



Effects of maternal toluene exposure on testosterone levels in fetal rats

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ABSTRACT

The goal of our study was to determine if toluene affected the synthesis and secretion of testosterone in fetal rats. Dams were exposed to atmospheres that contained 0.09 ppm, 0.9 ppm or 9 ppm of toluene for 90 min/day from gestational days (GDs) 14.5 to 18.5 via nasal inhalation. Fetal plasma testosterone concentrations determined by enzyme immunoassay were significantly reduced on GD18.5 after exposure to 0.9 and 9 ppm, but not to 0.09 ppm, of toluene in male, but not in female, fetuses. We measured, using real-time PCR methods, mRNA levels in fetal testes for several steroidogenic enzymes involved in testosterone synthesis and insulin-like 3 (Ins3), a marker of Leydig cell differentiation. The mRNA levels of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) were significantly reduced after exposure to 0.9–9 ppm toluene. However, the mRNA levels of cytochrome P450 cholesterol side-chain cleavage, cytochrome P450 17 α -hydroxylase/c17-20 lyase, 17 β -hydroxysteroid dehydrogenase, and Ins3 were not significantly altered by exposure to 0.9–9 ppm toluene. In addition, immunohistochemical analysis showed reduced 3 β -HSD-immunoreactive areas in the interstitial region of fetal testes after exposure to 0.9 and 9 ppm, but not 0.09 ppm, toluene. These findings indicate that toluene reduced the synthesis and secretion of testosterone in fetal testes from rats possibly as a consequence of reduced 3 β -HSD expression.

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1. Introduction

Toluene is a volatile organic compound widely used as an industrial solvent, and it can also be found as an air pollutant in homes and buildings. Exposure to higher levels of toluene from inhalation is known to induce reproductive and developmental toxicity such as intrauterine growth retardation, premature delivery, and testicular atrophy (Donald et al., 1991). Several lines of evidence suggest that toluene affects the endocrine function of reproductive organs in both humans and animals. For example, occupational exposure to toluene reduces blood testosterone levels in rostragrade printers (Svensson et al., 1992a,b). Inhalation of paint thinner that contains 66% of toluene also reduces the synthesis and secretion of testosterone in adult male rats (Yilmaz et al., 2001, 2006). Toluene exposure during the neonatal period reduces testosterone levels in male rats until sexual maturation (Hansson et al., 1985). Thus, while toluene inhibits testicular function in adults, its effects during development are largely unknown.

Leydig cells (LCs) of the testes synthesize testosterone in mammals. Two populations of LCs arise in the course of testes development (Hutaniemi and Pelliniemi, 1992). The fetal population of LCs appears during fetal life and disappears after birth. The

other population, the adult LC population, arises during puberty and sustains adulthood. Insulin-like 3 (Ins3) is a member of the insulin-IGF-relaxin family of growth factors and hormone and is produced by LCs of the testes (Adham et al., 1993; Pusch et al., 1996). The expression of Ins3 is proposed to be a useful marker of the status of LC differentiation (Balvers et al., 1998). LCs of the rat testes strongly express Ins3 from embryonic day (ED) 17.5 to ED19.5 and on postnatal day 35 or later, whereas Ins3 expression is weak from ED21.5 until puberty (McKinnell et al., 2005).

Testosterone synthesized from the fetal population of LCs is first detectable on ED15.5 and rises to reach peak levels around ED18.5 in rats (Warren et al., 1973). Biosynthesis from cholesterol to testosterone requires a series of steroidogenic enzymes that include cytochrome P450 cholesterol side-chain cleavage (P450sc), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), cytochrome P450 17 α -hydroxylase/c17-20 lyase (P450c17), and 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (Payne and Hales, 2004). Male rats have higher plasma testosterone levels than females starting in late fetal life (Slob et al., 1980; Weisz and Ward, 1980). Actions of testosterone and its metabolites during development are essential for genital development (Hutaniemi and Pelliniemi, 1992; Hutson et al., 1997) and sexual differentiation of the central nervous system (MacLusky and Naftolin, 1981; Morris et al., 2004).

The goal of our study was to determine if toluene inhibited the synthesis and secretion of testosterone in fetal rats. Accordingly, we examined the effects of exposure to toluene during late fetal life on plasma testosterone concentrations in fetal rats. Also,

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we examined whether exposure to toluene alter the expression of steroidogenic enzymes involved in testosterone synthesis (P450sc, 3 β -HSD, P450c17 and 17 β -HSD) and the expression of Ins3, as a marker for LC differentiation, in the fetal testes of rats.

2. Materials and methods

2.1. Animals

Pregnant Long-Evans rats (Institute for Animal Reproduction, Kumagaya, Japan) were bred in individual cages in a 12-h light/12-h dark cycle at 23 ± 1 °C with free access to water and food. Care of animals and experiments were performed according to the Guidelines for the Care and Use of Experimental Animals of the National Institute for Environmental Studies of Japan.

2.2. Inhalation exposure to toluene

Pregnant rats (n = 4 per group) were exposed to atmospheres containing 0.09 ppm, 0.9 ppm or 9 ppm of toluene for 90 min/day from gestational day (GD) 14.5 to GD18.5. The day after mating was designated GD0.5. We previously reported that 9 ppm toluene modulated mRNA expression of neurotrophin-related genes in the hippocampus of adult mice (Wito-Shire et al., 2007). Therefore, we selected 0.09, 0.9 and 9 ppm toluene as our low-, mid- and high-dose groups for the present study. In addition, a control group that consisted of six pregnant rats was exposed to filtered clean air under similar conditions as the experimental groups.

Toluene vapor was generated by an organic solvent gas generator (Silbata Scientific Technology Ltd., Tokyo, Japan), diluted with filtered clean air to achieve the desired concentration, and introduced into an acrylic exposure chamber. We adjusted the concentrations of toluene gas in the exposure chamber based on the results of portable gas chromatography mass spectrometry (Hagadic, Inficon, East Syracuse, NY, USA). The mean levels of toluene gas concentrations were 5.7 ± 4.9 ppb in the control group and 91.2 ± 9.2 ppb, 0.90 ± 0.02 ppm, and 9.10 ± 0.20 ppm in the experimental groups.

2.3. Sampling

Immediately after the final toluene exposure on GD18.5, pregnant rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), and fetuses were obtained by Cesarean section. Fetuses were counted, sexed and weighed, and blood was obtained to measure plasma testosterone concentrations with enzyme-linked immunosorbent assay. Testes from male fetuses of the control and 0.9 ppm-toluene-exposed groups were obtained to examine the mRNA levels of steroidogenic enzymes (P450sc, 3 β -HSD, P450c17, and 17 β -HSD) and Ins3 using real-time PCR methods. In addition, fetal testes of all groups were used for immunohistochemical analysis for 3 β -HSD to investigate the effects of toluene on the protein expression level of 3 β -HSD in the interstitial region of fetal testes.

2.4. Enzyme-linked immunosorbent assay for testosterone

For assay of testosterone, plasma samples obtained from male and female fetuses of four litters in each group (3–5 fetuses in each sex per litter) were used. The lipophilic fraction of plasma samples was extracted with diethyl ether. Plasma was diluted with a 24-fold volume of distilled water. The dilution was then mixed with a 5-fold volume of diethyl ether in a glass tube, and the diethyl ether fraction was collected and then evaporated at 37 °C for 20–30 min. The remaining water-soluble fraction was again mixed with diethyl ether, and extraction was repeated. Testosterone in extracted samples was assayed using a Testosterone EIA Kit (Cayman Chemical, Ann Arbor, MI, USA) in accordance with the manufacturer's protocol. The intraassay and interassay coefficients of variation were 6.8% (testosterone concentration, 144.28 pg/ml) and 10.7% (testosterone concentration, 68.5 pg/ml), respectively. Plasma concentrations of testosterone were averaged in each sex for each litter, and values of the litter means were statistically analyzed.

2.5. Real-time PCR analyses for steroidogenic enzymes and Ins3

We used testicular tissues in control and 0.9 ppm-toluene-exposed groups (three litters/group with 2–5 male fetuses in each litter) to measure mRNA lev-

els of steroidogenic enzymes in testes. Total RNA was extracted and purified from fetal testes with an RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using a TaKaRa PrimeScript RT reagent kit (TaKaRa Bio, Inc., Otsu, Japan). For each sample, total RNA (2 μ g) was reverse-transcribed to first-strand cDNA in a final volume of 40 μ l of PrimeScript buffer containing PrimeScript RT Enzyme Mix 1 (2 μ l), random 6-mers (200 pmol), and oligo dT primer (100 pmol) for 15 min at 37 °C. The reaction was terminated by incubation at 85 °C for 5 s. The reaction mixtures containing cDNA were stored at –80 °C until use for real-time PCR.

Real-time PCR was performed using the LightCycler ST300 (Roche Diagnostics, Mannheim, Germany). To prepare standard samples for each gene transcript, an equal amount of each cDNA sample was combined and then serially diluted with EASY Dilution (TaKaRa Bio). For the standards and single sample of unknowns, 2 μ l of RT product was amplified in 20 μ l of a reaction mixture containing 200 nM of each primer (see Table 1) and 10 μ l of 2 $\times$ SYBR Premix Ex Taq (TaKaRa Bio). The gene-specific primer sets were designed with LightCycler Probe Design Software 2.0 (Roche) with reference to Genbank sequences downloaded from the NCBI rat Genome Resources website (<http://www.ncbi.nlm.nih.gov/genome/guide/rat/>) so as to have ~500 bp melting temperature and ~50% GC content and generate amplicon lengths from 150 to 200 bp. The conditions of real-time PCR were an initial step to activate the Taq polymerase for 10 s at 95 °C, followed by 50 cycles at 95 °C for 5 s for denaturation and 60 °C for 20 s for annealing and extension, with a temperature transition rate of 20 °C/s. After real-time PCR, a melting curve analysis was carried out to demonstrate the specificity of the PCR products, and showed that the melting curve for the PCR products targeting on each gene transcript had a single peak (data not shown). The amounts of the mRNAs for steroidogenic enzymes and Ins3 in each sample were normalized by division by the amount of mRNA of the housekeeping gene cyclophilin B (CYPB) in the same sample. The normalized mRNA levels of each steroidogenic enzyme and Ins3 were averaged in each litter, and values of the litter means were then used for statistical analyses.

2.6. Immunohistochemical analysis for 3 β -HSD

Testes obtained from 2 to 5 male fetuses in each litter (control group and 0.9–9 ppm- and 9 ppm-toluene-exposed groups, four litters for each; 0.09–9 ppm-toluene-exposed group, three litters) were immersed in 10% formalin in saline for fixation, and dehydrated through a graded series of ethanol followed by xylene, and embedded in paraffin. Sections of tissues were cut at a thickness of 5 μ m and were mounted on glass slides coated with 3-aminopropyltriethoxysilane.

Tissue sections were processed at room temperature unless otherwise stated. Deparaffinized sections were placed in absolute methanol for 30 min and in 3% H₂O for 10 min. After rinsing in 0.05 M phosphate-buffered saline (PBS, pH 7.5), the sections were incubated with normal goat serum (1:100) for 1 h, followed by rabbit anti-3 β -HSD antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS containing 5% normal goat serum and 0.1% Triton X-100 overnight at 4 °C. Sections were then incubated with PBS and 0.1% Triton X-100 overnight at 4 °C. Sections were then incubated with rabbit anti-3 β -HSD antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was visualized using a Dako DAB Plus Liquid System (Dako, USA). Immunopositive sections were then counterstained with methyl green. In addition, some deparaffinized sections were stained with hematoxylin for histopathological observation. These sections were dehydrated through a graded series of ethanol, cleared by xylene, and then coverslipped with an embedding medium.

To determine the density of 3 β -HSD-immunoreactivity, photomicrographic digital images (final magnification: 35 $\times$; resolution: 300 dpi) were taken from 3 β -HSD-immunostained sections of fetal testes. Regions of these photomicrographs (total area: 0.185 mm²) were analyzed for measuring the density of 3 β -HSD-immunoreactivity in the interstitial region of testes. The interstitial region of testes (these images was trimmed with the aid of a computer and graphics software (Adobe Photoshop CS3, Adobe, San Jose, CA, USA). The surface area of the interstitial region and the 3 β -HSD-immunoreactive area in the interstitial region were then measured by using the ImageJ 31 (National Institutes of Health, Bethesda, MD, USA). As criterion of determination of 3 β -HSD-immunoreactive area, the area darker than 100 of 256 scales was measured. The 3 β -HSD-immunoreactive area was then normalized by dividing by the total area of the interstitial region of interest, and it is expressed as the 3 β -HSD-immunoreactive area per 1-mm² area of the interstitial region of fetal testes.

Table 1
Sequences of primers used for real-time PCR.

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product length (bp)	NCBI reference sequence
P450sc	GCTGATCTCTCTCTACACCA	GCATCTCTGGAATTTCTGC	150	NM_017286
3 β -HSD1	TGGCAGACAGACAGGA	TAMGACATAGTACAGTCTGTCAA	155	NM_001007719
P450c17	CGCTTTCAGCATCTCTGTA	CGCTTTCAGCATCTCTGTA	170	NM_012753
17 β -HSD3	CAGACAGACATCTCTGACATTTGAA	TACTACGAGATGATCTTCTGC	164	NM_054007
Ins3	CCGCTCTCTCTCTCTACTG	TACTACGAGATGATCTTCTGC	155	NM_053680
Cyclophilin B	AGACTGTCTCCAAACACATGGA	GATCTCTTCTCTCTCTCTCTGC	155	AF071225

2.7. Statistical analyses

Two-way factorial analysis of variance (ANOVA) was used to examine the effects of toluene or sex and the effects of interaction between toluene and sex on the number of fetuses, the body weight of fetuses, and plasma testosterone concentration. When significant overall effects were detected with two-way ANOVA, Fisher's PLSD test was performed for post hoc analysis. One-way ANOVA was performed to determine differences among groups in the body weight gain of pregnant rat during exposure to toluene, the total number of fetuses and the density of 3 β -HSD-immunoreactivity in fetal testes. Bonferroni/Dunn test was succeeded when significant overall effects were detected with one-way ANOVA. In the post hoc analysis, a probability value of $p < 0.05$ was considered to indicate statistical significance. Differences in the mRNA levels of steroidogenic enzymes and InsI3 in fetal testes between control and 0.9 ppm-toluene-exposed groups were examined by the unpaired *t*-test.

3. Results

3.1. Effects of toluene exposure on pregnant rats

One-way ANOVA indicated that toluene exposure during late pregnancy had no significant effect on the body weight gain of pregnant rats [F(3, 14) = 0.337, $p = 0.799$] and total number of fetuses [F(3, 14) = 0.179, $p = 0.909$] (Table 2). Two-way ANOVA indicated that there was no significant overall effect on toluene [F(3, 28) = 0.137, $p = 0.937$] and sex [F(1, 28) = 1.83, $p = 0.187$] and the interactive effect of toluene and sex [F(3, 28) = 1.42, $p = 0.259$] on the number of fetuses. There was no significant effect on toluene [F(3, 28) = 0.728, $p = 0.400$] and sex [F(1, 28) = 2.44, $p = 0.0849$] and the interactive effect of toluene and sex [F(3, 28) = 0.025, $p = 0.994$] on the body weight of fetal rats. While one fetal death was observed after exposure to 0.9-ppm toluene, it was not considered treatment related.

3.2. Effects of toluene on plasma testosterone concentration in fetal rats

There were significant effects of toluene [F(3, 24) = 3.59, $p < 0.05$] and sex [F(1, 24) = 53.6, $p < 0.0001$] on plasma testosterone concentrations in fetal rats, although the interactive effect of toluene and sex was not significant [F(3, 24) = 1.61, $p = 0.213$]. In post hoc analysis, plasma testosterone concentrations in male fetuses exposed to 0.9 ppm or 9 ppm of toluene were significantly ($p < 0.05$) less than control fetuses of the same sex, but there was no significant difference between the control and 0.09-ppm-toluene-exposed groups of male fetuses (Fig. 1). In female fetuses, toluene had no significant effects on plasma testosterone concentrations. When plasma testosterone concentrations were compared between sexes, concentrations of male fetuses were significantly ($p < 0.05$) higher than those of female fetuses in all groups.

3.3. Effects of toluene on the mRNA levels of steroidogenic enzymes and InsI3 in fetal testes

The mRNA levels of 3 β -HSD1 in fetal testes were significantly ($p < 0.05$) reduced after exposure to 0.9 ppm toluene (Fig. 2). However, there was no significant difference in the mRNA levels of other

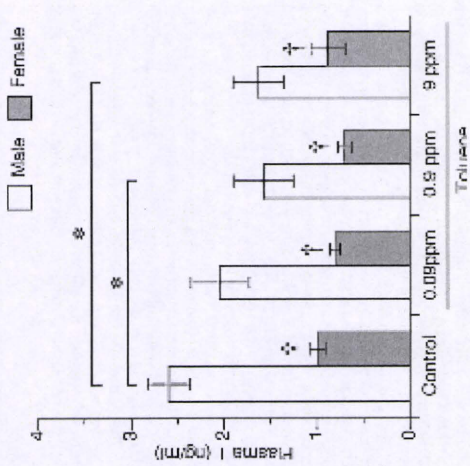


Fig. 1. Effects of exposure to toluene via maternal inhalation on plasma testosterone concentrations of fetal offspring on CD18.5. Values are means $\pm$ S.E.M. * $p < 0.05$ vs. males of the same group.

steroidogenic enzymes, P450c17 and 17 β -HSD3, and InsI3 between control and 0.9-ppm-toluene-exposed groups.

3.4. Effects of toluene on 3 β -HSD expression in fetal testes

Cells containing 3 β -HSD-immunoreactivity in the cytoplasm were found in the interstitial region of testes in male fetuses (Fig. 3). In light microscopy using hematoxylin–eosin sections, there was no striking difference in the histoarchitecture of fetal testes among control and toluene-exposed groups.

One-way ANOVA indicated that there were overall significant effects of toluene on the 3 β -HSD-immunoreactive area in the interstitial region of fetal testes among groups [F(3, 11) = 7.55, $p < 0.01$]. In post hoc analysis, the 3 β -HSD-immunoreactive area in the interstitial region of fetal testes was significantly ($p < 0.05$) reduced after exposure to toluene at 0.9 and 9 ppm, but not at 0.09 ppm (Fig. 4).

4. Discussion

Serum and testicular testosterone levels are reduced in adult rats that inhale 1500 ppm or 3000 ppm of paint thinner that contains 66% toluene (Yilmaz et al., 2001, 2006). Since these exposures levels contained 990–1980 ppm of toluene, toluene appears to have inhibitory effects on the synthesis and secretion of testosterone in sexually matured rats. Furthermore, exposure to 80 ppm

Table 2
Body weight gain of pregnant rats and the number and body weight of fetuses.

