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Oxytocin receptor is not required for social attachment in prairie voles

Kristen M. Berendzen[1,2,3,4,†], **Ruchira Sharma**[1,2,3,4,†], **Maricruz Alvarado Mandujano**[8,†], **YiChao Wei**[8,†], **Forrest D. Rogers**[5], **Trenton C. Simmons**[5], **Adele H.M. Seelke**[5], **Jessica M. Bond**[5], **Rose Larios**[1,2,3,4,6], **Nastacia L. Goodwin**[1,2,3,4], **Michael Sherman**[1,2,3,4], **Srinivas Parthasarthy**[8], **Isidero Espineda**[1], **Joseph R. Knoedler**[8], **Annaliese Beery**[10], **Karen L. Bales**[5,7], **Nirao M. Shah**[8,9,*], **Devanand S. Manoli**[1,2,3,4,*]

[1]Department of Psychiatry and Behavioral Sciences, University of California, San Francisco; San Francisco, CA 95158, USA

[2]Center for Integrative Neuroscience, University of California, San Francisco; San Francisco, CA 95158, USA

[3]Weill Institute for Neurosciences, University of California, San Francisco; San Francisco, CA 95158, USA

[4]Kavli Institute for Fundamental Neuroscience, University of California, San Francisco; San Francisco, CA 95158, USA

[5]Department of Psychology, Physiology, and Behavior, University of California, Davis; Davis, CA 95616, USA

[6]Neurosciences Graduate Program, University of California, San Francisco; San Francisco, CA 95158, USA

[7]Department of Neurobiology, Physiology, and Behavior, University of California, Davis; Davis, CA 95616, USA

[8]Department of Psychiatry and Behavioral Sciences, Stanford University; Stanford, CA 94305, USA

[9]Department of Neurobiology, Stanford University; Stanford, CA 94305, USA

[10]Department of Integrative Biology, University of California, Berkeley; Berkeley, CA 94720, USA

[*]Corresponding authors. Devanand.manoli@ucsf.edu and Nirao@stanford.edu.
[†]These authors contributed equally to this work
Lead Contact: Devanand.manoli@ucsf.edu

Author Contributions

Conceptualization, K.L.B., D.M. and N.M.S.; Methodology, A.B., K.L.B., K.M.B., M.A.M., D.M., F.D.R., N.M.S., R.S., Y.C.W.; Investigation, J.M.B., K.M.B., I.E., N.G., J.K., R.L., M.A.M., S.P., F.D.R., T.C.S., A.H.M.S., R.S., Y.C.W.; Visualization, K.M.B., M.A.M., R.S., Y.C.W.; Funding acquisition, K.L.B., D.M., N.M.S.; Writing –Original Draft, K.M.B., R.S.; Writing – review & editing, K.L.B., K.M.B., D.M., M.A.M., N.M.S., R.S., Y.C.W.

Declaration of interests

Dr. Nirao Shah is a member of the advisory board for Neuron. The authors declare no other competing interests.

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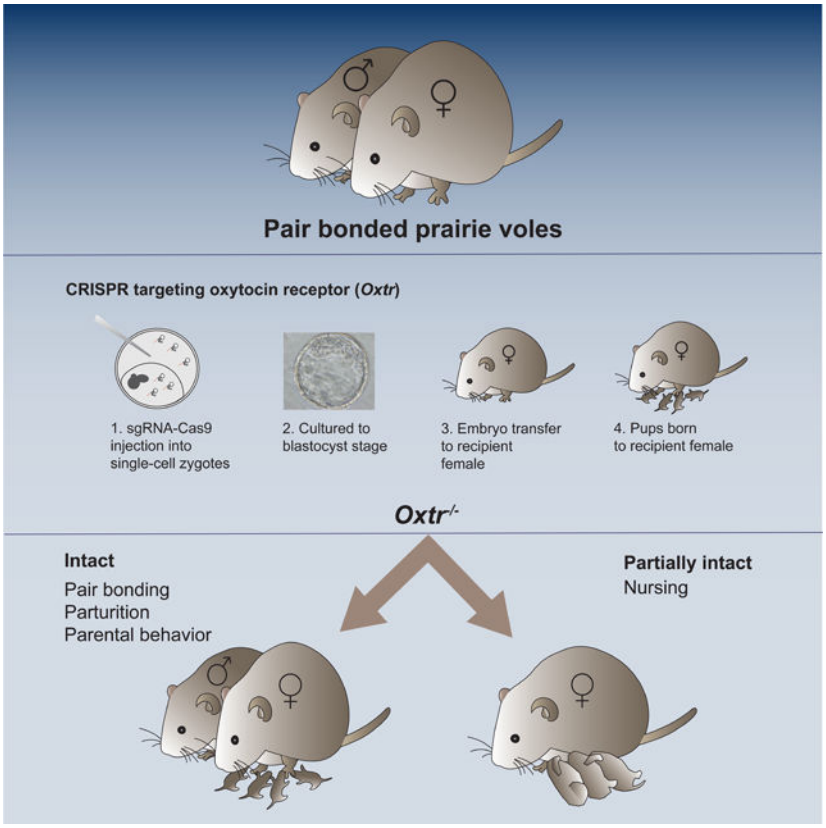
Summary

Prairie voles are among a small group of mammals that display long-term social attachment between mating partners. Many pharmacological studies show that signaling via the oxytocin receptor (Oxtr) is critical for the display of social monogamy in these animals. We used CRISPR-mutagenesis to generate three different *Oxtr* null mutant prairie vole lines. *Oxtr* mutants displayed social attachment such that males and females showed a behavioral preference for their mating partners over a stranger of the opposite sex, even when assayed using different experimental setups. Mothers lacking Oxtr delivered viable pups, and parents displayed care of their young and raised them to the weanling stage. Together, our studies unexpectedly reveal that social attachment, parturition, and parental behavior can occur in the absence of Oxtr signaling in prairie voles.

eTOC Blurb:

Berendzen et al. report the surprising finding that prairie voles lacking the oxytocin receptor (*Oxtr)* display pair bonding and parental behaviors, including nursing. Despite many pharmacological studies suggesting a requirement for Oxtr, these findings indicate that Oxtr is genetically dispensable for pair bond formation and parental behaviors in voles.

Graphical Abstract



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Introduction

Strong, specific, and sustained relationships between mates and kin are displayed by a fascinating, but limited, subset of species across the animal kingdom[1–3]. Such attachments, which form the basis of diverse and complex social systems, are observed in species that have evolved the capacity to form lasting bonds between individuals, suggesting that they are innate with a strong underlying genetic component[4–7]. Progress in understanding the molecular or neural networks that promote social attachment has been hindered because traditional genetic model organisms such as *C. elegans*, *D. melanogaster*, *D. rerio*, and *M. musculus* do not display enduring attachments as adults. Here we report the use of CRISPR-based targeting in prairie voles (*Microtus ochrogaster*) to probe signaling pathways implicated in social attachment.

