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Are age and sex differences in brain oxytocin receptors related to maternal and infanticidal behavior in naïve mice?

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ABSTRACT

This article is part of a Special Issue "SI: Parental Care".

There is significant variability in the behavioral responses displayed by naïve young and adult mice when first exposed to pups. This variability has been associated with differences in the expression of oxytocin receptors (OXTRs) in the brain in several species. Experiment I investigated the behavioral responses of juvenile, adolescent, and adult CB57BL/6 males and females when first exposed to pups. We found an age increase in maternal females (11% of juveniles, 20% of adolescents, and 50% of young adults), and infanticidal males (0% of juveniles, 30% of adolescents, 44.5% of young adults, and 100% of older adults). Experiment II investigated OXTR density in the brain of juvenile and adult mice. Our results revealed an age decline in the density of OXTR in several brain regions, including the lateral septum, cingulated and posterior paraventricular thalamic nucleus in both males and females. Adult females had higher OXTR density in the ventromedial nucleus/postero-ventral hypothalamus (VMH) and the accessory olfactory bulb (AOB), but lower density in the ventral region of the lateral septum (LSv) than juveniles. Males had lower OXTR density in the anterior olfactory area (AOA) compared to juveniles. No age or sex differences were found in the medial preoptic area, and amygdaloid nuclei, among other brain regions. This study suggests that 1) maturation of parental and infanticidal behavioral responses is not reached until adulthood; 2) the pattern of development of OXTR in the mouse brain is unique, region specific, and differs from that observed in other rodents; 3) either up or down regulation of OXTR in a few brain regions (VMH/AOB/LSv/AOA) might contribute to age or sex differences in parental or infanticidal behavior.

Introduction

There has been a controversy on the typical behavioral response displayed by naïve young and adult mice when first exposed to pups. Adult females and males are commonly described as spontaneously maternal or infanticidal respectively (Calamandrei and Keverne, 1994; Gandelman, 1973a,b; Gandelman and vom Saal, 1975; Leussis et al., 2008; Noirot, 1969, 1972; Stolzenberg and Rissman, 2011; Svare, and Mann, 1981). However, other authors have found that at least half of the females did not show maternal behavior, and a small percentage of naïve adult males showed paternal behavior (Alsina-Llanes et al., 2015; Brown et al., 1996; Hamaguchi-Hamadaa et al., 2004; Kennedy and Elwood, 1988; Kuroda et al., 2011; Lucas et al., 1998; McCarthy and vom Saal, 1986; Pedersen et al., 2006). In the case of young females and males, the literature suggested that they showed lower incidence of maternal or infanticidal behavior respectively (Gandelman, 1973a,b; McCarthy and vom Saal, 1986; Noirot, 1972). However, in contrast to rats and prairie voles (Brunelli and Hofer, 1990; Mayer and Rosenblatt, 1979; Olazábal and Morrell, 2005; Olazábal and Young, 2006a; Stern, 1987), the ontogeny of parental and infanticidal behavior in CB57BL/6 mice had not been investigated in detail.

Several factors have been proposed in the literature to explain age and sex differences in parental and infanticidal behavior (Alsina-Llanes et al., 2015; Kuroda et al., 2011; McCarthy and vom Saal, 1986; Svare and Mann, 1981). In the current study we investigated whether the oxytocinergic system could be related to age and sex differences in parental or infanticidal behavior in naïve mice. Oxytocin (OXT) is known to facilitate maternal behavior in many species (Olazábal et al., 2013; Pedersen, 2013). For example, knock out and pharmacological studies in mice suggested that OXT facilitated maternal behavior, while a reduction in OXT function promoted infanticidal behavior (McCarthy, 1990; Ragnauth et al., 2005). However, those approaches often produced global increases or decreases in OXT function, affecting multiple brain sites, and likely multiple OXT functions. Additionally, the results of knock out mice

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studies for OXTR and OXT genes have also been controversial and while some studies found no behavioral differences, others concluded that OXT facilitated maternal and inhibited infanticidal behavior in mice (Macbeth et al., 2010; Nishimori et al., 1996; Pedersen et al., 2006; Ragnauth et al., 2005; Takayanagi et al., 2005). Intra or inter-species natural variation in OXTR in the brain has also been used to study the role of OXT in parental and other social behaviors (Curley et al., 2012; Champagne et al., 2001; Dumais et al., 2013; Olazábal and Young, 2006a,b). Those studies found that both up or down regulation of OXTR in a few brain regions (instead of large networks) could be associated to parental behavior (Curley et al., 2012; Champagne et al., 2001; Olazábal and Young, 2006a,b).

Some of the natural variations in the OXT system are those that occur during development and sexual maturation. The OXT system undergoes significant developmental changes from weaning to adulthood in most species (Hammock and Levitt, 2013; Olazábal and Young, 2008; Shapiro and Insel, 1989; Tribollet et al., 1989). However, those developmental changes are species specific. For example, in contrast to rats (Tribollet et al., 1989; Shapiro and Insel, 1989), Hammock and Levitt (2013) found an age decline in OXTR density in the neocortex, but no change in the VMH in mice. That study analyzed few brain regions and used a small number of animals (n = 3). Therefore, we decided to compare OXTR density in the whole brain of juvenile weanlings and adults. We hypothesized that natural age variations in the density of OXTR in the mouse brain might contribute to age differences in the behavioral response to newborns when mice are exposed to pups for the first time.