Treatment group	Body weight gain of pregnant rat from GD14.5 to GD18.5 (g)	Number of fetus		Body weight of fetus (g)	
		Total		Male	Female
Control	28.6 $\pm$ 2.84	13.0 $\pm$ 0.97	6.33 $\pm$ 1.02	6.67 $\pm$ 0.56	1.62 $\pm$ 0.18
Toluene 0.09 ppm	31.7 $\pm$ 1.67	12.0 $\pm$ 0.82	7.25 $\pm$ 0.85	4.78 $\pm$ 0.85	1.36 $\pm$ 0.04
Toluene 0.9 ppm	26.2 $\pm$ 5.24	13.0 $\pm$ 0.71	6.25 $\pm$ 1.03	6.78 $\pm$ 0.63	1.44 $\pm$ 0.06
Toluene 9 ppm	29.3 $\pm$ 4.64	12.8 $\pm$ 1.65	7.25 $\pm$ 0.63	5.50 $\pm$ 1.19	1.35 $\pm$ 0.04

There was no significant difference in any parameters among groups.

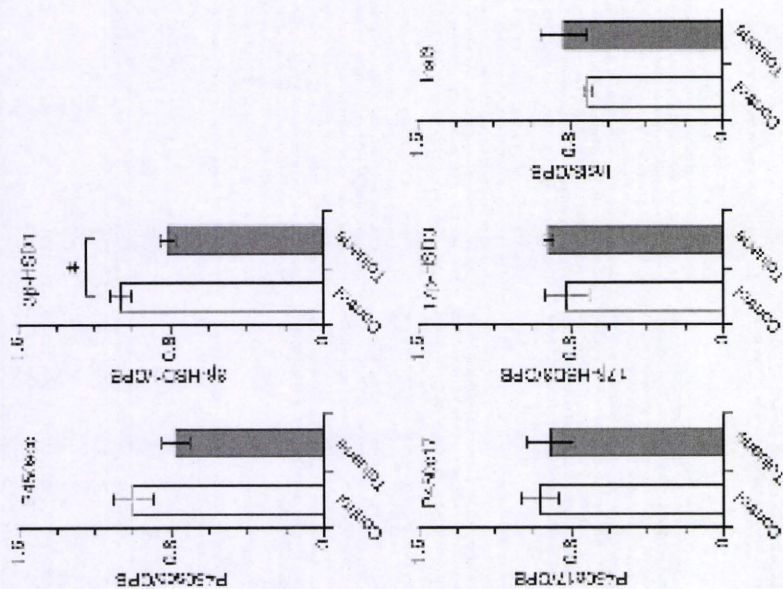


Fig. 2. Effects of exposure to toluene at 0.9 ppm on mRNA levels of P450c17, 17 β -HSD3, and InsI3 in fetal testes on CD18.5. Values are means $\pm$ S.E.M. * $p < 0.05$.

or 500-ppm toluene during the first week after birth reduces serum testosterone levels in 21-day-old-male rats, and 1000 ppm of toluene produces similar effects in 36-day-old-male rats (Hansson et al., 1985). In the present study, we exposed pregnant rats to toluene during late pregnancy and measured fetal plasma testosterone levels. The testosterone levels in male fetuses were significantly decreased after exposure to 0.9 and 9, but not 0.09 ppm of toluene. These findings with our present study suggest that exposure to toluene reduces the synthesis and secretion of testosterone not only in prepubertal and adult male rats but also in fetal male rats. In addition, fetal rats were more sensitive because the lowest effective dose of toluene was lower in fetal rats than either prepubertal or adult rats. This observation implies that testicular functions of fetal male rats are more sensitive and vulnerability to toluene than older rats.

The synthesis of testosterone in Leydig cells involves a series of steroidogenic enzymes (Payne and Hales, 2004). P450c17 is located in the inner mitochondrial membrane catalyzes the conversion of cholesterol to pregnenolone that is transported to the smooth endoplasmic reticulum in the cytoplasm where 3 β -HSD1 catalyzes the conversion of pregnenolone to progesterone (Omura and Morohashi, 1995; Payne and Hales, 2004). In an

effort to explain reduced fetal testosterone levels with exposure to toluene, we measured mRNA levels of steroidogenic enzymes related to testosterone synthesis after exposure to 0.9 ppm toluene, the lowest dose associated with this effect. Toluene significantly decreased the level of 3 β -HSD1 mRNA, although the mRNA levels of P450c17, P450c17 and 17 β -HSD3 were not significantly affected by toluene. In addition, immunohistochemical analysis showed that 3 β -HSD-immunoreactive area in the interstitial region of fetal testes was significantly decreased by exposure to toluene at 0.9 ppm or 9 ppm, but not at 0.09 ppm. These suggest that toluene decreases steroidogenic activity in testosterone synthesis by reducing 3 β -HSD expression in fetal testes. The inhibitory effects of toluene on 3 β -HSD may lead to the decrease in plasma testosterone concentrations in fetal testes.

We also examined the effects of toluene on the mRNA level of InsI3 in the fetal testes to test the possibility that toluene inhibits testosterone synthesis and secretion via altering LC differentiation. InsI3 can be useful for a marker of LC differentiation, because InsI3 is strongly expressed in mature LCs of both the fetal and adult populations (Balvers et al., 1998; McKinnell et al., 2005). InsI3 produced by the fetal population of LCs is involved in the regulation of trans-abdominal testicular descent (Nef and Parada, 1999; Zimmermann

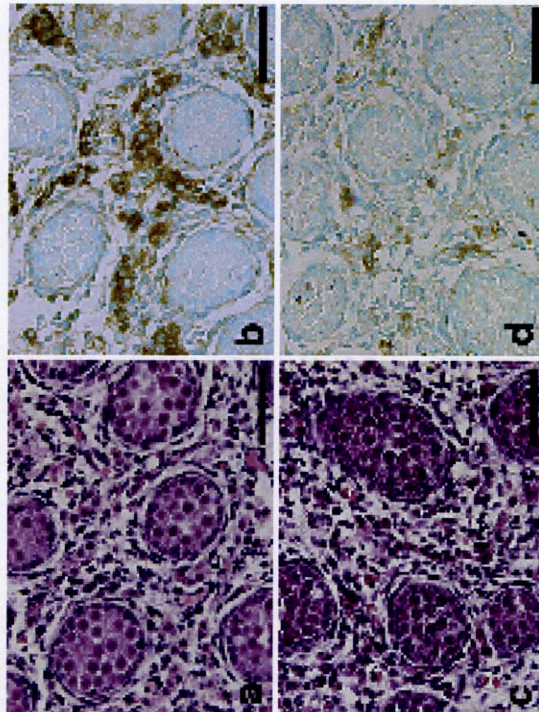


Fig. 3. Photomicrographs of hematoxylin–eosin-stained sections (a and c) and 3β-HSD-immunostained sections (b and d) of fetal testes on GD18.5 in control group (a and b) and 0.9-ppm-toluene-exposed group (c and d). Scale bars indicate 50 μm.

et al., 1999). In the present study, we showed that there was no significant difference in *Ins13* mRNA level of the fetal testes between control and 0.9-ppm-toluene-exposed groups. In addition, we did not observe any striking difference in the histoarchitecture of fetal testes among control and toluene-exposed groups. Accordingly, it appears that exposure to toluene in conditions of this study does not alter LC differentiation and/or induce death of LCs during fetal life in rats. Ineffectiveness of toluene on mRNA levels of *P450c17*

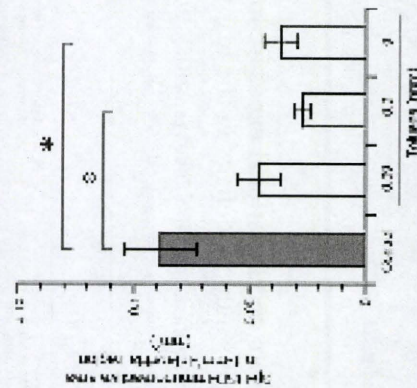


Fig. 4. Effects of exposure to toluene via maternal inhalation on 3β-HSD-immunoreactive area in the interstitial region of testes of fetal male rats on GD18.5. Values are means ± S.E.M. * $p < 0.05$.

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Conflict of interest statement

There are no conflicts of interest.

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In utero and lactational exposure to low doses of chlorinated and brominated dioxins induces deficits in the fear memory of male mice

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ABSTRACT

Environmental-level in utero and lactational exposures to dioxins have been considered to affect brain functions of offspring. Here, we determined whether in utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-tetrabromodibenzo-p-dioxin (TBDD), at the dose that does not harm the dams, affects the acquisition and retention of fear memory in mouse offspring. Pregnant C57BL/6j mice were administered by gavage TCDD or TBDD at a dose of 0 or 3.0 µg/kg body weight on gestation day 12.5, and their male offspring were examined for their behavior in adulthood. In the fear conditioning, a paired presentation of tone and foot shock was repeated three times, and retention tests for contextual and auditory fear memory were carried out 1 and 24 h after the fear conditioning. Groups of mice that were exposed to TCDD and TBDD in utero and via lactation showed deficits in the contextual and auditory retention tests at 1 and 24 h retention intervals. The present results suggest that maternal exposure to a low dose of TCDD or TBDD disrupts the functions of memory and emotion in male mouse offspring, and that the developmental toxicities of these chemicals are similar to each other.

1. Introduction

Dioxins are produced mainly by combustion, released into the environment and accumulated in human and wildlife populations largely via the food chain. Excessive exposure to dioxins relative to the current environmental level has been reported to increase the risk of cancer incidence and induce reproductive dysfunction in humans and wildlife (for reviews, see Mukerjee, 1998; Birnbaum and Tuomisto, 2000; Petroff et al., 2001; Schecter and Gasiewicz, 2003). In addition, since dioxins can be transferred from a mother to the brain of developing-offspring (Kakeyama et al., 2003), developmental neurotoxicity may be induced even when a mother has been exposed to a relatively low level of dioxins (Kakeyama and Tohyama, 2003; Darvas, 2008).

However, previous animal studies of dioxin neurotoxicity produced unexpected and still unexplained effects. Schantz and co-workers (Schantz et al., 1996; Seo et al., 1999, 2000; Widholm et al., 2003) reported that maternal exposure to a low dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic congener, does not affect spatial learning but induces a deficit in visual reversal task and apparently improves working memory in rats. In utero and lactational exposure to TCDD alters operant behavior

depending on the task schedule in rats (Markowski et al., 2001, 2002; Hojo et al., 2002, 2008), and the performance of active avoidance task in rats, in terms of prolonged latency to avoidance but not of avoidance rate (Nishijo et al., 2007). Such effects of maternal exposure to dioxins on neurobehavior in animal experiments were often expressed as 'altered' neurobehavioral functions, but not as a dysfunction of the brain.

A possible reason why the developmental neurotoxic effects of dioxins have not been clarified is that previous studies mainly focused on the hippocampus despite the possible involvement of other brain regions in the neurotoxicity of dioxins. In other words, to obtain a complete picture of the neurotoxicity of dioxins, not only memory function, but also emotional function that is related to neurobehavioral changes, should be studied. In order to do so, we adopted the fear conditioning task to study the neurotoxic effects of dioxins on behavior, because the formation of conditioned fear requires both memory and emotional functions.

In the fear conditioning, laboratory animals are initially exposed to an innocuous stimulus, typically an emotionally neutral stimulus such as tone (conditioned stimulus; CS) that is paired or followed by a noxious unconditioned stimulus (US), typically an electric shock on the foot (Davis, 1992; Maren, 2008). After one to several CS-US pairings, the CS elicits defensive behaviors such as freezing behavior in the animals. Conditioned fear is considered to be mediated mainly by N-methyl-D-aspartic acid (NMDA) glutamate receptors in the hippocampus and

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amygdala (LeDoux, 2000; Maren, 2001). We and others also reported that maternal exposure to TCDD reduces the gene expression of NMDA receptor subunit in the forebrain (Kakeyama et al., 2001; Nayyar et al., 2003; Hood et al., 2006). In the present study, the conditioned fear was examined after in utero and lactational exposure to TCDD in mice.

In comparison with the neurotoxicity of TCDD, we studied the possible effects of 2,3,7,8-tetrabromodibenzo-p-dioxin (TBDD), a brominated analogue of TCDD. Brominated dioxins have attracted social concerns in recent years, since the combustion of plastics and textiles that contain brominated flame retardants produces polybrominated congeners of dibenzo-p-dioxins/furans (Dilberto et al., 1993; Ao et al., 2009; Guruge et al., 2009). TBDD has been suspected to have dioxin-like effects (Ivins et al., 1992; Weber and Greim, 1997; Van den Berg et al., 2006; Ohbayashi et al., 2007; Samara et al., 2009). Although TBDD toxicity has been studied for various endpoints, such as delft palate, lethal dose 50 (LD₅₀), and thymic atrophy (for a review, see Birnbaum et al., 2003), it has not been clarified in the case of the developing brain.

In this study, we examined whether TCDD and TBDD affect neurobehavioral function, focusing on the formation and/or retention of fear memory, and compared the toxic potencies of TBDD relative to those of TCDD in adult male offspring exposed to TCDD or TBDD in utero and via lactation.

2. Materials and methods

2.1. Chemicals

TCDD and TBDD, the purities of which were higher than 99.5%, were purchased from Cambridge Isotope Laboratory (Andover, MA, USA). N-nonane and corn oil was obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Animals and exposure to chemicals

Time-mated pregnant C57BL/6j mice were obtained from CLEA Japan (Tokyo, Japan). They were housed in a temperature- and humidity-controlled room (22–24 °C, 30–60% humidity), and were maintained on a 12 h light–dark cycle (lights on at 8:00–20:00). Food and water were provided to the animals *ad libitum*. On gestation day 12.5, TCDD or TBDD dissolved in corn oil containing 0.4% N-nonane at a dose of 3.0 µg/kg body weight (9.32 mol/kg for TCDD, 6 mol/kg for TBDD) was administered by gavage to pregnant mice per group. Because the elimination half-lives of TCDD and TBDD in rats are between 2 and 3 weeks (Birnbaum et al., 2003), this form of exposure to rodent dams at late gestational periods results in in utero and lactational exposure to their offspring (Kakeyama et al., 2003; Fritz et al., 2005). Thus, offspring were exposed around the period of brain maturation (Dehay and Kennedy, 2007).

Pregnant mice that served as a control group were administered an equivalent volume of corn oil containing 0.4% N-nonane. Each group consisted of 8–9 pregnant mice. Pups were housed with their biological mothers until weaning at 21 days of age. At 25–28 weeks of age, one male offspring per litter was randomly selected for the behavioral experiment. Since in utero and lactational exposure to TCDD in rats affect contextual fear in male but not female offspring (Mitsui et al., 2006), only male offspring were used in this study. The number of animals for control and TCDD and TBDD exposures were 9, 9 and 8, respectively. At the start of the behavioral test, the average body weights (±SEM) of control, TCDD- and TBDD-exposed mice were 30.2 ± 0.4, 29.3 ± 0.5 and 29.4 ± 0.6, respectively. The animal experiments and handling of hazardous chemicals were performed in accordance with the guidelines for animal experiments and environmental safety, respectively, of the University of Tokyo.

2.3. Apparatus and stimulus

The experiments were conducted in a windowless room containing two conditioning chambers (320 mm (w) × 250 mm (h) × 250 mm (d) in size), equipped with a stainless-steel shock-grid floor having 36 rods (3 mm diameter, 6 mm apart) and a stainless-steel drop pan (Med Associates, St. Albans, VT, USA). These chambers were accommodated in a sound-attenuating box (550 mm (w) × 500 mm (h) × 500 mm (d)) equipped with a speaker in the side wall. To minimize the possible effects of contextual factor for auditory test, two types of chamber configuration, described as context A and B below, were used. For context A, the front, top and back walls of the chamber were made of transparent acrylic resin, and the two side walls were made of modular aluminum. For context B, a white, polyvinyl chloride floor covered the shock-grid bars and a white, polyvinyl chloride round-arched insert was placed inside the conditioning chamber. A tone (80 dB, 1800 Hz, 30 s) was presented as a CS via a speaker electrically connected to a computer. To control and present the stimulus, a computer software FreezeFrame (MED Associates) was used. An electric foot shock (0.75 mA, 2 s) as a US was delivered via a shock generator (Med Associates). Background noise (65 dB) was provided by internal fans. The behavior of each mouse was monitored and recorded using a digital video camera that was connected to a computer and placed in front of the chamber during the conditioning and test sessions, and was analyzed using FreezeFrame Video-Based Conditioned Fear System software (Actimetrics, Willmette, IL, USA).

2.4. Behavioral test procedures

Behavioral testing was conducted between 13:00 and 17:00. To minimize the possible effects of plasma corticosterone concentrations on animal behavior (Butte et al., 1976; Hui et al., 2004; Thompson et al., 2004) during the testing period, we counterbalanced the task by controlling the order of animals among control, TCDD and TBDD groups.

One day before the conditioning session, the mice were habituated to both context A and B for 10 min. On the day of the conditioning session, the mice were placed in context A of the chamber for 2 min for acclimation, followed by three co-terminating CS–US pairings. That is, at 120, 210 and 300 s after placing the mouse in the chamber, CS was provided for 30 s each time, followed by US in the last 2 s in a 30 s period of the CS. The mice remained in the chamber for 60 s after the third CS–US presentation. Retention tests for contextual and auditory fear memory were carried out 1 and 24 h after the conditioning session. In the contextual test, the mice were returned to context A for 3 min in the absence of the tone and foot shock. One hour after the contextual test, the mice were subjected to the auditory test. In this test, the mice were placed in context B without any stimuli for 2 min, and then subjected to CS for 2 min without a foot shock.

The percentage of time freezing after shock deliveries (1 min) in the conditioning session, and during the contextual (3 min) and auditory (the first and last 2 min) test sessions was calculated for each animal using the FreezeFrame software. Freezing was defined as the absence of movement except for breathing.

2.5. Statistics

Data were analyzed by two-way analysis of variance (ANOVA) for repeated measures followed by Tukey's HSD test (Figs. 1–3), and by Student's t-test (Fig. 4). All data in the text and figures are expressed as mean and standard error of the mean (SEM).

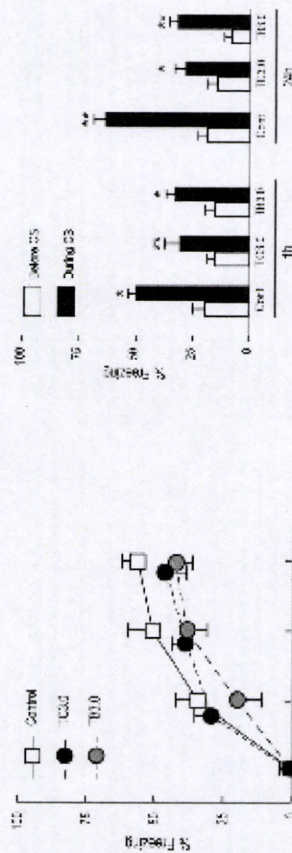


Fig. 1. Freezing responses after three shock deliveries (1 min) during the conditioning session. Control, offspring born to dams exposed to corn oil; TC3.0, offspring born to dams exposed to 3.0 µg/kg TCDD; TB3.0, offspring born to dams exposed to 3.0 µg/kg TBDD. Values represent mean ± SEM. Asterisks indicate significant differences between Before CS and During CS ($p < 0.05$; $p < 0.01$).

