Oxytocin receptor knockout mice display deficits in the expression of autism-related behaviors

Roger L.H. Pobbe[a,*], **Brandon L. Pearson**[a], **Erwin B. Defensor**[a], **Valerie J. Bolivar**[b,c], **W. Scott Young III**[d], **Heon-Jin Lee**[e], **D. Caroline Blanchard**[a,f], and **Robert J. Blanchard**[a,g]

[a] Pacific Biosciences Research Center, University of Hawaii,1993 East-west Road, Honolulu, HI 96822, USA

[b] Wadsworth Center, New York State Department of Health, Albany, NY 12208, USA

[c] Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Albany, NY, USA

[d] Section on Neural Gene Expression, NIMH, NIH, DHHS, Bethesda, MD 20892, USA

[e] Department of Oral Microbiology, School of Dentistry, Kyungpook National University, Daegu, South Korea

[f] Department of Genetics and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96822, USA

[g] Department of Psychology, University of Hawaii, 2430 Campus Road, Honolulu, HI 96822, USA

Abstract

A wealth of studies has implicated oxytocin (Oxt) and its receptors (Oxtr) in the mediation of social behaviors and social memory in rodents. It has been suggested that failures in this system contribute to deficits in social interaction that characterize autism spectrum disorders (ASD). In the current analyses, we investigated the expression of autism-related behaviors in mice that lack the ability to synthesize the oxytocin receptor itself, *Oxtr* knockout (KO) mice, as compared to their wild-type (WT) littermates. In the visible burrow system, *Oxtr* KO mice showed robust reductions in frontal approach, huddling, allo-grooming, and flight, with more time spent alone, and in self-grooming, as compared to WT. These results were corroborated in the three-chambered test: unlike WT, *Oxtr* KO mice failed to spend more time in the side of the test box containing an unfamiliar CD-1 mouse. In the social proximity test, *Oxtr* KO mice showed clear reductions in nose to nose and anogenital sniff behaviors oriented to an unfamiliar C57BL/6J (B6) mouse. In addition, our study revealed no differences between *Oxtr* WT and KO genotypes in the occurrence of motor and cognitive stereotyped behaviors. A significant genotype effect was found in the scent marking analysis, with *Oxtr* KO mice showing a decreased number of scent marks, as compared to WT. Overall, the present data indicate that the profile for *Oxtr* KO mice, including consistent social deficits, and reduced levels of communication, models multiple components of the ASD phenotype. This article is part of a Special Issue entitled Oxytocin, Vasopressin, and Social Behavior.

Keywords

Autism; Oxytocin receptor; Social behavior; Mouse models

*Corresponding author. Fax: +1 808 956 9612. rogerlh@hawaii.edu. .

Introduction

Oxytocin (Oxt) is a nine-amino acid peptide synthesized in the brain, more specifically in the hypothalamic supraoptic and paraventricular nuclei, and secreted mainly from the posterior pituitary gland into the peripheral circulation (for review see Lee et al., 2009; Ross and Young, 2009). The classical physiological roles of peripherally released Oxt include stimulation of smooth muscle contractions in the uterus during labor and facilitation of milk ejection during lactation (Burbach et al., 2006; Mann and Bridges, 2001). In addition to its classical functions, Oxt released within the central nervous system regulates complex behavioral functions, especially in the context of social interactions (Insel, 2010; Veenema and Neumann, 2008). Extensive studies using several different species established the key role of Oxt in the mediation of pro-social behavior, including the onset of maternal care (Leng et al., 2008; Pedersen and Prange, 1979), pair bonding (Donaldson and Young, 2008; Lim and Young, 2006) and social recognition (Ferguson et al., 2000; Insel and Fernald, 2004).

Oxt produces its biological effects by attaching to and activating its receptor (Gimpl and Fahrenholz, 2001). The Oxt receptor (*Oxtr*) is widely expressed in reproductive-related tissues including the mammary gland, uterus, ovary, testis, and prostate, as well as in many nonreproductive tissues such as the kidney, heart, vascular endothelium and brain (Gould and Zingg, 2003; Zingg and Laporte, 2003). In the brain, the *Oxtr* is abundant in areas such as the amygdala, hippocampus, olfactory lobe, and hypothalamus (Gould and Zingg, 2003), in which it has been implicated in the regulation of many of the social, emotional and neurondocrine behaviors that Oxt is believed to influence. Mice that lack the ability to synthesize the *Oxtr* itself, *Oxtr* knockout (KO) mice (for details see Lee et al., 2010), have further extended our knowledge of the role of Oxt as a central mediator of complex behaviors. Two groups have generated *Oxtr* KO mouse strains and subsequently characterized their behavioral performance (Lee et al., 2008; Takayanagi et al., 2005). Male *Oxtr* KO mice generatedby Takayanagi et al. (2005) emitted fewer ultrasonic vocalizations than wild-type (WT) littermates in response to social isolation. In addition, unlike WT controls, these male *Oxtr* KO mice continued to investigate a previously presented 'familiar' ovariectomized C57BL/6J (B6) female as if she were 'novel', indicative of a deficit in social recognition. Lee et al. (2008) developed two *Oxtr* KO mouse strains: one that lacked the Oxtr in all body tissues (*Oxtr*$^{-/-}$) and a relatively specific forebrain KO (*Oxtr*$^{FB/FB}$). The latter KO strain has reduced Oxtr binding beginning at postnatal day (PND) 21 in most if not all forebrain regions (central and medial amygdalar nuclei, hippocampus, perirhinal cortex, and piriform cortex) (Pagani et al., 2011). Macbeth et al. (2009) evaluated the performance of these two strains in a social discrimination paradigm and found that total *Oxtr* KO males are unable to differentiate individuals of the same strain but can discriminate between females from different strains, something the forebrain-specific *Oxtr* KO mice cannot do. Possible reasons for this difference may relate to the spatial or temporal differences in diminished Oxtr expression between total and forebrain-specific KO strains, as for instance postnatal delay and incomplete loss of forebrain binding in the latter strain. Importantly, studies examining the behavioral performance of mice with targeted mutation in the Oxt gene have consistently revealed deficits in social memory, reduced aggression, and normal social approach for this group, as compared to WT (Crawley et al., 2007; DeVries et al., 1997; Ferguson et al., 2001; Winslow and Insel, 2002).

