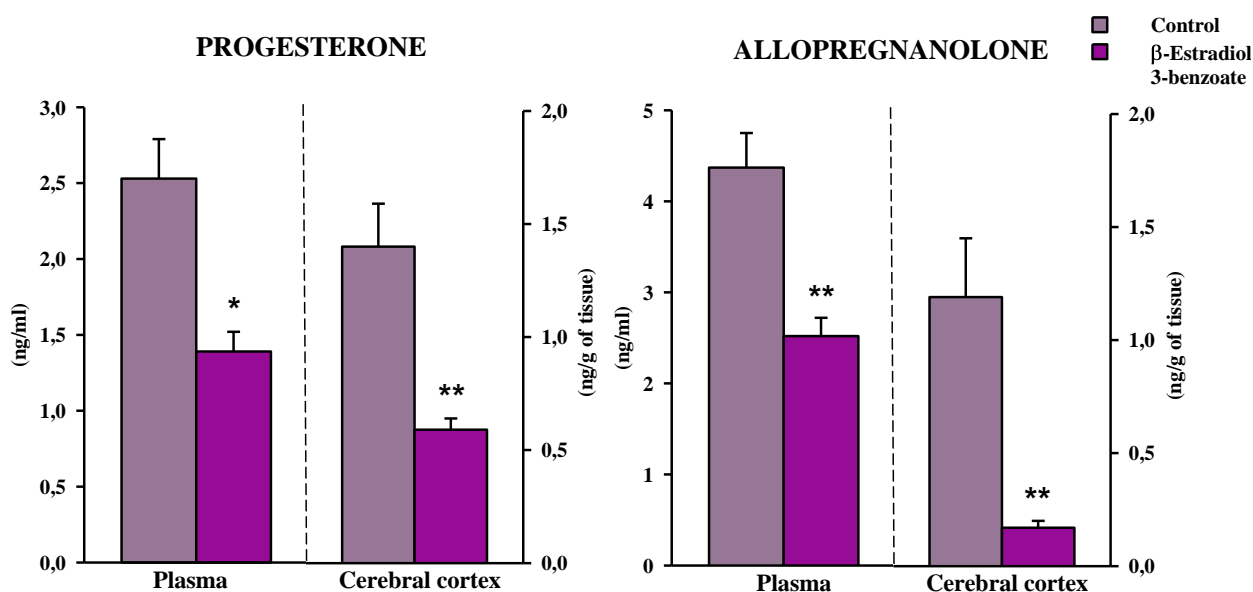
**Statistical analysis**

Data are presented as means  $\pm$  S.E.M. The statistical significance was assessed by t-test and one- or two-way analysis of variance (ANOVA) followed by the Newman-Keuls test. A *p* value of  $<0.05$  was considered statistically significant.

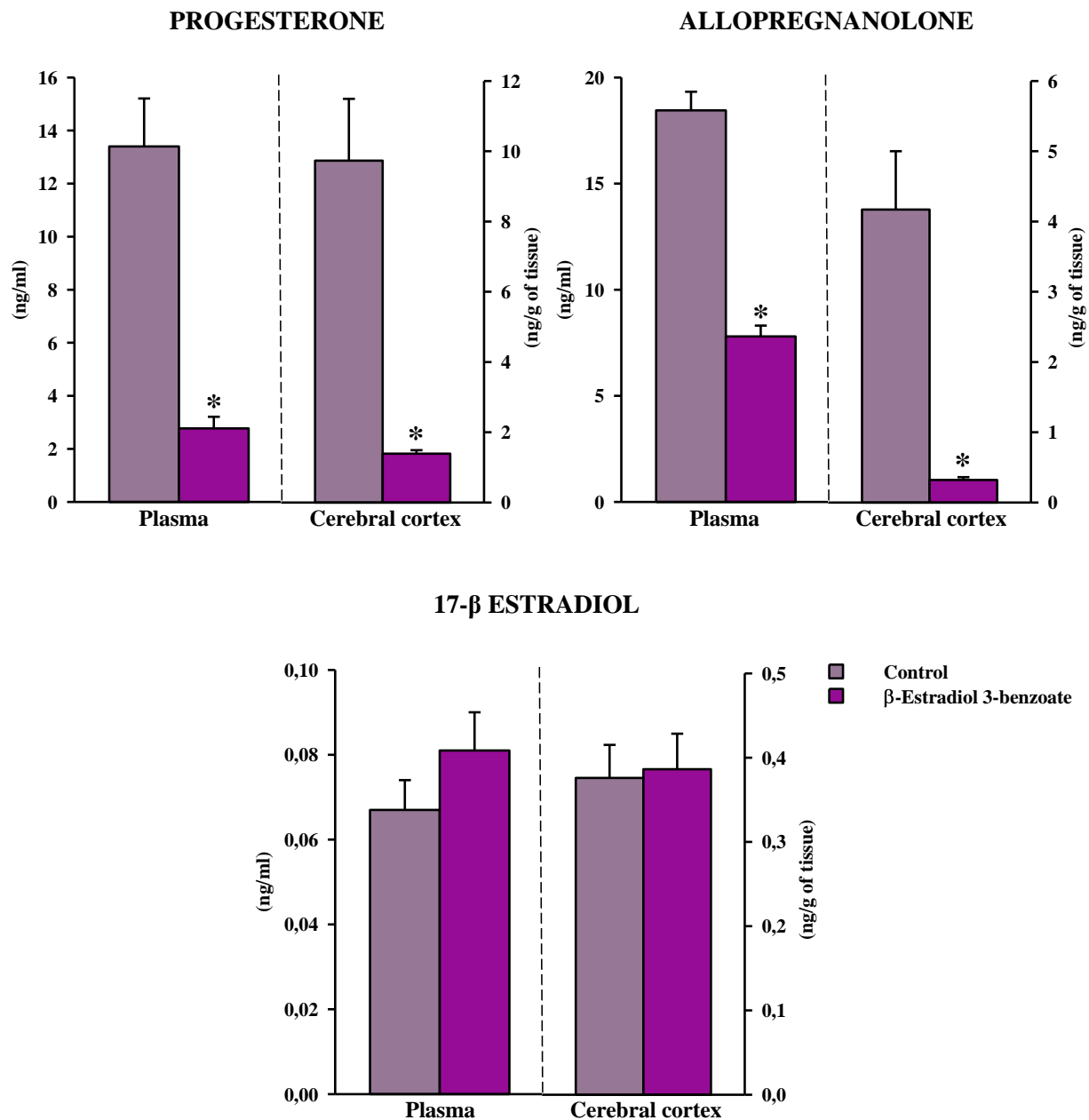
### 1.3 Results and discussion

#### Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on steroid hormone concentrations in rat plasma and cerebral cortex.

In addition to its previously demonstrated effect on peripheral steroidogenic tissue (Rodriguez et al. 1993), we have now shown that neonatal administration of  $\beta$ -estradiol 3-benzoate resulted in significant decreases in the plasma and cerebrocortical concentrations of progesterone (-94% and -58%, respectively,  $p < 0.005$ ) and its neuroactive metabolite allopregnanolone (-42% and -85%, respectively,  $p < 0.0005$ ) in female rats at 21 days after birth (Figure 1). Furthermore, administration of  $\beta$ -estradiol 3-benzoate to female neonates induced pronounced decreases in the plasma and cerebrocortical concentrations of progesterone (-86 and -73%, respectively,  $p < 0.001$ ) and allopregnanolone (-92% and -58%, respectively,  $p < 0.001$ ) in female rats at 60 days after birth (Figure 2). In contrast, the same treatment had no significant effect on the plasma and cerebrocortical concentrations of  $17\beta$ -estradiol (Figure 2).



**Figure 1.** Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on neuroactive steroid concentrations in rat plasma and cerebral cortex. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were sacrificed 21 days later for measurement of the plasma and cerebrocortical concentrations of progesterone and allopregnanolone. Data are expressed as nanograms of steroid per millilitre of plasma or as nanograms of steroid per gram of tissue and are means  $\pm$  S.E.M. of values from 10 rats per group. \* $p < 0.005$  and \*\* $p < 0.0005$  versus the respective control value.



**Figure 2.** Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on neuroactive steroid concentrations in rat plasma and cerebral cortex. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were sacrificed 60 days later for measurement of the plasma and cerebrocortical concentrations of progesterone, allopregnanolone and 17 $\beta$ -estradiol. Data are expressed as nanograms of steroid per millilitre of plasma or as nanograms of steroid per gram of tissue and are means  $\pm$  S.E.M. of values from 10 rats per group. \* $p < 0.001$  versus the respective control value.

Neonatal exposure to exogenous estrogen is thought to program hormonal cyclicity in adulthood, at least in part by promoting the development of neuronal circuits that are resistant to the positive feedback actions of estrogen (Robinson 2006). This effect of estrogen impairs the ability of this steroid to stimulate the GnRH surge that is required to trigger the LH surge and subsequent ovulation. Exposure of female rat pups to testosterone or estrogen on postnatal days 1-10 thus renders them permanently incapable of undergoing the LH surge (Korenbrod et al. 1975) and, consequently, results in a lack of ovulation and an altered formation of the corpora lutea, the endocrine structures responsible for the production of progesterone (Handa et al. 1985; Rodriguez et al. 1993). Accordingly, our results show that both plasma and brain levels of progesterone are markedly reduced in rats treated with  $\beta$ -estradiol 3-benzoate while the concentration of estradiol was not affected by this treatment. This result is in agreement with the evidence that treatment with androgens or estrogens decreases the secretion of progesterone by the ovaries (Rodriguez et al. 1993), while prenatal exposure of female rats to exogenous androgen was previously found not to affect the serum concentration of estradiol in adulthood (Foecking et al. 2005).

As a result of such impaired production of progesterone by the gonads, we have now found that the brain and plasma concentrations of its neuroactive steroid metabolite allopregnanolone are also reduced. Treatment with oral contraceptives, which reduces both the basal and stimulated serum concentrations of LH in rats, also results in decreased brain and plasma concentrations of progesterone and allopregnanolone in these animals (Follesa et al. 2002); it also prevents the increase in the serum concentrations of these steroids that normally occur in women during the luteal phase of the menstrual cycle (Rapkin et al. 2006).

**Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on anogenital distance, vaginal opening and estrous cycle pattern.**

A method used to investigate the endocrine events and the integrity of the hypothalamic-pituitary-ovarian reproductive axis in rodents is the assessment of changes in vaginal cytology during the estrous cycle. The reproductive cycle of female rats is called estrous cycle. Rats typically begin cycling immediately after the vaginal orifice opens, an event that occurs between postnatal days 32 and 36. Rats may initially show some irregular cycles (Goldman et al. 1985) before having a recurrent ovulation that occurs at either 4 or 5 day intervals. The vaginal opening does not reflect the onset of sexual maturity. Several days or weeks may pass before the first ovulation, which is characterized by the presence of cornified cells in the vaginal smear. The estrous cycle is characterized by four phases: proestrus, estrus, metestrus and diestrus. Each phase differs based on the proportion among three types of cells observed in the vaginal smears: epithelial cells, cornified cells and leukocytes (Marcondes et al. 2002). A proestrus smear consists of predominance of round nucleated epithelial cells, which often have a granular appearance under the microscope. Proestrus lasts for one day and is followed by vaginal estrus, identifiable by the presence of large numbers of anucleated cornified cells, or cells with jagged edges. The predominance of cornified cells may last one day in a 4-day cycle, or may be present for two consecutive days in a 5-day cycle. A metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells. A diestrus smear primarily consists of a predominance of leukocytes. The appearance of these cells typically correlates with the status of vaginal mucosa, uterus and ovaries and is linked to alterations in circulating concentrations of sex steroids and gonadotropins. In fact, the keratinization of vaginal epithelial cells that typically characterizes the day of estrus is a response to the rising level of estradiol that begins on the second day of diestrus and peaks around midday on proestrus (Pawluski et al. 2009). The term “estrus” refers to the “heat period” or sexual receptivity. The female rats are sexually receptive when small numbers of cornified cells begin to appear (Young et al. 1941; Rodgers 1970; Hardy 1972), an event that occurs at the end of the day of vaginal proestrus, exactly when the ovulation takes place.

In order to further characterize the effects of neonatal administration of  $\beta$ -estradiol 3-benzoate on the hypothalamic-pituitary-ovarian reproductive axis, we examined the development of the reproductive tract and the estrus cycle in the rats treated with  $\beta$ -estradiol 3-benzoate.

All animals have been monitored from the day of birth (day 0) until day 60 to check for anogenital distance, vaginal opening and to subsequently perform vaginal smears. There was no difference in anogenital distance between control and  $\beta$ -estradiol 3-benzoate-treated rats at 7 and 21 days after birth. 60 days after birth  $\beta$ -estradiol 3-benzoate-treated rats showed a significant increase in the anogenital distance with respect to control animals (from 16 to 18 cm,  $p < 0.05$ ) (Table 1).

**Table 1.** Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate (EB) on anogenital distance, vaginal opening and estrous cycle in female rats.

Neonatal treatments	N° of rats	Anogenital distance (cm)	Vaginal opening (Median day)	Estrous cycle		
				Regular	Irregular	
					Prolonged estrus	Persistent estrus
Control	10	16 $\pm$ 0.25	31 $\pm$ 0.58	10/10	0/10	0/10
EB	10	18 $\pm$ 0.67*	44 $\pm$ 1.15**	0/10	10/10	0/10

Rats were injected with EB (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth. Data are means  $\pm$  S.E.M. from 10 rats per group. \* $p < 0.05$ ; \*\* $p < 0.0005$  versus the respective control group.