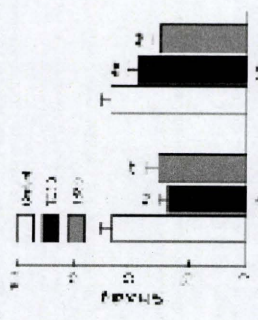


Fig. 2. Freezing responses during the contextual test (3 min). Control, offspring born to dams exposed to corn oil; TC3.0, offspring born to dams exposed to 3.0 µg/kg TCDD; TB3.0, offspring born to dams exposed to 3.0 µg/kg TBDD. Values represent mean ± SEM. Asterisks indicate significant differences from the control group ($p < 0.01$).

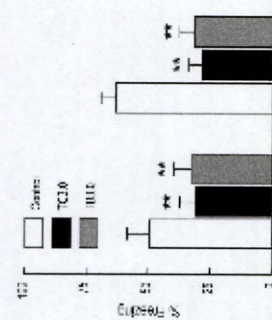


Fig. 3. Freezing responses in the auditory test (the last 2 min). Control, offspring born to dams exposed to corn oil; TC3.0, offspring born to dams exposed to 3.0 µg/kg TCDD; TB3.0, offspring born to dams exposed to 3.0 µg/kg TBDD. Values represent mean ± SEM. Asterisks indicate significant differences from the control group ($p < 0.01$).

3. Results

The mean percent freezing (±SEM) after foot shock deliveries during the conditioning session is shown in Fig. 1. In this session, all

4. Discussion

In utero and lactational exposures to dioxins have been reported to induce abnormal reproductive, learning/memory brain functions and immune functions in laboratory animals, such as rodents and primates (Schecter and Gasiewicz, 2003; Kakeyama and Tohyama, 2003), and these endpoints were used in the reevaluation of possible health risks of dioxin and related compounds by the World Health Organization (WHO, 2000). In particular, among these endpoints, alteration of brain functions such as learning and memory has gained much attention owing to epidemiological (Wigle et al., 2008) as well as laboratory animal studies (Wormley et al., 2004). Additionally, in recent years, the risk assessment of not only chlorinated dioxin congeners but also brominated congeners of dioxins has been required because brominated dioxins are produced unintentionally during the combustion of brominated flame retardants that include brominated biphenyls and other related compounds (Birnbaum et al., 2003; Van den Berg et al., 2006). In the present study, we obtained two novel findings on the health risks associated with dioxins. The first is that a very low dose of TCDD induced deficits in the formation and/or retention of fear memory in both the contextual and auditory fear conditioning. The second is a comparison between the risks associated with TCDD and TBDD.

Regarding the TCDD-induced developmental neurotoxicity, the dose used in *in utero* and lactational exposure to TCDD in the present study induced deficits in fear memory in both the contextual and auditory conditioning in mice under the situations wherein the overall health conditions of the dams and their pups were minimally affected; that is, no effects were observed in the body weight gain of dams during pregnancy, maternal death, number of live pups, and birth weight of pups. To the best of our knowledge, the dose used in the present study is the lowest dose ever reported at which the developmental neurotoxicity of TCDD in mice was demonstrated. That is, the dose (a single oral dose of 3.0 µg/kg b.w. to their mother on gestation day 12.5) was approximately 1/60 of the LD₅₀ of TCDD in C57/BL6 mice (Chapman and Schiller, 1985). An earlier report suggested that only a slight degree of immunotoxicity can be induced by a maternal dose of 5.0 µg/kg b.w. in C57/BL6 mice (Ito et al., 2008).

The observation of a deficit in contextual fear memory in mouse pups born to dams in *in utero* and lactationally exposed to TCDD is consistent with a previous finding in rats (Mitsui et al., 2006). Although the molecular mechanism of such toxicities is largely unknown, *in utero* and lactational exposure to TCDD has been reported to suppress the expression of two molecules, cyclic AMP response element-binding protein (CREB) (Mitsui et al., 2006) and NMDA receptor subunit NR2B (Kakeyama et al., 2001; Nayyar et al., 2003; Hood et al., 2006), both of which are considered to participate in memory function in the hippocampus (Morris et al., 1990; Collingridge and Bliss, 1995; Can, 1997; Josselyn et al., 2004; Alberini, 2009). In accordance with these findings, the hippocampus is reported to play a critical role in contextual fear memory, because a lesion or inactivation of the hippocampus impairs fear memory in mice (LeDoux, 2000; Maren, 2001). Taken together, the hippocampus is vulnerable to the neurotoxic actions of dioxins and is considered to be the brain region that mediates TCDD-induced memory impairment, at least, in the contextual fear conditioning.

Besides the toxic TCDD effects determined in the contextual test, we also found that the offspring born to dams in *in utero* and lactationally exposed to TCDD show impairments in the auditory test. Since TCDD-exposed mice had significantly higher freezing responses during CS presentation than before CS presentation in the auditory test, such deficit is not considered to be induced by the deficit in auditory perception. Although *in utero* and lactational

exposure to a dioxin-like PCB congener was reported to cause low-frequency hearing deficits (Crofton and Rice, 1999), exposure to TCDD under the present condition might not induce hearing deficits, at least at the level that could affect the performance of the auditory fear test. Thus, the impairments in the auditory test using TCDD-exposed mice in the present study are considered to be caused by the dysfunction of auditory fear memory.

Taken together, *in utero* and lactational exposure to TCDD induces deficits in common function of fear memory in both the contextual and auditory paradigms. Fear memory is required for the associative function between environmental cognition (contextual and/or auditory environment) and fear feeling, and thus consists of memory and emotional functions. *In utero* and lactational exposure to TCDD might have affected not only the hippocampus but also other brain regions, since the hippocampus is suggested to be less important for the auditory fear conditioning than the contextual fear conditioning (Phillips and LeDoux, 1992), although whether the hippocampus is involved in the formation of the auditory fear memory is still controversial (Maren, 2008). The amygdala is considered to be such a putative region which plays critical roles in the acquisition and expression of the fear conditioning (LeDoux, 1995; Fendt and Fanselow, 1999). This notion is supported by the experimental evidence that the lesion of the amygdala and blockade of NMDA receptors in this region hamper the fear memory in mice (Fendt and Fanselow, 1999; LeDoux, 2000; Rodrigues et al., 2001). It has been considered that the acquired information is converted from short-term memory to persistent long-term memory through a consolidation process (McGaugh, 2000), and that NMDA receptors in the amygdala are involved in short-term memory and consolidation process of fear memory. The present result of the TCDD group (Figs. 2 and 3) parallels with that of the blockade of NMDA receptors in the amygdala (Rodrigues et al., 2004). Thus, it is plausible to speculate that *in utero* and lactational TCDD exposure also disrupts NMDA receptor-dependent neural transmission in the amygdala, and thus leads to impairments in short-term and long-term memory in the fear conditioning. Further investigation is needed to elucidate the possible toxic effects of dioxins on brain regions, such as the amygdala.

In this study, we compared the relative neurotoxicities of TCDD and TBDD, and found that *in utero* and lactational exposure to TBDD disrupts fear conditioning at the same dose level and degree of effects as *in utero* and lactational exposure to TCDD. Regarding the toxic effects of TBDD, we found that the TBDD-exposed group showed deficits in the contextual and auditory fear conditioning, similarly to the TCDD-exposed group, indicating that toxic effect of TBDD on the developmental brain is a similar to that of TCDD. The presence of brominated dioxins in human tissue was reported in Japan (Choi et al., 2003), China (Li et al., 2007) and Sweden (Ericson Jorgensen et al., 2010), and the amounts of brominated dioxins were estimated to be up to 15% of the total dioxin TEQ, given that brominated dioxins have toxic equivalency factors similar to those of chlorinated dioxins (Ericson Jorgensen et al., 2010). Because the TBDD dose used in the present study is lower than that used in previous reports in mice, it is considered that the degree of toxicities should be investigated and that continuing efforts to clarify the developmental neurotoxic effects of not only TCDD but also TBDD are needed in the future.

As described above, in the present study, we observed an impairment in the formation of fear memory in adult male mice that were exposed to TCDD and TBDD *in utero* and via lactation. However, since TCDD and TBDD are planar chemicals, they are considered to bind to the arylhydrocarbon receptor (AHR) and elicit AHR-dependent toxicities. Because the structure of human AHR is more similar to that of the DBA/2 strain than to that of the C57BL/6 strain, and because a humanized AHR knock-in mouse study has

revealed that humans are less sensitive than the C57BL/6J to at least the teratogenicity of TCDD; it would be reasonable to assume that humans are less sensitive to TBDD toxicities (Moriguchi et al., 2003; Ramadoss and Perdev, 2004). Further studies are needed to address this issue in terms of pharmacokinetics, tissue distribution and dose dependence. In addition, the mice in this study (6 months old) were older than those in other previous studies. Although the performance of memory tasks and brain functions can be considered to similar at least until 9 months of age (Ben Abdallah et al., 2010), further analyses are necessary to clarify the age difference in terms of when the test was performed and when the exposure was carried out. It would also be ideal to confirm the possible effects of these compounds on dams' nursing behavior possibly by cross-fostering experiments.

5. Conclusions

In conclusion, *in utero* and lactational exposure to TCDD in mice at a very low dose caused deficits in the contextual and auditory fear conditioning, which is required for memory and emotional function in offspring. *In utero* and lactational exposure to TBDD showed that TBDD has a similar degree of potency as TCDD and it can induce impairments of contextual and auditory fear memory.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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EXPERIMENTAL PROCEDURES Subjects and behavioral manipulations

All procedures performed on animals were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996) and were approved by The Rockefeller University Animal Care and Use Committee. Adult male Sprague–Dawley rats (275–300 g on arrival; Charles River Laboratories, Wilmington, MA, USA) were housed with food and water available *ad libitum*, under a 12/12 h light/dark cycle in a temperature-controlled (22 °C) facility. Each animal was handled for 10 min daily for 3–4 weeks before the experiment. A radial eight-arm maze with only two arms attached was placed in a black painted room approximately 2.5 m² in diameter (Fig. 1A). Visual cues were placed distally to each of the arms. Proximal to one arm there was a single cue while proximal to the other arm there were several cues. The size of the animal's location on both arms was set to 10×30 cm² because place fields have been estimated to be on the average 20–25 cm region of space (Maurer et al., 2006; estimated from Wilson and McNaughton, 1993). Since place fields form rather quickly but are stabilized with repeated exposure (Kubie and Muller, 1991; Best and White, 1999), on two consecutive days before the experiments, each animal was exposed to

in situ hybridization (ISH) and immunohistochemical techniques have detected context-specific cell ensemble activity in the hippocampus and neocortex at the single cell level (Chaudhuri et al., 1997; Guzowski et al., 1999; Vazdarjanova et al., 2002). We have recently found that in animals exposed to a restricted part of an environment, zif268-immunoreactive cells formed clusters of a few (3–5) active cells adjacent to clusters of non-active cells in the CA1 and CA3 (Pavides et al., unpublished observation). A critical question that arises then is how this “spatial map” is formed in the hippocampus—does a cluster of neighboring neurons encode for each spatial component, or does the same cluster of cells become engaged in many different components of an environment, or a combination of both? The present study was aimed at investigating the precise configuration of IEG-expressing cell clusters, and determining how different clusters may participate in different parts of an environment. We have investigated patterns of active nearby CA1 neurons in animals exploring spatial environments, using ISH with *zif268* and *Homer1a* mRNAs and three dimensional (3D)-reconstruction imaging methods.

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HIPPOCAMPAL CELLS ENCODE PLACES BY FORMING SMALL ANATOMICAL CLUSTERS

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Abstract—The hippocampus has been hypothesized to function as a “spatial” or “cognitive” map, however, the functional cellular organization of the spatial map remains a mystery. The majority of electrophysiological studies, thus far, have supported the view of a random-type organization in the hippocampus. However, using immediate early genes (IEGs) as an indicator of neuronal activity, we recently observed a cluster-type organization of hippocampal principal cells, whereby a small number (~4) of nearby cells were activated in rats exposed to a restricted part of an environment. To determine the fine structure of these clusters and to provide a 3D image of active hippocampal cells that encode for different parts of an environment, we established a functional mapping of IEGs *zif268* and *Homer1a*, using *in situ* hybridization and 3D-reconstruction imaging methods. We found that, in rats exposed to the same location twice, there were significantly more double IEG-expressing cells, and the clusters of nearby cells were more “tightly” formed, in comparison to rats exposed to two different locations. We propose that spatial encoding recruits specific cell ensembles in the hippocampus and that with repeated exposure to the same place the ensembles become better organized to more accurately represent the “spatial map.” Published by Elsevier Ltd on behalf of IBRO.

Key words: place cells, functional organization, cell assemblies, nearest neighbor distance, *zif268*, *Homer1a*.
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Abbreviations: CON, home-cage control; D_{1-2} , the difference between first and second nearest-neighbor distances; D_{2-3} , the difference between the first and second nearest-neighbor distances; D_{3-4} , the difference between the second and third nearest-neighbor distances; D_{4-5} , the difference between the third and fourth nearest-neighbor distances; DAB, 3,3'-diaminobenzidine; DL, two different locations exposure; IEGs, immediate-early genes; ISH, *in situ* hybridization; K_{cup}, kurtosis; N₁, the nearest-neighbor distance; N₂, the second nearest-neighbor distance; N₃, the third nearest-neighbor distance; S_k, skewness; SL, the same location exposure twice; 3D, three-dimensional; A, the evenly scattered nearest-neighbor distance; ρ , the average cell density.

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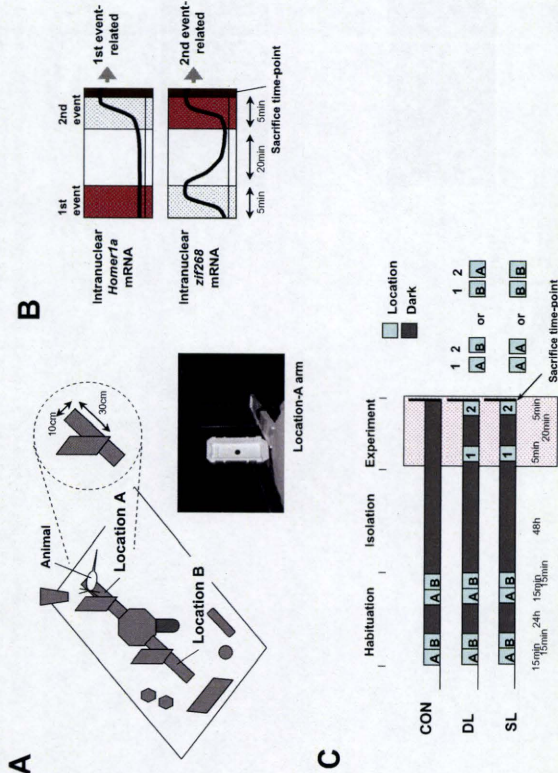


Fig. 1. Behavioral setup and experimental paradigm. (A) Environment. Behavioral testing was performed in a black painted room approximately 2.5 m² in diameter. An elevated eight-arm maze, with only two arms attached, was placed in the center of the room. The arms, which were 60 cm long, were sectioned off with a barrier in the middle of the arm and the animals were placed on Locations A or B (size: 10×30 cm² each) on the end of the arms. Distal to each arm were a number of cues. The room was always illuminated with red light. During place exposure of an animal, a white light was turned on. Included in the room was the isolation chamber in which the animals were kept. (B) Qualitative time course of peak expression of *Homer1a* and *zif268* mRNAs. The differential time course of these two genes allows for identification of neuronal activity of the two exposures. (C) Experimental Paradigm. Three experimental groups of animals were used—non-exposed controls (CON), exposed to two different locations once (DL), or exposed to the same location twice (SL). All animals were habituated to the arms for 15 min on two consecutive days. They were then returned to their home cage and placed in an isolation chamber for 48 h. On the day of the experiment, they were exposed to the arms as indicated and 30 min after the initial exposure were anesthetized and sacrificed. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

both of the arms for 15 min each, and then returned to their home cage, which was located in a dark isolation chamber within the test room.

Peak expression of *zif268* and *Homer1a* mRNAs (Fig. 1B) has been reported to be at approximately 5 and 30 min, respectively (Guzowski et al., 1999; Bottai et al., 2002; Vazdarjanova et al., 2002). Thus, for labeling cells in two environments using the two groups (Fig. 1C): (i) two different locations exposure (DL, $n=6$), in which each animal was exposed on one arm for 5 min, returned to its home cage in the dark for 20 min and exposed on the other arm for 5 min; (ii) the same location exposure twice (SL, $n=7$), in which each animal was exposed on one arm for 5 min, returned to its home cage in the dark for 20 min and exposed on the same arm for 5 min; and (iii) the home-cage control group (CON, $n=5$), in which each animal was kept in its home cage in the dark. Immediately following the second exposure, animals were anesthetized with ketamine (120 mg/kg) / Xylazine (12 mg/kg) under red light, and then decapitated. In addition, to conform the time course of peak expression of *zif268* and *Homer1a*, we performed exploratory studies with different groups of animals: (i) exposed for 5 min (5 min-group, $n=2$); (ii) exposed for 5 min and kept in dark for 15 min (20 min-group, $n=2$); and (iii) exposed for 5 min and kept in dark for 25 min (30 min-group, $n=2$). At the end of each time period, the animals were sacrificed, the brains were rapidly removed and frozen in isopentane at -30°C and then in powdered dry ice and at -80°C . Coronal sections on a cryostat (Jung Frigocut 2800N, Leica Microsystems, Barnack, Germany, USA) at -15°C . Sections were individually collected on a gelatin-coated slide and stored at -80°C before *in situ* hybridization. All efforts were made to minimize the number of animals used and their suffering.

Template and riboprobe preparation

Riboprobes were generated by RT-PCR. For the *zif268* DNA template, PCR primers (Integrated DNA Technologies, Coralville, IA, USA) were designed to amplify a fragment from bases 627–1266 of the rat *zif268* cDNA. For rat *Homer1a* DNA template, PCR primers were designed from the 1.3 kb sequence in the 3'-UTR region in the rat *Homer1a* sequence, modeled as previously described (Brakeman et al., 1997; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004). Non-isotopic riboprobes were synthesized with digoxigenin-labeled UTP (Roche Diagnostics, Indianapolis, IN, USA), and with a fluorescein-labeled UTP (Roche Diagnostics) mixture (Ishii et al., 2004). Isotopic riboprobes were synthesized with ^{32}S -labeled UTP (PerkinElmer, Boston, MA, USA) mixture (Ambion, Austin, TX, USA) (Nakamura and McEwen, 2005).