Adult prairie voles exhibit social attachment behavior such that mating partners form an enduring bond with each other. This attachment behavior, commonly referred to as pair bonding, has been observed in ethological studies in the wild as well as in the laboratory setting[8,9]. Pair bonded voles spend time together in close proximity (huddling behavior) and show a social preference for each other over a potential new mating partner. Neuropeptide signaling has long been known to control the display of social behaviors across diverse species[10–14]. Differences in neural populations regulated by these pathways correlate with interspecies variations in social structure[15–17]. Intriguingly, the evolution of varied complex social systems and affiliative behaviors, including social monogamy, has repeatedly converged upon the nonapeptide hormones oxytocin (Oxt) and arginine vasopressin (Avp) and their orthologs across phylogenies[18–20].

Ethological studies, using live-trapping of wild prairie voles, reported that mating pairs are more likely to be trapped together than is expected by chance[9]. Similar studies of closely related species such as meadow voles (*M. pennsylvanicus*), that do not pair bond, showed that live-traps contained single animals[9]. Subsequent studies demonstrated that pair bonding behavior can also be observed in a laboratory setting[21,22]. Pair bonding is displayed as a suite of behavioral traits, the most commonly measured of which is a preference for the familiar partner over a novel stranger. Pair bonded animals prefer to huddle with their partners compared to exploring unfamiliar conspecifics of the opposite sex[23]. Such partner preference is also accompanied by aggression toward unfamiliar opposite sex conspecifics, indicative of active rejection of potential new mates[24].

Comparative studies between socially monogamous and non-monogamous vole species revealed striking differences in Oxtr expression in brain regions thought to be important for social attachment, and implicated natural variation within species in specific aspects of pair bonding and attachment behaviors[15,16,25–28]. Pharmacological studies from multiple groups have shown that Oxt is sufficient to induce pair bonding behavior in otherwise naive voles and the administration of Oxtr-antagonists induces loss of these behaviors[12,17,21,29]. Viral manipulations of Oxtr expression in specific brain regions of prairie voles also recapitulate findings from such pharmacological studies[30,31]. Taken together, these findings suggest a critical role for Oxt signaling via its cognate receptor, Oxtr, in driving pair bonding behaviors in this species.

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Prairie voles, like many other animals that display pair bonding behavior, exhibit biparental care of their young, and oxytocin signaling is thought to control these behaviors as well[31,32]. Oxytocin is additionally critical for milk let-down, the reflexive release of milk triggered by sensory stimuli associated with suckling[33,34]. All pups born to female mice null for Oxt or Oxtr die shortly after birth because of complete failure of milk let-down[35,36]. In addition to this role in lactation, stimulation of Oxt-expressing neurons in virgin female mice induces pup retrieval behaviors typical of lactating females[37]. Thus, decades of research implicates both Oxt and its cognate receptor Oxtr in a large repertoire of parenting behaviors. typical of lactating females[37]. Thus, decades of research implicates both Oxt and its cognate receptor Oxtr in a large repertoire of parenting behaviors.

To test the genetic requirement of *Oxtr* in pair bonding and parental behaviors, we employed a CRISPR-based approach to generate mutant prairie voles null for this receptor. Surprisingly, male and female prairie voles homozygous for each of the three distinct loss-of-function *Oxtr* alleles displayed pair bonding. Moreover, we observed that *Oxtr* null females were capable of raising pups to weaning. We therefore conclude that, contrary to previous assumptions, pair bonding and parental behaviors in prairie voles do not require Oxtr function.

Results

CRISPR-targeting can reliably generate multiple null alleles of *Oxtr* in prairie voles

To perform CRISPR-based gene targeting in prairie voles, we developed a protocol to obtain single cell embryos for injection of Cas9 ribonucleoprotein complexes. We were unable to achieve successful superovulation using hormonal supplementation protocols including those previously reported[38], and therefore we implemented a timed-mating strategy to harvest embryos synchronized at specific developmental time points. We harvested 0.5 day single cell embryos using this protocol, an approach that yielded 3.7±1.2 embryos/female.

Because we maintain prairie voles in an outbred background, we were concerned about natural variation in *Oxtr* coding sequence that could potentially reduce targeting efficiency by specific small guide RNAs (sgRNAs). Indeed, we observed 3 synonymous substitutions in exon 1 of the *Oxtr* locus from 4 voles in our colony (Figure S1A). We designed 8 protospacer adjacent motif (PAM) site-anchored sgRNAs based on conserved sequences in exon 1. We initially tested whether these sgRNAs could generate mutations in exon 1 *in vitro*, injecting them as Cas9 ribonucleoprotein complexes individually into single cell embryos and genotyping 4 days later at the blastocyst stage (Figures S1B-D). Two sgRNAs consistently yielded mutations in exon 1, and sequencing revealed multiple mutations in individual blastocysts, suggesting that CRISPR-targeting had also occurred after the single cell stage (Figures S1E-F).

We proceeded to co-inject these two sgRNAs into single cell embryos, cultured them *in vitro* to the blastocyst stage, and then transferred these healthy blastocysts into recipient pseudopregnant prairie voles (Figure 1A). Each recipient female gave birth to 1–2 pups (~10% of embryos transferred) ~16 days following embryo transfer. Given the significant chimerism we observed following genotyping of blastocysts injected with our chosen

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sgRNAs (Figures S1E-F) and the low yield of liveborn pups following embryo transfer, we mated each founder (G0) to a wildtype (WT) partner even when genotyping tail samples from these founders revealed no mutations, as germline chimerism may not be reflected in the small, peripheral tissue samples used in genotyping[39]. This strategy yielded 3 distinct *Oxtr* alleles that transmitted via the germline of founders with WT tail DNA (Figures 1B,C). *Oxtr*^{1} has a 1 base pair (bp) insertion that is predicted to yield an 84 amino acid (aa) peptide, *Oxtr*^{4} contains 2 small, multi-bp deletions that are predicted to yield an 81 aa peptide, and *Oxtr*^{5} contains a large deletion spanning and extending 1.5 kbp beyond the sequence targeted by the two sgRNAs. There is currently no completely assembled and annotated prairie vole genome to aid in sequencing-based analysis of off-target events. Therefore, to isolate *Oxtr* alleles and minimize carry-over of potential off-target mutations in our lines, we independently outcrossed all lines of each of the three mutant alleles to WT voles in our colonies.