Sex differences in behavior have also been studied for decades and are well known to be species specific (Bridges et al., 1974; Lonstein and De Vries, 2000; Mayer and Rosenblatt, 1979; Olazábal, 2014; Shapiro et al., 1991). The hormonal regulation of the OXT system differs sometimes among species (Young et al., 1996), with potential implications in their behavioral development and maturation (Dumais et al., 2013; Lukas et al., 2010; Uhl-Bronner et al., 2005). Therefore, the behavior of juvenile and adult males and females is mainly a consequence of neural and physiological adaptations to different reproductive and social strategies in each species (Beach, 1967; Lonstein and De Vries, 2000; Noirot, 1972; Olazábal and Young, 2006a; Rosenblatt, 1967; Shapiro and Dewsbury, 1990). In the current study, we hypothesized that natural sex differences in the density of OXTR in the mouse brain might contribute to the sex differences in the behavioral responses toward pups.

In Experiment I, we described the behavioral responses toward newborns in juvenile weanling (21–22 day-old), adolescent (30–35 day-old), and adult (60–100 day-old) naïve females and males. In Exp II, sex and age differences in brain OXTR density in juvenile weanling and adult animals were determined, and the relationship of those differences with age and sex differences in parental and infanticidal behavior in mice discussed. The current approach was chosen to identify potential areas of the brain where OXTR fluctuations or OXT action could affect mouse behavioral responses toward newborns.

Material and methods

Animals

In Exp I we used juvenile weanling (21–22 day-old) males (n = 10) and females (n = 9); adolescent (30–35 day-old) males (n = 10) and females (n = 10); young adult (60–65 day-old) males (n = 9) and females (n = 10); and older adult males (100–105 day-old, n = 9). In Exp II we used 12 naïve juvenile (21–22 day-old, 7 females and 5 males); 8 adult female (60–70 day-old) and 6 older adult (100–105 day old) male CB57BL/6 mice. All animals were naïve mice, without any previous pup or sexual experience. Animals were originally obtained from Jackson Laboratory and inbred at the animal facility of the Facultad de Medicina (UdelaR, Montevideo, Uruguay) since 2010. All animals were weaned at age 20–21 days, and maintained in same-sex groups of 5–6 individuals per cage until used. Cages were 45 cm × 25 cm × 15 cm, with transparent plexiglas walls and wood shaving as bedding. Animals were kept under a 12:12 h light-dark cycle (light on from 6:00 am), at 22 °C, with ad libitum access to food (Vitaron, Montevideo, Uruguay) and water. All procedures carried out in this study were approved by the local committee of ethics in animal research (CHEA, No. 071140, December 26th, 2011) and followed the recommendations of the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health (2011) and the "Guidelines for Ethical Conduct in the Care and Use of Animals" (APA, Board of Scientific Affairs, Committee on Animal Research and Ethics, 2012).

Experiment I

Parental behavior test in naïve juveniles and adults

All subjects were individually housed and habituated to the cage (27 cm × 21 cm × 14 cm, floor area of 370 cm^2) 1 h before two newborns (1–3 days of age) were placed inside the cage for 15 min. We recorded frequency and duration of licking and grooming, crouching postures, and frequency of retrieval or pup transport. In case the subject attacked the pups, the test was immediately stopped and pups were sacrificed. Animals were then included in one of 4 categories: Infanticidal (I) if they attacked the pups. That is male or female bit a pup, and pup squealed. The attack was confirmed by inspecting the pups for wounds and bleeding after the subject was removed; Non-Parental (NP) if they failed to show at least two components of parental behavior (crouching, licking and grooming, or retrieval); Partial Parental Behavior (PPB) if they showed 2 of the 3 main components of parental behavior, and Full Parental Behavior (FPB) if they displayed all components of parental behavior: pup retrieval, licking and grooming (≥60 s), and crouching over at least one pup (≥30 s). All behavioral observations were recorded using the free software StopWatch http://www.cbn-atl.org/research/stopwatch.shtml.

Experiment II

Oxytocin receptor autoradiography

All animals were sacrificed, their brains removed, frozen in dry ice, and stored at −80 °C. The stage of the estrous cycle in adult females was determined (postmortem) using vaginal smears. All juveniles had been weaned at least one day, and no longer than 2 days before the sacrifice. Three series of 20 μm-thick brain sections for OXTR autoradiography were obtained using a cryostat. Tissue was processed as described in previous studies (Olazábal and Young, 2006a,b). Briefly, brain sections were left out of the −80 °C freezer for 1 h for the tissue to dry, and later fixed in 0.1% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 2 min at room temperature. Slides were then rinsed in Tris–HCl buffer (pH 7.4) and later incubated for 90 min in 50 pM ^{125}I-OVTA (Perkin Elmer, USA) in Tris with 10 mM $MgCl_2$, 0.1% bovine serum albumin (RIA grade, Sigma). Unbound ligand was removed by washes in 50 mM Tris pH 7.4, 10 mM $MgCl_2$. The slides were finally quickly dipped in dH_2O, rapidly dried using a hair dryer for 15 min, and exposed to BioMax MR film (Kodak, Rochester, NY, USA) along with I^{125} microscale standards (American Radiolabeled Chemicals) for 48 h. All slides were processed at the same time and sufficient amount of the solutions were prepared to use the same solutions for all slides. Previous studies have found that ^{125}I-OVTA signal is selective for OXTR in mice (Lee et al., 2008). Sections used for autoradiography were also stained with cresyl violet to better identify the location of the autoradiographic binding.

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Image processing and quantitative analysis

Stained slides and autoradiographic films were placed in a magnifier connected to digital camera and a computer with an image system that permitted to capture the images of the sections at high resolution. Pictures of the sections were taken, and signal in the brain regions of interest was analyzed. Optical density readings were obtained using ImageJ free software (NIH), and converted to decompositions per minute (d.p.m./milligram tissue equivalent) based on I^{125} autoradiographic standards. A squared shaped area was applied to the brain regions of interest only on one side of the section, OXTR binding within that area measured at least in 2 sections for each brain region or subregion, and the average reading recorded. Background reading, taken from areas of the tissue with no OXTR binding, was also recorded and subtracted to the signal. All further details of the quantitative analysis can be found in Olazábal and Young (2006a,b).