The pro-social actions of Oxt have become the focus of researchers working on therapeutic approaches for developmental psychiatric disorders characterized by impairments in social interactions such as autism spectrum disorder (ASD) (Green and Hollander, 2010; Insel et al., 1999). Indeed, several studies reveal associations between common genetic polymorphisms in the Oxtr gene and ASD (Campbell et al., 2011; Gregory et al., 2009;

Jacob et al., 2007). Substantial clinical trials have shown that administration of Oxt to subjects with ASD significantly reduces the number and severity of repetitive behaviors (Hollander et al., 2003), and improves the retention of social information (Hollander et al., 2007) and emotion recognition (Guastella et al., 2010). ASD is a neurodevelopmental disorder characterized by occurrence of a triad of behavioral symptoms: (1) deficits in social interaction, (2) impaired communication, and (3) ritualistic–repetitive behaviors; all typically detectable in early childhood but continuing throughout life (APA, 2000; Folstein and Rosen-Sheidley, 2001). Recently, much effort has gone into modeling the behavioral symptoms of ASD in mice aiming to further understand the underlying genetic mechanisms (Crawley, 2007; Silverman et al., 2010).

The purpose of the current study was to extend the existing research characterizing the social behaviors of mice lacking Oxtr in the whole body. To reach this goal, male *Oxtr* KO and their WT littermates were submitted to a battery of behavioral tests designed to fully assess the expression of autism-related behaviors. The visible burrow system (VBS), three-chambered and social proximity tests have proven most useful for assessment of measures associated with the first diagnostic symptom of ASD, deficits in social interaction (Arakawa et al., 2007; Defensor et al., 2011; Moy et al., 2007; Pobbe et al., 2010). Our study also provides information on the second diagnostic symptom, communication deficits, as mice were assessed for baseline and social scent marking (Arakawa et al., 2009). In addition, a detailed analysis of grooming microstructure and a repetitive novel object contact task (Pearson et al., 2011) were used to compare *Oxtr* WT and KO genotypes in regards to the third diagnostic symptom of ASD, repetitive behaviors.

Materials and methods

Experimental subjects

13 wild-type *Oxtr*^{+/+} (WT) and 16 knockout *Oxtr*^{−/−} (KO) male mice aged 10–14 weeks old at the beginning of behavioral testing were used as subjects. Animals for this study were offspring of a B6-backcrossed stock obtained from Dr. Scott Young's Laboratory (B6.129SJL-*Oxtr*tm1.1Wsy/J). All subjects were littermates from crosses of heterozygous mice. Mouse genotypes were determined as previously described (Lee et al., 2008) with purified DNA collected from tail biopsy after weaning at PND 25. Stimulus mice used for social behavior tests were adult CD-1 mice bred in-house from stock obtained from Charles River Labs (Wilmington, MA), and B6 mice bred from stock obtained from The Jackson Laboratory (Bar Harbor, ME). Subjects and stimulus mice were housed in individually ventilated cages, 35.5 cm×20 cm×13 cm (*H*), with up to seven same-sex littermates after weaning in a temperature-controlled room (22±1 °C). All subjects were maintained on a 12-h light/dark cycle (lights on at 06:00 am), with free access to food and water in their home cages. All procedures were conducted in accordance with protocols approved by the University of Hawaii Laboratory Animal Service Institutional Animal Care and Use Committee.

Behavioral tests

Unless otherwise noted, behavioral tests were performed under ambient fluorescent lighting (120 lx) during the light phase of the light/dark cycle from 9:00 am to 5:00 pm. Temperature (22 ±1 °C) and humidity (70%) were controlled in the experimental room.

VBS

Briefly, each VBS colony was housed in a rectangular, galvanized metal bin, 86 cm × 61 cm × 26 cm (*H*), as previously described (Pobbe et al., 2010). Three chambers were positioned behind a barrier wall extending across a short width of the bin, 30 cm from the end wall.

This barrier wall separated an open surface area from the chambers in the other compartment. A video camera was connected to a DVD recorder and mounted on the ceiling over the VBS allowing the recording of subject behavior from an overhead view. The experimental room was maintained on a 12-h light/dark cycle (lights on at 06:00 am), being illuminated by fluorescent lamps during the light period and by infrared light during the dark phase.

Forty-eight hours prior to colony formation, subjects were marked for individual identification with a commercial crème-based hair dye (Jerome Russel, extra strength crème hair bleach). On day 1, each group of three male WT or KO mice was moved from the rearing room to the testing room and placed in the VBS at the beginning of the dark period. Four VBS colonies were scored simultaneously in the experimental room. VBS colony grouping was maintained for 4 days. All animals of a particular colony were previously unfamiliar to each other. Four WT and four KO colonies were created and analyzed.

