DEVELOPMENTAL EXPOSURE TO ENDOCRINE DISRUPTERS: NEUROBEHAVIORAL EFFECTS IN AN ANIMAL MODEL

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To my parents
and sister
with love

1. INTRODUCTION

1.1 PREMISE

An area of research relatively new in the field of echotoxycology is the study of the effects of manmade substances, such as chemicals of industrial production, on the endocrine system of animals and humans. These compounds, also classified as "endocrine disrupting compounds" (EDCs), can alter the normal functions of the endocrine system by acting as agonists or antagonists or by simply interfering with the hormonal action. Furthermore, they may alter the levels of plasma proteins that bind both the hormones and, in target tissues, their receptors (Soto et al.,1995; Nagel et al., 1997).

It has been shown that EDCs bind to steroid hormone receptors, especially estrogen receptors, *in vitro* and *in vivo*. Although estrogen is a fundamental hormone for the female reproductive system, it plays an important role during normal fetal development and male reproductive organ function (Korach, 1994; vom Saal et al., 1997). Small perturbations of the hormonal milieu during the perinatal period can cause developmental alterations that in adulthood may result in phenotypic variation. EDCs can also interact at the DNA level and result in mutation or epigenetic mechanisms, such as the insertion of methyl groups on the histones. An organism exposed to EDCs is unable to detect any abnormal event and therefore unable to respond homeostatically. For example, when an estrogenic EDC, such as bisphenol A (BPA), binds to the estrogen receptors, there is an alteration of the normal fetal development.

Toxicological studies have always been based on the morphological effects of the exposure to unusually high and thus toxic doses of chemical compounds. These studies analyze higher doses than those present in the environment. During the last ten years, scientists shifted their attention to the effects of low, environmentally relevant doses of EDCs on behavior and neuro-physiology. In this context, behavioral study is a necessary step for understanding the effects of EDCs during ontogenesis since behavior is the result of a complex interaction between different systems, such as the neuroendocrine and the sensory-motor. Behavioral alterations could be used as indicators of the perturbations occurring during ontogenesis that are sometimes observable only during adulthood or precise and determined stages of development. This method takes the name of "ethotoxicology", a

discipline that studies behavior in an evolutionary and functional context. Ethotoxicology yields a better quantitative and qualitative understanding of the mechanisms of EDCs action and of their possible consequences on individual adaptive abilities and population dynamics.

The goal of my research is to analyze the effects of low doses of bisphenol A and diethylstilbestrol on the house mouse (Mus Musculus domesticus) during two critical developmental periods: pre-natal and post-natal.

1.2 THE CONCEPT OF "CRITICAL PERIOD" ON THE DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

The early developmental stage of an organism represents a sensitive period for the anatomical and functional organization of the central nervous system (Greenough et al.,1990; Liu et al., 1997). In fact, during this time genes and several environmental factors interact leading to the differentiation of the neuroendocrine tissues, but what is the influence of such factors on the activity of the central nervous system and behavior is not well known. To this end, events that happen during ontogenesis, such as small perturbations of the sensorial experiences or hormonal environment, are known to modulate and influence the development of an organism. Specific examples can be found in the vast literature on the ontogenesis of the neuroendocrine and behavioral system following maternal stress: with consistent evidence from rodents (King e Edwards, 1999; Reznikov et al., 1999; Secoli e Teixeira,1998; Szuran et al., 1991) to primates (Clarke e Schneider, 1993; Schneider, 1992). In this context, the most affected system is the hypothalamus-hypophysis-surreneal gland which regulates glucocorticoids secretion in response to stressful experiences. Specifically, adult animals that during fetal life were exposed to high concentrations of costicosteroids showed higher physiological and neuroendocrine response to stress than the controls. Same effects were observed in experimental models of parental care-giving (Liu et al., 1997; Peters, 1988). Recently it has been hypothesized that the behavior can be epigenetically transmitted by aspects of maternal behavior that would modify the DNA structure during the postnatal period (Francis et al., 1999). In general, numerous rodents studies showed the importance of the social environment for the expression of individual neuro-behavioral differences in adulthood. For example, factors such as sex and number of pups per litter, social interaction (play behavior and aggressive interactions), environmental enrichment and handling, have

profound developmental consequences on the central nervous system (Beach e Sadler, 1987; Denenberg e Morton, 1964; Dyer e Southwick, 1974; Martin e Bateson, 1985; Moore e Power, 1992; Namikas e Wehmer, 1978; Brain e Griffin, 1970; Lore e Stipo-Flaherty, 1984; Smythe et al., 1994; Manni et al., 1998; Veenman et al., 1999; Escorihuela et al., 1994; Plotsky e Meaney, 1993; Tucker et al., 1984). Moreover epidemiological data showed how humans are subjected to the same ontogenetic rules, since children of mothers consuming drugs before and during gestation showed dysfunctions of the nervous system and behavior (Peters et al., 1984; Tonkiss et al., 1996). The possible causes of these effects have been investigated using animal models. For example the prenatal exposure to cocaine had a profound effect on the dopamine and serotonin systems resulting in altered adult behavior (Wood et al., 1994; Giustino et al., 1998; Cirulli et al., 1997; Laviola, 1996; Gonda et al., 1996). From the aforementioned examples it is possible to conclude that:

- The perinatal development of an organism is sensitive to apparently little perturbations of the physiological environmental.
- The possible outcomes are irreversible alterations of the neurological and endocrine systems.
- These different behavioral profiles are often visible long after the perturbation took place. Thus, it can be suggested that the toxicological research needs to consider the developmental effects of EDCs exposure from the fetal to adulthood, with special focus in the behavior.

1.3 SEXUAL DIFFERENCES ON THE NEUROENDOCRINE SYSTEM AND ON THE BEHAVIOR: THE ROLE OF HORMONES

Sexual hormones regulate the development of several tissues, especially the ones belonging to the reproductive system. For example, in male fetuses, testosterone acts as a prohormone that is converted in the urogenital sinus to the more potent androgen 5α-dehydroxytestosterone (DHT). DHT acts along with estradiol regulating the prostate development in the urogenital sinus of mouse embryos (vom Saal et al., 1997; Gupta et al., 2000). This doesn't seem to happen during the differentiation of organs that originate from the Wolff's duct (epididymis, vas deferens and seminal vesicles), (vom Saal et al., 1992).

Thus estrogenic of EDCs may have different effects in different organ and, consequently, be tissue specific (vom Saal et al., 1998).

Sexual steroids influence the nervous system during sensitive periods of ontogenesis and adulthood, organizing and re-organizing the neural circuits involved in neuro-behavioral functions (Matsumoto, 1991). One of the epigenetic consequences of steroids is the development of behavioral, cognitive and neuro-anatomical sexual dimorphisms. In higher vertebrates, such as mammals and birds, the processes of gonadic sexual differentiation are determined by genes in the sex chromosomes. Gonads produce and release androgens and estrogens that influence the typical development of male and female brains. Without the gonadic effect, the brain is originally female (Phoenix et al., 1959; Whalen, 1974; McEwen, 1981). Once gonadic steroids are released, they act on the brain development binding to intracellular and membrane steroids receptors, namely androgens (AR), estrogens (ER) and progesterone (PR) receptors. After the steroid-dependent sexual differentiation of the brain, estrogens, androgens and progesterone play an important role in the activation of socio-sexual behaviors in adulthood. Typically, after puberty a new increase in sex steroids production reinforces the brain sexual differences predetermined during ontogenesis, leading to observable behavioral sexual differences (Crews, 1993; Gahr,1994). The effects of steroids during the development are referred as irreversible and are indicated as “organizational”, while the effects on the adults are considered revertible and are called “activational” (vom Saal, 1983; Arnold e Breedlove, 1985). Precisely, estrogens and aromatizable androgens (testosterone converted in estrogen by the enzyme aromatase) organize the neural development during the perinatal period leading to permanent sexual dimorphisms of several regions of the brain, synaptic formation, dendritic extension and distribution of serotoninergic fibers (Matsumto, 1991).

It is important to emphasize that a wide range of sexual dimorphic behaviors are not directly related to reproduction. These behaviors comprehend acquaintance, exploration, play, level of activity, aggression, social behavior, learning, vocalization, regulation of feeding metabolism, urinary posture, food preference and so on (Beatty, 1984; Beach, 1975; Holman e Hutchison, 1991; Harlan et al., 1979; Goy e McEwen, 1980; Haug et al., 1993). For example the exploratory behaviors of numerous mammalian species are sex specific and it has been hypothesized a correlation between sex and use of the space (Gaulin e Fitgerald 1986). In rats sexual differences of spatial learning occur around the onset of puberty, with females showing more failures (Krasnoff e Weston, 1976). Similarly in humans fluctuations

on the spatial ability, such as mental rotation, appear at puberty (Hampson, 1990). It is important to highlight the existence of specie-specific behavioral sexual differences: for example in rats females are normally more active than males in several tests, while mice these results are not consistent (Dixon e Defries, 1986). In other rodents, such as the praire vole, males are more active than females (Perrot-Sinal et al., 2000). Furthermore often a clear behavioral distinction in terms of "presence/absence" is impossible and differences in frequency are more useful (Goy e McEwen, 1980). The behavioral sexual dimorphisms listed so far are caused either by the activational or organizational effects of sex steroids (Perrot-Sinal et al., 2000; Beatty 1979; Arnold e Breedlove, 1985). A better example of behavior "organized" by sex hormones is the aggressive behavior of adults toward pups showed by rodents and primates (vom Saal, 1983; --, 1984). In a similar way sexual hormones "organize" the learning and acquisition of avoidance behaviors in the shuttle box (Beatty, 1979).

At this point it is noteworthy open a short parenthesis: recent experimental evidences indicated that some of the sexual dimorphisms in the CNS cannot be ascribed only to the action of gonadic hormones (Carruth, 2002). Indeed, genetic mechanisms can induce the behavioral and nervous systems differentiations so far described (Arnold, 1996). A further source of information about sexual differences comes from the non-hormonal action of genes in the sexual chromosomes. Another important role in this context is played by the environment (Juraska, 1991). For example, in rodents (Moore e Morelli, 1979) and humans (Reid, 1994) it is known that the quality and quantity of the maternal stimulation influences the following neuroendocrine and behavioral development of the offspring (Kelly et al., 1999).

An interesting phenomenon that revealed the ontogenetic sensitivity of tissues to sex steroids is the intrauterine position. A fetus of mice, rat and gerbil is subjected to variable levels testosterone and estradiol depending of which sex are the sibling next to him (for example a female between two males). Such hormonal variations causes evident differences on morphological, functional and behavioral features: genital morphology, regulation of puberty, lasting of the estrus cycle, territorial and aggressive behavior, sexual behavior and sexual attraction. (vom Saal, 1989; vom Saal et al., 1999). It is believed that these behaviors and the relative physiological system are evolved through the action of sexual selection. Animals that share a common phylogenesis show similar morphological, physiological and behavioral adaptive functions that probably underwent the same selective pressures.

In conclusion, the timing of exposure to hormones or EDCs in developing tissues is critical. During development of the reproductive organs and the CNS small concentration of chemical compounds may interfere and disrupt the endocrine function, leading to irreversible alterations (Bern, 1992; vom Saal et al., 1995; vom Saal and Sheehan, 1998)

1.4 THE THEORY OF SEXUAL SELECTION

Behavioral sexual differences are the reflection of adaptive behavioral strategies adopted by males and females that can be understood by the conceptual frame of evolution. The theory of evolution predicts that males and females will be equal in all those traits exposed to similar selective pressures, but they will differ in traits evolved under different selective conditions. Both sexes have mechanisms shaped by peculiar and specific selective pressures. Although natural selection (Darwin 1859) is considered the principal casual process that lead to the adaptation of specific traits, Darwin (1871) introduced a second evolutionary theory: the theory of sexual selection. Sexual selection is the evolutionary process that select traits on the basis of a direct, immediate reproductive advantage. In species where the parental investment differ between sexes (Trivers, 1971), the sex that shows the lower parental investement (usually the male) adopts a more risky behavioral strategy (more novelty and sensation seeking). The sex that invests more in parental behavior (generally the female) produce a low number of offspring increase the number of mates doesn't bring any advantage (Trivers 1971; Symons 1979; Buss e Schmitt 1993). Conversely, males have more advantages increasing the number of mates and this lead to a high intra-males competition for females. Males that win the intra-sexual competition are able to father more offspring than the defeated ones.

Since males have a greater reproductive potential than females, taking risks in order to gain a resource can be beneficial for them. When males are observed in approaching and intra-sexual competitive behaviors, such as sensation seeking, neophilia, explorative behavior, impulsiveness, aggressiveness, usually are bolder than females. Using the theory of sexual selection in interpreting this apparently heterogeneous list of behavioral traits, it become clear that they belong to conceptual unity: a males will shown more risky behaviors in situation where the cost of the risk is overcome by the higher reproductive benefit.

Phylopatry plays also an important role in this context: in the major part of Mammals cited so far, once females reach puberty they will stay with the parental group, while males leave

and either joint other groups or live solitary (Dobson, 1982). As a consequence males and females present different social roles. Naturalistic studies conducted by Crepau and Newman (1991) showed the sex-specific profiles of Primates responses to potential dangerous stimuli: the result was a relation between sex specific differences of defensive behaviors and the different social and reproductive roles. In light of the hypothesis and empirical studies based on the theory of sexual selection, it seems reasonable to suppose that behaviors responsible for the adaptive response to stressful and potentially dangerous situations have been the target of sexual selection.

1.5 ESTROGEN RECEPTORS IN VERTEBRATES: IMPLICATION FOR THE STUDY OF ENVIRONMENTAL ESTROGENS

While feminization of the brain structures and functions seems to depend on the moderate interaction between estrogens and the developing nervous system, defeminization and masculinization depends on androgens converted in estrogen by the enzyme aromatase (Dohler e Jarzab, 1992; Lephard, 1996; Hutchison et al., 1996). Estrogen receptors (ER), along with the support of androgen receptors (AR), mediate the differentiation of the male and female CNS. ERs are present in the CNS already during the first days of fetal development and their function is well supported by the literature (Beyer, 1999, Vito e Fox, 1981; Pasterkamp et al., 1996; O'Keefe et al., 1993; Friedman et al., 1983; Keefer e Holderegger, 1985; O'Keefe et al., 1995). It is demonstrated that the activation of intracellular ERs can induce gene transcription, causing changes of the cellular structure and function, resulting in the modification of substrates at the basis of the neural transmission: dendrites, axons, synapses and neurotransmitter receptors (Beyer, 1999; Woolley, 1998; Wooley e McEwen, 1993; Matsumoto et al., 1979; Miyakawa e Arai, 1987; Carrer e Aoki, 1982; Chung et al., 1988).

In the Subphylum Vertebrata, the neuroendocrine system underlying the socio-sexual functions shows an elevated level of homogeneity (Nelson, 1995). Estradiol is identical in all the vertebrates: steroid hormones are identifiable for a highly conserved structure, contrary to the protein hormones that can vary in the aminoacidic sequence but maintain the same name in different species. More specifically, one of the two classic estrogen receptors, ER-alpha, is the same in fish and humans (Pakdel et al. 1989). The high conservation of the alpha form of

the receptor during the hundreds of million of years of evolution of vertebrates, has profound implications for the study of chemical compounds with estrogenic activity. (vom Saal, 1995). The traditional hypothesis is that if a chemical compound is able to bind one vertebrate species ERs, then it could bind to the ERs of other vertebrates, humans included. But this doesn't necessarily imply the same effects in different species. Furthermore, because of the developmental and tissue specific action of estrogen, different cells or tissues of a species can show different responses. More specifically it has been suggested that the tissue response to estrogen can vary depending on the organism developmental stage. This is in part due by the complex interactions of other hormones, receptors and on the ontogenetic history of such an organism (Katzenellenbogen et al., 1996; Li et al., 1997). So, in this context it is acceptable suppose that EDCs binding to ERs may determine physiological changes of the cellular function. Since the role played by ERs receptors organizing and sensitizing the developing brain is critical, then the exposure to estrogenic compounds during this period would result unfavorable. This is hypothesis is supported by less efficient homeostatic mechanisms during perinatal life. We refer to "fragile fetus" to indicate a critical period when the exposure to EDCs can result in irreversible alteration of the CNS: such a phenomenon can happen also at very low concentrations that in the adult animal may not be supported by toxicologic evidences (Bern, 1992). It is thus possible to conclude that the exposure to estrogenic compounds during critical periods of fetus development may disrupt the physiological action of steroids, leading to permanent alterations of the CNS structure and function and consequently of the behaviors (Colborn et al., 1998).

1.6 EFFECTS OF EDC ON THE ENDOCRINE SYSTEM

Several chemicals that are widespread in the environment due to human activity are classified as "endocrine disrupters (EDs) or "hormonal active compounds". The common characteristic of these compounds is that they can bind to endogen hormones receptors. Very high attention have been moved to the study of EDs that bind to androgen receptors (Kelce et al., 1995), thyroid (Brouwer et al., 1998; Porterfield e Hendry, 1998) and through other cellular mechanisms (Colborn et al., 1998). Some of these compounds, the phytoestrogens, are naturally found in plants such as soy, cereal and in several fruits and vegetables (Welshons et al., 1990). Others are synthetic compounds sold for specific uses and to manufacture

products. The estrogenic active compounds belonging to the EDs family are found in pesticides (o'p'-DDT, methoxychlor, kepon, toxaphane), in plastics (bisphenol A, nonylphenol), in pharmaceutical products (ethynil estradiol, diethylstilbestrol (DES), in compounds for domestic use (octylphenol) and industrial chemicals such as PCBs (Soto et al., 1995; Nagel et al., 1998; Go et al., 1999). A wide literature on the ontogenetic developmental alterations caused by these environmental pollutants focused on behavior and reproductive capacity across different species. Some of the evidences are the interruption of the normal development, problems on the thyroid system, reproductive impairments, reduction of the immune response, abnormal mating and parental behavior and sudden disappearing of entire populations (Colborn et al 1993; 1998). Abnormality of the reproductive system linked to the exposure to EDs have been described in different class of vertebrates, such as fish, alligators, turtles, birds and mammals (Colborn et al., 1993, 1998). For example, anomalies of reproductive and socio-sexual behaviors have been observed in herring gulls nesting in the Great Lakes, where it has been registered a high incidence of abnormal clutches, decreased interest for mating, decreased expression of territorial behavior, decreased parental care, mating behavior between females, feminization and demasculinazion of sexual characters, with the resulting decrease of the reproductive success of entire colony (Fox et al., 1978; Fry et al., 1987; Rattner et al., 1984; Shugart, 1980). These anomalies were associated with impairments of the endocrine system caused by the exposure to organic-chloride contaminants such as DDT and PCBs and their active metabolites (Rattner et al., 1984; Fry e Toone, 1981).

