Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex-specific way

EARLY POSTNATAL GENISTEIN ADMINISTRATION PERMANENTLY AFFECTS NITRERGIC AND VASOPRESSINERGIC SYSTEMS IN A SEX-SPECIFIC WAY

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Abbreviations

ARC arcuate nucleus

AVP arginin-vasopressin

BLA basolateral amygdala

BPA Bisphenol A

BSTmv ventromedial part of the bed nucleus of the stria terminalis

E_2 17-β-estradiol

ERα estrogen receptor α

ERβ estrogen receptor β

EDC endocrine disruptors compound

GEN genistein

La lateral amygdala

LS lateral septum

MPOA medial preoptic nucleus

NO nitric oxide

nNOS neuronal isoform of Nitric oxide synthase

PaAP anterior parvicellular part of the paraventricular nucleus

PaDC paraventricular dorsal cap

PaLM lateral magnocellular part of the paraventricular nucleus

PaMM paraventricular medial magnocellular

PaMP paraventricular medial parvicellular

PaV ventral part of the paraventricular nucleus

PBS phosphate saline buffer

ROI region of interest

SCN suprachiasmatic nucleus

VMHvl ventromedial hypothalamus

Abstract

Genistein is a natural xenoestrogen (isoflavonoid) that may interfere with the development of estrogen-sensitive neural circuits. Due to the large and increasing use of soy-based formulas for babies (characterized by a high content of genistein), there are some concerns that this could result in an impairment of some estrogen-sensitive neural circuits and behaviors. In a previous study, we demonstrated that its oral administration to female mice during late pregnancy and early lactation induced a significant decrease of nitric oxide synthase positive cells in the amygdala of their male offspring's. In the present study, we have used a different experimental protocol mimicking, in mice, the direct precocious exposure to genistein. Mice pups of both sexes were fed either with oil, estradiol or genistein from birth to postnatal day 8. Nitric oxide synthase and vasopressin neural systems were analyzed in adult mice. Interestingly, we observed that genistein effect was time specific (when compared to our previous study), sex specific, and not always comparable to the effects of estradiol. This last observation suggests that genistein may act through different intracellular pathways.

Present results indicate that the effect of natural xenoestrogens on the development of the brain may be highly variable: a plethora of neuronal circuits may be affected depending on sex, time of exposure, intracellular pathway involved, and target cells. This raises concern on the possible long-term effects of the use of soy-based formulas for babies, which may be currently underestimated.

Keywords

Phytoestrogens; endocrine disruptors; sexual dimorphism; hypothalamus; limbic system.

INTRODUCTION

Many brain regions, including amygdala, hypothalamus and the bed nucleus of the stria terminalis are sexually dimorphic (for a recent review see McCarthy, 2015). Sex hormones, 17-β-estradiol (E_2) and testosterone, have important roles in their normal development (for recent reviews see Ball et al., 2014, Panzica and Melcangi, 2016), whereas several endocrine disruptors compounds (EDCs), at least those classified as xenoestrogens or xenoandrogens, may affect their normal development (Panzica et al., 2011). The presence of EDCs in the environment increased in the last 50 years and only recently their effects were under scientific evaluation and legislative regulation (Bourguignon et al., 2016, Kortenkamp et al., 2016, Slama et al., 2016). Many studies indicate that EDCs stimulation, during peri- and post-natal critical periods, may interfere with the formation of neuronal networks in a sex specific manner either binding or biasing the turnover of estrogen receptors (for a reviews see Panzica et al., 2007, Gore and Dickerson, 2012). Furthermore, blood brain barrier may be more permeable to EDCs than to estrogens in the perinatal period (Doerge et al., 2001). During this age, long lasting effects may be caused by a much lower dose than the one considered not toxic by laws that, in general take into account only acute toxic effects on adults (Richter et al., 2007).

The pre- or/and peri-natal exposure to EDCs may lead to permanent neuroanatomical changes in adults (Panzica et al., 2011) in particular in neuronal Nitric Oxide Synthase (nNOS) expression in bed nucleus of the stria terminalis and medial preoptic nucleus (MPOA) (Gotti et al., 2010, Martini et al., 2010) and in amygdala (Rodriguez-Gomez et al., 2014) that may affect aggressive (Wisniewski et al., 2005), anxiety-related (Rodriguez-Gomez et al., 2014) and sexual behaviors (Hull and Dominguez, 2006).

Among EDCs, phytoestrogens are a class of compounds widely distributed in the plant kingdom potentially able to influence all processes regulated by estrogens (Wang et al., 2002). Genistein (GEN) is a phytoestrogen of the group of isoflavones, highly present in soy (Mazur, 1998). GEN is considered a potential EDC due to the fact that is able to interact with the neural pathways related to estrogens in a complex and multidirectional manner. In particular, the exposure to GEN during developmental critical periods could have several long-term consequences on the nervous system of male and female higher vertebrates, as well as on related behaviors (Jefferson et al., 2012, Rodriguez-Gomez et al., 2014). In recent years there has been an ever-increasing consumption of soy because of its easy cultivation, and its

high protein levels. Moreover it is a substitute for dairy products for the feeding during childhood; this has caused a significant increase in exposure to phytoestrogens.

EDCs may interfere with the expression of many neuropeptides and enzymes (Panzica et al., 2011). Therefore, we focused here our morphological analysis on the nitrergic and vasopressinergic circuits. Both systems are regulated by gonadal hormones' signaling in adulthood [vasopressin, AVP, (Nomura et al., 2002, Grassi et al., 2010, Piet et al., 2015); nitric oxide, NO, (Scordalakes et al., 2002, Grassi et al., 2013b, Grassi et al., 2013c, Grassi et al., 2016)]. In addition they are sexually dimorphic in several regions (AVP, De Vries and Panzica, 2006; NO, Panzica et al., 2006), and are sensitive to hormonal fluctuation during the estrous cycle (NO, Gotti et al., 2009, Sica et al., 2009, Martini et al., 2011; AVP, Skowsky et al., 1979, Levin and Sawchenko, 1993).

NO is a gaseous neurotransmitter produced by three isoforms of Nitric Oxide Synthase: endothelial, inducible and neuronal NOS (Alderton et al., 2001), the latter being expressed in several regions of rodent brain (Rodrigo et al., 1994, Gotti et al., 2005). NO is involved in many physiological activities such as neuroprotection, neural degeneration, long-term potentiation and secretion of many neurotransmitters and neuropeptides (for reviews see: Nelson et al., 1997, Prast and Philippu, 2001, Hull and Dominguez, 2006). NO also modulates several behaviors including reproduction and sexual behavior (for reviews see Nelson et al., 1997, Panzica et al., 2006) and mediates AVP release (Zhang et al., 2009, Vega et al., 2010).

AVP is a nonapeptide produced in two major neural subpopulations: the magnocellular neurons of the supraoptic and paraventricular nucleus projecting to the neurohypophysis (where the peptide is released within the blood as antidiuretic hormone, regulating osmolarity and blood pressure) (Bourque, 1999), and the parvocellular neurons mainly located within the medial amygdala, bed nucleus of the stria terminalis, medial subdivision of the paraventricular nucleus (PVN), and the suprachiasmatic nucleus (SCN) (Rood and De Vries, 2011). These cells project to a number of brain sites where AVP acts as a neuropeptide modulating several behaviors, i.e., social memory, parental behavior, sex behavior, aggression, anxiety, circadian rhythms, body temperature regulation, and others (for a review see Caldwell et al., 2008). In rodents, many of those behaviors are affected by E_2 modulation of the AVP system (Scordalakes and Rissman, 2004, Grassi et al., 2010).

The critical age in which neural circuits may be permanently affected by EDC exposure such as bisphenol A (BPA) (Martini et al., 2010) or GEN (Wisniewski et al., 2005, Panzica et al., 2011), depends on the species and on the considered brain area. Many of these circuits develop prenatally (McCarthy, 2008). For instance prenatal GEN exposure resulted in a

demasculizing effect on AVP (or arginin vasotocin) system in MPOA and BST in mammals (Scallet et al., 2003) and birds (Viglietti-Panzica et al., 2007). Other neural systems are sensitive to steroid hormones during early postnatal development. In this period sexual hormones shape BST circuits and disrupt typical aspects of the female reproductive system in rats (Fukushima et al., 2013), as well as the SCN nucleus and circadian rhythms (Royston et al., 2016).

In our previous study we have shown that indirect exposure to GEN (administered with a pipette to the mother), during late gestation (through the placenta) and the first post-natal week (through the mother milk), resulted in changes in anxiety and aggressive behaviors of male offspring mice, paralleled with changes in the nNOS system in the amygdala (Rodriguez-Gomez et al., 2014). However, the developmental effects attributable to GEN exposure in our study are more likely to result from fetal exposures rather than from the postnatal one. In fact, GEN can easily cross the placental barrier (Doerge et al., 2001), while its accumulation in the milk is limited due to the mammary gland barrier (Doerge et al., 2006). Therefore, the purpose of this experiment was to focalize on the effects of a post-natal exposure (i.e. the first postnatal week) on both sexes, mimicking the exposure to soy-milk during infancy (Rozman et al., 2006). Moreover, we broadened our analysis also to the AVP system, since, in other models, it was affected by GEN treatment through E_2-similar mechanisms (Forsling et al., 2003; Scallet et al., 2003; Grassi et al., 2013a). Some of the nNOS and AVP sexually dimorphic circuits are controlled by estrogens for both their development and their activation in adulthood. Therefore, they are major putative targets for the disruptive action of GEN if this molecule is acting by interfering with the cellular mechanisms of estrogenic action.