Furthermore, neonatal  $\beta$ -estradiol 3-benzoate treatment induced a delay in the vaginal opening from day 31 to day 44 ( $p < 0.0005$ ). Changes in vaginal cytology during the estrous cycle were monitored from the day of vaginal opening until day 60 after birth. A regular estrous cycle was observed in all control animals. In contrast, ten out of ten  $\beta$ -estradiol 3-benzoate-treated rats showed a prolonged estrus, with a diestrous condition appearing occasionally, suggesting that a single administration of  $\beta$ -estradiol 3-benzoate on the day of birth compromises the estrus cycle in adult female rats. Given that the keratinization of vaginal epithelial cells that typically characterizes the day of estrus is a response to the rising level of estradiol (Pawluski et al. 2009), the persistent presence of

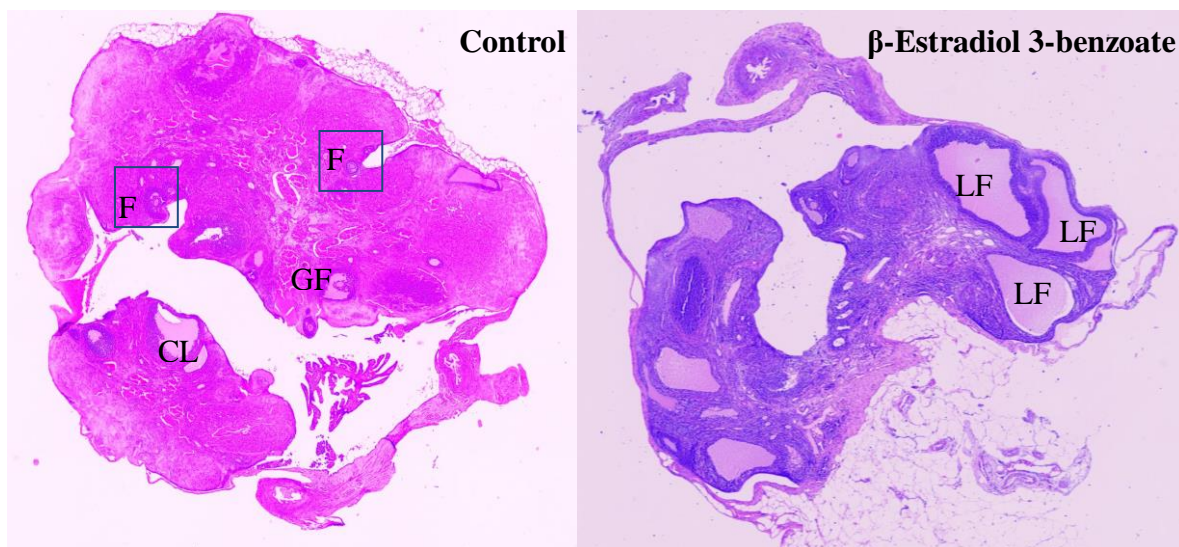
keratinized cells and the predominance of the estrous phase observed in  $\beta$ -estradiol 3-benzoate-treated rats could be related to the finding that  $17\beta$ -estradiol concentrations were not affected by this treatment. Moreover, we found that, the neonatal treatment with  $\beta$ -estradiol 3-benzoate decreased progesterone levels in the adult animal. Progesterone plays an essential role in the second half of the cycle, by promoting the optimal conditions for fertilization and implantation of the egg cell. Therefore, the persistence of the estrous phase and the lack of the cyclic alternation of the other phases, could be due to the unchanged estradiol concentrations and reduced levels of progesterone present in these animals.

### **Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on morphology and histology of the uterus and ovaries in adult female rats.**

Next we examined whether the dysregulation of the estrous cycle induced by neonatal treatment with  $\beta$ -estradiol 3-benzoate might result in alterations of the ovarian morphology. As expected (Bloch et al. 1995), there was no difference in weight between the left and right ovary. In the  $\beta$ -estradiol 3-benzoate-treated rats, mean uterus and ovaries weights were less than half compared to values observed in the control groups (EB:  $0.51\pm 0.08$ g; Co:  $1.71\pm 0.09$ g,  $p < 0.001$ ). Ovarian weight is influenced by the size and number of Graafian follicles and of corpora lutea which are in turn influenced by circulating LH and FSH.

Histological examination of the ovaries showed that all control rats showed a remarkable number of follicles at different stages of maturation and the presence of corpora lutea. On the contrary,  $\beta$ -estradiol 3-benzoate-treated rats exhibited a different histological pattern characterized by a marked reduction in the number of corpora lutea and an increase in the number of large follicles with a hypertrophic granulosa layer. Accordingly, administration of a high dose (100  $\mu$ g) of estradiol for five days after birth to female rats induced profound alterations in the ovarian histology, with a lack of corpora lutea and a predominance of large follicles (Kouki et al., 2003). Given that the primary function of the corpus luteum is the secretion of progesterone, the almost total absence of this endocrine structure in the ovary of  $\beta$ -estradiol 3-benzoate-treated rats could be

responsible for the dramatic reduction of brain and peripheral levels of progesterone and its metabolite allopregnanolone.



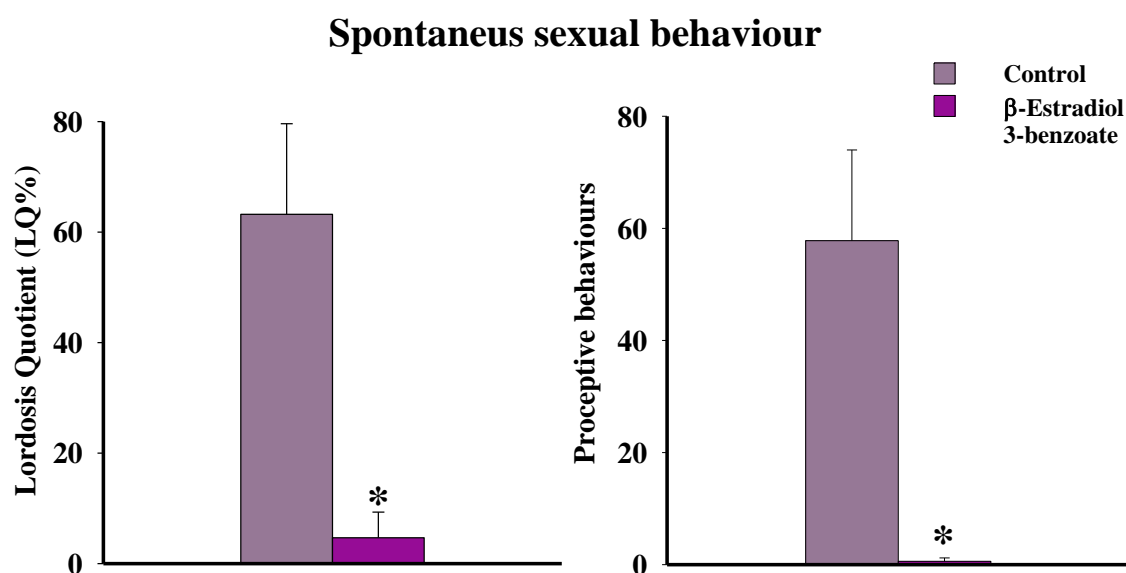
**Figure 3.** Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on histology of the uterus and ovaries in adult female rats. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth. The ovaries from  $\beta$ -estradiol 3-benzoate-treated rats and from control females exhibited a different histological pattern during the estrous cycle. Of note, the increased number of large follicles (LF) and the decreased number of corpora lutea (CL) in  $\beta$ -estradiol 3-benzoate-treated female rats. Control females showed follicles at different stages of maturation (F), in particular Graafian follicles (GF).

### **Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on spontaneous and induced sexual behaviour in adult female rats.**

Neuroendocrine and anatomical alterations induced by neonatal administration of  $\beta$ -estradiol 3-benzoate also affect sexual behaviour in the adult. This is known since 1963, when Whalen and Nadler showed that neonatal treatment with high doses of estrogen for five days significantly reduced lordosis behaviour in adult female rats. Thus, we assessed whether both spontaneous and induced sexual behaviours were affected in adult female rats following neonatal exposure to  $\beta$ -estradiol 3-benzoate. Female rats engage in proceptive and receptive sexual behaviours. Proceptive behaviours involve the solicitation of sexual activity (hopping, darting and ear wiggling), whereas receptive behaviours ensure that mating is successful (lordosis). Several papers have documented



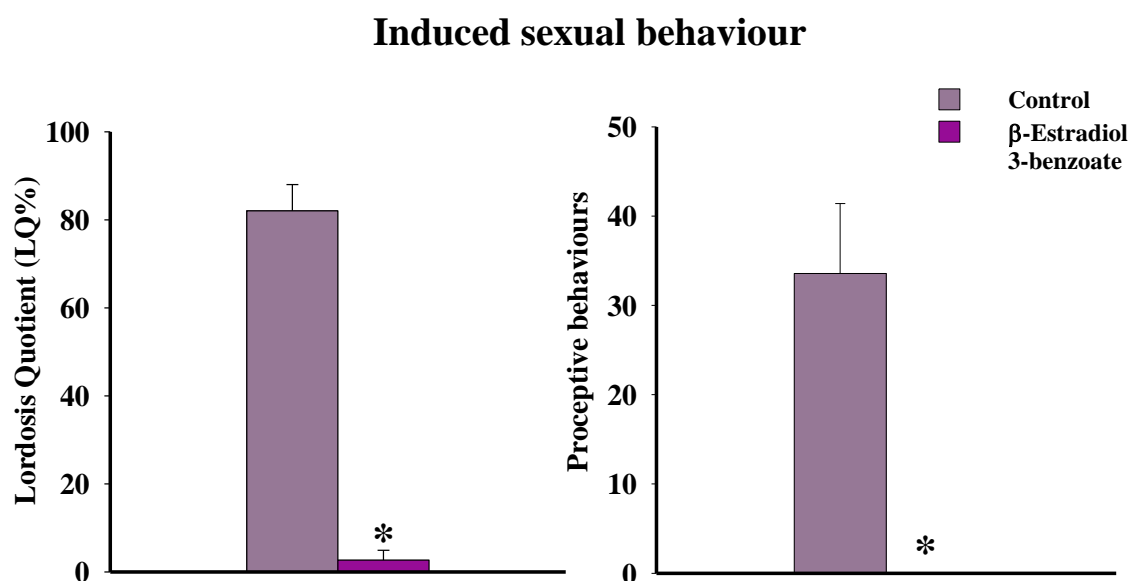
that proceptive behaviours were controlled by progesterone, while the expression of receptive behaviours was amplified by a combination of estrogen and progesterone (Feder 1984). In agreement, ovariectomized females do not display proceptive or receptive behaviours in the presence of a male (Boling and Blandau, 1939). As expected, neonatal administration of  $\beta$ -estradiol 3-benzoate to female rats affected the spontaneous sexual behaviour in adulthood. In fact, this treatment induced a marked decrease of the lordosis quotient (-93%,  $p < 0.05$ ) and of the proceptive behaviours (-99%,  $p < 0.05$ ) (Figure 4) with respect to control animals that showed a regular mating during their estrous cycle.



**Figure 4.** Effect of neonatal administration with  $\beta$ -estradiol 3-benzoate on spontaneous sexual behaviour of adult female rats. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested for sexual behaviour overnight, for 4 consecutive nights, between days 60 and 90 after birth. Each female was placed in a testing arena with a sexually experienced male. For each female, a lordosis quotient (LQ) (number of lordosis displayed/number of mounts  $\times$  100) was calculated and proceptive behaviours (ear wiggling, hops and darts) were monitored. Data are means  $\pm$  S.E.M. from ten rats per group. \* $p < 0.05$  versus the respective control value.

Next we evaluated whether the induction of estrus stage in the  $\beta$ -estradiol 3-benzoate-treated females was able to enhance sexual behaviour and actively promote mating with the male. All rats were bilaterally ovariectomized one month before the test. For the

experiment, they were injected with estradiol benzoate and progesterone 48 hours and 6 hours before mating, respectively. As expected (Xiao & Becker 1997; Blaustein & Wade 1977), this treatment induced lordosis and proceptive behaviours in control animals. On the contrary, neonatal  $\beta$ -estradiol 3-benzoate-treated rats, showed a severe decrease of the lordosis quotient (97%,  $p < 0.0001$ ) and of proceptive behaviours (-100%,  $p < 0.0001$ ), compared to control rats (Figure 5), an effect similar to that observed during the spontaneous sexual behaviour evaluation.



**Figure 5.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on induced sexual behaviour. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested for sexual behaviour for 60 min between days 60 and 90 after birth. To induce estrous, EB (200  $\mu$ l/rat, s.c.) and progesterone (500  $\mu$ g/rat, s.c.) were administered 48 hours and 6 hours before the test, respectively. Each female was placed in a testing arena with a sexually experienced male. The lordosis quotient (LQ) (number of lordosis displayed/number of mounts  $\times$  100) was calculated and the proceptive behaviours (ear wiggling, hops and darts) were monitored. Data are means  $\pm$  S.E.M. from ten rats per group. \* $p < 0.0001$  versus the respective control value.

Therefore, the injection of a low dose of  $\beta$ -estradiol 3-benzoate (10  $\mu$ g) to female rats on the day of birth, abolishes sexual receptivity in adulthood, similar to what has been previously reported for the higher dose of 100  $\mu$ g (Levine and Mullins, 1964). This effect persists, even when estrogen and progesterone are administered to induce estrus after ovariectomy. In contrast, in ovariectomized rats the administration of estradiol and progesterone restores sexual behaviour (Levine and Mullins, 1964).

Overall these results suggest that the administration of a low dose of  $\beta$ -estradiol-3-benzoate, such as 10  $\mu\text{g}$ , is sufficient to androgenize the sexually dimorphic brain regions responsible for sexual behaviour. Therefore, this model can be used to study the phenomenon of defeminization of the developing brain.

#### **1.4 Acknowledgements**

We thank Dr. Massimo Annis of the Department of Cytomorphology for his assistance with the hematoxylin & eosin staining of ovarian sections, and Dr. Pietro Paolo Secci for his assistance with analysis and acquisition of the histological images with the microscope.

## Chapter 2

### Effect of neonatal estradiol treatment on emotional, cognitive and social behaviours in adult rats

#### 2.1 Introduction

The  $3\alpha,5\alpha$ -reduced metabolite of progesterone, allopregnanolone, is a neurosteroid that exerts a rapid change in neuronal excitability and elicits behavioural effects of its administration to experimental animals (Biggio and Purdy 2001). Our understanding of the physiological role of this endogenous neuroactive steroid was greatly increased by the finding that allopregnanolone is the most potent and efficacious positive allosteric modulator of gamma-aminobutyric acid type A (GABA<sub>A</sub>) receptor function (Majewska 1992; Lambert et al. 1995). In fact, the interaction between allopregnanolone and the GABA<sub>A</sub> receptor appears to underlie the pharmacological actions of this steroid that are similar to those induced by classical positive modulators of GABA<sub>A</sub> receptors such as benzodiazepines. Allopregnanolone can be directly synthesised in the brain, in addition to peripheral sources; however, the corpus luteum contribution of progesterone and allopregnanolone to the peripheral plasma also augments brain concentrations of this neuroactive steroid (Biggio and Purdy 2001; Ottander et al. 2005). Given that GABA<sub>A</sub> receptors participate in the regulation of a variety of psychophysiological phenomena, including anxiety, depression, sleep, cognitive function, seizures, social and sexual behaviour, such fluctuations in the concentrations of allopregnanolone may play a crucial role in the cognitive and psychiatric manifestations of conditions characterized by marked changes in the hormonal milieu. In fact, animal studies have shown that allopregnanolone, administered systemically or intracerebroventricularly, elicits anxiolytic, antidepressive, anticonvulsant and sedative-hypnotic effects similar to those induced by benzodiazepines (Biggio and Purdy 2001). Moreover, allopregnanolone facilitates social and sexual behaviour and exhibits analgesic and neuroprotective actions (Frye et al. 2009; Jevtovic-Todorovic et al. 2009; Djeballi et al. 2005; Wang et al. 2005).

