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Abbreviations: OXT, oxytocin; OXTR, oxytocin receptor; ES cells, embryonic stem cells; AVP, arginine vasopressin; AVPR1a, vasopressin V1a receptor.

Abstract

The oxytocin receptor (OXTR) and its ligand, oxytocin (OXT), regulate reproductive physiology (i.e., parturition and lactation) and sociosexual behaviors. To further define the function of OXTR, we generated mice with a null mutation in the *Oxtr* gene (*Oxtr*$^{-/-}$). *Oxtr*$^{-/-}$ mice were viable and had no obvious deficits in fertility or reproductive behavior. *Oxtr*$^{-/-}$ dams exhibited normal parturition, but demonstrated defects in lactation and maternal nurturing. Infant *Oxtr*$^{-/-}$ males emitted fewer ultrasonic vocalizations than wild-type littermates in response to social isolation. Adult *Oxtr*$^{-/-}$ males also showed deficits in social discrimination and elevated aggressive behavior. OXT-deficient (*Oxt*$^{-/-}$) males from *Oxt*$^{-/-}$ dams, but not from *Oxt*$^{+/-}$ dams, showed similar high levels of aggression. These data suggest a developmental role for OXTR in shaping adult aggressive behavior. Our study demonstrates that OXTR plays a critical role in regulating several aspects of social behavior, and may have important implications for developmental psychiatric disorders characterized by deficits in social behavior.

Text

Oxytocin (OXT), a nonapeptide hormone, was the first peptide hormone to have its structure determined and the first to be chemically synthesized in a biologically active form (1, 2). OXT is produced primarily in the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (3) and secreted mainly from the posterior pituitary gland. In addition, OXT fibers project to various brain regions (4) where OXT functions as a neurotransmitter or neuromodulator. Besides its classical functions, i.e., induction of labor and milk ejection, OXT plays an important role in social behavior (i.e., sexual behavior, maternal behavior, affiliation and social memory), the estrous cycle, penile erection, and ejaculation (4-7).

The actions of OXT are mediated via binding to the oxytocin receptor (OXTR). OXTR contains seven transmembrane domains and belongs to the class 1 family of G protein-coupled receptors. In response to ligand binding, OXTR mainly leads to stimulation of phospholipase C by interacting $G\alpha_{q/11}$. OXTR is widely expressed in the reproductive tract (i.e., uterus, mammary gland, ovary, testis, and prostate), brain, and kidney in mammals (4).

OXT-deficient (*Oxt*$^{-/-}$) mice displayed impairments in milk ejection (8, 9) and social recognition (10), but no obvious defects in parturition (8). Although OXT

is a strong uterotonin and is considered to drive parturition, our observations unexpectedly and clearly prove that OXT is not essential for labour in mice. In contrast, *Oxtr* mRNA expression in the uterus is upregulated dramatically at term (11), uterine sensitivity to OXT increases just before parturition (12, 13), and OXTR antagonists delay parturition in mice (14), suggesting an indispensable role for OXTR in mouse parturition. We therefore suspected that a functional redundancy supporting the OXT system might compensate for the defective *Oxt* gene in *Oxt*$^{-/-}$ mice. Furthermore, growing evidence suggests a role for OXTR in modulation of social behaviors. To further study the function of OXT/OXTR system, we generated mice lacking OXTR (*Oxtr*$^{-/-}$) and evaluated the reproductive functions including parturition and sociosexual behaviours. In addition, we further compared their maternal and male aggressive behaviors with that of previously generated ligand *Oxt*$^{-/-}$ mice.

Materials and Methods

Generation of *Oxtr*$^{-/-}$ Mutant Mice and Genotyping. To construct the targeting vector, mouse 129/Sv strain-derived genomic clones (11) were used. The targeting vector was designed to substitute exons 2 and 3, containing most of the *Oxtr* coding region with the same sequence and phosphoglycerate kinase promoter-neomycin resistance cassette (PGK-Neo) flanked by three *lox*P sites (Fig. 1*A*). A 6.1 kb *Xho*I-*Bam*HI fragment was used as the 5' homology region; a 2.2 kb *Bam*HI-*Sph*I fragment containing exons 2 and 3 was inserted between two *lox*P sites; and a 2.8 kb *Sph*I-*Sph*I fragment was used as the 3' homology region. An MC1 promoter-herpes simplex virus-thymidine kinase cassette (MC1-TK) was used for negative selection. We linearized this construct with *Sal*I and electroporated it into E14TG2a embryonic stem (ES) cells. G418 and FIAU (Moravek Biochemicals) doubly resistant clones were screened by Southern blot analysis. We generated chimeric mice by microinjection of heterozygous ES clones into C57BL/6J blastocysts. We mated chimeric males to CAG-*cre* transgenic female mice (15) to yield *Oxtr*$^{+/-}$ mice. Offspring from intercrosses of heterozygous littermates were genotyped by Southern blot analysis (Fig. 1*B*). The care and use of mice in this study was

approved by the Institutional Animal Care and Use Committee of Tohoku University.