Studies led by the Jacobsons had shown an association between neurobehavioral deficits in babies and maternal consumption of PCB contaminated fish from Michigan Lake. Children exposed to higher concentration of PCBs were slower when elaborating information, had less quantitative and visual discriminative memory, reduced verbal ability and a IQ 6.2 points lower than the average (Jacobson e Jacobson, 1996; Jacobson et al., 1989). In one study led by Daly and coll. (1998), rats born from mothers that had eaten contaminated fish from Ontario Lake during gestation or lactation shown a greater frustration and depression in response to a lack or expected reward. These results suggest that the neuroendocrine deficits caused by EDs can be similar in humans and rats.

One of the most controversial EDs is the DES, a synthetic estrogen shown to cause long term effects after fetal/neonatal life exposure (Takasugi e Bern, 1988; Vannier e Raynaud, 1980). Mothers taking DES during gestation in order to avoid abortion, had daughters with

higher incidence of vaginal and cervix cancers and malformations of the reproductive organs (Nollar et al., 1990). Also the male reproductive system was affected, with higher incidence of chryptoorchidism, abnormal sperm but a normal fertility (Gill et al. 1976; Raloff, 1995). This is in countertendency now, since a decrease of the semen quality and a constant increase of genital malformations is being registered every year (Swan et al., 1997; John Radcliffe group, 1986; Paulozzi et al., 1997; Hass e Sakr, 1997; Sharpe e Skakkebaek, 1993). DES is not only a powerful estrogen, similar in its efficacy to the estradiol, but during the development it has also the unique ability to concentrate in target tissues, such as the reproductive organs of birds, reptiles and other animals, causing morpho-functional anomalies (Shah e McLachlan, 1976). This pharmacological drug is the example of what a potent estrogenic compound can do and, it illustrates the effects that this family of compounds can have on the health (Newbold, 1995).

An important issue in the study of EDs is the doses used in tests of risk assessment. Several experiments on laboratory animals that utilized environmental relevant concentrations of human exposure (Nagel et al., 1997; vom Saal et al., 1998, 2005; Welshons et al., 1998) demonstrated that perinatal exposure to EDs caused reproductive anomalies described also in humans (McLachlan et al., 1975; Grocock et al., 1988; Sharpe et al., 1996). In ecological experiments, organisms with temperature-dependent sexual determination have been shown to present a sexual inversion when exposed to EDCs. In these organisms (TDS) estrogens play a role in the normal development of the female sex, as in the turtle (Crews, 1993). In the red-ear turtle (Trachemys scripta elegans) the inhibition of the synthesis of estrogen produces males while the application of estrogens to eggs that presumably contains males produces females; the application of estrogenic compounds have shown to have effects of similar sexual inversion (Crews et al., 1989). For example when hydroxyl estrogenic PCBs were applied to males incubating eggs, females were born (Bergeron et al., 1994). It is important to notice that the estrogenic activity of different compounds can be species or gender-specific. Clark et al. (1998) found that p,p'-DDE acts as an estrogen in tiger salamander (Ambystoma tigrinum), but the technical category of DDT acts as an anti-estrogen. The exposure of rodents to high doses of environmental estrogens during development anticipates puberty and alters the reproductive function (Gray, 1992). Developmental exposure of rat females with high doses of o,p'-DDT causes a precocious puberty as well a loss of fertility (Welch et al., 1969; Heinrichs et al., 1971). In male rats developmental exposure to high doses of o,p'-DDT reduces fertility as well prostate and

seminal vesicles weight (Gellert et al., 1974). Furthermore anomalies of the daily sperm production, epydidimis, prostate and seminal vesicles have been caused by the prenatal exposure to low doses of BPA (Nagel et al., 1997; vom Saal et al., 1998; Welshons et al., 1999). Maternal exposure to low doses of BPA during pregnancy induces an increases of body growth of the offspring and accelerates puberty in females (Howdeshell et al.2000).

An increasing interest of the behavioral alteration after developmental exposure to EDs can be observed by the growing literature. Epidemiological and experimental studies reported deficits of attention, iperactivity, novelty seeking, aggression and sociality, learning and memory, as consequences of exposure to EDCs during the prenatal period of life (Jacobson & Jacobson 1996; Farabollini et al.1999, 2002; Dessì et al. 2002; Lephart et al.2002; Palanza et al.1999;2001;2002; Rice&Hayward 1999; Kobu et al. 2003). Our most recent research confirmed the potential danger to low doses of EDCs. Specifically our data indicates an effect of pesticides and BPA on the development of behavioral sexual differences: these effects can be highlighted in adult subjects only with a proper experimental design that predict the exposure only during a critical period (Palanza et al. 1999; 2001; 2002).

1.7 ETHOTOXICOLOGY: THE ETHOLOGICAL APPROACH TO THE STUDY OF THE BEHAVIOR IN THE TOXICOLOGICAL RESEARCH

The target of toxicological research in the field of hormonal active pollutants is the reproductive system, ignoring other systmes such as the neuroendocrine or the behavior. This is particularly true for the estrogenic compounds since the reproductive system are considered to be the end point for the action of steroids, both during sexual differentiation and adulthood. Furthermore results of clear and evident alterations at the anatomical and physiological level brought the majority of researchers to treat animals with high doses of chemical compounds, above the concentration present in the environment. Nonetheless the experimental evidences presented in the previous paragraphs indicate that very low doses of hormonally active compounds may have physiologically effects: this different approach take in consideration the precise timing and real exposure of humans and animals. In fact, it has been shown that the brain is rich of ERs already during the fetal life and that low doses of EDCs affect the organization and differentiation of the CNS. The lack of detailed studies on the effects of EDCs on behavior in the field of toxicology is an important issue, because of the importance

that is given to neurobehavioral toxicology in order to evaluate the risk of exposure to xenobiotic (MacPhail, 1994; Weiss e Elsner, 1996). In the 1975 in the article "Assessing the impact of low level chemicals on development: behavioral and latent effects", Spyker conclude that "longitudinal studies on the behavior of organisms exposed during development are essential for a careful evaluation of the impact on human health to low concentration of some coumpunds. (Spyker, 1975)

The nervous system is a complex system composed of several parts: brain, spinal cord and a wide network of peripheral nerves and sense organs. It is responsible for the reception, transmission and integration of all the information that allow an organism to react and to adapt to environmental challenges. Psychological processes such as perception, learning and memory, affection, voluntary and involuntary movement are all dependent on the nervous system. Furthermore the autonomic nervous system has the control of other organs and systems that regulate the homeostatic process of physiological functions such as blood pressure, heart rate, respiration. So, the nervous system, along with the endocrine system, executes the control of a wide body functions.

The alterations of the CNS caused by xenobiotics can be studied at different levels, such as the electrophysiological, neurochemical, morphological and behavioral. Of course none of these levels of study is able, alone, to answer completely to the basic questions, but a neurobiological approach of the behavior would be particularly helpful. In fact the behavior represents the integration of multiple informations coming from the nervous and endocrine systems. As a consequence, small perturbation of one of these systems can thus results in observable modifications of the behavior. Furthermore behavioral studies are not invasive and may be used either to study the effect of an acute treatment or to monitor the progressive developmental effects of a long term exposure to a neurotoxic agent. It is also important to highlight that the functions of the CNS cannot be determined only with histological of physiological methods without taking in consideration the behavioral aspects. In fact it can be argued that the exposure to EDCs can lead to alterations observable only when the animal is behaviorally challenged and maybe only for specific behavioral responses. This is a crucial argument for the evaluation of the risks assessment of an EDCs: since the expected and observable effects are typically expressed from a functional level, then an evolutionary approach allows to evaluate the adaptive value of altered behaviors, yielding to a better predictability of any individual and population consequences (Parmigiani et al., 1998)..

Historically two different schools of thought have contributed to the scientific development of a behavioral discipline, each one with different methodological approaches: ethology and comparative psychology. Ethology, mostly studied in Europe, put more emphasis on an evolutionary and natural observational methodology of the behavior (Thorpe, 1978). In North America comparative psychology focalized mainly on the study of processes underlying the conditioned and learned behavior. From a methodological point of view the ethological approach uses "ethograms" that consist of a detailed list of well defined behavioral responses, representing different categories of the behavioral system in exam (Hinde, 1973; Altman, 1974). This approach is particularly indicated in the studies of social behavior (Whishaw et al., 1983). Through the specie-specific study of behaviors with functional and adaptive value in the natural conditions, the ethological research may allow the evaluation of a sophisticate analysis of behavioral alterations in the field of neurotoxicology (Wiemayer e Lincer, 1987). Nonetheless the study of behavior in the toxicological research had been preformed using almost entirely the approach coming from comparative psychology: the animal is employed as an "instrument" in order to observe specific neural and endocrine mechanisms, the experimental situations are highly controlled but not appropriate for the adaptive and functional point of view (Cuomo et al., 1996; Parmigiani et al., 1998; Palanza et al., 1999).

Of course it is not the goal of this work try to acknowledge one methods over another. In fact the studies on conditioning obtained with comparative psychology are fundamental results in the field of animal behavior and neurobiology, such as on the visual system (Rice e Gilbert, 1982), acustic system (Crofton e Sheets, 1989), neuromuscular system (Meyer et al., 1979; Gerhart et al., 1982; Fowler et al., 1990; Kulig et al., 1985). Anyway it is thought that a better attention must be spent on the study of complex aspects of the behavior, such as the social and non- social behaviors.

1.8 THE HOUSE MOUSE AS AN ANIMAL MODEL

Not only human populations but also wild and domestic animals can be exposed to hormonal active EDCs that are present in the environment. Domestic animals, for example, can be used as environmental sentinels because they share the same environment of humans. For example, one of the effects following metyl mercury contamination in the Minimata Bay was

an evident level of intoxication of the CNS of domestic cats, that usually fed from contaminated fish caught by their owners (Chang, 1980). Another case was the effect on myelin observed in neonates and litters of dogs washed with soaps containing hexachlorophene (Edds e Simpson, 1974). Anyway the study of these alterations in the natural field is very difficult. For example, it is not possible make a fine qualitative and quantitative measure of several behavioral parameters, especially for behavior in the social sphere. Moreover, once an alteration of the behavior is detected, it would be hard to find if it has been caused by the action of one chemical or by the synergic/additive action of more compounds: this would require a highly and constant control of the experimental conditions. Nonetheless there are no doubts that in nature does exists peculiar areas of contaminations and that it is possible to monitor animal populations confined in such areas and compared them with neighborhood populations: this would allow the building up of a predictive risk for man and other species (Wiemayer e Lincer, 1987; Douglas, 1989). But because of the several experimental difficulties and high costs in related to research on field, the ethological approach in laboratory shows without any doubt more benefits, especially when the real toxicity of the compound, its modality of action and the systems affected are not well understood.

In this context, the house mouse (Mus musculus domesticus) is a species with numerous characteristics that make it a good model in the toxicological and neurobehavioral research. In fact, the mouse is an highly opportunistic specie that is subjected to multiple ecological pressures (Bronson, 1979), widely distributed all over the world and with a high degree of adaptation to extremely variable environments: from deserts to the andine peaks, from atolls in the pacific to the antartic islands, from farms to whalers. Even though it has been documented the existence of some feral populations totally separated from humans, the mouse is an human commensal specie and therefore both species may be exposed to several of the same pollutants (Bronson, 1979; Berry, 1989). Furthermore being the mouse predated both by terrestrial predators and birds of pray it could act as a vector of environmental contaminants. The social organization of the house mouse is extremely variable, depending on food availability and distribution and on the population and predator density. Lastly, the wide use of mice and rats in the neurobiological, pharmacological and behavioral research, provide a detailed literature on the cellular and physiological mechanisms at the basis of the expression of the behavior, allowing for a better interpretation of the results obtained and the

formulation of valid hypothesis on the possible biochemical and physiological effects of an EDCs, from where it can be draw an evaluation for the human risk.

2. EXPERIMENTAL PHASE

2.1 AIM OF THE RESEARCH: NEUROBEHAVIORAL SEXUAL DIFFERENCES RELATED TO THE ESTROGENIC COMPOUNDS ANALIZED

The goal of my research is to investigate, through a transgenerational study, if the exposure to low doses of BPA and DES leads to developmental alterations of the CNS and behavior in the house mouse. The previous research performed in our laboratories showed the following key points:

1. The existence during ontogenesis of critical periods where the CNS and behavior are extremely sensitive to the action of sexual steroids. During these periods exposure to substances that mime the action of such hormones, in concentration normally not dangerous to the organism, affect the neurobehavioral development.
2. On the basis of sexual differences related to the action of endogenous sexual steroids, males and females seem to be differently sensitive to the action of EDCs. Specifically we have noticed that the explorative-emotional and cognitive behaviors are the most affected after perinatal exposure to estrogenic EDCs (Palanza et al, 1999a,b; 2001; 2002a,b; Gioiosa et al., 2007). In relation to this, we found that the catecholaminergic system is a target point of such compounds, as it has been shown for the number and biochemical characteristics of alpha-2 adrenergic receptors of the hypothalamus and locus coeruleus.

Since catecholamines modulate the expression of several socio-sexual and cognitive behaviors that present sexual specific differences involved in mental and neurodegenerative disorders, my main focus is to investigate how the exposure to BPA and DES during both prenatal and post natal life can affect the sexual differentiation of specific brain regions and neural systems crucial for the control of the motor activity.

2.2 SEXUAL DIFFERENCES IN THE CATECHOLAMINERGIC SYSTEM

A sexual differences has been shown in the etiology of several mental health disorders such as neurodegenerative diseases, mood disorders and autism (Connel et al., 2004).Among the numerous neurotransmitters that are affected, monoamines play a major role, especially catecholamines as dopamine and noradreanaline. Thus, it is plausible the presence of a sexual dimorphism in the structural organization and neural functions of brain areas part of these systems. For example Parkinson and Alzheimer diseases are characterized by the depletion of noradrenergic (NA) and dopaminergic (DA) neurons respectively in the locus coeruleus (LC) and substantia nigra (SN). Both these regions show specific differences between sexes either in the number of neurons and volume of the nuclei (Pinos et al., 2001; Kubo et al., 2003) or in the uptake/ regulation of the neurotransmitter (Dluzen, 2005). Such differences may be related to differences of ERs distribution, type and mechanisms of action.

Sexual differences in the catecholaminergic system of developing and adult rats have been well characterized. Rat females have higher rate of cathecolamine synthesis, catabolism and turnover than males. Specifically, females show an higher turn-over of DA in the tubero-infudibularis, greater release of DA in response to amphetamine or estrogen and a higher number of TH immunoreactive cells in the anteroventral hypothalamus have. Conversely, greater levels of DA in the limbic system, striatum and hypothalamus have been reported in males. Also the NA system present sex differences: higher levels of NA in the hypothalamus have been found in males, while females had a higher level of NA in the striatum and limbic system (Reisert et al., 1991). As already mentioned, the epigenetic organizational effects of sexual steroids may be involved in the formation of these permanent sex specific differences on the anatomy and physiology of catecholamines. For example the neural-anatomical characteristics of regions innervated by catecholamines, such as the ventrolateral part of the mediobasal hypothalamus (VMH), important for the regulation of female sexual behavior, and the medial preoptic area of the hypothalamus (MPOA), important for sexual behavior of males, are under the regulation of neonatal sex steroids (Gorski, 1990; Fabre-Nys, 1998). Specific effects of perinatal sex steroids on the catecholaminenergic system have been found in the periventricular nucleus of the hypothalamus and in the locus coeruleus, where the number of cells and fibers expressing TH was higher in females than males and testosterone-

treated females (Fabre-Nyss, 1998). Same trend has been found in the cortex, where during early development females presented a higher concentration of catecholamines than males (Stewart et al., 1994). Moreover, the development of the biogenic amines in the midbrain and brainstem is under the control of estrogen: this has been clearly demonstrated in several studies, even though it does not always result in a sexual-specific phenotype (Beyer et al.,1991, 2000; Kuppers et a., 2000; Ivanova et al., 2003; Luque et a., 1992). Sexual differences of catecholaminergic regions are not limited to the developmental period. In fact, it has been observed that the activational effects of sex steroids vary among males and females, producing changes in volume, synaptic density and number of cells. This is due either by a different distribution of steroid binding receptors, nongenomic indirect mechanisms. Indeed, several brain regions important for the control of general brain output, such as the control of motor activity and sensory processes of cognitive function, don't show any binding sites for steroids, but either they are reached by sexual dimorphic projections or may function under the regulatory control of the estrogen membrane receptor (Fabre-Niss, 1998)

2.2.1 **THE LOCUS COERULEUS (LC)**

The locus coeruleus is a nucleus of the brain stem (A4) from which a small number of neurons (from around 1500 in rats to 10000-15000 in humans) send out their projections. Regardless the small number, these neurons have a multitude of ramifications that project caudally to the spinal cord, rostrally to all the forebrain through the noradrenergic bundle and dorsally to the cerebellum. Virtually each part of the CNS is reached by the LC: telencephalon, diencephalon, neocortex, hippocampus, amygdale and hypothalsmus; while this innervations is almost absent in the basal ganglia.

The LC is regulated by afferent fibers from the nucleus paragigantocellularis (PGI), that respond to stimuli eliciting the sympathetic nervous system, and from the nucleus prepositus hypoglossus (PH) that is related to the control of eye movements: this clearly showing how the activity of the LC is usually associated with states of attention and arousal. Other fibers that synapse with the LC come from the prefrontal cortex, amygdala, hypothalamus, stria terminalis and dorsal raphe. These connections happen at the level of the

periceorulear region and are of special interest because they link the LC activity with the cognitive and affective processes.

From a neurochemical point of view the LC is the major producer of NE, where virtually all of its neurons contain dopamine beta-hydroxylase (DBH), the enzyme that convert DA to NE. Nevertheless, other important neurotransmitters have been found within this region, mostly peptides such as vasopressin, somatostatin, enkephaline and galanin. From a functional point of view, an environmentally novel stimulus acts on the noradrenergic system in contrasting ways: for example, it has been shown that animals treated pharmacologically with NE stimulants increase the levels of exploration and contact toward a stimulus (i.e. an object) when tested in a familiar environment. When tested in an unfamiliar environment, the same treatment induces the animal to a general exploration of the environment and a decrease of the attention from the stimulus: these evidences suggest that the LC elicit states of arousal that leads to the investigation of novelties and salient stimuli.

How the noradrenergic system controls the central motor systems is not well understood. It is nonetheless know that in Parkinson disease, a neurodegenerative disease that among its symptoms has the impairment of voluntary motor control, the number and cytology of LC neurons are affected. When NA is applied *in vitro* locally in the cortex it enhances the excitability of the motor cortex pyramidal neurons. LC modulation of movements may be related with the cerebellar control of motor functions and it may regulate the motor performance acting at the level of spinal interneurons, possibly coordinating motor functions in relation to salient stimuli relevant for the survival of the organism (Berridge et al., 2003)

The LC is under the developmental control of sex steroids, specifically testosterone and estrogen, which lead to significant sexual differences in relation to volume and number of neurons in adulthood (De Blas et al., 1990; Guillamon et al., 1988,1998; Pinos et al., 2001; Segovia et al., 1999). Furthermore estrogen preserves the NA biosynthetic activity and functions as neuroprotectant of noradrenergic neurons. Estrogen increases the discharge from neurons of the A1 nucleus (Kaba, 1983) and it induces the transcription of C-Fos and progesterone receptors in neurons of the A2 nucleus (Jennes et al., 1992). These biochemical and physiological characteristics can be explained by the presence in the locus coeruleus of both the nuclear ERs, but specially the ERß (Mitra et al., 2003).