EXPERIMENTAL PROCEDURES

Animals

Two months old virgin CD-1 mice (10 females and 5 males) were purchased from HARLAN Italy and maintained as an outbreed colony at the University of Torino. Mice were housed and treated according to European guidelines (European Union Council Directive of 24th November 1986 n° 86/609/EEC). All the procedures were approved by the Italian Ministry of Health and by the Ethical Committee of the University of Turin.

Groups of 3-5 animals of the same sex were housed in 45x25x15 cm polypropylene mouse cages at 22±2°C, under a 12:12 natural light/dark cycle (light on at 8:00 am). Water and food were provided ad libitum (standard mouse chow 4RF25-GLP with certificated non-detectable estrogenic activity, Mucedola srl, Settimo Milanese, Italy).

After 2 weeks habituation period, females were mated with males in 2:1 ratio until vaginal plug was detected. Pregnant females were randomly housed in 3 groups (3-4 per cage, 45x25x15 cm). 2-3 days before delivery, females were separated in order to identify litters.

Estradiol and genistein treatment

Litters were reduced to 7 pups at birth, then, they were randomly allocated to each cage in a way that each mother fostered pups from the all litters. A total of 70 pups were divided into 10 cages belonging to three experimental (see below) groups since the day of birth. The size of the litter was established in order to ensure an appropriate number of males and females for the following analysis since we could not determine the sex of each pup at birth. Moreover this number allowed us to have enough pups despite few of them in each group died in the first week of age (about 10%). In each group, pups were fed (with a micropipette) either vehicle (10 µl/g sesame OIL; cat. Number S33547, Sigma-Aldrich, Milan, Italy), E_2 (50 µg/Kg; cat. Number E4876 Sigma-Aldrich, Milan, Italy) or GEN (50mg/Kg cat. number G6649, Sigma–Aldrich, Milan, Italy) diluted in sesame oil starting from birth (post-natal day 1, PND1) to PND8. GEN dose approximate the amount of GEN reported in soy-based baby formulas (Cimafranca et al., 2010). Pups were weaned at postnatal day 21 (PND21) and housed in treatment-differentiated monosexual groups of 3-5 animals.

Fixation and tissue sampling

At two months of age, 36 animals (6 males and 6 females per each group) were randomly chosen for immunohistochemical analyses.

Animals were deeply anesthetized with Pentothal Sodium (100 mg/kg, MSD Animal Health Srl, Segrate, Milano, Italy), monitored until the pedal reflex was abolished and killed by cervical dislocation. To ensure that females were in the same hormonal condition they were killed in estrus (tested by vaginal smear). Brains were quickly dissected and fixed in 5% acrolein in saline-phosphate buffer 0.1M, pH 7.3-7.4 (PBS) for 150 min at room temperature. After fixation, they were washed in PBS, and stored in a PBS–30% sucrose solution at +4°C, until their precipitation. The next day, brains were frozen in isopentane pre-cooled in dry ice at -30°C / –40°C and stored at –80°C.

Coronal section (40 μm thick) were cut with a cryostat (Leica CM 1900), obtaining 3 series of adjacent serial sections, collected for free-floating procedure in a cryoprotectant solution (Watson et al., 1986), and kept at −20 °C. Alternate sections were stained for nNOS or AVP. Brains were always processed in groups containing both female and male sections of each treatment, so that between-assays variance could not cause systematic group differences.

Immunohistochemistry

Before the procedures for nNOS or AVP immunohistochemistry, the sections were washed for 15 minutes in sodium borohydride (0.1% in PBS) to remove the acrolein, followed by overnight washings in PBS (at 4°C). Sections were then exposed to Triton X-100 (0.2% in PBS) for 30 min and treated for blocking endogenous peroxidase activity (PBS solution containing methanol/hydrogen peroxide, 20 min, at room temperature).

nNOS immunohistochemistry

After 30 min of preincubation in normal goat serum (Vector Laboratories, Burlingame, CA, USA; 1:100) sections were incubated overnight at room temperature with the rabbit polyclonal antibody against nNOS (Diasorin, Sillwater, MN, USA; 1:12.000). The next day, sections were incubated for 60 min in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1:200). The antigen-antibody reaction was revealed by 60 min incubation with biotin–avidin system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). The peroxidase activity was visualized with a solution containing 0.4 mg/ml of 3,3′-diamino-benzidine (Sigma–Aldrich, Milano, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene and cover slipped with Entellan mounting medium (Merck-Millipore, Milano, Italy). Specificity of the primary antibody was reported in previous papers (Gotti et al., 2005, Gotti et al., 2009).

AVP immunohistochemistry

Sections were pre-incubated for 60 minutes in a medium containing 2% normal goat serum (Vector Laboratories, Burlingame, CA, USA), 1% bovine serum albumin (Sigma–Aldrich, Milano, Italy), and 0.2% Triton X-100 in PBS, pH 7.4. After this step, sections were incubated for 48 hours in a polyclonal rabbit anti-vasopressin antibody [1:20.000, gift of Dr. Michael Sofroniew, (UCLA, Los Angeles, USA), 2% normal goat serum, 1% bovine serum albumin, 0.2% Triton X-100 in PBS pH 7.4] at 4°C. After incubation for 60 min in

biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA, diluted 1:200, in PBS containing 2% normal goat serum and 0.2% Triton X-100, pH 7.4), the antigen-antibody reaction was revealed with the same protocol described for nNOS immunohistochemistry.

Specificity of AVP antiserum for both radioimmunoassay and immunohistochemistry was reported in previous studies (Hayward et al., 1976, Sofroniew et al., 1978, Sofroniew et al., 1979).

Controls

For both nNOS and AVP immunoreactions we performed the following controls: (a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); (b) the secondary antibody was omitted. In these conditions, cells and fibers were totally unstained.

Quantitative analysis

For computer assisted quantitative analysis we selected two standardized serial sections of comparable level (according to the mouse brain atlas, (Paxinos and Franklin, 2001) per each nucleus of each animal. Selected sections were photographed with a Nikon eclipse 80i microscope equipped with a Nikon Digital Sight DS-L2 video camera using a 20X objective. Images were analyzed with Image J software (version 1.45s, Wayne Rasband, NIH, Bethesda, MD, USA). For each level of the analyzed nucleus immunoreactive cells were counted (using the cell counting *plug-in*) in a selected frame with a standardized area (region of interest, ROI) placed within the boundaries of the nucleus.

For the nNOS system, we analyzed the MPOA (0.14 mm and -0.10 mm from Bregma; ROI: 100,000 μm^2, Fig.1A), the ventromedial part of the Bed nucleus of the Stria Terminalis (BSTmv; -0.10 mm and -0.22 mm from Bregma; ROI: 30,000 μm^2, Fig.2A), the BasoLateral Amygdala (BLA; -0.7mm and -1.06 mm from Bregma, left hemisphere; ROI: 200,000 μm^2), the Lateral amygdala (La; -0.7mm and -1.06 mm from Bregma, left hemisphere; ROI: 150,000 $\mu m^{2)}$, three subdivision of paraventricular nucleus [ROI: 150,000 μm^2; anterior parvicellular part (PaAP; -0.58mm from Bregma), lateral magnocellular part (PaLM; -0.58mm and -0.94 mm from Bregma, and ventral part (PaV; -0.58 mm and -0.70 mm from Bregma), Fig.3A], the arcuate nucleus (ARC; -1.46 mm and -1.70 mm from Bregma; ROI: 130,000 μm^2, Fig.4A) and the ventrolateral subdivision of ventromedial hypothalamus (VMHvl; -1.46 mm and -1.70 mm from Bregma; ROI: 75,000 μm^2, Fig.4A).

AVP immunoreactivity was quantified in the following nuclei: LS (1.18 mm and − 0.10 mm from Bregma; ROI: 98,300 μm^2), BSTmv (0.50 mm to − 0.10 mm from Bregma; ROI: 98,300 μm^2, Fig.5), SCN, (−0.22 mm and −0.82 mm from Bregma; ROI: 85,000 μm^2), and four subdivision of the paraventricular nucleus [paraventricular dorsal cap (PaDC, −0.58 mm and - 0.94 mm from Bregma; ROI: 2700 μm^2); PaLM, (−0.58 mm and - 0.94 mm from Bregma; ROI: 20,000 μm^2); paraventricular medial parvicellular (PaMP, −0.58 mm and - 0.94 mm from Bregma; ROI: 30,250 μm^2); paraventricular medial magnocellular (PaMM, −0.58 mm and - 0.94 mm from Bregma; ROI: 30,300 μm^2, Fig.6A).

