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Prenatal Ethanol Exposure Increases Depressive-Like Behavior and Central Estrogen Receptor α and Oxytocin Expressions in Adult Female Mandarin Voles

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Feng-Qin He, Jie Zhang, and Xiang Guo (2012) Prenatal ethanol exposure increases depressive-like behavior and central estrogen receptor α and oxytocin expressions in adult female mandarin voles. *Zoological Studies* **51**(1): 1-11. Prenatal exposure to ethanol is considered to increase risk of depression in offspring. Herein, we tested the effect of prenatal ethanol exposure on adult female mandarin voles (*Microtus mandarinus*). We verified depression-like behavior of female offspring exposed to ethanol prenatally (exposure group), and control females (control group) during a forced swimming test. For the exposure group, ethanol doses (750 mg/kg body weight in 0.9% saline, 10 ml/kg) were orally administered by clean drinking tubes to 5 pregnant females from day 14 of their pregnancy until postnatal day 0. In the control group, 6 pregnant females received 0.9% saline (10 ml/kg) but no ethanol. At 90 d of age, the exposure and control groups were tested during a forced swimming, and levels of serum estradiol (E_2), estrogen receptor alpha-immunoreactive neurons (ERα-IRs) and oxytocin-immunoreactive neurons (OT-IRs) were measured. The exposure group exhibited significantly decreased locomotion and increased immobility during the swim test. The level of serum E_2 was higher in the exposure group, and numbers of ERα-IRs in the bed nucleus of the stria terminalis (BNST), medial amygdaloid nucleus (MeA), hypothalamic paraventricular nucleus (PVN), and supraoptic nucleus (SON) in the exposure group were significantly lower. Numbers of OT-IRs in the hypothalamic PVN and SON of the exposure group were lower than those of control animals. Our results suggest that prenatal ethanol exposure may lead to increases in serum E_2 levels and decreased ERα and OT in the central nervous system of adults and may be related to the development of depression-like behaviors. http://zoolstud.sinica.edu.tw/Journals/51.1/1.pdf

Key words: Prenatal ethanol exposure, Forced swimming test, Estradiol, Estrogen receptor α, Oxytocin.

Depression is a highly prevalent, chronic, recurring, and potentially life-threatening mental illness (Nestler et al. 2002, Berton and Nestler 2006), and prenatal ethanol exposure is associated with an increased risk of depression in offspring (Forrest et al. 1992, Larkby and Day 1997, Mancinelli et al. 2007, Hellemans et al. 2010). Both mouse and rat offspring, the mothers of which had consumed moderate quantities of ethanol throughout gestation, demonstrated dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis which is common in depression and is primarily reflected by increased HPA tone and activity (Bale and Vale 2004, Hellemans et al. 2010). Attention was recently focused on the role of neurohypophyseal hormones such as oxytocin (OT) on HPA axis activation in depression (Bao et al. 2008). In the central nervous system, the OT gene is primarily expressed in magnocellular neurons in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON). Release of OT via the neurohypophysis into the bloodstream and by extrahypothalamic fibers projecting into the brain exert a wide spectrum of central and peripheral

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effects (Le Mevel et al. 1993). For example, in addition to its regulatory role in social attachment, OT was shown to have stress-protective effects in animals, including humans (Heinrichs et al. 2003, Parker et al. 2005). One study showed that peripheral OT levels are reduced in depression, while another found no difference in OT plasma levels between depressed and control patients (van Londen et al. 1997, Slattery and Neumann 2010b). Although prenatal exposure to ethanol (PAE) increases the HPA tone and results in HPA dysregulation throughout life, paralleling many HPA changes associated with depression (Hellemans et al. 2010), neurobiological mechanisms of depression need to be further researched.

Estrogen is a well-known regulator of mood, and estradiol (E_2) treatment can result in both depressant and antidepressant effects (Morrison et al. 2006). The variable effects of estrogen on the mood may be explained by opposing actions of estrogen when mediated through estrogen receptor alpha (ERα) or beta (ERβ). Research into this area has yielded contrasting results. For example, ERα was shown to play a role in the susceptibility of females to major depressive disorders (Tsai et al. 2003). However, ERα appears to be unrelated to emotion in females (Malacara et al. 2004). Species differences in central (estrogen receptor) ER expression may also contribute to differences in the effects of E_2 on moods. For example, ERα-positive cells are present in the PVN of monogamous California mice, but not in polygynous house mice, whereas ERα-positive cells are present in the anterior hypothalamus (AHA) of house mice but not in California mice (Merchenthaler et al. 2004). Monogamous male pine voles (*Microtus pinetorum*) express lower levels of ERα-immunoreactive neurons (IRs) in the medial amygdaloid nucleus (MeA) than polygynous male montane (*M. montanus*) and meadow voles (*M. pennsylvanicus*) (Cushing and Wynne-Edwards 2006). In addition, immunocytochemical and in situ hybridization studies detected either low levels or the absence of ERα-IRs in the PVN and SON of rats (Hrabovszky et al. 1998).

