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Neuropeptides and enzymes are targets for the action of endocrine disrupting chemicals in the vertebrate brain

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Running title: Endocrine disruptors, neuropeptides, and enzymes

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Abstract.

Endocrine disrupting chemicals (EDCs), are molecules that can interfere with endocrine signalling pathways and cause adverse consequences on animal and human physiology, inducing infertility or diseases, including behavioral alterations. Some EDCs are acting through their binding to androgen or/and estrogen receptors primarily operating through a genomic mechanism regulating gene expression. This mechanism of action may induce profound developmental effects, and the major targets of the EDCs action are the gene products, i.e. mRNAs inducing the synthesis of various peptidic molecules, that include neuropeptides and enzymes related to neurotransmitters' synthesis. Here, we briefly review available immunohistochemical data on some of the systems that are affected by EDCs in lower and higher vertebrates.

Keywords. estrogens; androgens; diethylstilbestrol; genistein; DDE; bisphenol A; vasotocin; vasopressin; kisspeptin; neuropeptide Y; aromatase; tyrosine hydroxylase; nitric oxide synthase.

INTRODUCTION

Several chemical signals guide the development and differentiation of the central nervous system: many of them are originated by the endocrine system. In particular, among the hormonal signals with high impact on brain development, gonadal hormones, such as 17β -estradiol (E_2) or androgens, play key roles in the development of primary and secondary sex characteristics in higher vertebrates, as well as in that of several steroid dependent behaviors and/or neural circuits. In particular these hormones may modulate neural differentiation (i.e., cell migration, survival and apoptosis, synaptic plasticity) through their peculiar action at genomic level. Gonadal hormones may, in fact, bind to cytoplasmic receptors that, when activated, migrate to the nucleus where they act as transcription factors and regulate the expression of specific mRNAs, even if other non-genomic mechanisms are also present (for a recent review see (McCarthy, Wright, and Schwarz 2009)).

Several experimental studies demonstrated that disturbing the hormonal milieu during specific periods of pre- or postnatal development (critical periods), via exogenous hormonal treatment or gonadectomy may induce irreversible changes in the organization of the central nervous system as well as behavioral alterations in many vertebrate species (Wallen 2009). Therefore, the developmental exposure to some industrial pollutants or natural molecules, belonging to the category of endocrine disrupting chemicals (EDCs), that are able to bind to gonadal hormone receptors (xenoestrogens, XE, or xenoandrogens, XA) may induce adverse effects on endocrine structures development, and therefore may affect humans, farm animals and wildlife (McEwen 1987; Panzica, Viglietti Panzica, and Ottinger 2005).

Due to their estrogenic or androgenic effects, XE or XA could, even in very low concentrations, deeply influence the development and the function of neural circuits

and behaviors. In fact, behavioral responses represent the culmination of several integrated systems, and even small changes of neural or neuroendocrine components are likely to disrupt or modify behavior. Importantly, disturbances in normal behavior may influence the individual fitness and, therefore, acquire a real biological significance in both animal and human ecosystems. Due to their action as transcription factors, activated gonadal hormones' receptors regulate the synthesis of appropriate mRNAs for the production of peptides or propeptides that are involved in the control of several physiological activities (Scholz et al. 2010).

Neuropeptides and behavior

The ubiquitous nature of neuropeptides and of their respective receptors in the central and peripheral nervous systems suggested, from the time of their first identification as hypothalamic hormones, that they may play a key role in controlling several physiological processes (for a review see (Guillemin 2005). Investigations at a cellular level have demonstrated that neuropeptides exert powerful modulatory effects on neurons and neuronal circuits (De Graan, Schotman, and Versteeg 1990). In addition, several systemic studies have addressed the influence of neuropeptides on many behavioral processes as learning and memory [vasopressin and CRF(Gulpinar and Yegen 2004)], social behaviors [oxytocin and vasopressin (Carter 1998; Young and Wang 2004; De Vries and Panzica 2006; Goodson 2008; Heinrichs and Domes 2008; Veenema and Neumann 2008; Ferguson, Young, and Insel 2002; Skuse and Gallagher 2009)], sexual behavior and reproduction [GnRH, substance P, NPY (Argiolas 1999), Kisspeptin (Greives et al. 2008; Roa et al. 2008)], feeding behavior [NPY, POMC (Beck 2000; Magni et al. 2009)], or anxiety-related behaviors [vasopressin (Landgraf 2001), NPY (Kask et al. 2002)].

Endocrine disruptors and neuropeptides

Many studies investigated cellular effects of EDCs in *in vitro* systems (Pelissero et al. 1996) or in lower vertebrates (Sumpter 1998; Hutchinson et al. 2000). In recent years, several laboratories, including our, started to investigate the effects of precocious exposure to EDCs on both behavior and neural circuits in higher vertebrates [for reviews see (Gore 2008; Panzica et al. 2007; Panzica et al. 2009)]. In many cases neuropeptides or proteins were affected by the exposure to EDCs.

Arginine-vasopressin and related peptides

Arginine-vasopressin (AVP) in mammals or related peptides as arginine-vasotocin (AVT) in non-mammalian vertebrates, are present in magnocellular and parvocellular systems of hypothalamus and limbic system (De Vries and Panzica 2006). The AVP/AVT parvocellular system located within the bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA) is sexually dimorphic in mammals (De Vries, Buijs, and Van Leeuwen 1984) and other vertebrates [fishes, (Parhar et al. 2001); amphibians, (Moore and Lowry 1998); reptiles, (Hillsman, Sanderson, and Crews 2007); birds (Viglietti-Panzica et al. 1992; Panzica et al. 2001); for a review of comparative aspects see (Goodson and Bass 2001)], and it is involved in the control of male copulatory behavior in birds (Panzica et al. 2001) and in the modulation of aggressive behavior and other social behaviors in mammals and other vertebrates [rat (Everts, de Ruiter, and Koolhaas 1997; Popik and Van Ree 1998; Veenema and Neumann 2008); mouse (Scordalakes and Rissman 2004); zebra finch (Goodson and Adkins-Regan 1999; Goodson, Lindberg, and Johnson 2004); green anole lizard (Hattori and Wilczynski 2009); tungara frog (Kime et al. 2007; Kime et al. 2010); goldfish (Thompson et al. 2008)]. Administration of exogenous estrogens

demasculinizes this system in birds (Panzica et al. 1998), whereas in mammals the situation is more complex and both estrogens and androgens may contribute to the masculinization of the system (Han and De Vries 2003; Allieri et al. 2005; Plumari et al. 2002; Pierman et al. 2008). In adulthood, gonadal hormones modulate the expression of AVP or AVT both in the BST/MeA system [rat (De Vries, Buijs, and Sluiter 1984); Japanese quail (Viglietti-Panzica et al. 1994); bullfrog (Boyd 1994)], and in magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei [rat (Grassi et al. 2010)]. For these peculiar features, the avian AVT system (Panzica et al. 2002), and partially the mammalian AVP system (Kodavanti and Curras-Collazo 2010), has been considered as a potential target for the action of EDCs.

In the Japanese quail, embryonic exposure to estradiol benzoate (EB), prior to day 12 of incubation, irreversibly demasculinizes both adult male copulatory behavior and parvocellular AVT system of BST (Panzica et al. 1998). To test the estrogenic activity of different XEs we injected them into the eggs at day 3 of incubation [in fact, exogenous EDCs are eaten by the mother and accumulated within eggs (Lin et al. 2004)]. When adult (two-month old) we tested their effects on both male sexual behavior and AVT immunoreactivity.

Brain changes were specifically analyzed in our studies. In particular, we observed (Fig. 1) a significant demasculinizing effect of diethylstilbestrol (DES), genistein, or ethylene, 1,1-dichloro-2,2-bis-p-chlorophenyl (DDE) on the sexually dimorphic parvocellular AVT system of BSTm and on its projections to medial preoptic nucleus and lateral septum (Viglietti-Panzica et al. 2005; Viglietti-Panzica, Mura, and Panzica 2007; Mura et al. 2009). For other EDCs the effects on this neural system were not significant or absent. For example, ethynylestradiol (EE₂) and

methoxychlor (MCX) both affecting male sexual behavior (Halldin, Axelsson, and Brunstrom 2005), did not induce alterations of the AVT system (Mattsson et al. 2008). The lack of effect of EE₂ is surprising due to its large use in mammals as substitute of E₂. However, our studies suggested that behavioral demasculinization and demasculinization of the VT-ir system are not completely correlated and varies according to different estrogens or xenoestrogens. In fact, while EB-dependent behavioral demasculinization is complete at both 10 and 25µg/egg, the decrease of the VT-immunoreactivity in POM, BSTm and SL is less pronounced (60% instead of 90%) when eggs are exposed to lower levels of EB (Viglietti-Panzica et al. 2005). This difference in sensitivity is also evident when considering the effects of xenoestrogens: DES is fully demasculinizing the copulatory behavior (Viglietti-Panzica et al. 2005), whereas genistein or DDE have significant, but limited, effects (Viglietti-Panzica, Mura, and Panzica 2007; Mura et al. 2009). In spite of this, if we compare the effects of DES, genistein or DDE on the VT-ir system we found similar significant decrease which is not comparable to that induced by 25µg/egg of EB (Fig. 1). The dissociation between demasculinization of the behavior and demasculinization of the VT-ir system shown in these studies suggests that these effects are induced by estrogens via at least partly different pathways. In the study of Mattson et al. (Mattsson et al. 2008), 0.3 µg/egg of the positive control EE₂ significantly decreased but not totally abolished male copulatory behavior, at the same time it did not reduce the VT-ir in POM, BSTm and SL. Possibly, a dose of EE₂ which completely suppresses the behavior could induce an effect also on the VT-ir system.

In addition, in the same study, we demonstrated, through the administration of an ER α selective agonist, propyl pyrazole triol (PPT), that estrogen-induced effects on reproductive organ differentiation are mediated by ER α , whereas

demasculinization of male copulatory behavior and of the AVT-immunoreactive (ir) system is not induced by activation of ER α alone (Mattsson et al. 2008). Therefore, we hypothesized ER β should have a primary role for the development of these circuits and behavior. This is also supported by the observation that, during development of hypothalamic and limbic regions of Japanese quail, ER α appears later than ER β (Axelsson et al. 2007). Future experiments should test this specific point.

In mammals, acute exposure to E₂ stimulates the synthesis of the mRNA AVP (Roy, Reid, and Van Vugt 1999) and induces a significant increase of AVP immunoreactivity in magnocellular nuclei of female rat (Grassi et al. 2010). AVP system has been investigated in rats after dietary exposure to the phytoestrogen genistein (from prenatal day 7 up to the age of 2 months and a half) (Scallet et al. 2003), or after exposure to polybrominated or polychlorinated compounds (Coburn, Gillard, and Curras-Collazo 2005; Coburn, Curras-Collazo, and Kodavanti 2007). Dietary exposure to genistein induced an increase of vasopressin content in hypothalamic extract. Exposure to organoalogen compounds, generally believed to act as antiandrogens (Stoker et al. 2005), inhibited the release of AVP from SON punches and *in vivo*. These studies suggest that also in mammals the AVP system has been a target for the action of EDCs (Kodavanti and Curras-Collazo 2010) **and this special issue**.