CRISPR-generated mutations in *Oxtr* produce loss of function alleles

Each of the three *Oxtr* alleles we generated is predicted to generate non-functional Oxtr (Figure 1C). We tested this directly by performing a ligand binding assay *in situ*, using a radiolabeled small molecule competitive agonist for Oxtr. These studies showed a complete lack of binding in homozygous null mutants of both sexes of *Oxtr*^{1}, *Oxtr*^{4}, and *Oxtr*^{5} (Figures 2A-E, S2A-H), demonstrating absence of all ligand-binding Oxtr *in vivo*. Loss of Oxtr signaling may lead to changes in binding of the functionally related neuropeptide vasopressin (Avp) to its receptor, Avpr1a, or in expression of the Avp or Oxt peptides themselves. We could discern no differences in Avpr1a binding in a small number of Oxtr null mutants that we tested (data not shown). Furthermore, we found no increase in expression of Oxt or AVP peptide in *Oxtr* nulls compared with WT (Figure S2I-L). Consistent with the lack of requirement for Oxtr for survival, we observed Mendelian ratios of WT, heterozygous, and homozygous pups born to heterozygous parents (*Oxtr*^{1} 29:60:24, *Oxtr*^{4} 37:62:32 and *Oxtr*^{5} 25:55:26).

Female and male prairie voles lacking Oxtr exhibit pair bonding

Prairie voles are induced ovulators, and following a short period of cohabitation between opposite sex animals, co-housed pairs will mate and subsequently display pair bonding[23,40]. To test whether our Oxtr null mutants display deficits in pair bonding, we co-housed sexually naive males and females for 7 days, a period previously shown to be sufficient to induce pair bonding[23,40]. WT or homozygous mutants were paired with an unfamiliar, unrelated WT animal of the opposite sex. We used two different setups commonly used to assay partner preference and behaviors between the experimental subject and a cohoused partner or novel unfamiliar conspecific of the opposite sex[21,41]. In the branched chamber apparatus, the experimental animal, its pair bonded partner, and an unfamiliar, opposite-sex conspecific are housed in separate interconnected chambers such that only the experimental subject has free access to all chambers (Figure 3A)[21]. We also used a linear, partially-divided chamber in which the cohoused partner and unfamiliar conspecific are tethered at opposite ends from each other, and only the experimental subject has access to all chambers (Figure 3B). In both experimental setups, the experimental animal can exhibit affiliative or other behaviors toward either tethered animal[41,42].

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We tested pair bonding of *Oxtr*^{1} mutants and their control group in the linear chamber design and that of *Oxtr*^{4} and *Oxtr*^{5} and their control groups in the branched chamber apparatus. Surprisingly, we observed that males and females homozygous for each of the three, null *Oxtr* alleles all spent more time huddling with their partners. They also displayed aggression towards the unfamiliar conspecific of the opposite sex, indicative of rejection of other potential mates. These displays of huddling with partners and rejection of unfamiliar potential partners were comparable to those of WT animals in both experimental paradigms we deployed (Figures 3, S3). However, *Oxtr*^{1} mutant males showed no significant difference in aggression toward partner and stranger females, in contrast to WT controls who showed variable but significant aggression towards stranger females, (Figure 3H). In any event, we find that prairie voles demonstrate pair bonding behaviors in the absence of functional Oxtr.

Prairie voles lacking Oxtr bear viable pups and display bi-parental care similar to wild types

Given the concordance in pair bonding displays by animals homozygous for each of the three mutant *Oxtr* alleles, we tested a subset of these for performance in parenting. *Oxtr*^{4} or *Oxtr*^{5} homozygous mutants and their WT siblings were paired with WT animals of the opposite sex until parturition and then tested for parental care of their progeny. Both male and female prairie vole parents interact intensively with their pups, spending prolonged periods huddling, licking, and grooming them. Experimental interference during the pre-weaning period can disrupt both subsequent displays of parental care as well as the repertoire of adult social behaviors exhibited by the pups[43,44]. Accordingly, the standard assay is to observe the duration of parental interactions with their pups in the first few days after parturition and document pup survival and health at weaning[45].

We observed that *Oxtr* null parents interacted equivalently with their pups compared to their WT counterparts (Figures 4A-F). Both WT and mutant parents spent the majority of their time in the nest, in direct contact with their litters, and, in the case of mothers, nursing pups. Unlike mice lacking Oxtr[36], we never observed cages in which pups were scattered across the cage floor, indicative of effective retrieval to the nesting area of pups who had wandered away.

Surprisingly, we noticed that pups born of mothers homozygous for either of two *Oxtr* alleles survived past the first few hours of birth, with some weaning successfully, suggesting that mutant females could nurse some of their young (Figures 4G,H,J,K). *Oxtr*^{4} null mothers had significantly fewer surviving litters at weaning compared to WT mothers (Figure 4G). *Oxtr*^{5} null mothers also had fewer surviving litters at weaning, although this did not reach statistical significance (Figure 4J). Of the litters that survived to weaning to experimental females, *Oxtr*^{4} *and Oxtr*^{5} null mothers had fewer pups per litter (Figures 4H,K). Pups born to all mutant mothers weighed significantly less at weaning than pups born to WT mothers, suggesting a defect in milk let-down or subtle deficits in nursing behavior (Figures 4I,L, S4A). Consistent with a deficit in milk let-down, gross examination of the nipple area revealed less engorgement in females mutant for *Oxtr* compared to WT mothers (not shown).

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Both WT and mutant fathers successfully raised their litters to weaning (Figures 4G-L, S4). Pups raised by *Oxtr*^{4}, but not *Oxtr*^{1} or *Oxtr*^{5}, null fathers weighed less when compared to those raised by WT fathers, suggesting a variably penetrant role for Oxtr in fathers in their ability to raise thriving pups. Weights at birth and time of weaning were decreased from *Oxtr* null mothers compared with WT (Figure 4I, J, S4). Together, our findings show that, in prairie voles, biparental care and some milk let-down occur in the absence of Oxtr signaling[36].

Discussion

CRISPR-mutagenesis has been successfully used to manipulate a large variety of species of animals, allowing scientists the freedom to establish new model organisms for biological phenomena that are not observed in worms, fruit flies, zebrafish, or mice. We have utilized CRISPR-based targeting to test the role of Oxtr in prairie voles in modulating attachment and social behaviors, a suite of behaviors not displayed by standard genetic model organisms[46]. We find that pair bonding behaviors, nursing, and weaning of pups can all occur in the absence of Oxtr-mediated signaling. Our findings, consistent across multiple paradigms, three labs, and three null alleles of *Oxtr*, are in contrast to prior studies that highlight the importance of Oxtr signaling in pair bonding and parental behaviors in prairie voles[13,21,27,30,31,40,47].