Ethanol abuse during a woman's pregnancy produces a variety of deleterious effects in the offspring (Hollstedt et al. 1977). However, the mechanisms of those deleterious effects are not entirely clear. Among the various different animal models currently employed for screening antidepressant compounds, the forced-swim test (FST) is one of the most commonly used (Porsolt et al. 1978, Wallace-Boone et al. 2008). Herein, we utilized wild mandarin voles (*M. mandarinus*) as animal models and explored the neurobiological basis of depressive behavior. Mandarin voles are ideal models for exploring the neurobiological basis of various behaviors, which may provide abundant neurobiological knowledge (Wang et al. 1997). According to studies of mate preference and other related characteristics, it was found that mandarin voles have a monogamous mating system (Tai et al. 2001, Guo et al. 2011). So far, the depressive-like behavior of the animal and relationships among ERα, OT, and depressive-like behaviors have not been reported. Some epidemiological or experimental studies found that women have a higher prevalence of mood disorders than men (Hellemans et al. 2010), so we attempted to test the hypothesis that prenatal exposure of female pups to ethanol will affect the distribution of ERα and OT in parts of the brain associated with depressive-like behaviors.

MATERIALS AND METHODS

Subjects

Healthy adult female mandarin voles (*n* = 11, weighing 30-36 g, 90 d old) were obtained from an outbreed colony and reared in the Department of Life Science, Xian Univ. of Arts and Science, Xian, China. The colony of mandarin vole was established in 1997 with wild-captured animals (*n* = 300) from Lingbao City, Henan Province, China. The animals were individually housed in clear plastic cages (30 × 20 × 15 cm). The voles were maintained under a 14: 10-h light: dark photoperiod and at a temperature of 24-26°C. Hardwood shavings and cotton batting were provided as substrate and bedding. Rabbit chow (Laboratory Animals Center, Xian Medical Univ., Xian, China), carrot, and malt were provided ad libitum in this experiment. All methods for treating voles were approved by the Institutional Animal Care and Use Committee of Xian Univ. of Arts and Science.

Female voles were brought into estrus with estradiol benzoate (0.75 μg/g, 24 h before testing) and progesterone (0.015 mg/g, 4-6 h before testing), and the estrus state of the females was monitored by taking vaginal smears (He et al. 2008). Each female in estrus was paired with an adult male the bilateral or unilateral testes of which had descended (*n* = 11, total = 11 pairs) until 2 ejaculations were observed (day 0 of pregnancy)

(Ward et al. 2002). These pregnant females were allocated to either an ethanol or saline exposure group. The gestation period in voles is only about 21 d long. In the ethanol exposure group, we orally administered ethanol doses (750 mg/kg body weight in 0.9% saline, 10 ml/kg, Sigma, St. Louis, MO, USA) using clean drinking tubes to 5 pregnant females from day 14 of the pregnancy until postnatal day 0, so the animals were intubated for 7 consecutive days. In the saline treatment group, 6 pregnant females received 0.9% saline (10 ml/kg) but no ethanol (Macenski and Shelton 2001, Erlwanger and Cooper 2008). The weight of both ethanol- and saline-exposed pregnant females 1 d before parturition was 55-65 g, the number of live offspring per pair born was 1-5, and the sex ratio was 1.87: 1 female: male (Tai et al. 1999). Because the offspring were weaned 21 d after birth, all offspring were kept with their mothers until postnatal day 21. Subsequently, 11 female offspring with similar weights (12-14 g) were selected from the 11 pairs of vole individuals and maintained in our laboratory under the same conditions: 5 female offspring of 5 pregnant females with ethanol exposure were the exposure group, and 6 female offspring of 5 pregnant females with saline treatment were the control group. These female offspring were allowed to grow up (weighing 30-36 g, 90 d old), and after the behavioral testing, they were euthanized.

Forced swim test (FST)

From postnatal day 90, adult female offspring were tested using the FST following Porsolt et al. (1978), in which each animal was forced to swim for 6 min in an open cylindrical container (10 cm in diameter and 25 cm high) (Peng et al. 2007), containing 15 cm of water at 25 ± 1°C. The water on an animal's body was cleaned off with a towel, and the body temperature was allowed to return to normal next to a heater after each test. The interval between each animal experiment was 10 min. The investigator was blinded to the treatment groups. The total duration of immobility was scored following Zomkowski et al. (2004 2005). Each vole was judged to be immobile when it ceased struggling and remained floating and motionless in the water, making only movements necessary to keep its head above water. A decrease in the duration of immobility during the FST was taken as a measure of antidepressant activity.