In addition to AVP a second nonapeptide (oxytocin, OT) is produced by PVN and SON in mammals. OT and AVP are closely related molecules: they are transcribed from adjacent genes and differ by only two amino acids, suggesting that they arose from an ancestral gene by gene duplication (Urano, Hyodo, and Suzuki 1992). OT, as AVP, is released both centrally into the brain, and peripherally into the circulation. Peripherally, OT acts in the uterus to facilitate parturition and in the breast

to facilitate milk ejection during lactation. Centrally, OT fibers are found in a variety of specific regions of the brain and are chiefly originated by the parvocellular cells of the PVN (De Vries and Buijs 1983). OT is involved in social recognition (Ferguson, Young, and Insel 2002; Heinrichs and Domes 2008; Young, Wang, and Insel 1998; Young and Wang 2004) and several studies have also found that the central OT system may be responsible for social impairments in schizophrenia (Lee et al. 2007). Reduced oxytocin receptors downregulate reelin that may contribute to social behaviors of schizophrenia and autism (Lee et al. 2005). Oral BPA exposure reduces certain maternal behaviors in female rat such as licking-grooming and arched back posture that are related to OT (Della Seta et al. 2005). At the same time, BPA may induce an increase in OT-immunoreactive cell number in female rat PVN (Adewale et al. 2010). The behavioral effects of EDCs exposure could also be related to alteration of the expression estrogen-inducible central OT receptors (Bale and Dorsa 1995) as observed in the cingulate cortex of female pine vole perinatally exposed to MXC(Engell et al. 2006).

Kisspeptin and related peptides.

Kisspeptin (and related peptides identified in several vertebrates (Greives et al. 2008)) is universally recognized as essential activator of the gonadotropic axis, with key roles in puberty onset and in the control of gonadotropin secretion (Smith, Clifton, and Steiner 2006). While these fundamental functions are now well-known, novel aspects have emerged, including the involvement of kisspeptin in the neuroendocrine control of ovulation and the metabolic gating of reproductive function (Roa et al. 2008; Tena-Sempere 2010). Several studies demonstrated sexual differences (Clarkson and Herbison 2006; Kauffman 2009) and hormonal regulation

of the kisspeptin system (Greives et al. 2007). Due to the fact that many EDCs have been reported to alter sexual behavior, reproduction and puberty, kisspeptin system is an obvious target to investigate the effects of EDCs with sex steroid-like activities (Tena-Sempere 2010). Recently, a few studies documented the potential impact of putative XEs or mixtures of EDCs, on the expression of Kiss1 gene and/or kisspeptin content at the hypothalamus in rats, mice and sheep. These experimental evidences are summarized below.

In rats, neonatal exposure to synthetic estrogens, including EB and the selective ligand of ER α , PPT, as well as the phytoestrogen genistein, induced also variable degrees of suppression of kisspeptin fibers' density at anteroventral-periventricular region (AVPV) and/or arcuate nucleus (ARC) in adult female (Bateman and Patisaul 2008). Neonatal exposure to the XE bisphenol A (BPA), induced a significant decrease in hypothalamic kisspeptin mRNA levels at puberty (Navarro et al. 2009), and kisspeptin immunoreactivity within ARC, as estimated in terms of fibers' density in adulthood (Patisaul et al. 2009) **and this special issue**.

In mice, our preliminary data demonstrate that perinatal exposure to BPA induces an increase of kisspeptin immunoreactivity in males, attenuating in this way the sex dimorphism of the system (Fig. 2). In fact, in control mice kisspeptin immunoreactivity is higher in females than in males, BPA exposure increases kisspeptin expression in male arcuate, periventricular and anteroventral periventricular nuclei (Miceli et al. 2008; Panzica et al. 2009). In the sheep, recent data (Bellingham et al. 2009) demonstrated that prenatal exposure to a cocktail of EDCs induces a significant decrease in kisspeptin mRNA levels at the rostral, mid and caudal regions of the hypothalamus of exposed foetuses. However, no significant

effects were detected in exposed adult ewes, suggesting that such regulatory actions take place selectively during critical periods of maturation.

Overall, these data emphasizes the potential importance of alteration of normal differentiation of the hypothalamic kisspeptin system in reproductive endocrine disruption and it could be the direct cause of alteration in puberty onset (Howdeshell et al. 1999) or of GnRH system (Bateman and Patisaul 2008; Ottinger et al. 2008; Ottinger et al. 2009; Urbatzka et al. 2006) that have been reported among the consequences of precocious exposure to EDCs. In support to this view, there are also the data that the lowering in pubertal expression of Kiss1 gene following neonatal exposures to estrogenic compounds is linked to decreased gonadotropin secretion both in basal and post-gonadectomy conditions, and that such defective hormone patterns could be almost normalized by administration of exogenous kisspeptin (Navarro et al. 2009).

Neuropeptide Y

Neuropeptide Y (NPY) is an orexigenic molecule, made of 36 aminoacids, including a tyrosine (Y) at each end (giving the name “Y” to this peptide) (Allen 1990). NPY is widely distributed within the brain (Chronwall and Zukowska 2004) and, in the hypothalamus, it is mainly synthesized in neurons of ARC projecting to other hypothalamic regions involved in the regulation of food intake (Valassi, Scacchi, and Cavagnini 2008): PVN, ventromedial (VMH), and dorsomedial (DMH) nuclei. When centrally injected, NPY induces increase of food intake (Clark, Kalra, and Kalra 1985), and when chronically administered it produces hyperfagia, decrease of thermogenesis and obesity (Stanley et al. 1986). The action of NPY is mediated by several receptors, and in particular Y1 and Y5 receptors are mainly related to the food intake control (for reviews see (Eva et al. 2006). In addition, neurons that produce

NPY have been implicated in the regulation of the reproductive axis. Recent studies demonstrated that loss of the NPY gene cause a decrease in luteinizing hormone surge levels because of the absence of the facilitating effects of neuropeptide Y on proestrus, and consequent impairment of fertility (for a review see (Hill, Xu, and Levine 2002); these effects are due to close synaptic relationships among NPY-ir fibers and GnRH neurons at the level of the median eminence (Advis et al. 2003).

It has been shown that EDCs, as the BPA, may induce increase in body weight (Rubin et al. 2001; Howdeshell et al. 1999). These results suggest that obesity might be the consequence of exposure to endocrine disrupting substances during development, in particular those with estrogenic activity (Ruhlen et al. 2008). In recent years, many studies predicted the existence of chemicals “obesogens”, molecules that inappropriately regulate lipid metabolism and adipogenesis to promote obesity. It has been demonstrated, for example, that organotin (largely employed as antifouling agents in paints), in particular tributyltin (TBT), can promote obesity activating peroxisome proliferator-activated receptor γ (PPAR γ) and retinoid X receptor (RXRa, RXRb, and RXR γ). Actually it's known that *in vitro* stimulation of PPAR γ and RXR induces pre-adipocytes differentiation to adipocytes, while *in vivo* can lead to an increase of body fat depots (for reviews see (Grun et al. 2006; Newbold et al. 2008).

However, alterations of neural circuits involved in the control of food intake have not been investigated, even if the leptin (a circulating hormone produced by adipose tissue)-NPY axis seems to be one of the possible candidates for “obesogenic disruption”. In our recent preliminary study (Bo et al. 2009) we tested the hypothesis if TBT could also interfere with the nervous pathways controlling animal food intake. To this purpose, TBT, diluted in olive oil, was orally administered for 4 weeks at a

dose of 0.025 $\mu\text{g/g}$ body weight/day to adult male and female *Y1R/LacZ* transgenic mice, carrying the murine Y1R promoter linked to the *LacZ* gene (Oberto et al. 1998). During the treatment the body weight, circulating leptin levels, brains, and body fat were analyzed. TBT-treated adults of both sexes show statistically significant reduction of food intake in comparison to controls, but no differences were found in body weight and fat deposition. In addition, in treated animals we observed a highly significant reduction of blood leptin levels compared to controls. These results suggest the induction of an indirect obesogenic effect: treated animals show a reduction in food intake, but the body weight doesn't change and blood leptin levels collapse in comparison to controls. **This effect suggests a possible alteration of brain circuits controlling food intake.**

Brain sections were stained for NPY immunocytochemistry and the expression of Y1 receptors was determined by the expression of Y1/LacZ through β -galactosidase histochemistry. Due to the decrease of circulating levels of leptin, in TBT-treated adults we expected a parallel increase in NPY immunoreactivity. On the contrary, the computerized quantitative analysis of NPY immunoreactivity distribution demonstrated a statistically significant reduction of NPY expression in PVN, DMH, and ARC nuclei of treated-male mice in comparison to controls (Fig. 3). Also Y1/LacZ expression was significantly decreased in the whole system. In conclusion, these results indicate that adult exposure to TBT is profoundly interfering with the neural mechanisms involved in the control of food intake.

Endocrine disruptors, enzymes and neurotransmission.

In addition to neuropeptides, other proteins, i.e. enzymes, are directly involved in neurotransmission or neuromodulation, and may have profound behavioral effects. Some enzymes are, in many cases, the key molecules for the synthesis of

neurotransmitters, therefore alterations of their expression after exposure to EDCs may have a direct impact on neurotransmission. For example the enzyme aromatase [converting testosterone (T) into E₂] is important to regulate sexual behavior and neural circuits through the local synthesis of E₂ (Balthazart et al. 2009). Whereas, the enzymes tyrosine hydroxylase (TH) or neuronal nitric oxide synthase (nNOS) are needed for the synthesis, respectively, of catecholamines and nitric oxide (NO), that control several behaviorally relevant neural circuits.

Aromatase. Aromatization of T into E₂ by neural tissue for the intervention of the enzyme aromatase (CYP19 cytochrome P450) has classically been associated with the regulation of sexual differentiation, gonadotropin secretion, and copulatory behavior in all vertebrates (Naftolin 1994). However, new data indicate that the capacity for aromatization is not restricted to the endocrine brain and demonstrate roles for locally formed estrogens in neurogenesis and in responses of brain tissue to injury (Garcia-Segura 2008; Roselli, Liu, and Hurn 2009), as well as in neural transmission (Balthazart and Ball 2006). Several studies suggested that both androgens and estrogens may regulate aromatase expression in the adult and during ontogenesis [rat (Roselli, Abdelgadir, and Resko 1997; Zhao et al. 2008), Japanese quail (Balthazart, Tlemçani, and Ball 1996; Aste et al. 1994)].

Experimental modification of the ontogenetic differentiation or of the adult regulation of aromatase in the brain may result not only in the partial or total impairment of reproductive behavior and reproduction, but also in a wide spectrum of cerebral effects. Due to its properties, the aromatase-producing system is a potential target for the action of XE and XA. The CYP19 gene that encodes aromatase was cloned in human and other mammalian species (Wilson, McArthur, and Stegeman 2005). Most of the higher vertebrates possess a single CYP19 gene, and the control of

its tissue-specific expression has been extensively studied and shown to depend on the use of an alternative splicing of promoter and untranslated exon I, resulting in the generation of transcript variants with different tissue-specificity, but identical coding sequences (Simpson et al. 2002). In contrast, two different CYP19 genes are found in many teleosts, CYP19a and CYP19b, and several studies illustrated alterations of these systems in this class of vertebrates after exposure to EDCs (for reviews see (Cheshenko et al. 2008) **and in this special issue**). In general, the presence of several isoforms of this enzyme, or the alternative splicing mechanisms, determining different expression in various organs and species, produces a variety of results that are difficult to compare.

In the teleost brain, the aromatase B gene (CYP19b) is mainly expressed in radial glia along the entire lifespan (for a review see (Diotel et al. 2010), and aromatase expression or immunolocalization is altered after exposure to a variety of EDCs, including DES or BPA (Kishida et al. 2001; Lee et al. 2006), EE₂ or phytoestrogens (Le Page et al. 2006; Pelissero et al. 1996), dioxin (Cheshenko et al. 2007), DDT or organotin compounds (Lyssimachou, Jenssen, and Arukwe 2006; Kuhl and Brouwer 2006). In addition to the control of reproduction, aromatase activity in radial glia of nonmammalian vertebrates may be involved in the regulation of cell proliferation under physiological and regenerative conditions.