Mice. In addition to *Oxtr*$^{-/-}$ mice generated in the present study, we also used *Oxt*$^{-/-}$ mice generated previously (8), in order to distinguish between the role of the ligand, OXT, and that of the receptor, OXTR, in the regulation of maternal and aggressive behaviors. *Oxtr*$^{-/-}$, *Oxtr*$^{+/-}$ and *Oxtr*$^{+/+}$ mice were maintained on a mixed 129 × C57BL/6J background. *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice used in this study were descended from a mixed 129 × C57BL/6J strain as previously described (8). In the maternal behavior and aggressive behavior tests, we used *Oxtr*$^{-/-}$ and *Oxtr*$^{+/+}$ mice, and *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice from heterozygous intercrosses. For analysis of the potential effects of maternal OXT, we also used intercrosses of homozygous *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice to generate *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice from homozygous parents followed by cross-fostering with C57BL/6J females.

Southern Blot and Northern Blot Analysis. We isolated genomic DNA from mouse tail and poly(A)$^{+}$ RNA from tissues using TRIzol Reagent (Invitrogen) and oligotex-dT30/super (Takara). For Southern blot analysis, about 3 μg of DNA, digested with *Sac*I, was loaded on 1% agarose gels. For Northern blot analysis,

equal amounts of RNA (uterus, 2 μg; brain, 5 μg and 20 μg per lane) were loaded on formaldehyde agarose gels. These were subjected to electrophoresis, and transferred to Byodyne B nylon membranes (Pall). The membranes were hybridized to ^{32}P-labeled probes. Probes for Southern blots were obtained by digestion with restriction enzyme, and probes for Northern blots were obtained by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) [*Oxtr* probe A and B, spanning from 170 nucleotides (nt) to 429 nt (259 base pairs (bp)) and from 500 nt to 787 nt (288 bp) in the mouse *Oxtr* mRNA (GenBank accession number D86599) coding region, respectively; glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) probe, spanning from –8 nt to +1065 nt (1073 bp) of the mouse *Gapd* mRNA (GenBank accession number NM_008084) coding region] and labeled with Megaprime DNA labeling systems (Amersham Biosciences) with [^{32}P]-dCTP. Membranes were stripped and reprobed for *Gapd* to ensure equal loading.

Maternal Behavior Test. Maternal behavior was tested on both postpartum and virgin females. All postpartum females (10-15 weeks old) were individually housed once pregnant. Nest material was provided 1 day prior to testing. Births were recorded each morning. Each new mother was observed for 20

minutes with minimal disturbance. 1 hour after the removal of her pups, each female was exposed to three 1-3-day-old foster pups from an *Oxtr*$^{+/+}$ dam, which were placed in each corner of the cage distant from her nest. During the next 30 minutes, each female was continuously observed, and the following data points were recorded: latency to sniff and retrieve each pup, and latency and duration of crouching over all three pups in the nest. Different pups were used in each test. All virgin females (7-9 weeks old) were individually housed for 2 days prior to testing. For 2 consecutive days, each female was exposed to three 1-3-day-old foster pups for 30 minutes as described above. Only the second test was scored.

Ultrasonic Vocalization Test and Measurement of Locomotor Activity in Pups. 7-day-old male pups from 7 *Oxtr*$^{+/-}$ breeding pairs were tested between 1-4 hours before the dark phase. The parents were removed from the home cage 20 minutes prior to testing and the cage was placed on a heated surface at 35°C until testing was completed. Each pup was placed into a Plexiglass recording chamber (40 $\times$ 40 cm) for 3 minutes. Vocalizations were recorded using an ultrasonic detector and analyzed as WAV files (16). The number of 4.5 $\times$ 4.5 cm grids crossed during the test was also noted.

Social Discrimination Test. Prior to testing, males (4-7 months old) were individually housed and exposed to ovariectomized females for varying periods of time (15-40 minutes) for 2 days in order to reduce mating bouts during test sessions. The social discrimination test (17) consisted of placing an ovariectomized C57BL/6J stimulus female into the home cage of the experimental male for 5 minutes. After a 30 minute interexposure interval, the female (SAME) was then placed back into the cage along with another C57BL/6J ovariectomized stimulus female (NOVEL) for 5 minutes. The amount of time spent investigating each females anogenital or perioral area was then scored from the video-recorded session. Interactions including sexual behaviour were excluded from the analysis. Females were only exposed to one male per day to reduce male odour contamination.

Resident-Intruder Aggression Test. 10-week-old resident males were individually housed for about 4 weeks before testing. 10-week-old C57BL/6J mice, housed in groups, were used as intruders. Two tests of 5 minutes each with a 5 minute interval were performed. New intruder mice were used in each

test. The following data points were recorded: attack duration, frequency and latency.

For further details, see *Supporting Text*, which is published as supporting information on the PNAS web site.

Results

Generation of *Oxtr*$^{-/-}$ Mutant Mice. To analyze the roles of OXTR in the reproductive and central nervous systems, we generated OXTR-deficient mice by gene targeting (Fig.1*A*). The disruption of the *Oxtr* gene locus (Fig. 1*B*), the absence of *Oxtr* transcripts (Fig. 1*C*), and the absence of OXTR binding (Fig. 1*D*) in *Oxtr*$^{-/-}$ mice confirmed that the recombined allele is null. A 1:2:1 Mendelian distribution of the progeny was observed [(*Oxtr*$^{+/+}$:*Oxtr*$^{+/-}$:*Oxtr*$^{-/-}$), 65:133:69 (males); 79:133:78 (females)].