DVD recordings were made for 4 h each, on days 1, 2 and 3 in the dark period and days 2, 3 and 4 in the light period. Behaviors were scored from these recordings by time sampling, with a 30-s sample being taken every 10 min for each mouse of a particular colony. Frequencies of behaviors were the basic measures evaluated, and the mean frequency of each behavior for each genotype was compared across the four days of testing.

The occurrence of the following behaviors was scored: huddle: lying in contact with another animal for more than 10 s of the 30-s time sample; being alone: remaining three body lengths away from the nearest neighbor, for more than 10 s of the 30-s time sample; allo-grooming: lick or rub with paws, another animal; self-grooming: lick or rub self; approach to the front or back of another animal was defined in terms of a line bisecting the approached mouse, perpendicular to the long axis of its body; flight: rapid locomotion away from an approaching animal; chase/follow: rapid locomotion toward another animal, or a slow approach toward an animal that was moving away. Frequencies of huddling and being alone were calculated as percentages of total occurrence within a session by dividing the frequency of each behavior by 24, the highest possible number of occurrences.

Three-chambered social approach test

Twenty-four hours after removal from the VBS, mice were tested for social approach behavior in the three chamber apparatus, which was constructed according to published studies (Nadler et al., 2004). Briefly, a subject mouse was placed into the middle chamber of the divided $41 \times 70 \times 28$ cm (*H*) apparatus. An empty, inverted wire cup (Galaxy Pencil/ Utility Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH) was placed in each of the two outer compartments. Empty glass jars of the same diameter were placed on top of the base of the wire cups to prevent movement of the enclosures, or escape by stimulus mice. For the habituation phase, the sliding doors were elevated and the mouse given free access to the three chambers during a 10 min session. Following the habituation phase, the mouse was placed back into the center, the sliding doors were closed and an unfamiliar male CD-1 mouse was placed into one of the two cups, with the doors being again lifted and the subject mouse permitted to explore the entire apparatus for 10 min; this constituted the sociability phase. The stimulus mouse placement was successively alternated between trials. The time spent in each compartment during both sessions was collected in real-time with two stopwatches by a single observer who was blind to the genotype of the subject. During both the habituation and sociability phases, cameras were mounted in front of both outer compartments and connected to a DVD recorder. The frequency and duration of rear, contact, sniff, quick-withdraw, nose to nose, and self-grooming were scored off-line using Noldus Observer software (Noldus Information Technology, Wageningen, The Netherlands) in each of the two outer compartments during the sociability phase.

Self-grooming

Twenty-four hours after the three-chambered test, mice were individually assessed for grooming microstructure as previously described (Pearson et al., 2011). Briefly, each mouse was placed in a $14 \times 7 \times 30$ cm (*H*) Plexiglas chamber for 20 min under normal fluorescent lighting. An aluminum lid that permitted air circulation but prevented escape was placed over the top. Two digital cameras were used to collect video from the front and side aspects so that the mouse's grooming behavior was always visible. Videotapes were scored using Noldus software for the frequency and duration of paw licking, head washing, body grooming, leg licking, and tail/genital grooming. In addition to the collection of frequencies of body directed grooming, the following variables were determined according to Kalueff et al. (2007): a bout was defined as at least one episode of any category of grooming, or an uninterrupted sequence of grooming types. Bouts are divided by at least 6 s of inactivity or by an activity other than grooming. An interrupted bout was defined as a grooming bout that is interrupted by less than 6 s; the proportion of bouts that were interrupted was calculated as interrupted bouts divided by total bouts multiplied by 100. Transitions were transfers between regional grooming subtypes. Incorrect transitions were transfers which do not follow the cephalo-caudal progression (0—no grooming, 1—paw licking, 2—head wash, 3—body groom, 4—leg licking, 5—tail/genital groom). The proportion of incorrect transitions was calculated as incorrect transitions divided by total transitions multiplied by 100.

Social proximity

Twenty-four hours after collection of data for the self-grooming analysis, mice were tested in the social proximity test as previously described (Defensor et al., 2011). Social proximity testing was conducted in a clear rectangular chamber (7 cm L × 14 cm W × 30 cm H) constructed of acrylic plastic. For testing, the subject mouse and an unfamiliar male B6 mouse were placed simultaneously into this chamber and an aluminum lid was placed over the top to prevent escape. Video from two cameras providing front and side views was transferred to a video merge processor which combined both channels into a single side-by-side output. The availability of both views aided in the discrimination of behaviors by reducing occlusion of one animal view by the other. The output from the video processor displaying both the front and side view was transmitted to a DVD recorder for storage and subsequent analysis.

The frequencies of the following behaviors were manually quantified by an observer blind to the subject's genotype: nose to nose: subject's nose tip and/or vibrissae contact the nose tip and/or vibrissae of the other mouse; anogenital sniff: subject's nose or vibrissae contacts the base of the tail or anogenital region of the other mouse; crawl over: subject's forelimbs cross the midline of the dorsal surface of the other mouse; push under: subject's head/snout push under the ventral surface of the other mouse to a depth of at least the ears of the subject animal crossing the midline of the other mouse's body; allo-grooming: lick or rub with paws the other mouse; upright: subject displays a reared posture oriented towards the other mouse with head and/or vibrissae contact.