In physiological conditions, mammalian and avian aromatase is chiefly expressed in neurons (Naftolin, Horvath, and Balthazart 2001), whereas, under pathological conditions, neural damage induces aromatase expression in astroglia (García-Segura et al. 1999; Peterson, Saldanha, and Schlinger 2001). Aromatase activity in glial cells down-regulates gliosis, enhances cell proliferation and promotes neuronal survival, contributing to the reorganization and repair of neural tissue (for

reviews see (Garcia-Segura 2008; Saldanha, Duncan, and Walters 2009).

The exposure of terrestrial vertebrates to different EDCs resulted in a variety of response for aromatase expression or activity. Nonylphenol and BPA (two XEs) (Mosconi et al. 2002), as well as Clotrimazole (a pharmaceutical used for treatment of fungal infections) or EE₂ (Gyllenhammar et al. 2009), do not alter brain aromatase expression in adult amphibia (*Rana temporaria*), whereas exposure to DDE, a metabolite of DDT with antiandrogenic activity, resulted in a significant decrease or increase of aromatase mRNA, according to the different doses (Arukwe 2006). A transient increase of aromatase mRNA at postnatal day 21 has been observed in male rats exposed to PCB during pregnancy, whereas other enzymes, as the 5 α reductase, demonstrated a permanent decrease of mRNA in both sexes (Colciago et al. 2009). Genistein, a phytoestrogen that selectively binds ER β , is a tyrosine kinase inhibitor that can regulate the calcium-dependent phosphorylation processes controlling brain aromatase activity (Balthazart et al. 2003). However, in the rat, genistein *in vivo* does not alter brain aromatase levels (Lephart, Adlercreutz, and Lund 2001; Weber, Setchell, and Lephart 2001).

In the Japanese quail, we tested, on sections from our previous experiments on the AVT system (Viglietti-Panzica, Mura, and Panzica 2007; Mattsson et al. 2008; Mura et al. 2009), the effects of prenatal exposure to E₂, genistein, DDE, EE₂ or to the ER α agonist PPT on the differentiation of the aromatase system of the medial preoptic nucleus (POM), the key center for the control of male copulatory behavior in quail (Panzica, Viglietti-Panzica, and Balthazart 1996). These compounds were administered *in egg* early during development (day 3 of incubation) and quail were sacrificed at the age of 8 weeks. Sections of the preoptic region were immunostained with the Harada's (Toyoake, Aichi, Japan) antibody against quail recombinant

aromatase (Foidart et al. 1995). For each animal we quantified the volume of the POM by tracing the boundaries of the nucleus on each section, following the boundaries of the main ARO-ir cell cluster (a method previously validated for measuring POM volume, (Balthazart, Tlemçani, and Harada 1996). To quantify the number of the ARO-ir cells of the POM, we chose 4 consecutive sections below the anterior commissure and cells were counted using the particle counting protocol of ImageJ. Results of this quantification are summarized in Fig.4. Embryonic exposure to E₂, genistein, DDE, EE₂ or PPT resulted in non-significant variations of both POM volume and number of aromatase-positive cells. The fact that E₂ and xenoestrogenic compounds do not affect POM volume is a confirmation of our previous study on the effects of *in egg* administration of E₂. In fact, we have not observed significant changes of POM volume identified in Nissl-stained sections (Aste et al. 1991). Aromatase-positive cells represent a large population of POM elements (about 40% of POM cells are expressing the enzyme, (Aste et al. 1994), it is therefore not surprising that also their number shows not significant variations for the different treatments.

In conclusion, it seems that the aromatase system is an important target for EDCs action in fishes, where the majority of positive elements are radial glial cells. In higher vertebrate, where the expression of the enzyme is generally limited to neurons, the exposure to EDCs seems ineffective, whereas alteration of the aromatase system have been reported for non-nervous organs (Gyllenhammar et al. 2009; Sakimura et al. 2002; Ye and Leung 2008). Future studies in mammals and birds should consider if EDCs could modulate aromatase expression in the glial compartment during reactive processes. A recent study demonstrated that soy (a species rich in phytoestrogens, particularly genistein) has neuroprotective effects on the rat brain (Azcoitia et al. 2006), but it is unknown if this is a direct action of the phytoestrogen

or if it is mediated by some stimulation of glial aromatase activity.

Tyrosine Hydroxylase. Catecholamines are ubiquitous neurotransmitters and they are involved in the central control of several behaviors, including sexual behavior (Hull and Dominguez 2006; Hull et al. 1999). The synthesis of catecholamines depends by the action of a key enzyme called tyrosine hydroxylase (TH), which has been largely employed to immunohistochemically detect the catecholaminergic system. TH-immunoreactive neurons have a sexually dimorphic distribution in the preoptic area and brainstem of rats. In particular, the female shows more immunoreactive cell bodies than the male in the AVPV of the preoptic area (Simerly, Swanson, and Gorski 1985), and in the locus coeruleus (LC) of the brainstem (Luque et al. 1992).

Gonadal steroids play an important role for the organization of sex differences in the catecholaminergic systems (Simerly et al. 1985; Fabre-Nys 1998). Perinatal treatment with T-propionate masculinize the number of TH-positive cells in female rat AVPV, destroying at the same time the phasic pattern of luteinizing hormone secretion in response to estrogen and progesterone injections (Simerly et al. 1985), the action of T is mediated in this region by its aromatisation to E₂ and requires both types of ERs (Bodo, Kudwa, and Rissman 2006).

On the contrary, the sexual dimorphism of the LC, a brainstem region where ARs are particularly expressed (Hamson, Jones, and Watson 2004), is dependent by the AR. In fact, males with the testicular feminization mutation (Tfm), characterized by a spontaneous mutation of the AR receptor preventing the androgen action, demonstrate a greater number of neurons than their control littermate males, thus suggesting that androgens are involved in the control of neuron number in the LC perhaps decreasing cell survival (Garcia-Falgueras et al. 2005).

The TH systems of AVPV and LC, **as well as their total volume** are targets for the action of EDCs. The significant sex differences in TH neuron number observed in control rat offspring AVPV were diminished or obliterated in offspring exposed to BPA primarily because of a decline in TH neuron number in BPA-exposed females. In addition the sexually dimorphic behaviors in the open field observed in the vehicle-exposed offspring were not observed in the BPA-exposed offspring (Rubin et al. 2006). Similarly to BPA, perinatal administration of genistein abolishes the sexual dimorphism of TH-ir system of AVPV, in addition also the number of TH cells expressing ER is significantly decreased (Patisaul, Fortino, and Polston 2006). **Exposure to BPA during the fetal and suckling periods has a similar effect on the rat LC total volume, disrupting the sexual differentiation of the nucleus (Kubo et al. 2001; Kubo et al. 2003).** Some preliminary studies in 1 month-old CD1 mice perinatally exposed to BPA demonstrated the disappearance of the sexual dimorphism **of the TH system** observed in control offsprings (Ponzi et al. 2006) **and a significant reduction of the volume of LC (Miceli et al. 2010).** Thus EDCs may affect both dopaminergic (AVPV) and noreadrenergic (LC) systems, thus influencing the related behaviors.

Nitric oxide synthase. Nitric oxide (NO) is an ubiquitous gaseous messenger molecule produced mainly by the endothelial cells of blood vessels through the action of an enzyme called nitric oxide synthase (NOS). After its discovery, three principal isoforms of NOS have been identified: the endothelial (eNOS), the macrophage (mNOS) and the neuronal (nNOS) (Forstermann et al. 1994).

Within the central nervous system NO is a neuronal messenger, whose action takes place primarily by inducing an increase of soluble cyclic guanosine monophosphate (cGMP) in target cells (Bredt and Snyder 1992; Snyder, Jaffrey, and

Zakhary 1998; Vincent 2010). The localization of nNOS in diverse cell types, belonging to a variety of neuronal systems, suggests a widespread role in neuromodulation for the free radical NO. The effects on blood vessel tone and neuronal function form the basis for the important role that NO has on neuroendocrine function and behavior: it is involved in a variety of physiological activities, such as long-term potentiation, neuroprotection, neural degeneration, and the regulation of peptidergic secretion. In particular, NO plays a crucial role in reproduction at various level in the organism, from mounting behavior to lordosis and ovulation. Finally, it influences several motivated behaviors including aggressive behavior (Nelson et al. 2006), learning and memory (Nelson et al. 1997; Hull and Dominguez 2006).

NO-producing neurons have been localized, with both histochemical and immunohistochemical methods, in several parts of the mammalian and non-mammalian central nervous system including the olfactory system, the cerebral cortex, the diencephalon, the brainstem, the cerebellum and the spinal cord [fishes (Brüning, Katzbach, and Mayer 1995), amphibians (Brüning and Mayer 1996), reptiles (Brüning, Wiese, and Mayer 1994), birds (Atoji, Yamamoto, and Suzuki 2001; Brüning 1993; Panzica et al. 1994; Cozzi, Massa, and Panzica 1997), mammals (Vincent and Kimura 1992; Gotti et al. 2005)]. In particular, nNOS-immunoreactive (nNOS-ir) neurons and fibers were described in several hypothalamic and limbic nuclei of rodents (Sica et al. 2009): e.g., medial preoptic nucleus (MPOM), PVN, SON, arcuate nucleus (ARC), ventromedial nucleus (VMH), bed nucleus of the stria terminalis (BST), and amygdaloid complex. Many of these nuclei are implicated in the control of reproduction (Hadeishi and Wood 1996). In rodents, some of these structures show a sex dimorphism in the number of nNOS-positive cells (Collado et al. 2003; Carrillo et al. 2007; Edelmann et al. 2007; Gotti et al. 2009; Sica et al.

2009), as well as fluctuations related to physiological changes of gonadal hormones level during the estrous cycle (for a review see (Gotti et al. 2010). Several studies have suggested complex relationships among nNOS system and gonadal hormones: the regionally specific distribution of nNOS immunoreactive (nNOS-ir) elements and their co-existence with gonadal hormones' receptors suggest the existence of significant neuroendocrine relationships (for a review see (Panzica et al. 2006). Experiments involving gonadectomy and hormone replacement therapy demonstrated that the expression of nNOS is strictly related to circulating gonadal hormone levels (Ceccatelli et al. 1996; Chu, Sarkar, and Etgen 2004; Du and Hull 1999; Martini et al. 2009). In addition, the analysis of mutant animals knockout for ERalpha (Sica et al. 2002) or aromatase (Sica et al. 2007), or Tfm rats with spontaneous mutation of the AR (Martini et al. 2008) demonstrated that both estrogens and androgens have a relevant role for the sexual differentiation of nNOS system in the hypothalamus and BST. Therefore, the nitrinergic system appears to be a good candidate to study behavioral and neural alterations after exposure to endocrine disruptors with xenoestrogenic activity.