In situ hybridization double-labeling with DAB and silver-grains

ISH double-labeling with DAB and silver-grains was performed as described previously (Stone et al., 1999; Yang et al., 1999; Nakamura and McEwen, 2005) with modifications. Fresh-frozen sections were processed at the same time with two riboprobes using identical conditions. In brief, sections were fixed with 4% formaldehyde in sterile 1× PBS. Sections were rinsed in PBS, incubated with proteinase K in digestion buffer (50 mM Tris-HCl pH 7.4, 5 mM EDTA), and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl. Sections were rinsed in sterile 2× standard saline citrate (SSC), dehydrated with increasing ethanol concentrations, delipidated with chloroform, and allowed to air-dry.

Hybridization solution (50% formamide, 600 mM NaCl, 10 mM Tris-HCl pH 7.4, 1× Denhardt's solution, 1 mM EDTA, 10 mM DTT, 10% dextran sulfate, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, and 0.1 $\mu\text{g}/\text{ml}$ of digoxigenin-labeled *Homer1a* probes and 1.2×10⁶ cpm of ^{32}S -labeled *zif268* probes), was applied to each

slide (150 μl), and the sections were coverslipped and incubated in a humidified environment at 68°C for 17 h.

Following the hybridization, the coverslips were removed and the sections were rinsed in 50% formamide and 2× SSC at 64°C and in 2× SSC alone. Sections were incubated with 20 $\mu\text{g}/\text{ml}$ RNase A in digestion buffer (500 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) at 37°C , and rinsed in 0.5× SSC, 0.25× SSC, and TN buffer (0.1 M Tris-HCl pH 7.4, 0.15 M NaCl) at room temperature. Sections were incubated with 3% H_2O_2 in TN buffer and incubated with 3% bovine serum albumin (BSA) in TN buffer (0.1 M Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20) at room temperature, and then incubated with anti-digoxigenin-POD antisera (1/500 dilution, Roche Diagnostics) in BSA/TNT at 4°C for 16 h. Sections were rinsed in TNT, and incubated using the Tyramide Signal Amplification (TSA) biotin system (PerkinElmer) at room temperature for 10 min. Sections were incubated with streptavidin-horseradish peroxidase (PerkinElmer) for 1 h, reacted with 0.02% 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) and 0.006% H_2O_2 in 0.05 M Tris-HCl for 15 min, dehydrated in increasing ethanol, and allowed to air dry. Dried sections were dipped in Kodak NTB emulsion (Kodak, Rochester, NY, USA) and stored at 4°C . Emulsion slides were developed with Kodak D-19 developer (Kodak) at 15°C , air-dried, counterstained with 0.2% Cresyl Violet, dehydrated using graded concentrations of ethanol and xylene, and coverslipped.

In situ hybridization double-labeling with two-color fluorescence

ISH double-labeling with two-color fluorescence was performed with fresh-frozen sections as described previously (Ishii et al., 2004) with modifications. In brief, hybridization solution, consisting of 50% formamide, 600 mM NaCl, 10 mM Tris-HCl pH 7.4, 1× Denhardt's solution, 1 mM EDTA, 10% dextran sulfate, 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, 0.1 $\mu\text{g}/\text{ml}$ of digoxigenin-labeled *zif268* *Homer1a* probes and 0.1 $\mu\text{g}/\text{ml}$ of fluorescein-labeled *zif268* probes, was applied, and sections were coverslipped and incubated in a humidified environment at 68°C for 17 h. After stringent hybridization washes, sections were incubated in BSA/TNT at room temperature, and then incubated with both anti-digoxigenin-AP antisera (1/500 dilution, Roche Diagnostics) and anti-fluorescein-POD antisera (1/100 dilution, Roche Diagnostics) in BSA/TNT at 4°C for 16 h. Sections were rinsed in TNT, incubated using TSA biotin system (PerkinElmer), and then incubated with streptavidin Alexa 488 (1/800 dilution, Molecular Probe, Eugene, OR, USA) for 1 h in the dark. Sections were also incubated with HNPP and Fast Red (Roche Diagnostics) in detection buffer (0.1 M Tris-HCl pH 8.0, 0.15 M NaCl, 10 mM MgCl₂) at room temperature for 30 min, counterstained with TOPRO-3 (1/10,000 dilution, Molecular Probe), and coverslipped. Sections were captured using laser scanning confocal microscope (LSM510, Carl Zeiss, Thornwood, NY, USA).

Signal detection of IEG's

The spatial distribution of *Homer1a* and *zif268* expressing cells was analyzed in the CA1 field of the right dorsal hippocampus (3.4–4.0 mm posterior from bregma), according to the rat atlas (Paxinos and Watson, 1998). The pyramidal cells were visualized on a computer screen using a 40× objective under bright-field microscopy (Olympus BX41, Japan) and a CCD camera (Olympus Q-Color3, Japan). Double IEG-labeled cells were analyzed by identifying DAB-labeled dots and silver grains for *Homer1a* and *zif268*, respectively. The cells were also stained with Cresyl Violet for positive identification of nuclei. DAB-labeled dots in the nuclei were identified as brown dots, while silver grains in nuclei were identified by small black dots over the nuclei. Since the grains are localized on the emulsion, they are at a higher focal point than the DAB stain which is localized on the tissue. To distinguish

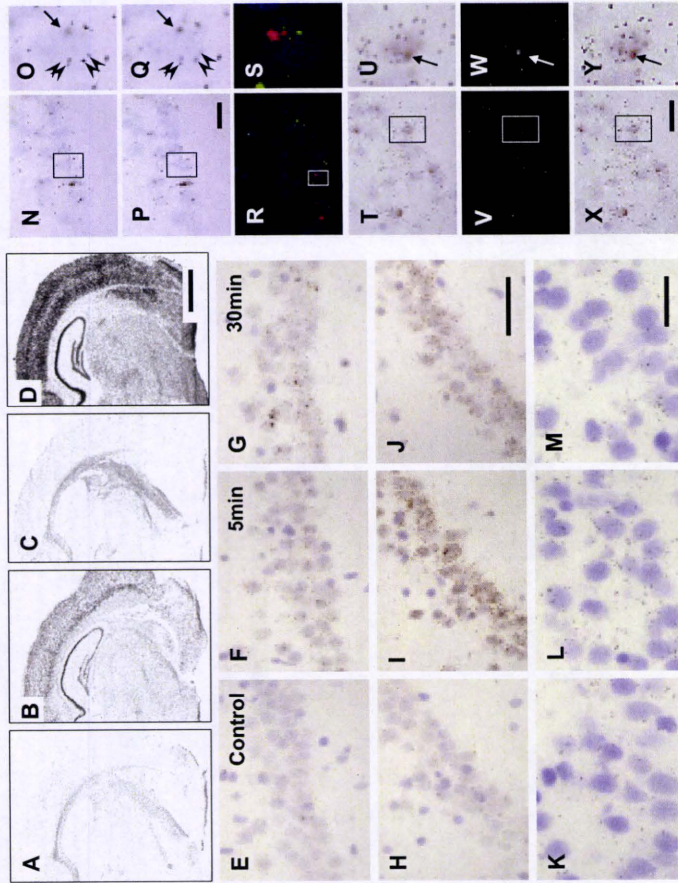


Fig. 2. Specificity, time-course, and dual-labeling *in situ* hybridization for *Homer1a* and *zif268* mRNAs. (A–D) Autoradiographs showing expression of *Homer1a* (B) and *zif268* (D) mRNAs with ^{32}S -labeled antisense, and sense riboprobes for *Homer1a* (A) and *zif268* (C). Contrary to the sense signals, strong antisense signals for *Homer1a* and *zif268* were found in the cortex, and the principal cell layers of the hippocampus. (E–M) Expression of DAB-labeled *Homer1a* (E–G), and DAB- (H–J) and grain-labeled (K–M) *zif268* in the CA1 region. There were three experimental groups: control (E, H, K), and groups exposed to one arm for 5 min (5 min-group, F, I, L) and exposed for 5 min and kept in dark for 25 min (30 min-group, G, J, M). (N–Q) Double-labeling ISH showing expression of DAB-labeled *Homer1a* (arrow) and grain-labeled *zif268* (arrowheads) in CA1 pyramidal cell nuclei (violet) "on-emulsion" (N, O), and "on-tissue" (P, Q) focal planes. (R, S) Expression of *Homer1a* (red) and *zif268* (green) mRNAs in nuclei (blue) by two color-fluorescent ISH showing a similar distribution as with double DAB/grain-emulsion (red and green) mRNAs in nuclei (blue) by (brown) and Cresyl Violet staining. (V, W) Brown DAB-labeled signals appear as white signals when the "on-emulsion" and "on-tissue" images in the same frame were subtracted (see Experimental procedures). (X, Y) Signal-detection image overlaid with the on-tissue image and the subtracted image, in which the signals were colored in red. The higher magnification of the insets shows the nucleus with the staining in O, Q, S, U, W, and Y. Scale bars: A–D: 2 mm; E–J: 100 μm ; K–M: 25 μm ; N–P: 20 μm ; R, T, V, X: 50 μm .

grains from DAB-labeled dots, we performed image processing as follows: (i) the same frame images (each image: $351 \times 263 \mu\text{m}^2$) were captured at two different focal planes. One focal plane was "on-emulsion" level for grains (small black; Fig. 2N, O), and the other was "on-tissue" level for DAB (brown) and Cresyl Violet staining (Fig. 2P, Q); (ii) When on-emulsion grains were focused, on-tissue DAB-labeled signals caused a diffused reflection (Fig. 2N, O, T, U) because of the focal range; (iii) Once the two images with on-emulsion level and on-tissue level were subtracted (images appeared as white signals; Fig. 2V, W) and these represented the DAB-labeled signals; (iv) This subtracted image was labeled red and then overlaid on the on-tissue image (Fig. 2X, Y) and used as "a signal-detection image" for further analysis.

For the IEG-labeling analysis, adjacent signal-detection images were captured along a 1200 μm length of the CA1 pyramidal

cell layer (1.0–2.5 mm lateral from midline suture) in each section. According to grain and DAB-labeled signal detection, the position of a positive cell was marked on the signal-detection image by a person blind to the experimental groups.

Reference images and 3D-reconstructed images

For 3D image reconstructions, alternate 12 μm -thick sections were used in order to avoid double counting of cells whose nucleus may appear in two neighboring sections since cell nuclear diameter is at most 12 μm . A hippocampal image, containing the CA1, CA3 and dentate gyrus regions, was captured as follows: (i) each region of interest was visualized with a 20× objective using a bright-field microscope (Olympus BX61, Japan), and was captured with a CCD camera (Olympus Q-camera Religa 1300, Japan); (ii) The signal-detection images consisted of 90 adjacent

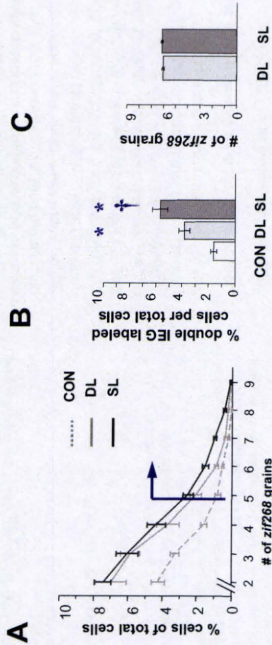


Fig. 3. Quantification of zif268- and Homer1a-labeled CA1 neurons. (A) Line-plots showing the distribution of the number of zif268 grains in CA1 neurons that also contain DAB-labeled Homer1a and Cresyl Violet stained nuclei in non-exposed home-cage animals (CON), exposed to two different locations once (DL), or exposed to the same location twice (SL). Arrow shows the threshold of the number of zif268 grains in the nuclei used for the double IEG-labeling analysis. (B) Bar-plots showing the cell population with cumulative values of more than five zif268 grains in the CON, DL, and SL groups. (C) Bar-plots showing the average numbers of zif268 grains in the double IEG-labeled cells between the DL and SL groups. Error bars indicate SEM in each group. * $P < 0.01$, significant difference between control and experimental groups. † $P < 0.05$, significant difference between the DL and SL groups. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

position of a positive cell was marked, according to the signal-detection imaging described above; (v) Then, eight serial reference images per animal (each image: $4646 \times 2464 \mu\text{m}^2$, pixel size: $1 \times 1 \mu\text{m}^2$) were sequentially aligned slice-by-slice, based on con-

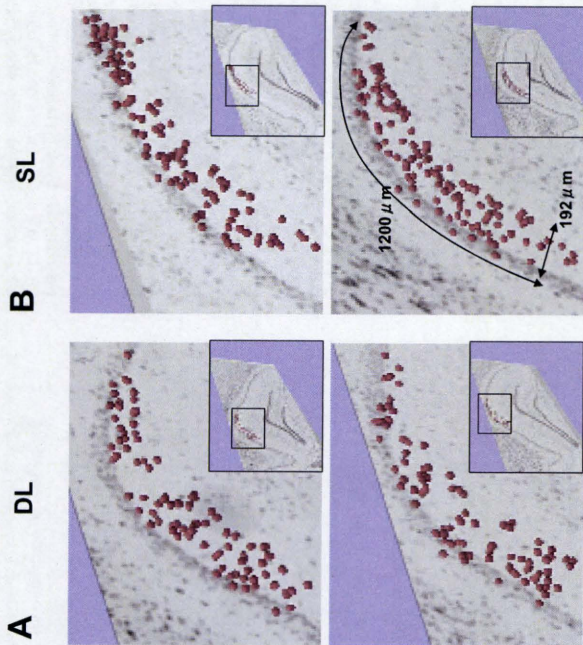


Fig. 4. Representative high magnification 3D-reconstructed images of zif268 and Homer1a double-labeled cell populations (red dots) in the dorsal CA1 field in DL (A) and SL (B) exposed animals. The upper and lower levels showing two representative 3D-reconstructed images in each group. The inset in the corner shows a lower magnification of the image. The total number of double IEG-labeled cells was 87 (A upper), 91 (A lower), 110 (B upper), and 133 (B lower) per $2.4\text{--}2.9 \times 10^3 \mu\text{m}^2$ ($192 \mu\text{m}$ thickness in the antero-posterior axis by eight sections and $1200\text{-}\mu\text{m}$ -long CA1 pyramidal cell layer in coronal section). There appears to be clusters of nearby cells in both of the DL and SL groups.

trast/brightness of cellular distribution containing hippocampal cell-layer curves, using the image registration software FLIRT 5.3 (University of Oxford, Oxford, UK) (Jenkinson and Smith, 2001); (vi) The aligned reference images and marked dots were then 3D reconstructed, volume rendered and visualized using the software 3D Slicer (<http://www.slicer.org>). An interval between the reference images was $24 \mu\text{m}$ for 3D image reconstruction. We measured areal cell density ρ ($1/\mu\text{m}^2$) in the CA1 field of each animal, using the following formula:

$$\lambda = 1/n \sum_{i=1}^n \lambda_i = 1/n \sum_{i=1}^n \rho_i \quad (1 \leq i \leq n; \text{ animal})$$

where λ is the evenly scattered, nearest-neighbor distance (μm).

Distances based on nearest neighboring cells

Based on methods used to identify spatial patterns in 2D and 3D (Ripley, 1977, 1979), we characterized clusters of the nearby cells in the CA1 field. Using software DL 6.2 (RSI Inc., Boulder, CO, USA), we quantified a distance from a cell to the nearest neighboring cell using the following formula:

$$N_i = \sum_{j=1}^n N_{i,j} \quad (1 \leq i \leq n; \text{ cell}; \quad 1 \leq j \leq n; \text{ animal})$$

where N_i (μm) is the nearest-neighbor distance, and n_i is the maximum cell number per animal (Fig. 5A). Individual values of the N_i ($N_{i,j}$) were quantified from each position of positive cells in each 3D-reconstructed image, and then in the DL and SL groups. We successively quantified the following values:

$$N_1 = \sum_{j=1}^n N_{1,j} \quad (1 \leq i \leq n; \text{ cell}; \quad 1 \leq j \leq n; \text{ animal})$$

$$N_2 = \sum_{j=1}^n N_{2,j} \\ \vdots \\ N_{i-1} = \sum_{j=1}^n N_{i-1,j}$$

where N_i is the distance from a cell to the second nearest neighboring cell (second nearest-neighbor distance), N_3 is the distance from a cell to the third cell $\dots$; and, $N_{i,j}$ is the distance from a cell to the furthest cell.

Furthermore, for the further second-order process, we calculated with the following formulas:

$$D_{1-2} = \sum_{j=1}^n D_{1-2,j} = \sum_{j=1}^n (N_{2,j} - N_{1,j}) \quad (1 \leq i \leq n; \text{ cell}; \quad 1 \leq j \leq n; \text{ animal})$$

$$D_{2-3} = \sum_{j=1}^n D_{2-3,j} = \sum_{j=1}^n (N_{3,j} - N_{2,j})$$

$$D_{3-4} = \sum_{j=1}^n D_{3-4,j} = \sum_{j=1}^n (N_{4,j} - N_{3,j})$$

$$\vdots$$

$$D_{i-1-i} = \sum_{j=1}^n D_{i-1-i,j} = \sum_{j=1}^n (N_{i,j} - N_{i-1,j})$$

where D_{1-2} (μm) is the difference between the first and second nearest-neighbor distances in each animal, D_{2-3} is the differences between the second and third nearest-neighbor distances, D_{3-4} is the differences between the third and fourth nearest-neighbor distances $\dots$ and D_{i-1-i} is the difference between subsequent order nearest-neighbor distances.