Repetitive novel object contact task

On the day following the social proximity test, mice were relocated to the experimental room at least 30 min before habituation for the repetitive novel object contact task. The habituation session consisted of placing a mouse in a clean standard mouse cage, 26.5 cm × 17 cm × 11.5 cm (*H*), with the floor covered by a layer of sawdust bedding (1 cm), during a 10 min session on the day prior to testing. A micro-isolator lid was modified by removing the filter element and frame, and thick gauge wire bisecting both horizontal planes of the lid was added dividing the overhead image into four equal sized compartments. The number of

transitions between quadrants was scored using Noldus Observer software. This permitted assessment of any possible baseline differences in motor activation or exploratory tendencies between genotypes. On the following day, and at the same time as the habituation session, mice were then individually placed within an identical clean cage containing fresh sawdust bedding as well as four novel objects located approximately 3 cm from each of the four corners. The objects were four distinct small children's toys: a 4 cm long jacks piece, a 1.5 cm^3 multicolored die, a multicolored 3 cm long arrangement of Lego blocks, and a 3.5 cm long white and red bowling pin which were all made out of high-density plastic to prevent chewing. Each mouse was then able to investigate the environment and objects during a 10 min session. A video camera mounted above the cage was used to record the test. The arrangement of objects was identical for all subject mice, and each object was thoroughly cleaned with 70% ethanol and dried between trials. Recorded DVDs were scored for the occurrence of investigation for each of the four toys. Investigation was defined as clear facial or vibrissae contact with or burying of the novel objects; merely passing or pausing by an object was insufficient for investigation. The occurrence of repetitive contacts with three and four toys and the total frequency of contacts with each of the four toys were calculated. In order to determine if there was a genotype effect on the tendency to display preferences for particular toys, frequencies of contact with each object were ranked in decreasing order from maximum to minimum preference values for each subject, averaged by genotype and compared.

Urinary scent marking

Immediately following removal from the cage used in the repetitive novel object contact task, mice were individually housed for at least seven days before assessment of baseline scent marking. Previous studies have demonstrated that single housing is critical to establish motivation to engage in detectable levels of scent marking (Arakawa et al., 2009). The scent marking arena was an inverted rat cage with a steel mesh divider wall installed to bisect the arena. This apparatus was placed on top of a 30×45 cm section of drawing paper, and the subject mouse was placed on one side for a 20 min baseline session. Twenty-four hours later, the mouse was tested for urinary scent marking to an unfamiliar male CD-1 mouse that had just been placed in the opposite half of the arena. The drawing paper's orientation in the test compartment was marked to ensure that only subject markings were counted, and the paper was allowed to dry overnight. It was then fixed and stained with a 6% solution of ninhydrin (Fisher) in methanol to label urinary scent marks, and dried. To quantify the amount of urinary scent marking, a 1×1 cm printed transparency grid was placed over the paper and the number of squares containing a stained mark was counted manually by an assistant blind to the genotype of the subject.

Statistical analysis

The behavioral data obtained in the VBS were analyzed by three-way analyses of variance (ANOVA) with days of testing (1–4) and lighting period (dark or light phase) as within-subjects factors, and genotype (WT or KO) as the between-subjects factor. Significant effects were followed by unpaired t-tests. Data from the three-chambered test were analyzed using within-genotype repeated measures ANOVA for comparison of time spent in the empty cup side with time spent in the CD-1 mouse side. Unpaired t-tests were performed to compare the behavioral measures obtained in the self-grooming analysis, social proximity test and repetitive novel object contact task, with genotype (WT or KO) as the grouping variable. Similarly, mean frequencies of behaviors scored in the three-chambered test were compared with unpaired t-tests. Scent marking scores were compared by two-way analyses of variance (ANOVA), with condition (baseline or CD-1 scent marking) as the within-subjects factor, and genotype (WT or KO) as the between-subjects factor. For all statistical analyses, a probability level of $p<0.05$ was considered significant.

Results

VBS

Table 1 provides results of statistical tests for all measures of the VBS analysis, by genotype, lighting period, and day, along with interaction effects.

Approach: front and back

Figs. 1A and B present the mean frequency of frontal and back approaches for both genotypes over the 4 days. The main effect of genotype was significant for frontal approaches, but not for back approaches, reflecting a significant reduction of the former measure in the *Oxtr* KO group. No interactions were statistically significant (Table 1).

Flight

Fig. 1C presents the mean frequencies of flight for both genotypes over the 4 days. Flight was significantly reduced in the *Oxtr* KO group. The interactions between days and genotype, and lighting period and genotype were statistically significant (Table 1), with *Oxtr* KO mice showing less flight during the dark period, on day 1.

Chase/follow

The mean frequencies of chase and follow behaviors for *Oxtr* WT and KO mice over the 4 days of testing are displayed in Fig. 1D. The main effect of genotype was significant for such behaviors, reflecting a significant reduction of this measure in the *Oxtr* KO group. No interactions were statistically significant (Table 1).

Self-grooming

Fig. 2A presents the mean frequencies of self-grooming for both genotypes over the 4 days. The main effect of genotype was significant for self-grooming, revealing a significant increase of this behavior in the *Oxtr* KO group. No interactions were statistically significant (Table 1).

Allo-grooming

The mean frequencies of allo-grooming for *Oxtr* WT and KO mice over the 4 days of testing are shown in Fig. 2B. The main effect of genotype and the interaction between lighting period and genotype were statistically significant (Table 1), with *Oxtr* KO mice exhibiting less allo-grooming during the light period.

Huddle

Fig. 2 C presents the percentage of observations of huddling behavior for both genotypes over the 4 days. Huddling was significantly reduced in the *Oxtr* KO group in both dark and light periods. All subjects showed more huddling behavior during the light period. Interactions between lighting period and genotype as well as days by lighting period were statistically significant (Table 1).

Alone

The percentage of observations of *Oxtr* WT and KO mice spending time alone over the 4 days is presented in Fig. 2D. Time spent alone was significantly increased in *Oxtr* KO mice. For all subjects, being alone was more prevalent during the dark period. Interactions between lighting period and genotype as well as days by lighting period were statistically significant (Table 1).