We have therefore studied the long-term effects of early exposure of mice of both sexes to BPA on the nitrinergic system (Martini et al. 2010). Mice of both sexes were exposed for 10 prenatal and 8 postnatal days to BPA that was administered to the mothers. The maternally exposed mice were sacrificed at the age of 2 months and their brains were sectioned and immunohistochemically treated for the detection of nNOS. Significant effects of BPA exposure were detected for the number of immunoreactive cells in the MPOM and in the ventromedial subdivision of BST, in a sex-oriented and dose-dependent way (Fig. 5). These results indicate that BPA has a powerful effect on specific portions of the nNOS-ir system belonging to the accessory

olfactory system that are particularly important for the control of sexual behavior. In addition, they confirm that precocious exposure to EDCs, in particular to BPA, may have a high impact on the organization of specific neural pathways in a selective and non-unidirectional way, and this can later affect complex behaviors or functions as those related to reproduction. Exposure of adult rats to dioxin (a compound with antiestrogenic activity, (Faqi and Chahoud 1998), induced a marked decrease of nNOS expression in the PVN, lateral hypothalamic area and perifornical nucleus, regions where nNOS has a role as an important regulator of food intake, a function that is affected by acute dioxin exposure (Cheng et al. 2003).

CONCLUSIONS

Data reported in this short review clearly confirm that EDCs can exert subtle effects by interfering with gene expression and other cellular activities which can cause transient activational responses, or permanent impairment (vom Saal and Welshons 2006; Welshons, Nagel, and vom Saal 2006; Welshons et al. 2003). The impact of EDCs will vary depending upon a variety of factors, including the doses (in general the studies reported in this review used doses lower than the laws' limits), the duration of the exposure and when in the life-cycle of an organism exposure occurs. In particular, developmental stages are typically far more vulnerable to signal disruption than adult stages and the consequences of fetal exposure may be drastically different from those of adult exposure, as a consequence, levels of exposure that have been considered as "background" and thus "safe" can have deleterious effects early in life (Panzica et al. 2009).

The molecular targets of EDCs are multiple, but many of them act through steroid hormone receptors and may directly interfere with the regulation of gene

expression. This heavily impact the production of key molecules for the functions of neural circuits, as neuropeptides or enzymes involved in the synthesis of neurotransmitters. Alteration of the expression of these compounds has a wide range of effects not only in neural transmission, but also in the neuroendocrine regulation of cerebral activities and may explain the behavioral alterations observed in higher vertebrates exposed to XEs or XAs. In fact, as demonstrated by our and others' studies, in birds and in mammals the exposure to these compounds during the critical period (early embryonic period in birds, perinatal period in rodents) may alter the differentiation of relevant sexually dimorphic pathways inducing the appearance of a sex-reversed neurochemical phenotype that is the most probable cause of the final alteration of sexually differentiated behaviors in the adult animal. In mammals, the protection mechanism of the alpha-feto protein (AFP, that binds the circulating estrogens, preventing their transport through the blood-brain barrier) may be bypassed by EDCs, such as BPA, that generally exhibit a lower affinity for plasma estrogen binding proteins including AFP (Milligan, Khan, and Nash 1998). EDCs can thus interfere with the male- and female-typical development of brain systems (e.g. dopaminergic, NO producing and kisspeptin producing systems) that control the occurrence of a wide range of behaviors required for reproduction such as sexual behaviors, social and non-social behaviors in adult life.

In conclusion, the data collected so far should stimulate further studies on the direct alteration of neuropeptides' expression or of the enzymes involved in the synthesis of neurotransmitters (or in neuroendocrine regulation), as one important molecular mechanism that is at the basis of EDCs adverse effects.

LEGENDS TO FIGURES

Fig. 1 – Modifications of the expression of the arginine-vasotocin (AVT) in male Japanese quail exposed to different xenoestrogens during embryonic development.

Top – Sections illustrating the variations of the AVT immunostaining within the lateral septum (SL) and the bed nucleus of the stria terminalis (BST). Bar=300 μ m.

Bottom – Fractional area covered by AVT-immunoreactivity in different nuclei (SL, BST, and medial preoptic nucleus, POM) and different experimental groups (OIL: controls; EB, estradiol benzoate; DES, diethylstilbestrol; GEN, genistein; DDE, ethylene 1,1-dichloro-2,2-bis-p-chlorophenyl). Fractional area of controls has been conventionally put to 100. Data from (Viglietti-Panzica et al. 2005; Viglietti-Panzica, Mura, and Panzica 2007; Mura et al. 2009).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison to controls.

Fig. 2 - Modifications of the expression of Kisspeptin immunoreactivity in mice perinatally exposed to bisphenol A (BPA).

Bar charts illustrate the fractional area covered by Kisspeptin immunoreactivity in three hypothalamic regions: periventricular region (Pe), antero-ventral-periventricular region (AvPv), and arcuate nucleus (ARC). Fractional area of control female mice (F-Oil) has been conventionally put to 100. In all nuclei we have observed a strongly significant sex dimorphism in control animals. BPA exposure increases significantly Kisspeptin immunoreactivity in males (M-BPA) and not in females (F-BPA). ** $p < 0.01$, *** $p < 0.001$ in comparison to F-Oil. M-BPA and F-BPA, ° $p < 0.01$, °° $p < 0.001$ in comparison to male controls (M-Oil). Sections illustrate the sex

dimorphism in the AvPv of control mice (F-Oil and M-Oil) and the increase of immunoreactivity in males exposed to BPA (M-BPA). Data from (Miceli et al. 2008).

Fig. 3 – Modifications of the expression of neuropeptide Y immunoreactivity in hypothalamic nuclei of adult mice acutely treated with trybutyltin (TBT).

Bar charts illustrate the fractional area covered by NPY immunoreactivity in four hypothalamic regions: paraventricular nucleus (PVN), arcuate nucleus (ARC), dorsomedial nucleus (DMN), and ventromedial nucleus (VMN). Fractional area of control male mice has been conventionally put to 100. In all nuclei we have observed a significant sex dimorphism in control animals, $^{\wedge}p<0.05$, $^{\wedge\wedge}p<0.01$ in comparison to male controls. Adult exposure to TBT induces a significant decrease of NPY immunoreactivity only in males, $^{**}p<0.01$, $^{\circ}p=0.054$ in comparison to control males. Data from (Bo et al. 2009).

Fig. 4 - Expression of the enzyme Aromatase in the medial preoptic nucleus (POM) of male Japanese quail exposed to different xenoestrogens during embryonic development.

Top – Sections illustrating aromatase immunoreactivity in some of experimental groups (OIL , Controls; EB, estradiol benzoate; GEN, genistein 100 μ g; DDE, ethylene 1,1-dichloro-2,2-bis-p-chlorophenyl 40 μ g). CA, anterior commissure; Asterisk, third ventricle. Bar=200 μ m.

Bottom – Bar charts illustrating the results of cell count and POM measurements. Cell number and POM volume of controls have been conventionally put to 100. Both parameters show only non-significant changes. Groups: OIL , Controls; EB, estradiol benzoate; GEN100, genistein 100 μ g; GEN1000, genistein 1000 μ g; DDE low, 20 μ g;

DDE high, 40µg; EE2 ethinylestradiol; PPT propyl pyrazole triol. Details of different experimental groups in (Mattsson et al. 2008; Mura et al. 2009; Viglietti-Panzica, Mura, and Panzica 2007).

Fig. 5 - Modifications of the expression of nNOS immunoreactivity in mice perinatally exposed to bisphenol A (BPA).

Left – Distribution of nNOS in male mouse nuclei affected by perinatal BPA exposure. MPOM. Medial preoptic nucleus; BSTmv, bed nucleus of the stria terminalis, medio-ventral part; AC, anterior commissure; f, fornix; asterisk, third ventricle; oc, optic chiasma. Bar=300 µm.

Right – Bar charts illustrating changes of nNOS immunoreactivity in MPOM and BSTmv. *p<0.05 in comparison to male controls (M-Oil). °p<0.05 in comparison to female control (F-Oil). Data from (Martini et al. 2010).