Reproductive Functions in *Oxtr*$^{-/-}$ Mice. In *Oxtr*$^{-/-}$ mice, OXT-induced contractions in pregnant myometrium were not evident (Fig. 2*A*). Since arginine vasopressin (AVP), another nonapeptide hormone synthesized in the PVN and SON and secreted from the posterior pituitary, acts as a partial agonist of OXTR (18, 19) and also stimulates uterine contractions (20, 21), we also examined AVP-induced myometrium contractions in *Oxtr*$^{-/-}$ mice. The myometrium of *Oxtr*$^{-/-}$ mice did not respond to AVP (Fig. 2*B*). Receptor autoradiography confirmed the absence of OXTR binding binding in pregnant myometrium of *Oxtr*$^{-/-}$ mice (Fig. 1*D* and *Supporting Text* which are published as supporting information on the PNAS web site). We previously reported the inability to

detect *Avpr1a* mRNA in the uterus of wild-type mouse using RT-PCR (21). Taken together, these findings prove that AVP-induced uterine contractions in pregnant wild-type mice are mediated solely by OXTR, consistent with our previous report (21).

To examine reproductive function, $Oxtr^{-/-}$ and $Oxtr^{+/+}$ mice were mated in all possible combinations. Contrary to our prediction, the onset and the duration of labor were normal in $Oxtr^{-/-}$ females (Fig. 2*C*). Furthermore, $Oxtr^{-/-}$ mice exhibited normal rates of mating and pregnancy, and litter sizes, demonstrating that OXTR is not essential for either male or female reproductive function. However, all offspring from $Oxtr^{-/-}$ dams died within 24hr after birth, regardless of the genotype of the offspring (Fig. 2*C*). This mortality was likely due to defects in lactation since milk was not observed in the digestive tract of pups from $Oxtr^{-/-}$ dams. All offspring from $Oxtr^{-/-}$ mice were successfully fostered to $Oxtr^{+/+}$ mice, and thus the survival defect in the pups lies entirely with the $Oxtr^{-/-}$ dams. Histological analysis of mammary glands in $Oxtr^{-/-}$ females indicated that the development of mammary tissues during gestation and milk production were normal, except accumulation of milk in ducts of the postpartum mammary glands (data not shown). Thus, $Oxtr^{-/-}$ females failed to lactate, similar to $Oxt^{-/-}$ females (8).

***Oxtr*$^{-/-}$ Female Mice Display Defects in Maternal Nurturing.** Since OXT plays a role in maternal behavior (7), we examined maternal behavior in *Oxtr*$^{-/-}$ mice. Initially, the behavior of postpartum females was observed for 20 minutes in their home cages. Both *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ females built nests and spent the majority of this period crouching over their pups ($P > 0.05$), but pups of *Oxtr*$^{-/-}$ females were often found scattered around the cage ($P < 0.01$) (Fig. 3*A*). Following this initial observation, we monitored the dams' responses to three pups placed in different corners of the cage for 30 minutes. *Oxtr*$^{-/-}$ dams displayed a significantly longer latency to retrieve the pups (first retrieval, $P > 0.05$; second retrieval, $P < 0.05$; and complete retrieval, $P < 0.01$) or to crouch over the pups ($P < 0.01$), and spent less time crouching over the pups ($P < 0.05$), than *Oxtr*$^{+/+}$ females (Fig. 3*B*). The impairment of retrieval was not due to the failure of the mother to detect the pups, since latency to approach and sniff their offspring was similar to wild-type mothers (11.7 ± 5.0 s compared with 9.9 ± 4.2 s; $P > 0.05$). Additionally, virgin *Oxtr*$^{-/-}$ females displayed a similar phenotype (Fig. 3*C*), suggesting that OXTR is required for nurturing responses to pups outside the physiological context of pregnancy and parturition. Interestingly, we also demonstrated that both postpartum and virgin *Oxt*$^{-/-}$ females displayed normal

maternal behavior (data not shown), consistent with an earlier study (22). This could be explained by other ligands, such as AVP, activating this receptor.

The decrease in maternal behavior of postpartum *Oxtr*$^{-/-}$ females could be explained as reflection of the inability to lactate. However, our data showed that virgin *Oxtr*$^{-/-}$ females that have not experienced lactation, displayed an impairment of maternal behavior (Fig. 3*C*), and *Oxt*$^{-/-}$ females showed normal maternal behavior despite their inability to lactate (data not shown). These results strongly suggest that any deficits in maternal behavior in these mice would be a clear indication of a specific behavioral deficit.

Decreased Ultrasonic Vocalizations and Increased Locomotor Activity in Infant *Oxtr*$^{-/-}$ Males. Next, we examined isolation-induced ultrasonic vocalizations and locomotor activity of infant *Oxtr*$^{-/-}$ males. *Oxtr*$^{-/-}$ males, like *Oxt*$^{-/-}$ males (16), emitted significantly less calls than did wild-type littermates ($P < 0.05$) (Fig. 4*A*), while displaying significantly higher levels of locomotor activity during the test ($P < 0.05$) (Fig. 4*A*). These results suggest that perhaps *Oxtr*$^{-/-}$ males are less distressed by social isolation and shift their behavior toward more exploratory activity than do wild-type littermates.