Statistical analysis

Data in Exp II was analyzed using the statistical package StatView (SAS Institute Inc, Cary, NC) and using ANOVA with sex and age as factors. One-way ANOVA followed by Fisher post hoc test was used to compare all age and sex groups after finding sex, age, or sex and age interaction effects. A conservative analysis with Bonferroni (p value < than .0083) to correct for multiple comparisons is also shown in the text when appropriate. The olfactory bulb was lost in 3 adult males while removing the brain from the skull and processing the tissue. Therefore data on AOB and MOB were analyzed separately to avoid losing statistical power in the other brain regions. The statistical significance level was $p < .05$. Data are expressed as mean ± SE.

Results

Exp I

Ontogeny of parental and infanticidal behavior

Juvenile males and females did not show infanticidal or full parental behavior. Only one of the 9 juvenile females showed some components of maternal behavior and reached the criteria for PPB. At the age of 30–35 days, 2 females showed PPB while 3 of the males attacked pups. The rest of adolescent mice were non-parental. One male reached the criteria for licking (1/10). However, none of them reached the criteria for PPB or FPB. Fifty percent of young adult females showed either FPB or PPB, while 44.5% of males were infanticidal. The rest of young adult animals were non-parental (Table 1). However, 3 young adult males showed substantial licking behavior (data not shown), but did not reach the criteria for PPB. 100% of 100–105-day-old males attacked the pups.

Table 1
Percentage of parental, non-parental or infanticidal animals.

	Behavioral response	FPB	PPB	NPB	IB
Females					
Age	20–22 days	0.0%	11.1%	88.9%	0.0%
	30–35 days	0.0%	20%	80.0%	0.0%
	60–62 days	20.0%	30%	50.0%	0.0%
Males					
Age	20–22 days	0.0%	0.0%	100.0%	0.0%
	30–35 days	0.0%	0.0%	70.0%	30.0%
	60–62 days	0.0%	0.0%	55.5%	44.5%
	100–105 days	0.0%	0.0%	0.0%	100.0%

FPB = Full Parental Behavior; PPB = Partial Parental Behavior; NPB = Non-Parental Behavior; IB = Infanticidal behavior. Date is expressed as percentage of animals showing behavior during the first exposure to newborns (15 min test).

Exp II

Brain regions with an age difference in OXTR density

A significant decline in the density of OXTR in males and females was found in the dorsal/intermediate lateral septum (LSdi, age effect, $F = 20.3$ $p < .001$; Figs. 1 and 2), cingulated cortex (CGC, age effect, $F = 28.0$ $p < .001$), and posterior paraventricular thalamic nucleus (PVP, age effect $F = 29.0$ $p < .001$; Figs. 3 and 5). Bonferroni correction (p value < than .0083) did not find significant difference between AM and juveniles in the LSdi ($p = .02$).

In contrast, adult females and males had higher OXTR density in the accessory olfactory bulb (AOB, 1200 ± 79 and 1305 ± 22 respectively) than juvenile females and males (989 ± 52 and 925 ± 22 respectively, Fig. 4). No statistical significant difference in the VMH was reached when all females were included in the comparison (sex effect $F = .2$ $p = .64$; age effect $F = 4.1$ $p = .06$; sex and age interaction $F = .48$ $p = .50$). When only females in the proestrous and estrous stage of the estrous cycle were included in the analysis, females had higher OXTR density in the VMH and postero-ventral hypothalamus than juveniles (age effect $F = 7.4$ $p = .01$; Fig. 5). Adult females and males were not different in the density of OXTR in the VMH ($p = .12$; Fig. 5).

Several other brain regions showed a decline in OXTR density, but not in all sex groups. The piriform cortex and septohippocampal region (Pir, Shi, Table 2), also showed decline in OXTR density from weaning to adulthood but the difference was significant mainly in females (see Table 2). Bonferroni correction revealed only a significant difference

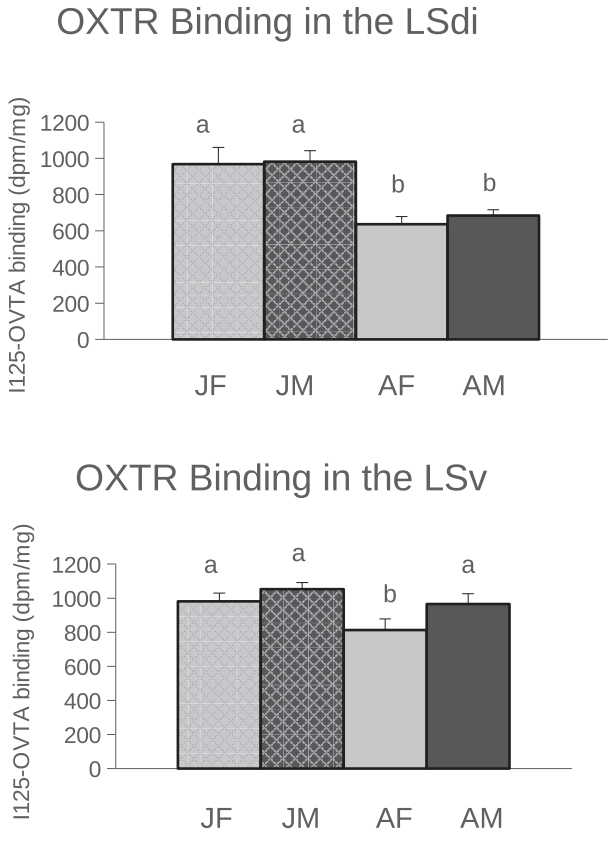

Fig. 1. OXTR binding in the lateral septum of juveniles and adults. OXTR binding in the lateral septum dorsal/intermediate (LSdi) and ventral (LSv) was lower in adults than in juveniles. JF = juvenile females; JM = juvenile males; AF = adult females; AM = adult males. Different letters represent significant differences between the groups. Data are expressed as means ± S.E. ($p < .05$).