Serum E_2 quantification

Blood was taken from the retro-orbital sinus at diestrus 2 d after each FST. All serum samples were carefully collected and separated from the blood by centrifugation, and serum samples were stored at -80°C for 1 wk until being assayed. Serum E_2 assays were performed using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Serum samples were diluted 1: 20 to measure the optical density of E_2 at 450 nm. Enzyme-specific activities were determined using a linear regression analysis ($r > 0.95$).

Tissue collection and ERα and OT immunocytochemistry

ERα and OT expressions were examined 1 h after behavioral testing. Voles were deeply anesthetized and perfused with a 0.1 M phosphate-buffered solution (PBS, pH 7.4) and 4% paraformaldehyde in 0.1 M PBS. The brain was removed within 3 min and placed in 4% paraformaldehyde overnight. Prior to dissection, brains were immersed in 30% sucrose until saturated. Coronal sections (40 μm) were cut on a cryostat, and consecutive sections were collected in 2 vials containing 0.01 M PBS for 2 different immunohistochemical stainings. The ERα antibody (sc-542; Santa Cruz Biotechnology, Santa Cruz, CA, USA) is an affinity-purified rabbit polyclonal antibody raised against a peptide mapped at the C-terminus of ERα of mouse origin. The OT antibody (AB911; Upstate Biotechnology, Lake Placid, NY, USA) is also a rabbit polyclonal antibody.

Floating sections were processed using the primary antibody and a streptavidin/peroxidase method (Bioss Co., Beijing, China). We incubated 1 vial per brain for 7 min with 3% H_2O_2 and then washed them twice for 10 min each with distilled water. We shrank the tissue in 0.01 M PBS. Sections were preincubated for 90 min with normal goat serum (SP-0023) and incubated at 4°C overnight with the primary antibody solution (ERα antibody 1: 100; OT antibody 1: 5000) diluted by antibody diluent (0.01 M PBS containing 20% bovine serum albumin and 1.7% Trition-X-100).

The next day, sections were washed 4 times for 5 min each with 0.01 M PBS and incubated for 60 min in a 37°C water bath with a biotinylated goat anti-rabbit antibody (SP-0023), followed by another round of 4 washes for 5 min each

with 0.01 M PBS. After 60 min of incubation with S-A/HRP and 4 washes for 10 min each with 0.01 M PBS, sections were stained with 3, 3'-diaminobenzidine tetrahydrochloride to visualize the immunoreactivity. Because ERα was included in nuclei and OT was contained in the cytoplasm, we counted stained nuclei and cytoplasm using an Olympus microscope (Olympus microscope is made in Tokyo, Japan). Slides were randomized and coded for microscopic analysis so that the counters were blinded to the experimental treatment. Numbers of cells that showed immunoreactivity were quantified by eye per a standard area (200 × 200 μm) using a grid sampling. We counted the number of ERα-IRs in the anterior hypothalamus (AHA), bed nucleus of the stria terminalis (BNST), lateral septum (LS), medial amygdaloid nucleus (MeA), hypothalamic paraventricular nucleus (PVN), and supraoptic nucleus (SON); we selected these areas of the brain because they are involved in emotion (Zhai et al. 2008, Song et al. 2010). Different brain areas were determined according to Nissl-stained brain sections from mandarin voles and a stereotaxic atlas of the rat brain.

For each brain nucleus, the criteria of 3 sections from anterior to posterior anatomically matched between subjects were chosen and counted to minimize variability. Individual means for each animal were obtained by counting positive neurons bilaterally in 3 sections from each nucleus. Counts were separately performed for each hemisphere, and results were averaged between hemispheres. Numbers of OT-IR neurons were quantified in 2 areas with a distinctive population of OT-IR neurons: the PVN and SON. Sections were chosen by their correspondence to the reference atlas plate and not by the level or intensity of ERα-IR or OT-IR labeling. All immunohistochemical procedures included negative controls (in which the primary antibody was not added). For all subjects, positive neurons were counted by a trained experimenter blinded to the experimental treatment. Sections were photographed with a Nikon (Tokyo, Japan) camera attached to a Nikon microscope.