Three-chambered social approach test

Fig. 3 shows the main effect of genotype on the duration of time that *Oxtr* WT and KO mice spent in both side compartments of the three-chambered apparatus. *Oxtr* WT mice showed a significant preference for spending time in the side of the test box containing the unfamiliar CD-1 mouse (stimulus mouse) vs. the opposite side ($F_{1,12} = 6.68$; $p < 0.05$). *Oxtr* KO mice did not show a significant preference for one side over the other ($F_{1,15} = 1.58$; $p = 0.22$ n.s.). The >frequencies of behaviors scored during the sociability phase of the three-chambered test are displayed in Table 2. *Oxtr* KO mice showed a significant reduction in the frequencies of sniff behavior oriented to the unfamiliar CD-1 mouse [$t(27) = 2.69$; $p < 0.05$], as compared to their WT littermates. No other significant differences were found.

Self-grooming

The mean frequencies of self-grooming subtypes for both *Oxtr* WT and KO genotypes are displayed in Fig. 4. The statistical analysis revealed no significant differences between groups for any of these subtypes. Similarly, no statistically significant differences in the number of bouts, number of interrupted bouts, proportion of interrupted bouts, in the number of transitions between grooming stages, number of incorrect transitions, and proportion of incorrect transitions were found.

Social proximity

Fig. 5 displays the mean frequencies of various types of social behaviors in *Oxtr* WT and KO mice in response to an unfamiliar B6 mouse. *Oxtr* KO mice showed a significant reduction in the frequencies of nose to nose [$t(27) = 2.90$; $p < 0.01$] and anogenital sniff [$t(27) = 2.18$; $p < 0.05$] behaviors oriented to the B6 mouse, as compared to their WT counterparts, in the social proximity chamber. No other significant differences were found.

Repetitive novel object contact task

Fig. 6 shows the mean frequencies for all measures assessed in the repetitive novel object contact task for both *Oxtr* WT and KO genotypes. During the habituation phase, no significant genotype difference was found in the number of transitions between quadrants. In addition, the frequency of total contacts with unfamiliar novel objects did not significantly differ between these two groups. Similarly, when proportional preferences for each toy were ranked and averaged for each genotype, no significant difference in object preferences was found. Finally, the statistical analysis revealed that when the number of identical three- and four-object sequences was compared between genotypes, no differences were observed.

Urinary scent marking

The mean number of squares containing scent marks in the base-line and social scent marking conditions for both genotypes is displayed in Table 3. The main effect of genotype was significant ($F_{1,27} = 4.43$, $p < 0.05$), reflecting a significant reduction of scent marking in the *Oxtr* KO group. There was no reliable condition effect ($F_{1,27} = 2.99$, $p = 0.095$ n.s.) nor a statistically significant interaction between genotype and condition ($F_{1,27} = 0.041$, $p = 0.84$ n.s.).

Discussion

The purpose of the current study was to characterize the behavioral phenotype of *Oxtr* KO mice, as compared to their WT counterparts, more specifically in regards to the expression of autism-related behaviors. Three tests (VBS, three-chambered and social proximity tests) were used to assess the levels of sociability in both genotypes. The VBS is a semi-natural context in which colonies of mice or rats live for extended periods in situations affording

multiple tunnels and burrows in addition to an open surface area (Arakawa et al., 2007; Blanchard et al., 2001), and therefore allows for a more naturalistic measurement of social behavior. The three-chambered test provides a simple design with a high-throughput approach to compare sociability measures between different strains and genotypes (Moy et al., 2007; Yang et al., 2011); however, this situation effectively limits many overt forms of interactions that are possible in semi-natural environments (Halladay et al., 2009; Pratte and Jamon, 2009). The social proximity test was specifically devised to provide a description of social behaviors in situations of forced contact, i.e. when avoidance was not an option. In this extremely small test enclosure, both mice of a pair can normally stand on the substrate without contacting each other; however, any locomotor movement would put the moving mouse in contact with the other pair member (Defensor et al., 2011). The comparison of genotype effects on sociability measures assessed in these three specific situations enabled a more detailed and ethologically valid analysis of social interactions in these mutants.

The effects of genotype on these three situations were consistent and highly significant. In the VBS, *Oxtr* KO mice showed robust reductions in interactive behaviors: frontal approach, huddling, allo-grooming, and flight, with more time spent alone, and in self-grooming, when compared to the WT group. These results were corroborated in the three-chambered social approach test: unlike WT controls, male *Oxtr* KO mice failed to spend more time in the side of the test box containing the unfamiliar CD-1 mouse. In this test, *Oxtr* KO mice showed a clear reduction in the frequencies of sniff behavior oriented to the unfamiliar CD-1 mouse. In the social proximity situation, *Oxtr* KO mice displayed a significant decrease in the frequencies of nose to nose and anogenital sniff behaviors oriented to the B6 mouse, as compared to their WT littermates. Taken together, these results indicate consistent social deficits observed in mice lacking *Oxtr* in all tissues.

These results support and extend the behavioral phenotype of *Oxtr* KO mice by adding clear social deficits to the previously reported impairments in social recognition described for these mice (Lee et al., 2008; Macbeth et al., 2009; Takayanagi et al., 2005). In this context, a recent published study by Sala et al. (2011) described similar social deficits for *Oxtr* KO mice in the three-chambered test that are normalized by intra-encephalic administration of either Oxt or vasopressin (AVP). These authors showed that the Oxt restoration of social approach in *Oxtr* KO mice was abolished by pretreatment with a selective V1a receptor antagonist. Within the brain, Oxt and AVP interact with three G-protein coupled receptors that include *Oxtr* and the two AVP1 receptors (V1a and V1b) (Manning et al., 2008); taking into account the highly similar sequences of these two neuropeptides, and the high homology of their receptor sequences it is possible that at the concentrations used by Sala et al. (2011) Oxt acted through activation of V1a receptors (for further details see Ring, 2011).