Social Amnesia in *Oxtr*$^{-/-}$ Mice. Since *Oxt*$^{-/-}$ mice display social amnesia (10), we examined social discrimination in adult *Oxtr*$^{-/-}$ males. Males were initially exposed to an ovariectomized C57BL/6J female. After a 30 minute separation, the male was simultaneously exposed to this same female and a novel female of the same strain. As expected, *Oxtr*$^{+/+}$ males spent significantly more time investigating the novel compared to the familiar female ($P < 0.05$) (Fig. 4*B*), and therefore were able to discriminate between the two. However, *Oxtr*$^{-/-}$ males spent a similar amount of time investigating both females ($P > 0.05$) (Fig. 4*B*) suggesting an impairment of social discrimination. There was no difference between the two genotypes in the amount of time spent investigating the initial female ($P > 0.05$), indicating that the *Oxtr*$^{-/-}$ males' deficit was not due to a difference in exposure time or motivation to investigate a female (data not shown). However, *Oxtr*$^{-/-}$ males were able to discriminate outbred CD-1 stimulus females (data not shown), suggesting that the deficit represents an impairment rather than a complete disruption in social recognition.

***Oxtr*$^{-/-}$ Males Display High Levels of Aggression Due To the Lack of OXTR Activation During Prenatal Development.** Since we observed more wounded mice in group-housed males from cages containing *Oxtr*$^{-/-}$ mice than in cages

containing only *Oxtr*$^{+/+}$ mice (Fig. 5*A*), we assessed aggressive behavior using the resident-intruder test. *Oxtr*$^{-/-}$ resident males attacked the intruder with shorter latency ($P < 0.05$), for longer duration (test1, $P < 0.05$; and test2, $P > 0.05$), and with higher frequency (test1, $P < 0.05$; and test2, $P < 0.05$) compared to *Oxtr*$^{+/+}$ residents (Fig. 5*B*). In contrast, in adjacent cages containing male *Oxt*$^{-/-}$ mice, the rate of wounded mice was similar to that in *Oxt*$^{+/+}$ mice (Fig. 5*A*). Furthermore, aggressive behavior of *Oxt*$^{-/-}$ mice in the resident-intruder test was indistinguishable from *Oxt*$^{+/+}$ males (Fig. 5*C*).

To investigate this discrepancy in aggression phenotypes between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice, we examined possible compensatory effects of the *Oxtr* mutation. OXTR and AVPR1a autoradiography in the brain showed no OXTR binding in *Oxtr*$^{-/-}$ mice and the density of AVPR1a -binding was similar between *Oxtr*$^{+/+}$ (5826 $\pm$ 602.3 dpm/mg) and *Oxtr*$^{-/-}$ (4787 $\pm$ 463 dpm/mg) mice (Fig. 6*A* and *B*). Northern blot analysis failed to detect any RNA products with sequence similarity to *Oxtr* mRNA in the brain (Fig. 6*C*). In addition, *Oxtr*$^{-/-}$ mice showed no differences in *Oxt* and *Avp* mRNA expression in the hypothalamus (data not shown), pituitary OXT (*Oxtr*$^{+/+}$, 202.6 $\pm$ 10.7 ng/pituitary, n=9; *Oxtr*$^{-/-}$, 222.8 $\pm$ 9.3 ng/pituitary, n=7) and AVP (*Oxtr*$^{+/+}$, 471.6 $\pm$ 70.9 ng/pituitary, n=9; *Oxtr*$^{-/-}$, 453.3 $\pm$ 28.3 ng/pituitary, n=7), plasma OXT (*Oxtr*$^{+/+}$, 36.3 $\pm$ 5.2 pg/ml, n=5; *Oxtr*$^{-/-}$, 39.3 $\pm$

6.1 pg/ml, n=5), or plasma testosterone levels (*Oxtr*$^{+/+}$, 1329.4 ± 277.7 pg/ml, n=10; *Oxtr*$^{-/-}$, 1148.7 ± 225.5 pg/ml; n=10). These results indicated that there were no apparent dysregulations of AVP, AVPR1a or testosterone, each of which are known to influence aggression.

OXT in the dam can transfer through the placental barrier (23-25), and *Oxtr* mRNA is present in the mouse brain during embryonic development (Fig. 6*D*). Furthermore, perinatal injections of OXT in prairie voles have an impact on adult behavior (26). Therefore, we hypothesized that *in utero* exposure to OXT might have rescued the aggression phenotype in *Oxt*$^{-/-}$ mice derived from heterozygous matings. Therefore, we analyzed aggression in *Oxt*$^{-/-}$ mice generated from homozygous matings (Fig. 6*E*), creating an OXT-free developmental environment. Like *Oxtr*$^{-/-}$ males, *Oxt*$^{-/-}$ males generated from homozygous matings exhibited highly aggressive behavior, as previously reported (16). Our findings indicate that embryonic exposure to OXT affects the development of aggression in adulthood, although other intrauterine factors in *Oxt*$^{-/-}$ dams could also influence aggression. However, since increased aggression is seen in *Oxtr*$^{-/-}$ mice from *Oxtr*$^{+/-}$ mothers, it suggests that the defect lies in the pup.