On the other hand, a number of reports have indicated that allopregnanolone may also have some non-beneficial effects. In fact, allopregnanolone has been shown to induce irritability/aggression (Miczek et al. 1997; Fish et al. 2002), and it has been reported to induce anxiety following a short-term treatment (Gulinello et al. 2001). Moreover, allopregnanolone has been described to have a detrimental learning profile when injected systemically (Johansson et al. 2002) or directly into the brain (Mayo 1993; Ladurelle et al. 2000). Accordingly, it has been shown that the administration of allopregnanolone impairs episodic memory in women (Kask et al. 2008).

Several findings indicate that allopregnanolone plays an important role during brain development (Mameli et al. 2005). In fact, it has been described that neonatal allopregnanolone promotes the establishment of neuronal circuitry and supports the survival of developing neurons (Griffin et al. 2004). Also, previous studies demonstrated that alterations in neonatal allopregnanolone levels have a profound effect on the morphology and the structure of several brain areas such as the cortex and the thalamus (Grobin et al. 2003; Grobin et al. 2004; Grobin et al. 2006); in addition they also critically alter the normal development of the hippocampus (Cooper et al. 1999). Accordingly, alterations in adult behaviour have also been reported. Thus, manipulation of neonatal allopregnanolone levels alters the performance in the elevated plus maze and the aversive learning in the passive avoidance test (Martin-Garcia et al. 2008). In addition, allopregnanolone administration from postnatal day 5 to postnatal day 9 has been shown to alter responses to GABA<sub>A</sub> modulators (such as benzodiazepines) during adulthood (Darbra et al. 2009), and to induce an anxiolytic profile in the elevated plus maze (Darbra et al. 2012). All together these evidences suggest that alteration of allopregnanolone during critical developmental periods have important consequences in adult behaviour.

On the basis of these evidences, given that treatment of female rats with  $\beta$ -estradiol 3-benzoate on the day of birth induces a dramatic reduction of the brain and plasma levels of allopregnanolone in adulthood, one of the aims of this thesis was to assess whether this treatment might modify several emotional, cognitive and social behaviours. Specifically, rats underwent the elevated plus maze test to evaluate anxiety levels, the motility meter test to evaluate the locomotor activity, the Porsolt test to assess depression,

pentylentetrazole (PTZ)-induced convulsions to assess seizures sensitivity, the Morris water maze test to evaluate learning and memory and the resident-intruder test to investigate the agonistic and social behaviours.

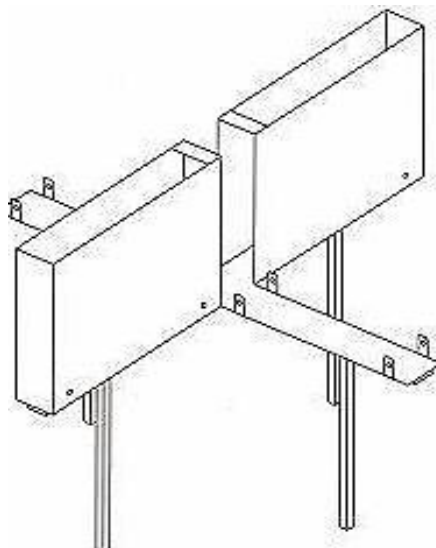
## 2.2 Materials and methods

### Animals

Animal care and handling throughout the experimental procedures and neonatal treatment with  $\beta$ -estradiol 3-benzoate were performed as described in chapter 1. The animals were subjected to behavioural tests between days 60 and 90 after birth. To avoid effects of estrous cycle status we tested control animals in randomized phases of the estrous cycle. The female rats used as intruders, as mates of interaction and as receptive female were bilaterally ovariectomized (OVX) as described in chapter 1.

### Elevated plus-maze test

The plus-maze was made of black polyvinyl chloride and comprised two open and two closed arms (12 x 60 cm) connected by a central square (12 x 12 cm) that served as the start point (Figure 1). The apparatus was mounted 50 cm above the floor of a quiet, dimly lit room. On the test day, rats were allowed to acclimate to the experimental room for 1 hour before the test. Each rat was tested only once. The animal was placed at the start point of the maze facing an open arm.



**Figure 1.** Elevated Plus-Maze apparatus

During the 5-min test, the number of entries into open and closed arms and the time spent in each type of arm were monitored for each rat by two observers unaware of the treatment group; arm entry was defined as the presence of all four feet of the animal in the arm. Rats were tested in a randomized order between 09.00 and 14.00 hours. Tests were performed during the light period of the light-dark cycle. The maze was cleaned thoroughly at the end of each test.

### **Locomotor activity test**

Locomotor activity of rats was assessed with the use of a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, OH, USA). The test chamber consists of a cubicle made of clear Perspex (48 x 50 cm) and with 50-cm high walls (Figure 2). Two facing blocks containing an infrared array record horizontal activity, and a similar system assesses vertical activity. Each animal was gently placed at the centre of the chamber and allowed to explore the apparatus in an illuminated and quiet room. Locomotor activity was recorded during the light cycle between 09.00 and 14.00 hours. Rats were tested in a randomized order, and locomotor activity was assessed for each animal individually during a 10-min period. The total distance travelled (cm) by each animal was accumulated over consecutive 2-min time windows, and the number of movements was recorded. The arena was cleaned thoroughly at the end of each test.



**Figure 2.** Motility meter apparatus.



### Porsolt test

The forced swim test or Porsolt test (Porsolt et al. 1977) is also called behavioural despair test and it is usually used to evaluate the antidepressant activity of drugs. The apparatus consists of a clear acrylic cylinder, 18 cm in diameter and 40 cm in height, filled with water at room temperature (25°C) to a depth of 15 cm (Figure 3). The rat is placed into the cylinder from which there is no chance to escape, so he is forced to stay afloat or swim for 5 minutes. After a short period of hyperactivity, the animals stay in an immobile position and they no longer try to escape. Immobility in the water is considered an index of depressed mood and antidepressant drugs tend to reduce the immobility time. Testing was conducted over two consecutive days. On day 1 (*pre-test*), rats were placed in the cylinder for 10 minutes, to adapt to the experimental room before the test. On day 2, 24 hours after the pre-test, rats were placed in the cylinder for 5 minutes. Movements were recorded using a video camera and DVD recorder. Scoring was performed by an observer unaware of treatment group. Immobility duration was scored on day 2 as time spent not swimming or reaching at the walls. The test was performed during the light cycle between 09.00 and 14.00 hours. The acrylic cylinder was cleaned thoroughly at the end of each test.



**Figure 3.** Porsolt apparatus

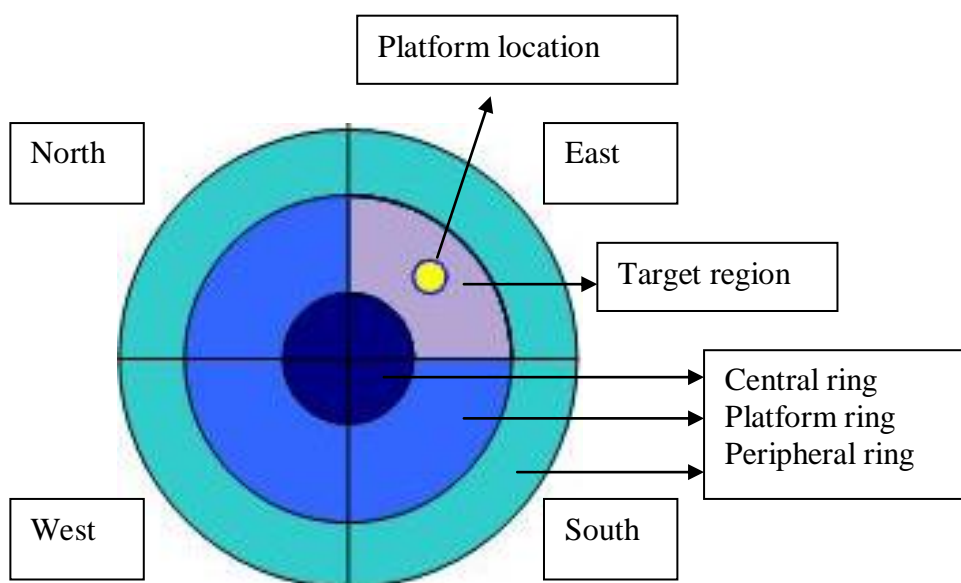
### **PTZ-induced seizures**

The sensitivity of the animals to seizures was evaluated using pentylentetrazole (PTZ) (Depoortere et al. 1986). This convulsant drug, when injected intraperitoneally at the dose of 60 mg/kg induces generalized tonic-clonic convulsions sometimes leading to death within a few seconds in control rats. PTZ was dissolved in distilled water and was injected i.p. at a dose of 60 mg/kg in a volume of 3 ml/kg. Control animals received the same volume of vehicle. Rats were observed for 30 minutes following PTZ injection. The latency to appearance of seizures, the number of rats showing seizures and the number of deaths were recorded.

### **Morris water maze**

The Morris water maze is a test of spatial learning introduced almost 30 years ago by Richard Morris (Morris 1984). It consists of a circular pool (150 cm in diameter, 60 cm in depth) whose interior is painted black. The apparatus was located at the centre of a room dedicated to measurement of this behavioural paradigm. The water temperature was maintained at  $25 \pm 2^\circ\text{C}$  with the use of a submersible digital water-heating system. The pool was divided into four virtual quadrants, and a removable circular escape platform (10 cm in diameter, 32 cm in height) was introduced into one of the quadrants (target quadrant) at a depth of 2 cm below the water surface. The protocol we followed consisted of a training phase and a probe trial. Over five consecutive days, each rat was subjected to four training trials, in which it was placed into the pool in the quadrant next to the target quadrant. Once the animal had climbed onto the platform, it was allowed to remain there for 15 seconds before the next trial; if it had not found the escape platform by 120 seconds, it was gently guided to the platform and allowed to rest there for 15 seconds. The time elapsed (latency) before the animal climbed onto the platform, swim speed, and distance travelled during each trial were recorded; animals that did not climb onto the platform before the end of the trial period were assigned a latency value of 120 seconds. To assess the long-term spatial memory at 24 hours after the last training trial, each rat was subjected to a probe trial, in which the escape platform was removed from the pool and the animal was released from the quadrant opposite to the original platform location and allowed to freely swim for 60 seconds. Behavioural data from the training and probe

tests were acquired and analyzed using an automated tracking system (Ethovision, Noldus, Wageningen, The Netherlands). Using this software, the precise rat location (in  $x, y$  coordinates) was recorded throughout the probe test (capture rate 10 frames/seconds). From this spatial distribution, proximity measure (Gallagher et al. 1993) was calculated automatically. For the probe trial, the tracking software virtually divided the pool into four quadrants, three concentric annuli and a target region consisting of the intersection of the platform quadrant and the platform annulus (Figure 4).



**Figure 4.** Schematic representation of the virtual division of the water maze used to assess memory performance.

### Resident-intruder test

Animals were assessed for the display of offensive aggressive behaviours against an unfamiliar conspecific intruder using a standard resident-intruder aggression test (Koolhaas et al. 1980). This type of aggression test is the most frequently used methodological approach in the laboratory because resident-intruder paradigms have a very wide species generality (Adams 1979; van Hooff 1977; Wilson 1975). Subjects were 80 female rats. Forty rats, ovariectomized 3 weeks before the experiment, were used as intruders. The use of OVX female intruders eliminates the estrous cycle variability that

has been shown to affect aggression in rats (Ho et al. 2001). The remaining 40 female rats were used as residents. 7 days before the test, the female rats to be tested as residents were isolated and their cages were no longer changed or cleaned in order to allow them to establish a territory in their home cages. On the test day, the OVX female intruder (same size as the resident) was introduced in the resident's home cage. The rats were left undisturbed to freely interact for 10 minutes while being videotaped. Social interactions between residents and intruders took place during the dark phase of the light/dark cycle. Clear Plexiglas lids were used to allow videotaping from above with an 8 mm Sony Handicam (in nightshot) for subsequent behavioural analysis of the interactions. To assist in identification, the intruder rats were painted with black magic marker at least 12 hours prior to testing. At this time, all residents and intruders were moved into the testing room and left undisturbed for at least 12 hours. The videotaped social interactions were scored by one trained observer that was unaware of the animals' group. The interactions were analyzed for 21 behaviours based on the ethogram by Grant and Mackintosh (1963; see Table 1, modified from Clipperton et al. 2008, for behaviour descriptions) using the Behaviour Tracker software. The behavioural analysis focused on the treated rat, the resident. The behaviour of the intruder was collected only in relation to the behaviour of the resident (i.e., attacks received, reciprocal attacks, social inactivity), and in the reciprocal pairs of behaviours (dominant/submissive behaviour and chasing/avoidance of the intruder). Moreover, different categories of individual behaviours were formed (modified from Clipperton et al. 2008) to provide an overall view of the animals' behaviour.

### **Statistical analysis**

Data are presented as means  $\pm$  S.E.M. The statistical significance was assessed by t-test and one- or two-way analysis of variance (ANOVA) followed by the Newman-Keuls test. A *p* value of  $<0.05$  was considered statistically significant.