A key difference between our work and preceding studies is that the latter were conducted in adult animals, using pharmacological or viral misexpression strategies to determine a role for Oxtr function in behavior. Despite efforts to demonstrate the specificity of pharmacologic approaches to manipulate Oxtr activity, it remains possible that the compounds employed in these studies alter yet-to-be-identified pathways that contribute to pair bonding. In such a model, exogenous ligands that activate Oxtr bind to and stimulate additional receptors, while compounds that antagonize Oxt binding to Oxtr could also inhibit either its binding to additional receptors or other ligand-receptor interactions[48,49]. It is possible that compensatory pathways activated due to constitutive loss of function of Oxtr in our studies obscure a functional role for this receptor in pair bonding and parenting[50,51]. We did not find an increase in Oxt or Avp expression or discernible changes in Avpr1a expression indicative of such compensation, though we cannot exclude more subtle changes in the expression of these neuropeptides or other pathways that substitute for the loss of Oxtr signaling. Beyond Avp, additional neuroendocrine or neuromodulator pathways may be engaged following the initial social interactions that promote bonding[52–54]. Irrespective of these pathways, which now must be determined, the neural circuits mediating pair bonding and parental behaviors in prairie voles can facilitate the display of these behaviors independent of Oxtr signaling.

Future studies with targeted loss of Oxtr in specific brain regions of adult voles will reveal whether it is important for these behaviors. Alternatively, Oxtr and Avpr1a signaling might redundantly regulate pair bonding and parenting; Oxt can also signal via Avpr1a and it may therefore modulate these behaviors entirely through Avpr1a rather than Oxtr[55,56]. These possibilities can be disambiguated by testing the behavioral performance of voles null for *Avpr1a* or *Avpr1a* and *Oxtr*. Determining identity of the non-Oxtr targets of the

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pharmacological agents that have been used to modulate pair bonding might also lead to insights into the underlying neural circuits and molecular signaling pathways. In any event, our studies rigorously demonstrate that, in three different loss of function mutations generated and tested independently, Oxtr signaling is not genetically required in male and female prairie voles for either pair bonding or multiple aspects of parenting.

Female mice null for *Oxtr* show a complete failure of milk let-down and nursing behavior such that none of their pups survives beyond the day of birth[34,36,57]. In contrast, many pups born to prairie vole mothers lacking Oxtr survive to weaning, albeit with reduced weight. Furthermore, both female and male *Oxtr* null voles display intact parental behavior, consistent with recent findings indicating preserved alloparenting behavior in male prairie voles lacking Oxtr[38]. It is possible that the phenotypic difference between mice and voles mutant for *Oxtr* is a consequence of rapid evolution of pathways that regulate nursing and parenting in mammals[6,46,58]. Alternatively, this difference in phenotypes could reflect the fact that the *Oxtr* mutant mice were maintained on an inbred background whereas our colony is consistently outbred to wild voles[59,60]. It will be interesting to determine whether *Oxtr* null mice on an outbred, wild background can also exhibit parental behaviors. If true, this would suggest that inbreeding has led to reliance on Oxtr signaling for parenting in mice rather than a fundamental difference in species-specific roles for Oxtr in parental displays.

Oxt signaling has also been implicated in affiliative displays in humans suggesting a conserved role for this neuropeptide hormone in these social behaviors across ~90 million years of evolution[28]. Based on observations in prairie voles and other mammals, including humans, clinical trials have used exogenous oxytocin or small molecule ligands to Oxtr to ameliorate the deficits in social attachment and cognition seen in multiple psychiatric conditions; these studies however have yielded mixed results[25,61–65]. Together with these clinical studies, our observation that Oxtr signaling is not required genetically for pair bond formation or parenting in prairie voles suggests that we require a more refined understanding of the molecular pathways underlying social attachment behaviors. New genetic models such as the *Oxtr* prairie vole mutants we have generated may better allow the rigorous dissection of the molecular and circuit mechanisms mediating attachment behavior and its disruption in disease. Whole-animal mutants better represent what may occur in patients with mutations associated with neuropsychiatric disorders, and molecular genetic approaches in prairie voles now permit us to test directly the impact of such genetic disruptions in the context of complex social and attachment behaviors.

Star Methods

Resource availability

Lead contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Devanand Manoli (devanand.manoli@ucsf.edu).

Materials availability—All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

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Data and Code Availability—All data reported in this paper will be shared by the lead contact upon request.

Code

This paper does not report original code.

Experimental model and subject details

Animals—Subjects were laboratory-bred prairie voles (*Microtus ochrogaster*) which originated through systematic outbreeding of a wild stock captured near Champaign, Illinois. Prairie voles were bred, maintained, and tested at three distinct sites: University of California San Francisco, University of California Davis, and Stanford University. Sexually naïve male and female animals were group weaned at 21 ± 1 days and separated to pair-housing with either a same-sex sibling or an age-matched same-sex non-sibling (about half to each type of pairing). Voles were maintained under a 14:10 h light-dark cycle in clear plastic cages (45 × 25 × 15 cm) with bedding, nesting material (nestlet), and a PVC hiding tube. Rooms were maintained at approximately 20°C, and food and water were available *ad libitum*.

Breeding pairs were established between two heterozygotes or a homozygous *Oxtr* mutant male and heterozygous female partner from a breeding line maintained by the respective labs. Pups from $Oxtr^{1}$ breeding pairs were weighed at weaning, and those from $Oxtr^{4}$ and $Oxtr^{5}$ pairs (mutant line and WT partner) were weighed at birth and at weaning date. Percent of pups surviving is calculated based on the number of pups alive at weaning at 21 days out of the total number of pups born in a litter, with two litters assessed per pair. Voles were assigned into experimental or control groups based on genotype when they reached 7–9 weeks of age at the start of testing. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Research Council. The protocol was approved by the Institutional Animal Care and Use Committee at each respective institution.

Method details

Isolation of prairie vole embryos for gene targeting—Unlike mice, ovulation in prairie voles is behaviorally induced. All attempts to artificially super-ovulate prairie voles yielded poor results in our hands, differing from findings of a prior study[38]. We therefore developed a timed mating protocol where we placed 6–8 week old females with adult males for 12 hours and then placed a physical barrier between them and housed them for another 24 hours. We empirically determined that this induced estrus in all the females we paired, and the females all yield fertilized embryos approximately 18 hours after the removal of the barrier.