Statistical analyses

All data were checked for normality using a one-sample Kolmogorov-Smirnov test. The majority of data were normally distributed and analyzed using independent-sample *t*-tests. Data are presented as the mean ± standard error (SE) and alpha was set to $p < 0.05$. All statistical analyses were conducted using SPSS 10.0 (SPSS, Chicago, IL, USA).

RESULTS

The exposure group spent less time engaged in locomotion (duration: $F_{(1,9)} = 13.1$, $p = 0.031$; frequency: $F_{(1,9)} = 5.015$, $p = 0.034$) and more time immobile (duration: $F_{(1,9)} = 13.204$, $p = 0.037$; frequency: $F_{(1,9)} = 6.620$, $p = 0.021$) during the FST than the control group (Fig. 1). The exposure group had significantly higher serum E_2 levels than the control group ($F_{(1,9)} = 6.667$, $p = 0.021$) (Fig. 2).

We found a significant effect of ethanol on the number of ERα-IRs in different brain regions of adults. Numbers of ERα-IRs in the BNST ($F_{(1,9)} = 2.295$, $p = 0.045$), MeA ($F_{(1,9)} = 0.345$, $p = 0.036$), PVN ($F_{(1,9)} = 15.345$, $p = 0.001$), and

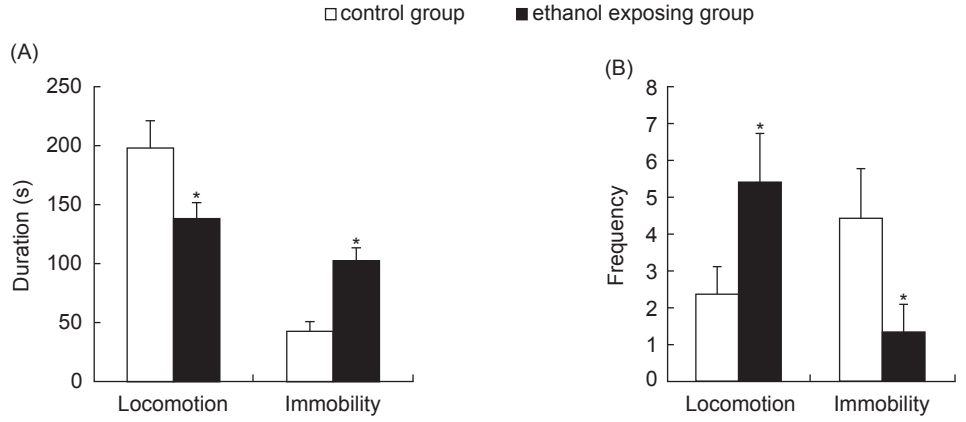

Fig. 1. Locomotion and immobility in the forced swimming test. (A) Durations of locomotion and immobility. (B) Frequencies of locomotion and immobility. *Indicates a significant difference, $p < 0.05$. Values are the mean (± standard error).

SON ($F_{(1,9)}$ = 14.447, p = 0.002) of the exposure group were significant lower than those of the control group (Figs. 3, 4, 6), and those in the AHA ($F_{(1,9)}$ = 0.271, p = 0.377) and LS ($F_{(1,9)}$ = 3.992, p = 0.642) of the exposure group were slightly lower than those of the control group (Figs. 3, 4). We found reduced numbers of OT-IRs in the PVN ($F_{(1,9)}$ = 2.379, p = 0.041) and SON ($F_{(1,9)}$ = 0.357, p = 0.026) of the exposure group compared to the control group (Figs. 5, 6).

DISCUSSION

Prenatal ethanol exposure increases depressive behavior in adult female offspring

Ethanol exposure resulted in a reduction in locomotion time and an increase in immobility during the FST for adult females, the mothers of which were administered ethanol orally. Therefore, prenatal ethanol exposure leads to increased depressive-like behavior in adult offspring, and results suggest that ethanol exposure during brain growth spurts has long-term effects on behavior, neurochemistry, and neuroendocrinology. These findings confirm that alcohol increases depressant-like behaviors via neurochemical and neuroendocrine mechanisms. Although the etiology of depressive disorders is at present unknown, prenatal ethanol oral administration during pregnancy can be considered in the context of early life adversity or environments. Programming of the fetal HPA axis by ethanol can result in long-term alterations in the physiological and behavioral profiles of offspring (Hellemans et al. 2010), and we confirm results of much literature that HPA programming ultimately confers increased susceptibility to developing depression disorders if stressors are encountered later in life (Macri et al. 2007), e.g., during the FST as employed herein.