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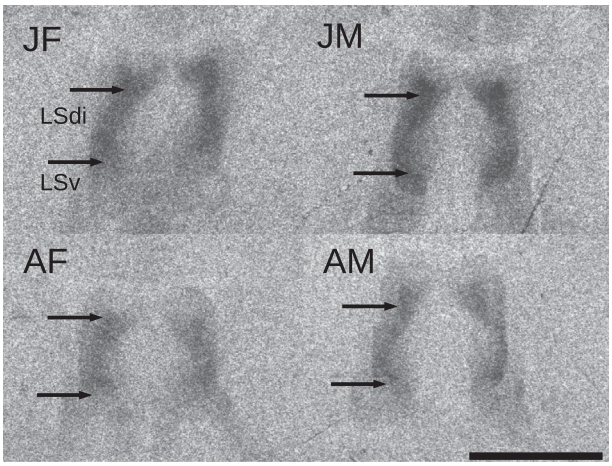


Fig. 2. Pictures of sample brain sections showing the autoradiographic signal for I^{125} OVTA for animals representative of each age and sex group. Juvenile females (AF); juvenile males (JM); adult females (AF); adult males (AM). Arrows show the area where OXTR density in the dorsal/intermediate (LSdi) and ventral region (LSv) of the lateral septum was measured. Scale bar = 2 mm.

between AF and JF in Pir, and AF and JM in SHi. Similarly, OXTR density in the periaqueductal gray (PAG) and the posteroventral hippocampus (mainly in CA1 region and ventral subiculum) was lower in adults, but only when compared to juvenile females (Table 2). Bonferroni correction (p value < than .0083) revealed only a significant difference between AM and JF in HiPpv and PAG. The anterior olfactory area (AOA), showed a significantly lower OXTR density in male adults

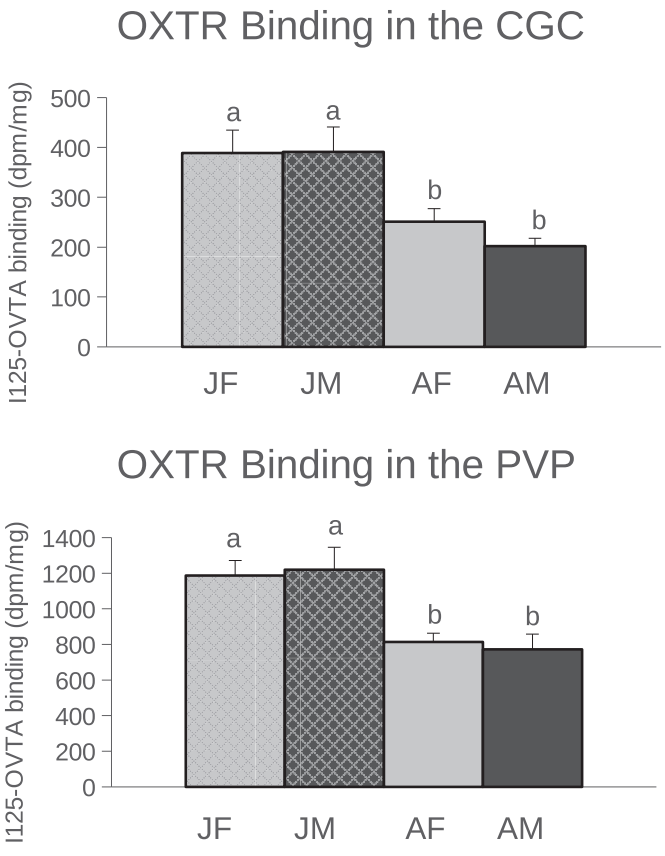
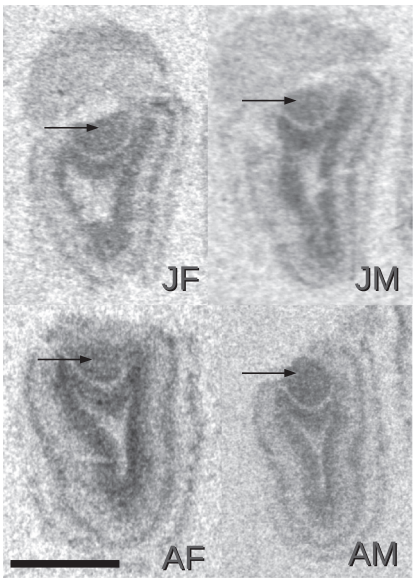


Fig. 3. OXTR binding in the cingulated cortex (CGC) and the posterior paraventricular thalamic nucleus (PVP) of juveniles and adults. OXTR binding in the CGC and the PVP was lower in adults than in juveniles. Different letters represent significant differences between the groups. Data are expressed as means ± S.E. ($p < .05$).



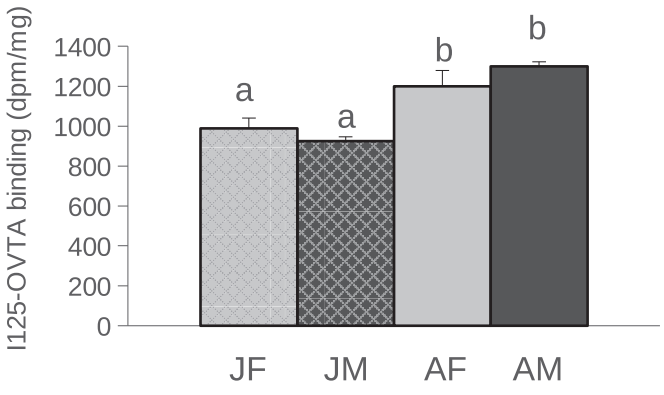


Fig. 4. OXTR binding in the accessory olfactory bulb (AOB) of juveniles and adults. The upper side of the figure shows pictures of sample brain sections showing the autoradiographic signal for I^{125} OVTA in the AOB for animals representative of each age and sex group. Juvenile females (AF); juvenile males (JM); adult females (AF); adult males (AM). Arrows show the area where OXTR density was measured in the AOB. Scale bar = 1 mm. At the bottom, the figure shows OXTR binding in the AOB for each group. In the AOB, OXTR binding was higher in adults compared to juveniles. Different letters represent significant differences between the groups. Data are expressed as means ± S.E. ($p < .05$).

when compared to juveniles ($p < .05$). Female adults did not differ from juveniles or adult males and Bonferroni correction showed no significant differences among the groups in this brain region (see Table 2 and Fig. 6).