2.2.2 THE SUBSTANTIA NIGRA (SN)

The substantia nigra (SN) is anatomically placed in the mesenchephalon and is characterized by neurons that produce DA that innervate several structures of the basal ganglia, mainly the striatum (nigro-striatal pathway), and thalamus. It is anatomically and functionally formed by two different parts, the *pars compacta*, that contains the DA neurons, and the *pars reticulate*, that gives to the SN the peculiar brown color.

Basal ganglia are a set of nuclei that extend from the mesencephalon to the telencephalon carrying out several functions. These involve the control and fine tuning of the extrapyramidal motor system through the nigro striatal pathawy, cognitive and reward related behaviors through the meso-cortico-limbic pathway. From a pathological point of view, the best characterized disease that involved the SN is Parkinson, a neurodegenerative disorder that involved the impairment of voluntary movements and cognitive/affective behaviors. Parkinson is characterized by the depletion of DA neurons (virtually all of them disappear) in the SN and it seems to affect the sexes differently with a higher incidence in males than females (Dluzen et al.,1998; Shulman, 2007). It has been known since the 80s the importance of the action of estrogen in the development of the dopaminergic system and its possible neuroprotective action toward its neurodegeneration: presence of sexual differences in the neurotoxic effects of methamphetamine exposure. Dopamine neurons expressing TH in the midbrain are present during the preinatal life and estrogen plays an important trophic, regulatory and protective function for the expression of the final phenotype of these neurons (Kipp et al., 2006). In fact the nigrostriatal system presents a transient expression of the enzyme aromatase during the embryonic and neonatal stages of life. Furthermore in this region during the neonatal period nuclear ERs are present (Raab et al., 1995) and developmental sex differences with respect to their expression seems to exist (Kipp et al., 2006; Ravizza et al., 2002). These estrogenic effects seem to be due by both embryonic production of estrogen at the level of the neurons and by the gonads. It has been suggested that the sexual differences that are seen at the level of the dopaminergic system are genetically based, but that gonadic hormones may modulate or implement further these sex biases. *In vivo* and *in vitro*, estrogen stimulates neurite growth and TH expression but it inhibits the expression of the dopamine transporter (DAT) in the midbrain of developing mice (Ivanova et al., 2003; Kipp et al., 2006). Recently, it became clear that even though the expression of classic ERs in the SN seems to be relative absent, sex differences can be found in other characteristics such as in the function of DAT (Dluzen 2005).

2.3 BISPHENOL A (BPA)

Bishenol A (BPA -2,2-bis(4-idroxyiphenyl)propan) is an essential compounds in the production of polycarbonates plastic and epoxy resins. It is used in the manufacture of dental sealants, food containers and it is also added in other plastics in order to make toys (polyvinyl chlorides-PVC) and soda and mineral water containers (polyethylene teraphtalates-PET). Polycarbonates are formed by the link of several monomer of BPA through ester bonds. Even though among the characteristics of polycarbonates there are chemical and high temperature resistance (www.bisphenol-a.org), it has been shown that these bonds are really sensitive to heat and either acidic or basic conditions, leading to their hydrolysis and consequent release of BPA. More precisely, cleavage of containers, storing of acidic or basic food, repeated washing and multiple use of products made of polycarbonates lead to the leaching of BPA (Brotons et al., 1995; Howdeshell et al., 2003; Carey, 2003; Olea et al., 1996). The pollutant action of BPA is not only related to direct human exposure through food and beverages, but is widespread in all the environments, reaching and affecting the wildlife through landfill leached, water sewage and natural degradation of plastics (Crain et al., 2007).

The estrogenic activity of BPA has been under discussion by the observation that in some bioassay its activity is weaker than estrogen (Welshons et al., 1999). But it is now well known that BPA is hormonally active, acting at low concentration as estrogen in vitro and in vivo (Krishnan et al., 1993; Nagel et al., 1997; Steinmetz et al., 1997) and anti- androgen and thyroid hormones (Lee et a., 2003; Zoeller et al., 2005). BPA can also act as an antagonist of estrogen, even in the same tissue, by binding differently to the ERs, having different affinity for the receptors and being active in different tissue at different doses, properties that conferred to BPA the term of SERM. (for a review see Welshons et al., 2006). A growing literature showing in *vitro* and *in vivo* effects of BPA and epidemiologically relevant information on human exposure to this EDC have been published in the last 5 years. These studies show a wide series of consequences due to the exposure to low but environmentally relevant doses of BPA during critical periods of development: the effects span from alteration of cellular mechanisms in reproductive and nervous tissues to behavioral effects in different animal species (Richter et al., 2007; vom Saal et al., 2005; Welshons et al., 2006; Wetherill et al., 2007).The fundamental issue is that these effects happen after exposures to doses lower

than the 50 µg/kg day considered the safe by the US Food and Drug Administration (FDA) and by the US environmental Protection Agency (EPA). The levels used by these agencies in order to calculate the reference dose of human exposure is based on the toxicological approach which divide a 50 mg/kg/day lowest observable adverse effect dose (LOAEL) for a 1000 safety factor. Instead the new approach of "low dose" uses environmentally relevant doses in the range of wildlife and human exposure.

2.3.1 BPA, ITS EFFECTS ON THE CATECHOLAMINES AND RELATED BEHAVIORS

The exposure to low doses of BPA during periods of life sensitive to the organizational effects of steroid hormones has been reported in several studies that analyzed different aspects brain and behavioral function. Specifically it has been reported that 5 days old male rats subjected to the injection of 15 µg/kg of BPA increased their spontaneous motor activity when tested at 1 month of age. This hyperactive profile was paired with the midbrain down-regulation of the dopamine receptor D4 and dopamine transporter DAT, an effect also reported in studies conducted on estrogen regulation of the developing dopamine system (Kipp et al., 2006). Furthermore when these animals where 2 months old a clear reduction of TH immune-reactive cells in the SN was observed (Ishido et al., 2004), giving rise to the speculation of a neurotoxic effect of BPA associated with the possible neurodegenerative features of Parkinson. Other evidences highlighted how the dopaminergic system seems to be very sensitive to the developmental exposure to BPA: developmental doses ranging from 10 to 300 µg/kg/day influenced behavioral and molecular aspects of the psychostimulant action of methamphetamine and amphetamine: locomotion, sensitization toward the rewarding effects of these drugs, explorative behaviors and DA receptors function (Adriani et al., 2003; Laviola et al., 2005; Suzuki et al., 2003). Also the noradrenergic system has been reported to be affected by the developmental exposure to BPA: when rats of both sexes were exposed to 30 µg/kg/day, they showed an inversion of the sexual differences in the LC. Interestingly when these animals where tested in a new environment, this anatomical result was paired with an effect on risk taking behaviors in both sexes and with an increase of explorative behaviors in male, with a consequent loss of sexual differences (Kubo et al., 2003). Other evidences come from aspects related to male and female sexual behavior under the

dopaminergic and noradrenergic control. Dopaminergic neurons and noradrenergic terminals have been found in the anteroventral periventricular region of the hypothalamus and in the medial preoptic area, where they modulate gonadotropine releasing hormones (GnRH). In these regions, developmental exposure to low doses of BPA disrupted the sex differences of TH immunopositive neurons between (Rubin et al., 2006). Further evidences of a BPA action on these systems come from my research as an undergraduate: prenatal exposure to 10 µg/kg/day of BPA affected the pharmaco-kinetic aspects of the binding to alpha$_2$-adrenergic receptors in the locus coeruleus and preoptic area (POA), and a loss of sexual differences in the density of these receptors in the POA. Considering behavioral aspects to some extent under the regulation of the monoaminergic system, it has been demonstrated in several species that BPA may interact with the organizational effects of gonadic steroids, reducing or in some case altering the sexual differences at the basis of novelty seeking, displacement activity, exploration, locomotion, risk taking behaviors and anxiety related behaviors, aggressiveness and sexual behaviors (Dessi-Fulgheri et al., 2002; Farabollini et al., 2002; Gioiosa et al., 2007; Kawai et al., 2003; Panzica et al., 2007; Porrini et al., 2005; Rubin et al., 2006).

3. PRENATAL EXPERIEMENT: EFFECTS OF BPA AND DES ON THE CENTRAL CATECHOLAMINERGIC SYSTEM AND NON SEXUAL BEHAVIORS.

3.1 INTRODUCTION

Bisphenol A (BPA) is the monomer used in the synthesis of polycarbonate plastics, the macromolecules involved in the production of the lining for food and beverage cans, epoxy resins for the manufacture of dental sealants and as an additive in products of different nature and use. The huge amount of BPA produced every year, its virtually constant presence in the human environment and its ability to act as an endocrine disruptor had rise awareness

in the scientific community. BPA can bind to the intracellular and membrane estrogen receptors leading to the activation of genomic and nongenomic mechanisms acting either as an antiestrogenic and androgen antagonist compound. As a consequence it affects different neuroendocrine systems such as the gonadic and the thyroid. The reference dose for BPA exposure is 50 ug/kg/day, but its ability to pass through the placenta, the blood brain barrier and the presence of low level of drug-metabolizing enzymes in the brain, makes lower doses to be risky for the fetal CNS development (VomSaal 1998; Kawato 2004). It is in fact during the fetal life that gonadic steroids, especially testosterone through its metabolite 17β-estradiol, influence the programming of the brain. This happens through the regulation of neural proliferation, apoptosis and differentiation, axonal and dendritic growth and neural plasticity, with the consequent structural and functional sexual dimorphism of the CNS (Panzica, Aste et al. 1995; Beyer 1999; McEwen and Alves 1999). Early exposure to BPA in utero involves a decrease in sexual differences in several regions of the brain such as the anteroventral periventicular nuclues (Rubin, Lenkowski et al. 2006), locus coeruleus (Kubo 2001,2003), medial preoptic area (Ponzi, unpublished data), bed nucleus of the stria terminalis (Funabashi 2004).

I chose to evaluate the effects of the prenatal exposure low doses of BPA and DES on the number of neurons expressing tyrosine hydroxylase (TH) in the locus coeruleus (LC) of the house mouse. TH is the rate-limiting enzyme for dopamine (DA) and norepinephrine (NE) synthesis. During early postnatal life, estrogen stimulates the expression and the synthesis of midbrain TH in males and females mice (Ivanova 2002), regulating in a sexually dimorphic way the gene expression of TH in the LC of transgenic LacZ mice (Thanky 2002). The LC is a region of the brain stem that counts for the higher number of neurons producing noradrenaline (NA). It is known that rat LC presents sexual dimorphic features in regard of volume, number of neurons and number of dopmanine-β-hydroxylase-immunoreactive cells, with females being higher in such morphological patterns (Pinos et al., 2001; Guillamon et al., 1988; Luque et al., 1992). Because of the neuromodulatory nature of NA and the wide connections that LC has with the higher and lower parts of the CNS, its activity is widely involved in the control and modulation of several neurobehavioral systems. Being the behavior the endpoint of the brain activities it was reliable to expect effects of BPA on this aspect. In literature, early exposure to low doses of BPA has impacts on the emotional and affective behavior of several rodent species: for example female mice became less sensitive to the reinforcing effect of d-amphetamine (Laviola et al., 2005), perhaps affecting the brain

reward system targeted by the drug; explorative, ambulatory and novelty preference behaviors are affected in female rats, while anxiety levels and the neural pathways sensitive to the stimulatory effects of d-amphetamine are affected in males (Farabollini 1999, Adriani 2003). In rats, developmental exposure to BPA has been shown to decrease the sexual differences of exploration and learning behaviors in a passive avoidance test in rats (Kubo, 2001, 2003, Rubin 2006,Fujimoto 2006) while in mice exploratory, locomotory and maternal behaviors and to alters the levels of anxiety in mice (Rubin et al., 2006; Palanza et al., 2002; Ryan et al., 2006).

Thus beside the immunohistological description of the neurons positive for TH in two different stages of the house mouse life, I observed the effects of the prenatal exposure to BPA on the open field test and spontaneous activity, paradigms useful in order to understand the activational and emotional levels and the drive to exploration.

3.2 METHODS

3.2.1 Procedure

Cd-1 mice (*Mus musculus domesticus*) were maintained in an outbred colony at the University of Missouri. The animals were housed in 18 x 29 x 13 cm polypropylene mouse cages on corncob bedding. Pregnant and lactating females were fed Purina 5008 (soy-based) breeder chow, and after weaning, animals were fed Purina 5001 (soy-based) chow. Water was provided *ad libitum* in glass bottles and was purified by ion exchange followed by a series of carbon filters. Rooms were maintained at 25 ±2 C under 12:12-hr light:dark (L:D) cycle, with lights on at 1100 hr.

Adult 4 months old virgin female mice were time-mated by being placed into the cage of a stud male for 4 hours beginning at 0800hr. Mating was verified by the presence of vaginal plug (day 0 of gestation) and pregnant females were housed three per cage.

3.2.2 Chemical Administration

From the day 11 of gestation to the day 18 time-mated (17^{th} for Des treated females) pregnant mice underwent the following feeding schedule: corn oil alone (MP biomedicals, Aurora, OH, USA; control group; n=9), two different doses of BPA (Sigma Chemical, St. Louis, MO, USA) of 50 μg/kg body weight (low dose, n=9) and 5 mg/kg body weight (high

dose, n=10) and two different doses of DES (Sigma Chemical, St. Louis, MO, USA?) of 0.5 μg/Kg body weight (low dose; n= 9) and 50 μg/Kg body weight (high dose; n=10) dissolved in corn oil. Pregnant mice were weighted on day 11, 13 and 16 of gestation and the weight was used for calculate the volume of chemicals the mice were administered; the high DES dose feeding was stopped at day 17of gestation to avoid the pregnant mice abortion. The doses were delivered directly into the mouth of the animals. Mice were picked up by the skin between the shoulders and held upright. The pipette tip was placed into the mouth with the pipette gently touching the roof of the mouth, and the oil was ejected from the pipetter. Mice readily consume corn oil, and this procedure is not stressful for the dams (see Palanza, Parmigiani, vom Saal 2001). On day 17 of pregnancy females were individually housed in 18 x 29 x 13 cm polypropylene mouse cages. The dams gave birth on day 19 of pregnancy (the DES high dose two days later). At birth litters were reduced at 12 pups (6±1 males, 6±1 females). The offspring were weaned on PND 21 (birth day is PND1) and moved in a room maintained at 25 ± 2 C under a 12 : 12 hr light : dark cycle, with lights on at 0800.

3.2.3 Tissue preparation

As 30 and 70 days old (average) both female and male mice from each litter were administered 100 ul of chetamine and perfused transcardially with 20 ml saline solution (PBS) followed by 20 ml of 5% of formaldehyde in phosphate/buffered saline solution (pH 7.2). The sacrifice of the adult female happened when they presented the diestrus. Diestrus was checked by vaginal smear. After perfusion brains were quickly removed, immersion fixed in formaldehyde/PBS at 5% for 24hr and washed in 50 % alcohol for one day. After dehydration brains were embedded in paraffin.

Serial 10um-thick coronal sections were cut on a microtome. One section out of six (for locus coeruleus and substantia nigra) and one out of three (for AVPe) were then immunohistochemically stained and used for quantitative analysis. The regions under study were identified in eight of these sections for the LC and the SNpc and in twelve for the AVPe: a subgroup of these sections was then chosen for statistical analysis (for more details see paragraphs below). The distance among sections is preventing the possibility of counting the same neuron twice.

3.2.4 Immunostaining

Sections were processed for immunohistochemistry by using the ABC method: they were deparaffinized and rehydrated by series of xylene deparaffinization (3 X 2 minutes) and of alcohol re-hydratation (100% x 3, 95 % x 3, 70 % x 3 each for 2 minutes), washed in distilled water, incubated in trypsine for 30 minutes (0.1% in PBS), rinsed in tap water and incubated with 0.1 M Borax for 10 minutes and then washed in tap water for 1 minute and rinsed in PBS 2 times for 5 minutes.

3.2.4.1 Neurons positive for tyrosine hydroxylase:

Nonspecific binding was blocked with 10% of normal goat serum for 20 minutes. This was followed by the application of a polyclonal rabbit anti-Tyrosine Hydroxylase serum (Chemicon AB152, 1:500 in PBS with addition of 0.3% Triton-X (PBS-T) and 1% normal serum) overnight at 4 ^{o}C. After washing the sections 3 times with PBS-T, sections were incubated with a biotinylated goat anti rabbit (vector laboratories) diluted 1:200 in PBS-T and 1% normal serum for 1 h. Sections were then washed with PBS-T for 3 times and incubated with ABC reagents (Biomedia) for 1 h and washed again in PBS-T. 10 mg of 3,3'-Diaminobenzidine (DAB) diluited in a solution made with 25 ml of PBS, 8% NiCl and 0.4% H_2O_2 was applied for 5 minutes.

3.2.4.2 Neurons positive for dopamine ß-hydroxylase:

Nonspecific binding was blocked with 10% of normal horse serum for 20 minutes. This was followed by the application of a polyclonal sheep anti-dopamine ß-hydroxylase serum (Affinity Bioreagents PA3-925, 1:10000 in PBS with addition of 0.3% Triton-X (PBS-T) and 1% normal serum) overnight at 4 ^{o}C. After washing the sections 3 times with PBS-T, sections were incubated with a biotinylated rabbit anti-sheep (ICN biomedicals,Inc) diluted 1:1000 in PBS-T and 1% normal serum for 1 h. Sections were then washed with PBS-T for 3 times and incubated with ABC reagents (Biomedia) for 1 h and washed again in PBS-T. 10 mg of 3,3'-Diaminobenzidine (DAB) diluited in a solution made with 25 ml of PBS, 8% NiCl and 0.4% H_2O_2 was applied for 5 minutes.

3.2.5 Cell count

Cell counting was made by using a microscope at 10X of resolution and a hand-counter.

3.2.5.1 Locus Coeruleus:

The first histological section showing the mesencephalic trigeminal nuclei laterally to the LC was selected as the benchmark section, approximately at -5.68 mm from bregma. Because of either artifacts or weak staining, several sections were missed. So, for the statistical analysis, we choose to use the three sections following the benchmark, in this way we could provide values for each animal. All the black-stained neurons with a recognizable nucleus or with an evident neural branch were counted in both the right and left LC and an average for each section was made. The total number of neurons for this region was obtained summing the average of neurons from the three sections.