Decreased expression of ERα-IRs may increase depressive-like behavior

Our experiment is the 1st to show a positive association between prenatal ethanol treatment and serum estrogen levels. Previous studies showed an increase in estrogenic levels after ethanol treatment in women (Muti et al. 1998, Juarez et al. 2002), men (Couwenbergs 1988), and male rats (Esquifino et al. 1989). However, why prenatal ethanol treatment increases serum estrogen levels in adult female offspring is not yet completely understood; it may due to increased aromatization of E_2 from testosterone, so ethanol increases serum estrogen levels (Hilakivi-Clarke et al. 1997).

Numbers of ERα-IRs in the BNST, MeA, PVN, and SON significantly declined in females exposed to ethanol in the present results, and the BNST, MeA, PVN, and SON were demonstrated to be involved in depression (Swaab et al. 2005, Lee et al. 2009). Research from our own lab also showed that these brain areas are correlated with animal emotions (Zhai et al. 2008, Song et al. 2010) and concurs with other studies which showed that ERα is related to mood including depression in females (Osterlund et al. 1999, Tsai et al. 2003). In the present experiment, serum

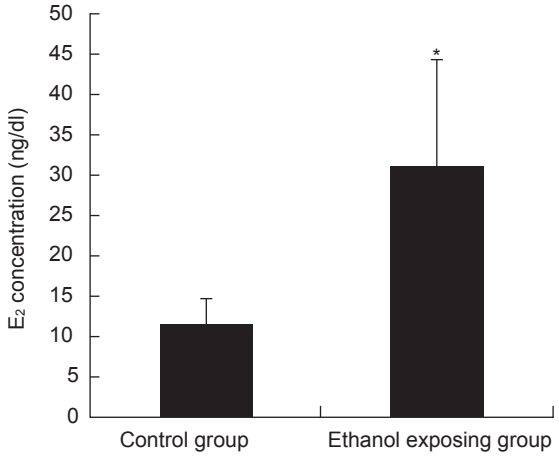

Fig. 2. Mean (± standard error) level of circulating serum estradiol (E_2) concentrations (ng/dl) according to an ELISA. *Indicates a significant difference, $p < 0.05$.

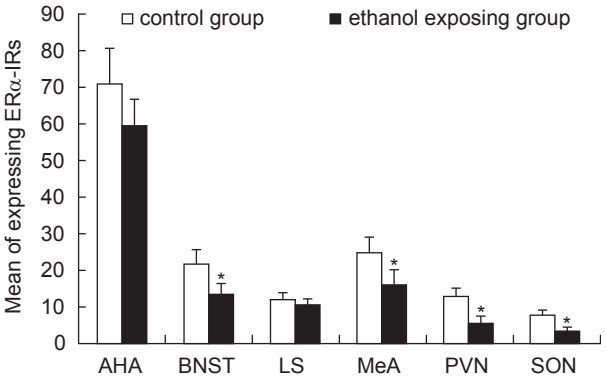

Fig. 3. Mean (± standard error) number of estrogen receptor (ER)α immunoreactive cells following prenatal exposure to ethanol in female voles. *Indicates a significant difference, $p < 0.05$.