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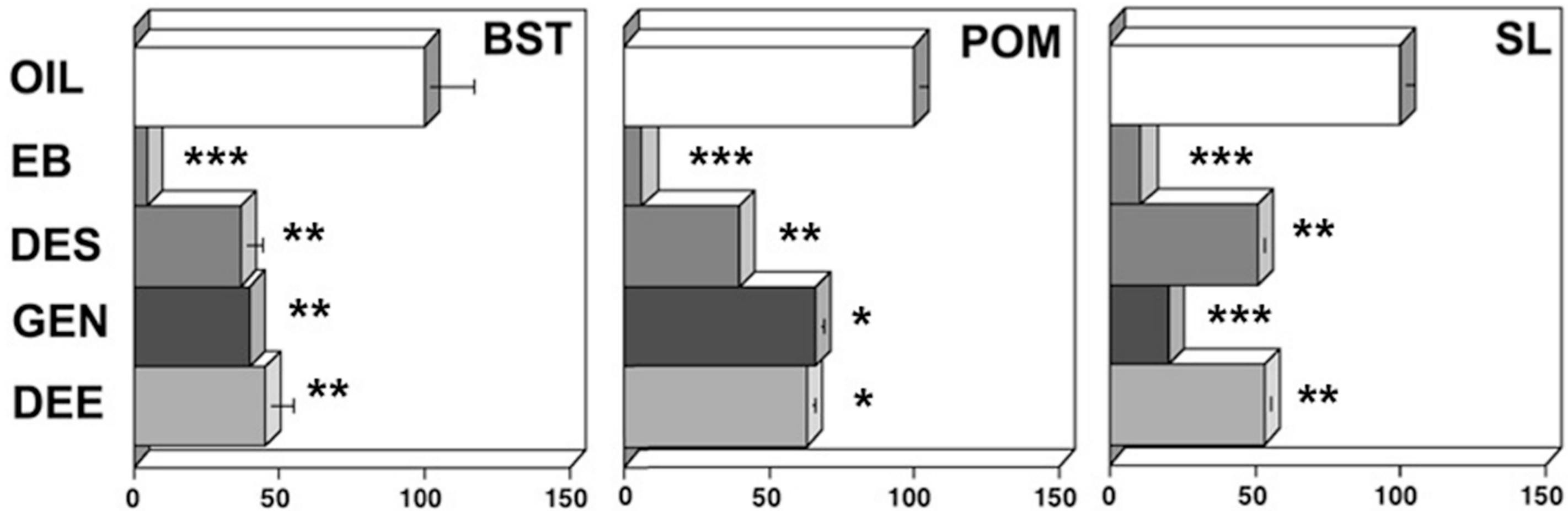
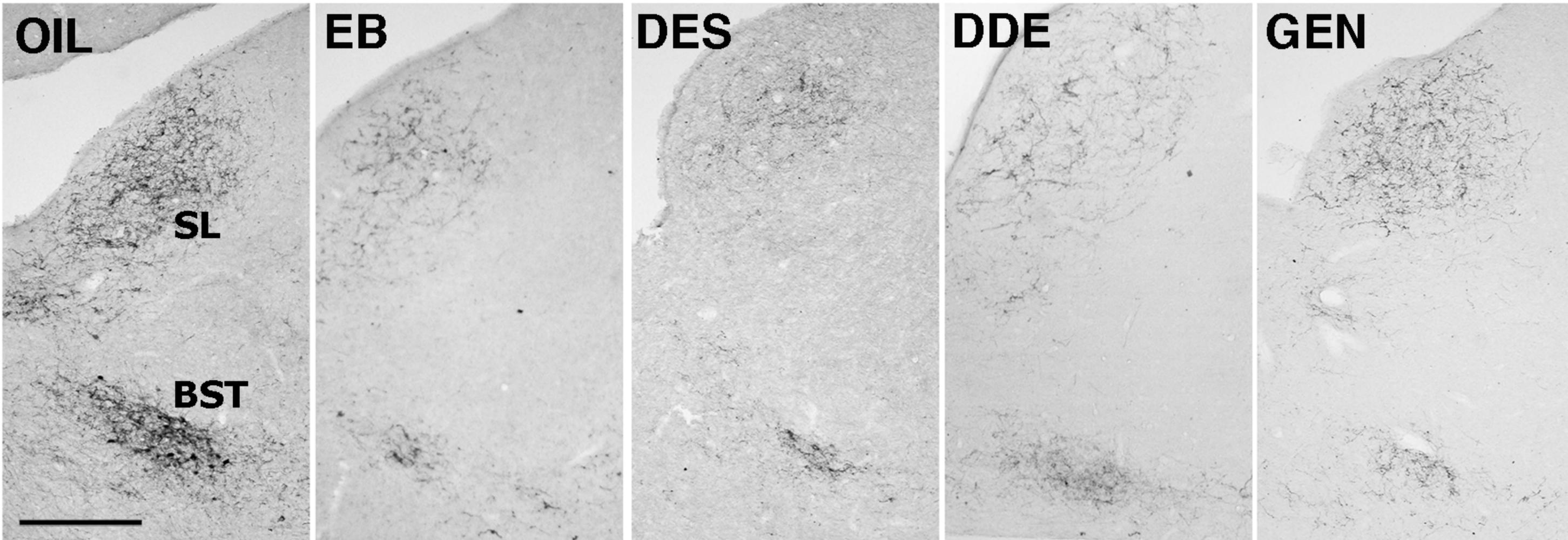
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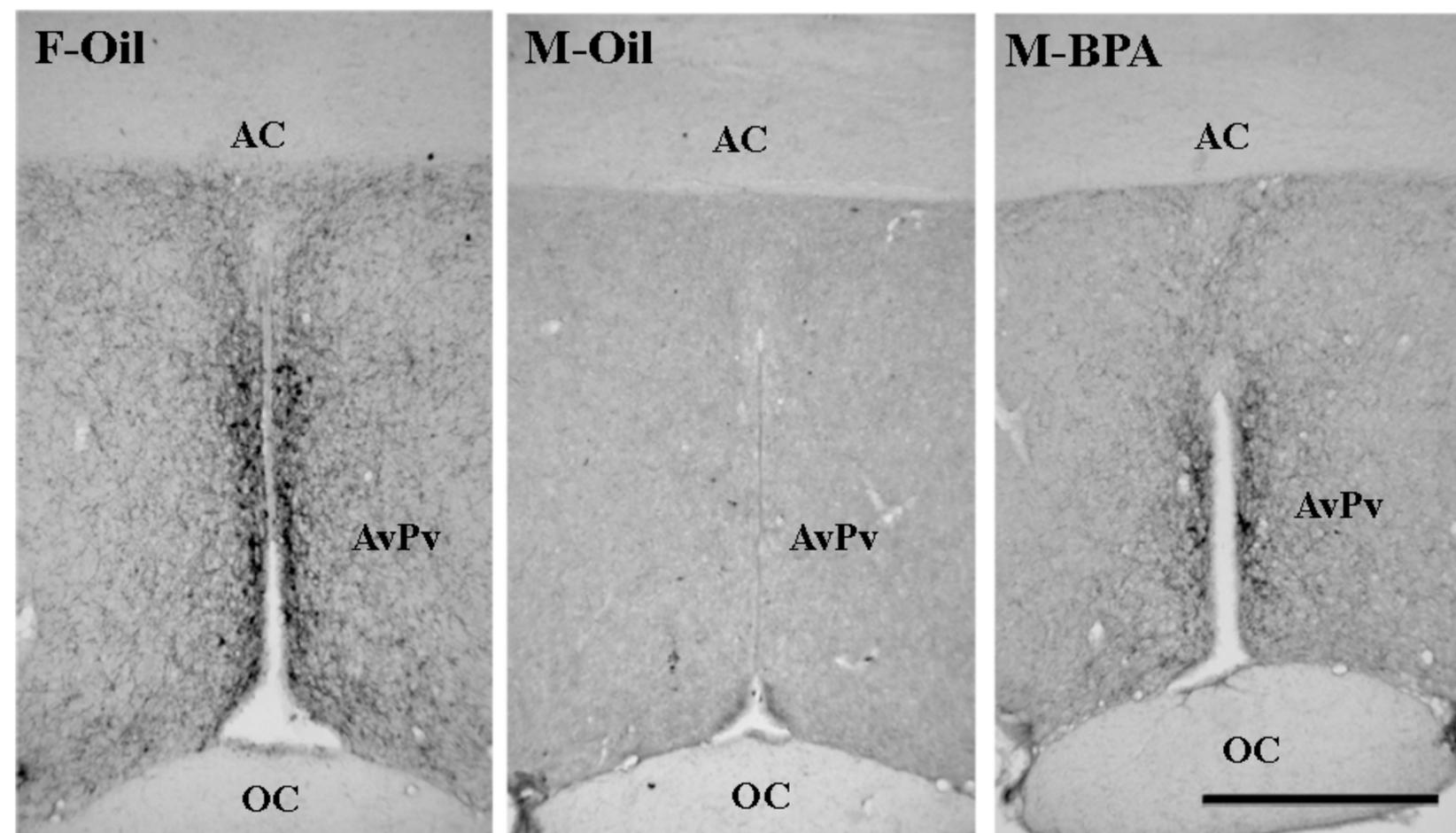
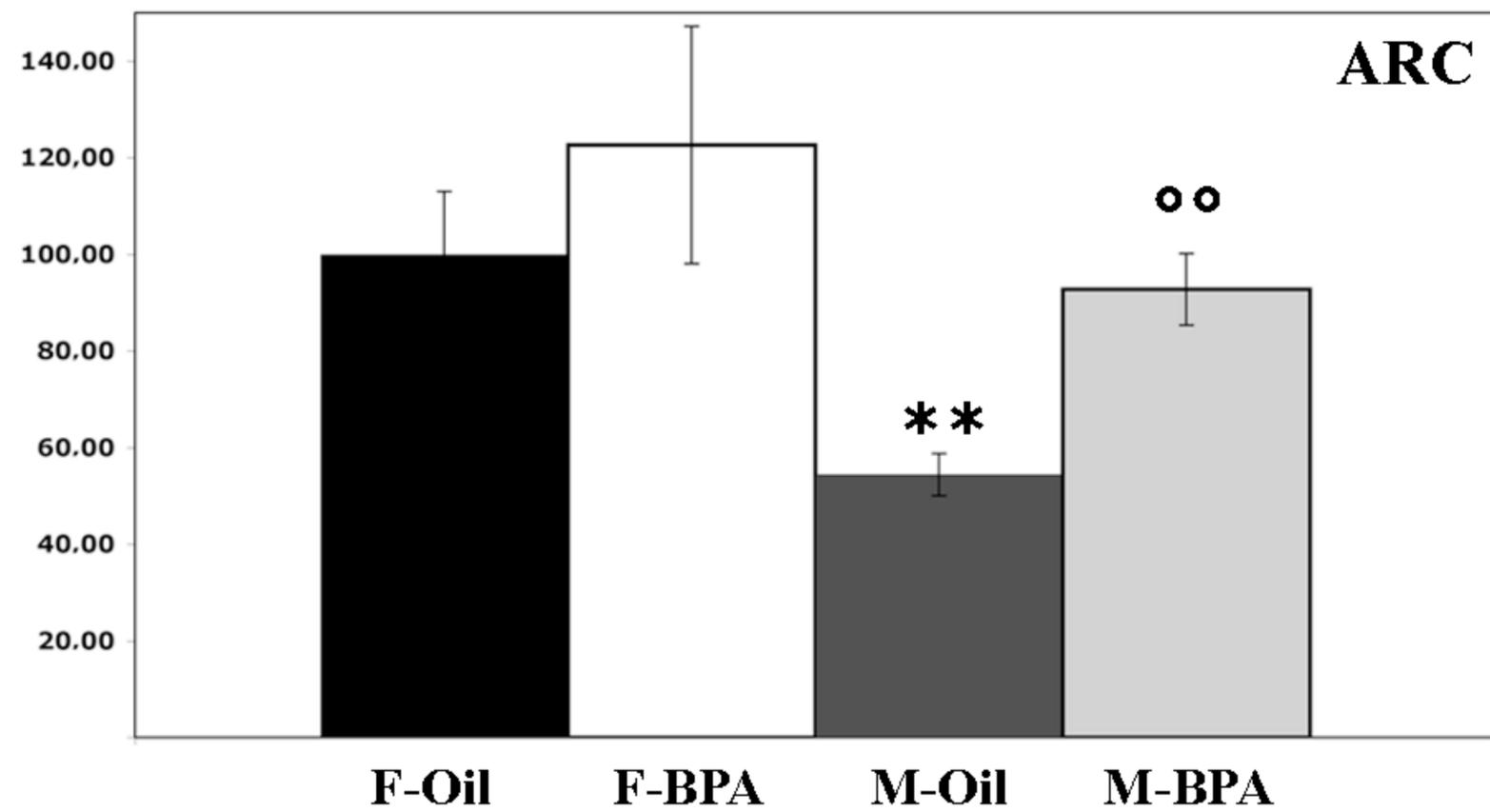