3.2.5.2 Substantia nigra:

The number of nigro-striatal dopaminergic neurons from prepubertal and adult mice was counted in the substantia nigra pars compacta (SNpc), the region that stains for TH. Exactly only three sections were counted from each animal, the benchmark of which was chose approximately at the level – 3.16 from the bregma (Paxinos and Franklin), where the medial terminal nucleus of the accessory optic tract (III nerve) separate the TH immunopositive neurons of the SNpc from the ones of the ventral tegmental area (VMT). Then the other two sections were chose so that for each animal were counted sections at the same anatomical level: one section was chosen 60 µm more rostral to the benchmar, the other one was chosen 180 µm more caudal. All the black-stained neurons with a recognizable nucleus or with an evident neural branch were counted in both the right and left emispheres and an average for each section was made; then the total mean cell count for each animal within a group of treatment was pooled to give group means.

3.2.5.3 AVPe:

The number of neurons stained for TH of adult mice was counted in the AVPe. The range of sections that were analyzed extended from approximately 0.14 mm to 0.38 mm from the bregma, essentially through a region that follows ventrally the anterior commissure until it splits in two branches. Of these twelve sections a subpopulation, ranging from approximately from 0.26 mm to 0.38 mm from the bregma, was chose for the data analysis in order to obtain enough values for each animal. All the black-stained neurons with a recognizable nucleus or with an evident neural branch were counted in both the right and left side of the third

ventricle and an average for each section were made; then the total mean cell count for each animal within a group of treatment was pooled to give group means.

3.2.6 Behavioral experiments

3.2.6.1 Spontaneous activity:

At 28 days and at three months of age one male and one female from each litter were randomly chosen for the spontaneous activity test. Each mouse was isolated 12 hours before the start of the observation and placed in an 18x29x13 cage with walls made of transparent polypropylene. Observations started at the onset of the light phase (8:00 in the morning) and at the onset of the dark phase (8:00 in the evening) and last for two hours. Each animal was provided with 4 pellets of Purina 5008 and water ad libitum. Observations were collected every 4 minutes for a total of 30 observations registered in an ethogram comprehensive of the following class of behaviors:

- behaviors of maintenance, such as grooming and feeding
- active behaviors, such as ambulation, grabbing cage lid and rearing.
- inactive behaviors, such as sleeping.

As grooming are considered the behaviors were the animal grooms itself inside and outside of the nest.

Feeding is when the mouse eats pellets or drinks.

Locomotor activity is when the mouse moves around the home cage.

The mouse executes a grabbing behavior anytime it jumps to grab the grilled lid of the cage.

Rearing is considered when the mouse assumes a vertical posture in order to explore the walls of the cage and the air.

Total activity was calculated as the summation of ambulation, grabbing and rearing.

In the dark phase the collection of the observed behaviors were obtained by means of a red light in order to avoid stress and disruption of the circadian cycle to the mice. At the end of the cycle of observation each mouse was marked with non-toxic markers and re-joints its siblings until the day of sacrifice.

3.2.6.2 Open Field Test

The open field test (OFT) was carried out in a 54x54x45 cm arena made in plexiyglass and with a black floor partitioned in 25 boxes. A dim light were positioned in a

way to obtain half of the arena in shadow and the other half bright. At the age of three months one male and one female from each litter were placed in the centre of the field and video recorded for ten minutes. Behaviors were collected and organized in classes in a ethogram. Each class was composed of specific behavioral patterns and the frequency and time of each behavior were recorded by means of "The Observer" software (NOLDUS). A list of the behaviors is the following one:

- time spent in ambulation close to the walls of the arena
- time spent in ambulation in the centre of the arena
- time spent exploring the environment assuming a vertical posture: rearing, close to the walls of the arena
- time spent exploring the environment assuming a vertical posture: rearing, in the centre of the arena
- time spent exploring the environment sniffing the walls
- time spent exploring the environment sniffing the air in the centre of the arena
- time spent grooming in the centre of the arena
- time spent grooming close to the walls of the arena
- total time spent in the centre of the arena: index of anxiety
- total time spent close the walls of the arena: index of anxiety

For each behavior it has been calculated the total time spent executing it, pooling the time spent in doing such behavior in the centre and walls of the arena.

The apparatus were cleaned up with 70% ethanol between each animal test in order to avoid olfactory cues. Furthermore, two identical OPF apparatus were used so that all males were tested in one and all females in the other. No vaginal smears were taken so that I cannot tell which female was in which estrus cycle.

3.2.7 *Statistical analysis*

T student statistical analysis was performed in order to observe if any sexual difference in the same group of treatment was present for each variable studied. All the probabilities reported are tested for an alpha levels (α) less than $p=0.05$ only in presence of normal distribution and equal variance. When this was not the case a log or square transformation was used. Planned comparison of same sex means across the different

treatments were made using a one way ANOVA and in presence of a $p< 0.01$ it was followed by the Bonferroni post hoc test.

Any effect of the treatment and any interaction of treatment with sex were analyzed by a factorial analysis of variance (ANOVA) as indicated using the program Statistica (StatSoft). In case of statistically significant effects, post hoc comparisons of differences between group means were made using the Fisher least significant difference (LSD).

3.3 RESULTS

3.3.1 Effects of prenatal exposure to BPA and DES on spontaneous motor activity

- *Effects on juvenile mice:*

Pre-pubertal mice were tested for spontaneous motor activity in their home cages and several behaviors were observed. A sexual dimorphism was found in terms of time spent by the mouse exploring the home cage in vertical posture (rearing) during the nocturnal phase ($t = -3.14$; $df = 16$; $p<0.01$; males(9)= 2.55± 0.60, females(9)= 0.44± 0.29). This sexual dimorphism disappeared with all three prenatal treatments. Females exposed prenatally to DES are overall more active than males underwent the same treatment ($t= 2.41$; $df= 16$; $p= 0.02$; males(9)= 13.44± 1.21, females(9)= 21.55± 3.03), while no such a difference is found among the sexes of the controls and BPA doses.

- *Effects on adult mice*

When observed for rearing during the night, males exposed prenatally to the low dose of BPA are more explorative than females ($t=-3.45$; $df= 16$; $p=0.002$; males= 5.88± 0.94; females= 2.00± 0.55). The same effect is present in the group exposed to the higher dose of BPA ($t= -2.56$; $df= 18$; $p= 0.01$; males= 4.30± 0.80; females= 1.90± 0.48).

Planned comparison of males mean differences analyzed by one way ANOVA resulted almost significant ($F_{(3,33)}= 2.8$; $p= 0.05$) and the following Bonferroni post hoc test showed that males exposed prenatally to the low dose of BPA are more explorative than males of the control group ($p= 0.04$; males oil= 3.77± 0.87; males BPA= 5.88± 0.94). Females from the prenatal DES treatment were overall more active than males from the same group ($t=2,97$; $df= 16$; $p=0.008$; females (9)=36.11±2.13; males(9)=23.66±3.59). This sexual dimorphism was not observed in any of the other three groups of treatment. One way ANOVA looking for

males differences showed a trend ($F_{(3,33)}$=2.32; p=0.09) and the following Bonferroni post hoc showed that males from the DES group of treatment were slightly less active than same sex animals from the control group (p=0.08).

3.3.2 Effects of prenatal exposure to BPA and DES on induced motor activity

- *Explorative behaviors*

Males exposed prenatally to the higher dose of BPA are more explorative than same group females when observed performing the rearing behavior (t=-2.39; df=16; p=0.02; males(8)=29.99±1.78; females(8)=21.30±3.40). This difference between sexes is present neither in the control nor in the other two treatments. Rearing behavior was analyzed by means of a factorial ANOVA that resulted statistically significant for the interaction between the two parameters sex and treatment ($F_{(3,56)}$= 2.80; p= 0.04). Fisher LSD post hoc showed how females exposed prenatally to the higher dose of BPA were les explorative than all the other animals of all the treatments (p<0.05). When then I analyzed the explorative behavior of sniffing, one clear sexual dimorphism was present in the DES group: males resulted more explorative than females (t=-4.57; df=12; p=0.0006; males(7)=13.90±1.06; females(7)=8.03±0.71). This effect was not present in the other three treatments. A factorial ANOVA for this behavior resulted statistically significant for the interaction between the parameters sex and treatments ($F_{(3,56)}$= 2.80; p= 0.04); the following Fisher LSD post hoc showed that males exposed to the higher dose of BPA are less explorative than control males (p=0.04; males oil(7)=29.52±1.99; males bpa(8)=9.38±1.04).

- *Locomotor activity*

Females exposed prenatally to the higher dose of BPA tended to be more mobile than males of the same treatment (t=1.77; df=16; p=0.09; males(8)=56.99±1.65; females(8)=63.44±3.51). A factorial ANOVA was run to analyze as dependent variable the overall locomotor activity and resulted in a statistically significant effect of the treatment ($F_{(3,56)}$= 2.95; p= 0.04): when the four treatments where compared by means of a Fisher LSD post hoc it was evident that all the three different estrogenic treatments induced an increase of locomotor activity when compared to the control group (p<0.05; oil(14)=54.25±1.48; desl(14)=59.19±1.41; bpal(16)=58.62±1.67; bpah(16)=59.85±1.91).

- *Behaviors associated with emotionality/risk taking*

Females from the group of control were slightly more active in the center of the arena than males of the same group (t=1.78; df=14;p=0.09; males(9)=8.57±0.96; females(7)=11.31±1.26). Same but stronger effect was present in the group exposed to DES (t=2.28; df=12; p=0.04; males(7)=8.15±1.17; females(7)=12.28± 1.37), while animals exposed prenatally to the two doses of BPA didn't differ between the sexes. Instead, females exposed to the higher dose of BPA spent more time moving close the walls of the arena than males from the same group (t=2.01; df=16; p=0.06; males(10)=46.85± 1.81; females(8)=57.17± 2.29). Mice exposed to the prenatal dose of DES showed sexual differences in terms of total time spent in the center and close to the walls of the arena. Females of this treatment stayed longer in the center (t=2.09; df=12; p=0.05; males(7)=9.73±1.5; females(7)=14.61 ±1.77) while males spent slightly more time near the walls of the arena (t=-2.07; df=12; p=0.06; males(7)=89.61±1.54; females(7)=84.67±1.81). No such an effect had been found for any of the other treatments.

3.3.3 Effects of prenatal exposure on the number of TH-positive neurons in the locus coeruleus

- *Prepubertal mice*

In the LC a sexual dimorphism was present in the group of control: females showed significantly more neurons stained for TH than males (t=2.26; df=10; p=0.04; males(6)=31.05±2.29; females(6)=41.00±3.73). A similar effects was found in mice prenatally exposed to the lower dose of BPA (t=2.44; df=9; p=0.03; males(7)=31.63±3.09; females(4)=49.70±7.71). A one way ANOVA resulted statistically significant for males across treatments ($F_{(3,25)}$= 5.08; p= 0.006). The following Bonferroni post hoc test highlighted how males exposed prenatally to DES had less neurons than all the other treatments, especially when compared with males from the group of control (p=0.04; males oil(6)=31.05±2.29; males DES(6)=41.83±2.56).

- *Adult mice*

Adult mice from the group of control were not different between sexes, but males prenatally exposed to the low dose of BPA showed to have less neurons than females of the same treatment (t=2.57; df= 11; p=0.02; males(7)=40.41±3.56; females(6)=56.33±5.22). The increase of sexual differences was slightly present also in mice exposed to DES, even tough

with an opposite direction (t=-2.24; df=8; p=0.05; males (5)=53.00±3.14; females(5)=41.05±4.08). To further investigate this point a factorial analysis of the variance was run and it resulted statistically significant for the interaction between the parameters sex and treatments ($F_{(3,25)}$= 5.08; p=0.02). The following Fisher LSD test showed how females exposed to DES had significantly less neurons then females exposed to BPA (p=0.02), while the opposite was true for males (p=0.05).

3.3.4 Effects of prenatal exposure on the number of DBH-positive neurons in the locus coeruleus

No effects were found for the number of DBH positive neurons in pre pubertal mice, so that males and females across the four conditions had the same number of neurons and the different treatments didn't show any form of effects.

3.3.5 Effects of prenatal exposure on the number of TH-positive neurons in the substantia nigra

No effects were found for the number of TH positive neurons in pre-pubertal and adult mice, so that among the two life stages, males and females across the four conditions had the same number of neurons and the different treatments didn't show any form of effects.

3.3.6 Effects of prenatal exposure on the number of TH-positive neurons in the AVPe

In this experiment I had enough tissue to be able of consider also the higher dose of DES.

Females of the group of control showed to have a higher number of neurons than males from the same group (t=6.01; df=8; p=0.0003; males(5)=14.78±0.81; female(5)=28.29±1.82). The same dimorphic trend was present in the low dose of DES (t=2.85; df=7; p=0.02; males(4)=13.63±1.16; females(5)=27.04±5.05), low dose of BPA (t=3.40; df=8; p=0.009; males(5)=14.24±1.21; females(5)=33.27±7.44), high dose of BPA (t=2.72; df=8; p=0.02; males(5)=15.06±2.83; males(5)=28.45±4.00).

The high dose of DES decreased the sexual dimorphism as much that males and females were equal in terms of number of neurons.

3.4 DISCUSSION

The results of this experiment can be grouped depending on the effects caused by the prenatal exposure to the environmental estrogens: a) effects that eliminated the sexual differences present in the control group, b) effects that created sexual differences, c) non estrogenic effects of BPA and d) effects that can be comparable, thus estrogenic, between DES and the different doses of BPA. The two doses of DES were chosen has positive controls for the two doses of BPA. More precisely the two low doses and the two high doses were expected to be comparable. Data from the high dose of DES were eliminated because of the toxic effects caused by this compound during gestation, leading to a very small number of mice. Nevertheless, I chose to show its effects on the AVPe since it was clear its effect of masculinization.

In prebupertal mice, unexposed males were more explorative than females in their home-cage, while the exposure to all the three different treatments eliminated this effect. When adult mice where observed for risk taking behaviors in a new environment, the exposure to both the doses of BPA reduced the time and activity spent by females in the center of the arena to the levels of males. A similar effect was found in a caudal region of the locus coeruleus of these mice, where the prenatally exposure to 5 mg per kg body weight of BPA and to the low dose of DES induced a decrease in the sexual differences related to the number of neurons expressing TH. At my knowledge this is the first report of a sexual dimorphism in the LC of mice.

Several aspects of the behavior and of the neuroanatomy have emphasized how EDCs used in this experiment can also create sex specific differences; this is the case of exploratory behaviors in the home-cage and in the open field. BPA and DES exposure generally seems to increase the time males spent investigating the environment, even though the picture is more complicated by the fact that these environmental estrogens act specifically on different aspects of the explorative behavior, such as rearing and sniffing. Interestingly this sex specific effect is evident also for measures of locomotor activity, where DES exposure increased the home-cage activity of females both in prepubertal and adult mice, while the higher dose of BPA had the same effect in the open field. It is worth mentioning that in the locus coeruleus of the adults no sex specific differences were found in the controls: the explanation of this developmental effect will be given further below. By now I want to stress the effects of the low dose of BPA and DES. Adult female mice prenatally exposed to

50µg/kg/day showed a higher number of TH immunoreactive neurons than males, a result consistent with the one found for the prebupertal study and that stresses the fact that this dose of BPA seems to delay the maturation of the male LC. Surprisingly, for the same anatomical feature the exposure to DES seems to have an effect later in life when males exceed females in number of TH neurons, an effect absent in the prepubertal mice. It is relevant to point out that in this case the two compounds are creating a sexual dimorphism, but they act in opposite directions: females exposed to DES has less TH stained neurons and Des males has more TH stained neurons than their respective same sex mice exposed to BPA.

The effects of the developmental exposure to low doses of BPA and other EDCs on the catecholaminergic system and non reproductive behaviors have already been established in literature (Fujimoto et al., 2006; Gioiosa et al., 2007; Ishido et al., 2003, 2005; Kubo et al., 2001, 2003; Mizuo et al., 2004; Panzica et al., 2007; Ryan et al., 2006; Rubin et al., 2006). In the rat, the LC has been shown to be sexually dimorphic in several patterns such as volume and number of neurons, with females overrating males starting from PND 15 (Pinos 2001,Guillamon 1988,Luque 1992). Furthermore, the expression of TH and TH protein levels are subjected to a different regulation during different stages of early postnatal life (Bezin 1994 a,b). The organizational effects of sex steroids on the dimorphism of the LC has been shown also for the neurons producing dopamine-β-hydroxylase (DBH), the enzyme that convert dopamine (DA) in norepinephrine (NE), which is dependent by the biosynthetic chain starting with the oxidation of tyrosine to L-dihydroxyphenilalanine (L-DOPA). In my experiment I was unable to find such an effect in mice. Nonetheless the effect found in the LC of the prebubertal mice seems somehow to agree with the effect found in rats exposed developmentally to a low dose of BPA, which reversed the sex differences relative to the volume and number of neurons in the LC (Kubo, 2003). From my work it seems that the development of neurons expressing TH in the LC follows a sex specific pattern: females seems to reach the adult number already before puberty, while it could be argue that males need the activation effect of gonadic steroids that happen at this stage of life. This is of course a speculation because from my data it cannot be inferred what happen and when. But it is interesting the fact that perinatal estrogen stimulates the expression of TH in the midbrain of mice (Ivanova et al., 2003) and that in the LC of rats, the number of TH immunoreactive neurons fluctuates from early post natal days to adulthood, with adult rats having a lower number of neurons then pre-weaned ones (Bezin et al., 1994). Benzin' experiment was done on males, and it would not agree with my results since the peak in TH neurons was reached at

PND 21. But it is relevant to draw the attention to the presence of a developmental plasticity in this nucleus, that it maybe species and, possibly, sexual specific, with a particular organizational effect of gonadic hormones during the perinatal life. What seems to be interesting at this regard is the effect of the prenatal low dose of BPA in my experiment: observing the data, it seems to affect mostly the development of the TH population in males. Although the number of neurons increase for males od all the treatments after puberty, males exposed to the low dose of BPA doesn't reach the number of control adults. This effect is not probably related to a possible estrogenic activity, since DES doesn't seem important for this feature: males reach the same number of TH neurons of the adults of the group of control. Actually these two compounds appear to have opposite effects leading to the hypothesis that an early exposure to estrogens, such as DES, would affect primarily females, making them to be phenotipically more similar to the males. In fact females exposed to DES reach puberty with the same number of TH neurons of the males from the group of control (resulting in a decrease of the sexual differences at this time when compared to controls), a number that is approximately maintained also in adulthood, leading to a sexual dimorphism. The presence of BPA at low doses seems to affect mainly the males that fail to reach the number of TH immunoreactive neurons of the males from the control group. In the locus coeruleus of rats Guillamon (1988) found that the determination of a sexual dimorphism is in relation to the presence of developmental sex hormones. In his study, treatment with testosterone propionate (TP) during the 1st day of life caused adult females to decrease the volume and number of neurons, reaching thus the males. Again this is hard to find in my results, but it must be take in consideration the time of exposure and the species used. Nonetheless, I could be able to show that early exposure to BPA affect the development of a part of the catecholaminergic system.