**Table 1. Description of scored behaviours in the resident-intruder test.**

<b>Agonistic behaviours delivered</b>	
Follow intruder	The resident rat actively follows, or pursues and chases the intruder; reciprocal to avoid.
Dominant behaviours	The resident rat is in control; includes pinning of the intruder, aggressive grooming, crawling over or on top, and mounting attempts.
Attacks delivered	Physical attacks, including dorsal/ventral bites. Only the frequency of attacks was measured.
<b>Agonistic behaviours received</b>	
Avoidance of the intruder	The resident withdraws and runs away from the intruder while the intruder is chasing.
Submissive behaviours	The intruder rat is in control; includes crawl under, supine posture (ventral side exposed), prolonged crouch, and any other behaviour in which the intruder is dominant (e.g., the intruder pins, aggressively grooms, etc., the resident).
Attacks received	Physical attacks including bites to dorsal/ventral regions. Only the frequency of attacks was measured.
Defensive upright posturing	Species-typical defensive behaviour; upright with the head tucked and the arms ready to push away.
<b>Dominance score</b>	Total agonistic behaviour delivered minus total agonistic behaviour received. A negative score indicates that the resident was the submissive animal in the pair, while a positive score signifies that the resident was the dominant animal.
<b>Social investigation</b>	
Oronasal investigation	Active sniffing of the intruder's oronasal area.
Body investigation	Active sniffing of the intruder's body.
Anogenital investigation	Active sniffing of the intruder's anogenital region.
Approaching and/or attending to the intruder	Often from across the cage; the resident's attention is focused on the intruder, head tilted toward the intruder and movements toward the intruder; this becomes "chasing the intruder" once along the tail or sniff if resident is within 1.5 cm of the intruder.
<b>Non-social behaviours</b>	
Horizontal exploration	Movement around the cage; includes active sniffing of air and ground.
Vertical exploration	Movement to investigate upwards, both front feet off the ground; includes sniffing, wall leans and lid chews.
Digging	Rapid stereotypical movement of forepaws in the bedding.
Abnormal stereotypies	"Strange" behaviours, including spinturns, repeated jumps/lid chews/head shakes.
Solitary inactivity	No movement; includes sit, lie down and sleep.
Self-groom	Rapid movement of forepaws over facial area and along body.

## 2.3 Results and discussion

### **Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on the behaviour of rats in the elevated plus maze, motility, Porsolt test and PTZ-induced seizures**

Neonatal administration of  $\beta$ -estradiol 3-benzoate did not affect the behaviour of adult female rats in the elevated plus maze test. In fact, the percentage of time spent in the open arms and the percentage of open-arm entries did not differ between control and  $\beta$ -estradiol 3-benzoate-treated rats (Table 2). This test is based on the normal rat's aversion toward an open environment. Usually, rodents spend less time and make fewer entries into the open arms of the maze; therefore, a higher percentage of time spent in the open compartment implies a lower level of anxiety. The lack of changes in anxiety like-behaviour between  $\beta$ -estradiol 3-benzoate- and vehicle-treated rats may indicate that the decrease in the concentrations of allopregnanolone and progesterone, induced by neonatal  $\beta$ -estradiol 3-benzoate treatment, might not account for changes in the emotional state of the animals in adulthood. These results are in contrast with previous observations showing that decreased levels of allopregnanolone induced by ovariectomy or by administration of the  $5\alpha$ -reductase blocker finasteride increase anxiety-like behaviour in the elevated plus-maze paradigm (Zimmerberg and Farley 1993; Smith et al. 1998b). Accordingly, administration of oral contraceptives, a treatment that markedly decreases brain allopregnanolone concentrations, enhances anxiety levels in the elevated plus maze paradigm (Follesa et al. 2002). Furthermore, both progesterone and allopregnanolone, when injected systemically or intracerebroventricularly, induce anxiolytic effects in various behavioural tests (Bitran et al. 1991, 1993; Wieland et al. 1991; Zimmerberg et al. 1994).

Consistent with the results obtained in the elevated plus maze test, neonatal estradiol treatment did not affect the locomotor activity of adult female rats in the motility meter test. In fact, we did not find statistically significant differences between the two experimental groups in the total distance travelled and in the number of movements (Table 2).

Clinical and animal studies support a role of allopregnanolone in depressive behaviours. Indeed, decreased plasma and cerebrospinal fluid concentrations of allopregnanolone

have been detected in major depressive disorder (Uzunova et al. 2003). Moreover, it has been shown that administration of drugs with clinical relevance in the treatment of these pathologies influences the secretion of these steroids (Dubrovsky 2005; Pisu and Serra 2004).

**Table 2.** *Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on the behaviour of adult female rats in different tests*

<b>Tests</b>	<b>Parameters</b>	<b>Control</b>	<b><math>\beta</math>-Estradiol 3-benzoate</b>
Plus maze	% time in open arms	13.2 $\pm$ 2.7	11.2 $\pm$ 2.5
	% open-arm entries	17.1 $\pm$ 2.9	13.4 $\pm$ 2.9
Motility	Total distance travelled (cm)	1935.1 $\pm$ 283.9	2471.7 $\pm$ 303.2
	No. of movements	70.2 $\pm$ 6.4	77.7 $\pm$ 7.4
Porsolt	Immobility (sec)	86.1 $\pm$ 10.2	95.2 $\pm$ 8.5
	Diving	1.5 $\pm$ 0.6	0.9 $\pm$ 0.4
	Swimming (sec)	213.9 $\pm$ 9.8	204.8 $\pm$ 7.8
PTZ seizures	Animals showing convulsions	7/7	8/8
	Latency (sec)	112 $\pm$ 12	111 $\pm$ 15

Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and underwent to the elevated plus maze test, motility meter test, forced swim test and PTZ-induced seizures between days 60 and 90 after birth. Data are means  $\pm$  S.E.M. of values from 15 rats per group (elevated plus maze and forced swim tests), 10 rats per group (motility meter test), 8 rats per group (PTZ-induced seizures).

In addition, allopregnanolone has been shown to elicit an antidepressant effect in the forced swim test (Kisthi et al. 2000). Therefore, given that neonatal treatment with  $\beta$ -estradiol 3-benzoate decreases allopregnanolone levels in the adult female rats, we

evaluated whether this treatment would affect the forced swim test that measures behavioural despair in rodents as an index of depression. As shown in table 2, we observed no significant alterations in the time of immobility and in the number of dives, measured in adult females that had been treated with  $\beta$ -estradiol 3-benzoate on the day of birth, suggesting that the decrease in brain allopregnanolone levels, induced by neonatal treatment with  $\beta$ -estradiol 3-benzoate, does not affect behavioural despair in adult rats. In contrast, ovariectomy, which also decreases brain allopregnanolone content (Follesa et al. 2002), increases depressive behaviour in female rats (Frye and Wawrzycki 2003) and administration of progesterone and allopregnanolone can reverse these effects (Frye and Walf 2002).

Finally, neonatal administration of  $\beta$ -estradiol 3-benzoate did not affect the sensitivity of the animal to the convulsant effect induced by PTZ. In fact, both  $\beta$ -estradiol 3-benzoate- and vehicle-treated rats showed seizures after administration of PTZ (60 mg/kg, i.p.), with a similar latency of onset (Table 2). Again, this result is at odd with the evidence of the protective actions of allopregnanolone against several standard convulsive tests in rodents (Gasior et al. 1997).

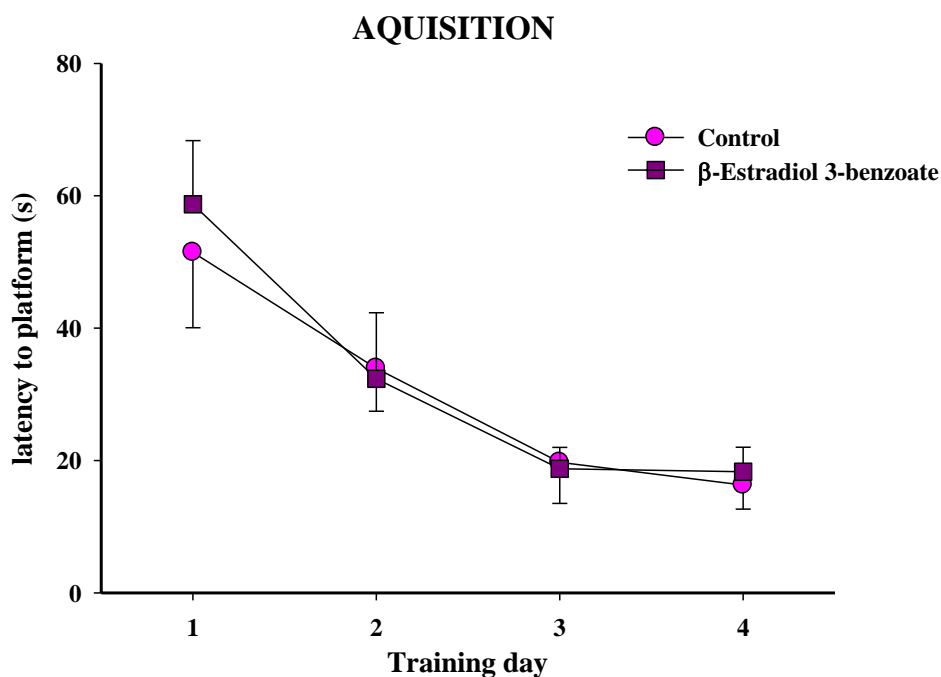
Overall, although allopregnanolone seems to be involved in different psychiatric and neurological disorders including anxiety, depression and epilepsy, the lack of differences in the above behavioural analyses between neonatal  $\beta$ -estradiol 3-benzoate-treated rats and the respective controls does not support a role for allopregnanolone in mediating the behaviour in these animals.

### **Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on the behaviour of rats in the Morris water maze test in adult female rats.**

Given that allopregnanolone administration decreases spatial learning in the Morris water maze (Johansson et al. 2002) and rats treated on the day of birth with  $\beta$ -estradiol 3-benzoate have lower levels of this neurosteroid, we evaluated the effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on this behavioural test. The Morris water maze is a test used to study spatial learning and memory, introduced almost 30 years ago by Richard Morris (Morris 1984), where rats are trained to locate a hidden escape platform



that is positioned just below the water surface. In our experiment, all rats appeared to swim normally and showed no difficulty in locating the hidden platform provided. During the 4 training trials both control and neonatal  $\beta$ -estradiol 3-benzoate-treated rats showed a decrease in the latency to locate the hidden platform, but we did not observe any treatment differences (Figure 5).



**Figure 5.** Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on learning performance in the Morris water maze test in adult female rats. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the Morris water maze test between days 60 and 90 after birth. The time elapsed (latency) to reach to the platform was recorded as an acquisition parameter. Data are means  $\pm$  S.E.M. of values from 9 rats per group (repeated-measures ANOVA).

Accordingly, during the probe trial, when the hidden platform was removed to evaluate spatial memory retention, neonatal  $\beta$ -estradiol 3-benzoate-treated rats did not show a better performance in their ability to locate the original platform location. In fact, although in neonatal  $\beta$ -estradiol 3-benzoate-treated rats there was a trend towards a lower latency to reach the area where the platform used to be, and  $\beta$ -estradiol 3-benzoate-treated rats spent more time in the platform quadrant, these differences did not reach

statistical significance. Moreover, there were no differences between the two experimental groups in the other parameters measured (Table 3).

**Table 3.** Effect of neonatal treatment with  $\beta$ -estradiol 3-benzoate on memory performance in the Morris water maze test in adult female rats.

<b>PROBE TRIAL</b>	<b>Control</b>	<b><math>\beta</math>-Estradiol 3-benzoate</b>
<b>Whole water maze parameters</b>		
Distance (m)	12.50 $\pm$ 0.59	13.09 $\pm$ 0.64
<b>Platform parameters</b>		
No. of entries	2.62 $\pm$ 0.67	2.29 $\pm$ 0.18
Latency (s)	21.17 $\pm$ 7.30	8.59 $\pm$ 1.56
<b>Platform quadrant parameters</b>		
No. of entries (m)	3.88 $\pm$ 0.48	4.14 $\pm$ 0.34
Time (s)	15.35 $\pm$ 2.00	21.73 $\pm$ 2.46 <sup>†</sup>
Latency (s)	5.82 $\pm$ 1.10	4.96 $\pm$ 0.59
<b>Target region parameters</b>		
Time (s)	7.99 $\pm$ 1.40	8.54 $\pm$ 1.00
Latency (s)	6.53 $\pm$ 1.12	6.21 $\pm$ 1.49
<b>Peripheral ring parameters</b>		
Time (s)	25.27 $\pm$ 0.80	27.33 $\pm$ 1.35

Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the Morris water maze between days 60 and 90 after birth. Parameters measured during the probe trial were recorded as storage capacity. Data are means  $\pm$  S.E.M. of values from 9 rats per group (t-test, <sup>†</sup> $p=0,06$  versus the respective control value).

These results demonstrate that during platform training, neonatal estradiol-treated rats showed similar learning abilities to those of control animals. Likewise, they did not show differences in the storage capacity and in the ability to retain information on the position of the platform, acquired during the training. Although numerous evidences have shown that allopregnanolone administration impairs spatial memory (Frye and Sturgis 1995; Joansson et al. 2002; Matthews et al. 2002), these results, in agreement with the previous behavioural data, suggest that the drastic and permanent reduction in the endogenous levels of allopregnanolone, induced by neonatal treatment with  $\beta$ -estradiol 3-benzoate, does not influence the cognitive behaviour of adult female rats.