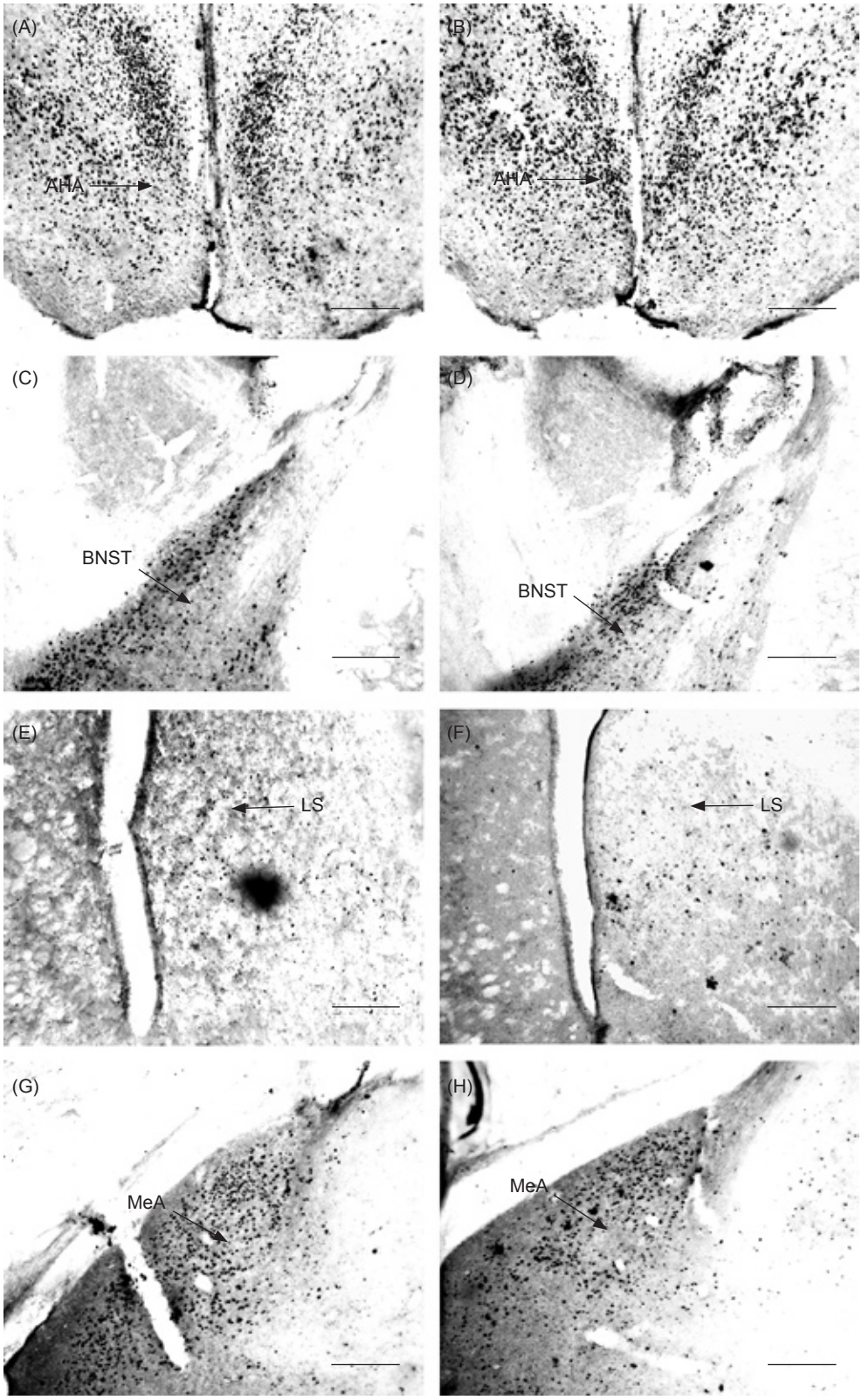

Fig. 4. Estrogen receptor (ER)α immunoreactivity in *Microtus mandarinus* females exposed to ethanol prenatally and a control group. Anterior hypothalamus (AHA) of the control group (A) and ethanol-exposed group (B); bed nucleus of the stria terminalis (BNST) of the control group (C) and ethanol-exposed group (D); lateral septum (LS) of the control group (E) and ethanol-exposed group (F); medial amygdaloid nucleus (MeA) of the control group (G) and ethanol-exposed group (H). Scale bar = 200 μm.

E_2 levels of adult female offspring exposed to prenatal ethanol treatment were higher than those exposed to saline treatment, but numbers of ERα-IRs in certain brain regions declined. E_2 caused the downregulation of ERα, which is consistent with previous findings (Oliveira et al. 2004). Species differences in central ER expression may also contribute to differences in the effects of E_2 on moods. For example, ERα-positive cells are present in the PVN of monogamous California mice, but not in polygynous house mice, whereas ERα-positive cells are present in the AHA of house mice but not in California mice (Merchenthaler et al. 2004). Monogamous male pine voles (*M. pinetorum*) express lower levels of ERα-IRs in the MeA than polygynous male montane (*M. montanus*) and meadow voles (*M. pennsylvanicus*) (Cushing and Wynne-Edwards 2006). In addition, immunocytochemical and *in situ* hybridization studies detected either low levels or the absence of ERα-IRs in the PVN and SON of rats (Hrabovszky et al. 1998). Consequently, the varying effects of ethanol on depression may result from different properties of central ERα expression in different species.

ERα is thought to modulate the activity of corticotropin-releasing factor (CRF) neurons in depression, and CRF expression is increased in depression (Wang et al. 2008). Reduced numbers of ERα-IRs in these brain regions raise the possibility that a disturbed balance in the production of the CRF may contribute to activation of the HPA axis in depression. In contrast to our study, another study found a significantly increased expression of the ERα gene in the PVN of depressed patients (Wang et al. 2008). This discrepancy between studies may have been the result of differences between expressions of the ERα gene and protein between species.