The possibility that BPA can interact with the activities mediated by estrogen in the brain is supported by several studies, where neonatal and acute exposure to low doses of BPA affected the neurosteroid synthesis in presynaptic terminals of the CA1-CA3, in the granule neurons of DG region and stimulated the Ca++ cell influx in the hippocampus of rats (Kawato, 2004). Concentration of BPA in the range of human exposures can mimic and inhibits the stimulatory effect of estradiol on the ERK expression and signaling in the cerebellum (Zsarnovsky et al., 2005). In the range of 40-400ug/kg, inhibits hippocampal synaptogenesis (MacLusky et al., 2005). My results on the effect of the high dose of BPA finds a parallel with the work by Rubin et al. (2006), where very low doses of BPA deleted

the sex differences regarding TH-positive neurons of the anteroventral periventricular hypothalamus. They also have agreed with Funabashi study, done with a different biochemical system (Funabashi et al. 2004): he described how BPA exposure induced a loss of sex differences in a population of neurons positive for CRH in the bed nucleus of the stria terminalis. Interestingly in all these studies the trend seems to be a decrease in the number of neurons in the females group that reach the number of males, suggesting an estrogenic activity of BPA: this cannot be inferred by my study.

All three the EDs affected the behavioral responses in the open field (OPF), leading animals exposed prenatally to ambulate more regardless of the sex. This result can be interpreted as an increase in the activity of prenatally exposed mice. Other groups have found that the exposure to developmental doses of BPA made rats hyperactive, possibly through a specific action of this EDC on the midbrain dopaminergic system (Ishido et al., 2004, 2005; Masuo et al., 2004; Mizuo et al., 2004; Suzuki et al., 2003). Interestingly, I failed to find any action of BPA or DES on the substantia nigra, but it is possible that the differences in dosage and the period of exposure could account for it. Nonetheless, the effects that BPA showed to have on the ctaecholaminergic system of the LC in this present study and in previous (Ponzi, unpublished data) could explain some of the behavioral effects observed.

The few sexual differences that I found for the untreated animals in the behaviors analyzed is inconsistent with other studies. The reason of that can be related to the small number of animals I used or to a lack in controlling the estrous cycle in the females tested, since the estrous cycle affect the level of anxiety in female mice (Galeeva 2001).

One result that seems to agree with other studies is that females are more active in the central region of the open field and that the exposure to BPA reduced this sex specific effect (Panzica et al., 2007). What is relevant from my result is that this effect doesn't seem to be caused by an estrogenic effect of BPA, since the prenatal exposure of DES doesn't influence this phenotype.

It is noteworthy that sexual differences of several behavioral aspects in the OPF had been demonstrated primarily in rats but for mice results are quite contrasting and probably they depend on the experimental setting (Archer, 1975; Blizard, 1973, 1975; Morgan et al., 2004). In preadolescent and peripuberal CD1 mice BPA decreased the sexual differences relative to time spent in the center of the arena and rearing (Rubin et al., 2006) so as in rats it reversed the sexual differences related to the exploratory behaviors (Kubo et al., 2003; Fujimoto et al., 2006). Interestingly, in a perinatal study it has been shown that a low dose of DES lead to an

increase of activity in both males and females of mice (Tanaka et al., 2004), an effect that somehow agrees with my study. Estrogen levels are positively correlated with striatal dopamine release (Laviola 2005) and a stimulation or a modulation of this neurochemical system could be an explanation of the higher activity in the BPA and DES group, but this is a speculation that need to be tested. Furthermore taking in consideration that the studies to which I am referring used acute administrations of E in OVX adult females. What can be generalized is that, even though some of my results don't present the sexual differences showed by others, this seems to be related to a different experimental set up. Nevertheless I was still able to demonstrate the specific effects that the BPA exposure has on behaviors related to emotionality and exploration, known to be modulated by the monoamines and so by the cathecolamines.

Notwithstanding, my result put once again more emphasis on the effects of exposure to environmental estrogens during sensitive developmental periods, during which it has been hypothesized that undefined toxic environmental agents may contribute to the evolution of neuropathologies later in life (Panzica-Viglietti-Ottinger 2005, Landrigan 2005,Logroscino 2005,Opler 2005, Jacobson 1996).

Last but not the least is the modality of exposure to BPA: the great amount of BPA produced every year and its ubiquitary presence in the human environment, even if it is a non persistent substance in the body, makes it reliable that human are chronically exposed to it. This is a point that we must keep in mind in light of the fact that steroid hormones can be produced locally in neurons and glial cells (Garcia-ovejero 2005, Melcangi and Panzica, 2006), raising the possibility of a continued interference at the level of the central nervous system by the continuous exposure to BPA for all the lifetime of the organism.

3.5 PICTURES AND GRAPHS

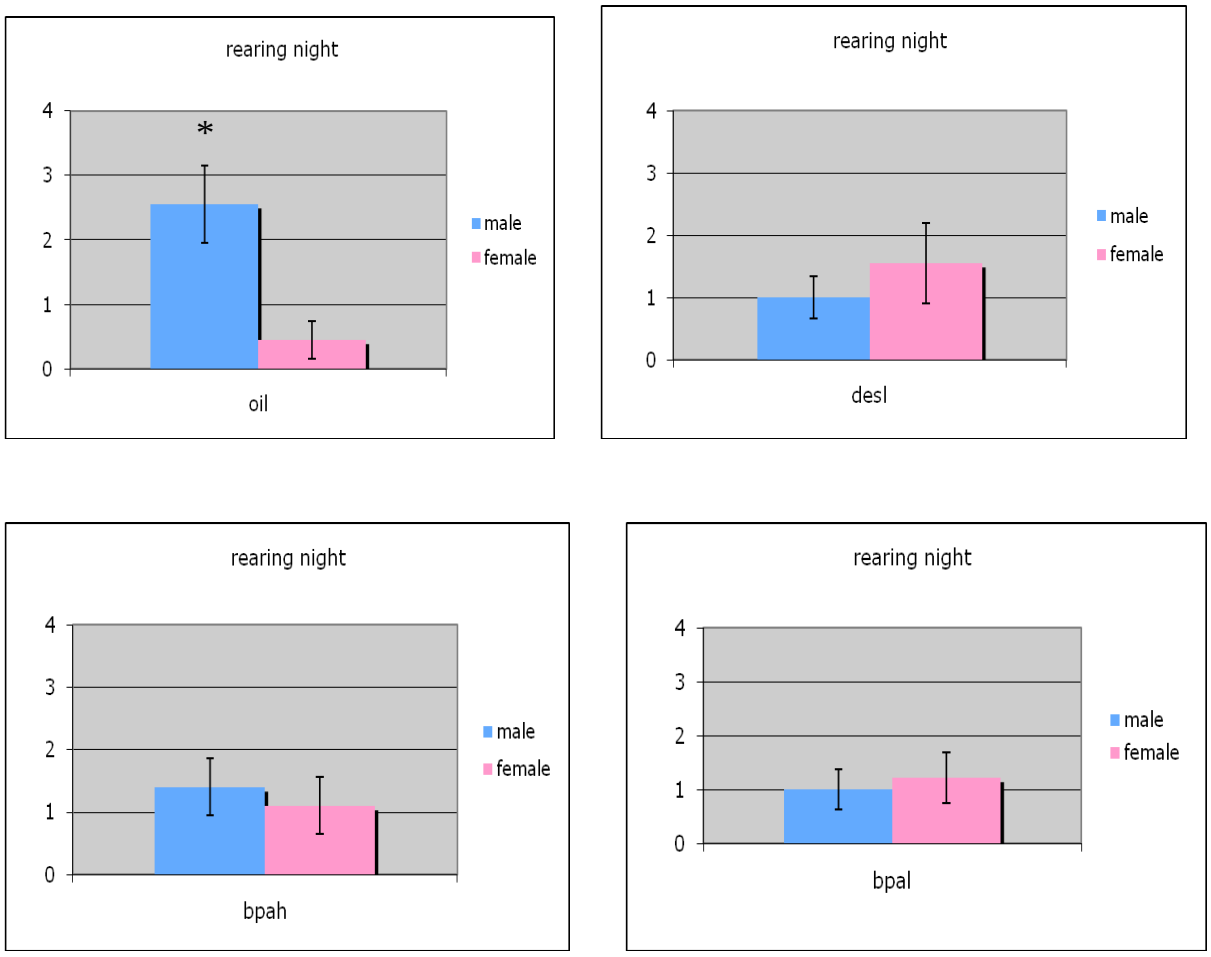


Fig1. Levels of rearing in prepubertal mice during an experiment of home cage activity *p<0.01

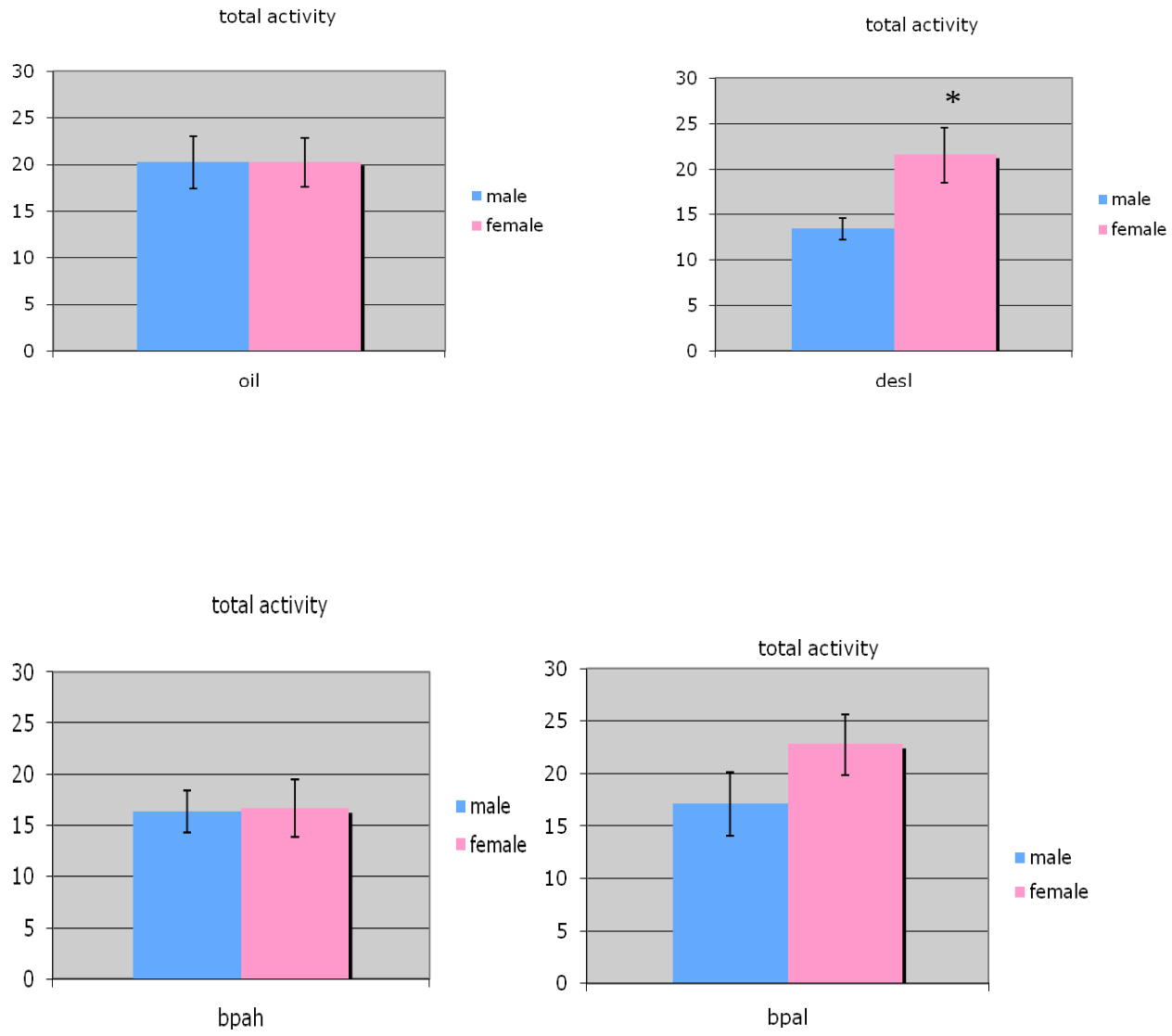


Fig 2. Total activity in prepubertal mice tested in an experiment of home cage activity *p<0.05

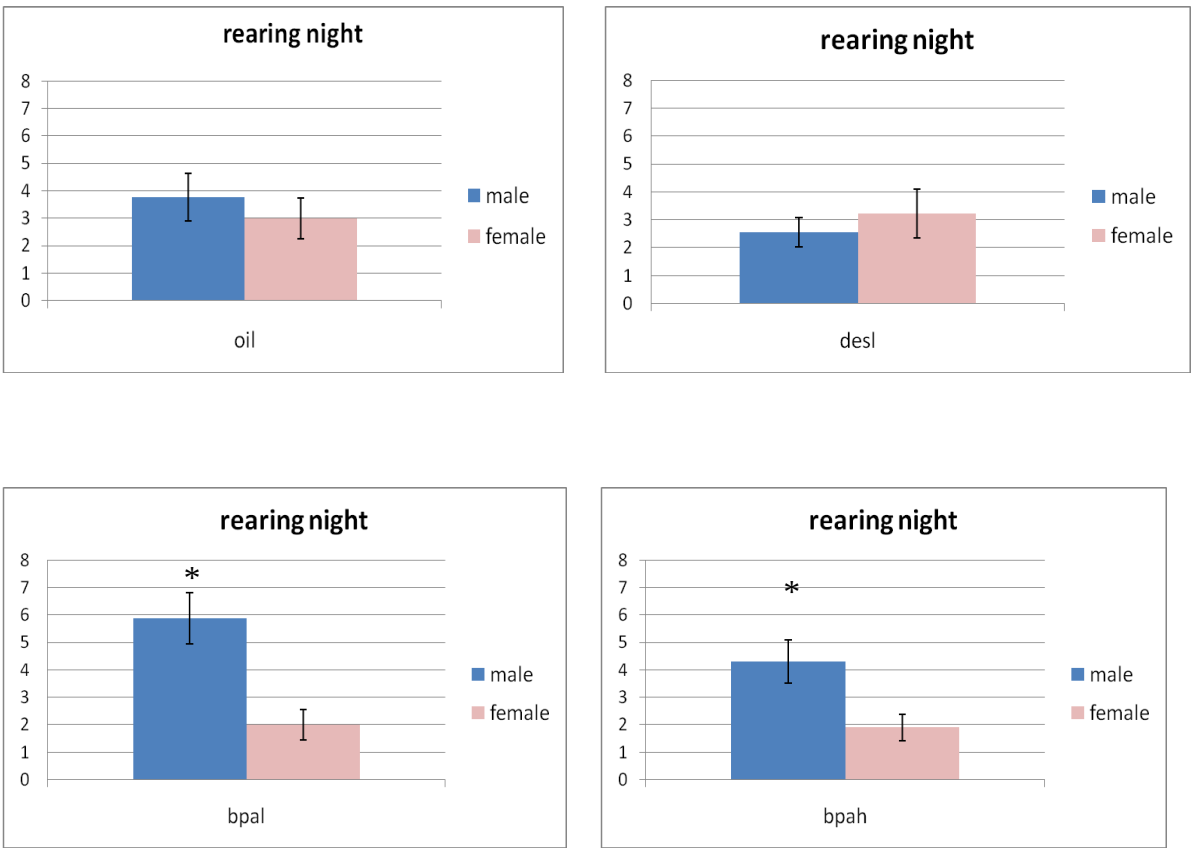


Figure 3. Nocturnal rearing in adult mice tetsed for spontaneous activity in the homecage *p≤0.01

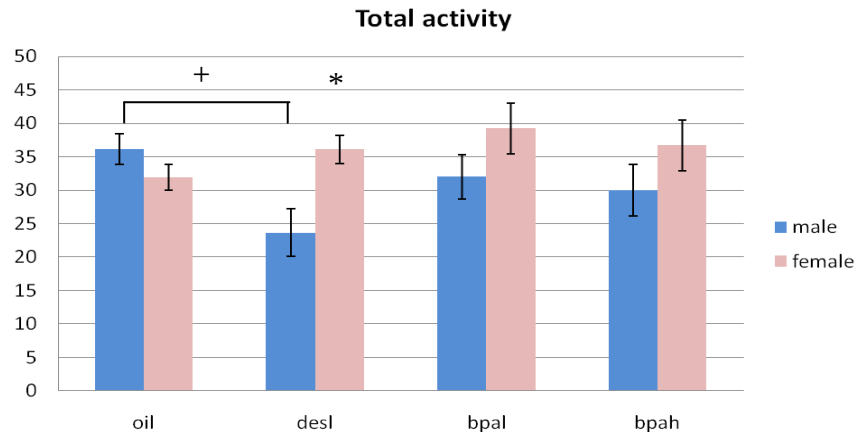


Figure 4. Total activity in adult mice tested for spontaneous activity in the home cage.

*p<0.05

+p=0.08

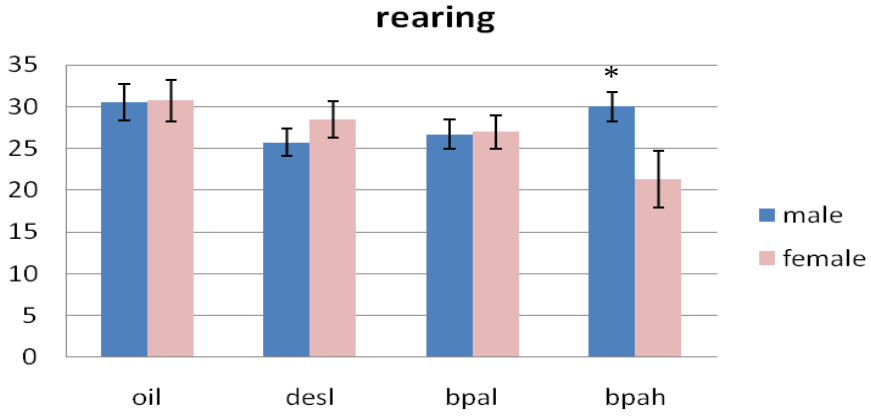


Figure 5. Vertical exploration of adult mice in a OPF. Values are in %time.

*p<0.05

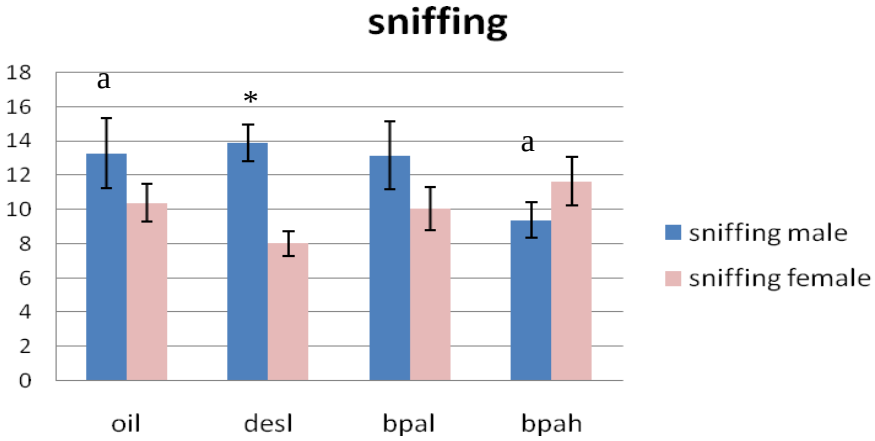


Figure 6. Sniffing behavior of adult mice in a OPF test. Values are in %time.