Mutagenesis and embryo manipulation for gene targeting—The current assembly of the prairie vole genome, which includes just over 70% annotation, reveals only one region for *Oxtr* that currently maps to scaffold sequence. Additionally, we screened a BAC library (CHORI BACPAC) that covers the entire vole genome for clones containing sequences similar to the prairie vole *Oxtr* coding sequence[66]. Of the 10 clones we

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obtained, all contain a single locus identical to the region scaffold that aligns to *Oxtr* in the current version of the genome, suggesting that prairie voles contain only one copy of *Oxtr*. We used these sequences and obtained the genomic sequence of exon 1 of *Oxtr* from 4 randomly chosen animals from our colony and identified polymorphisms that correspond to 3 synonymous substitutions. Based on these sequences, we designed 8 sgRNAs targeting exon 1, and adopted microinjection protocols commonly used to manipulate embryos from mice to test varying concentrations of the guides along with either 20 ng/ul or 100ng/ul of Cas9 mRNA based on studies in mice, rats, and other rodent species[67,68]. We cultured embryos to the blastocyst stage, with ~45% of embryos surviving following injection. We then harvested genomic DNA from individual blastocysts, and assayed for mutagenesis of *Oxtr* by Surveyor PCR, and sequencing of genomic PCR products[69]. These analyses revealed significant mosaicism in blastocysts, suggesting that mutagenesis in vole embryos occurred significantly later than the first division. Based on these studies, we identified 2 sgRNAs (sgRNA1: GAGCATCGCTGACCTGGTGGTGGC, sgRNA2: CAGCTGCTGTGGGACATCACCTTC) that reliably yielded detectable mutations in embryos and selected these for further use. We performed pronuclear injections of the 2 sgRNA guides, 2μl Cas9 protein (PNA BIO, INC CP02, 5μg/μl) and 2μl cas9 mRNA (TRILINK BIOTECHNOLOGIES, INC L-7206, 1μg/μl) and cultured the manipulated embryos to the blastocyst stage. We generated pseudopregnant recipient females using our timed mating protocol with vasectomized males and surgically implanted the manipulated embryos into their oviduct[68]. Using such an approach, we developed a protocol whereby ~10 embryos are transferred to each uterine horn to obtain 3–5 live born pups per female.

Isolation and Outcrossing of *Oxtr* Alleles—Tail samples were taken from the founders (G0s) and screened for mutations in the Oxtr locus. Based on the chimerism we observed in our embryo experiments, it was possible that the G0 carried a mutation in their germ cells but not their tails. We therefore paired all G0s with wild types and screened F1 tails to look for germ-line mutations. We were able to isolate F1s with Oxtr mutations and design PCR genotyping schemes to identify the mutation in subsequent generations, hence verifying germ-line transmission. Every F1 was outcrossed (paired with a wild type of the opposite sex, *Oxtr*^{4} and *Oxtr*^{5}, >3 generations; Oxtr1, 7 generations) to remove off-target effects of CRISPR mutagenesis. As there is currently no completely assembled and annotated prairie vole genome to aid in sequencing-based analysis of off-target events, we established an outcrossing protocol based on standard genetic practices in mice and flies to isolate specific mutations. Assuming that the fraction of the original background that is unlinked is ½^N for N generations, we outcrossed *Oxtr*^{1} for 7 generations to the wild type strain.

Autoradiography—Wildtype and homozygous mutant siblings were sacrificed using CO_2 and their brains were dissected and rapidly frozen on powdered dry ice before storing at −80ºC. 20μm sections were thaw mounted onto super mount frost plus slides. Slide mounted sections were thawed until dry and fixed for two minutes in 0.1% paraformaldehyde (0.1% paraformaldehyde in 0.1M PBS). Slides were rinsed 2×10 minutes in 50mM Tris pH 7.4, then incubated for 90 minutes at room temperature in a solution (50 mM Tris, 10 mM MgCl2, 0.1% BSA, 0.05% bacitracin, 50 pM

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radioligand) containing the radioactively labeled 125I-ornithine vasotocin analog vasotocin, d(CH2)5[Tyr(Me)2,Thr4,Orn8,(125I)Tyr9-NH2] (125I-OVTA, PerkinElmer, Inc.). Slides processed for non-specific binding were incubated with an additional 50 uM non-radioactive ligand [Thr4Gly7]-oxytocin (Bachem). All slides were washed 3×5 min in chilled Tris-MgCl2 (50mM Tris, 10mM MgCl2, pH 7.4), followed by a 30-minute soak in Tris-MgCl2 on ice. Slides were quickly dipped in cold, distilled water and then air dried and exposed to Kodak BioMax MR film (Kodak, Rochester, NE, USA) for 3 days and subsequently developed. To quantify ligand binding, we first defined anatomical centers using the Allen Mouse Brain Atlas. Acetylcholine esterase was stained using the enzyme's conversion of thiocholine ester.

The 125I-OVTA binding was quantified directly (measured as optical binding density, OBD) from the film using a light box, a top mounted camera, and the MCID Core Digital Densitometry system (Cambridge, UK) according to methods previously described[70]. Values for OBD from a set of 1251 autoradiography standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) were loaded into MCID in order to generate a standard curve, from which 5 OBD values for each ROI were extrapolated (*Oxtr*^{4} and *Oxtr*^{5}). Average OBD values were calculated for each ROI within each individual specimen, as well as for one background area where no binding was detected, which served as a measure of non-specific binding. This average background/non-specific binding was subtracted from the ROI measurements to yield normalized OBDs across specimens and correct for individual variation in non-specific binding across individuals. Autoradiography performed on *Oxtr*^{1} was calculated with respect to the background before adjusting the background to zero. Non-standard binding controls performed on *Oxtr*^{1} showed that there was no difference between signal observed in the mutant and background. The signal intensities were compared using the student's T test across males and females of both wild type and mutant siblings of a given mutant strain.

RT- quantitative PCR—Adult WT and *Oxtr* mutant voles were deeply anesthetized by IP injection with 2.5% Avertin and euthanized by decapitation. The brain was quickly dissected out and sectioned into 500 μm coronal slices using a brain matrix mold (BrainTree Scientific) chilled on ice. Slices were floated in ice-cold phosphate buffered saline (PBS) and the paraventricular nucleus (PVN) was identified using anatomical landmarks and dissected using a Zeiss microscope. For *Oxtr*^{4}, tissue was dissected and RNA purified from individual animals. For *Oxtr*^{5}, tissue from 2–3 animals was pooled prior to RNA extraction to reduce variability across samples. Tissue was flash-frozen on dry ice and stored at −80°C until further processing. Total RNA was extracted using TRIzol according to manufacturer's instructions and quantified using NanoDrop (Thermo Scientific). We used 5 μl total RNA to program a 20 μl reverse transcription reaction using the ProtoScript cDNA synthesis kit with random hexamer priming. We used 2μl of the RT reaction to run real-time quantitative PCR using FastStart SYBR Green Master Mix and the StepOnePlus™ Real-Time PCR System. To quantify mRNA levels we used a relative quantitation method wherein we constructed a standard curve for each gene using six serial 1:10 dilutions of cDNA pooled from all of our samples. We ran two technical replicates for each standard curve and biological sample and normalized relative

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Avp and Oxt levels to the housekeeping gene Gapdh after verifying that Gapdh mRNA level did not vary by genotype. The following oligonucleotide primers were used: Avp, TGCGTGTTTCCTGAGCC (forward) and ATGTTGGGTCCGAAGCAG (reverse); Oxt CTGCTACATCCAGAACTGTCC (forward) and AAACAGCCCAGCTCATCG (reverse); Gapdh, GGTAAAGTCATCCCAGAGCTG (forward) and CCTGCTTCACCACCTTCTTG (reverse). Before the RT-qPCR reaction we sequenced the Oxt and Avp PCR products to confirm the specificity of our primers.