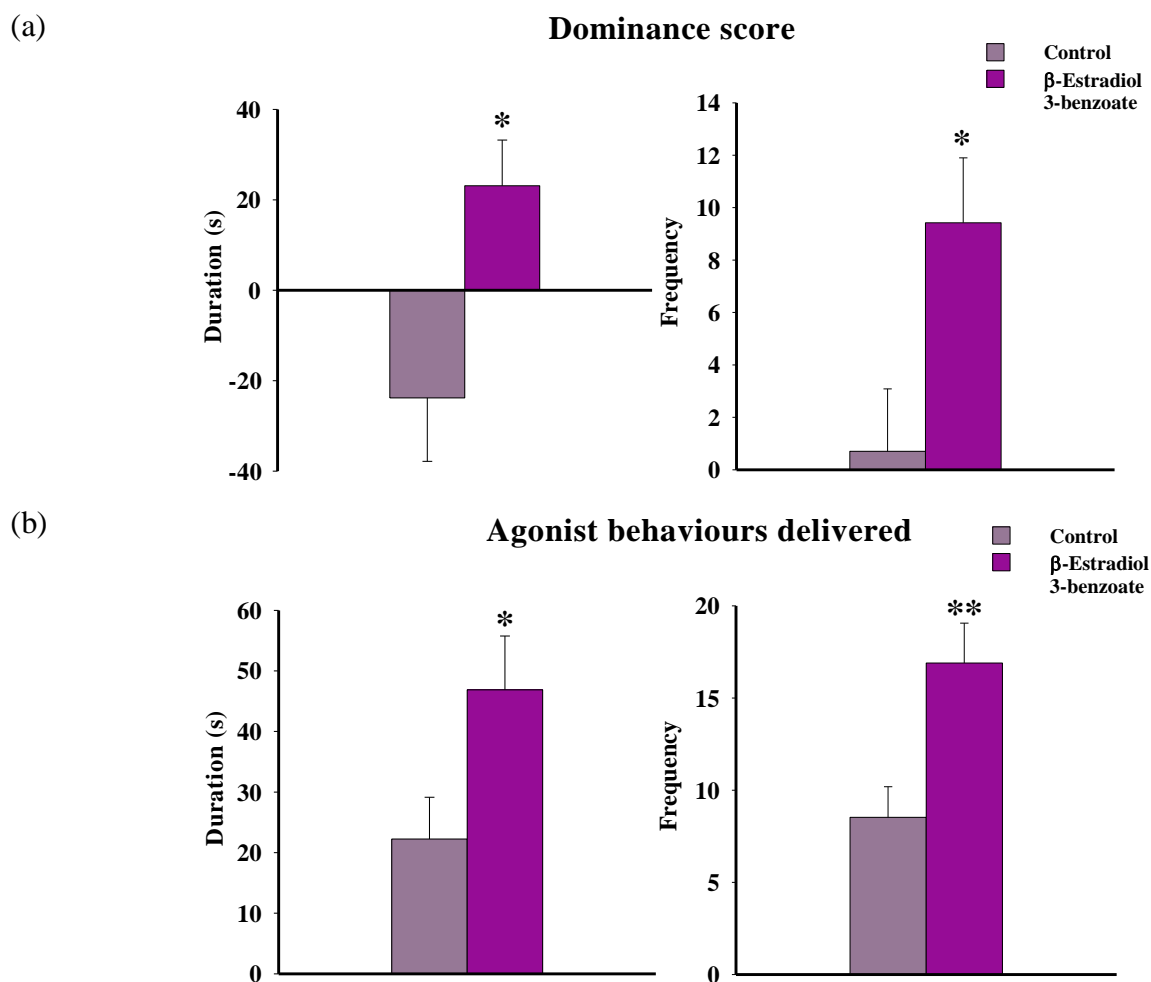
### **Effect of neonatal administration of $\beta$ -estradiol 3-benzoate in the resident-intruder test on adult female rats.**

It is well established that gonadal hormones mediate agonistic social interactions. In male rodents, castration radically reduces inter-male aggression and the administration of testosterone, the main male gonadal hormone, or one of its metabolites, estradiol or dihydrotestosterone, restores aggressive behaviours. Furthermore, male mice that cannot convert testosterone to estradiol show an estradiol-reversible reduction in aggression (Toda et al. 2001). Thus both androgen and estrogens appear to be involved in the mediation of aggressive behaviour in males. Moreover, a few studies in males have reported that the neurosteroid allopregnanolone is implicated in aggressive behaviour. Similar to benzodiazepines, its effect is biphasic, with low doses that increase aggression, while high doses are were sedative and likewise reduce aggressive behaviour (Miczek et al. 2003).

There has been much less research conducted on aggression or agonistic behaviour in females than male mice. Non reproduction-related agonistic behaviour in females has been shown to be qualitatively different from the typical intrasexual aggression studied in males, which is less ritualized and more violent (Svare and Gandelman 1973; Al-Maliki et al. 1980). In fact, virgin females may use other agonistic behaviours rather than attacks, such as chasing, pinning or aggressively grooming the intruder (Grant and Mackintosh 1963; Alleva 1993, Clipperton et al. 2008). These behaviours do not lead to the violent exclusion of the intruder from the territory, but rather are aimed at establishing dominance over the intruder. The study of female agonistic interactions in aggression tests therefore requires the use of a comprehensive analysis based on the rat's full behavioural repertoire and thus including more than just measures of attacks (Mos and Oliver 1989; Olivier et al. 1989; Miczek et al. 2001; Pietropaolo et al. 2004; Branchi et al. 2006).

In the present study, we used the resident-intruder test to investigate the effects of neonatal treatment with  $\beta$ -estradiol 3-benzoate on the response of adult female rats to an unfamiliar, gonadectomized conspecific intruder (Koolhaas et al. 1980). Neonatal administration of  $\beta$ -estradiol 3-benzoate did not affect the non-social behaviours of adult female rats. In fact, horizontal and vertical exploration, digging, solitary inactivity and

self-grooming were not altered (data not shown). In contrast, neonatal  $\beta$ -estradiol 3-benzoate treatment increased the dominance score (total agonistic behaviours delivered minus total agonistic behaviours received) with respect to control animals (duration: +197%  $p < 0.01$ ; frequency: +1235%  $p < 0.05$ , Figure 6a).



**Figure 6.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the behaviour of adult rats in the resident-intruder paradigm. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the resident-intruder paradigm for 10 min between days 60 and 90 after birth. Behaviours were analyzed from the time the intruder was introduced into the home cage. The duration and frequency of each behaviour were measured as: (a) duration and frequency of dominance score. (b) duration and frequency of agonistic behaviours delivered. Data are means  $\pm$  S.E.M. from 20 rats per group. \* $p < 0.01$ ; \*\* $p < 0.005$  versus the respective control value (t-test).

In fact, agonistic behaviours delivered (follow the intruder, dominant behaviours and attacks) increased (duration: +111%  $p < 0.05$ ; frequency: +98%  $p < 0.005$ ; Figure 6b) in adult female rats treated with  $\beta$ -estradiol 3-benzoate on the day of birth. In particular,

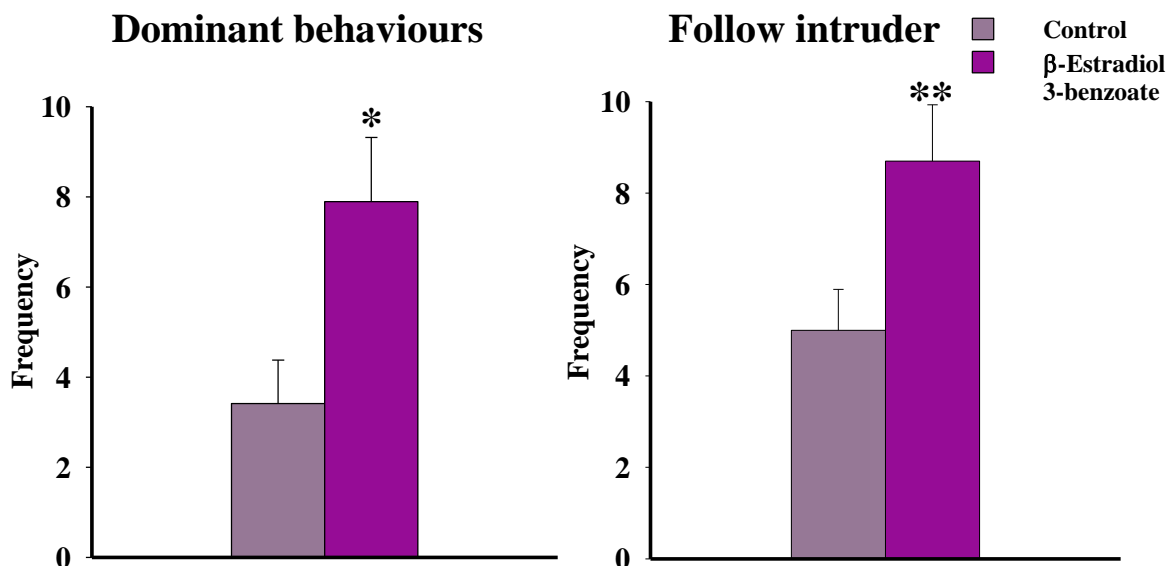
there was an effect of treatment on dominant behaviours (frequency: +131%  $p < 0.05$ , Figure 7; duration: +152%  $p < 0.05$ , Table 4) and on following the intruder (frequency:

**Table 4.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the behaviour of adult rats in the resident-intruder paradigm.

Behaviours	Control	$\beta$ -Estradiolo 3- benzoato	<i>p</i>
<b>Agonistic behaviours received</b>			
Avoidance			
Duration (s)	3.56 $\pm$ 1.14	3.74 $\pm$ 0.89	0,90
Frequency (n)	1,81 $\pm$ 0.59	2.00 $\pm$ 0.48	0,80
Submissive			
Duration (s)	42.00 $\pm$ 12.99	17,58 $\pm$ 5.52	0,08
Frequency (n)	4,63 $\pm$ 1.44	3,37 $\pm$ 0.99	0,47
Defensive posture			
Duration (s)	2,19 $\pm$ 0.73	2,84 $\pm$ 0.63	0,50
Frequency (n)	1,50 $\pm$ 0.55	2,11 $\pm$ 0.39	0,36
<b>Agonistic behaviours delivered</b>			
Follow intruder			
Duration (s)	3,38 $\pm$ 0.74	5,79 $\pm$ 0.98	0,07
Dominant behaviours			
Duration (s)	14,56 $\pm$ 5.29	36,63 $\pm$ 8.15	<b>0,04</b>
Attacks delivered			
Frequency (n)	0.00 $\pm$ 0.00	0,16 $\pm$ 0.12	0,22
<b>Social investigation</b>			
Oronasal investigation			
Duration (s)	23,81 $\pm$ 5.63	21,26 $\pm$ 2.94	0,68
Frequency (n)	10,31 $\pm$ 1,57	9,11 $\pm$ 1,13	0,53
Body investigation			
Duration (s)	84,94 $\pm$ 11.04	84,84 $\pm$ 8.24	0,99
Frequency (n)	27,12 $\pm$ 2,49	30,84 $\pm$ 2,25	0,28
Approach/Attend			
Duration (s)	1.50 $\pm$ 0.52	2.11 $\pm$ 0.72	0,51
Frequency (n)	1.81 $\pm$ 0.59	2.32 $\pm$ 0.52	0,53

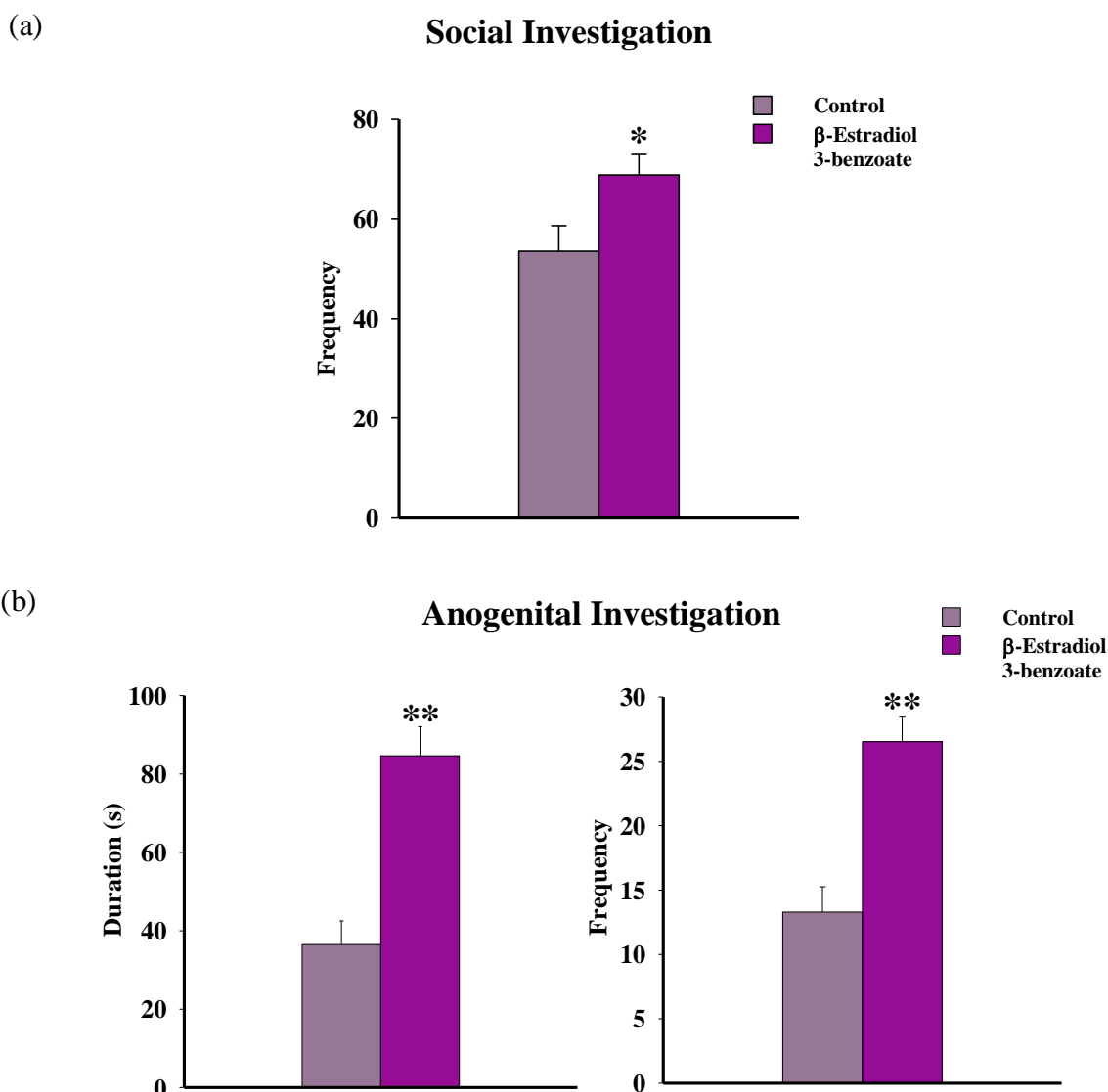
Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the resident-intruder paradigm for 10 min between days 60 and 90 after birth. Behaviours were analyzed from the time the intruder was introduced into the home cage. The duration and frequency of each behaviour were measured. Data are means  $\pm$  S.E.M. from 20 rats per group (t-test).

+77%  $p < 0.05$ , Figure 7), while attacks were not statistically different between groups (Table 4).