*p<0.01

[a]p<0.05

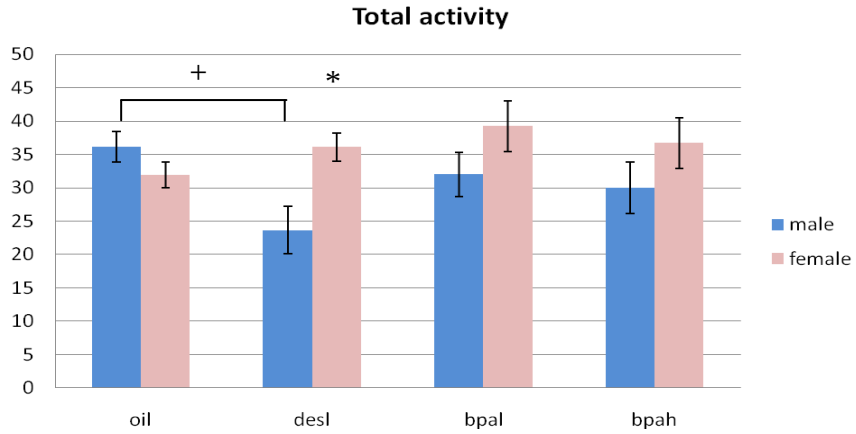


Figure 4. Total activity in adult mice tested for spontaneous activity in the home cage.

*p<0.05

+p=0.08

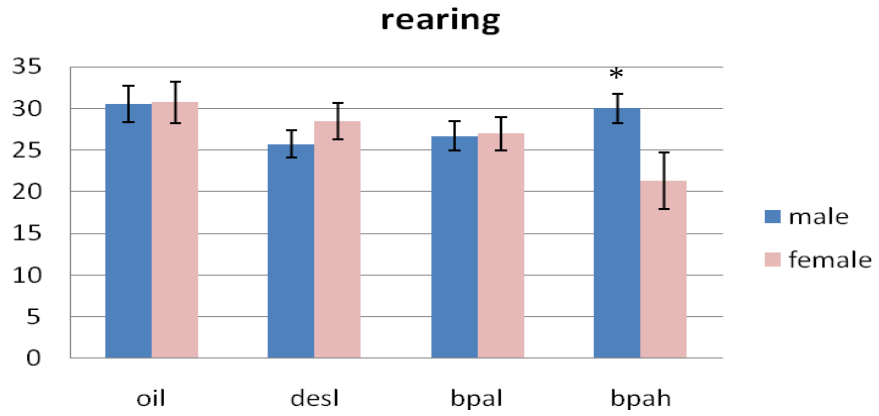


Figure 5. Vertical exploration of adult mice in a OPF. Values are in %time.

*p<0.05

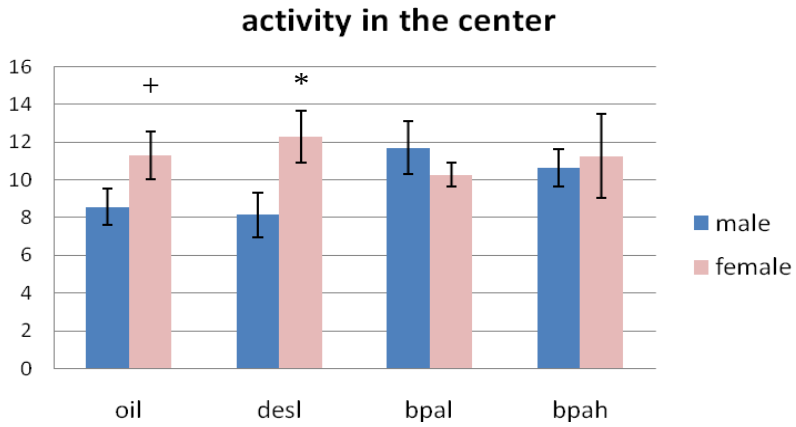


Figure 7. Activity in the center of the arena by adult mice, measured as %time spent in ambulation.

*p<0.05

+p<0.1

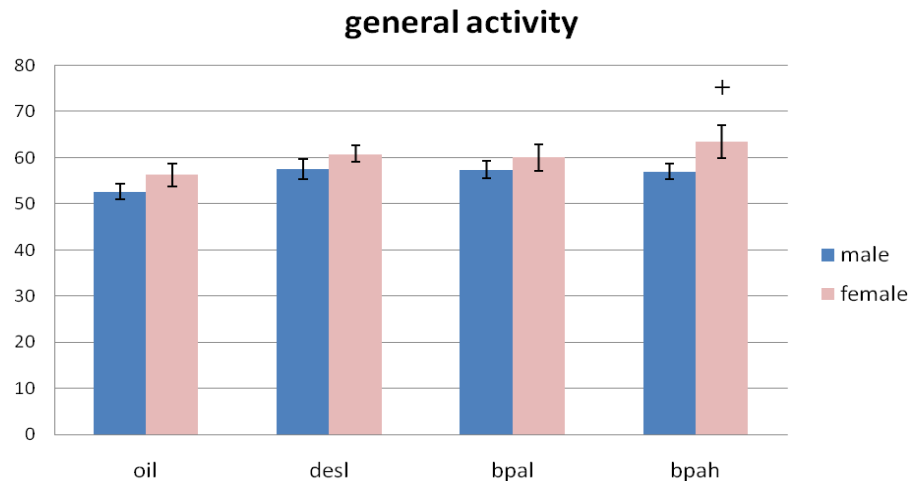


Figure 8. Sexual differences in general activity in a OPF. Values are in %time.

+p<0.1

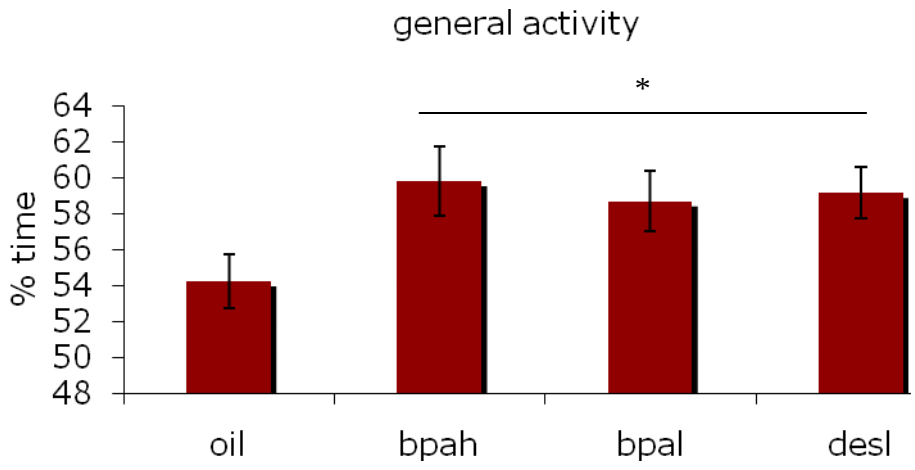


Fig 9. Levels of activity in the OPF. Values are in %time.

*p<0.05

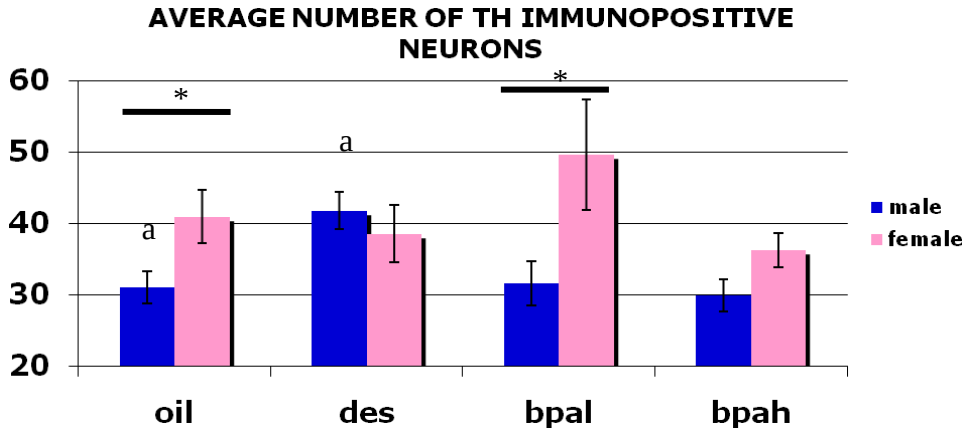


Fig 10. Number of TH immunoreactive neurons in LC of prepubertal mice.

*,[a] p< 0.05

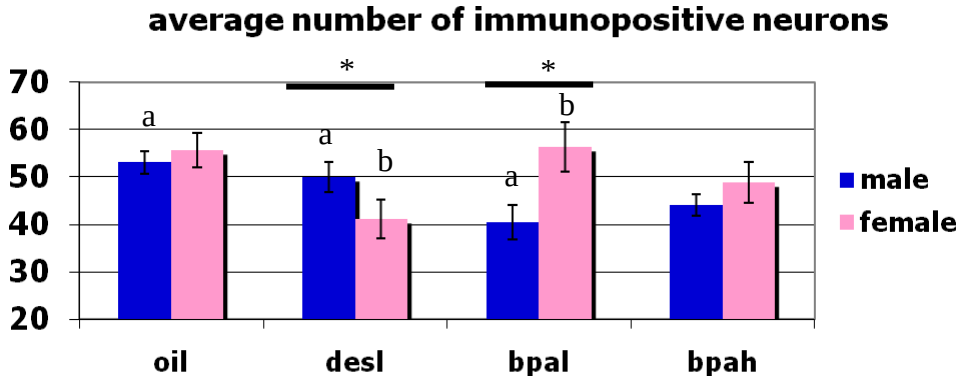


Fig 11. Number of TH immunoreactive neurons in the LC of adult mice.

*,[a,b] p≤0.05

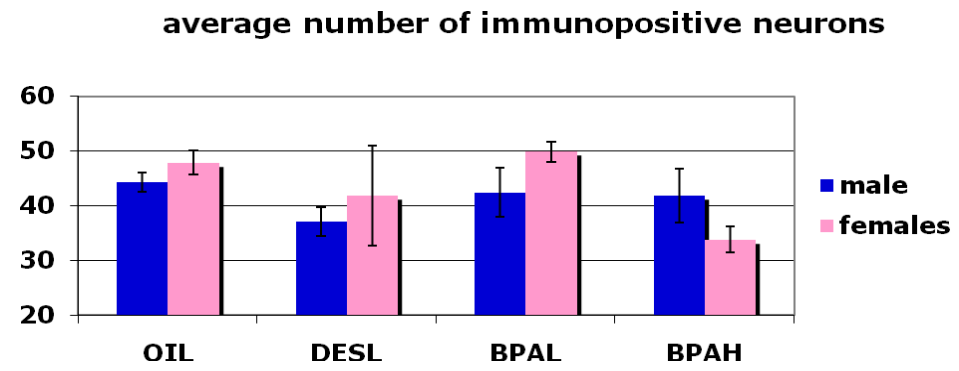


Figure 12. Number of DBH immunoreactive neurons in the LC of young mice.

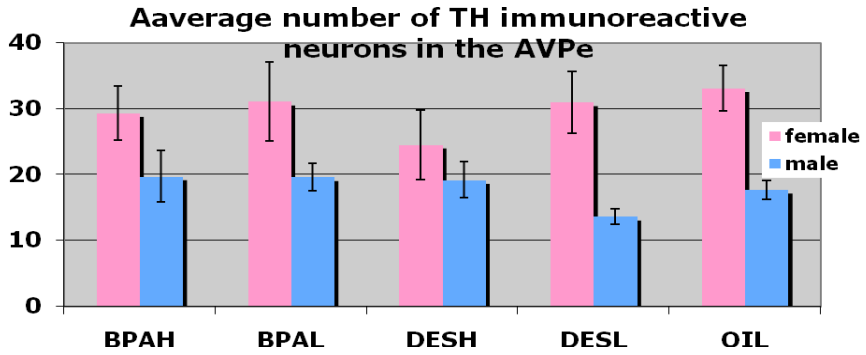


Figure 13. number of TH immunoreactive neurons in the AVPe of adult mice.

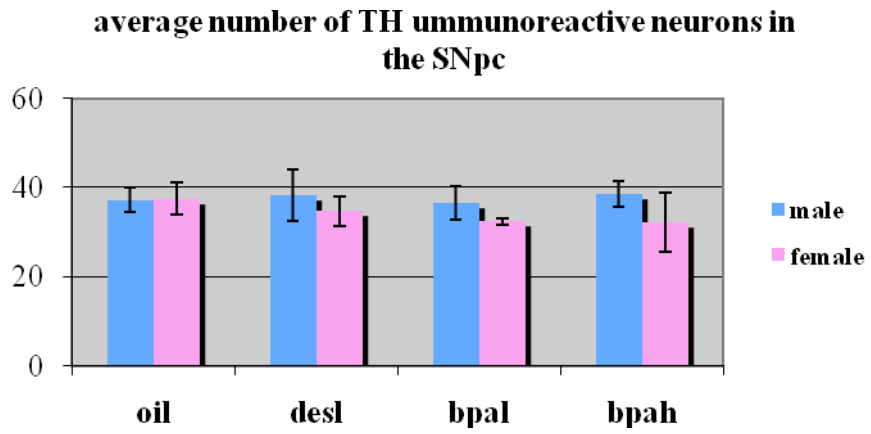


Figure 14. Number of TH immunoreactive neurons in the SNpc of prepubertal mice

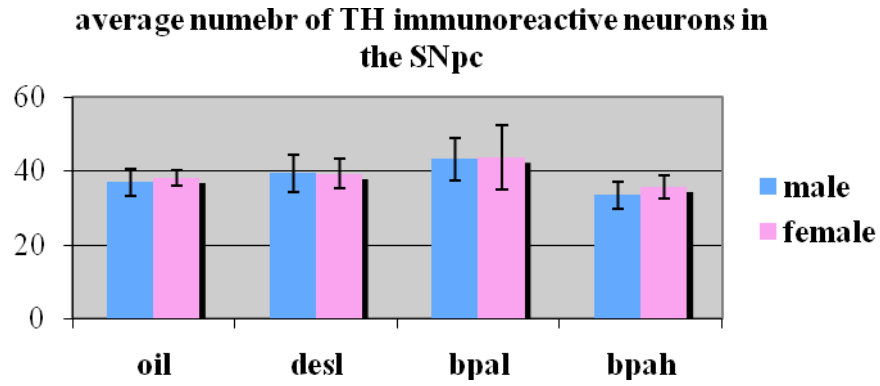
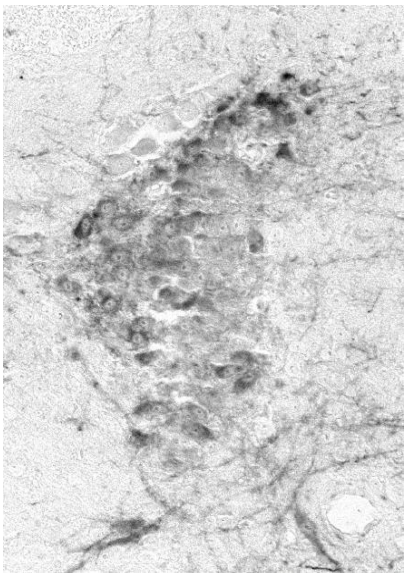
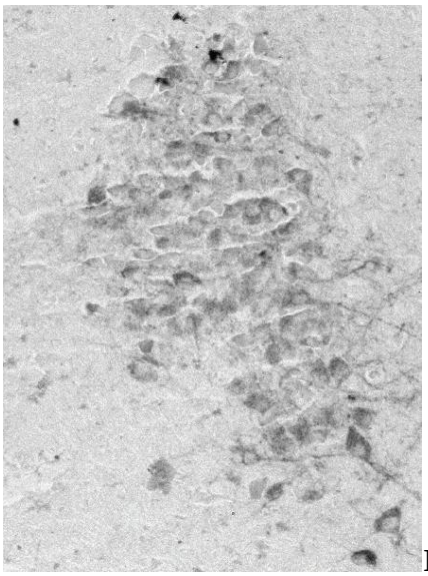


Figura 15. Number of TH immunoreactive neurons in the SNpc of adult mice.



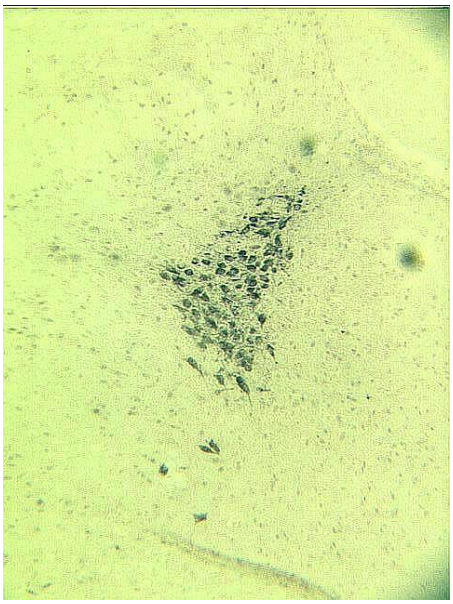
A



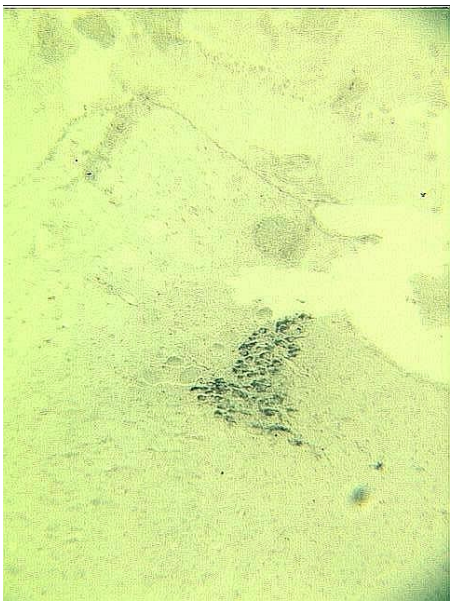
B

Picture 1. TH immunopositive neurons in the locus coeruleus of adolescent mice.