Brain region with a sex difference in OXTR density

A sex effect was found only in the ventral subregion of the lateral septum (LSv, sex effect $F = 6.2$ $p = .03$; age effect $F = 3.1$ $p = .10$; sex * age $F = 1.5$ $p = .25$). Adult females had lower OXTR density than adult males and juveniles (see Figs. 1 and 2). Bonferroni correction only revealed a significant difference between AF and juveniles.

Brain regions without age or sex differences in OXTR density

Data analysis revealed that juveniles had higher or similar density of OXTR in most brain regions compared to adults. No age or sex differences were found in the main olfactory bulb (MOB), NA core (NAc) or shell (Nash), medial preoptica area (MPOA) subregions, zona incerta (ZI), or medial (MA), cortical (CoA), basolateral (BLA), and central (CeA) amygdala, among other brain regions (see Table 3).

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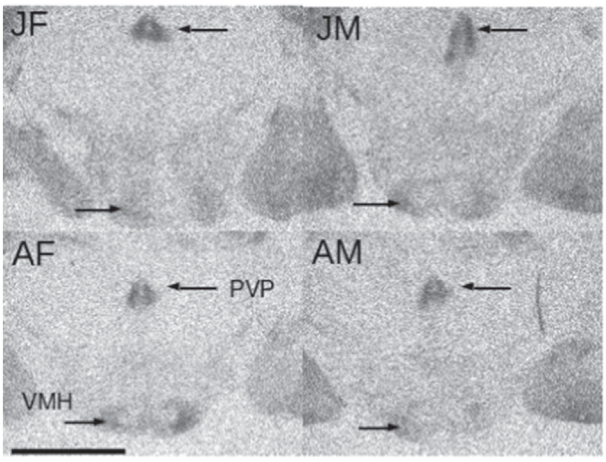

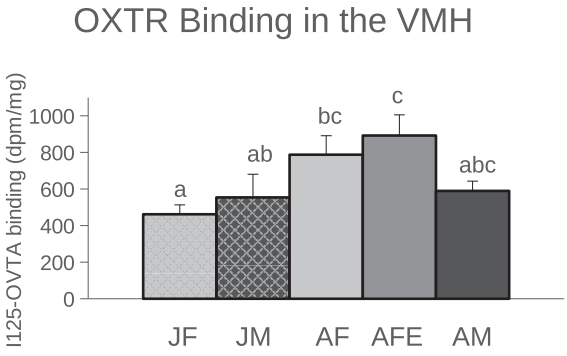

Fig. 5. OXTR binding in the ventromedial nucleus of the hypothalamus (VMH) of juveniles and adults. The upper side of the figure shows pictures of sample brain sections showing the autoradiographic signal for I^{125} OVTA in the VMH for animals representative of each age and sex group. Juvenile females (AF); juvenile males (JM); adult females (AF); adult females in estrous/proestrous (AFE); adult males (AM). Group AF includes all females but statistical comparison included only AF or AFE. Arrows show the area where OXTR density was measured in the VMH and PVP. At the bottom, the figure shows OXTR binding in the VMH for each group. In the VMH, OXTR binding was higher in AFE than in juveniles. Different letters represent significant differences between the groups. Data are expressed as means ± S.E. ($p < .05$). Scale bar = 2 mm.

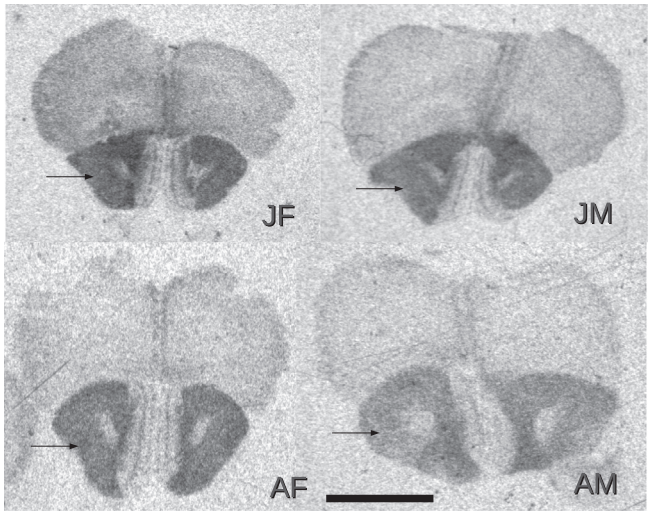

Fig. 6. OXTR binding in the anterior olfactory area (AOA) of juveniles and adults. The figure shows pictures of sample brain sections showing the autoradiographic signal for I^{125} OVTA in the AOA for animals representative of each age and sex group. Juvenile females (AF); juvenile males (JM); adult females (AF); adult males (AM). Arrows show the area where OXTR density was measured in the AOA. Scale bar = 2 mm.

Discussion

The present study described the development of parental and infanticidal behavior, and brain OXTR distribution in male and female CB57BL/6, one of the most used strains of mice. In Exp I we found that infanticidal and parental responses in naïve mice developed gradually, were absent in juveniles, and not fully expressed until adulthood. In Exp II we showed that the pattern of development of OXTR in the mouse brain is unique and region specific and that either up or down regulation of OXTR in a few brain regions could contribute to age or sex differences in parental or infanticidal behavior.