Discussion

This study provides a comprehensive analysis of mice lacking the *Oxtr* gene. Although OXT has been used to induce or augment labor in humans, and the OXT antagonist delays labor in wild-type outbred mice (14), parturition is initiated and proceeds normally in *Oxtr*$^{-/-}$, similar to *Oxt*$^{-/-}$ mice (8). Although this unexpected phenotype may be due to functional redundancy in the OXT signaling system or a compensatory effect resulting from the absence of *Oxtr* throughout development, it is clear that the OXT signaling pathway is not essential for normal parturition in mice.

The impairment in social discrimination in *Oxtr*$^{-/-}$ in our study is consistent with previous results from ligand knockout mice. *Oxt*$^{-/-}$ females as well as *Oxt*$^{-/-}$ males also show significant deficit in social recognition (10, 27). The comparison of social discrimination in *Oxtr*$^{-/-}$ mice between genders would be important for understanding the regulation of social discrimination that is related to estrogen, gonadal steroid (27).

In contrast to our results, it was reported by another group that *Oxt*$^{-/-}$ males from *Oxt*$^{+/-}$ dams displayed reduced aggression (28). This contradiction could be caused by differences in the targeting construct used, which did not result in the complete loss of Oxt peptide, or in the testing paradigm used (9, 28). The

study reporting decreased male aggression performed the tests in a neutral arena, while our study and others reporting increased aggression used a resident intruder paradigm. Therefore the changes in aggression due to disruption of the OXT system may be context dependent"

In both aggressive behavior and maternal behavior, phenotypes were different between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice. *Oxtr*$^{-/-}$ males from *Oxtr*$^{+/-}$ dams displayed elevated aggressive behavior (Fig. 5*B*). *Oxt*$^{-/-}$ males from *Oxt*$^{-/-}$ dams, but not from *Oxt*$^{+/-}$ dams, displayed similar high levels of aggression (Fig. 5*C* and 6*E*). This indicates that *in utero* exposure to maternal OXT may affect adult aggressive behavior. In addition, maternal behavior in *Oxtr*$^{-/-}$ females from *Oxtr*$^{+/-}$ dams was impaired (Fig. 3*A*-*C*), but *Oxt*$^{-/-}$ females from *Oxt*$^{+/-}$ or *Oxt*$^{-/-}$ dams showed normal maternal behavior (data not shown). These results suggest that although prenatal activation of OXT/OXTR system may significantly affect adult aggressive behavior in males, it is not sufficient for the establishment of maternal behavior. Thus, these causative mechanisms seem to be different between aggressive behavior and maternal behavior. We speculate that the difference in phenotypes of maternal behavior between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice can be

explained by a possibility that other ligands than OXT that activate OXTR can compensate for the defect of the *Oxt* gene.

Semi-natural environment-housed *Oxt*$^{-/-}$ females from *Oxt*$^{+/-}$ dams showed high levels of aggression and infanticidal behaviors, unlike cage-housed *Oxt*$^{-/-}$ females from *Oxt*$^{+/-}$ dams (29). These results indicate that postnatal environment also affects the behavior via OXT/OXTR system. In semi-natural environment, phenotypes of *Oxtr*$^{-/-}$ mice, such as impaired maternal behavior or elevated aggressive behavior might be altered, and *Oxt*$^{-/-}$ males from *Oxt*$^{+/-}$ dams might be aggressive. These studies suggest an important interaction between environment and the OXT/OXTR system in regulating social behavior.

Our observations demonstrated that the OXT/OXTR system plays an important role in regulating social behavior, and might have important implications for human behavioral disruptions. Further comprehensive investigation of *Oxtr*$^{-/-}$ mice may provide new insight into the neurobiological mechanisms resulting in psychiatric disorders associated with disruptions in social behavior, including autism.

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Figure Legends

Fig. 1. Generation of *Oxtr* $^{-/-}$ mice. (*A*) The *Oxtr* locus and gene targeting constructs. Exons (E) are indicated by boxes (white boxes, 5' and 3' UTRs; gray boxes, coding regions). Positions of restriction enzyme sites and the probes used for Southern blot analysis are shown (*X*, *Xho*I; *S*, *Sph*I; *Sa*, *Sac*I; *B*, *Bam*HI). The *lox*P sites are represented by arrowheads (not to scale). PGK-Neo, phosphoglycerate kinase promoter-neomycin resistance cassette; MC1-TK, thymidine kinase cassette. (*B*) Southern blot analysis of genomic DNA from littermate progeny from *Oxtr* heterozygote crosses. *Sac*I-digested tail DNA was hybridized with the radiolabeled probes indicated in (*A*). (*C*) Northern blot analysis of poly(A)$^{+}$ RNA from the pregnant uteri (day 19 of gestation) of *Oxtr*$^{+/+}$, *Oxtr*$^{+/-}$ and *Oxtr*$^{-/-}$ mice. The blot was sequentially hybridized with *Oxtr* probe A and a *Gapd* probe. (*D*) OXTR-binding autoradiograms in the pregnant uteri (day 19 of gestation) of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice.