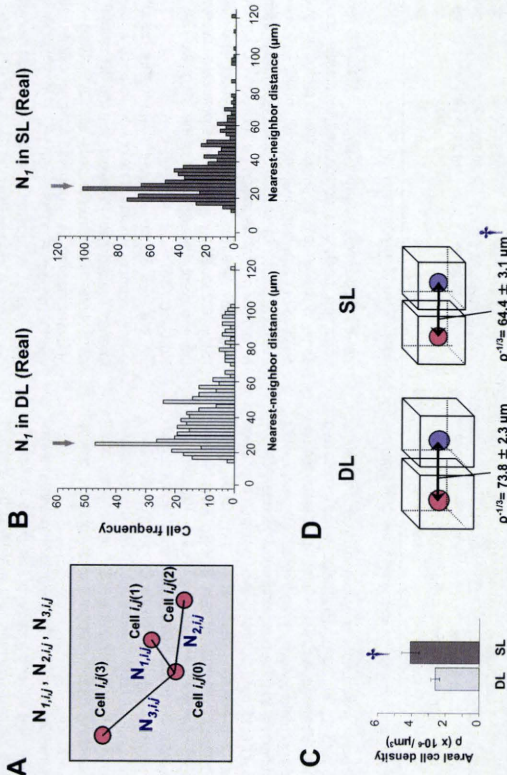


Fig. 5. Nearest-neighbor distances and cell density of the double IEG-labeled cells. (A) Schematic drawing showing the nearest neighbor distance between the cells ($N_{1,j}$), the second nearest neighbor distance ($N_{2,j}$), and the third nearest neighbor distance ($N_{3,j}$). Cell $i(1)$ is shown as the nearest neighboring cell from cell j in animal j ($N_{1,j}(0)$) and Cell $j(2)$ as the second neighboring cell, and Cell $j(3)$ as the third nearest neighboring cell. (B) Histograms showing the distribution of the nearest-neighbor distance (N_1) cell frequency in the DL (left) and SL (right) groups. (C) Bar-plots showing cell density ρ in CA1 pyramidal cell layers in the DL and SL groups. (D) Schematic drawings showing sizes of evenly scattered, nearest-neighbor distances λ ($\lambda = \rho^{-1/2}$, animal: $1 \leq j \leq n$; see Experimental procedures) in the DL and SL groups. Error bars indicate SEM in each group. * $P < 0.05$, significant difference between the DL and SL groups. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Distributions of the N_1 and D_{1-2} , D_{2-3} , D_{3-4} , ..., D_{9-10} were simulated for the DL and SL groups using Monte Carlo simulations on software IDL 6.2. Monte Carlo simulations were performed in the CA1 pyramidal layer of each animal. Based on the "signal-detection images," positions of all cells were identified and extracted on a 1200 μm length in the CA1 for each "reference image" and these "reference images" were reconstructed in 3D montages. In total, 9946 cells in DL ($n=6$ animals) and 11,566 cells in SL ($n=7$ animals) were used. Monte Carlo simulation samples of positive cells were positioned randomly in a set of all cells identified in space in CA1 pyramidal cell layers in each animal. The simulation was performed 100 times in each animal by repeated sampling in identical cell positions. Then, we measured the distance between the simulated samples. To directly compare the values between the DL and SL groups, we used the following values:

$$\begin{aligned} N_1' &= \sum (1/\lambda_i) \sum N_{1,i} (1 \leq i \leq n; \text{cell}; 1 \leq i \leq n; \text{animal}) \\ D_{1-2}' &= \sum (1/\lambda_i) \sum D_{1-2,i} = \sum (1/\lambda_i) \sum (N_{1,i} - N_{2,i}) \\ D_{2-3}' &= \sum (1/\lambda_i) \sum D_{2-3,i} = \sum (1/\lambda_i) \sum (N_{2,i} - N_{3,i}) \\ D_{3-4}' &= \sum (1/\lambda_i) \sum D_{3-4,i} = \sum (1/\lambda_i) \sum (N_{3,i} - N_{4,i}) \\ &\dots \\ D_{9-10}' &= \sum (1/\lambda_i) \sum D_{9-10,i} = \sum (1/\lambda_i) \sum (N_{9,i} - N_{10,i}) \end{aligned}$$

where N_1' is the normalized nearest-neighbor distance, and D_{1-2}' , D_{2-3}' , D_{3-4}' , ..., and D_{9-10}' are the normalized differences between subsequent-order nearest-neighbor distances.

Nearest neighbor topographical arrangement

In many instances, the clusters appeared to consist of a small number (2–4) of double-labeled cells. To more precisely analyze this arrangement, we grouped these cells into what we labeled a "triad" which consisted of three closely located cells. We determined the nearest neighbor of each of the cells and calculated the distances between the cells as the "triad size." Differences in triad size were then compared between the DL and SL groups.

Statistical analysis

For double IEG-labeled cells, one-way analysis of variance (ANOVA) was carried out to evaluate the significance of the main effect on the three experimental groups. Further, *post hoc* comparisons by Tukey–Kramer test were performed between relevant pairs. Student's two-tailed *t*-test was also used to compare between the DL and SL groups.

The values of the nearest-neighbor distances and the differences between subsequent-order nearest-neighbor distances were not normally distributed; hence, the data were analyzed using the nonparametric two-sample Kolmogorov–Smirnov test (two-tailed), to compare between relevant pairs. Moreover, skewness (SK_0) and kurtosis (Ku_0) of the distribution (Q_1 : lower quartile, Q_2 : median, Q_3 : upper quartile, D_1 : first decile, D_9 : ninth decile) were defined as follows:

$$\begin{aligned} SK_0 &= (Q_3 - 2Q_2 + Q_1) / (Q_3 - Q_1) \\ Ku_0 &= (Q_3 - Q_1) / (D_9 - D_1) \end{aligned}$$

All statistical analyses were carried out using the software StatView (SAS, Cary, NC, USA) and R 2.4.1 (R Foundation for Statistical Computing, Vienna, Austria).

Specificity of expression for Homer1a or zif268 mRNAs

To investigate IEG expression in CA1 neurons encoding spatial environments, we employed ISH labeling of *zif268* and *Homer1a* mRNAs. It has previously been reported that peak expression of *zif268* mRNAs can be detected within 2–5 min at the genomic sites of transcription as intranuclear foci after an animal has been exposed to a "place or event" (Guzowski et al., 1999), and this intranuclear active transcription disappears within 15–20 min. Peak *Homer1a* mRNAs appear within 25–40 min as intranuclear foci, which can be detected after exposure of an animal to a "place or event" (Bottai et al., 2002; Vazdarjanova et al., 2002). Our experimental protocol, therefore, involved exposing animals for 5 min to one location (e.g., Location A, shown in Fig. 1A), placing them in isolation for 20 min and then exposing them either to the same (i.e., Location A) or to a different location (i.e., Location B, shown in Fig. 1A). Thus, intra-nuclear *Homer1a* expression should correspond to the first "place or event" while intra-nuclear *zif268* expression should correspond to the second "place or event" (Fig. 1B). We hypothesized that in animals exposed to the same location twice, there should be a larger number of double *zif268*- and *Homer1a*-expressing cells, in comparison to exposing animals to two different locations once (Guzowski et al., 1999; Vazdarjanova et al., 2002).

The patterns of *zif268*- and *Homer1a*-expressing cells were detected by single isotopic ISH autoradiography (Fig. 2A–D). We also examined the time course of expression of intra-nuclear mRNAs using single ISH for DAB-labeled *Homer1a* (Fig. 2E–G), DAB-labeled *zif268* (Fig. 2H–J), and grain-labeled *zif268* (Fig. 2K–M). Expression of DAB-labeled *Homer1a* mRNAs in nuclei increased in the 30 min-group (Fig. 2G) as compared to the 5 min-group (Fig. 2F). 20 min-group, and non-exposed controls (Fig. 2E). Meanwhile, similar to expression of DAB-labeled *zif268* (Fig. 2H–J), expression of grain-labeled *zif268* mRNAs increased in the 5 min-group (Fig. 2L), as compared to the 20 min-group, 30 min-group (Fig. 2M), and non-exposed control group (Fig. 2K). These time peaks of *Homer1a* and *zif268* mRNA expression are comparable to what has been previously reported (Guzowski et al., 1999; Bottai et al., 2002; Vazdarjanova et al., 2002).

Double-labeling with Homer1a and zif268 mRNAs

We employed ISH double-labeling for DAB-labeled *Homer1a* and grain-labeled *zif268* (Fig. 2N–Q), in three experimental groups of animals: (i) non-exposed controls (CON); (ii) exposed to two different locations once (DL); and (iii) exposed to the same location twice (SL) (Fig. 1C). We then analyzed the population of single (Table 1) and double (Table 2) IEG-labeled cells for *Homer1a* and *zif268*. *zif268*-expressing cells were analyzed by counting the cumulative number of intra-nuclear grains. IEG expression levels in non-exposed animals were taken as background. For a cell to qualify as double IEG-labeled, cells with less than five *zif268* grains were eliminated due to the fact that

Table 1. Percent single IEG-labeled cells of total CA1 cells

IEG	Criterion	ANOVA		Group		SL
		<i>F</i> (2,15)	<i>P</i> -value	CON	DL	
<i>zif268</i>	From 2 grains	10.1	<i>P</i> <0.005	41.91±2.55	50.38±1.93*	55.42±1.93*
	From 3 grains	8.87	<i>P</i> <0.005	23.36±2.11	30.60±1.40*	34.62±1.99*
	From 4 grains	8.64	<i>P</i> <0.005	11.09±1.32	16.24±1.09*	19.06±1.47*
<i>Homer1a</i>	From 5 grains	10.55	<i>P</i> <0.005	4.89±0.71	7.88±0.52*	10.00±0.93*
		8.92	<i>P</i> <0.005	19.46±1.29	31.28±2.83*	32.92±2.31*

* Shows a significant difference, as compared to the CON group, by *post hoc* comparisons (Tukey–Kramer test).

the background ratio became minimum from five grains (Tables 1 and 2, Fig. 3A). The cells, which contained DAB-labeled *Homer1a*, a Cresyl Violet-counterstained nucleus, and cumulative values from five *zif268* grains, were then quantified for the three experimental groups. ANOVA showed that the DL and SL groups had a higher population of double IEG-labeled cells than the CON group (*F*(2,15)=17.69, *P*<0.0001; Table 2, Fig. 3B). Furthermore, *post hoc* comparisons by Tukey–Kramer test showed that the SL group had a significantly higher double IEG-labeled cell population than the DL group (Table 2). In terms of single IEG-labeled cell population, there were no differences in either the number of *Homer1a* or *zif268* alone expressing cells between the SL and DL groups (Tables 1 and 2).

To determine whether *zif268* expression was at a similar level following either the same place exposure twice or two different place exposures, we quantified the average number of *zif268*-labeled grains in the double IEG-labeled cells and compared them between the DL and SL groups. Student's *t*-test did not reveal a difference in the average number of *zif268* grains in the double IEG-labeled cells between the DL and SL groups (Fig. 3C). Thus, *zif268* expression was similar in the two experimental groups.

Nearest-neighbor distance

For the 3D analysis, the scanned hippocampal images with double IEG-labeled cells were first sequentially aligned (Fig. 4A, B). We then used previously described methods for spatial mapping patterns in 2D and 3D (Ripley, 1977, 1979) that involved calculating the nearest neighbor distance N_1 (μm ; Fig. 5A), and the further second-order process. One of the peaks at 24 μm (arrow) in the DL group corresponded to a peak (arrow) in the SL group. This peak appears to contain aligned values of N_1 when each 24

Table 2. Percent double IEG-labeled cells of total CA1 cells

IEG (criterion)	ANOVA		Group		DL	SL
	<i>F</i> (2,15)	<i>P</i> -value	CON	DL		
<i>Homer1a</i> and <i>zif268</i> (from 3 grains)	11.7	<i>P</i> <0.001	6.46±0.48	13.35±1.54*	16.05±1.55*	
<i>Homer1a</i> and <i>zif268</i> (from 4 grains)	12.94	<i>P</i> <0.0005	3.18±0.39	7.48±1.03*	10.00±1.03*	
<i>Homer1a</i> and <i>zif268</i> (from 5 grains)	17.69	<i>P</i> <0.0001	1.57±0.23	3.79±0.42*	5.63±0.58**	

* Shows a significant difference, as compared to the CON group, by *post hoc* comparisons (Tukey–Kramer test).

** Shows a significant difference, as compared to the DL group, by *post hoc* comparisons (Tukey–Kramer test).

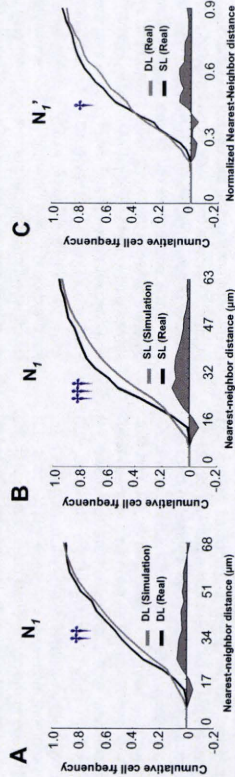


Fig. 6. Nearest-neighbor distances of the double IEG-labeled cells. (A–C) Cumulative line-plots showing the N_1 distribution between real and simulated samples in the DL (A) and SL (B) groups. Histograms also show the subtracted values of the N_1 distribution between real and simulated samples (dark-gray area). (C) Cumulative line-plots showing the normalized N_1 (N_1') distribution between the DL and SL groups. Histograms also show the subtracted values of the N_1' between the DL and SL groups (dark-gray area). † $P < 0.05$, †† $P < 0.005$, ††† $P < 0.0005$. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

each CA1 space size was normalized to the cell density (see Experimental procedures) to directly compare the N_1 between the DL and SL groups of real samples. The SL group had a lower N_1' distribution than the DL group (two-sample Kolmogorov–Smirnov test, $P < 0.05$, Fig. 6C). This indicates that the clustering of double IEG-expressing cells is tighter in the SL group, even though the total number of cells is larger.

Differences between subsequent-order nearest-neighbor distances

To further characterize a 3D spatial mapping of double-IEG labeled cells, we estimated the second-order process, which indicates differences between subsequent-order nearest-neighbor distances (Ds; e.g., the difference between the first and second nearest-neighbor distances, see Experimental procedures). If a D shows a higher distribution, it can be interpreted that the gap between the active neighboring cells is due to formations of clusters of active cells in the hippocampus (Fig. 7). The DL group had a higher distribution of D_{1-2} , D_{2-3} , and D_{3-4} than simulated samples (two-sample Kolmogorov–Smirnov test, D_{1-2} : $P < 0.05$, D_{2-3} : $P < 0.0005$, D_{3-4} : $P < 0.05$; Mann–Whitney U-test, D_{1-2} : $P < 0.005$, D_{2-3} : $P < 5 \times 10^{-4}$, D_{3-4} : $P < 0.01$; Fig. 8A). There was no difference in the distributions of D_{4-5} , D_{5-6} , D_{6-7} , D_{7-8} , D_{8-9} , and D_{9-10} between real and simulated samples in the DL group. The SL group had a higher distribution of D_{1-2} , D_{2-3} , and D_{3-4} than simulated samples (two-sample Kolmogorov–Smirnov test, D_{1-2} : $P < 0.00005$, D_{2-3} : $P < 0.005$, D_{3-4} : $P < 0.05$; Mann–Whitney U-test, D_{1-2} : $P < 5 \times 10^{-4}$, D_{2-3} : $P < 0.001$, D_{3-4} : $P < 0.05$; Fig. 8B). There was no difference in the distributions of D_{4-5} , D_{5-6} , D_{6-7} , D_{7-8} , D_{8-9} , and D_{9-10} between real and simulated samples in the SL group. These results show that the SL and DL groups had clusters of nearby double IEG-expressing cells.

As for the normalized Ds (D_s'), the DL group had a higher distribution of D_{2-3}' and D_{5-6}' than the SL group (two-sample Kolmogorov–Smirnov test, D_{2-3}' : $P < 0.05$; and D_{5-6}' : $P < 0.05$; Mann–Whitney U-test, D_{2-3}' : $P < 0.01$; and D_{5-6}' : $P < 0.05$; Fig. 8C). Therefore, the SL and DL

groups of animals showed a differential distribution of subsets of active nearby CA1 pyramidal cell clusters.

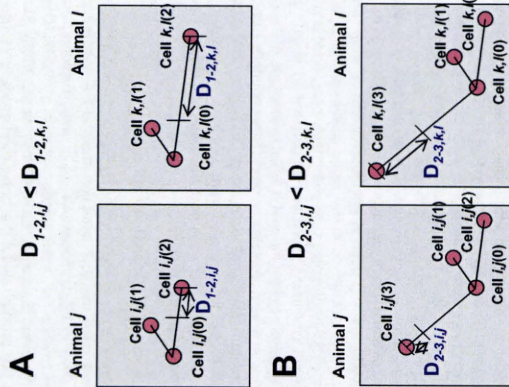


Fig. 7. Schematic drawing comparing cluster formation in two different animals (animals I vs. J). (A) Representative comparisons of D_{1-2} ($D_{1-2, IJ} < D_{1-2, k, I}$). In animal J, cell $l(1)$ is shown as the nearest neighboring cell, and cell $l(2)$ is shown as the second nearest neighboring cell from cell I, which is cell $l(10)$ as a starting point. As compared to animal J, animal I has a larger gap between cell $k(2)$ and cell $k(10)$. This can be interpreted as either forming a cluster of the two nearby cells in animal J or forming a cluster of the three nearby cells in animal I. Another example of comparisons of D_{2-3} ($D_{2-3, IJ} < D_{2-3, k, I}$) is shown. As compared to animal J, animal I has a larger gap between cell $k(3)$ and cell $k(10)$. This can be interpreted as either forming a cluster of the four nearby cells in animal J or forming a cluster of the five nearby cells in animal I. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

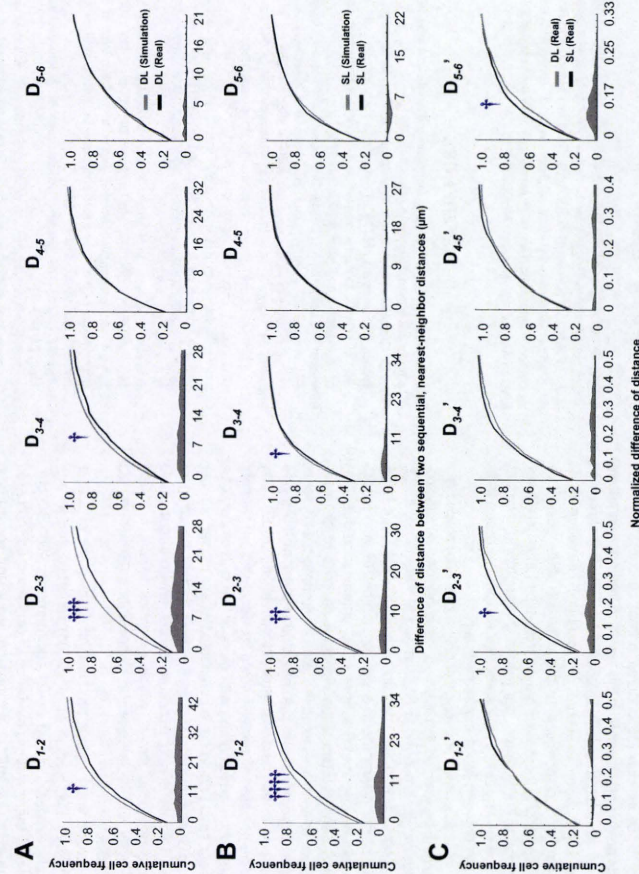


Fig. 8. Differences between subsequent-order nearest-neighbor distances. (A–B) Cumulative line-plots showing differences between subsequent-order nearest-neighbor distances (D_{1-2} , D_{2-3} , ..., and D_{9-10}) for real and simulated samples in the DL (A) and SL (B) groups, and normalized differences of distance between two sequential nearest-neighbors (D_{1-2}' , D_{2-3}' , ..., and D_{9-10}') of real samples between the DL and SL groups (C). Histograms also show the subtracted values of these differences between real and simulated samples (dark-gray area). † $P < 0.05$, †† $P < 0.005$, ††† $P < 0.00005$, significant difference between relevant pairs by Two-sample Kolmogorov–Smirnov Test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Nearest neighbor topographical arrangement

We further estimated a basic cluster arrangement of nearby cells which consisted of a triad (three cells) that is closely arranged neighboring cells. The triad size analysis showed that the SL group had a lower distribution of triads with closer located cells than the DL group (two-sample Kolmogorov–Smirnov test, $P < 0.05$; Mann–Whitney U-test, $P < 0.05$; Fig. 9). Thus, these results showed that the SL group had subsets of tighter clusters of active nearby cells than the DL group.