As previously described for both nNOS (Gotti et al., 2005, Sica et al., 2009, Martini et al., 2010) and AVP (Rood and De Vries, 2011), positive neurons were identified for the presence of a clearly labeled cell body and proximal processes. nNOS positive neurons were counted within MPOA, BSTmv, BLA, La, PVN, and ARC. AVP positive cells were counted within PVN and SCN. The values are reported in Fig 1-4 and 6 as cell number/10^5 μm^2.

In the nuclei where the cells were absent or difficult to discriminate from the background (nNOS, VMHvl, AVP, LS and BST), we quantified the presence of immunoreactive material with the method of the fractional area, by calculating, in binary transformation of the images (threshold function of Image J), the percentage of pixels covered by the positive structures in the predetermined ROIs (as previously described by Viglietti-Panzica et al., 2007).

Statistical analysis

Morphometrical data were analyzed using the statistic software SPSS 22.0. Differences were considered significant when *p value* was less than 5% ($p \leq 0.05$). Before statistical tests, to choose whether to use parametric or non-parametric tests, we evaluated the normality of data with the Shapiro-Wilk test. All the data have shown a normal distribution: therefore, we used the analysis of variance (ANOVA) to check for the presence of significant differences.

For the quantitative analysis of histochemically stained sections, we applied the two-way ANOVA for repeated measures in order to determine whether the rostral and caudal levels selected for each nucleus could be considered comparable or significantly different from each other. In the first case we proceeded by calculating the average values between the two sections: in the second case we continued the analysis by considering separately the two levels and running the two-way ANOVA to highlight any significant differences. If the values of the two sexes are significant different we performed independent one-way ANOVA on both sexes. If the ANOVA significance was $p<0.05$, we applied the Tukey's post hoc test to reveal significant differences among groups.

RESULTS

NOS system

Qualitative observation of our specimens demonstrated that the nNOS immunostaining has a distribution of cells and fibers consistent with our previous studies (Gotti et al., 2005). The following quantitative analyses report for each nucleus the value of cell density (when applicable) or of the fractional area covered by immunoreactive material, calculated on the basis of the applied ROI for each region (see Materials and Methods).

In all examined nuclei, with the exception of the ARC, a preliminary two-way ANOVA for repeated measures (being the different positions, rostral and caudal, the repeated measures) reported no effects of the position. Therefore, we proceeded by calculating the average values between the two sections. For the ARC we continued the analysis by considering separately the two levels.

Medial preoptic area (MPOA)

As expected (Martini et al., 2010), in the medial preoptic area (MPOA) of control males we observed many rounded nNOS+ cells in a dense mash of fibers (Fig. 1F) and their number was significantly higher than in control females. We observed significant effects of both sex ($F_{(1,32)}$= 60.270, p<0.0001) and treatment ($F_{2,32)}$= 14.130, p<0.0001), but no interaction ($F_{(2,32)}$= 0.425, p= 0.658), over the density of nNOS+ cells in MPOA (Fig.1).

In all groups of males the density of labeled cells was higher than in the groups of females (Female OIL vs Male OIL: p=0.027; Female E_2 vs Male E_2: p=0.001; Female GEN vs Male GEN: p<0.0001).. In females we observed no effects of treatment ($F_{(2,7)}$= 2.915, p=0.145). On the contrary, in males-the treatment had significant effects ($F_{(2,23)}$= 20.277, p<0.0001): in fact, GEN males have significantly higher density of nNOS cells in comparison to both E_2 (p<0.0001) and OIL (p<0.0001) males (Fig.1B; Tab. 1).

Bed nucleus of the stria terminalis, ventromedial part (BSTmv)

As reported in previous studies (Martini et al., 2010), a comparable number of clearly labeled neuronal somas and fibers were present in BSTmv of both males and females (Fig.2C, F). Statistical analysis highlighted that nNOS+ cell density was not influenced by sex or treatment (Sex: $F_{(1, 32)}$= 3.536, p=0.071; Treatment: $F_{(2,32)}$=0.744, p=0.485), but there was an

effect of the interaction among sex and treatment (Interaction Sex*Treatment: $F_{(2,32)}$= 3.991, p=0.031). In fact E_2 and GEN treatments induced a no significant decrease of the density of nNOS+ cells in females while in males produced very limited increase. Comparisons among groups revealed only a tendency to significance for the comparison GEN males vs GEN females (p=0.074) (Fig.2B; Tab.1).

Amygdala (Lateral Amygdala and BasoLateral Amygdala)

In the amygdala we analyzed the lateral (La) and the basolateral (BLA) nuclei, two regions where no sex dimorphism was described for the nitrergic population, but where our previous investigation demonstrated an effect of prenatal treatment with GEN (Rodriguez-Gomez et al., 2014). In both regions, no obvious differences were observed among different groups. For La, no effect of sex, treatment and interaction of the two factors was observed (Sex: $F_{(1,30)}$= 3.103, p=0.091; Treatment: $F_{(2,30)}$= 0.069, p=0.933; Interaction Sex*Treatment: $F_{(2,30)}$= 0.291, p=0,750) (Tab.1). For BLA, only a significant effect of the interaction Sex-Treatment (Sex: $F_{(1,32)}$=0.798, p=0.380; Treatment: $F_{(2,32)}$=1.026, p=0.372; Interaction Sex*Treatment: $F_{(2,32)}$= 3.502, p=0.045) was detected, but the following post hoc analysis did not reveal a significant difference among treated and control groups (Tab.1).

Paraventricular Nucleus

The paraventricular nucleus showed an intense labeling for nNOS throughout the nucleus. In previous studies we performed a less detailed study of the nitrergic population and we have not detected any sex difference (Sica et al, 2009: Martini et al., 2010). Labeled neurons were rounded, grouped in small clusters, with sparse dendritic ramifications with a heterogeneous distribution within the different subdivisions that were thus separately analyzed. The highest population of nNOS+ cells was in the PaLM and extended laterally above the fornix like a cap. Along the rostro-caudal axis, PaLM changed its size, and the number of cells decreased. The PaMP showed the lowest number of nNOS+ cells, while the anterior magnocellular (PaAP) and PaV regions had an intermediate number of cells (Fig.3C, F). Size and shape of these subdivisions did not change considerably towards rostro-caudal axis**.** In PaLM area, we observed differences in the density of nNOS+ cells among the various treatment in both sexes (Sex: $F_{(1,23)}$=10.090, p=0.006; Treatment: $F_{(2,23)}$=4.468, p=0.028; Interaction Sex*Treatment: $F_{(2,23)}$= 1.366, p=0.282, Fig.3B). In males we observed no effects ($F_{(2,13)}$= 0.948, p=0.417), while females displayed a significant difference between experimental groups ($F_{(2,8)}$= 11.193, p=0.009) having E_2 and GEN females a significantly higher density of nNOS cells in

comparison to OIL females (female OIL vs female E_2: p=0.009; female OIL vs female GEN: p=0.040, Fig.3B). In PaAP statistical analysis revealed a different density of the nNOS+ cells, influenced only by sex but not by treatment (Sex: $F_{(1,23)}$= 14.677, p=0.001; Treatment: $F_{(2,23)}$= 1.438, p=0.265; Interaction Sex*Treatment: $F_{(2,23)}$= 3.027, p= 0.075). In fact a significant difference was observed between males and females for GEN groups (p=0.044), and only in male, GEN produces a significant increase in comparison with OIL group (p=0.029) (Fig.3B, Tab.1).

Finally, in PaV area, no effects of all factors was detected (Sex: $F_{(1,23)}$= 0.072, p=0.792; Treatment: $F_{(2,23)}$= 0.382, p=0.688; Interaction Sex*Treatment: $F_{(2,23)}$= 1.653, p= 0.221, Tab.1)

Ventromedial nucleus, ventrolateral part (VMHvl)

In ventrolateral part of the ventromedial hypothalamus (VMHvl) no labelled cells were observed and nNOS immunoreactivity was restricted to fibers (Fig.4D, G). We, thus, evaluated the fractional area occupied by immunoreactive material in the ROI (see Material and Methods). The intensity of labeling was not affected by the treatment but only by sex (Sex: $F_{(1,36)}$= 23.808, p<0.0001; Treatment: $F_{(2,36)}$= 1.111, p=0.342, Interaction Sex*Treatment: $F_{(2,36)}$= 2.293, p=0.118, Fig.4B). Namely, female treated groups showed a lower immunoreactivity in comparison with the same male treated groups, as expected (Martini et al., 2010), this sexual dimorphism is not present in control groups (female OIL vs male OIL: p=0.815; female E_2 vs male E_2: p=0.037; female GEN vs male GEN: p=0.006, Fig.4B).