Fig. 2. Reproductive functions in *Oxtr* $^{-/-}$ mice. (*A* and *B*) The amplitude of OXT (*A*)- or AVP (*B*)-stimulated contractions of myometrial strips isolated from pregnant mice (day 19 of gestation) of each genotype. These investigations were performed as previously described (21). (*C*) The profile of reproductive

functions in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice (male, 10-25 weeks old; female, 10-15 weeks old). Each genotype was mated and females were selected without reference to ovulatory cycle. Mating rate denotes the ratio of plugged females to matings and pregnancy rate denotes the ratio of pregnant females to plugged females. The morning of finding the copulation plug was designated as day 0.5 of gestation. The data represents mean ± SEM.

Fig. 3. Maternal nurturing in female *Oxtr* $^{-/-}$ mice. (*A*) Observation of newly postpartum *Oxtr*$^{-/-}$ (n=9) and *Oxtr*$^{+/+}$ (n=10) females before tests for maternal behavior. Time crouching over pups and percentage of newborns scattered was recorded. (*B* and *C*) Tests for maternal behavior. Latency to retrieve each pup, and latency and duration of crouching over all three pups of *Oxtr*$^{-/-}$ (n=9) and *Oxtr*$^{+/+}$ (n=10) postpartum females (*B*), and *Oxtr*$^{-/-}$ (n=15) and *Oxtr*$^{+/+}$ (n=7) virgin females (*C*) from heterozygous intercrosses. Failure to retrieve or crouch was assigned as 30 minutes, the length of observation period. *$P < 0.05$ and **$P < 0.01$ (Mann-Whitney *U*-test). Error bars, standard error.

Fig. 4. Infant ultrasonic vocalization and adult social discrimination. (*A*) Measurements of social isolation-induced ultrasonic vocalizations (left) and

locomotor activity (right) in *Oxtr*$^{-/-}$ (n=8) and *Oxtr*$^{+/+}$ (n=10) male pups from heterozygous intercrosses. (*B*) Test for social discrimination test. After the first exposure to a female and an interexposure interval, this female (SAME) was placed back along with another female (NOVEL). *Oxtr*$^{-/-}$ (n=14) and *Oxtr*$^{+/+}$ (n=10) males were examined for investigation times directed to the SAME or NOVEL females. *$P < 0.05$ (Mann-Whitney *U*-test). Error bars, standard error.

Fig. 5. Aggressive behavior as measured by the resident-intruder test. (*A*) The number of wounded mice (3-9 months old) in cages including each genotype. (*B* and *C*) Aggressive behavior of *Oxtr*$^{-/-}$ (n=9) and *Oxtr*$^{+/+}$ (n=9) mice (*B*), and *Oxt*$^{-/-}$ (n=11) and *Oxt*$^{+/+}$ (n=9) mice (*C*) from heterozygous intercrosses in the resident-intruder test. Attack duration, frequency and latency, and latency to first attack were recorded. *$P < 0.05$ (Mann-Whitney *U*-test). Error bars, standard error.

Fig. 6. Causal analysis of aggressiveness between *Oxtr*$^{-/-}$ and *Oxt*$^{-/-}$ mice. (*A* and *B*) OXTR (*A*) and AVPR1a (*B*)-binding autoradiograms in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ brain. (*C*) Northern blot analysis of *Oxtr* mRNA expression in *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ brain using *Oxtr* probe A and B. The same membrane was rehybridized with a

Gapd probe. Indicated amounts of poly(A)$^{+}$ RNA were used. (*D*) RT-PCR analysis for *Oxtr* and *Arbp*, in the brain of male fetus (C57BL/6J) and adult *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ males. (*E*) Aggressive behavior of *Oxt*$^{-/-}$ (n=8) and *Oxt*$^{+/+}$ (n=7) mice, from intercrosses of each of *Oxt*$^{-/-}$ and *Oxt*$^{+/+}$ mice and fostering by C57BL/6J females, in the resident-intruder test. **P* < 0.05 (Mann-Whitney *U*-test). Error bars, standard error.

Fig.1.