A. Female OIL; B. Male OIL.



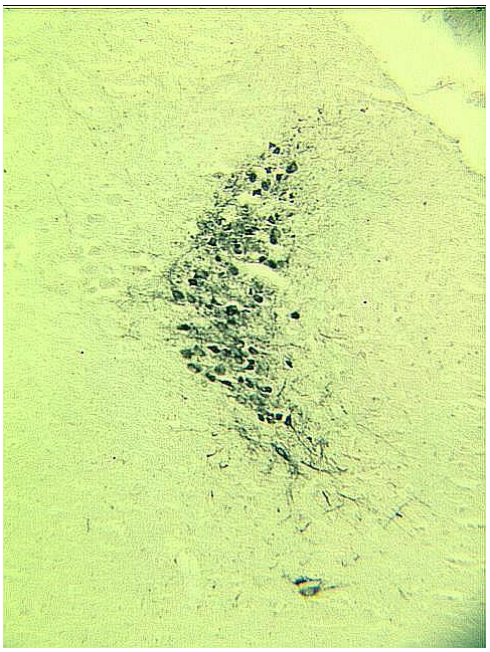
A



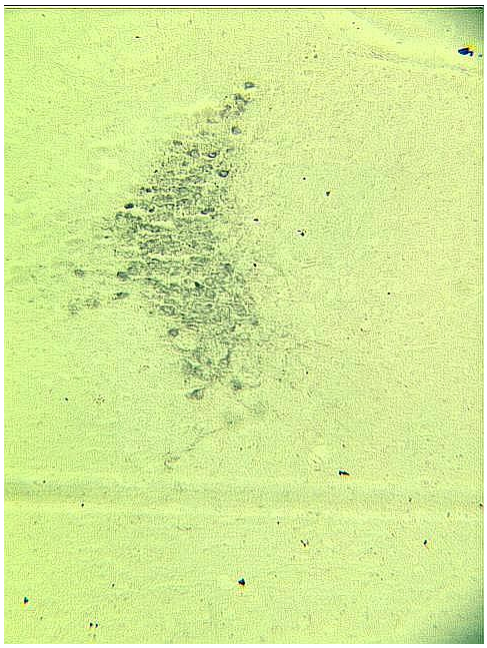
B

Picture 2. DBH immunopositive neurons in the locus coeruleus of adolescent mice.

A. Female OIL. B. Male OIL.



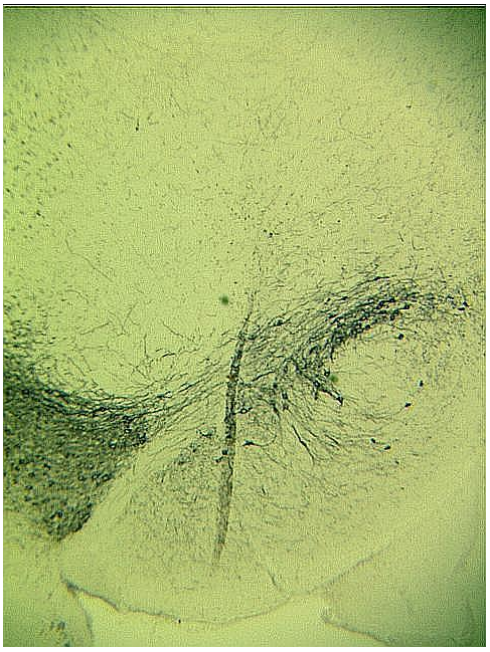
A



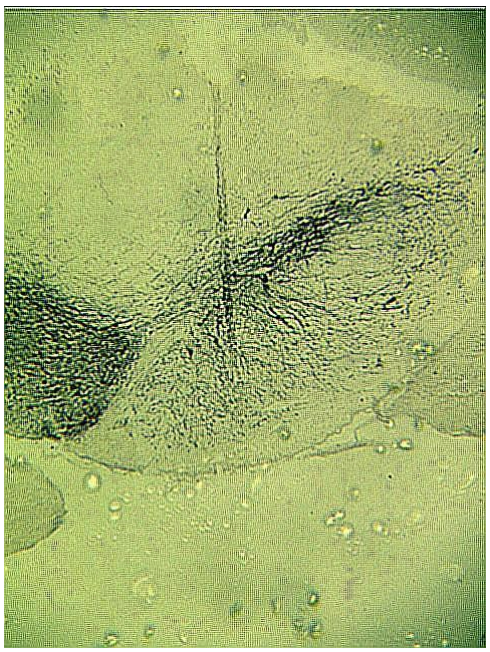
B

Picture 3. TH immunopositive neurons in the locus coeruleus of adult mice.

A. Female OIL. B.Male OIL.



A



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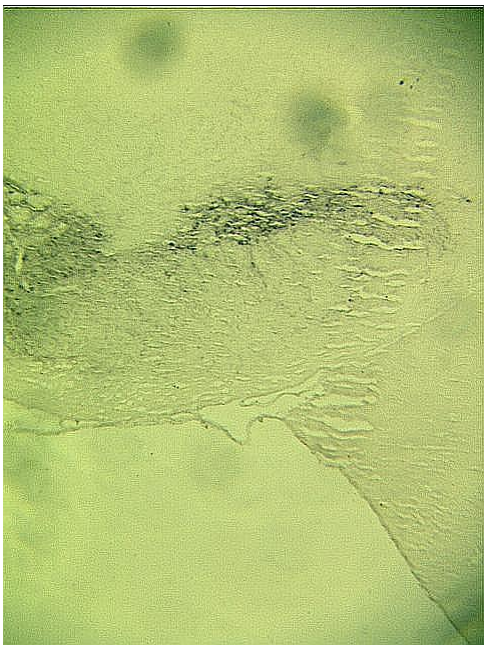
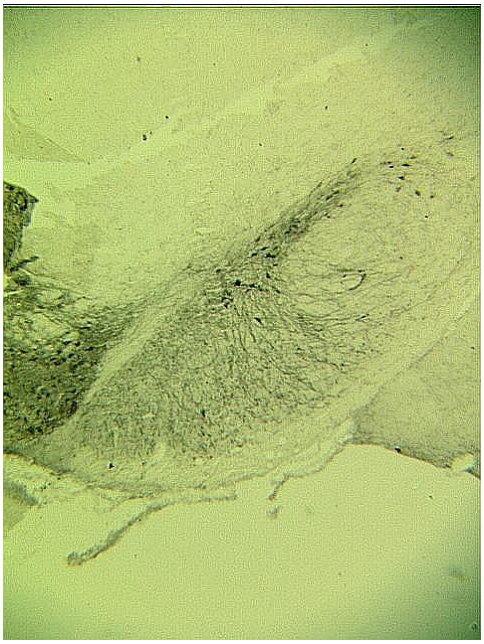


C

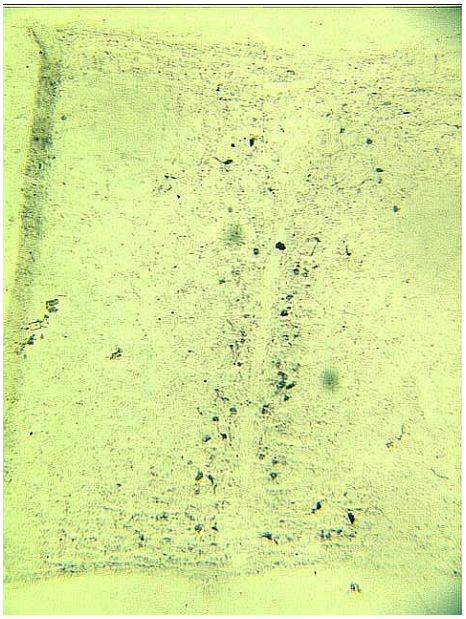


D

Picture 4. TH immunopositive neurons in adolescent and adult mice

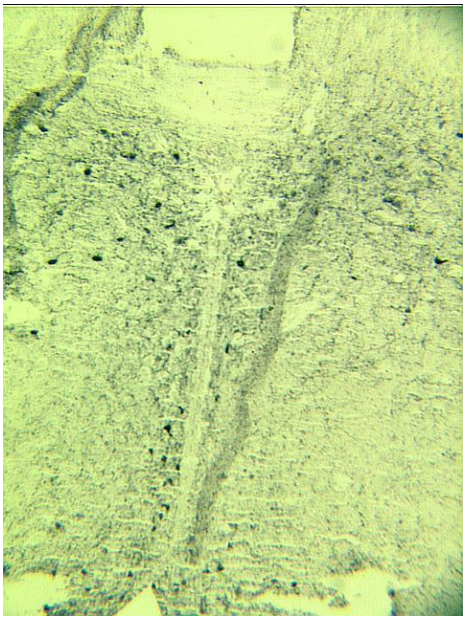
A. Young female, OIL. B. Young male OIL.

C. Adult female OIL. D. Adult male OIL.

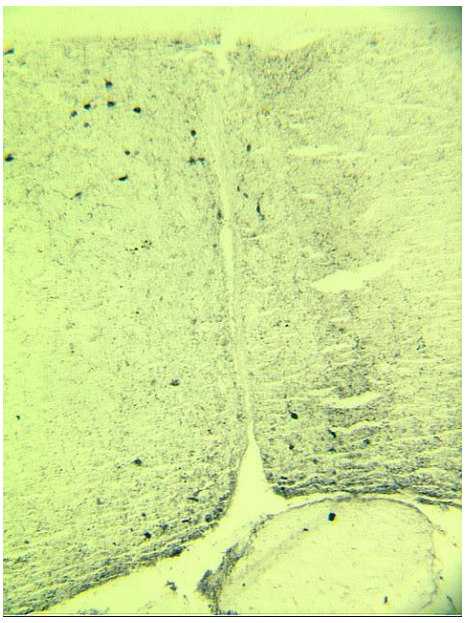


A

B



C



D

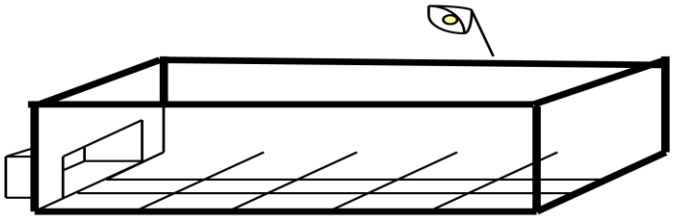
Picture 5. TH immunopositive neurons in the AVPe neucleus.

A. Adult female, OIL. B. Adult male, OIL.

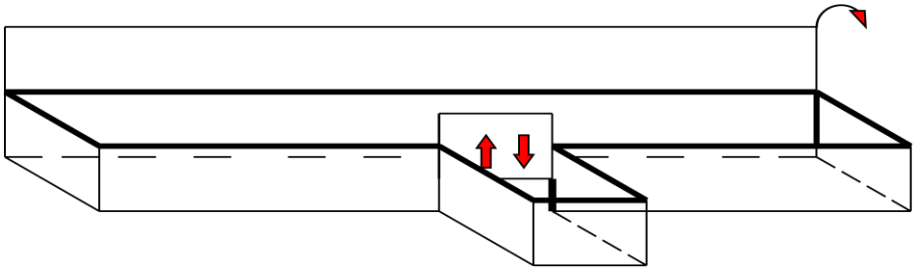
C. Adult female, DES high dose. D.Adult male, DES high dose.

APPARATUS USED FOR BEHAVIORAL STUDIES.

OPF



T-maze



4. POSTNATAL EXPERIMENT: EFFECTS OF TWO DIFFERENT DOSES OF BPA ON THE DEVELOPMENT OF NON SEXUAL BEHAVIORS

4.1 INTRODUCTION

Since the Barker' epidemiological study on the origin of diseases early in life that led to the definition of "the Barker hypothesis" (Barker et al., 1993; Osmond et al., 1993; Barker and Osmond, 2000), more interests have been put by the scientific community on the possible relation between developmental exposure to environmental pollutants and mental disease manifested in adulthood. Several studies and reviews showed a relation between the exposure to several toxicants and the incidence of degenerative and pervasive diseases such as Parkinson, Alzheimer and autism. A sexual specific incidence of these diseases has been already shown. As in the case of Parkinson and autism, (Dluzen et al.,1998; Shulman, 2007; Simon Baron-Cohen at al., 2005) such sex bias rose the hypothesis that the organizational action of gonadic steroids during perinatal life may play an important role. In these pathologies aspects of the affective and motor behaviors are impaired. Specifically one of the neural system mainly involved is the catecholaminergic system, sensitive to the action of prenatal and developmental exposure to low doses of BPA. This modality of exposure can affect the monoaminergic system and related behaviors, inducing to hyperactivity and to impaired aspects of emotionality (Gioiosa et al., 2007; Ishido et al., 2004, 2005; Masuo et al., 2004; Mizuo et al., 2004; Suzuki et al., 2003).

Another anatomical structure that is damaged in Parkinson and Alzheimer disease is the olfactory system (Doty, 2003), a system that present dopaminergic and noradrenergic innervations. The survival and development of newborn rodents depend on environmental cues that come from other conspecific. More precisely the chemical signals coming from the nest, which is impregnated of the maternal and littermates odors, play an important role in learning social behaviors. A pup that is taken away from its mother will react emitting distress calls that will suddenly cease when physical or olfactory maternal cues are presented. At the same time, when the motor skills of the pup are mature enough to allow her a more

independent locomotion, the newborn reacts chemotactically moving toward the maternal odor source, such as the maternal soiled bedding (Hofer et al., 1980; Shair et al., 1997; Leon et al., 1971, 1972). The ability to discriminate important surviving odor cues seems to occur during late gestation, leading to the idea that this skill is in part inborn. In fact newborn rodents can discriminate and shows preference between cues, odors, associated with the amniotic fluid, a process termed intrauterine learning (Hepper, 1987; Pedersen and Blass, 1982). They then sharp this ability postnatally experiencing odors belonging to the maternal nest, a phenomenon known as neonatal learning. Based upon such discriminative skills it can be inferred that animals are able to recognize individuals or classes of conspecifics in order to increase their own survival and future socio-reproductive success.

The precise neural-anatomical systems playing a role in such developmental learning behavior are not well clear yet: both the main olfactory system (MOS) and the accessory olfactory system -vomeronasal (AOB)-may participate (Porter and Schaal, 2003). Nevertheless, it has been suggested that an important neural circuit between the olfactory bulb (OB) and the locus coeruleus (LC) is essential for the rapid odor preference learning. This system would play an fundamental role during a sensitive learning period, when the pup is unable to move and protect himself (Moriceau and Sullivan, 2004; Sullivan, 2003). It is not clear which developmental effect the gonadic steroids have on the olfactory system, even though it is well known that androgens organize the rat vomeronasal system in a sexually dimorphic way (Guillamon and Segovia, 1997). Nonetheless, since the developmental effect of estrogen on dopaminergic and noradrenergic systems have been demonstrated(Beyer et al., 2000; Ivanova et al., 2003; Kipp et al., 2006), it could be suggested an indirect effect of sex steroids on the olfactory system and maternal odor conditioning development through the catecholaminergic system.

4.2 METHODS

4.2.1 Procedure

Cd-1 mice (*Mus musculus domesticus*) were maintained in an outbred colony at the University of Missouri. The animals were housed in 18 x 29 x 13 cm polypropylene mouse cages on corncob bedding. Pregnant and lactating females were fed Purina 5008 (soy-based) breeder chow, and after weaning, animals were fed Purina 5001 (soy-based) chow. Water was

provided *ad libitum* in glass bottles and was purified by ion exchange followed by a series of carbon filters. Rooms were maintained at 25 ±2 C under 12:12-hr light:dark (L:D) cycle, with lights on at 1100 hr. Adult 4 months old virgin female mice were time-mated by being placed into the cage of a stud male for 4 hours beginning at 0800hr. Mating was verified by the presence of vaginal plug (day 0 of gestation) and pregnant females were housed three per cage.

4.2.2 Chemical Administration

At postnatal day 1 (PND1), each litter was reduced to 12 pups (6±1 males, 6±1 females), weighted and left with their lactating mother. Each litter have been weighted at PND5, PND15 and PND21. From PND1 to PND15, each pup underwent the following feeding schedule depending on the group of treatment: corn oil alone (MP biomedicals, Aurora, OH, USA; control group; n=12), two different doses of BPA (Sigma Chemical, St. Louis, MO, USA): 20 μg/kg body weight (low dose, n=12) and 200 μg /kg body weight (high dose, n=12) dissolved in corn oil. The doses were delivered directly into the mouth of the animals. After have removed the mother from the home cage in order to reduce as much as possible the stressful experience, pups were picked up by the skin between the shoulders and held upright. The pipette tip was placed into the mouth with the pipette gently touching the roof of the mouth, and the oil was ejected from the pipetter. Mice readily consume corn oil.

4.2.3 Behavioral experiment

4.2.3.1 pup's selectivity approach

In this experiment one 10 days old pups of each sex from each experimental litter where randomly chosen to be tested for the discrimination of a maternal or unrelated odor cues. Pups were placed in a small T-maze made of plastic, with a 7 X 4 cm small arm crossed at one of its end by a 34 X 7 cm long arm. The small and long arms were separated by a guillotine. Prior to each test, the maze was carefully cleaned with 50% alcohol and well dry. The small tube and the entrance, central part of the big tube, were covered with clean bedding each time while in each of the two sides of the big arm was placed either maternal or unfamiliar nest bedding. The unfamiliar nest bedding was picked from the nest of a lactating female with offspring of the same age. The maternal side was switched alternatively between the right and left sides of the maze: in this way the position was balanced between sexes and

treatments, to avoid any visual and position preference. Then, one pup was placed in the short arm for 1 minute in order to familiarize with the apparatus. After the familiarization period, the guillotine was removed and the latency to enter the big arm and the time spent on each side were recorded. The total experiment lasted 5 minutes and the following behaviors have been observed:

- latency to enter the maze;
- latency to reach the maternal side;
- Time spent in the maternal arm;
- Time spent in the unfamiliar arm;
- Time spent in the centre;
- Number of transitions.

4.2.3.2 Spontaneous activity

One adult mouse from each sex and litter was randomly chosen for the test of spontaneous activity. Each female was tested for vaginal smears and females in clear estrous, presenting cornified cells, were chosen for the experiment. Each mouse was isolated in a home cage at 4 oclock in the afternoon, 4 hours before the onset of the nocturnal phase, and placed in the experimental room with 4 pellets of food and water *ad libitum*. Each experiment started at 8000 clock, dark phase onset. The locomotor behaviors where automatically analyzed by the program ANYMAZE in a dark room with red lights. The observations lasted for 5 hours and the following behaviors where recorded:

- Total distance covered;
- Frequency of mobile episodes.

4.2.4 Statistical analysis

T student statistical analysis was performed in order to observe if any sexual difference in the same group of treatment was present for each variable studied. All the probabilities reported are tested for an alpha levels (α) less than $p=0.05$, only in presence of normal distribution and equal variance. When this was not the case a log or square transformation was used. Planned comparison of same sex means across the different treatments were made using a one way ANOVA and in presence of a $p< 0.1$ it was followed by the Bonferroni post hoc test. Any effect of the treatment and any interaction of treatment with sex were analyzed by a factorial analysis of variance (ANOVA) as indicated using the program Statistica

(StatSoft). In case of statistically significant effects, post hoc comparisons of differences between group means were made using the Fisher least significant difference (LSD).