Arcuate nucleus

Few nNOS+ cells were present in the arcuate nucleus (ARC) of both females (Fig.4D-F) and males (Fig.4G-I). In our previous studies we have not detected a significant sex difference in control animals (Sica et al., 2009; Martini et al., 2010). The ANOVA for repeated measures revealed that in this nucleus, there was a significant difference between the two levels considered for the quantitative analysis ($F_{(1,23)}$= 77.494, p<0.0001; Tab. 1). Therefore, we considered the two levels separately and the statistical analysis revealed that, in the rostral level of ARC there was an effect only of sex (Sex: $F_{(1,29)}$= 18.499, p<0.0001; Treatment: $F_{(2,29)}$= 2.315, p=0.121; Interaction Sex*Treatment: $F_{(2,29)}$= 2.641, p=0.093). In fact, we detected a sexual dimorphism in animals treated with E2 but no differences among sexes in control and GEN groups (female OIL vs Male OIL: p=0.965, female E_2 vs male E_2, p=0.008

and female GEN vs male GEN, p=0.119, Tab.1). Moreover, an effect of the treatment was observed only in males ($F_{(2,19)}$= 6.663, p=0.007) in which both E_2 and GEN treatment produced a significant increase of NOS+ cells in comparison with control group (male OIL vs male E_2: p=0.010, male OIL vs male GEN: p=0.028, Tab.1).

In the caudal level, statistical analysis revealed effects of both sex and interaction among sex and treatment (Sex: $F_{(1,29)}$= 21.885, p<0.0001; Treatment: $F_{(2,29)}$= 0.170, p=0.844; Interaction Sex*Treatment: $F_{(2,29)}$= 8.526, p=0.002). In both GEN and E_2 treated groups we found a sex difference in the density of nNOS+ cells, while in control groups there was no dimorphism (female OIL vs male OIL: p= 0.992, female E_2 vs male E_2: p= 0.001, female GEN vs male GEN: p=0.014, Fig.4C). Moreover, a significant effect of treatment in both males ($F_{(2,19)}$= 6.663, p=0.007) and females ($F_{(2,8)}$= 16.208, p=0.004). However, in females, E_2 or GEN treatments induced a decrease of NOS+ cell density (female OIL vs female E_2: p=0.003, female OIL vs female GEN: p=0.019, Fig.4C, D-F), whereas in males they induced an increase (male OIL vs male E_2: p=0.010, male OIL vs male GEN: p=0.028, Fig.4C, G-I).

AVP system

The distribution of the AVP-ir structures was highly comparable to the previous descriptions in mouse (Rood and De Vries, 2011). In all examined nuclei, with the exception of the SCN, a preliminary two-way ANOVA for repeated measures reported no effects of the position. Therefore, we report here the results of the two-way ANOVA performed on the average values. For the SCN we continued the analysis by considering separately the two levels.

Lateral septum

According to numerous previous studies in almost all vertebrates (see for a review De Vries and Panzica, 2006) the vasopressin innervation of the LS show a strong sex difference with a higher density of fibers in males than in females. In the present experiment we confirmed this, while no effects of Treatment and Interaction was evident (Sex: $F_{(1,29)}$= 86.626, p<0.0001; Treatment: $F_{(2,29)}$= 0.433, p=0.654; Interaction Sex*Treatment: $F_{(2,29)}$= 0.485, p=0.622). A strong dimorphism was observed in all groups (Male OIL vs Female OIL, p<0.0001; Male GEN vs Female GEN, p=0.0001; Male E_2 vs Female E_2, p<0.002) while no effect of treatment in both males ($F_{(2,14)}$= 0.375, p=0.696) and females ($F_{(2,14)}$= 0.901, p=0.432), was found (Tab.1)

Bed nucleus of the stria terminalis, ventromedial part (BSTmv)

Even if positive cell bodies were not clearly visible in our specimens, the qualitative observation of the BST confirmed the existence of a strong sex dimorphism as previously demonstrated in other studies (see for a review De Vries and Panzica, 2006), with a higher density of AVP+ structures in males. We detected a significant effect of sex and no effects of treatment and of the interaction (Sex: $F_{(1,29)}$= 128.487, p<0.0001; Treatment: $F_{(2,29)}$= 2.219, p=0.131; Interaction Sex*Treatment: $F_{(2,29)}$= 0.599, p=0.558). A sex dimorphism was evident in all groups (female OIL vs male OIL: p<0.0001, female E_2 vs male E_2: p<0.0001 female GEN vs male GEN: p<0.0001, Fig.5).

Interestingly the treatment had a significant effect only in females (females: $F_{(2,14)}$= 7.373, p=0.008; males $F_{(2,13)}$= 1.174, p=0.345, Fig.5B, F-H), where we evidenced a significant effect for GEN in comparison to OIL (p=0.007 Fig.5B-E).

Suprachiasmatic nucleus (SCN)

In the SCN we observed a large group of AVP+ parvocellular neurons, with no apparent differences among sex (as reported in human in a previous study by Swaab in 1995). Moreover, we did not detect any evident difference in the treatment groups. The two-way ANOVA for repeated measures revealed a significant effect of the position ($F_{(1,23)}$= 14.116, p=0.001). Therefore, we proceeded to analyze each levels separately. In the rostral level of SCN we observed a weak, but not significant, effect of sex, but no effect of treatment or of interaction (Sex: $F_{(1,29)}$= 4.386, p=0.047; Treatment: $F_{(2,29)}$= 2.598, p=0.096; Interaction Sex*Treatment: $F_{(2,29)}$= 0.502, p=0.612; Tab.1).

In the caudal level of SNC, we observed an effect of both sex and treatment (Sex: $F_{(1,29)}$= 5.168, p=0.033; Treatment: $F_{(2,29)}$= 3.865, p=0.036; Interaction Sex*Treatment: $F_{(2,29)}$= 0.952, p=0.401), but, again, the post-hoc test did not reveal any significant differences among groups (Tab.1).

Paraventricular nucleus

The paraventricular nucleus is a complex structure containing several subgroups of parvo- and magnocellular AVP+ neurons; no study reported a significant sex difference for the AVP populations of this nucleus. We have performed, at first, a two-way ANOVA including all the subnuclei. This analysis did not detect any significant effect (Sex: $F_{(1,29)}$= 0.397, p=0.535; Treatment: $F_{(2,29)}$= 1.604, p=0.223; Interaction Sex*Treatment: $F_{(2,29)}$= 0.269, p=0.766, Tab.1). Therefore, we analyzed separately each individual sub-nucleus. No significant effect

of both sex and treatment was observed in PADC, PALM, and PAMM (see Tab.1 for the results). Only in the medial parvicellular part (PaMP) we detected an effect of the interaction sex-treatment (Sex: $F_{(1,29)}$= 0.404, p=0.531; Treatment: $F_{(2,29)}$= 0.430, p=0.656; Interaction Sex*Treatment: $F_{(2,29)}$= 9.236, p=0.001, Fig.6). An effect of GEN treatment was evident both in females ($F_{(2,14)}$= 4.104, p=0.044), with a significant differences among female OIL vs female GEN (p=0.036), and in males ($F_{(2,13)}$= 5.472, p=0.022), being GEN males significantly different from both OIL group (p=0.032) and E_2 group (p=0.049) (Fig.6). GEN treatment had a different effect in the two sexes: it increased the number of AVP+ cells in females and decreased the cell number in males (this sex difference is very close to significance, p=0.054).

DISCUSSION

Our results indicate that direct treatment with GEN of mouse pups, at a dose comparable to that used in infant formulas (Cimafranca et al., 2010) during the first postnatal week had organizational effects on nNOS and AVP limbic-hypothalamic circuits. Those effects were sexually dimorphic and only partially related to the estrogenic nature of GEN since some of them were not mimicked by E_2 treatment.

Estrogen signaling modulates many sexually dimorphic neuronal circuits and related behaviors (Kastenberger and Schwarzer, 2014, Guimaraes et al., 2015). These sex differences may arise from a hormonal imprinting early in life (McCarthy, 2015, Panzica and Melcangi, 2016). Stress (Roque et al., 2014) or endocrine disruptors (Rebuli and Patisaul, 2016), including phytoestrogens as GEN (Lephart et al., 2004), may permanently interfere with this process, changing subsets of neuronal circuits in the adult.

Among others, AVP and nNOS systems are involved in the control of a wide spectrum of behaviors (see for reviews Nelson et al., 1997, Caldwell et al., 2008) and are sensitive to estrogenic signaling in adulthood [nNOS (Panzica et al., 2006), AVP (Young et al., 2011)] and during the development (Campbell and Herbison, 2014). These circuits may thus be affected by the exposure to hormones and EDC during specific time window (critical period) (Panzica et al., 2009, Sullivan et al., 2014). In our study we observed alterations of both nNOS and AVP systems.

The pattern of nNOS immunoreactivity in control animals was consistent with our previous studies (Sica et al., 2009; Martini et al., 2010). In fact, as expected, sex differences in nNOS-ir were observed in the MPOA but not in the PVN, ARC, or BSTmv. However, a

previous study reported a higher cell density in C57/BL6 male's mice BSTmv (Sica et al., 2009) suggesting the existence of a strain difference in this region.

Similarly, the distribution of AVP immunoreactivity in control animals was consistent with previous studies in mouse (Rood and De Vries, 2011). As expected, we observed a significant dimorphism in the BST and LS with a higher density of immunoreactive material in males than in females (De Vries and Panzica, 2006, Rood et al., 2013).