As previously described and discussed in detail in Alsina-Llanes et al. (2015), the higher incidence of maternal behavior found in adult compared to juvenile female mice agreed with previous studies (Gandelman, 1973a,b; Noirot, 1969, 1972). However, the small percentage of full maternal animals (20%) found on the first exposure to pups in our study disagreed with several previous studies (70 to 100%; Calamandrei and Keverne, 1994; Gandelman, 1973a; Gandelman and vom Saal, 1975; Leussis, et al., 2008; Noirot, 1969, 1972; Stolzenberg and Rissman, 2011). In a recent publication (Alsina-Llanes et al., 2015), we discussed that the disagreement might be due to different criteria used in the different studies to define an animal as maternal (presence or not of all component of maternal behavior), and also to the different time the animals were pre-exposed to pups before testing (e.g. during rearing or habituation to testing cage). However, there is still a clear agreement that adult females are more prone to show maternal behavior components than juvenile females.

Our findings on the ontogeny of male infanticidal behavior also agreed with previous studies that used animals from different strains or ages. For example, McCarthy and vom Saal (1986) found that young (40–45 days of age) were less infanticidal (~36%) than old (~80%) male wild house mice. Svare and Mann (1981) also found an increase in infanticidal behavior from 25 to 65 days of age in CB57BL/6 and DBA strains. Finally, Gandelman (1973b) found that juvenile Rockland–Swiss mice (22 days of age) did not kill pups. Therefore, as seen in females, there is a clear agreement in the literature that adult males are more infanticidal than juvenile males.

Table 2
Brain regions with an age decline in OXTR density.

	Brain region	AOA	Pir	SHi	HiPpv (CA1)	PAG
Age and sex groups	AF; n = 8	1002 ± 61ab	914 ± 45a	630 ± 31a	778 ± 49a	271 ± 19ab
	AM; n = 6	923 ± 46a	923 ± 55a	678 ± 87ab	753 ± 94a	216 ± 24a
	JF; n = 7	1134 ± 49b	1128 ± 41b	721 ± 24bc	1053 ± 49b	337 ± 27c
	JM; n = 5	1126 ± 51b	1063 ± 31ab	823 ± 31c	942 ± 110ab	303 ± 38bc
p (F) values	Sex	.77 (.1)	.67 (.2)	.26 (1.4)	.42 (.7)	.19 (1.9)
	Age	.00 (15.0)	.04 (5.2)	.01 (11.6)	.03 (5.8)	.02 (7.4)
	Sex * age	.32 (1.1)	.19 (2.0)	.63 (.2)	.70 (.2)	.91 (.0)

AF = adult females; AM = adult males; JF = juvenile females; JM = juvenile males. Age, sex and sex * age stand for two way ANOVA p (F) values for sex, age and sex and age interaction effects. Different letters represent statistical significant difference between those groups ($P < .05$).

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Table 3
Brain regions with no difference in OXTR density in Juveniles and Adults.

	Brain Region	MOB	MOC	NAc	NAsh	MPOAm	MPOAl	MPOAvl	PVA	ZI	MA	CoA	BLA	CeA	HiPpd (CA1)
Age and sex groups	AF; n = 8	1201 ± 57	604 ± 85	307 ± 30	412 ± 38	343 ± 40	255 ± 18	348 ± 28	650 ± 35	380 ± 20	596 ± 82	719 ± 57	497 ± 40	522 ± 44	715 ± 58
	AM; n = 6	(1224 ± 47)	492 ± 92	251 ± 35	306 ± 46	235 ± 22	233 ± 23	381 ± 65	581 ± 33	397 ± 36	632 ± 68	834 ± 57	496 ± 63	591 ± 37	575 ± 99
	JF; n = 7	1053 ± 85	643 ± 83	319 ± 49	365 ± 45	300 ± 33	243 ± 22	376 ± 78	511 ± 100	354 ± 24	578 ± 59	814 ± 67	548 ± 67	520 ± 56	658 ± 125
	JM; n = 5	1074 ± 81	715 ± 70	322 ± 37	384 ± 49	376 ± 71	265 ± 46	415 ± 95	552 ± 109	370 ± 35	536 ± 22	837 ± 24	491 ± 31	521 ± 15	782 ± 127
p (F) values	Sex	.90 (.0)	.45 (.6)	.74 (.1)	.71 (.2)	.78 (.1)	.74 (.1)	.81 (.1)	.92 (.0)	.58 (.3)	.84 (.1)	.74 (.1)	.34 (1.0)	.95 (.0)	.80 (.1)
	Age	.21 (1.8)	.01 (8.0)	.22 (1.7)	.41 (.7)	.45 (.6)	.96 (.0)	.27 (1.4)	.62 (.3)	.69 (.2)	.64 (.2)	.54 (.4)	.57 (.4)	.74 (.1)	.38 (.8)
	Sex * age	.99 (.0)	.29 (1.2)	.57 (.3)	.67 (.2)	.19 (1.9)	.67 (.2)	.72 (.13)	.07 (4.1)	.66 (.2)	.49 (.5)	.56 (.4)	.69 (.2)	.79 (.1)	.29 (1.3)

AF = adult females; AM = adult males; JF = juvenile females; JM = juvenile males. Age, sex and sex * age stand for two way ANOVA p (F) values for sex, age and sex and age interaction effects.