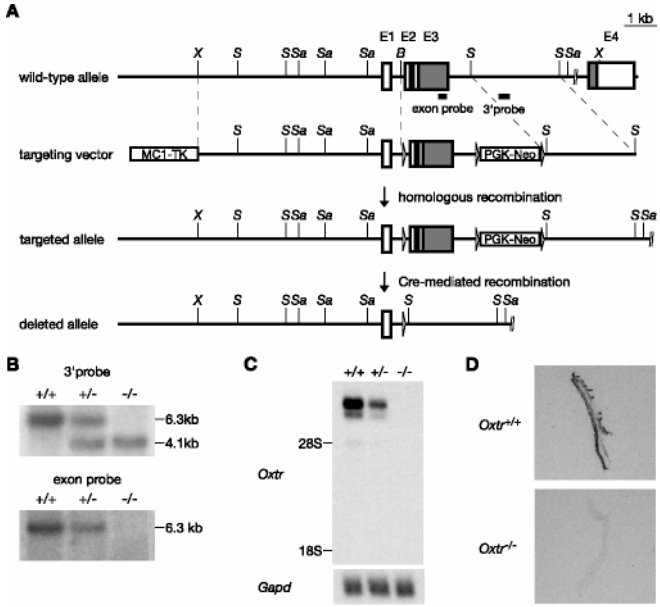
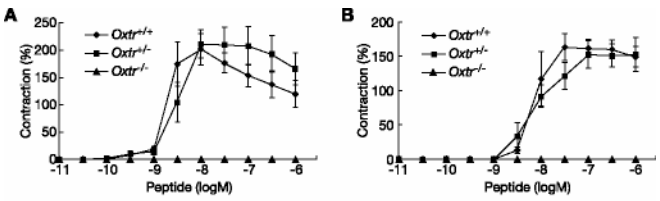


Fig.2.



C

Effect of genotype on reproductive functions

Fertility and sexual behavior in male and female mice

Pairing Male	Pairing Female	No. of mating trials	Mating rate (%)	Pregnancy rate (%)	Average litter size
Oxtr$^{+/+}$	*Oxtr*$^{+/+}$	79	35	71	7.5±0.7
Oxtr$^{-/-}$	*Oxtr*$^{+/+}$	63	41	65	8.2±0.9
Oxtr$^{+/+}$	*Oxtr*$^{-/-}$	43	42	67	8.6±0.5
Oxtr$^{-/-}$	*Oxtr*$^{-/-}$	82	33	59	8.4±0.8

Parturition and maternal behavior in female mice

Pairing Male	Pairing Female	No. of pregnant females	Onset of labor (day)	Duration of labor (hour)	Postnatal survivors per total births (%)
Oxtr$^{+/+}$	*Oxtr*$^{+/+}$	11	19.1±0.1	3.8±0.4	96
Oxtr$^{-/-}$	*Oxtr*$^{+/+}$	10	19.2±0.1	4.8±0.9	92
Oxtr$^{+/+}$	*Oxtr*$^{-/-}$	10	19.2±0.1	4.8±0.9	0
Oxtr$^{-/-}$	*Oxtr*$^{-/-}$	13	19.3±0.1	3.2±0.2	0

Fig.3.

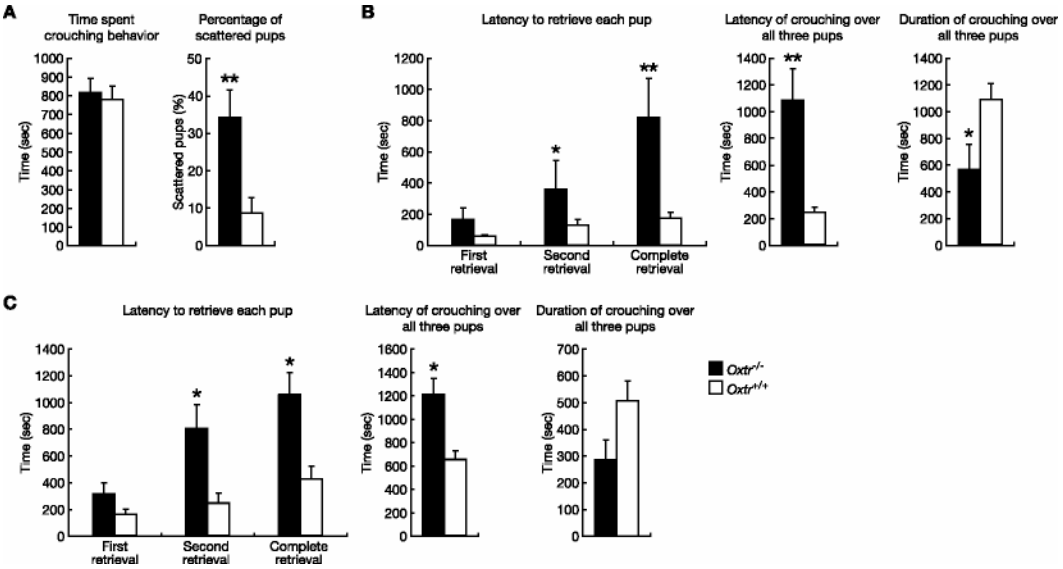

A
Time spent crouching behavior
Time (sec)
Percentage of scattered pups
Scattered pups (%)
B
Latency to retrieve each pup
Time (sec)
First retrieval
Second retrieval
Complete retrieval
Latency of crouching over all three pups
Duration of crouching over all three pups
C
Latency to retrieve each pup
Latency of crouching over all three pups
Duration of crouching over all three pups
Oxtr-/-
Oxtr+/+

Fig.4.

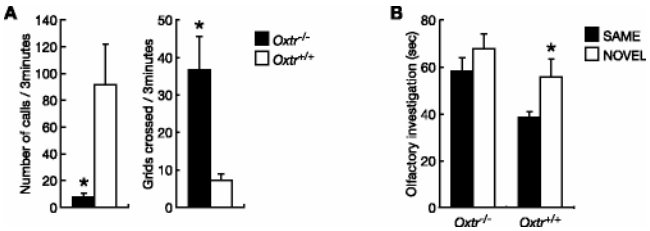

A
Number of calls / 3minutes
Grids crossed / 3minutes
Oxtr-/-
Oxtr+/+
B
Olfactory investigation (sec)
SAME
NOVEL
Oxtr-/-
Oxtr+/+

Fig.5.