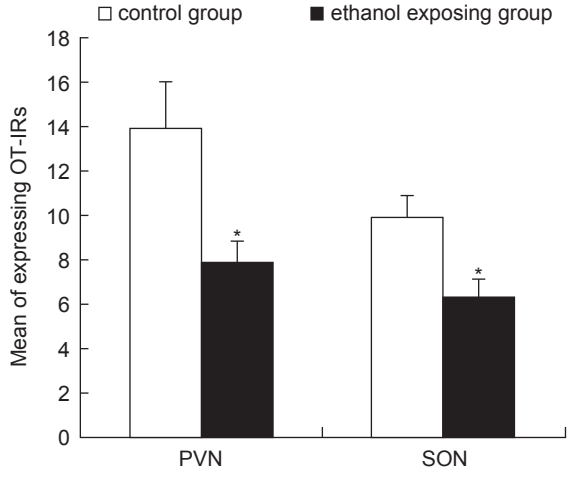

Fig. 5. Mean (± standard error) number of oxytocin (OT)-immunoreactive cells following experimental treatment in adult female mandarin voles. *Indicates a significant difference, $p < 0.05$.

Decreased expression of OT-IRs may lead to increase depressive-like behavior

Numbers of OT-IRs in the ethanol-exposed group were significantly lower in the PVN and SON. Reduced numbers of OT-IRs in certain brain regions may be one of the causes of depressive-like behavior. OT injected intraperitoneally decreased immobility in rats undergoing the FST, and this effect was even stronger following long-term treatment with OT (Arletti and Bertolini 1987). An intracerebroventricular injection of OT did not affect the behaviors of male and female rats during an FST, even after a chronic treatment regimen that did not affect depression behavior (Slattery and Neumann 2010a). OT administered centrally or systemically decreased the immobility time in mice during an FST, but this effect was not blocked by a non-peptide OTR antagonist (Ring et al. 2010). It is possible that results from one species cannot be generalized to others (Insel et al. 1993). Herein, prenatal ethanol exposure increased serum estrogen levels, and OT significantly decreased in the PVN and SON; but in rats, OT binding in the brain showed a positive correlation with serum estrogen (Larcher et al. 1995, Breton and Zingg 1997). Again, these effects may be species-specific (Insel et al. 1993).

Although in the present experiment, numbers of cells that showed immunoreactivity were quantified per a standard area (200 × 200 μm), a limitation of this study is that the densities of ERα and OT were measured and not total cell numbers, and co-localization of ERα with OT occurred in the hypothalamic PVN and SON in both the ethanol-exposed and control groups. The central roles of OT in behaviors and physiology are strongly dependent on steroid hormones, and estrogen can upregulate the production of OT messenger (m) RNA (Caldwell et al. 1989) and the release of OT (Johnson 1992). A membrane-bound receptor for estrogen may regulate OT expression within the PVN and SON (Sakamoto et al. 2007). Estrogen-induced OT binding in the brain is abolished in ERα knockout mice (Young et al. 1998). Additional evidence for OT regulation of E_2 sensitivity comes from studies of human breast cancer cell lines in