Partner preference assay—The partner preference test[23] was used to assess pair bond formation[71]. Subjects were cohoused with an opposite-sex, wildtype animal (partner). Analysis of partner preference between wild type siblings from the *Oxtr*^{1} background and wild type animals from this colony showed no difference on the partner preference test. Pairing of *Oxtr*^{1} was followed by a timed mating protocol for synchronization of estrous in which paired animals are separated by a clear, plastic barrier 18 hours following pairing. The barrier was left in place for 24 hours and then removed allowing the subject free access to the partner animal thereafter. Regardless of which laboratory performed testing, all animals were allowed to cohabitate for one week prior to behavioral testing. As *Oxtr*^{1} was generated and maintained independently from *Oxtr*^{4} and *Oxtr*^{5}, we used two different, well-established apparatuses for testing partner preference in the *Oxtr*^{1} line vs *Oxtr*^{4} and *Oxtr*^{5} based on the apparatus commonly used by the generating laboratory. In the branched design used to test *Oxtr*^{4} and *Oxtr*^{5}, the subject, their partner, and an unfamiliar opposite-sex conspecific (stranger) were placed in an apparatus made of three small polycarbonate cages (27×16×16 cm) joined by clear Plexiglas tubes. Strangers were selected to be of similar age and size as the partner animal. Both the partner and the stranger were tethered in two separate, end cages, and the test subject was placed, untethered in a central cage. The test subject was free to access any of the three cages over a period of 3 hours. All behaviors were video recorded from an approach centered on the two end cages. In the apparatus design used to assay *Oxtr*^{1} animals, the subject, partner, and an opposite sex stranger are placed in a linear apparatus with open top and 10 × 32 in walls. The partner and stranger animals are tethered on either end of the three-chamber arena and the subject allowed free access, again over a period of 3 hours. Behaviors are recorded from a top view camera capturing the entire apparatus. Videos were scored post-test by validated scorers blind to condition. Observed behaviors included location (i.e., duration of time in partner, stranger, and neutral cages), duration of stationary huddling or >50% side-by-side contact between the partner and stranger animals, and frequency of aggressive behavior (i.e., lunges).

Parental behavior—A total of 80 individuals, distributed across sex (male or female), strain (*Oxtr*^{4} or *Oxtr*^{5}) and genotype (wild-type or mutant) were paired with a wild type colony animal of the other sex. Following partner preference testing, pairs were left in large polycarbonate cages (44×22×16cm) with sterilized cotton for nesting material. All litters from 21 breeder pairs were observed on two separate days in the morning (08:00–11:00) and two separate days in the evening (15:00–18:00) for a total of four, 20-minute focal observations during the neonatal period (P1-P3). Parenting behavior was observed according to methods previously outlined[45]. Briefly, behaviors recorded included maternal and paternal huddling, non-huddling contact, licking/grooming, retrievals, hunching, nest building, and

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autogrooming. The time spent in behaviors that involved direct contact with pups (all those listed besides nest building) were combined for the total duration of pup-directed contact. In the mothers, lateral, active, and neutral nursing postures were scored. Active nursing is defined as pups attached while mother is locomoting around the cage, lateral nursing as mother laying on side with pups laying in front, and neutral nursing as standing over pups in a relaxed position without locomotion. The time in all of these nursing positions were combined for analysis of nursing duration. Eight individuals were not observed because of their death or the death of their partner (2 sires, 6 dams). Notably, of the two sires, one was wild type and one was mutant; and of the six dams, all six were mutant, split between *Oxtr*^{A} (n = 5) and *Oxtr*^{5} (n = 1). Increased rates of culling or natural death may have been due to reproductive complications in which oxytocin plays a significant role (pregnancy, birthing, and lactation). All observations were made in the home cage. Each parent was observed and left undisturbed during each 20-minute observation. The duration (seconds) of each behavior was recorded, and durations of all direct parenting behaviors (e.g. licking/grooming, huddling, etc.) were recorded within each observation period. All behaviors were live recorded using behavioral software (www.behaviortracker.com) or ScoreVideo (matlab).

Data analyses and statistics—The number of animals used was based on previous studies in the field by our group and others, combined with a power analysis. Assumptions of independence, homogeneity of variance, and normality were considered with multiple tests. The assumption of homogeneity of variance was considered through visual inspection of box plots and plots of residuals, and were further tested for homogeneity of variance using Bartlett's test of homogeneity of variances. Partner preference behaviors in all three mutant backgrounds were considered with paired two-tailed T-test for parametric data or Wilcoxon signed-rank test for non-parametric. Differential time between partner and stranger was calculated as partner huddle duration – stranger huddle duration. The level of statistical significance for each test was set at $p = 0.05$. Outliers were detected using a combination of z-score (values +/− 3 or greater were considered outliers) and plots of original and log-transformed data. One WT *Oxtr*^{5} male (duration with stranger = 3287 seconds), one WT *Oxtr*^{1} male (duration with stranger = 6928 seconds) and one WT *Oxtr*^{1} female (duration with stranger = 7356 seconds) were excluded as outliers due to durations in partner preference assay. Analyses were completed in R (version 1.1.423) and GraphPad Prism (version 7.03).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Inclusion and Diversity

We worked to ensure sex balance in the selection of non-human subjects.

One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper self-identifies as living with a disability. One or more of the authors of this paper received support from a program designed to increase minority representation in their field of research. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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Highlights:

- Prairie voles lacking oxytocin receptor (*Oxtr*) generated with CRISPR-targeting.
- *Oxtr*$^{-/-}$ voles form pair bonds or social attachments.
- *Oxtr*$^{-/-}$ voles show parental behavior.
- *Oxtr*$^{-/-}$ females nurse many of their pups to weaning.