Moreover, we have previously demonstrated that GEN prenatal administration interferes with anxiety-related behaviors and part of the nitrergic system in adulthood (Rodriguez-Gomez et al., 2014). Other EDCs (i.e. chlorobenzenes) may affect similar behaviors and the AVP system (Nagyeri et al., 2012). Similarly, BPA exposure during development may alter the nitrergic system (Martini et al., 2010). In neurons, GEN may bind primarily ERβ mimicking the E_2 effect (Routledge et al., 2000), therefore, in our experiment, as positive control, we included early post-natal treatment with E_2 that induced some alteration of the investigated neural circuits. Interestingly, GEN mimicked E_2 treatment only in part of the neuronal populations we have examined, (e.g. on nNOS+ cells in the PaLM, on the caudal part of ARC of females and on rostral part of the ARC in males), whereas, in other circuits GEN and E_2 have different effects (e.g. AVP+ labeling in BST and in PaMP). This suggests the GEN may have multiple way of action partly based on E_2-receptors and partly independent.

A crucial variable that may explain some of the discrepancies among studies on the effects of EDCs is the timing of exposure to these compounds. In our previous study, the perinatal administration of GEN to the mothers affected nNOS+ cells in amygdala but not in PVN (Rodriguez-Gomez et al., 2014). In the present study the postnatal treatment of pups affected nNOS+ cells in PVN but not in amygdala indicating that the critical period for the development of these circuits is probably different, as reported in other models (Wisniewski et al., 2005, Panzica et al., 2011) as AVP system in MPOA and BST (Scallet et al., 2003). In addiction, the experimental conditions are strongly different from our previous paper (Rodriguez-Gomez et al., 2014). In fact, in our first experiment the embryos were exposed to GEN during prenatal development (GEN easily crosses the placental barrier; Doerge et al., 2001), but the supply after birth was presumably reduced, because GEN is almost totally blocked by the mammary barrier in rodents (Doerge et al., 2006). In the present experiment (performed on both sexes) the pups were directly fed with GEN for 8 days, thus directly interfering with the organizational processes that take places in the course of the first post-natal week of life. The divergence in the effects between perinatal and postnatal GEN

administration indicates that those circuits are still plastic at early postnatal age and may be altered by EDCs exposure (Frye et al., 2012).

Finally, we observed that postnatal GEN administration has different effects depending on sex. In fact, it induced a significant but small effect in the BST of female (but this is not inverting the sexual dimorphism of the system), and a larger effect in the PVN inducing an inverted phenotype among males and females. The lack of effect on the sexual differentiation of the AVP system of the BST is largely in accord with previous studies demonstrating that this sexually dimorphic circuit is depending by a direct genomic effect or to androgen receptor rather than to the presence of E_2 in critical period (Pierman et al., 2008, Allieri et al., 2013).

CONCLUSIONS

Phytoestrogens are able to act as endocrine disruptors. While there is an increasing concern on the effects of exposure to EDCs of anthropogenic origin, and of their regulation (Slama et al., 2016). These plant-derived compounds are not perceived as potentially dangerous elements. However, an increasing number of studies have shown that also these compounds, in particular GEN, may interfere with the development of neural circuits in laboratory animals (Dinsdale and Ward, 2010). The present study, as well as the previous one (Rodriguez-Gomez et al., 2014), indicates that exposure of male and female pups to low doses of GEN, comparable to those present in formulas of soy milk, may alter in a sexually dimorphic way, nNOS and AVP circuits in hypothalamic structures that are involved in the control of several behaviors. Moreover, as previously described for other EDCs as the BPA (Rebuli and Patisaul, 2016), we observed important differences between the action of GEN and E_2 emphasizing that these molecules are not simply acting as "estrogen-like" or "weak estrogens" but rather have more complex and dynamic mechanisms of action.

The fact that GEN may have such a long-term effect for a short term- and low dose treatment raises concern for the use of soy based formulas for infant and soy based supplements for pre weaning animals.

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Figure Legends

Figure 1. Histograms and microphotographs illustrating the immunohistochemical immunoreactivity for nNOS (nNOS-ir) in the medial preoptic area. (A) Schematic diagram of the coronal section where the photographs were taken. The original drawings were taken, with permission, from the atlas of Paxinos and Franklin (2001) (B) Histograms representing nNOS-ir in MPOA (expressed as cells/$10^5\mu m^2$; OIL, white bars; E_2, grey bars; GEN, black bars). In each group, males had consistently more nNOS labeled cells than females. Moreover, GEN males have significantly higher density of nNOS cells in comparison to both E_2 and OIL males. The significant differences (see results) are denoted by different letters (a,b,c). Photomicrographs illustrating the MPOA of females (C-E) and males (F-H) treated with either OIL (C and F), E_2 (D and G) or GEN (E and H). Magnification bar =100 μm

Figure 2. Histograms and microphotographs illustrating the immunohistochemical immunoreactivity for nNOS in the bed nucleus of the stria terminalis. (A) Schematic diagram of the coronal section where the photographs were taken. (B) Histograms representing nNOS-ir in BST (expressed as cells/$10^5\mu m^2$; OIL, white bars; E_2, grey bars; GEN, black bars). E_2 and GEN treatments induced a no significant decrease decrease of the density of nNOS+ cells in females while in males produced a no significant increase. Photomicrographs illustrating the BST of females (C-E) and males (F-H) treated with either OIL (C and F), E_2 (D and G) or GEN (E and H). Magnification bar =20 μm.

Figure 3. Histograms and microphotographs illustrating the immunohistochemical immunoreactivity for nNOS in the paraventricular nucleus. (A) Schematic diagram of the coronal section where the photographs were taken. (B) Histograms representing nNOS-ir in PaLM. Females reported a significant difference between experimental groups: E_2 and GEN females have significantly higher density of nNOS cells in comparison to OIL females. (C) Histograms representing nNOS-ir in PaAP. There is a significant difference between males and females for GEN groups, and only in male, GEN produces a significant increase in comparison with OIL group. Histograms for B and C are expressed as cells/$10^5\mu m^2$; OIL, white bars; E_2, grey bars; GEN, black bars. The significant differences (see results) are

denoted by “a” or “b”.

(D-I) Photomicrographs illustrating the PVN of females (E-G) and males (H-I) treated with either OIL (D and G), E_2 (E and H) or GEN (F and I). Magnification bar =100 µm.

Figure 4 Histograms and microphotographs illustrating the immunohistochemical immunoreactivity for nNOS in the ventromedial and arcuate nuclei. (A) Schematic diagram of the coronal section where the photographs were taken. (B) Histograms representing nNOS-ir in VMH. Female treated groups showed a lower immunoreactivity in comparison with the same male treated groups, but this sexual dimorphism is not present in control groups. (C) Histograms representing nNOS-ir in a caudal level of ARC. In females, E_2 or GEN treatments induced a decrease of NOS+ cell density whereas in males they induced an increase. In both GEN and E_2 treated groups we found a sex difference in the density of nNOS+ cells, while in control groups there was no dimorphism. Histograms for B are expressed as percentage of area covered by stained elements. Histograms for C are expressed as cells/10^5µm^2; OIL, white bars; E_2, grey bars; GEN, black bars. The significant differences (see results) are denoted by “a”, “b”, or “c”.

Microphotographs illustrating the VMH-ARC of females (D-F) and males (G-I) treated with either OIL (D and G), E_2 (E and H) or GEN (F and I). Magnification bar =100 µm.

Figure 5. Histograms and microphotographs illustrating the immunohistochemical immunoreactivity for AVP in the bed nucleus of the stria terminalis. (A) Schematic diagram of the coronal section where the photographs were taken. (B) Histograms representing AVP-ir in BST (expressed as percentage of area covered by stained elements; OIL, white bars; E_2, grey bars; GEN, black bars). There is a strong sex dimorphism (having males a higher presence of AVP-ir materials in all groups. Only in female, GEN treatment increased AVP expression in comparison to OIL.

The significant differences (see results) are denoted by “a”, “b”, or “c”.

Microphotographs illustrating the BST of females (C-E) and males (F-H) treated with either OIL (C and F), E_2 (D and G) or GEN (E and H). Magnification bar =50 µm;

Figure 6. Histograms and microphotographs illustrating the immunohistochemical

immunoreactivity for AVP in the Paraventricular nucleus. (A) Schematic diagram of the coronal section where the photographs were taken. (B) Histograms representing AVP-ir in PaMP (expressed as cells/$10^5 \mu m^2$; OIL, white bars; E_2, grey bars; GEN, black bars). There is a significant differences among female OIL vs female GEN. In males, GEN group was significantly different from both OIL and E_2. The significant differences (see results) are denoted by "a" or "b".

Microphotographs illustrating the PVN of females (C-E) and males (F-H) treated with either OIL (C and F), E_2 (D and G) or GEN (E and H). Magnification bar =100 µm.

Table 1. Summary of the quantitative analysis of nNOS and AVP system. Data refers to cell density (number of cells/$10^5 \mu m^2$) except for nNOS labeling in VMHvl and AVP labeling in BST where it was expressed as fractional area. The data are the average of the two levels, when statistical analysis did not highlight significant differences. The significant differences (see results) are denoted by "a", "b", or "c".