4.3 RESULTS

4.3.1 Effects of postnatal exposure to BPA on pup selectivity approach

Ten days old female mice treated post-natally with 200µg/kg/d of BPA where slower to reach the maternal side of the maze than the same group males (t=2,16; p=0.04; male(10)=0.26±0.10; female(11)=0.67±0.16). This sexual dimorphism was observed neither in the control group nor in the animals from the lower dose of BPA. When the time spent in the maternal side, in the centre and the number of transitions were analyzed no effect of the sex and treatment was found. When the latency to enter the maze was analyzed by a two way ANOVA, it was clear an effect of the treatment ($F_{(2,52)}$=3.20; p=0.04). A Fisher LSD post hoc showed that mice that had being exposed to 20µg/kg/d of BPA entered the maze faster (p=0.02).

4.3.2 Effects of postnatal exposure to BPA on spontaneous activity in adult mice

Adult males exposed posnatally to the lower dose of BPA were more active than the females from the same group of treatment (t=-2.65; p=0.01; males (8)=30.70±2.19; females(9)= 20.08±2.99). When the frequency of mobile episodes was considered as another measure of activity, again males exposed to the lower dose of BPA resulted more active than the females of the same group of treatment (t=-1.79; p=0.09; males=22.87±4.79; females=13.57±4.23).

4.4 DISCUSSION

The overall result of this experiment is, once again, that the developmental exposure to low but relevant doses of BPA alters some behavioral sexual dimorphism of the. In this experiment my main concern was to analyze behavioral models of learning and activity, characteristics that are negatively involved in neurodegenerative and developmental diseases. In the first experiment, I observed that females developmentally treated with the higher dose of BPA reached maternal side of the maze later than males from the same group. This is an

interesting finding because the performance of this behavior is not expected to be sexually dimorphic, since the the maternal odor imprinting has the fundamental evolutionary meaning of increase the survival of an helpless pup. Pups must learn to associate the maternal odor cues with nest protection, feeding and all the related maternal behaviors important for neurobehavioral development. This will probably occur through an autonomic activation or hedonic response that reduces the pup stress. Stop of distress calls and decrease of sympathetic activity are seen in animal and human newborns when presented with odors from the mother (Doty, 2003; Porter and Shaal, 2003). From a functional and anatomical point of view, a major role for the rat maternal odor learning during the first 10 days of life seems to be played by three areas: the olfactory bulb and locus coeruleus (Sullivan, 2004). As pointed out in the introduction, it is not clear when and how gonadic steroids play a role in the development of the olfactory system and so if the exposure to the higher dose of BPA is acting as an estrogen or anti-androgen. Another possibility is that the higher dose of BPA is acting as a neurotoxicant only in females: this would either affect mainly the olfactory system or the development of the noradrenergic system.

A speculative observation of the data shows that these females have higher latency to enter the maze; this could mean either that they don't react to the maternal stimuli or that they can effectively react to the maternal odor orientating themselves toward it once they enter the maze, but that they have some impairment of the adaptive distressful reaction of maternal displacement. Such observation would be interesting, since mice exposed prenatally to the lower dose of BPA show an opposite, hyper-reactive trait: they enter the maze quicker than any other group. Unfortunately I couldn't be able to record either any behavior of risk assessment during the test or perform an experiment of retrieving: measures that would have been very helpful inorder to unravel this question. In such a frame work, a suggestion for future studies would be to consider the development of the vagal-parasympathetic and sympathetic systems and the relative coping response to stress in BPA treated mice. Another consideration can be pointed to the mother-offspring interaction as a source of this variability, with a differential disposition of the caregiver toward one sex and not the other, as in the case of the higher dose of BPA. It is worth to note that a decrease of maternal odor conditioning response was shown in animal developmentally exposed to polychlorinated biphenyls (PCBs) (Cromwell et al., 2007), chemicals that has already been shown to affect several neurobehavioral aspects of human babies such as IQ and hyper-hyporeflexia, (Young-Chen and Chen-Chin, 1992; Rogan et al.,1986; Jacobson and Jacobson, 1996). Although PCBs are

different from BPA in their structure and modality of action, some negative effects on rats learning ability in active and passive avoidance tests after BPA perinatal exposure have been demonstrated (Negishi et al., 2004). When adult mice were observed for levels of spontaneous activity, I found a clear effect of the lower dose of BPA: males were more active than females, a dimorphism that was not evident in the control group. The result that developmental exposure to low but environmentally relevant doses of BPA induce hyperlocomotion in rats has already been shown (Ishido et al., 2004, 2005; Masuo et al., 2004; Mizuo et al., 2004; Suzuki et al., 2003) and in the previous study I couldn't be able to observe any effect on the sex by the prenatal exposure to BPA. Nonetheless it is interesting to observe that once again, BPA acted in a sex specific way, creating for this particular behavior a sexual dimorphism that was absent in the untreated animals.

4.5 PICTURES AND GRAPHS

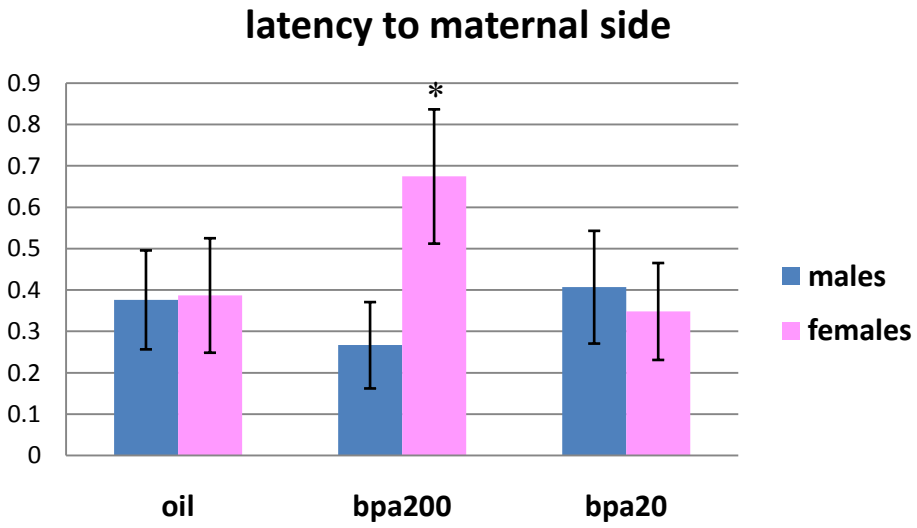


Figure 16. Latency to reach the maternal side of the t-maze in 10 days old mouse pups. Values are in %time.

*p<0.05

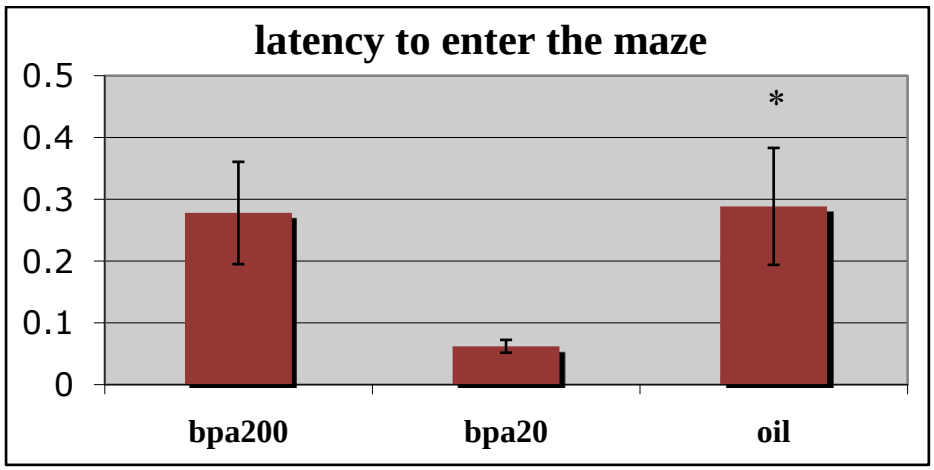


Figure 17. latency to enter the maze in 10 days old mouse pups. Values are in %time.

*p<0.05

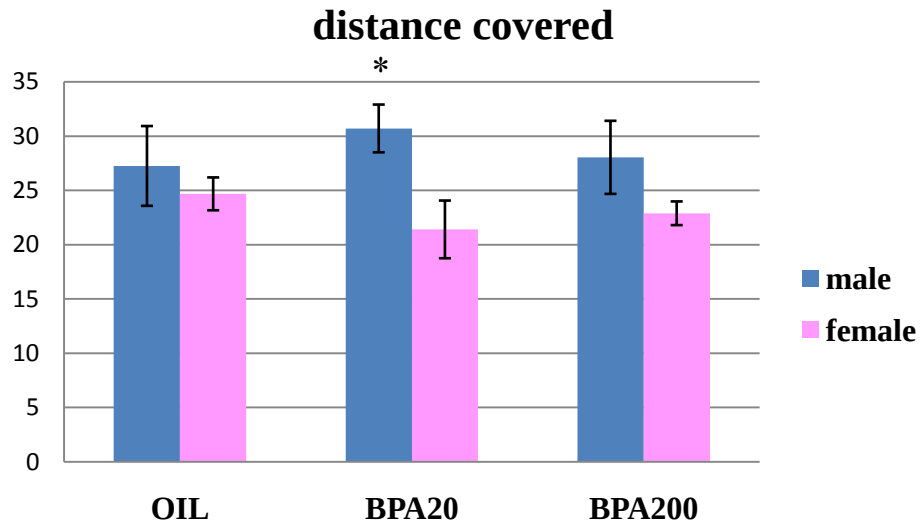


Figure 18. Distance covered by adult mice in a test for spontaneous activity in the home cage. Values are in meters. *p<0.05

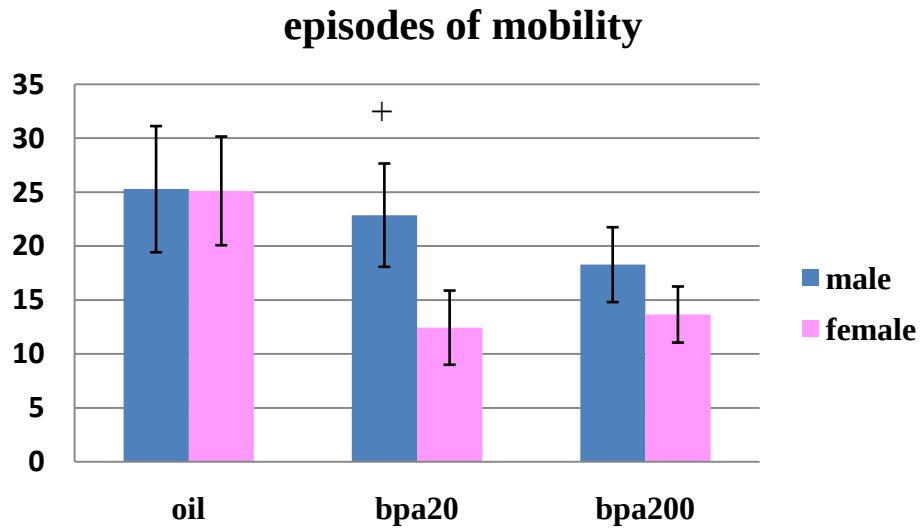


Figure 19. episodes of mobility in a test of spontaneous activity in the home cage in adult mice. Values represent absolute frequencies.

+p<0.1

5. GENERAL CONCLUSIONS

In my work of thesis I decided to analyze the effect of the pre- and post-natal exposure to low and environmentally relevant doses of Bisphenol A (BPA), two specific and sensitive periods for neurobehavioral development in the house mouse (*Mus musculus domesticus*). BPA is a known environmental estrogen used in the manufacture of polycarbonate plastic, the resin used in the lining of food and beverage containers, dental sealants, and as an additive in other products such as house furnitures and domestic tools. Its yearly high volume production and its virtual presence everywhere in the environment place a remarkable alarm for the risks that come from its exposure. For example, the main source of humans exposure comes are beverages and the foods contained in polycarbonate cans and landfills leached water. Human exposure to significant amount of BPA is continuous (Crain et al., 2007; vomSaal and Hughes, 2005). Studies on BPA estrogenic activity appeared first at the beginning of the last century. In the 1950s , BPA was for the first time used in the synthesis of polycarbonates; although for long time its estrogenic activity was considered "weak", growing literature is showing how very low BPA doses can mimic estradiol activity through both classical and non classical receptors. Furthermore it is starting to be accepted the fact that BPA, at levels that are in the range of human exposure, acts as a SERM: interacts differently from estradiol within the ligand-binding domain of ERs, has different binding affinity toward ERs and cell specific effects within the same tissue (vom Saal and Welshons, 2006; Welshons et al., 2006). This rises concern to its metabolically and physiologically activity on humans. During the embryonic life gonadic steroids, such as estrogen and testosterone converted to estrogen by the enzyme aromatase, permanently organize the brain in a sexually dimorphic way. The development of a sexual dimorphism in regard to behavior, cognitive functions and specific brain regions depends upon the epigenetic actions of estrogens and androgens, even though in some cases the effects seems to modulate or redefine a genetic based sexual difference (Kipp et al., 2006; Simerly 2002; Reisert and Pilgrim, 1991). During the sexual differentiation of higher vertebrates, such as mammals and birds, the sex determination depends on genes that lead the gonadic development. Gonads produce and release gonadic hormones, androgens and estrogens, which the final action is to determine the typical sex specific development of the brain (Phoenix et al., 1959; Whalen, 1974; McEwen, 1981). The gonadic steroids act on the development of the brain binding to

the intracellular and membrane receptors for steroids, namely androgens (AR), estrogens (ER) and progesterone (PR). After the steroid-dependent sexual differentiation of the brain, estrogens, androgens and progesterone play an important role activating socio-sexual behaviors in adulthood. Typically after puberty, the neural modifications induced by hormones reinforce the ontogenetic predetermined sexual differences in the brain, which are the source of the sexual differences observed in the behavior (Crews, 1993; Gahr ,1994).

Thus in this theoretic and empirical framework my general hypothesis was based on the following: given the BPA estrogenic and SERM activity, its ability to pass through the placenta and the blood brain barriers and the presence of low levels of drug-metabolizing enzymes in the embryonic brain (Kawato 2004), its low, environmentally relevant, dose exposure can affect the correct development of the fetus CNS, eliminating the sexual differences. More specifically, since it has been shown that prenatal and developmental exposure to BPA in rodents can affect the catecholamine modulated non sexual behaviors such as general levels of activity, exploration and emotions (Gioiosa et al., 2007; Kubo et al., 2001, 2003, Rubin et al., 2006; Fujimoto et al., 2006; Negishi et al., 2004), my main goal has been to observe how such BPA and DES specific exposure could negatively influence the catecholaminergic development of specific nuclei and related activity levels and learning behaviors. The general results of this work could be summarized in three main points:

a) Sexual dimorphism: looking at the two different experimental phases of exposure, a common trend can be drawn among the effects caused by BPA and DES. Both compounds act specifically and differently upon the sexes. I demonstrated how two doses of BPA and DES can reduce the sexual differences of some neurobehavioral aspects of the mouse, such as explorative behaviors and number of neurons synthetizing Noradrenaline (NE). Conversely I also demonstrated that when no sex specific differences were presented in the control group, the developmental exposure to EDCs acted specifically on one sex determining a sexual dimorphism, such as in some aspects of the explorative, learning and locomotor activity. in conclusion, these effects give the potentiality of the endocrine disrupters used to eliminate or increasing the differences between the sexes.

b) Hyperactivity: a consistent effect on the general activity has been demonstrated when BPA has been administered either pre- or postnatally: in a test of Open Field, mice treated prenatally with both the EDCs increased the time spent being mobile. This is an interesting and important point because it is consistent with previous finding from other laboratories, where the post-natal injection of a low dose of BPA affected the

dopaminergic system yelding to hyperactive rats (Ishido et al., 2004; Masuo et al., 2004, 2004; Mizuo et al., 2004). When observing the results from the other behavioral experiments it is clear that the exposure to BPA affected also the sexual differences at the basis of explorative and affective behaviors: although this outcome is generally consistent with some results from other studies, dipping down in the specific of my experiments some levels of conflicting data become evident. For example, the incapability to find females more active than males during the OPF and spontaneous activity tests: this is evidently caused by differences in the experimental settings and possibly for a lack of statistical power.

c) Estrogenic activity of BPA: analyzing my results it is clear that the action of BPA cannot be considered always estrogenic. In order to asses this point I used a low dose of DES as a positive control: an evident discrepancy between DES and BPA actions was present in several neurobehavioral endpoints took in consideration. For example in some cases, the prenatal exposure to DES increased the sexual differences while animals treated with BPA were not statistically different from the control group (spontaneous activity). In other cases I observed the opposite effects (explorative behaviors). In some other cases it was clear a sex specific effect of DES, but BPA action was upon the opposite sex, as in the case of the development of locus coeruleus TH positive-neurons. These results are even more complicated if we consider the different experimental contexts, since the functional value of a behavior changes if the natural or experimental situation changes. For example let's take in consideration the levels of activity: in the OPF, DES and the two doses of BPA seem to have a similar hyperactive effect in both sexes. Analyzing the identical variable but in a different setting, it is also evident how these compounds affect the sexes in a different way: this is the case of the test for spontaneous activity, were the estrogenic effect of DES is directed to the males, making them hypoactive, while no such effect was evident for BPA. At this time one point must be highlighted, namely that analyzing behavior, the dose of DES used may not be comparatively proper for the BPA doses. The estrogenic activity of BPA is 10 to 1000 time lower than DES (Richter et al., 2007); we used a 100 fold lower dose of DES compared to the lower dose of BPA. This level can be use to predict the estrogenic activity of low doses of BPA, but that it may not be the right one to test the same prediction, or at least to some how elucidate it, in a behavioral model.

In general it can be concluded that based on this behavioral animal model, I can refused the initial hypothesis since the prediction was based on the presumed neurobehavioral effects of BPA estrogenicity. Although some effect on the sexes has been observed, it is not

clear if they are caused by the estrogenic activity of BPA. Furthermore, while I found effects of BPA developmental exposure on the motor and emotional behaviors and on the catecholaminergic system, there wasn't a causal link between them. This once again brings up concern about the usefulness of my animal model in order to study the effects of BPA: evidences from the literature shows that the animal behavioral model cannot explain the proximal mechanisms at the basis of BPA organizational effects. Thus, until the exact tissue specific, molecular and biochemical actions of BPA are not clarified, any behavioral model risks to produce results difficult to unravel. For example, the setting test resulted to be fundamental as it was expected to be from the literature. For example, levels of activity in a safe environment and in a open field are expected to be different and dependent on estrogen levels (Archer, 1975; Morgan et al., 2004), possibly by the organizational effects of sex steroids. Lacking the knowledge of a BPA specific estrogenic activity resulted not useful in order to explain the different results obtained in this study.

Nonetheless, a wide literature consistent finding of BPA exposure on the levels of activity, emotionality and on the catecholaminergic system was found. This agrees with observations made by others and makes no doubt of such existent BPA effects. What it needs to be done is understand how BPA act at the molecular level within the noradrenergic or dopaminergic system, if it mimics estradiol, and maybe try to connect the effect of BPA and hyperactivity with some other system sensitive to the behavioral response in a novel environment, such as the autonomic nervous system and investigate its development.

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