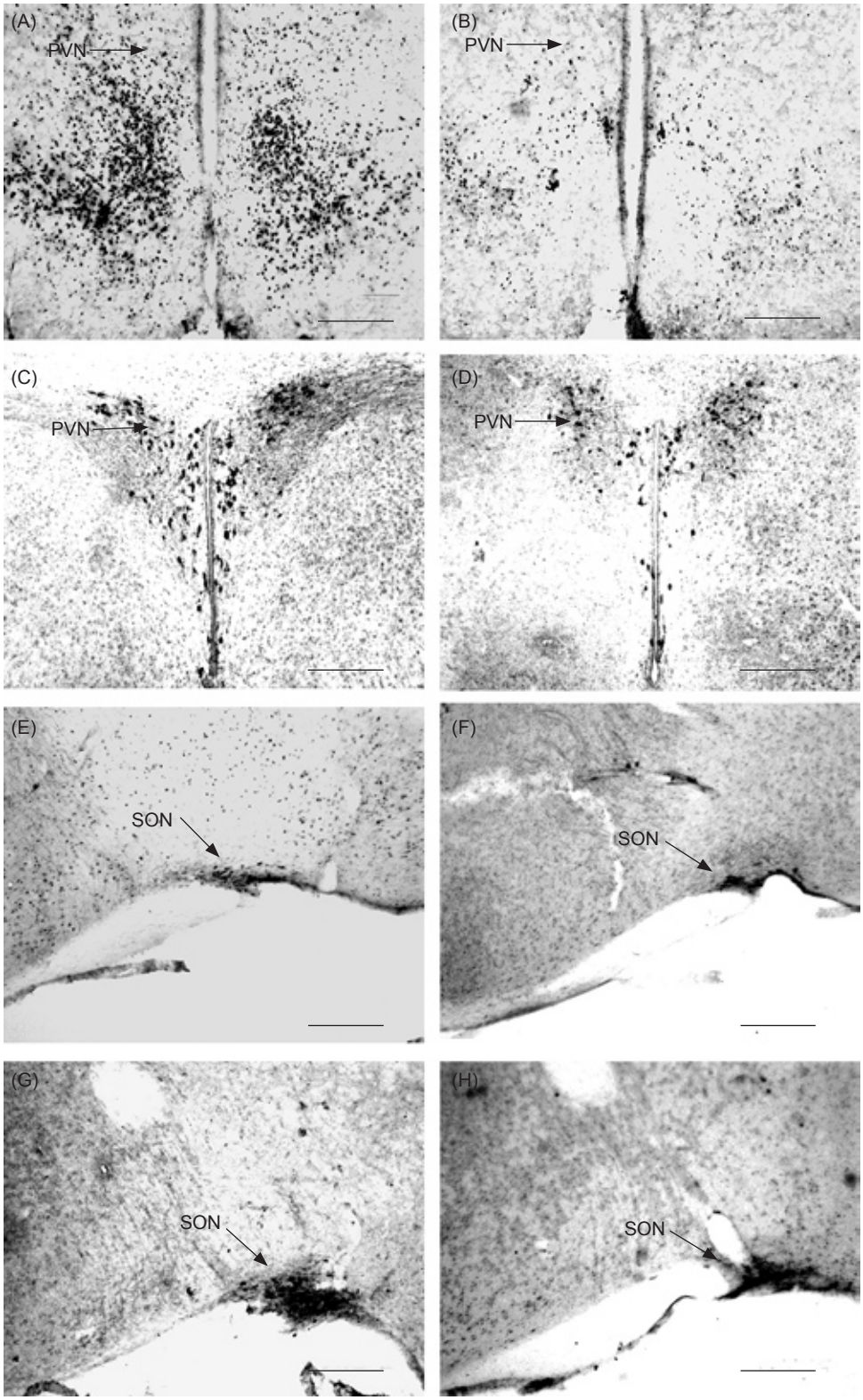

Fig. 6. Estrogen receptor (ER)α and oxytocin (OT) immunoreactivity in *Microtus mandarinus* females exposed to ethanol prenatally and the control group. ERα-immunoreactive neurons (IRs) in the paraventricular hypothalamic nucleus (PVN) of the control group (A) and ethanol-exposed group (B); OT-IRs in the paraventricular hypothalamic nucleus (PVN) of the control group (C) and ethanol-exposed group (D); ERα-IRs in the supraoptic nucleus (SON) of the control group (E) and ethanol-exposed group (F); OT-IRs in the supraoptic nucleus (SON) of the control group (G) and ethanol-exposed group (H). Scale bar = 200 μm.

which OT downregulates both ERα mRNA and ERα protein expressions (Cassoni et al. 2002). It is becoming increasingly clear that neuropeptides interact with steroids to regulate moods (Cushing et al. 2005). The fact that manipulations of ethanol early in life alter ERα-IRs in adulthood suggests that ethanol might not simply regulate sensitivity to E_2 through activational effects, but that organizational effects of OT produce lasting changes in sensitivity to steroids. Our data provide an additional mechanism by which neuropeptides might act to alter or regulate sensitivity to steroids. This in turn has the potential to alter depressive-like behaviors mediated by OT and ERα. However, in the female rat, the PVN contains only a negligible quantity of ERα, and the estrogen-dependent regulation of oxytocin synthesis in the PVN is mediated by ERβ (Patisaul et al. 2003). Therefore, regulating the responses to OT may be critical to 'proper' or species-specific expression (Wang and Vries 1993, Winslow et al. 1993, Williams et al. 1994), and understanding the relationship between the hormonal and peptide regulation of depressive-like behavior and central ERα and OT expression might differ in various mammalian species (Wang et al. 1997).

Depression concerns hyperactivity of the HPA axis, and this axis is driven by CRF release by neurons located in the PVN, which causes adrenocorticotropic hormone (ACTH) release at the level of the pituitary (Meynen et al. 2007). Because OT significantly inhibits the potentiating effect of CRF-stimulated ACTH release (Suh et al. 1986), OT seems to be able to stimulate and inhibit activity within the HPA axis with short- and long-term perspectives (Yegen 2010). In our experiment, numbers of OT-IRs in the ethanol-exposed group were significantly lower in the PVN and SON, and reduced numbers of OT-IRs might cause the release of CRF and ACTH, causing hyperactivity of the HPA axis. Therefore, it is easy to cause depressive-like behavior in these adult female offspring prenatally exposed to ethanol.

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