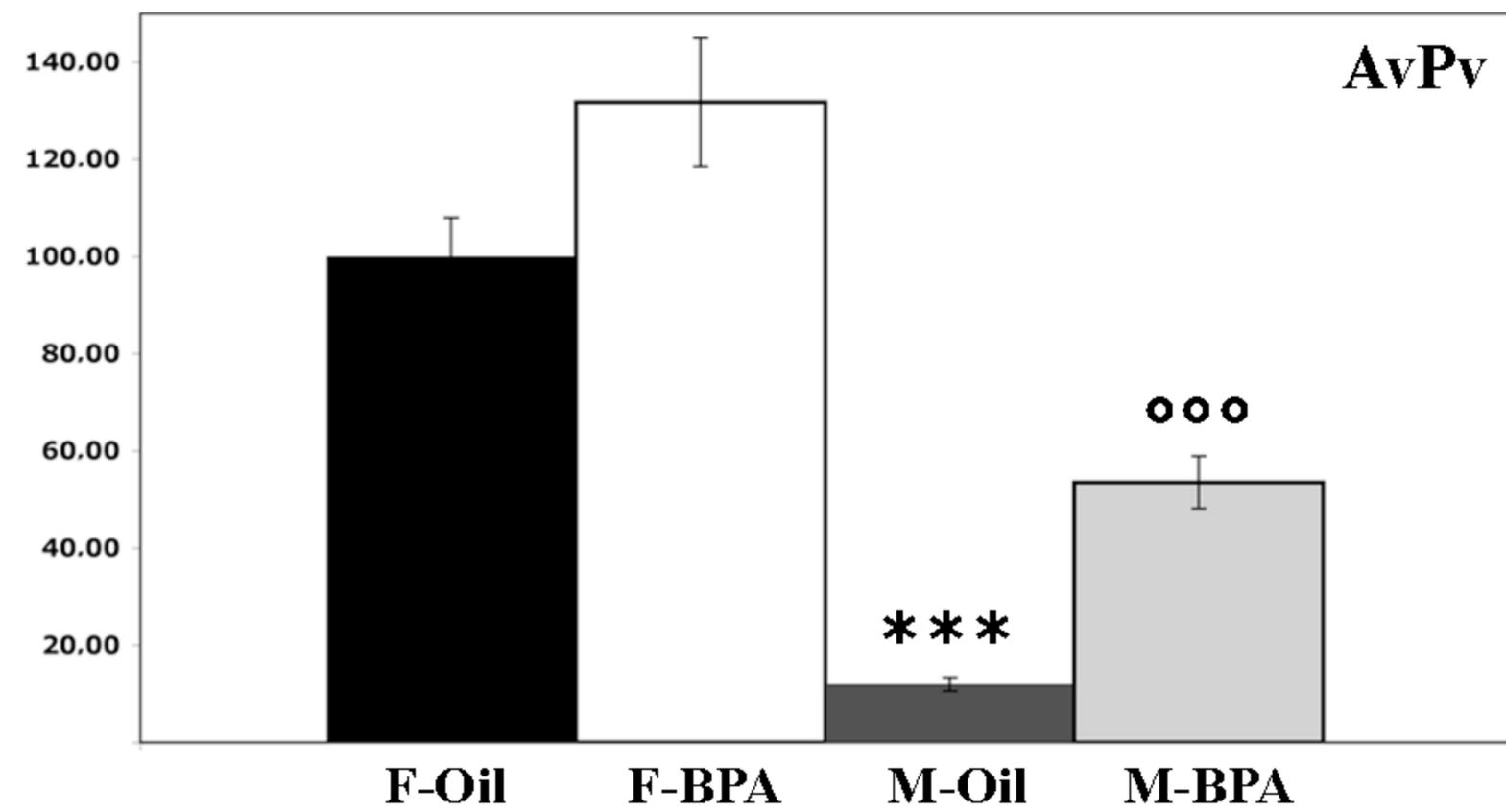
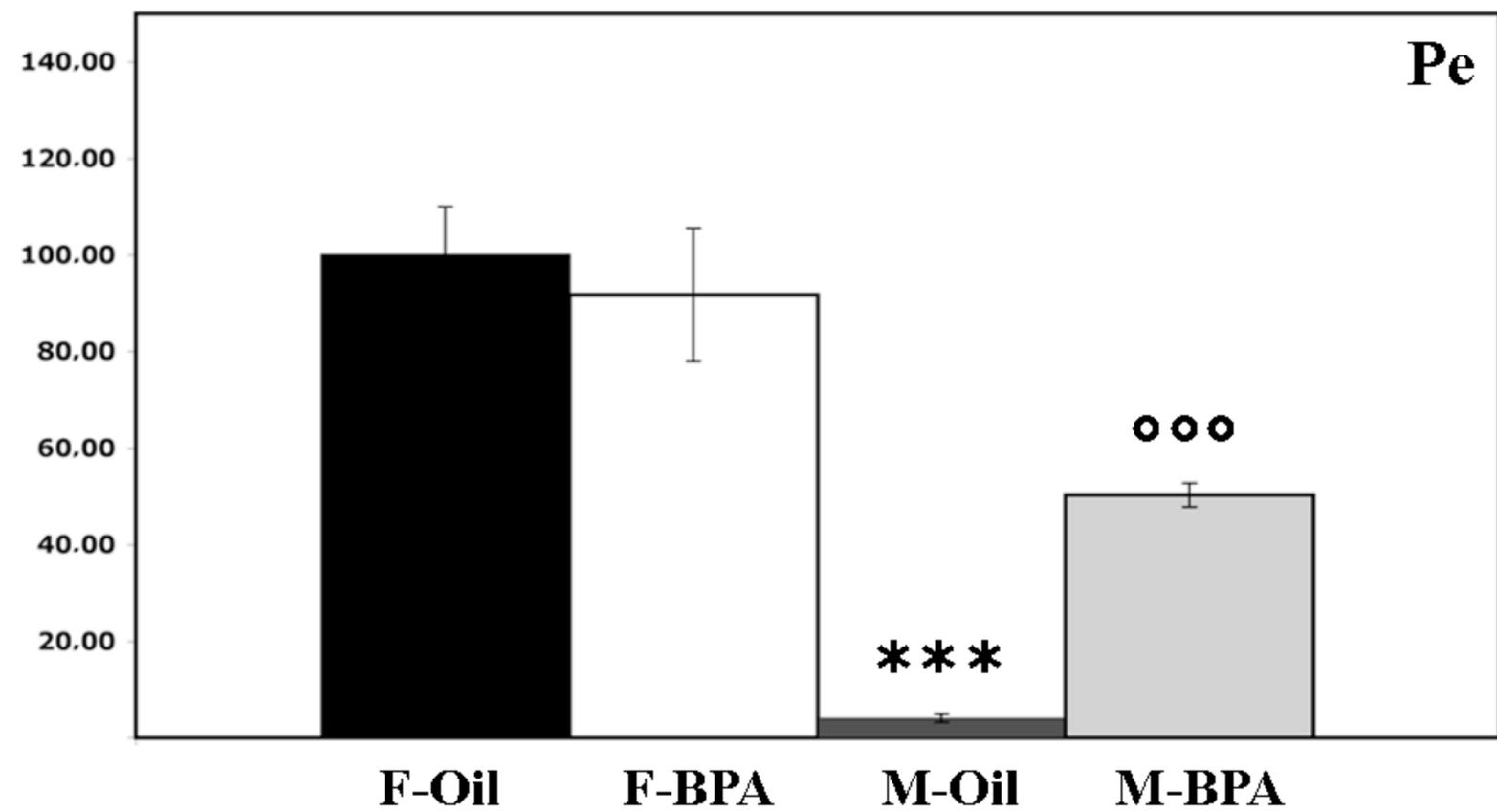
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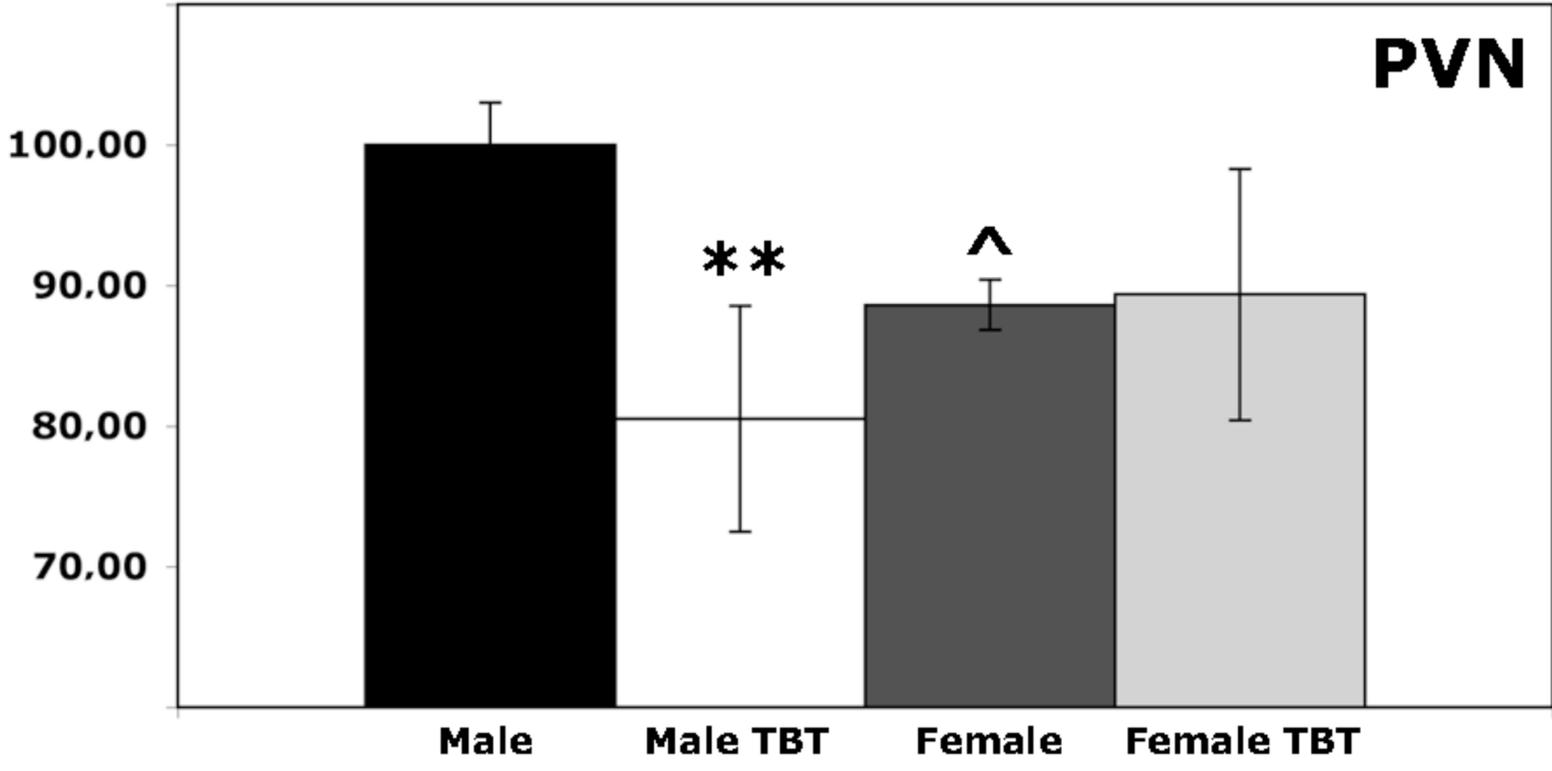
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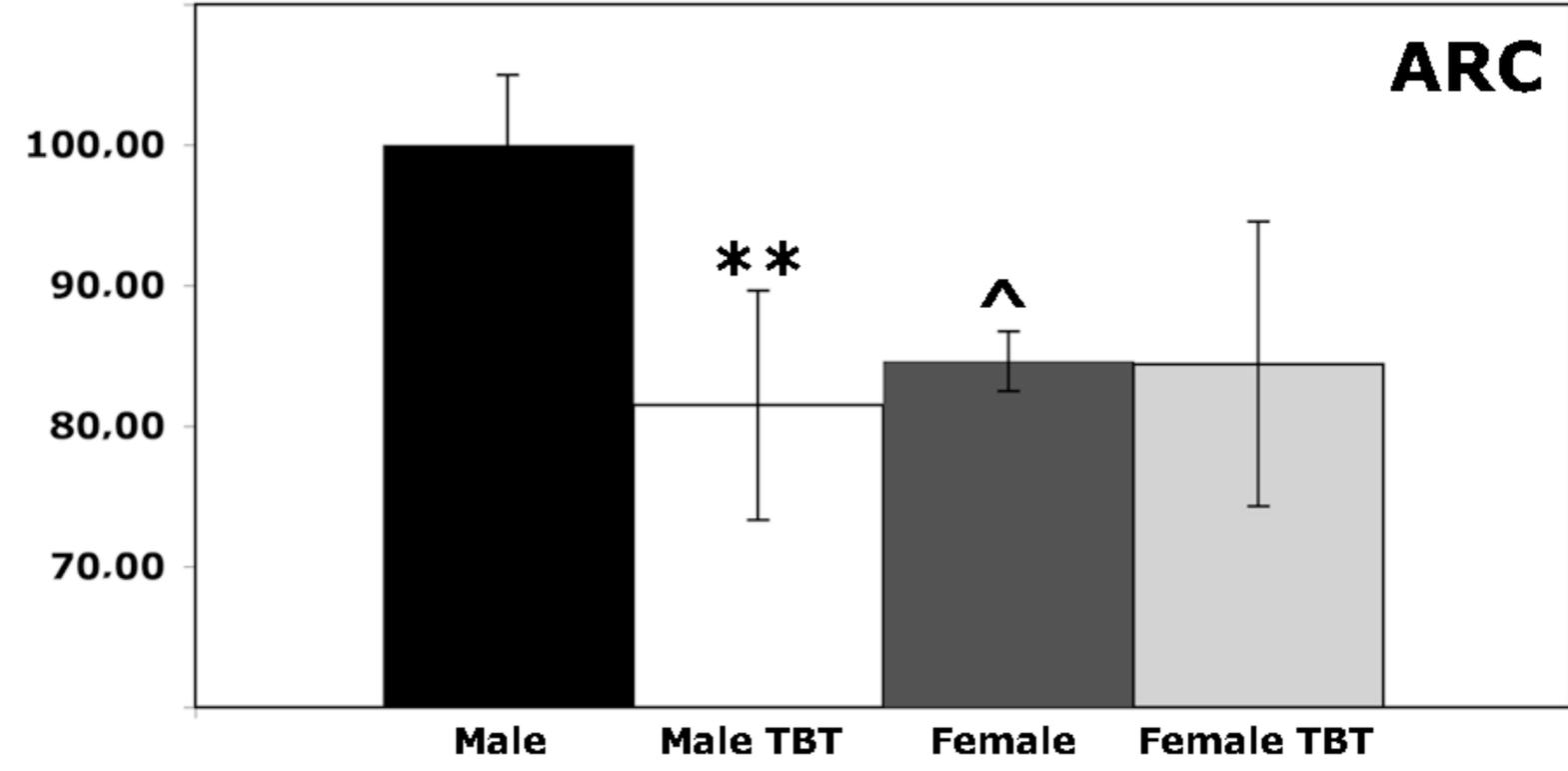