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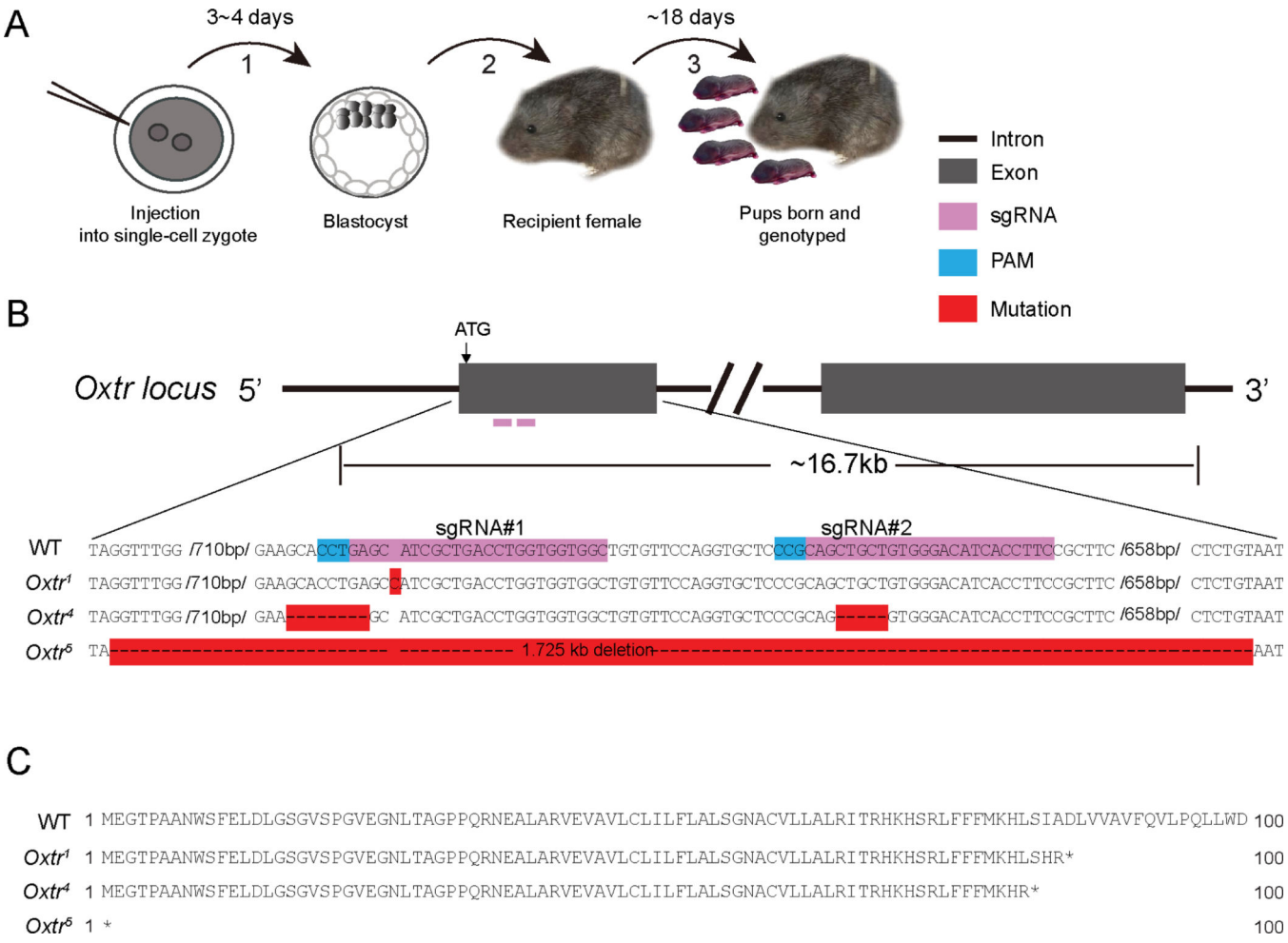


Figure 1. CRISPR mutagenesis yields multiple null alleles of *Oxtr* in prairie voles

A. Schematic of CRISPR-based targeting to generate *Oxtr* mutant prairie voles. Single cell embryos injected with Cas9-sgRNA ribonucleoprotein were cultured (1) to the blastocyst stage and transferred (2) to pseudopregnant recipient females who carried the embryos to term (3).

B. Schematic (top) of *Oxtr* locus encompassing first two exons. DNA sequence of WT and targeted *Oxtr* alleles. Dash/missing nucleotide represents a deletion (*Oxtr*^{4}, *Oxtr*^{5}) and red highlighted "C" (*Oxtr*^{1}) is an insertion. PAM, protospacer adjacent motif.

C. Predicted amino acid sequence of WT Oxtr, Oxtr^{1}, Oxtr^{4}, and Oxtr^{5} (only first 100 amino acids shown).

See also Figure S1.

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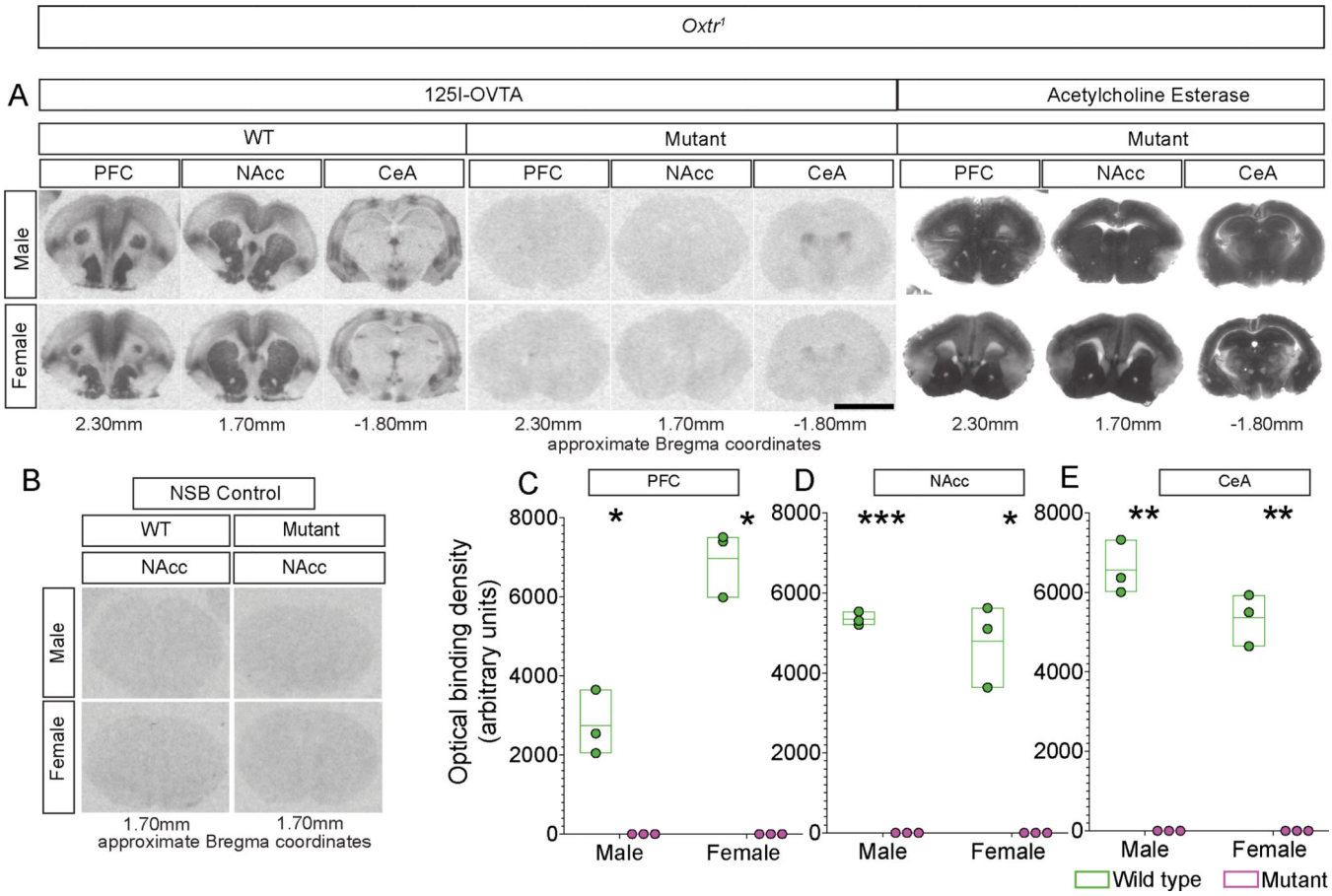