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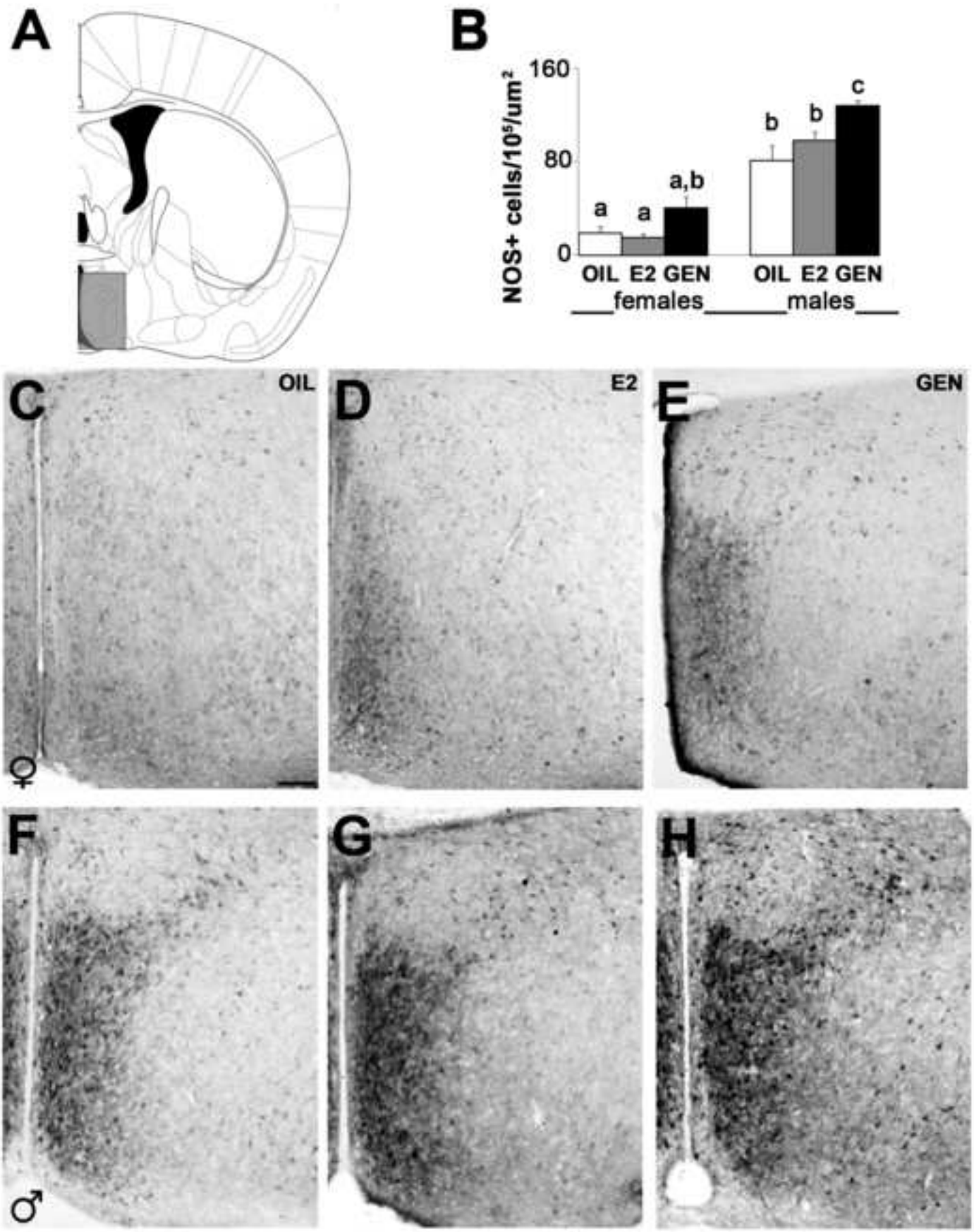

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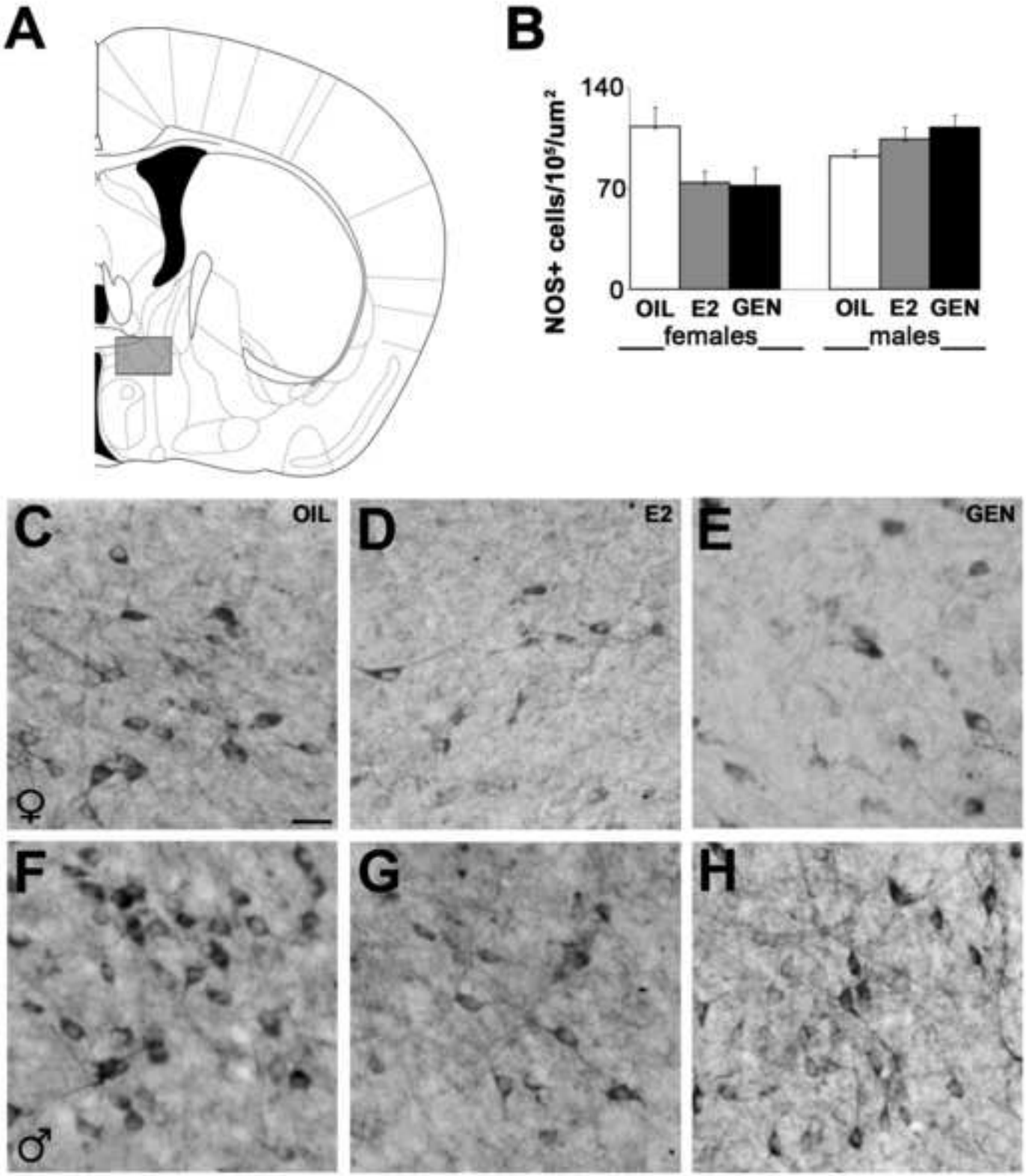