Interestingly, the behavioral profiles of *Oxtr* KO mice in the VBS and the three-chambered test, as well as nose to nose behaviors in the social proximity test are similar to those found in BTBR T+tf/J (BTBR) mice (Defensor et al., 2011; McFarlane et al., 2008; Pobbe et al., 2010), an inbred strain that consistently displays behavioral traits with face validity for all three diagnostic symptoms of ASD (Blanchard et al., 2011). It is noteworthy to mention that *Oxtr* KO mice displayed normal scores on measures of general health, sensory reflexes, exploratory locomotion and emotional-like reactivity (Lee et al., 2008; Sala et al., 2011), as compared to their WT littermates, indicating a specific deficit in sociability in this group of mice.

Existing mouse models of ASD commonly display patterns of restricted repetitive behaviors: when compared to B6, BTBR mice displayed an increase in the frequency of all subtypes of self-grooming, and a stronger preference for specific objects as well as a significantly higher number of visits that include a repetitive sequence of three or four

objects (Pearson et al., 2011). The present study revealed no significant differences between *Oxtr* WT and KO genotypes in regards to the occurrence of motor and cognitive stereo-typed behaviors: neither the frequency of self-grooming subtypes nor the patterns of object investigation changed between both groups. Taken together, these results indicate that lack of *Oxtr* in all tissues is not directly associated with the expression of restricted repetitive behaviors.

Prior evidence indicates that deposition of urinary scent marks toward conspecifics and emission of ultrasonic vocalizations appear to be the two major models of mouse communication (Arakawa et al., 2008; Scattoni et al., 2009). In this context, a previous study revealed that when compared to B6, FVB/NJ, and 129X1/SvJ mouse pups, BTBR pups produced an unusual repertoire of ultrasonic vocalizations in response to separation from the mother, suggesting a link to communication deficits (Scattoni et al., 2008). Also, adult male BTBR mice displayed lower scent marking and minimal ultrasonic vocalization responses to female urinary pheromones, as compared to B6 (Wöhr et al., 2011). In the present study, when compared to their WT littermates, adult male *Oxtr* KO mice displayed a reduced number of urinary scent marks across social and non-social contexts, indicating an overall decrease in communication levels. It is noteworthy mentioning that albeit assessed in a different developmental period (PND7), male *Oxtr* KO pups emitted fewer ultrasonic vocalizations than their WT counterparts in response to social isolation from the parents (Takayanagi et al., 2005).

In summary, the present study confirmed and extended previous findings indicating that *Oxtr* KO mice display consistent deficits in social recognition, and reduced, albeit still robust, levels of communication, as compared to their WT littermates. Furthermore, the current analysis illustrates the importance of a detailed description of social behaviors in inbred mouse strains that can be used as a model to investigate background genes specifically involved in ASD.

Acknowledgments

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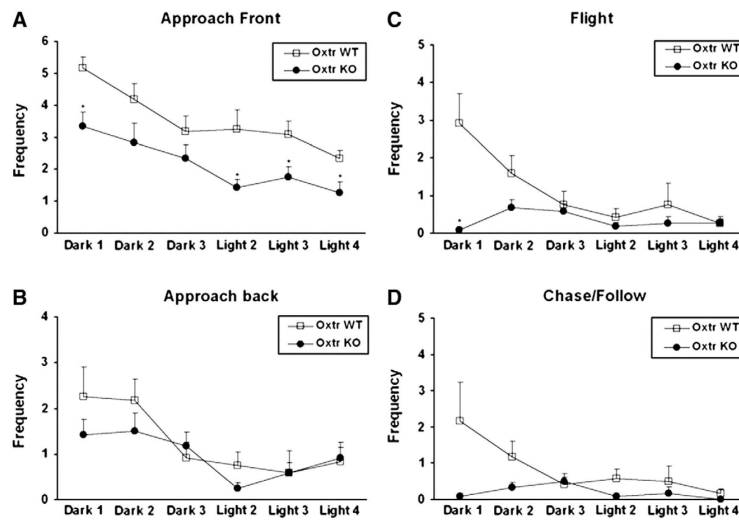Zingg HH, Laporte SA. The oxytocin receptor. Trends Endocrinol. Metab. 2003; 14:222–227. [PubMed: 12826328]