**Figure 7.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the behaviour of adult rats in the resident-intruder paradigm. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the resident-intruder paradigm for 10 min between days 60 and 90 after birth. Behaviours were analyzed from the time the intruder was introduced into the home cage. The duration and frequency of each behaviour were measured as: frequency of dominant behaviours and of following the intruder. Data are means  $\pm$  S.E.M. from 20 rats per group. \* $p < 0.01$ ; \*\* $p < 0.005$  versus the respective control value (t-test).

Therefore these results show that adult female rats, treated on the day of birth with estradiol, showed a dominant, but not aggressive, behaviour towards a conspecific intruder. Furthermore, neonatal estradiol-treated rats showed an increase in social investigation (oronasal investigation, body investigation, anogenital investigation, stretched approaches) (frequency: +29%  $p < 0.05$ , Figure 8a). In particular, there was an effect of treatment on the anogenital investigation (duration: +132%  $p < 0.0005$ ; frequency: +100%  $p < 0.0005$ ; Figure 8b), with no significant effects on the other behaviours (Table 4).



**Figure 8.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the behaviour of adult rats in the resident-intruder paradigm. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the resident-intruder paradigm for 10 min between days 60 and 90 after birth. Behaviours were analyzed from the time the intruder was introduced into the home cage. The duration and frequency of each behaviour were measured as: (a) frequency of social investigation (b) duration and frequency of anogenitale investigation. Data are means  $\pm$  S.E.M. from 20 rats per group. \* $p < 0.05$ ; \*\* $p < 0.0005$  versus the respective control value (t-test).

The increase in anogenital investigation observed in neonatal  $\beta$ -estradiol 3-benzoate-treated rats could be an index of sexual interest toward an intruder of the same sex. This behaviour could be attributed to the evidence that neonatal estradiol treatment induces a

masculinization of the brain as well as of sexual behaviour (Foecking et al. 2008). In fact, anogenital investigation is a typically male appetitive behaviour that serves to establish, maintain and promote sexual interaction. Accordingly, we also observed that neonatal  $\beta$ -estradiol 3-benzoate-treated rats showed numerous mounts with pelvic movements similar to the male intromission. It is known that females implemented this male-like behaviour for fun, but neonatal  $\beta$ -estradiol 3-benzoate-treated rats showed this behaviour in a persistent way. These results are in agreement with the evidence that pre- and post-natal treatment of female rats with testosterone showed a greater increase in the expression of male-like sexual behaviour (Pollak and Sachs 1975; Ward 1969). Moreover, females that *in utero* develop between two males show more male-like sexual behaviour during adulthood, compared to females located between two females, and this effect is blocked by prenatal treatment with an anti-androgen (Clemens et al. 1978). Therefore, these results further support the notion that treatment of females with exogenous estradiol or testosterone during development results in masculinization of adult brain and behaviour.

It has been shown that positive allosteric modulators of the GABA<sub>A</sub> receptor, such as allopregnanolone, can affect aggressive behaviour in a dose-dependent manner; high doses of these compounds diminish aggressiveness and induce sedation while moderate doses may increase aggressiveness (Miczek et al. 2003). Moreover, in the resident intruder test, female mice treated with progesterone show a lower aggressive behaviour towards the intruder, compared to animals treated with vehicle (Frye et al. 2006). This effect appears to be mediated by allopregnanolone, since knockout mice for the 5 $\alpha$ -reductase enzyme (the enzyme responsible for the conversion of progesterone into allopregnanolone) treated with progesterone or vehicle, show a similar aggressive behaviour towards the intruder, an event associated with the low levels of allopregnanolone present in these mice (Frye et al. 2006). The evidence that treatment of female rats with  $\beta$ -estradiol 3-benzoate on the day of birth induces a marked reduction in the brain and plasma levels of progesterone and allopregnanolone (Chapter 1), suggests that this steroids may play a role in the increased agonistic behaviour shown by  $\beta$ -estradiol 3-benzoate-treated rats.



## Chapter 3

### Neonatal exposure to $\beta$ -estradiol 3-benzoate enhances the pharmacological effects of diazepam in adult female rats.

#### 3.1 Introduction

GABA type A ( $\text{GABA}_A$ ) receptors with glycine receptors mediate fast inhibitory neurotransmission in the vertebrate CNS by gating  $\text{Cl}^-$  ions through an integral membrane channel.  $\text{GABA}_A$  receptors consist of five subunits surrounding a central channel selective for chloride ions in the plasma membrane, with the precise subunit composition determining the physiological and pharmacological properties of each receptor subtype (Olsen and Sieghart 2009).  $\text{GABA}_A$  receptors manifest a high level of structural heterogeneity, much greater than that of any other ligand-gated ion channel, with the five subunits belonging to various classes ( $\alpha_1$  to  $\alpha_6$ ,  $\beta_1$  to  $\beta_4$ ,  $\gamma_1$  to  $\gamma_3$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$ ,  $\rho_1$  to  $\rho_3$ ). This variety of subunits, each with a specific pattern of expression within the brain, suggests the existence of many subtypes of  $\text{GABA}_A$  receptor, each with different potential functions and sensitivities to various drugs.

Several different compounds act on the  $\text{GABA}_A$  receptor, e.g. agonists/antagonists for the GABA binding site and allosteric modulators such as benzodiazepines. For instance, diazepam is clinically used for its anxiolytic, sedative, hypnotic, anticonvulsant, and myorelaxant actions. Several studies have examined the contribution of the different  $\text{GABA}_A$  receptor subunits to the effects of benzodiazepines. Transgenic mouse-lines with mutations, postulated to alter individual amino acid residues in the benzodiazepine binding site, have revealed that receptors composed of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  subunits together with a  $\beta$  and  $\gamma$  subunit are sensitive to benzodiazepines, are located predominantly at synapses, and mediate most phasic inhibition in the brain. By contrast, most receptors composed of  $\alpha_4$  or  $\alpha_6$  subunits together with a  $\beta$  and  $\delta$  subunit are insensitive to benzodiazepines, are located extrasynaptically, and mediate tonic inhibition. Moreover, the differential pharmacology of diazepam is largely determined by variations in the expression of specific  $\alpha$  subunits (Mohler et al. 2006). For example, neurons expressing

$\alpha 1$  GABA<sub>A</sub> receptors have been found to mediate the sedative, amnesic and, at least in part, the anticonvulsant effects of diazepam, whereas those expressing  $\alpha 2$  GABA<sub>A</sub> receptors mediate anxiolysis (Mohler et al. 2006).

Long-term administration of sedative-hypnotic, anxiolytic, or anticonvulsant drugs or of certain drugs of abuse results in marked changes in the expression of specific GABA<sub>A</sub> receptor subunits that lead to the assembly of receptors with different subunit compositions and, consequently, different drug sensitivities. Neuroactive steroids that are active at GABA<sub>A</sub> receptors also affect GABA<sub>A</sub> receptor gene expression and activity in various regions of the brain in rats (Concas et al. 1998; Biggio et al. 2007; Maguire and Mody 2007; Smith et al. 2007). In particular, the progesterone metabolite allopregnanolone induces pharmacological effects similar to those elicited by classical positive allosteric modulators such as benzodiazepines (Majewska 1992). Given that allopregnanolone is produced, both in the periphery and in the brain, from endogenous progesterone (Mellon and Griffin 2002), physiological or pharmacologically induced fluctuations in the concentrations of this gonadal steroid are paralleled by changes in the synaptic concentration of allopregnanolone, which contribute to the regulation of GABA<sub>A</sub> receptor plasticity. As GABA<sub>A</sub> receptors are implicated in a variety of neuropsychophysiological phenomena, including anxiety, sleep, seizures, and depression, such fluctuations in the concentrations of neuroactive steroids may contribute to the cognitive and psychiatric manifestations of conditions characterized by marked changes in the hormonal milieu. Pregnancy, delivery, the estrous cycle, and inhibition of gonadal function are thus all associated with pronounced changes in the expression of specific GABA<sub>A</sub> receptor subunits in various regions of the brain and with consequent changes in receptor function (Concas et al. 1998; Follesa et al. 1998; Griffiths and Lovick 2005; Maguire and Mody 2007, 2008; Sanna et al. 2009).

We have recently demonstrated that neonatal administration of  $\beta$ -estradiol 3-benzoate induced changes in the expression of specific GABA<sub>A</sub> receptor subunits in the cerebral cortex of adult female rats (Calza et al. 2010). In particular, immunoblot analysis revealed increases in the abundance of  $\alpha 1$ ,  $\alpha 2$ , and  $\gamma 2$  subunits, whereas the amounts of  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ , and  $\delta$  subunits were not affected (Calza et al. 2010).

Given that the presence of an  $\alpha$  subunit, a  $\beta$  subunit, together with the  $\gamma 2$  subunit is required for sensitivity of GABA<sub>A</sub> receptors to benzodiazepines (Pritchett et al. 1989), these molecular changes might be expected to affect the pharmacology of GABA<sub>A</sub> receptor-mediated neurotransmission.

Therefore, we investigated whether the behavioural effects of diazepam in adulthood might be affected by neonatal administration of  $\beta$ -estradiol 3-benzoate.

## 3.2 Materials and methods

### Animals

Animal care and handling throughout the experimental procedures and neonatal treatment with  $\beta$ -estradiol 3-benzoate were performed as described in chapter 1. The animals were subjected to behavioural tests between days 60 and 90 after birth. To avoid effects of estrous cycle status, control animals were tested in randomized phases of the estrous cycle.

### Drug treatment

Diazepam was dissolved with one drop of Tween 80 in distilled water and was injected intraperitoneally (i.p.) at a dose of 0.5, 1, 2 or 6 mg per kilogram of body weight in a volume of 3 ml/kg. Control animals received the same volume of vehicle. Pentylentetrazole (PTZ) was dissolved and administered as described in chapter 2.

### Elevated plus-maze test

The plus-maze was conducted as described in the materials and methods section of chapter 2. Rats were tested in a randomized order between 09.00 and 14.00 hours beginning 30 min after the administration of diazepam.

### Locomotor activity test

Locomotor activity of rats was assessed as described in chapter 2. Locomotor activity was recorded during the light cycle, between 09.00 and 14.00 hours beginning 15 min after the administration of diazepam .

### PTZ-induced seizures

The anticonvulsant activity of diazepam (1 to 6 mg/kg) was tested against PTZ-induced seizures (Depoortere et al. 1986), as described in chapter 2. Diazepam was administered intraperitoneally 30 min before PTZ.

**Morris water maze**

Spatial learning was determined by the Morris water maze test as described in chapter 2. To assess the amnesic effect of diazepam, rats were injected with diazepam (2 mg/kg, i.p.) or vehicle over four consecutive days, 20 minutes before the each training trial (Savić et.al. 2009). A drug-free probe trial (McNamara & Skelton 1993) was chosen because diazepam impairs acquisition, but not retrieval of escape platform in the water maze (Anand et al. 2007; McNamara & Skelton 1991).

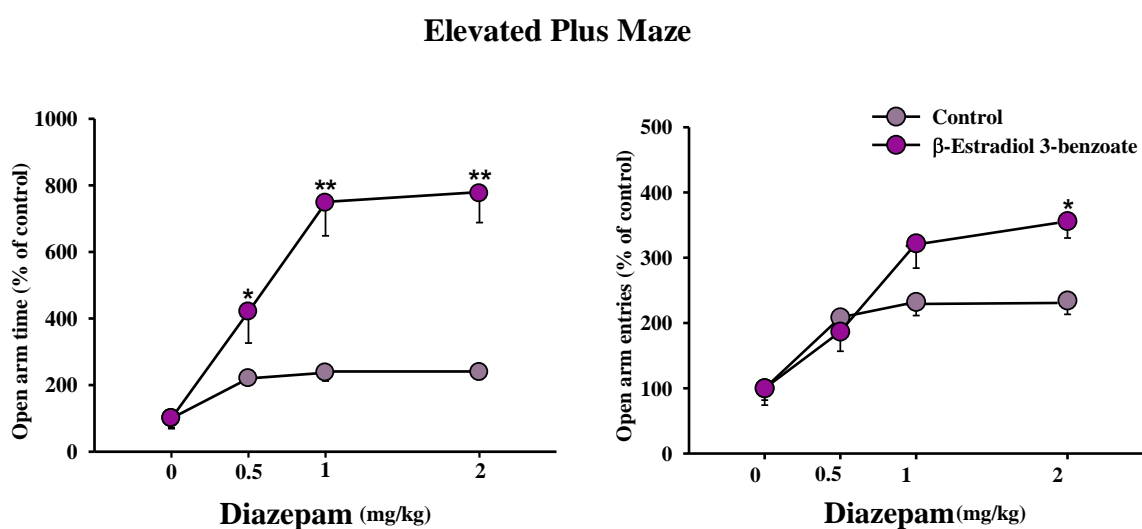
**Statistical analysis**

Data are presented as means  $\pm$  S.E.M. The statistical significance of differences was assessed by one- or two-way ANOVA and repeated-measure ANOVA with post hoc analysis by Newman-Keuls test. A *P* value of  $<0.05$  was considered statistically significant.

### 3.3 Results and discussion

#### Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on the anxiolytic and sedative actions of diazepam in adult female rats.

As previously described (Chapter 2), neonatal administration of  $\beta$ -estradiol 3-benzoate did not affect the behaviour of adult female rats in the elevated plus-maze test. In contrast, neonatal administration of  $\beta$ -estradiol 3-benzoate increased the sensitivity of rats to the effects of diazepam in this test (Figure 1).



**Figure 1.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the anxiolytic-like action of diazepam in female rats subjected to the elevated plus-maze test. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were subjected to the elevated plus-maze test for 5 minutes between days 60 and 90 after birth. Diazepam (0.5 to 2 mg/kg) or vehicle were administered i.p., 30 minutes before the test. The time spent in the open arms and the number of entries into the open arms of the maze are expressed as a percentage of the corresponding value for control animals and are means  $\pm$  S.E.M. of values from 10 rats per group (Two-way ANOVA followed by Newman-Keuls test).

Consistent with previous observations (Bitran et al. 1991), diazepam (0.5 to 2 mg/kg, i.p.) induced an anxiolytic-like action in control rats subjected to the elevated plus-maze test. This anxiolytic effect was more pronounced in rats that had been neonatally treated with  $\beta$ -estradiol 3-benzoate. The percent increase in the time spent in the open arms and in the number of entries into the open arms, following diazepam administration, were thus greater in  $\beta$ -estradiol 3-benzoate-treated rats than in control animals (at the diazepam

dose of 2 mg/kg: open-arm time, +675 versus +139%, respectively,  $p < 0.001$ ; open-arm entries, +256% versus 134%, respectively,  $p < 0.01$ ).