DISCUSSION

The main finding from the present study was the observation that in animals exposed to a restricted part of an environment (30 cm long by 10 cm wide), IEG-expressing cells in the dorsal hippocampus formed clusters of a few active cells, adjacent to non-IEG-expressing cells. Further, exposing animals to the same environment twice produced a larger number of double IEG-labeled (zf268 and *Homeo1a*) cells than exposing them to two different envi-

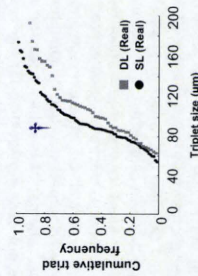


Fig. 9. Nearest neighbor topographical arrangement. (A) Cumulative dot-plots showing triad sizes of between the DL and SL groups. † $P < 0.05$, significant difference between relevant pairs by two-sample Kolmogorov–Smirnov Test. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

posed to the same place twice, there was a "tighter" cohesion of the clusters of active nearby cells, as revealed by the shorter distance to the nearest neighbor analysis. These results suggest a topographic organization of hippocampal pyramidal neurons to encode for space.

Topographic organization in the hippocampus

Our findings suggest that small clusters of active nearby hippocampal cells (~4) encode a specific place of an environment. Unlike the results with the functional IEG mapping method, recordings of hippocampal neurons have failed to provide conclusive evidence with regards to any type of topographical organization. It has been estimated that the majority (perhaps >80%) of pyramidal cells within the CA1 and CA3 hippocampal fields respond to an animal's position in space (for a review, see O'Keefe, 1999). However, as stated above, how these cells may be organized to represent a "spatial map" remains a mystery. A common observation from a number of multi-unit recording studies is that neighboring cells typically do not have adjacent place fields. Perhaps more intriguing is the fact that knowing the place fields in one environment, does not allow one to predict the relationship of place fields to cells in a different environment. Nonetheless, there is some evidence suggesting that hippocampal units are functionally organized (O'Keefe and Speakman, 1987; Eichenbaum et al., 1989; Deadwyler et al., 1996; Hampson et al., 1996, 1999, 2002). Anatomical evidence also supports cluster-type organization in the hippocampus. For example, CA3 neurons, which are upstream to CA1 neurons, have robust associational connections to neighboring CA3 neurons (Ishizuka et al., 1990; Li et al., 1994). Neighboring CA3 neurons were reported to project inputs to topographically organized nearby CA1 neurons at the septo-temporal axis (Brivanlou et al., 2004). This would indicate that neighbor-to-neighbor relations of pyramidal neurons are preserved in the CA3 and CA1 fields. Therefore, it is reasonable to suggest that in the CA1 field there is a high probability that neurons can be active in clusters following CA3 cell activation.

Hippocampal place cell characteristics

In animals restricted to a 10×30 cm² region of space, we observed approximately 2% of double IEG-expressing cells in the CA1, in response to the same place exposure twice. These IEG results are supported by single unit recording studies showing that 2–5% of all dorsal CA1 neurons, that are place cells, are active within a 20–25 cm region of space (Maurer et al., 2006; estimated from Wilson and McNaughton, 1993). Many of the firing characteristics of place cells have been described (Kubie and Muller, 1991; Best and White, 1999) and some of these characteristics pertinent to this discussion are as follows: (1) place fields form rather quickly but are stabilized with repeated exposure; (2) once stabilized, exposure to the same environment activates the same place cells; (3) place cells depend mainly on distal cues, although in the absence of prominent distal cues they can be affected by proximal cues; (4) place cells can have place fields in

multiple environments. Thus, space is represented by the activity of a network of cells, although, empirically, the likelihood of a cell having place fields in two sampled environments is low (9–16%) (Muller and Kubie, 1987). It is also relevant to mention that the remapping of place fields, which occurs when an animal is placed in different environments or the environments are intentionally changed, usually requires that the two environments are significantly different (Kubie and Muller, 1991; Willis et al., 2005; Leutgeb et al., 2005). Given these observations, it would be safe to speculate that exposing an animal to a restricted environment with limited cues would activate a subset of place cells. Also, exposing an animal to the same space twice would activate the same place cells twice. In the present study, the two locations were relatively different, but the general environment (e.g., room) was the same. Thus, clusters of active cells were also observed in the different place exposure but the cluster size was smaller and the nearest neighbor distances were farther than those observed in the same place exposure. While the majority of active cells may have been encoding the overall environment or other undetermined stimuli, it is reasonable to assume that a small percentage of the cells were activated specifically due to the animal's location. Therefore, the small percentage and tighter clusters of active cells we observed from repeated exposure to the same place could result from the place cell activity having more stable place fields.

Functional IEG mapping methods

A number of previous studies by Guzowski and associates have used the IEG detection method to map neuronal activity in the hippocampus, in animals exploring space (Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004; Chawla et al., 2005). Similar to the present study, the behavioral procedures used in these studies has been to let animals explore an environment for 5 min, return them to their home cage for 20 min, expose them to either the same or a different environment for 5 min and sacrifice them immediately. A number of differences, however, should be noted between their paradigm and ours: (1) A major difference was that they deliberately exposed animals to all parts of the environment (picking them up by hand and placing them to a different location every 15 s; see Vazdarjanova et al., 2002). In contrast, our objective was to minimize the extent of the environment that the animal was exposed to, in an attempt to reduce the space to be encoded and thus the number of active place cells; (2) Related to this, the size of the space and complexity of the sensory stimuli were different between the two studies: (10×30 cm² space with simple distal cues in the present study, and 61×61 cm² and complex proximal and distal cues in their studies); (3) Furthermore, while in their studies the two environments were completely different, in the present study animals were exposed to two different arms of an elevated maze which was located in the same room, with only the visual cues facing each of the arms being different; (4) A more subtle, but potentially significant, difference between the

paradigms was the fact that in their studies the animals were returned to the vivarium following exposure to the first environment, while the animals in the present study were returned to an isolation chamber in complete darkness; (5) in the present study animals were exposed to the environment previously—repeated exposure to an environment has been shown to stabilize place fields (Kubie and Muller, 1991; Kentros et al., 1998; Best and White, 1999); (6) Finally, in their studies IEG expression was determined using fluorescent ISH, with either *Arc* or *Arc/Homer1a* mRNAs, while in the present study we used grain- and DAB-labeled ISH with *zif268* and *Homer1a* mRNAs, respectively.

While the results from Guzowski and associates and the present study agree in certain aspects, they differ in others. The previous studies reported that following exploration in a novel environment, 40–45% of CA1 neurons were either *Arc* (intracellular or cytoplasmic) or *Homer1a* positive (Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004). In contrast, we observed that following exposure to an environment, 10% of CA1 cells were *zif268* positive. Vazdarjanova and Guzowski (2004) also reported that exposure to two different environments, produced a significantly higher number of double IEG labeled cells (15% increase from control), in comparison to exposure to the same environment which was twice as high (30% increase from control). Our findings are in agreement with this result, in that approximately twice as many double IEG-labeled cells were observed in the same place exposure group (4.06% increase from control), in comparison to the different place exposure group (2.22% increase from control), albeit at much lower numbers. Furthermore, our results show tighter clusters of active nearby cells in the same place exposure group than in the different place exposure group.

Within a small environment only a subset of place cells would be expected to be active, as confirmed by electrophysiological recordings (Wilson and McNaughton, 1993; Maurer et al., 2006). Thus, in an animal placed in a large part of the environment, a greater number of active cells should be engaged (Pavides et al., unpublished observations). Sensory stimulation would cause cells to be more active. In an animal exposed to an environment with richer cues, a greater expression of *zif268* and *Arc* mRNAs was induced in the hippocampus (Pinard et al., 2002). The active handling of animals would provide sufficient sensory stimulation, which along with more active exploration of a larger space could have contributed to the higher number of IEG-expressing cells. Furthermore, active handling of the animals during exposure to the environment could have produced stress, which increases levels of *zif268* mRNAs and proteins in the hippocampus (Revest et al., 2005).

In contrast to our observation of clusters of active nearby cells, Redish et al. (2001) reported that in animals exposed to two novel environments (60×60 cm² boxes) there was no evidence of clustering as indicated either by *Arc* mRNA expression or by unit recordings. What are possible reasons for the discrepancies? The

most plausible explanation is that in the previous study, free exploration of the environments by the animals activated 40–45% of the CA1 pyramidal cells, thus obscuring the observation of any cluster-type functional organization. Restricting animals to a small part of the environment allowed for a small number of cells to be activated and revealed the formation of small clusters. The spacing between cells within a cluster may still be sufficiently far apart for cells to be recorded simultaneously from a single electrode. Another problem is that tetrodes produce relatively large damage and deformation of the brain, especially when multiple tetrodes are used. Thus, whether cells within a cluster are being sampled is not certain. Assuming for the moment that the functional organization of the hippocampus to encode for space is that of clusters, a question that still remains is the arrangement of the "spatial map." The present findings reveal a functional unit of cell ensembles in the hippocampus, however, it remains unclear whether each cluster of cells encodes for each spatial component or multiple spatial components. Knowing the anatomical configuration of the clusters, is only the first step in deciphering the "spatial map" which could only be achieved by determining the interactions of unit discharges within the clusters. More refined unit recordings from the clusters would be necessary to decode the foundation of the "cognitive map."

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2.5.3. Resident–intruder test

After the light/dark choice test, the animals were returned to the home cage for a 60 min rest. Then, a castrated male was introduced into the cage and left there for 10 min. The light intensity was about 5 lux. The rats were then removed from the room, and were videotaped for subsequent analysis. The following behaviours were determined from the videotapes with the help of the Observer XT software (Noldus, Wageningen, the Netherlands): Chasing; tail writhe (simultaneous movement of the tail or part of the tail, with or without banging on the floor); piloerection, nose-off (the subject and the intruder stand immobile facing each other); open mouth display (open mouth displaying the incisors); kicking; sniffing (one rat approaches the other sideways); belly groom; pushing; kicking; boxing; biting; wrestling; exploration (horizontal and vertical activity); digging; self-grooming.

2.6. Immunocytochemistry

The subjects were euthanized with carbon dioxide the day following the behavioural tests and immediately perfused with PBS followed by 4% paraformaldehyde (PFA). The brain was removed and postfixed overnight at 4 °C in 4% PFA. It was then rinsed with PBS and cryoprotected in PB containing 30% sucrose for 24 h at 4 °C. Then, the brains were frozen in isopentane cooled on dry ice, and transferred to a –80 °C freezer.

Brains were frozen-sectioned at 50 µm and the VMN or the MePDA sections were collected as appropriate. Sections were treated by antibodies against the ERα (polyclonal, 1:25,000; Upstate, Lake Placid, NY) and EGFP (GFP, Abcam, Cambridge, MA) in combination with secondary antibodies (biotinylated rabbit) and avidin–biotin peroxidase complex (ABC elite kit from Jackson ImmunoResearch, West Grove, PA) to identify cells containing ERα and injection localization, respectively. After antibody reactions and several washings in PBS, sections were stained with diaminobenzidine (DAB). DAB revealed injection localization by brown coloration of EGFP while the ERα was stained in purple by the addition of nickel.

The number of ERα stained cells in the VMN and the MePDA was counted. Slices with anteroposterior coordinates from –3.10 to –3.20 for the MePDA and those according to atlas coordinates [60]. Only those with the greatest ERα staining have been chosen. Photographs of each nucleus were taken by a combination of microscope (Nikon Eclipse E400) and camera (Nikon Coolpix 990). Then, pictures were transferred to a computer and opened by Photoshop software. Based on atlas coordinates with the skull level [60] a virtual pen was used to delineate the VMN and the MePDA. The number of stained cells was counted and divided by the surface of each nucleus in order to obtain a density (number of ERα/mm²).

2.7. Data analysis

Only rats with bilateral brown coloration of EGFP in the MePDA or the VMN were included in the analysis. This coloration indicated successful bilateral virus infusions. For the social recognition test, rats killing the juvenile were excluded from analysis. This applied to 1 animal in the ERα shRNA VMN and to 2 animals in the AAV control VMN group.

A *t* test was used to evaluate the number of ERα immunoreactive cells in the groups treated with ERα shRNA and AAV control in the MePDA or the VMN. Behavioural data from the two groups infused with ERα shRNA and AAV control into the MePDA were analyzed separately from the groups infused with ERα shRNA and AAV control into the VMN.

Social recognition data were analyzed with two-factor ANOVA for repeated measures on one factor. The between-groups factor was treatment (ERα shRNA and AAV control) while the within-groups factor was exposure (1–5). In case of nonhomogeneous error variances, as determined by Levene's test, data were subjected to a logarithmic transformation. After a significant interaction, tests for simple effects within treatments as well as within exposures were performed. Following significant ANOVA, Tukey's HSD test was employed for determining differences between exposures. Data from the light/dark choice test and the resident–intruder tests were analyzed with *t* tests.

A one-tailed significance level of .05 was used to compare the first exposure to the other exposures. A two-tailed significance level of .05 was used for all other tests. The use of a one-tailed significance level was justified by the fact that exploration of the juvenile always reduced upon repeated exposure in intact rats. A two-tailed significance level of .05 was used for all other tests.

3. Results

3.1. Histology

Four rats in the group infused with ERα shRNA into the VMN had infusion sites outside of the intended nucleus. Consequently, there remained eight females in this group.

The number of ERα in the MePDA was significantly reduced in the animals infused with ERα shRNA compared to those infused

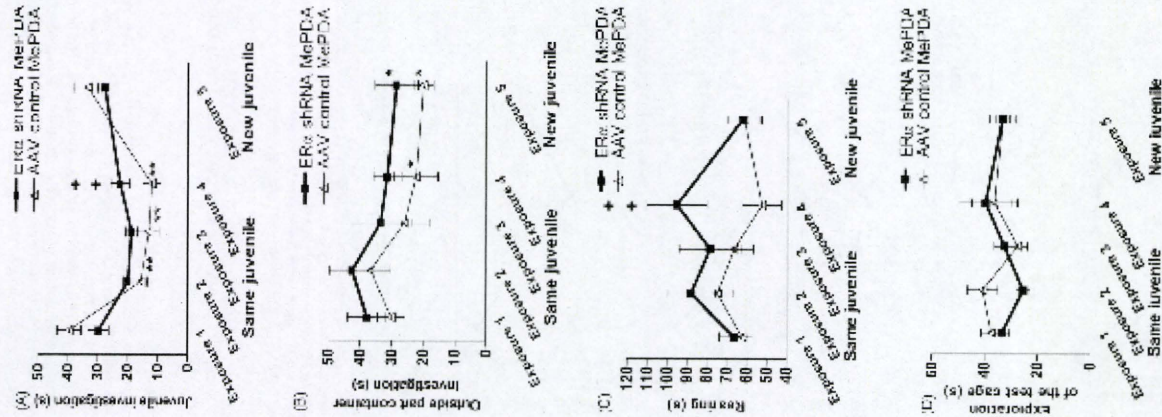


Fig. 2. Mean ± SEM of parameters of behaviors in the social recognition test. (A) Time (in s) spent in juvenile investigation; (B) time (in s) spent in outside part of the container (with juvenile inside); (C) time (in s) spent in rearing and exploration of the test cage in female rats with a reduced number of ERα in the MePDA and the corresponding controls. For further details, see legend.

3.2. Behavioral effects of ERα knockdown

3.2.1. Social recognition

There was no effect of exposure or treatment and no interaction between exposure and treatment with regard to locomotion (all *p*s > .05). Hence, this parameter is not further mentioned.

As illustrated in Fig. 2A, social recognition was affected in the subjects with reduced ERα expression in the MePDA. There was a significant main effect of exposure on juvenile investigation, $F(4,96) = 19.99$, $p < .001$, and a significant interaction treatment × exposure, $F(8,96) = 3.95$, $p < .01$. There was no main effect of treatment, $F(1,24) = .61$, *ns*. Tests of simple effects of exposure within treatment showed that the females infused with ERα shRNA into the MePDA spent about the same time investigating the juvenile at each exposure, $F(4,96) = 2.90$, *ns*, even at the 5th exposure, where the familiar juvenile was replaced by a new one. In contrast, animals infused with AAV control into the MePDA spent less time investigating the juvenile during the second, third and fourth presentation compared to the first, $F(4,96) = 21.04$, $p < .001$. When a new juvenile was introduced at the fifth exposure, the time the females spent investigating the juvenile was similar to that spent at the first presentation. Thus, the control group demonstrated no impairment of social recognition. The analysis of the effect of treatment within each exposure showed that the group infused with ERα shRNA into the MePDA investigated the familiar juvenile longer than the group infused with AAV control into the MePDA at the 4th exposure. No group differences were found at the other exposures.

There was a significant effect of exposure on the time spent investigating the container, $F(4,96) = 9.33$, $p < .001$ but there was no interaction treatment × exposure, $F(8,96) = .08$, *ns*, and no main effect of treatment, $F(1,24) = 1.23$, *ns*. Thus, the reduction in investigation of an inanimate object associated with repeated exposures was not affected in the subjects with ERα knockdown in the MePDA. Data are illustrated in Fig. 2B.

Regarding rearing there was a significant interaction treatment × exposure, $F(8,96) = 2.53$, $p < .05$ but no main effect of exposure, $F(4,96) = 2.28$, *ns*, or treatment, $F(1,24) = 1.58$, *ns*. Tests of simple effects of treatment within exposure showed that the subjects infused with ERα shRNA into the MePDA spent more time rearing than the animals infused with AAV control at Exposure 4, $F(1,24) = 5.65$, $p < .05$. Data are summarized in Fig. 2C.

Concerning exploration of the test cage, no significant effect was found for exposure, $F(4,96) = .80$, *ns*, or treatment, $F(1,24) = .35$, *ns*. The interaction treatment × exposure was also nonsignificant, $F(8,96) = 1.13$, *ns*. Data are shown in Fig. 2D.

Social recognition was not affected in the subjects infused with ERα shRNA into the VMN. There was a significant effect of exposure on juvenile investigation, $F(4,68) = 20.02$, $p < .001$ while there was no interaction treatment × exposure, $F(8,68) = .91$, *ns*, and no effect of treatment, $F(1,17) = 1.65$, *ns*. Data are summarized in Fig. 3A.