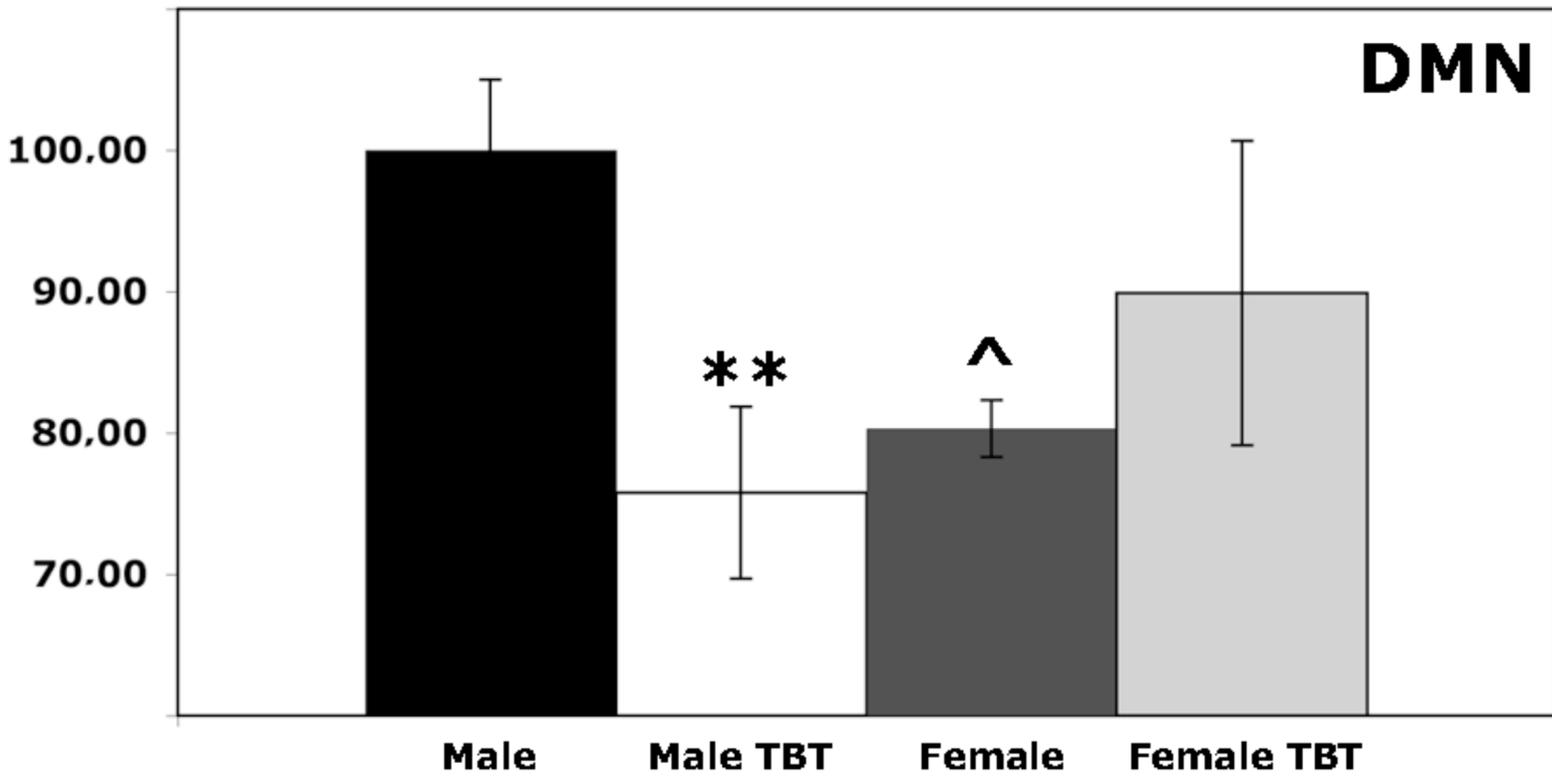
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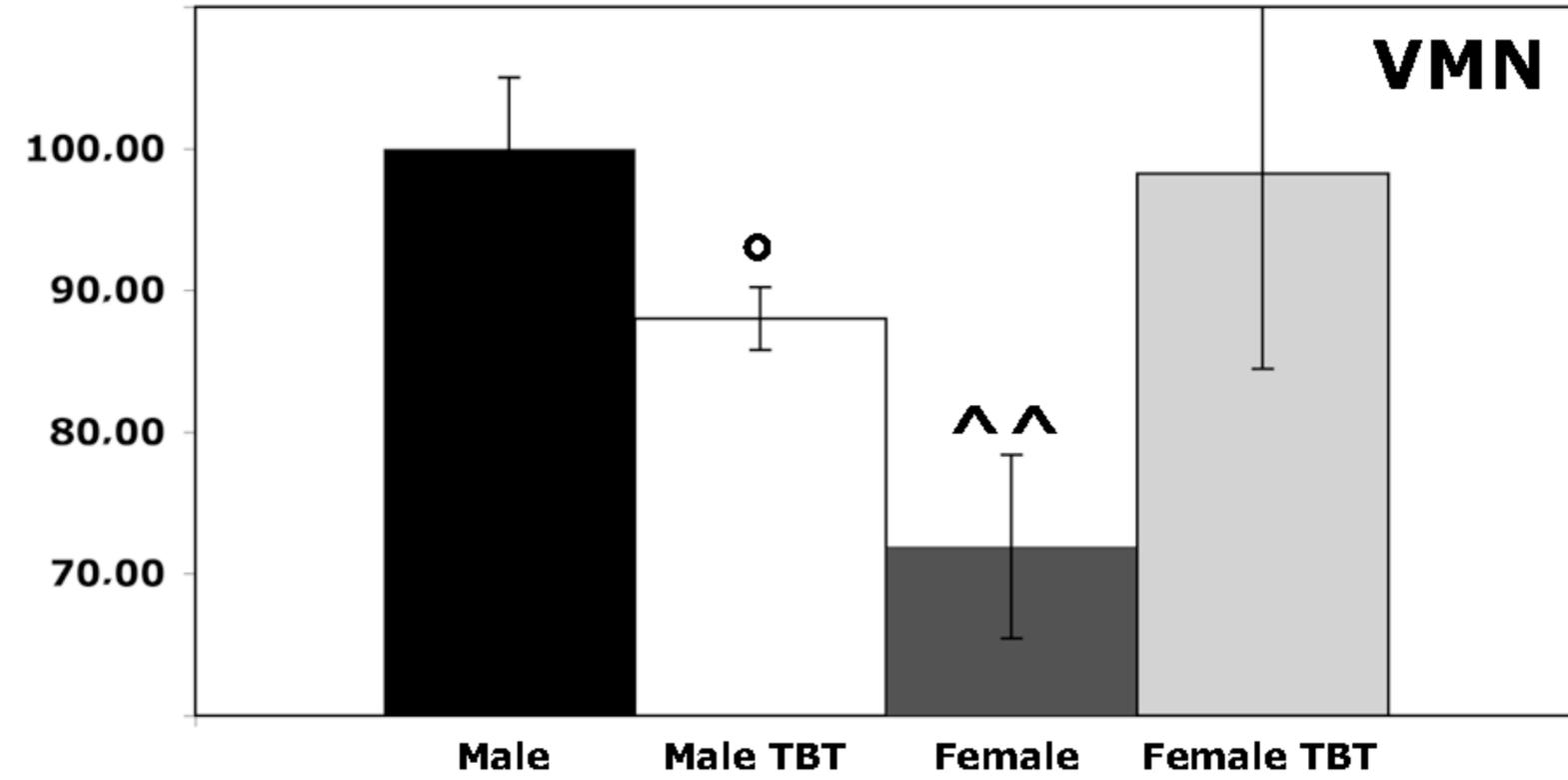
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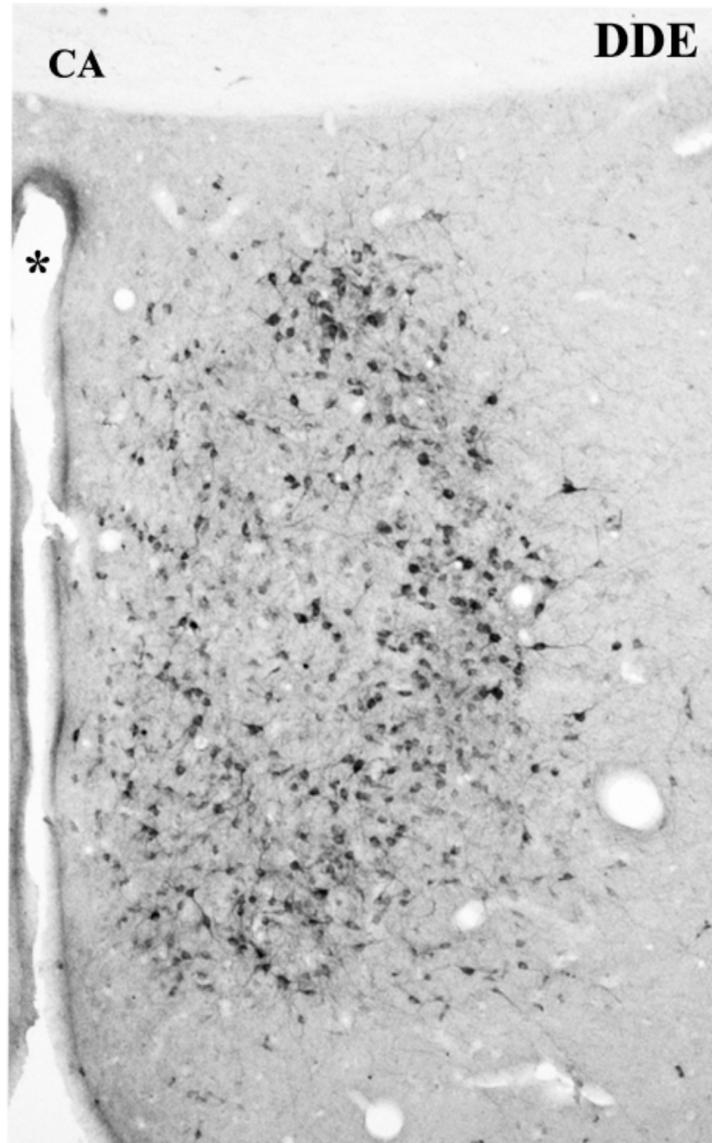
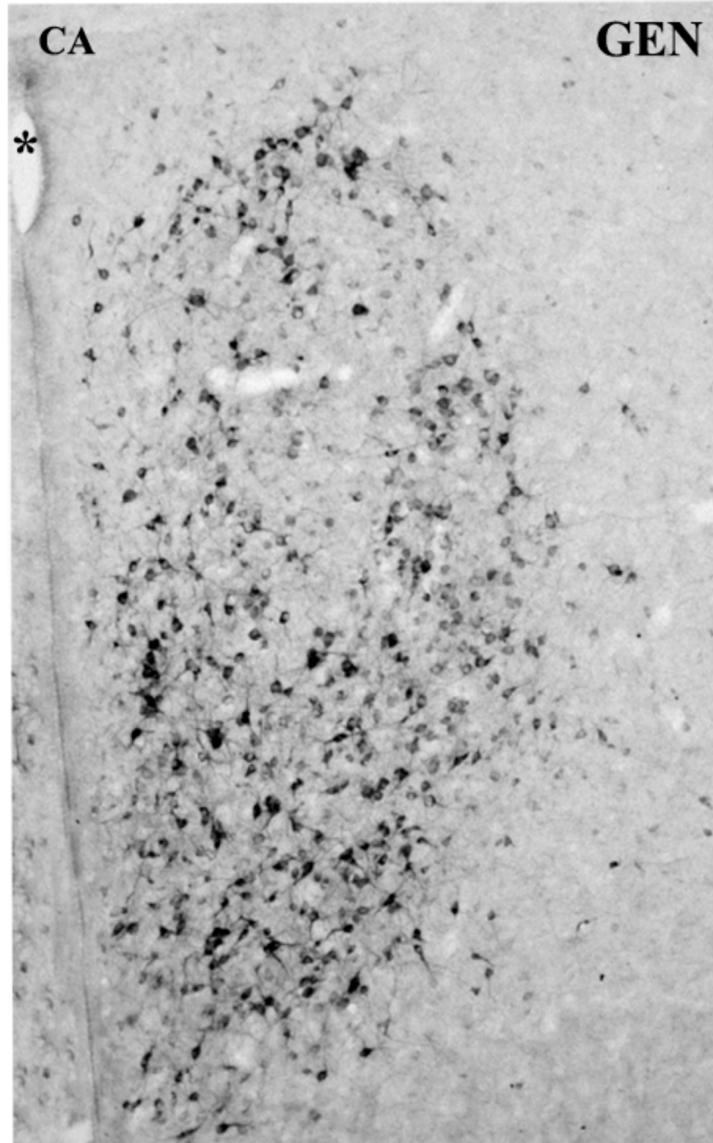
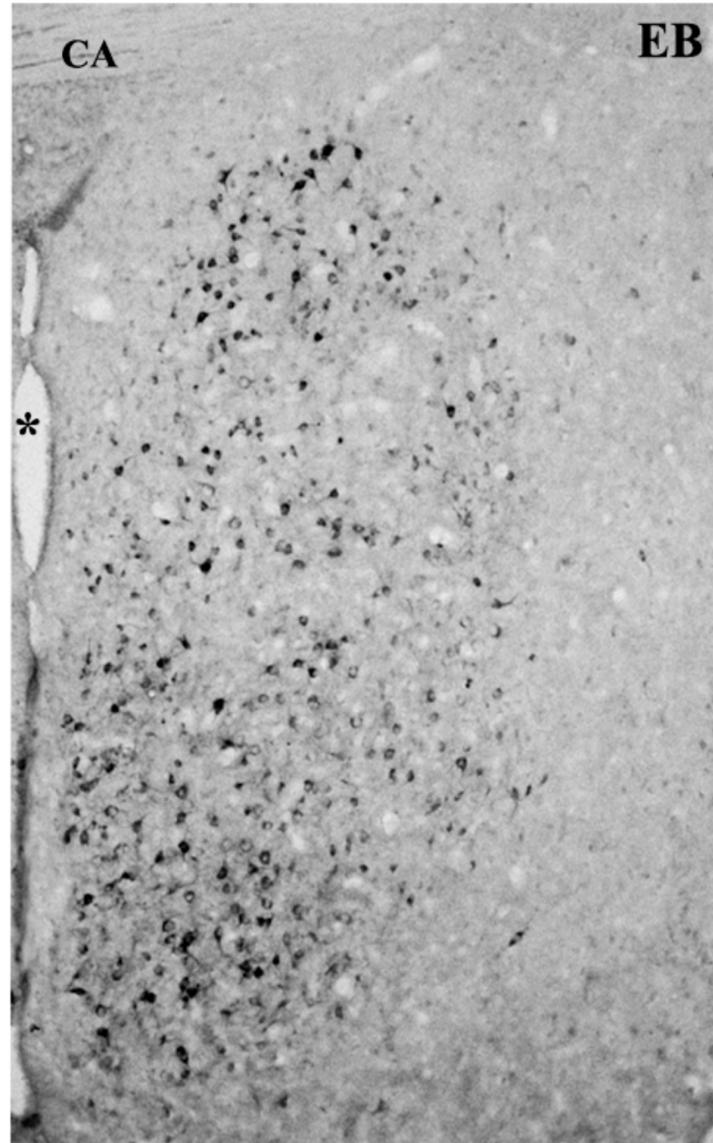
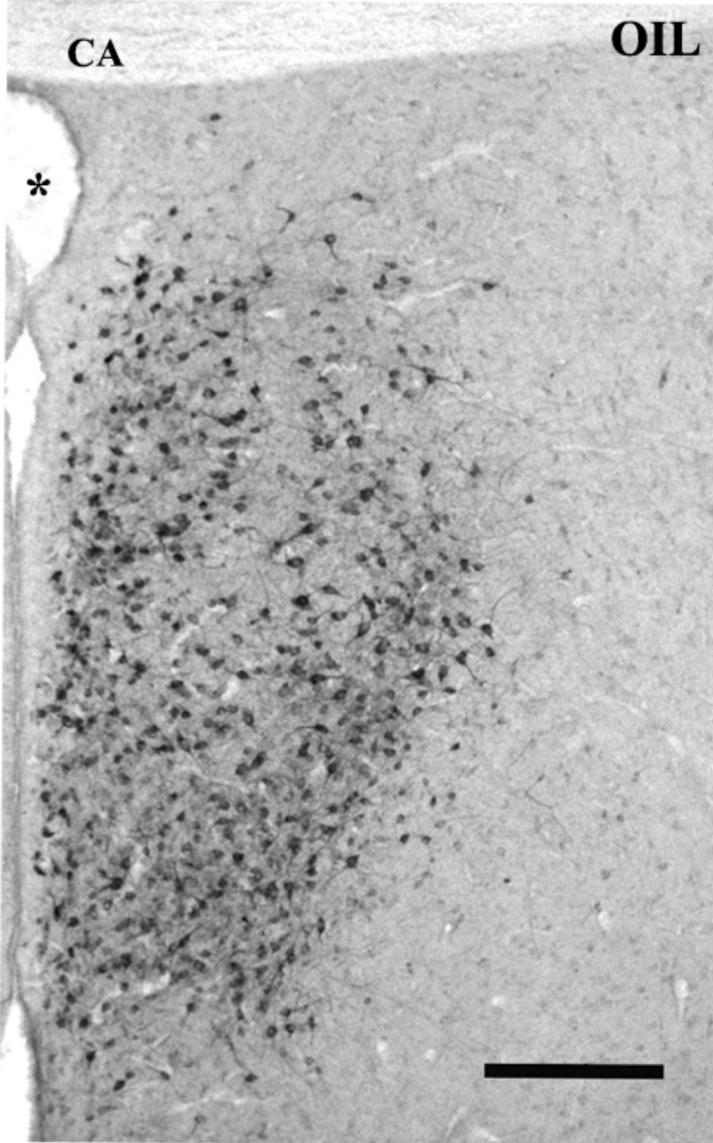