Fig. 1.
Individual frequencies (mean ± S.E.M.) of approaches to the front (A), approaches to the back (B), flight (C), and chase/follow (D) behaviors of WT and *Oxtr* KO mice during the dark and light periods in the VBS; n = 12 for each group; *p < 0.05 compared to WT by unpaired *t*-test.
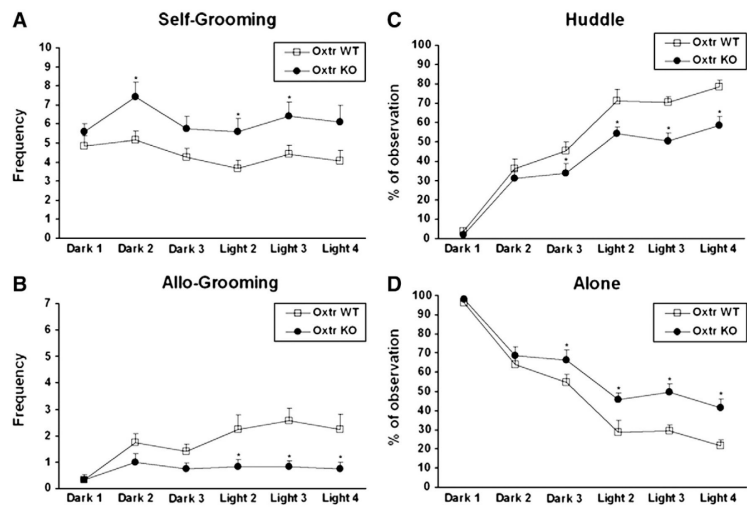
Fig. 2.
Individual frequencies (mean ± S.E.M.) of self-grooming (A), and allo-grooming (B), and percentage of observations (mean ± S.E.M.) of huddling behavior (C) or being alone (D) of WT and *Oxtr* KO mice during the dark and light periods in the VBS; n = 12 for each group; *p < 0.05 compared to WT by unpaired *t*-test.
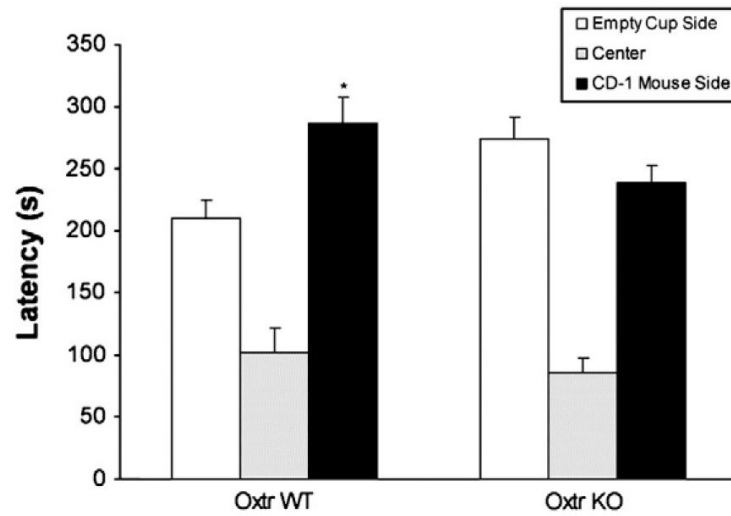
Fig. 3.
Time spent in each side (mean ± S.E.M.) during the three-chambered social approach test.
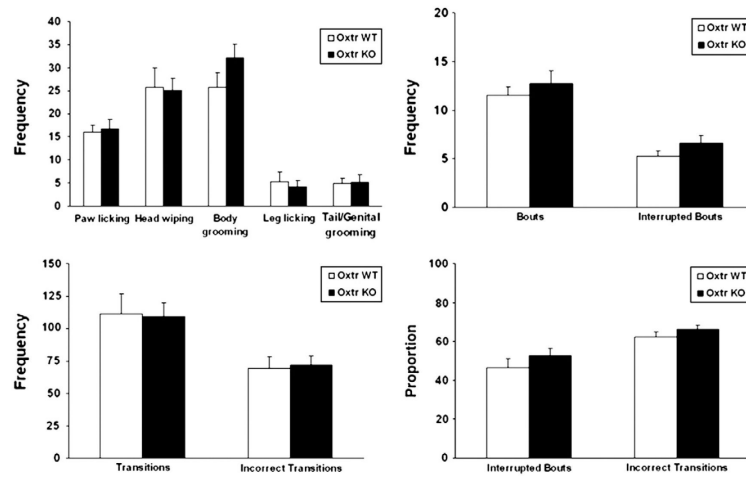*p < 0.05, within-genotype comparison; n = 13 for WT and 16 for *Oxtr* KO.

Fig. 4.
Frequency (mean ± S.E.M.) of variables assessed in the self-grooming analysis; n = 13 for WT and 16 for *Oxtr* KO.
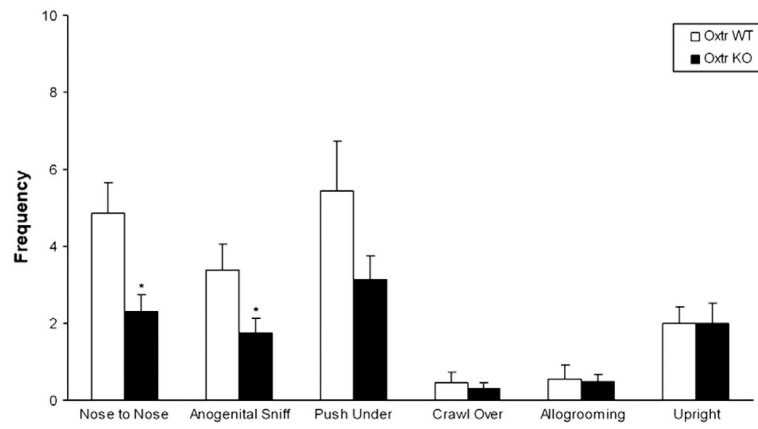
Fig. 5.
Frequency (mean ± S.E.M.) of social behaviors obtained when *Oxtr* WT and KO mice were confined with an unfamiliar B6 mouse in the social proximity chamber; n = 13 for WT and 16 for *Oxtr* KO; *p < 0.05 compared to WT by unpaired *t*-test.
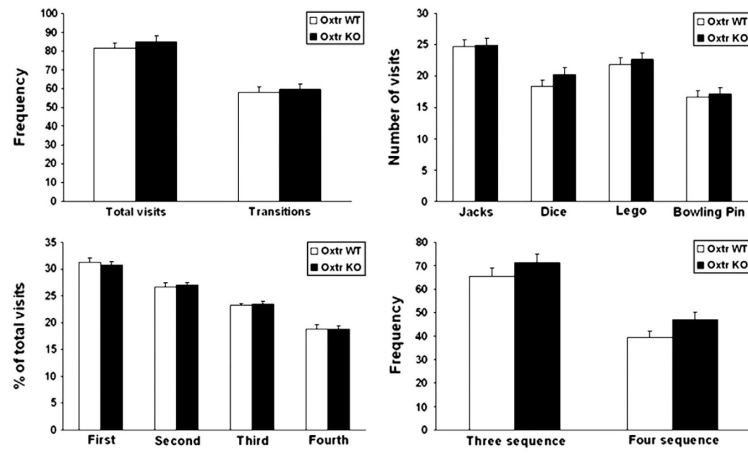
Fig. 6.
Frequency (mean ± S.E.M.) of variables assessed in the repetitive novel object contact task; n = 13 for WT and 16 for *Oxtr* KO.