There was a significant effect of exposure on the time spent investigating the outside of the container, $F(4,68) = 3.17$, $p < .001$ but there was no interaction treatment × exposure, $F(8,68) = .31$, *ns*, and no effect of treatment, $F(1,17) = .97$, *ns*. Data are illustrated in Fig. 3B.

Concerning rearing, there was no significant interaction treatment × exposure, $F(8,68) = .42$, *ns*, no main effect of exposure, $F(4,68) = 1.90$, *ns*, or of treatment, $F(1,17) = .97$, *ns*. Data are summarized in Fig. 3C.

Fig. 1. * represent a significant decrease between each session as compared to the first session, $p < .05$; **, $p < .01$. Lines indicate significant differences between groups; **, $p < .01$.

There was no main effect of exposure on exploration of the test cage, $F(4, 68) = 1.9$, ns, and there was no effect of treatment, $F(1, 17) = 97$, ns, and no interaction treatment $\times$ exposure, $F(8, 68) = 42$, ns. Data are shown in Fig. 3D.

3.2.1.1. Aggression, self-grooming and freezing during social recognition test. In the groups infused with ER α shRNA and AAV control into the MePDA, there was no effect of exposure or treatment and no interaction between exposure and treatment with regard to aggression, self-grooming and freezing (all p 's > .10; data not shown). Interestingly, the frequency of aggressive behaviors in these groups was close to 0. Hence, this parameter is not further mentioned in the MePDA groups.

Like the groups treated in the MePDA, subjects infused with the AAV control in the VMN displayed almost no aggressive behaviors. However, the group infused with ER α shRNA into the VMN displayed an increased higher frequency of these behaviors. ANOVA comparing this group to the one infused with AAV control into the VMN showed that there was a significant main effect of exposure, $F(4, 68) = 6.10$, $p < .001$, and treatment, $F(1, 17) = 7.50$, $p < .05$ as well as a significant interaction treatment $\times$ exposure, $F(8, 68) = 2.40$, $p < .05$. Tests for simple effects of treatment within each exposure showed that aggression was enhanced at the first two presentations of the juvenile (Exposures 1 and 2) $F(1, 17) = 4.53$, $p < .05$, and $F(1, 17) = 4.20$, $p < .05$, respectively, and at presentation of the new juvenile (Exposure 5), $F(1, 17) = 7.87$, $p < .05$. Data are illustrated in Fig. 4A.

Concerning grooming, there was a significant main effect of exposure, $F(4, 68) = 3.39$, $p < .01$, and a significant interaction treatment $\times$ exposure, $F(8, 68) = 3.75$, $p < .01$. There was no main effect of treatment, $F(1, 17) = 2.14$, ns. Tests of simple effect of exposure within treatment revealed that the subjects infused with ER α shRNA into the VMN spent more time grooming at the last two presentations of the familiar juvenile (Exposures 3 and 4) than they did at the first presentation (Exposure 1), $F(4, 68) = 5.83$, $p < .001$. Moreover, Tukey's HSD test demonstrated that the ER α shRNA VMN group spent more time grooming at the third and the fourth presentation of the juvenile (Exposures 3 and 4) than its control group, $F(1, 17) = 4.52$, $p < .05$, and $F(1, 17) = 5.12$, $p < .05$, respectively. Thus, grooming showed a pattern that was the inverse of pattern that which was observed with regard to aggression in the ER α shRNA VMN group. Data are summarized in Fig. 4B.

Regarding freezing, there was a significant main effect of exposure, $F(4, 68) = 3.39$, $p < .05$, and a significant interaction treatment $\times$ exposure, $F(8, 68) = 3.50$, $p < .01$. There was no main effect of treatment, $F(1, 17) = 1.86$, ns. Tests of simple effect of exposure within treatment revealed that the subjects infused with ER α shRNA into the VMN spent more time freezing at the last two presentations of the familiar juvenile (Exposures 3 and 4), and at presentation of the new juvenile (Exposure 5) than they did at the first presentation (Exposure 1), $F(4, 68) = 10.59$, $p < .01$. Likewise, Tukey's HSD test demonstrated that freezing was enhanced at the last presentation of the familiar juvenile (Exposure 4) in the subjects infused with ER α shRNA into the VMN compared to those infused with AAV control into the VMN, $F(1, 17) = 7.16$, $p < .01$. Data are shown in Fig. 4C.

3.2.2. Light/dark choice test

The subjects with reduced number of ER α in the MePDA showed an increase in the time spent in as well as the number of visits to the light chamber, $t(24) = 2.49$, $p < .05$, and $t(24) = 2.95$, $p < .01$, respectively. Data are summarized in Fig. 5A and B. Likewise, this group moved a longer distance than the control, $t(24) = 2.60$, $p < .05$. Data are illustrated in Fig. 5C.

The time spent in the light chamber or the ambulatory activity was not affected in the group infused with ER α shRNA into the VMN

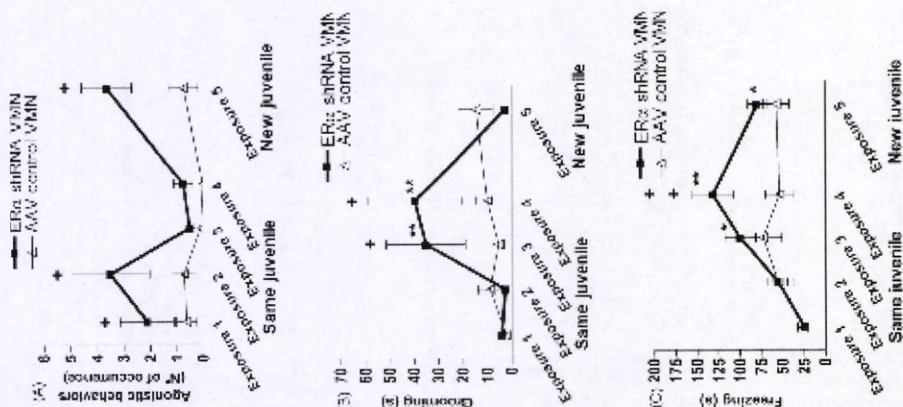


Fig. 4. Mean $\pm$ SEM of parameters of behaviors in the social recognition test. (A) number of agonistic behaviors; (B) time (in s) spent in grooming; (C) time (in s) spent in freezing in female rats with a reduced number of ER α in the VMN and the corresponding controls. For further details, see legend to Fig. 1. * represent a significant decrease between each session as compared to the first session, $p < .05$; ** $p < .01$. Lines indicate significant differences between groups.

(all comparisons between the VMN groups had p 's > .90). Data are shown in Fig. 5A–C.

3.2.3. Resident-intruder test

Aggressive behaviors were not affected in the group infused with ER α shRNA either into the VMN or into the MePDA. Indeed, the t test revealed that the number of aggressive behaviors in the subjects infused with ER α shRNA into the VMN was not significantly different from the number of aggressive behaviors displayed by the subjects infused with AAV control into the VMN, $t(17) = .487$, ns. Likewise, the t test showed that the number of aggressive behav-

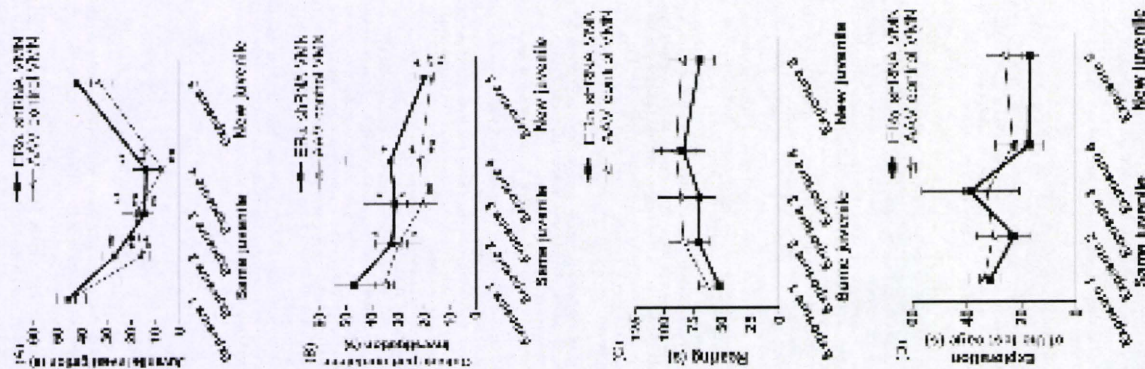


Fig. 3. Mean $\pm$ SEM of parameters of behaviors in the social recognition test. (A) time (in s) spent in juvenile investigation; (B) time (in s) spent in outside part of the container (with juvenile inside) investigation; (C) time (in s) spent in inside part of the container (with juvenile inside) investigation; (D) time (in s) spent in exploration of the test cage in female rats with a reduced number of ER α in the VMN and the corresponding controls. For further details, see legend to Fig. 1. * represent a significant decrease between each session as compared to the first session, $p < .05$; ** $p < .01$. Lines indicate significant differences between groups.

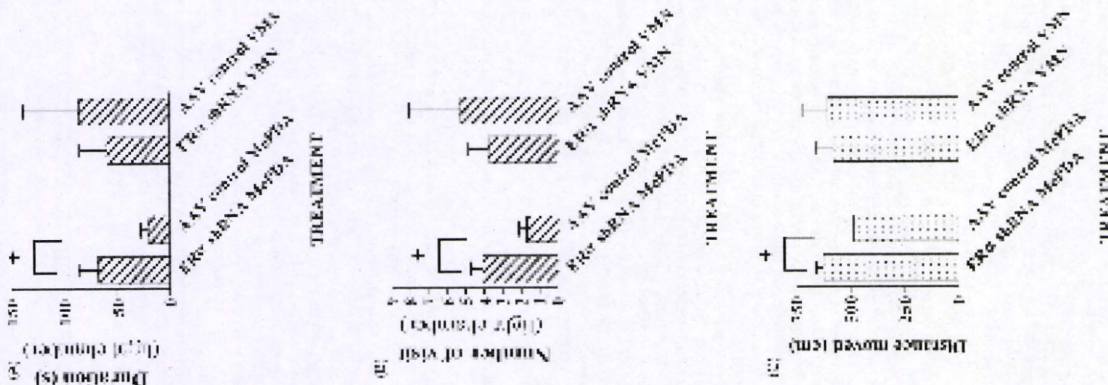


Fig. 5. (A) time (in s) spent; (B) number of visit in the light chamber and (C) distance moved (in cm) in the light and dark chambers by female rats with a reduced number of ER α in the MePDA or the VMN and the corresponding controls. For further details, see legend to Fig. 1. Data are mean $\pm$ SEM. Lines indicate significant differences between groups; * $p < .05$.

Table 1
Mean $\pm$ SEM aggressive behaviors in the resident-intruder test in female rats with a reduced number of ER α in the MePDa or the VMN and the corresponding controls.

Groups	Aggressive behaviors
ER α shRNA VMN	2.7 $\pm$ 1.2
AAV control VMN	4.5 $\pm$ 2.9
ER α shRNA MePDa	2.4 $\pm$ 1.4
AAV control MePDa	3.5 $\pm$ 1.7

iors in the group infused with ER α shRNA into the MePDa was similar to the group infused with AAV control into this structure, $t(24) = 472$, ns. No significant difference was found when the number of kickings and bitings was analyzed separately (all $p > 21$). Moreover, no difference was found in other social and exploratory activities, or anxiety-related behaviors. Data are shown in Table 1.

4. Discussion

4.1. Efficiency and specificity of ER α knockdown

The shRNA used in this experiment produced a substantial reduction of ER α expression in the VMN and the MePDa. At the same time the number of receptors was unaffected in structures adjacent to the target sites, thereby demonstrating the efficiency and specificity of the shRNA. The shRNA employed here does not modify ER β expression [55], making it reasonable to propose that the reduction of ER α expression in the VMN and the MePDa accounts for all behavioral effects observed in the present experiments. Nevertheless, it is not possible to exclude that the remaining ER α had some effect. The effects reported here might be amplified by a total suppression of this receptor.

4.2. Effects of ER α knockdown on social recognition

A specific reduction of the ER α in the MePDa suppressed social recognition in female rats. Indeed, such females showed no reduced response to repeated presentation of a conspecific juvenile and no increased response when the familiar juvenile was replaced with a new one. In contrast, the control group demonstrated habituation and dishabituation of juvenile exploration. Moreover, females with few ER α in the MePDa showed a reduced exploration of the exterior of the container upon repeated exposure. In fact, they were not different from the control group. This suggests that the deficient habituation to the juvenile was not due to a generalized lack of object recognition, but to a specific failure to habituate to a social object. It is worth noting that the decreased exploration of the non-social object was of less magnitude than the decrease of investigation of the juvenile. This observation could be explained by the fact that the direct social interaction between the female and the juvenile has a baseline level far above that of the exterior part of the container.

In order to display the habituation/dishabituation response underlying the paradigm employed here the experimental subject needs to recognize the juvenile used at the repeated exposures, and distinguish the new juvenile from the previous one. Present data show that the ER α within the MePDa is necessary for this. To the contrary, the ER α in the VMN does not appear to contribute to social recognition. Present data also reinforce the notion that recognition of social and non-social objects depend on different processes as suggested earlier [11].

Knockdown of ER α in the MePDa did not modify horizontal exploration. Hence, the absence of habituation to the juvenile in these females cannot be explained by a general alteration of exploratory activities. Furthermore, it is doubtful whether exploratory activities interfere with juvenile exploration, since

females with few ER α in the MePDa displayed more vertical activity than controls at the last presentation of the familiar juvenile, yet they spent more time exploring it than controls.

Social recognition in rodents has been shown to be based on olfaction [17,66], although auditory and visual signals may contribute under some circumstances [62]. The main and accessory olfactory systems project to the anterior and posterior cortical nuclei of the amygdala, respectively. The accessory olfactory system also projects to the anteromedial nuclei of the amygdala [68]. The MePDa is then reached by intrinsic connections [12,41]. Indeed, activation of the olfactory bulbs as well as the medial and cortical amygdala was demonstrated in female rats after an encounter with a conspecific pup [22] and in mice exposed to a juvenile [65].

It is conceivable that the ER α in the MePDa somehow modulates olfactory input. However, studies in aromatase knockout mice have shown an intact fos response to sexually relevant olfactory cues [8]. Likewise, adult ovariectomized mice show the same fos response to male odors regardless of whether they are treated with estradiol or not [27]. These data suggest that estrogens are not important for olfactory processing. Alternatively, activity at the ER α receptor may facilitate memory. Social recognition does not only depend on the capacity to distinguish one individual from another, but also on the capacity to remember the individual characteristics from one exposure to the next. While long-term treatments with estradiol have been repeatedly reported to improve some kinds of memory [16,44], there were no changes in performance on a memory task during the estrous cycle [10] suggesting that short-term variations in estrogen availability are not likely to have functionally relevant effects on memory. Although there is one study reporting short-latency effects of estradiol on memory, the involvement of ER α is doubtful since 17 α - and 17 β -estradiol were equally effective [43]. The intact habituation/dishabituation response to the cylinder observed even in the shRNA group reinforces the idea that ER α effects on memory are not involved. It is, indeed, possible that the effect of ER α silencing in the MePDa is specific for the recognition of a social stimulus.

4.3. Effects of ER α knockdown in the VMN on aggression toward the juvenile and anxiety-related behaviors

From the MePDa, the olfactory information reaches the preoptic area and the VMN mainly through the bed nucleus of the stria terminalis [12]. ER α knockdown in the VMN enhanced aggression towards a conspecific juvenile at the first two exposures. The ER α shRNA females showed also enhanced aggression when the novel juvenile was introduced. Since females with ER α knockdown in the VMN showed normal social recognition, we can reasonably infer that the juvenile became familiar after several exposures and did not, therefore, elicit aggression from the female. At exposures 3 and 4 the ER α shRNA females showed a high level of grooming and freezing. Since enhanced grooming and freezing is thought to be a response to anxiogenic stimuli [72], this observation may suggest that the juvenile had lost its capacity to evoke aggressive responses and instead produced anxiety.

4.4. Effects of ER α knockdown in the light/dark choice test

The silencing of ER α in the MePDa reduced anxiety. The females with few ER α in the MePDa spent more time in the light chamber and entered it more often than control females. They also presented an augmentation of distance moved in the entire device (light and dark chambers) compared to controls. Thus, females with intact ER α in the MePDa showed more anxiety-like behavior than females with few ER α . This fact suggests that stimulation of the ER α in the MePDa has an anxiogenic effect. This could explain why females with ER α knockdown in the MePDa showed more vertical activity

ity than controls at the last presentation of the familiar juvenile during the social recognition test. Although rearing is not strictly determined by anxiety, it is more frequent when the level of anxiety is low [25,40]. The absence of a group difference with regard to horizontal activity may be explained by the fact that this kind of activity is less sensitive to variations in anxiety.

The activity of estrogens-treated female rats has been shown to increase in safe environments [9,76] while it is reduced in threatening contexts [18]. It has been proposed that these opposing behavioral effects are consequences of estrogens' role in increasing arousal [61]. Heightened arousal increases locomotor activity in safe environments while it produces an anxiety response in threatening environments. Present results reinforce this idea because females with reduced anxiety also showed enhanced locomotor activity. The mechanisms by which estrogens act on anxiety in the MePDa remain unknown.

4.5. The possible role of progesterone receptors

It could be argued that some or all of the effects reported here are not directly dependent on the ER α but on a reduced availability of progesterone receptors in animals with ER α knockdown. Consequently, what has actually been observed here are the consequences of deficient progesterone receptor activation in the VMN or the MePDa. In the case of anxiety-like behaviors, this is unlikely, though, since estradiol, progesterone and estradiol + progesterone have similar effects in several tests of anxiety (e.g. [42]). This suggests that progesterone actions on non-estrogen inducible progesterone receptors are important, and these receptors should be unaffected in the animals used in the present experiment. With regard to aggression it is difficult to attribute a specific function to progesterone in view of the fact that both reduction and increase have been reported after progesterone treatment [4,5]. The role of progesterone in social recognition is essentially unknown. Nevertheless, it seems that alterations in progesterone receptors cannot be excluded as the ultimate explanation for some of the effects observed here. Since the reduction of progesterone receptor availability would be limited to estrogen inducible receptors, therefore a direct result of ER α knockdown, it is not unwarranted to maintain that the ER α also is involved in these behaviors. Present data do not allow for any evaluation of the specific role of progesterone receptors, but this was not the purpose of these experiments.

4.6. The possible role of ER β

There are many examples of interactions between the ER α and the ER β , both cooperative and opposing (e.g. [24,79]). It could be argued that some of the effects observed after reduction of the ER α are due to estrogen action at the ER β . The ER β -mediated effects may be obscured by an opposing action at the ER α in the intact animal. Although theoretically possible, opposing estrogen actions on the ERs would make little physiological sense. In the intact animal, both receptors would be activated simultaneously and the net effect would be nil. Even though some experimental manipulations may reveal opposing actions, their importance should not be exaggerated. In the particular case of social interaction, there is actually evidence against opposing actions of the ERs [13,15,70].