However, our findings differed from previous studies in the incidence of paternal behavior in males. While in our study most males ignored or killed the pups, several previous studies have reported the occurrence of paternal care. For example, the classic studies of Leblond (1938) found high levels (50%) of paternal behavior in males 22–28 days of age and adults. However, it is unclear if those were animals exposed to pups for the first time, because the authors also reported that juveniles required about 4 days of exposure to pups (sensitization) to display parental behavior. McCarthy and vom Saal (1986) also found a few animals (20–40% of adolescent and adult males) that hovered over the pups in the nest (test of 30 min). They also found strain differences; more parental (30–40%) and less infanticidal animals in CF-1 mice compared to wild house mice. Finally, also in contrast to our study (also see Svare and Mann, 1981), Gandelman (1973b) reported that around half Rockland–Swiss juveniles were parental Unfortunately, in those two previous studies the authors did not record all components of parental behavior or showed a detailed description of juvenile behavior.

Then, the disagreement in the incidence of parental behavior in adults and juveniles found in the literature, could be due to strain differences as shown by several studies (McCarthy and vom Saal, 1986; Svare and Mann, 1981). However, it is also possible that those differences were a consequence of the different criteria used to consider an animal as parental (sometimes only one behavioral component was used, SPriestnall and Young, 1978; vare and Broida, 1982), or different rearing conditions of juveniles (not clearly specified in many studies). For example, in a study by Gubernick and Laskin (1994), 80% of 40-day-old *Peromyscus californicus* (males and females pooled together) previously housed with their parents, and also receiving only 4 h of exposure to newborn siblings, failed to show parental behavior. However, if they cohabitated with parents and younger siblings for 30 days, 70% showed parental behavior. In that study, 65-day-old animals (males and females pooled together) showed very low incidence of infanticidal behavior (5–15%) that increased to 66% at the age 120 days. Then, having cohabitated with younger siblings had impacted on the incidence of parental behavior but not in the age-dependent increase of infanticidal behavior (consistent across different studies and strains). Therefore, our results in Exp I provided evidence that suggests that maturation of parental and infanticidal behavioral responses toward newborns in mice is not complete until adulthood, and also that adult males and females differ significantly in their behavioral responses to pups when first exposed to them.

In Exp II, we decided to compare adult males (mostly infanticidal) and females (mostly maternal) with juveniles (mostly indifferent to pups). OXTR density in the brain of juvenile weanling and adult mice was described in detail for the first time. Most brain regions showed a higher or similar OXTR density in juveniles compared to adults, suggesting a change in OXT function or a neural adaptation involving the OXT system after the time of weaning. This general pattern of higher OXTR density in juvenile mice agreed with previous findings in infant and juvenile rats (Shapiro and Insel, 1989; Tribollet et al., 1989). However, considering OXTR change in each brain region, the data revealed that the pattern of development of OXTR in mice was unique and also differed from that found previously in rats. For example, OXTR density in the CGC, thalamic (PVP), and hippocampal (CA1, CA2, and CA3) regions is very low or absent in juvenile rats, but abundant in mice. Besides, OXTR density in these brain regions declined significantly after weaning in mice, but not in rats (Shapiro and Insel, 1989; Tribollet et al., 1989). Similarly to rats, an age decline was also found in the LSdi and the Shi region, suggesting that the action of OXT in the LS-HiP system contributed to the transition from juvenile to adult life in mice, perhaps participating in changes in learning/neurogenesis, or social/defense/stress behavioral responses after weaning (Leuner et al., 2012; Owen et al., 2013).

To my knowledge there was not a detailed description of the development of OXTR in the MOB or AOB in other rodents. In the present

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study, high density of OXTR was found in both regions in females and in the 3 males investigated. Although adults showed no change in OXTR density in the MOB, the increased OXTR in the AOB and VMH of females could be of a significant behavioral relevance. AOB processes mainly non-volatile substances that could be relevant to accelerate the onset of parental behavior or inhibit infanticidal behavior in mice (Canavan et al., 2011; Huilgol et al., 2013; Tachikawa et al., 2013). The VMH has been proposed to inhibit maternal behavior in rats, and also receive olfactory and sensory information related to newborns from the MA (Bridges et al., 1999; Olazábal et al., 2013). Thus, the AOB and VMH are good candidates, or potential sites, where OXT action or OXTR fluctuations can influence mouse behavioral responses toward newborns. Maturation of gonads and increased circulating estrogen are likely responsible for changes in the density of OXTR in the VMH and AOB in adult females (de Kloet et al., 1985; Dominguez-Salazar et al., 2006; Dumais et al., 2013; McCarthy, 1995; Tribollet et al., 1990). Therefore, age and sex differences in maternal behavior in mice could be in part due to changes in the expression of OXTR in the VMH and/or AOB (also high in the three males analyzed). These areas could modify how olfactory information from the pups is processed in females, removing a potential inhibitory behavioral response, or eliciting the onset of maternal behavior in mice. On the other hand, males might need high OXTR density in the AOB to process information related to the pregnant or parturient female in order to inhibit attacks to their offspring after delivery (Elwood, 1985; Tachikawa et al., 2013). Higher OXTR density in the VMH had also been found in adult rats (Lukas et al., 2010; Shapiro and Insel, 1989; Tribollet et al., 1989; Uhl-Bronner et al., 2005), but not in mice (Hammock and Levitt, 2013). However, in the study of Hammock and Levitt (2013) the authors used a low number of animals per age group, and concentrated their analysis in few brain regions (mainly hippocampus, neocortex, and septum). It is also important to note that the current study might have also been unable to detect smaller differences among age or sex groups due to the low number of animals (5–8) used in most comparisons (see also Bonferroni correction values). However, the absence of sex differences in VMH OXTR density in mice (apparently higher in male rats, Dumais et al., 2013; Uhl-Bronner et al., 2005) is likely the result of a species difference.