Table 1

Summary of the statistic results obtained in the current VBS analysis.

| | ANOVA results | | | | | |
| | Main effect | | | | | |
	Genotype	Lighting period	Days	Days×genotype	Lighting×genotype	Days×lighting	Days×lighting×genotype
Approach front	F(1,22)=18.16; p<0.01 **	F(1,22)=27.98; p<0.01 **	F(2,21)=8.71; p<0.01 **	F(2,21)=1.66; p=0.21 n.s.	F(1,22)=0.02; p=0.86 n.s.	F(2,21)=1.42; p=0.26 n.s.	F(2,21)=0.03; p=0.96 n.s.
Approach back	F(1,22)=1.01; p=0.32 n.s.	F(1,22)=22.39; p<0.01 **	F(2,21)=0.68; p=0.51 n.s.	F(2,21)=1.44; p=0.25 n.s.	F(1,22)=0.51; p=0.48 n.s.	F(2,21)=2.53; p=0.10 n.s.	F(2,21)=0.28; p=0.75 n.s.
Flight	F(1,22)=6.83; p<0.05 *	F(1,22)=17.09; p<0.01 **	F(2,21)=3.68; p<0.05 *	F(2,21)=6.95; p<0.01 **	F(1,22)=8.46; p<0.01 **	F(2,21)=1.25; p=0.30 n.s.	F(2,21)=3.32; p=0.055 n.s.
Chase/follow	F(1,22)=5.48; p<0.05 *	F(1,22)=5.97; p<0.05 *	F(2,21)=1.67; p=0.21 n.s.	F(2,21)=2.46; p=0.11 n.s.	F(1,22)=2.00; p=0.17 n.s.	F(2,21)=0.29; p=0.75 n.s.	F(2,21)=1.93; p=0.16 n.s.
Self-grooming	F(1,22)=11.97; p<0.01 **	F(1,22)=1.87; p=0.18 n.s.	F(2,21)=3.89; p<0.05 *	F(2,21)=0.63; p=0.54 n.s.	F(1,22)=0.49; p=0.48 n.s.	F(2,21)=0.71; p=0.50 n.s.	F(2,21)=0.48; p=0.62 n.s.
Allo-grooming	F(1,22)=14.98;p<0.01 **	F(1,22)=15.62; p<0.01 **	F(2,21)=3.96; p<0.05 *	F(2,21)=0.81; p=0.45 n.s.	F(1,22)=10.75; p<0.01 **	F(2,21)=1.99; p=0.16 n.s.	F(2,21)=0.18; p=0.83 n.s.
Huddle	F(1,22)=14.81; p<0.01 **	F(1,22)=230.06; p<0.01 **	F(2,21)=37.09; p<0.01 **	F(2,21)=1.12; p=0.34 n.s.	F(1,22)=6.54; p<0.05 *	F(2,21)=34.24; p<0.01 **	F(2,21)=0.59; p=0.56 n.s.
Alone	F(1,22)=14.81; p<0.01 **	F(1,22)=230.06; p<0.01 **	F(2,21)=37.09; p<0.01 **	F(2,21)=1.12; p=0.34 n.s.	F(1,22)=6.54; p<0.05 *	F(2,21)=34.24; p<0.01 **	F(2,21)=0.59; p=0.56 n.s.

*
p<0.05.

**
p<0.01.

Table 2

Frequency (mean±S.E.M.) of behaviors obtained when Oxtr WT and KO mice explored each of the two outer compartments of the three-chambered apparatus during the sociability phase.

	Oxtr WT (empty cup)	*Oxtr* WT (stimulus mouse)	*Oxtr* KO (empty cup)	*Oxtr* KO (stimulus mouse)
Rear	23.85±3.06	23.15±3.57	31.81±3.4	24.31±2.38
Contact	1.69±0.7	2.54±0.69	2.19±0.58	2.56±0.47
Sniff	13.92±1.72	32.08±2.86	13.25±1.42	23.38±1.75 [*]
Quick withdraw	0±0	3.54±0.73	0±0	3.19±0.97
Nose to nose	0±0	4.00±1.03	0±0	2.31±0.45
Self-grooming	4.62±0.99	2.15±0.86	3.56±1.0	2.06±0.69

n=13 for WT and 16 for *Oxtr* KO.

[*] p<0.05 compared to WT by unpaired *t*-test.

Table 3

Number (mean±S.E.M.) of squares with scent marks of *Oxtr* WT and KO mice assessed in two experimental conditions.

	Oxtr WT	*Oxtr* KO
Baseline condition	145.77±26.77	100.13±16.75
Social condition	170.08±15.08	130.88±13.01

n=13 for WT and 16 for *Oxtr* KO.