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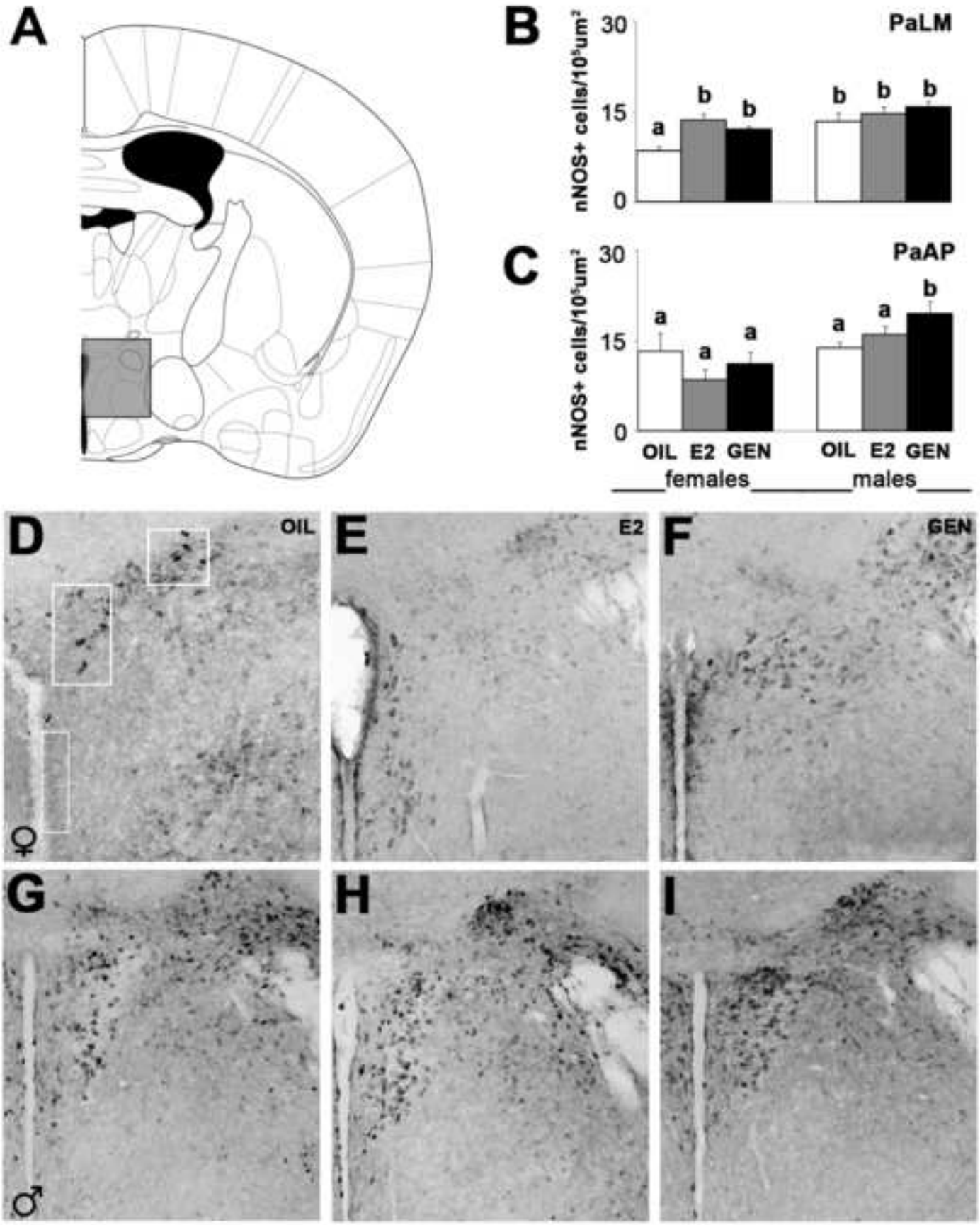

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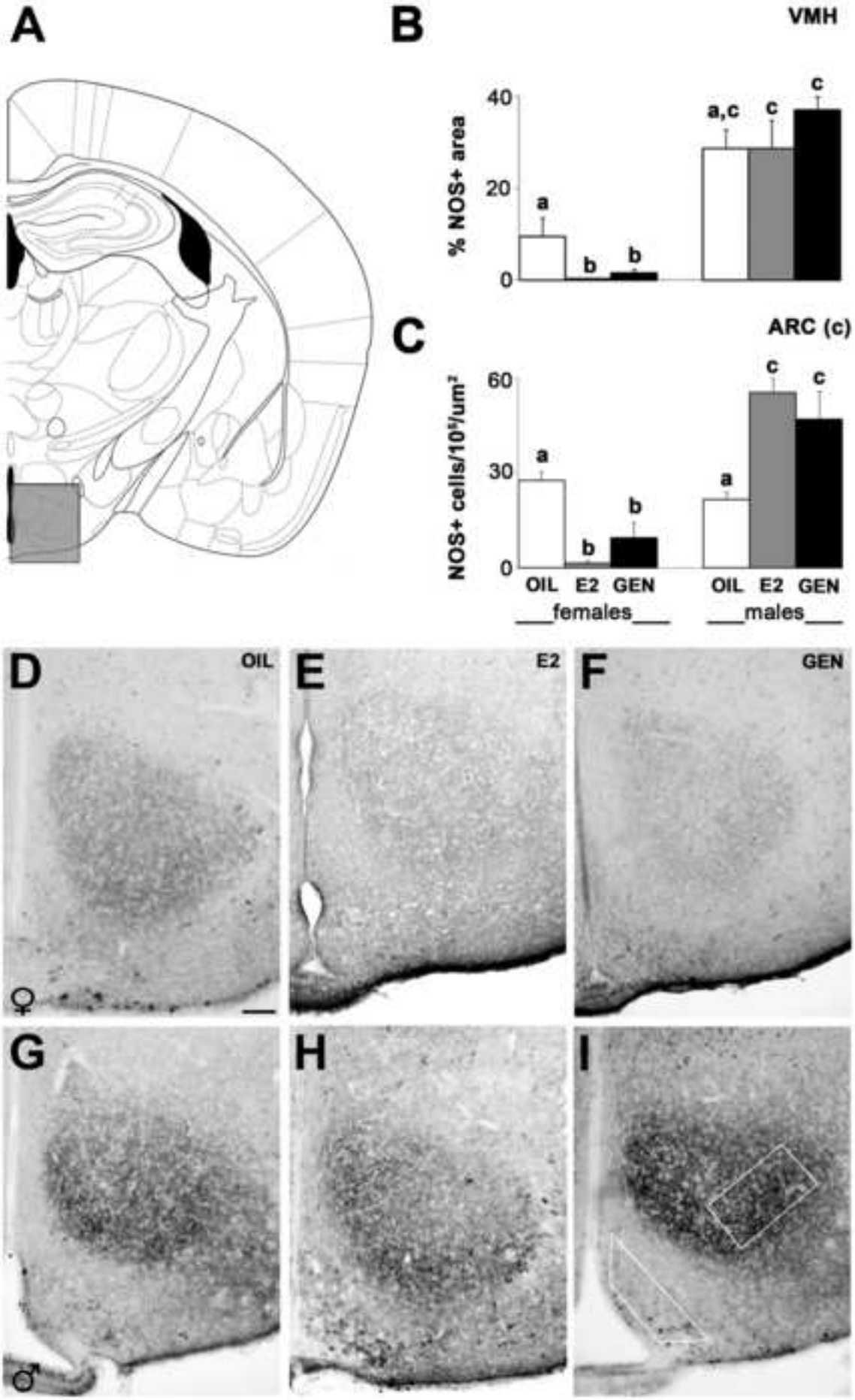

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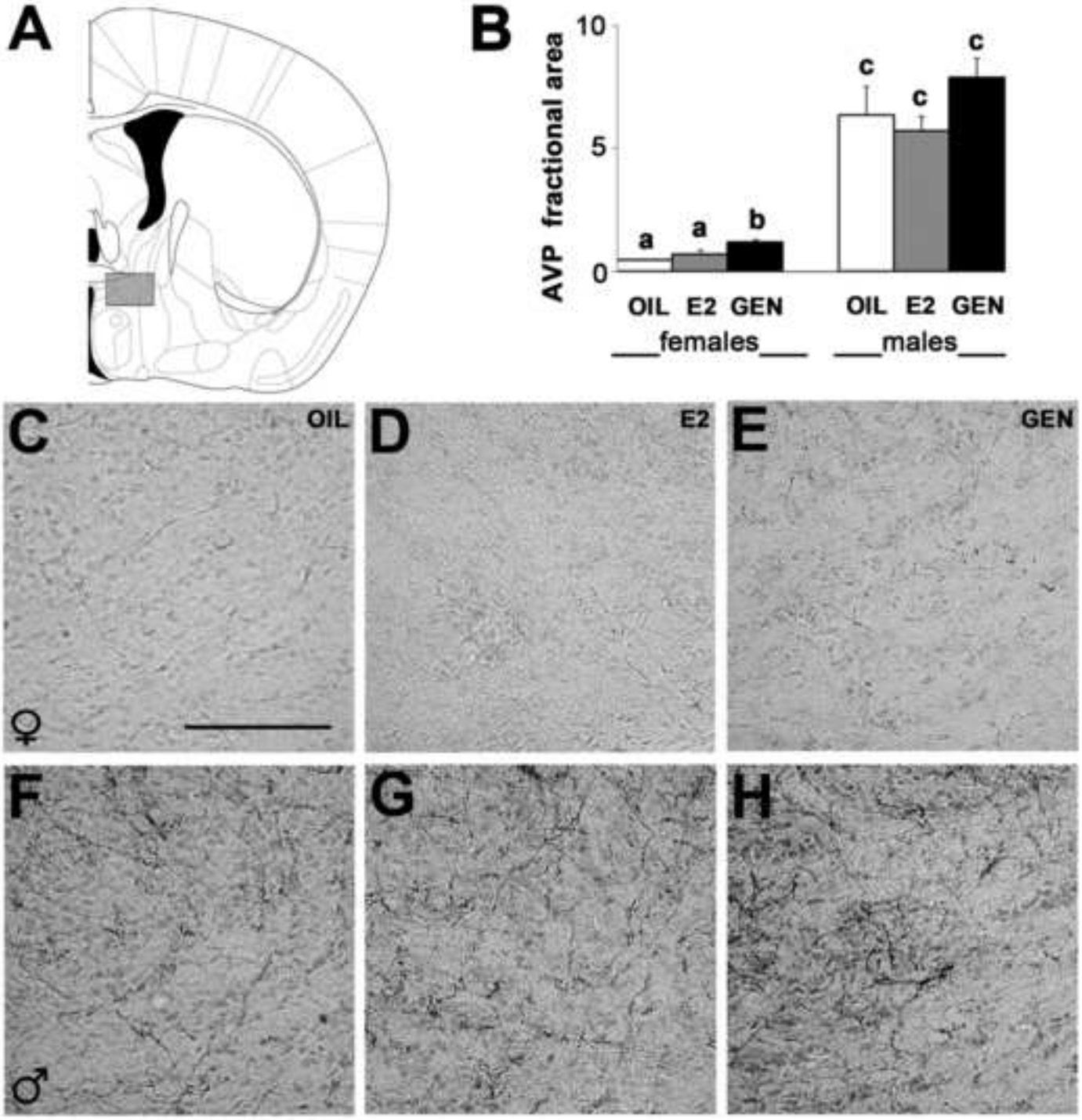