Moreover, administration of diazepam (6 mg/kg, i.p.) resulted in a significant decrease in the total distance travelled and in the number of movements relative to those apparent for vehicle-treated animals in both control and  $\beta$ -estradiol 3-benzoate-treated rats (Figure 2). However, in rats neonatally treated with  $\beta$ -estradiol 3-benzoate, diazepam induced a more pronounced effect on the total distance travelled compared to controls (-99% vs -70%; respectively,  $p < 0.05$ ) and on the number of movements (-96% vs -64%, respectively,  $p < 0.05$ ). As previously shown (Chapter 2), neonatal administration of  $\beta$ -estradiol 3-benzoate did not affect the total distance travelled and the total number of movements of adult female rats.

These results clearly demonstrate that diazepam induced a more pronounced anxiolytic-like effect in the elevated plus-maze test and a greater reduction in locomotor activity in rats subjected to neonatal administration of  $\beta$ -estradiol 3-benzoate compared to the vehicle-treated controls. A similar increased sensitivity to the anxiolytic effect of diazepam was previously observed in female rats subjected to neonatal androgen treatment (Fernandez-Guasti and Picazo 1997).

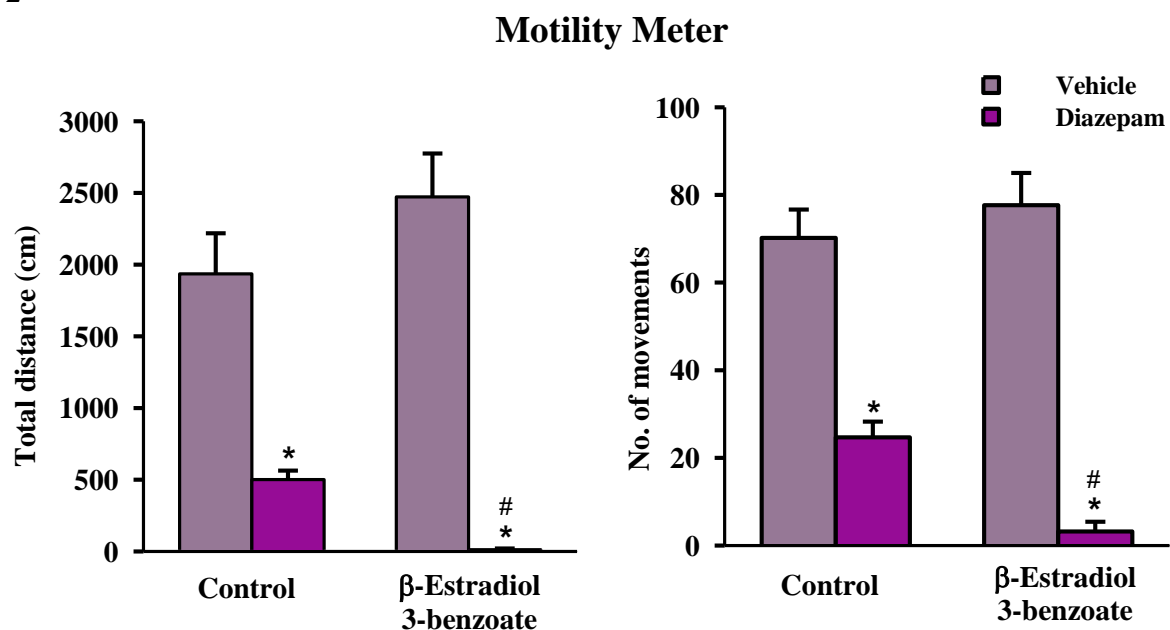
Given that the sedative effect of benzodiazepines requires the  $\alpha 1$  subunit of the GABA<sub>A</sub> receptor (Rudolph et al. 1999) whereas the  $\alpha 2$  subunit appears to be a major determinant of the anxiolytic effect of these drugs (Low et al. 2000), the enhancement of the locomotion-suppressing and anxiolytic effects of diazepam observed in rats subjected to neonatal administration of  $\beta$ -estradiol 3-benzoate may be attributable to the increased expression of the  $\alpha 1$  and  $\alpha 2$  subunits, respectively, in the cerebral cortex.

Expression of the  $\gamma 2$  subunit also appears to be a determinant of the action of benzodiazepines at GABA<sub>A</sub> receptors, given that the binding site for these compounds is located at the interface between  $\alpha$  and  $\gamma 2$  subunits (Olsen and Sieghart 2009). Diazepam thus fails to induce sedation or loss of the righting reflex in mice with targeted disruption of the  $\gamma 2$  subunit gene (Gunther et al. 1995). In addition, transgenic mice that express reduced levels of the  $\gamma 2$  subunit exhibit enhanced anxiety-like behaviour in the elevated plus-maze and forced novel exploratory tests (Crestani et al. 1999). Our observations thus

suggest that the increased expression of  $\alpha 1$ ,  $\alpha 2$ , and  $\gamma 2$  subunits in the brain of adult female rats subjected to neonatal administration of  $\beta$ -estradiol 3-benzoate results in an increased assembly of GABA<sub>A</sub> receptors, containing these specific subunits, which therefore exhibit an increased sensitivity to benzodiazepines.

This greater sensitivity to the anxiolytic effect of diazepam in rats subjected to neonatal administration of  $\beta$ -estradiol 3-benzoate does not seem to be in relation with the  $\alpha 3$  subunit. In fact, immunoblot analysis revealed that neonatal exposure to  $\beta$ -estradiol 3-benzoate did not affect the cerebrocortical abundance of this subunit of the GABA<sub>A</sub> receptors (Calza et al. 2010). Indeed, our result is in agreement with experimental evidence from the literature showing that GABA<sub>A</sub> receptors containing the  $\alpha 3$  subunit mediate only in part the anxiolytic effect of diazepam (Löw et al. 2000; Crestani et al.

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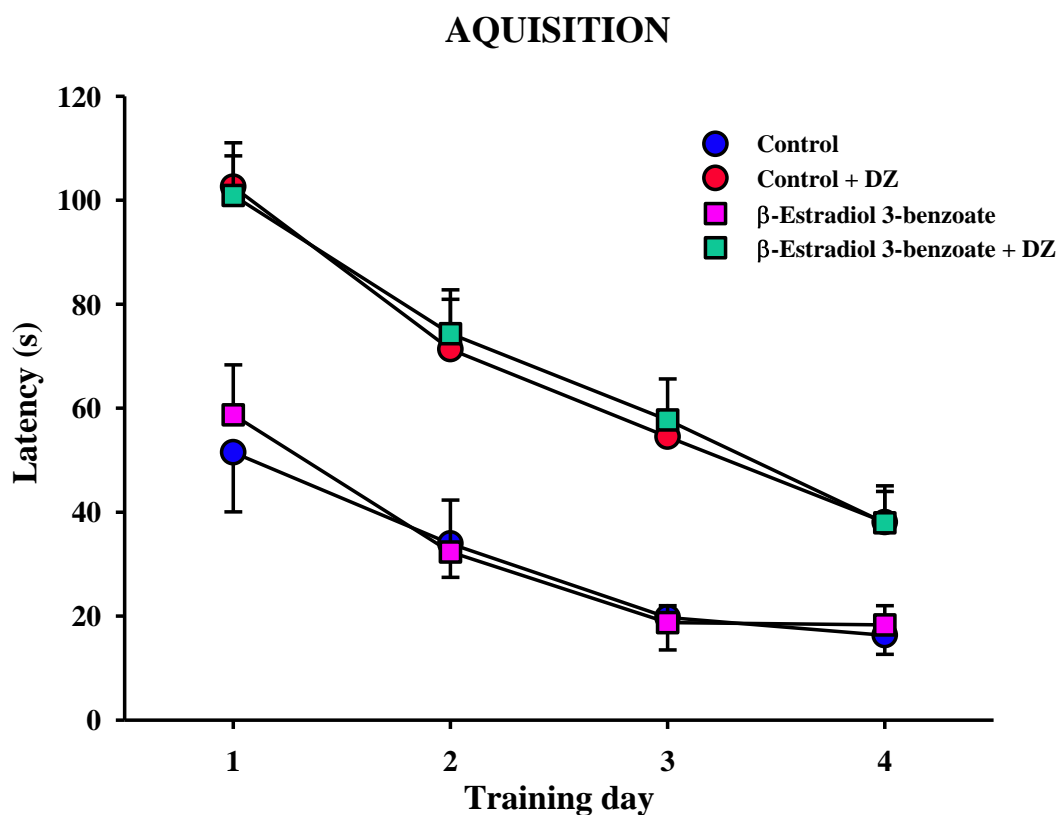


**Figure 2.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on locomotor activity in female rats subjected to the motility meter test. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested for spontaneous locomotor activity for 10 min between days 60 and 90 after birth. Diazepam (6 mg/kg, i.p.) or vehicle was administered 15 min before the test. The number of movements and the total distance travelled were determined. Data are means  $\pm$  S.E.M. from 10 rats per group. \* $p$ <0.001 versus the corresponding vehicle-treated group; # $p$ <0.05 versus control rats injected with diazepam (Two-way ANOVA followed by Newman-Keuls test).



### Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on the amnesic effect of diazepam.

Benzodiazepines have been repeatedly found to impair memory acquisition; in fact, they cause anterograde amnesia. Amnesic effects in humans were first recognized by anesthesiologists using benzodiazepines as pre-medication (Brandt & Oakes 1965; Haslett & Dundee 1968). Amnesia induced by benzodiazepines is also reported in animal studies (Hinrichs et al. 1984).



**Figure 3.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the amnesic action of diazepam in female rats subjected to the Morris water maze test. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested in the Morris water maze test between days 60 and 90 after birth. Diazepam (2 mg/kg, i.p.) was administered each training day 20 minutes before each trial. The time elapsed (latency) to reach to the platform was recorded as an acquisition parameter and is expressed in seconds. Data are means  $\pm$ S.E.M. of 10 animals per group. \* $p$ <0.05; \*\* $p$ <0.005 versus the respective control value (Two-way repeated measures ANOVA, followed by Newman-Keuls test).

We thus evaluated whether there was a different sensitivity to the amnesic effect of diazepam, in rats subjected to neonatal administration of  $\beta$ -estradiol 3-benzoate. As expected, diazepam (2 mg/kg), administered 20 minutes before training over four consecutive days, altered the acquisition process as shown by the increased latency to find the hidden platform in both control and neonatal  $\beta$ -estradiol 3-benzoate-treated rats (Figure 4). However, we did not find any differences between these two groups.

In the probe trial, performed without any pre-treatment because diazepam impairs acquisition but not retrieval of spatial information in the water maze, animals treated with diazepam during the training showed a decreased number of entries in the area where the platform used to be during training; none of the other parameters was significantly modified (Table 1). In contrast, neonatal administration of  $\beta$ -estradiol 3-benzoate increased the sensitivity of rats to the amnesic effects of diazepam, as shown by a significant increase in the latency to reach the area where the platform used to be during training (+444%,  $p < 0.01$ ), the latency to reach the platform (east) quadrant (+182%,  $p < 0.01$ ) and the latency to reach the target region (+339%,  $p < 0.01$ ). Moreover, the administration of diazepam during training decreased the time spent in the platform (east) quadrant (-43%,  $p < 0.05$ ) and in the target region (-67%,  $p < 0.01$ ) as well as the number of entries in the area where the platform used to be during training (-81%,  $p < 0.01$ ).

These results demonstrate that neonatal  $\beta$ -estradiol 3-benzoate-treated rats showed a greater sensitivity to the amnesic effects of diazepam, compared to the neonatal vehicle-treated controls. In fact, upon learning the position of the platform just after diazepam administration during the training days, recall of the platform position in the subsequent probe test was strongly impaired in neonatal  $\beta$ -estradiol 3-benzoate-treated than in vehicle-treated control animals. The difference in anterograde impairment, apparent in the Morris water maze test, between  $\beta$ -estradiol 3-benzoate-treated and vehicle-treated control animals was not linked to a change in overall locomotor activity induced by diazepam at the dose of 2 mg/kg, given that the distance travelled through the whole water maze did not significantly differ between the two groups.

Behavioural studies with subtype selective ligands for benzodiazepine receptors (Savić et al. 2005a, b, 2008b) and genetically modified animals (Collinson et al. 2002; Crestani et

al. 2002; Rudolph et al. 1999) have indicated that the  $\alpha 1$  subunit appears to be involved in the molecular mechanisms responsible for the induction of anterograde amnesia by diazepam (Rudolph and Mohler 1999). In fact, in mice that do not express a diazepam-sensitive  $\alpha 1$  subunit, the memory-impairing effect of diazepam was strongly reduced. Accordingly, our results suggest that the greater diazepam-induced amnesia observed in neonatal  $\beta$ -estradiol 3-benzoate-treated rats is mediated by the increase in  $\alpha 1$  subunit expression found in these animals.