As previously shown by comparative and individual difference studies (Curley et al., 2012; Champagne et al., 2001; Olazábal and Young, 2006a,b), both up and down regulations of OXTR were found correlated to parental behavior. Interestingly, female mice show lower levels of OXTR in the LSv, an area associated with the inhibition of maternal behavior, where previous studies found a negative correlation between OXTR density and maternal behavior (Olazábal and Young, 2006a; Olazábal et al., 2013; Sheehan et al., 2000). Although, male and female adults did not significantly differ in the density of OXTR in the AOA, it is also interesting to mention that a lower OXTR density in males, but not females, compared to juveniles could have consequences in the processing of olfactory information, generating sex differences in the behavioral response toward newborns, perhaps related to the higher incidence of infanticidal behavior in adult males. Infanticidal behavior in males, but not females, could also be explained in part by the general reduction of OXTR in several brain regions, in particular the AOA, and perhaps also affected by the absence of a higher OXTR density in the VMH to stimulate maternal behavior (Ragnauth et al., 2005). However, an increase in infanticidal behavior is not always associated to a reduction in the density of OXTR in the brain. For example, higher infanticidal behavior in adult, compared to juvenile females, was not associated with changes in brain OXTR density in prairie voles (Olazábal and Young, 2006a,b, 2008).

Although, fluctuations in OXTR density in the AOB/AOA in males, and AOB/VMH/LSv in females, could eventually promote sex and developmental differences in behavior, those hypothesis need to be carefully investigated in experiments specifically designed to address those possibilities. The changes in OXTR density observed in the present study could be also related to other of the multiple functions in which OXT participates (e.g. sexual behavior, social recognition, and regulation of anxiety, Gimpl and Fahrenholz, 2001). Interestingly, a recent study in rats showed significant sex differences in OXTR density in many brain regions, some of them associated with social interest (Dumais et al., 2013). In contrast, in the current study in mice we did not find sex differences in most areas of the brain investigated. This disagreement is likely the result of species differences (e.g. prairie voles also show no sex differences, Olazábal and Young, 2008).

No OXTR developmental change was observed in other areas of the brain known to process olfactory and sensory information related to maternal behavior. As shown in rats (Lukas et al., 2010; Tribollet et al., 1989), OXTR density in the MA, brain region relevant for social recognition, maternal, and sexual behavior, did not change after weaning in mice. In fact, similar to the current finding in mice, previous studies in rats found little or no change in the expression of OXTR in all amygdaloid nuclei (Lukas et al., 2010; Tribollet et al., 1989). Other areas of the brain, known to be critical for maternal behavior (NA, MPOA), did not show any change in OXTR after weaning in mice. Obviously, an absence of change in OXTR density does not exclude the possibility that OXT acts in the NA, MPOA, or other areas of the brain, to facilitate maternal behavior. However, the current study showed that changes in OXTR density in those specific brain regions are not necessary to change the behavioral response toward newborns in mice. Obviously, we do not ignore the possibility that a different synthesis or local/synaptic release of OXT in these brain regions could contribute to age and sex differences in behavior. Post-weaning developmental changes in oxytocinergic fibers or release could be expected in certain brain regions, but unfortunately, that aspect has not been investigated so far. Besides, other neurochemical systems could be acting in areas of the brain where OXTR density does not change (MA, MPOA), modulating the information received or sent to the AOB, VMH or LSv. It is also possible that OXT acts on V1AR to induce some of its behavioral effects (Song et al., 2014). Maternal behavior is the result of the coordinated action of a large neural network and multiple neurochemical systems (Olazábal et al., 2013), but local and region specific changes in OXTR might be critical to modify the processing of information by this large network.

As previously mentioned, a higher OXTR density in juveniles was not correlated to higher parental behavior in mice. This result also suggested that significant increases or decreases in brain OXTR density would not necessarily affect parental behavior positively or negatively respectively. One possibility is that juvenile parental behavior in mice is blocked by OXT-independent mechanisms or for the lack of activation of OXTR in the immature neural substrate that supports maternal behavior in mice (e.g. VMH, AOB). We have recently shown that if juvenile females are exposed to maternal fluids of parturition and newborn siblings in an overlapping litter context, most juvenile females can rapidly display maternal behavior when tested individually housed in a novel cage (Alsina-Llanes et al., 2015). Whether OXT, acting in the MOB/AOB, mediates that behavioral change observed in juvenile mice exposed to maternal fluids is unknown. But that clearly shows that maternal behavior in juveniles can be displayed without the major developmental changes in OXTR density described in this study, and likely by modifying the neural substrate that supports maternal behavior in alternative ways to those hypothesized in adults.

The findings of the current study are also useful to understand the limitations of gene knock out and systemic pharmacological studies. Down or up regulation of the components of the OXT system in the whole brain, as used in knock out or transgenic studies, might generate mixed or not specific effects. This kind of global up and down regulations of the OXT system can produce effects that facilitate maternal behavior in certain areas of the brain while inhibit it in others. The resulting behavior could then be a mixture of inhibitory or stimulatory effects that eventually compensate. This might in part explain why OXT and OXTR knock out studies have been so controversial in terms of their findings. Minor, transient, inconsistent, or no deficit in maternal behavior has been found in several OXT and OXTR in KO mice (Kuroda

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K.O., personal communication; Macbeth et al., 2010; Nishimori et al., 1996; Pedersen et al., 2006; Takayanagi et al, 2005). Therefore, more naturalistic approaches, such as those used in Champagne et al. (2001), Olazábal and Young (2006a,b), and Dumais et al. (2013) could be more appropriate to reveal how the oxitocinergic system adapts in different species or individuals to achieve its biological functions.

In summary, these few brain regions (VMH, AOB, LSv, AOA), where correlations between OXTR density and parental or infanticidal behavior were found, need to be investigated in more detail. The current data suggest that behavioral responses can be the result of regional specific regulation of OXTR, rather than global increases or decreases in the expression of this receptor in many areas of the brain. It is also important, in order to understand how the OXT system works, to know or study the biology of the species in which the system is investigated, and consider that those species, even rats and mice, can differ significantly. This is particularly important considering the rapid and direct translations from rodent to humans commonly read in the scientific literature.

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