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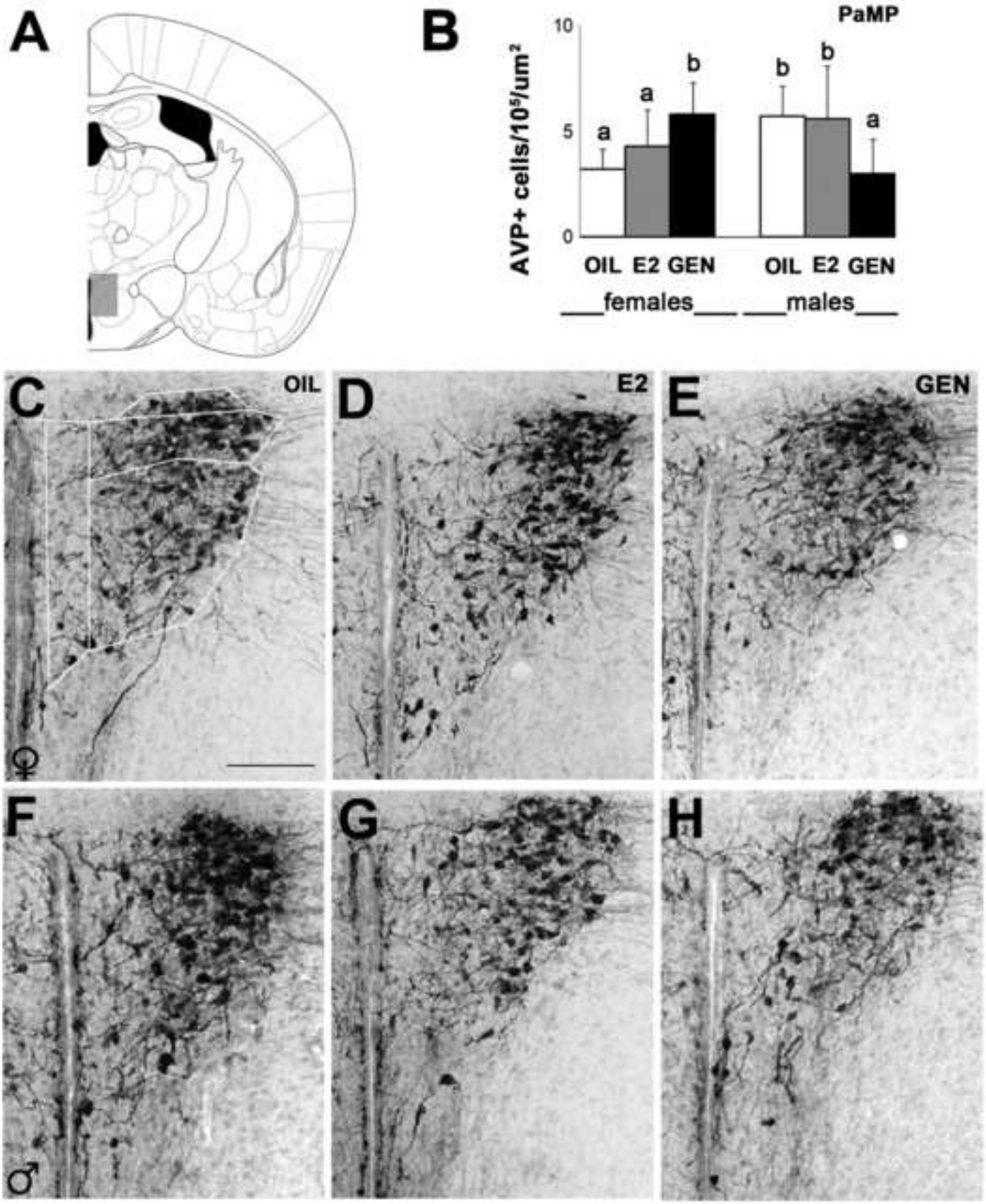

Table

Table 1

	females			males		
nucleus	**OIL**	**E2**	**GEN**	**OIL**	**E2**	**GEN**
			NOS			
MPOA	28.50 +/- 25.5[a]	25.5 +/- 1.00[a]	83.00 +/- 25.36[a,b]	**110.00 +/- 15.25**[b]	**121.87 +/- 8.19**[b]	**193.20 +/- 8.91**[c]
BSTmv	113.33 +/- 13.33[a]	74.44 +/- 7.78[a]	72.22 +/- 12.37[a]	92.78 +/- 4.34[a]	104.58 +/- 8.21[a]	113.00 +/- 8.16[a]
BLA	12.31 +/- 3.12[a]	5.55 +/- 1.52[a]	6.22 +/- 2.09[a]	5.19 +/- 3.36[a]	6.47 +/- 1.23[a]	8.12 +/- 2.54[a]
La	21.78 +/- 7.53[a]	21.02 +/- 8.02[a]	18.76 +/- 2.53[a]	12.19 +/- 2.03[a]	15.76 +/- 2.99[a]	15.31 +/- 2.16[a]
PaLM	85.02 +/- 6.94[a]	**136.31 +/- 10.76**[b]	**121.38 +/- 4.75**[b]	133.37 +/- 13,99[b]	145.95 +/- 12.72[b]	158.37 +/- 8.84[b]
PaAP	132.11 +/- 3.08[a]	85.24 +/- 1.72[a]	111.84 +/- 1.97[a]	138.63 +/- 0.93[a]	160.97 +/- 1.39[a]	**195.32 +/- 1.91**[b]
PaV	174.49 +/- 4.79[a]	105.62 +/- 1.20[a]	122.58 +/- 2.01[a]	125.54 +/- 1.83[a]	153.63 +/- 3.64[a]	142.35 +/- 1.67[a]
VMHvl	18.44 +/- 9.56[a]	0.25 +/- 0.14[b]	6.15 +/- 0.79[b]	28.62 +/- 4.17[a,c]	**28.69 +/- 6.15**[c]	**36.64 +/- 2.93**[c]
ARC (r)	8.20 +/- 4.89[a]	5.64 +/- 4.89[a]	11.28 +/- 5.78[a]	14.07 +/- 1.52[a]	**36.00 +/- 2.98**[b]	**30.58 +/- 5.74**[b]
ARC (c)	27.61 +/- 2.84[a]	**1.58 +/- 0.79**[b]	**9.47 +/- 4.93**[b]	21.64 +/- 2.33[a]	**55.38 +/- 4.59**[c]	**47.04 +/- 8.84**[c]
			AVP			
LS	0.84 +/- 2.19[a]	1.65 +/- 0.71[a]	1.01 +/- 0.23[a]	**6.94 +/- 0.63**[b]	**7.24 +/- 1.45**[b]	**8.21 +/- 1.23**[b]
BSTmv	0.45 +/- 0.07[a]	0.69 +/- 0.20[a]	**1.19 +/- 0.11**[b]	**6.38 +/- 1.15**[c]	**5.98 +/- 0.73**[c]	**7.90 +/- 0.77**[c]
SCN (r)	70.80 +/- 2.92[a]	78.20 +/- 5.16[a]	91.80 +/- 11.18[a]	65.20 +/- 4.99[a]	68.25 +/- 5.57[a]	73.40 +/- 6.17[a]
SCN (c)	58.00 +/- 6.53[a]	72.40 +/- 7.65[a]	84.80 +/- 8.04[a]	43.40 +/- 4.81[a]	68.00 +/- 11.80[a]	57.20 +/- 10.68[a]
PaDC	4.10 +/- 0.29[a]	6.20 +/- 0.84[a]	6.10 +/- 0.64[a]	4.90 +/- 0.58[a]	5.13 +/- 0.92[a]	4.90 +/- 1.18[a]
PaLM	17.50 +/- 2.11[a]	23.10 +/- 3.08[a]	24.80 +/- 4.14[a]	21.3 +/- 2.02[a]	20.50 +/- 1.32[a]	14.40 +/- 3.36[a]
PaMM	30.30 +/- 3.88[a]	28.60 +/- 4.18[a]	35.70 +/- 5.48[a]	28.40 +/- 2.81[a]	28.13 +/- 1.09[a]	27.70 +/- 5.65[a]
PaMP	3.20 +/- 0.44[a]	4.30 +/- 0.77[a]	**5.80 +/- 0.68**[b]	5.70 +/- 0.64[b]	5.63 +/- 0.59[b]	**3.00 +/- 0.72**[a]