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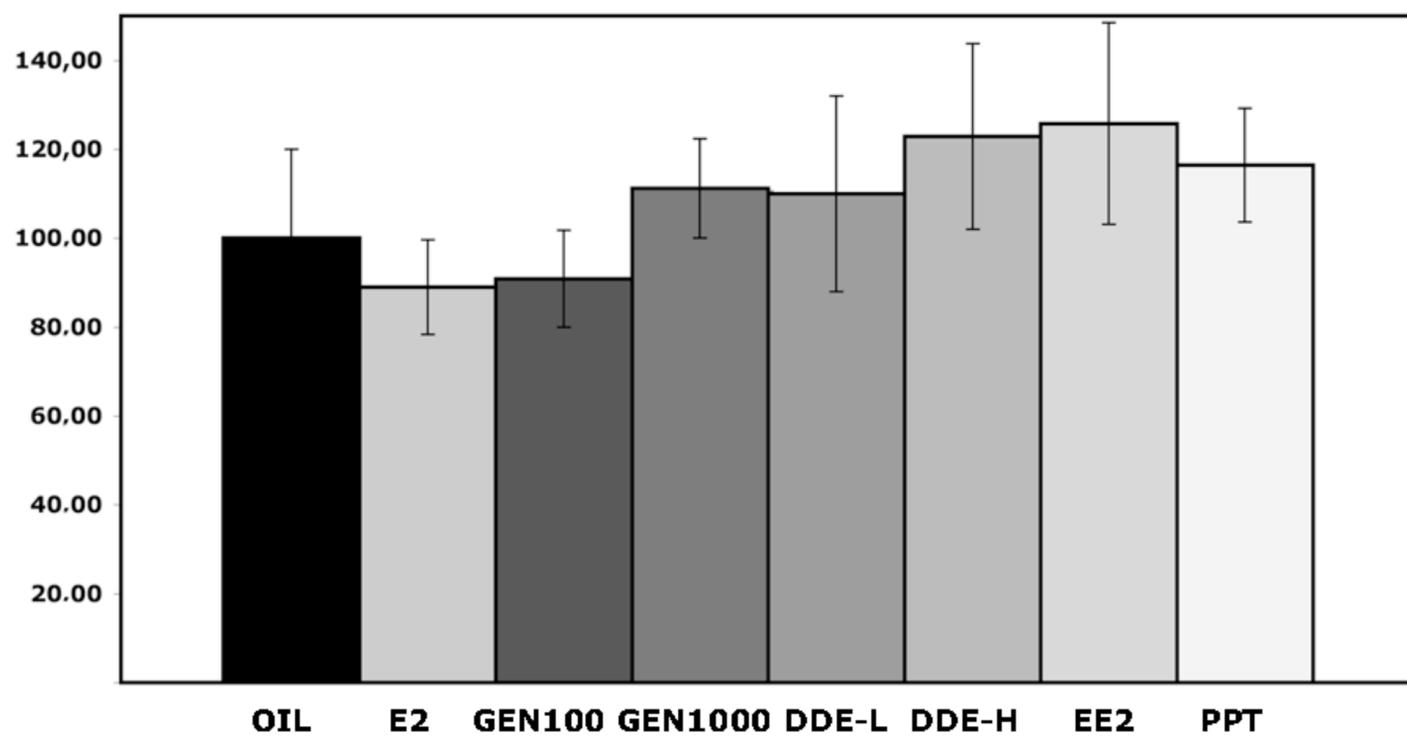


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