**Table 1.** *Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate (EB) on the behaviour of adult female rats in the Morris water maze test: different sensitivity to diazepam.*

<b>PROBE TRIAL</b>	<b>CO</b>	<b>CO+DZ</b>	<b>EB</b>	<b>EB+DZ</b>
Whole water maze parameters				
<b>Distance(m)</b>	12.50 $\pm$ 0.59	15.11 $\pm$ 1.19	13.09 $\pm$ 0.64	13.28 $\pm$ 0.88
Platform parameters				
<b>No. of entries</b>	2.62 $\pm$ 0.67	<b>0.83 <math>\pm</math> 0.17#</b>	2.29 $\pm$ 0.18	<b>0.43 <math>\pm</math> 0.30**</b>
<b>Latency(s)</b>	21.17 $\pm$ 7.30	31.17 $\pm$ 6.20	<b>8.59 <math>\pm</math> 1.56#</b>	<b>46.70 <math>\pm</math> 8.77**</b>
Platform quadrant(est) parameters				
<b>No. of entries(m)</b>	3.88 $\pm$ 0.48	4.67 $\pm$ 0.42	4.14 $\pm$ 0.34	3.57 $\pm$ 0.69
<b>Time(s)</b>	15.35 $\pm$ 2.00	12.68 $\pm$ 1.16	21.73 $\pm$ 2.46+	<b>12.41 <math>\pm</math> 1.67*</b>
<b>Latency(s)</b>	5.82 $\pm$ 1.10	7.43 $\pm$ 1.74	4.96 $\pm$ 0.59	<b>13.99 <math>\pm</math> 4.02**</b>
Target region parameters				
<b>Time(s)</b>	7.99 $\pm$ 1.40	4.44 $\pm$ 1.31	8.54 $\pm$ 1.00	<b>2.78 <math>\pm</math> 1.33**</b>
<b>Latency(s)</b>	6.53 $\pm$ 1.12	17.25 $\pm$ 8.71	6.21 $\pm$ 1.49	<b>27.26 <math>\pm</math> 7.14**</b>
Peripheral ring parameters				
<b>Time(s)</b>	25.27 $\pm$ 0.80	30.73 $\pm$ 5.68	27.33 $\pm$ 1.35	33.78 $\pm$ 6.07

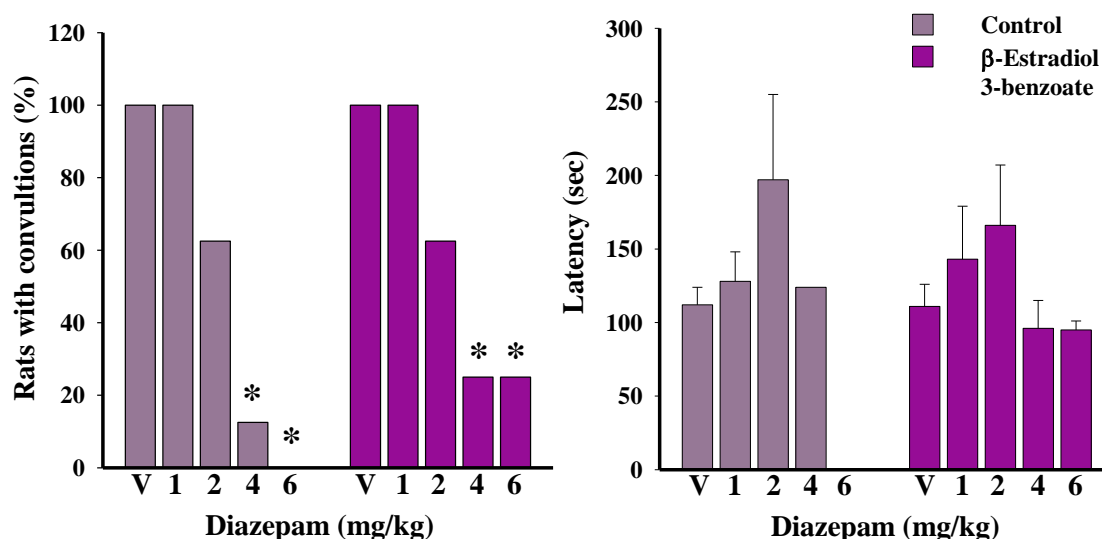
Data represent probe trial parameters and are means  $\pm$  S.E.M. of 10 animals per group. CO: control; DZ: diazepam. # $p$ <0.01 vs CO; \* $p$ <0.05, \*\* $p$ <0.01 vs EB (Two-way ANOVA, followed by Newman-Keuls test).

### **Effect of neonatal administration of $\beta$ -estradiol 3-benzoate on the anticonvulsant action of diazepam.**

Diazepam is a conventional antiepileptic drug, being a very active compound against seizures induced by convulsant agents impairing the GABAergic neurotransmission, such as PTZ, a drug that induces tonic convulsions in rats (Crestani et al. 2001).

We thus investigated the anticonvulsant activity of diazepam against PTZ-induced seizures in control and in  $\beta$ -estradiol 3-benzoate-treated rats (Figure 3). Injection of diazepam (1-6 mg/kg), 30 minutes before administration of PTZ (x mg/kg), decreased the percentage of animals showing seizures in a dose-dependent manner in both control and neonatal  $\beta$ -estradiol 3-benzoate-treated rats. The same treatment failed to affect the latency of convulsions. At the most efficacious doses tested (4 and 6 mg/kg), diazepam decreased the number of animals showing convulsions to a similar extent in both control and neonatal  $\beta$ -estradiol 3-benzoate-treated rats (Figure 3).

### PTZ-seizures



**Figure 4.** Effect of neonatal administration of  $\beta$ -estradiol 3-benzoate on the behaviour of rats in the PTZ-seizures tests. Rats were injected with  $\beta$ -estradiol 3-benzoate (10  $\mu$ g, s.c.) or vehicle (Control) on the day of birth and were tested for PTZ-induced seizures between days 60 and 90 after birth. Diazepam (1-6 mg/kg, i.p.) or vehicle were administered 30 minutes before PTZ (x mg/kg, i.p.). Animals were observed for 30 minutes; the latency to the appearance of seizures and the number of rats showing convulsions were recorded. Data are means  $\pm$  S.E.M. of values from 8 rats per group. \* $p < 0.05$  versus the respective control value (Two-way ANOVA followed by Newman-Keuls test).

Therefore, neonatal administration of  $\beta$ -estradiol 3-benzoate did not affect sensitivity to the anticonvulsant action of diazepam in adulthood. The evidence that the anticonvulsant effect of diazepam is similar in both experimental groups seems not to be related to the increased expression of the  $\alpha 1$  subunit, observed in neonatal  $\beta$ -estradiol 3-benzoate-

treated rats. In fact, the anticonvulsant activity of diazepam, assessed by its protection against PTZ-induced tonic convulsions, was reduced in mice that do not express a diazepam-sensitive  $\alpha 1$  subunit (Rudolph and Möhler 2004). The anticonvulsant effect of diazepam, which persisted in these mice, was due to GABA<sub>A</sub> receptors other than  $\alpha 1$  subunit type, since it was antagonized by flumazenil, suggesting that the anticonvulsant activity of benzodiazepines is partially but not fully mediated by receptors containing the  $\alpha 1$  subunit (Rudolph and Möhler 2004). Accordingly, the expression of other subunits ( $\alpha 3$ ,  $\alpha 4$ , and  $\alpha 5$ ) was not affected by neonatal treatment with  $\beta$ -estradiol 3-benzoate.

Overall, the results of this study demonstrate that neonatal administration of  $\beta$ -estradiol 3-benzoate induced an alteration in the behavioural response to diazepam in adulthood. Specifically, these animals showed a greater sensitivity to the anxiolytic, sedative and amnesic effects of diazepam but not to its anticonvulsant effect. Given that these effects of benzodiazepines seem to be mediated by specific GABA<sub>A</sub>  $\alpha$  and  $\gamma$  subunits, our previous observation that neonatal treatment with  $\beta$ -estradiol 3-benzoate increases the expression of  $\alpha 1$ ,  $\alpha 2$  and  $\gamma 2$  subunits in the cerebral cortex might imply that this treatment is capable to increase, in the adult brain, the expression of assembled cortical GABA<sub>A</sub> receptors containing these specific subunits, which exhibit a greater sensitivity to benzodiazepines. Moreover, these results further demonstrate that estradiol plays a major role in the modulation of GABAergic transmission during development.

## Summary and Conclusions

Circulating steroid hormones synthesized either from peripheral endocrine glands or from the brain can directly regulate brain function and modulate behaviour. Gonadal steroid hormones, especially during ontogeny, cause many sex differences that are not manifested behaviourally until sexual maturity is attained. In summary, we observed that the single administration of a very low dose of  $\beta$ -estradiol 3-benzoate on the day of birth might represent a useful experimental model to further investigate the physiological role of steroid hormones in the modulation of adult behaviour. Thus, female rats treated with  $\beta$ -estradiol 3-benzoate showed a delay in vaginal opening, acyclicity characterized by prolonged estrus, and ovarian failure. Moreover, this treatment abolished sexual receptivity in adulthood, even following estrogen and progesterone replacement after ovariectomy. Most importantly, these estrogenized animals showed a dysregulation of the hypothalamic-pituitary-gonadal axis that triggered a pronounced decrease in the cerebrocortical and brain concentrations of progesterone and its neuroactive metabolite allopregnanolone, that were apparent in both juvenile and adult animals. In contrast, this treatment did not affect  $17\beta$ -estradiol levels. To our knowledge, this is the first evidence that perinatal exposure to estrogen in female rats induced a selective and long lasting alteration in the plasma and, most important, in the brain concentrations of progesterone and allopregnanolone.

Although allopregnanolone seems to be involved in the regulation of a variety of psychophysiological phenomena, including anxiety, depression, sleep, cognitive function, seizures, social and sexual behaviour, the drastic and persistent reduction in the brain and peripheral concentrations of this neuroactive steroid failed to affect several behaviours measured in adult female rats neonatally treated with  $\beta$ -estradiol 3-benzoate. In fact, this treatment did not affect locomotor activity, anxiety- and mood-related behaviours, seizures sensitivity and spatial memory. In contrast, neonatal  $\beta$ -estradiol 3-benzoate-treated rats showed a dominant, but not aggressive, behaviour and an increase in body investigation, especially anogenital investigation, characteristic of a male appetitive behaviour. Given that the decrease in allopregnanolone concentration induced by neonatal  $\beta$ -estradiol 3-benzoate treatment appeared early (3 weeks) in development, it is

possible that estrogenized female rats have developed an adaptive mechanism to counteract the persistent decrease in brain levels of this neuroactive steroid. Accordingly, we have previously shown that neonatal  $\beta$ -estradiol 3-benzoate treatment did not affect the anxiolytic-like action of allopregnanolone in the elevated plus-maze (Calza et al. 2010). Moreover, neonatally androgenized or ovariectomized female rats were previously shown to be insensitive to the anxiolytic action of allopregnanolone in the elevated plus-maze test (Fernandez-Guasti and Picazo 1999; Laconi et al. 2001). The lack of sensitivity to the behavioural effect of allopregnanolone might be explained by the failure of  $\beta$ -estradiol 3-benzoate treatment to affect expression of the  $\alpha 4$  and  $\delta$  subunits of the GABA<sub>A</sub> receptor (Calza et al. 2010). Moreover, we have found a decrease in the expression of  $\delta$  subunit in the hippocampus of neonatal  $\beta$ -estradiol 3-benzoate-treated rats (Concas et al. personal communication). Receptors that contain these subunits are located at extrasynaptic sites in various brain regions, mediate tonic inhibition of neuronal activity, and are highly sensitive to modulation by allopregnanolone but not by benzodiazepines (Smith et al. 2007). Ablation of the  $\delta$  subunit influences the behavioural profile of neuroactive steroids in mice, with the anxiolytic-like effect in the elevated plus-maze test induced by allopregnanolone or ganaxolone being reduced in animals lacking the  $\delta$  subunit (Mihalek et al. 1999).

Moreover, neonatal administration of  $\beta$ -estradiol 3-benzoate to female rats increases sensitivity to the anxiolytic, sedative, and amnesic effects of diazepam in adulthood. Given that these effects seem to be mediated by specific GABA<sub>A</sub> receptor subunits ( $\alpha 1$ ,  $\alpha 2$  and  $\gamma 2$ ), the greater sensitivity of neonatally  $\beta$ -estradiol 3-benzoate-treated females to diazepam could be related to the changes in the expression of these subunits observed in the brain of these animals (Calza et al. 2010). Furthermore, the evidence that neonatal administration of  $\beta$ -estradiol 3-benzoate did not modify the sensitivity to the anticonvulsant action of diazepam indicates that this treatment does not alter the rate of diazepam metabolism. Given that the  $\alpha 1$  subunit only partially mediates the anticonvulsant effect of benzodiazepines, other mechanisms may underlie the sensitivity of neonatal  $\beta$ -estradiol 3-benzoate-treated rats to the anticonvulsant effect of diazepam.

Overall, these results indicate that the marked and persistent reduction in the cerebrocortical and peripheral concentration of the neuroactive steroid allopregnanolone induced by neonatal treatment with  $\beta$ -estradiol 3-benzoate does not change baseline behaviours in adult rats. On the contrary, the low levels of allopregnanolone seems to be associated to changes in the behavioural sensitivity to the positive allosteric modulator of the GABA<sub>A</sub> receptor, diazepam. The differences in the behavioural efficacy of diazepam may be due to the increased expression of specific GABA<sub>A</sub> receptor subunits, induced by neonatal  $\beta$ -estradiol 3-benzoate treatment, which therefore, may lead to the formation of receptor' subtypes that show a greater sensitivity to diazepam. Given that allopregnanolone exposure alters GABA<sub>A</sub> receptor subunit expression, the changes in allopregnanolone concentrations induced by neonatal  $\beta$ -estradiol 3-benzoate treatment might have implications for the structure and function of GABA<sub>A</sub> receptors during development. Indeed, perinatal administration of allopregnanolone was found to participate in the normal development of GABAergic neurotransmission by altering the GABA<sub>A</sub> receptor-mediated Cl<sup>-</sup> influx (Grobin and Morrow, 2001) and by influencing the localization of GABAergic interneurons in the prefrontal cortex of adult rats (Grobin et al. 2003). Allopregnanolone levels in the cerebral cortex of rats have previously been shown to undergo dynamic changes during development, with maximal concentrations apparent around the second postnatal week (Grobin and Morrow 2001). In the present study, we found that neonatal administration of  $\beta$ -estradiol 3-benzoate induced a marked decrease in allopregnanolone concentrations in the cerebral cortex of rats at 21 days of age. Such treatment might thus prevent the surge in allopregnanolone levels during the first weeks of life and thereby affect the developmental plasticity of GABA<sub>A</sub> receptors and consequently the pharmacology of these receptors.

In conclusion, female rats neonatally treated with  $\beta$ -estradiol 3-benzoate may represent a potential animal model in which to study the effects of estrogen on reproductive function and on the reproductive neuroendocrine system, and to examine the role of gonadal steroids in behaviour. These results further demonstrate that estradiol plays an important role in the modulation of GABAergic transmission during development of the central nervous system.



Moreover, this animal model could be useful to study one of the most common endocrine disorders in women such as the polycystic ovary syndrome (PCOS). Evidence from both clinical and animal studies suggests that PCOS has a developmental origin, in which androgen excess during fetal or prepubertal life can reprogram multiple tissues to manifest the syndrome in adolescence and adulthood (Abbott et al. 2005; Xita and Tsatsoulis 2006). This disorder is often accompanied by anovulation, amenorrhea and polycystic ovaries of infertility in women. In female rats, neonatal estrogenization induces some of these characteristics, such as poly-follicular ovarian histology, ovarian failure and a lack of estrous cyclicity.

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