4.7. The indirect role of ER α in the MePDa and the VMN for reproduction

The ER α in the VMN has been reported to be necessary for the display of receptivity and proceptivity as well as for sexual incentive motivation while knockdown of this receptor in the MePDa does not affect any of these behaviors [71]. However, the ER α in the MePDa is necessary for social recognition,

It is well known that sexually receptive female rats are capable to recognize their sexual partners [21], and this recognition may have consequences for their sexual behavior. For example, a sexually exhausted female will reinstate copulation with a novel male [1]. More subtle effects of mate recognition are likely to exist and to be important for reproductive efficiency. We suggest that estrogens assure appropriate sexual behavior through actions on the ER α within the VMN while this receptor in the MePDa participates in other functions, indirectly important for reproduction, like social, including mate, recognition.

In addition to the assurance of appropriate sexual behavior, the ER α in the VMN modulates the response to a threatening situation and some kinds of aggression. It is noteworthy that no effect of ER α knockdown was found in the resident-intruder test. The intruder, a castrated male, should not provoke a reaction much different from that produced by the juvenile. However, while the castrated male was introduced into the female's home cage, a safe environment, the juvenile was introduced into an observation cage where the female had been placed shortly before the test, in an unknown room. It is most likely that this is a threatening situation. Another interesting finding is that the silencing of ER α in the VMN had no effect in the light/dark choice test. Thus, the anxious reaction to a threatening environment seems to be limited to a social context. This could be another manifestation of the different effects of estrogens in different contexts. It appears to be most adaptive to display fear and aggression rather than sexuality in threatening contexts, but to be active and sociable in safe contexts. Present results together with earlier observations suggest that the combined actions of the ER α in the VMN and MePDa assure that this will be the case.

In conclusion, present data do not allow for any definite conclusion as to the exact mechanisms by which ER α knockdown modifies social recognition, aggression and anxiety. Nevertheless, it is hoped that present results contribute to an understanding of the quite intricate effects of estrogens, particularly within the VMN, underlying the behavioral modifications necessary for successful reproductive behavior in the female rat.

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Estrogen Receptor- α in the Bed Nucleus of the Stria Terminalis Regulates Social Affiliation in Male Prairie Voles (*Microtus ochrogaster*)

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Abstract

Estrogen receptor alpha (ER α) typically masculinizes male behavior, while low levels of ER α in the medial amygdala (MeA) and the bed nucleus of the stria terminalis (BST) are associated with high levels of male prosocial behavior. In the males of the highly social prairie vole (*Microtus ochrogaster*), increasing ER α in the MeA inhibited the expression of spontaneous alloparental behavior and produced a preference for novel females. To test for the effects of increased ER α in the BST, a viral vector was used to enhance ER α expression in the BST of adult male prairie voles. Following treatment, adult males were tested for alloparental behavior with 1–3-day-old pups and for heterosexual social preference and affiliation. Treatment did not affect alloparental behavior with 73% of ER α -BST males and for heterosexual social preference and affiliation. Increasing ER α in the BST affected heterosexual affiliation, with ER α -BST males spending significantly less time in side-by-side contact with females relative to time spent with control males. ER α -BST males did not show a preference for either the familiar or novel female. These findings differed significantly from those reported in ER α -MeA enhanced males, where ER α inhibited alloparental behavior and produced a preference for a novel female. The findings from this study suggest two things: first, that increased ER α in the BST decreases social affiliation and second, that altering ER α in different regions of the social neural circuit differentially impacts the expression of social behavior.

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Introduction

Estrogen acting via estrogen receptor alpha (ER α) masculinizes male behavior [1–4], which is typically associated with low levels of prosocial (positive affiliative) behavior and high levels of aggression. In male rats, treatment with a selective ER α agonist increased male aggression and anxiety [5] and masculinized serotonergic (5-HT) projections in female rats [6]. Conversely, data suggest that low levels of ER α are associated with high levels of male prosocial behavior. Highly social males, such as prairie voles (*Microtus ochrogaster*), pine voles (*M. pinetum*), and Djungarian hamsters (*Phodopus campbelli*), display low levels of ER α in two regions of the brain that play a critical role in the expression of social and sociosexual behavior, the bed nucleus of the stria terminalis (BST) and medial amygdala (MeA) [7–9]. In male prairie voles, increased ER α is associated with decreased prosocial behavior. Neonatal castration inhibits the expression of prosocial behavior in male prairie voles [10,11], and significantly increases ER α expression in the BST and MeA [12]. Enhancing ER α in the MeA of male prairie voles decreased spontaneous alloparental behavior and resulted in a preference for a novel female over a familiar female [13]. These findings are significant for a couple of reasons. First, spontaneous

have a similar function in both highly social and less social species. Many of the studies that have examined the role of the BST in regulating social behavior have either found responses in both regions, which is not unexpected given the intimate relationships and interconnection, or have examined the response in only one region. One of the difficulties of interpreting the effects on behavior through the manipulation of a single region is that it is often unclear whether the change is due to the direct effect of the region or a response of the neural circuit to the manipulation. Therefore, one of the goals of this study is to use the same manipulation in a different region within the same neural circuit to determine if the effects are the same or if they vary by region.

Given that the BST and MeA have efferent and afferent neural connections and are part of the social neural network [15], ER α expression is low in both areas in male prairie voles [8], and ER α expression in the MeA decreased social behavior [13], we sought to determine the role of ER α in the BST. It also has been suggested that studying the role of ER α in the BST in regulating male social behavior is an essential next step [26]. Therefore the objectives of this study were to test the prediction that increasing ER α in the BST would reduce the expression of male prosocial behavior and to determine if the enhancing ER α in the BST had the same or different effects on male prosocial behavior as it did in the MeA. To accomplish this, a viral vector was used to enhance ER α expression in the BST of adult male prairie voles. This is a powerful technique for teasing apart the roles of specific brain regions as viral vectors can be delivered to specific sites using stereotaxic injection and they have limited spread, compared to the chronic and wide spread effect in knock out models. We replicated the design of Cushing et al. [13]. Treated males, along with the appropriate controls, were then tested for the expression of spontaneous alloparental behavior and heterosexual social preference and affiliation.

Methods

Subjects

Animals used in this study were laboratory-reared prairie voles that originated from wild stock trapped near Urbana, Illinois. Animals were housed under a 14:10 light/dark cycle and provided Purina high fiber rabbit chow (cat # 5326) and water *ad libitum*. Pairs were weaned at 21 days of age and housed in same-sex pairs until treatment in polycarbonate cages (28.3 cm $\times$ 17.5 cm $\times$ 12.5 cm) with wood shavings for bedding. At the time of testing all subjects were sexually naive adults, 60–90 days of age. Animals were maintained in accordance with USDA and NIH guidelines and all procedures were approved by the University of Memphis Institutional Animal Care and Use Committee prior to conducting any study.

Viral Vector

Adeno-associated viral (AAV) vectors were used to enhance the expression of ER α in the BST. The following is a brief description of the vector for complete details see Musatov et al. [27]. The vectors express shRNA containing human ER α (AAV-ER α) or luciferase (AAV-luciferase) target sequences. Vectors also express enhanced green fluorescent protein (GFP) as a reporter that allows for visual detection of transduced neurons. These vectors have been previously demonstrated to permit effective transfection in prairie voles [13].

ER α Adenoviral Vector Transfection

To achieve site-specific over-expression of ER α in the BST, adult males (60–70 days of age) were stereotactically injected,

bilaterally, with AAV-ER α . A site-specific control was generated by transfecting the BST of males with a vector encoding firefly luciferase cDNA, and an ER α control was generated by transfecting the caudate putamen with ER α , as the caudate does not express ER α . At approximately 60 days of age, a stereotaxic apparatus was used for site-specific injections of the AAV vector into the brain of experimental males. Males were randomly assigned to one of three treatment groups: 1) AAV-ER α into the BST ($n=17$), 2) AAV-ER α into the caudate putamen (ER α transfection control) ($n=9$), or 3) AAV-luciferase into the BST (injection control) ($n=17$). Prior to the procedure, animals were deeply anesthetized with a combination of Ketamine (67.7 mg/kg) and Xylazine (13.3 mg/kg). The viral vector (1 μ l) was infused over a 5-min period with a micropump injector, and the infusion needle was left in place for an additional 3 min. The injection coordinates were determined to be AP -0.17 mm, ML ± 1.75 mm, 4.6 ventral from Bregma and, for the caudate, AP -1.3 mm anterior, ML ± 2.0 mm lateral, and 4.0 mm ventral. These coordinates specifically target the medial division of the BST. Animals were given 2–3 wks to recover and allow for expression of the vector prior to behavioral testing. Based upon a pilot study with prairie voles and other published results this amount of time is sufficient for the expression of transacted ER α and expression lasts at least 13 weeks [27,28]. Following completion of testing, animals were euthanized and their brains collected to verify the accuracy of the injection and successful transfection. Only animals with bilateral expression of transacted ER α were included in the analysis.

Verification of Transfection

Upon completion of the social preference test, brains from experimental animals were fixed using immersion fixation, sectioned at 30 μ m on a freezing-sliding microtome, and then the free-floating sections were stained for ER α using standard AB immunocytochemistry (for complete details see [8]). The human-specific primary antibody RM9101-s (Ncomarkers, Fremont CA; 1:1000 dilution) was used to label transacted ER α and then visualized using DAB. RM9101-s does not label prairie vole ER α . Therefore, all ER α observed with this antibody were the product of transfection. Successful transfection was determined qualitatively by visually examining ER α expression using a Nikon E-500 microscope. It should be noted that there were no animals that displayed only a few transacted-ER α immunoreactive cells; transacted males either displayed no or a significant amount of transacted-ER α cells. Fig 1 shows a typical level of transacted ER α expression. Control BST transfection was determined by staining for GFP expression, as described in [13].

Alloparental Behavior Test

Subjects were removed from the home cage and placed into a testing apparatus for 30–45 min to acclimate; food and water were provided during the acclimation period. The testing apparatus consisted of two standard size mouse cages (29 cm $\times$ 19 cm $\times$ 13 cm) connected by an 8-cm long clear acrylic tube. After the acclimation period, during which time all subjects investigated both mouse cages, an unclaired vole pup 1–3 days of age was introduced. The vole pup was placed in the cage without the subject so that latency to approach the pup could be assessed. Recording began as soon as the stimulus pup was introduced. The test continued for 10 min after the subject entered the cage with the pup. If an attack occurred, the test was ended immediately so that, if necessary, the pup could be treated and to prevent further injury. Subjects were given 30 min to make an approach before ending the test. All tests were recorded with a digital video camera and scored by the same

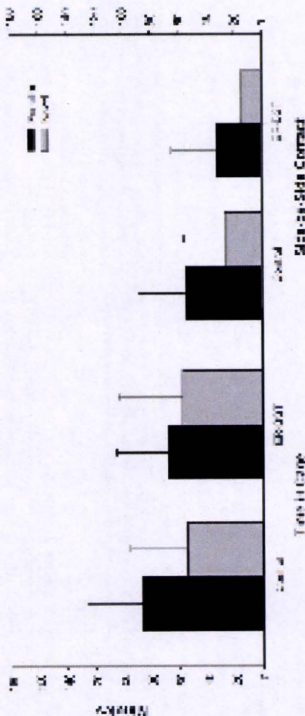


Figure 3. Shows the mean ($\pm$ s.e) time spent in the cage of and in physical (side-by-side) contact with the familiar and novel stimulus female by treatment. While there was no significant difference either within or between treatments for time spent in the cage or in contact with the familiar versus the novel female ER α -BST treated males spent significantly less total time (combined familiar and novel) than control males (see text).
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Discussion

The results from this study support the hypothesis that low levels of ER α in the BST play a role in the expression of male prosocial behavior. Increasing ER α in the BST significantly reduced heterosexual social affiliation, but did not impact the expression of alloparental behavior. These findings are significant on several levels. First, they suggest that mechanisms underlying different types of affiliation differ, as increasing ER α in the BST decreased physical contact with females, but did not alter the amount of time spent licking or huddling with pups. Second, the results differed significantly from the effects of enhancing ER α expression in the MeA, where enhanced ER α inhibited the expression of alloparental behavior and resulted in the formation of a preference of the novel female [13]. Although the BST and MeA are both part of the extended amygdala, these results indicate that they regulate different aspects of social behavior and that the MeA is more important in regulating the expression of alloparental behavior than is the BST. Finally, the differential effects of increasing ER α in the BST versus in the MeA suggest the possibility that the suite of prosocial behaviors seen in male prairie voles is, at least in part, a product of the interaction between these two critical regions of the social neural circuit.

Enhancing ER α in the BST did not affect the expression of alloparental behavior. Male prairie voles typically display high levels of spontaneous alloparental behavior, ranging from 70 to 100% of males displaying alloparental behavior [13,32]. While at the lower end of the range, 73% of ER α -BST males were alloparental. This is compared with 33% of males in which ER α was enhanced in the MeA [13]. The expression of male parental behavior has been associated with a number of regions in the brain, including the BST and the MeA. In prairie voles, male parental behavior was associated with increased fos expression in both the MeA and BST [16]. Also in male prairie voles, adult castration reduced the expression of male parental behavior and the expression of arginine vasopressin, which has been shown to play a significant role in male social behavior [33,34], in the BST and MeA; treatment with testosterone restored both [23]. While Wang and De Vries [23] hypothesized that this resulted from the effect of testosterone on vasopressin expression, there is evidence that estrogen may play a direct role, with estrogen receptors

Statistical Analyses

For all data sets, the two control groups were compared using a *t*-test. In no case were there differences between the two control groups; these were combined into a single control group for all subsequent analyses. A Fisher's exact test was used to analyze whether or not there was a difference between the proportion of control and ER α -BST males that expressed alloparental behavior. A one-way ANOVA was used to compare alloparental behaviors, licking and huddling, by treatment. A one-way ANOVA was used to analyze between treatment effects on social preference, while a paired *t*-test was used to analyze within treatment effects. For all statistical tests, the criterion for significance was $P \leq 0.05$.

Results

Eleven males were successfully transfected with ER α in the BST. Figure 1 shows a representation of successful transfection.

Alloparental

There was no significant difference in number of males that express alloparental behavior; 8 of 11 ER α -BST males were alloparental compared to 10 of 16 control males (Fisher's Exact $P = 0.69$). Non-alloparental behavior was comprised of, for ER α -BST males: 2 pup attacks and 1 with no contact; for controls: 2 pup attacks and 4 that did not enter the pup cage. In animals that expressed alloparental behavior, there was no effect of treatment on time spent licking or huddling with the pup (Fig. 2).

Heterosexual Social Preference & Affiliation

There was a significant effect of treatment on affiliation (Fig. 3). ER α -BST males (48 ± 7.3 s.e. min) spent significantly less total time in side-by-side with both familiar and novel females than did control males (81.9 ± 5.6 min; ANOVA $F_{1,25} = 13.4$ $P < 0.005$; $t_{6} = 3.43$; $P = 0.004$). There was no significant difference between or within treatment groups for time spent in side-by-side contact with the familiar versus the novel female. Although a preference for the familiar animal is not expected after only 1 hr of cohabitation, control males did display a trend toward a preference for the familiar female (Control paired $t_{46} = -2.01$; $P = 0.06$; ER α -BST paired $t_{9} = -1.64$; ns; Fig. 3).

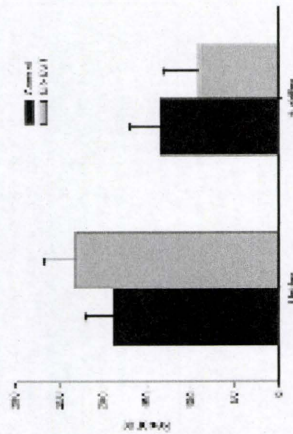


Figure 2. Shows the mean ($\pm$ s.e) time spent in the cage of and in physical (side-by-side) contact with the familiar and novel stimulus female by treatment. While there was no significant difference either within or between treatments for time spent in the cage or in contact with the familiar versus the novel female ER α -BST treated males spent significantly less total time (combined familiar and novel) than control males (see text).
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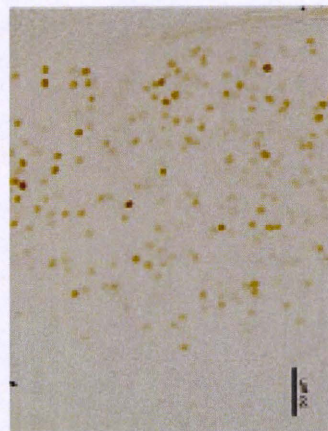


Figure 1. Shows photomicrograph of AAV-mediated ER α expression at 200x in the medial division of the BST. ER α was labeled using the human-specific antibody RM9101-s (Neomarkers, Fremont CA), which does not bind to endogenous prairie vole ER α . Immunoreactivity was visualized with DAB.
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experimentally-blind observer using the JWatcher program (UCLA). Behaviors scored and analyzed included latency to enter the pup cage, duration of pup-directed licking and huddling, and pup-directed attacks. Animals that displayed pup-directed aggression were classified as non-alloparental.

Heterosexual Social Preference and Affiliation

One week after the alloparental test, treated males were tested for heterosexual social preference. This was done using the same apparatus as that used in the standardized vole partner preference test [11,29]. Each subject was placed in a clean cage with a sexually naive female for a 1-hr period of cohabitation. This female was designated as the "familiar." Immediately after the cohabitation period, social preferences of the subject were assessed. The social preference arena consists of three polycarbonate standard size mouse cages in a modified Y-shape. The two cages housing stimulus animals are in parallel and a third cage (neutral) is attached separately to each stimulus cage by Plexiglas tubes. The familiar female is gently tethered in one of the stimulus cages while an age- and size-matched sexually naive female, classified as "novel," that is unrelated to both the familiar female and the subject is tethered in the other stimulus cage. At the start of the test, immediately after the cohabitation period, the experimental animal is placed in the neutral cage and is free to move among the three cages. The social preference test lasted 3 hrs and was recorded using a digital video camera and then scored using the JWatcher program at a 10:1 temporal reduction. Behaviors scored included: time in each cage, time investigating each stimulus animal, and time spent in side-by-side contact with each stimulus animal. It should be noted that without hormonal manipulations, 1 hr of cohabitation is insufficient for prairie voles to form a preference for the familiar partner [14,30] and so control males were expected to spend equal amounts of time with the familiar and novel females. All stimulus females were sexually naive adults, 60–90 days of age, and mating is not a concern as female prairie voles do not undergo a spontaneous estrous cycle, requiring 24 or more hours of contact with a novel male before becoming sexually receptive [31].