A

Wounded mice					
Genotypes of members in the same cage	No. of observed mice	Wounded mice	No-wounded mice	Wounded / total (%)	Mice / cage
Oxtr$^{+/+}$	37	1	36	2.7	4.1
Oxtr$^{+/+}$, *Oxtr*$^{+/-}$, *Oxtr*$^{-/-}$	54	18	36	33.3	4.0
Oxt$^{+/+}$	21	0	21	0	3.5
Oxt$^{+/+}$, *Oxt*$^{+/-}$, *Oxt*$^{-/-}$	62	2	60	3.2	4.1

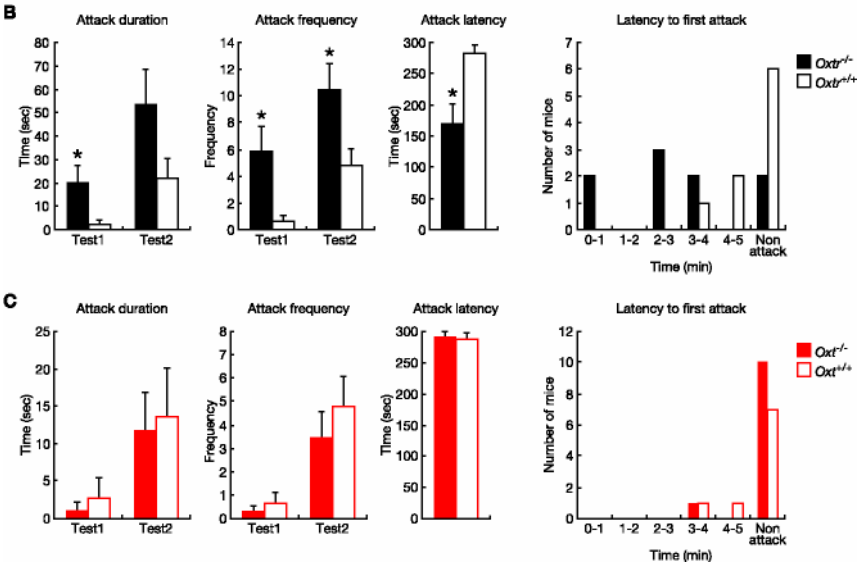
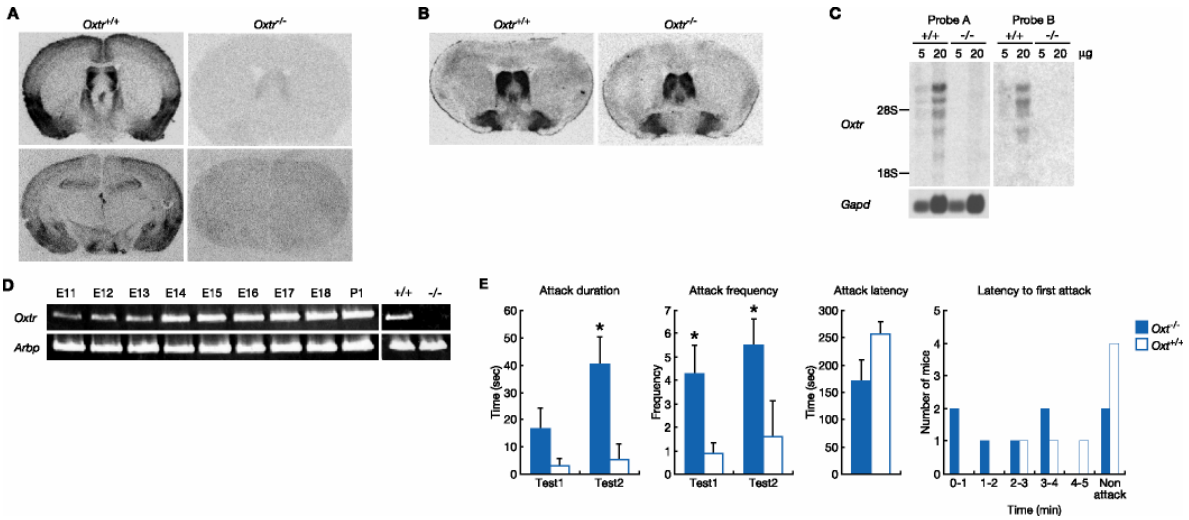


Fig.6.



Supporting Figure 7 Legend

Fig. 7. ^{125}I Linier-AVP binding autoradiograms in the pregnant uteri (day 19 of gestation) of *Oxtr*$^{+/+}$ and *Oxtr*$^{-/-}$ mice. The binding present in *Oxtr*$^{+/+}$ mouse uterus likely reflects binding of the radioligand to the OXTR since this ligand has a low affinity for the OXTR and RT-PCR revealed no vasopressin receptor mRNA in the pregnant uterus.