Figure 2. *Oxtr* mutant voles lack functional, ligand-binding Oxtr

A. Loss of binding with the competitive agonist 125I-OVTA visualized in coronal sections through the rostral telencephalon: Left: Labeling in PFC (prefrontal cortex), NAcc (nucleus accumbens), and CeA (central amygdala) of WT sibling controls; Middle: Labeling in equivalent sections through PFC, Nacc, and CeA from *Oxtr*^{1} homozygous mutant voles; Right: The same mutant sections as in the middle panels, stained for Acetylcholine esterase to demonstrate equivalence of sections chosen for WT and mutants.

B. Non-specific binding (NSB) control shows no off-target binding in WT or *Oxtr*^{1} mutant sections.

C-E. Optical density-based quantification of binding to 125I-OVTA shows that binding is essentially undetectable in mutants null for *Oxtr*^{1} in PFC (C), NAcc (D), or CeA (E). Scale bar =5 mm; boxplot depicts max-minScale bar =5 mm; boxplot depicts max-min, midline denotes mean; n=3 for WT and mutant males and females (C-E). Scale bar =5 mm; boxplot depicts max-minSee also Figure S2.

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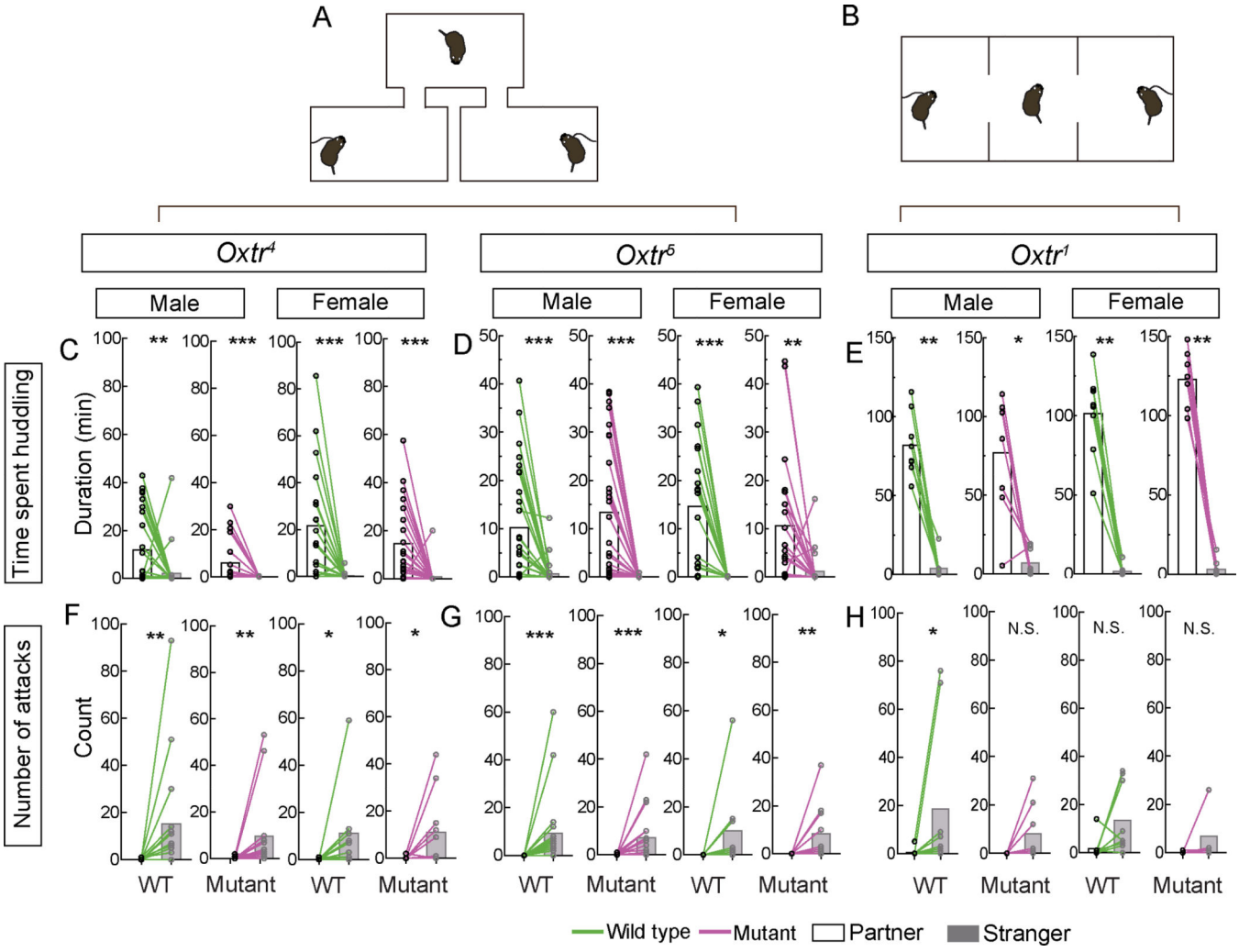


Figure 3. Female and male prairie voles lacking Oxtr exhibit pair bonding Scale bar =5 mm; boxplot depicts max-min

A-B. Schematic of partner preference test performed in a branched (A) or linear chamber with two partitions (B). Experimental vole is free to move between chambers (A) or partitioned space (B) whereas stimulus voles (opposite sex partner and stranger) are restrained by tethers. Scale bar =5 mm; boxplot depicts max-min

C-E. WT, *Oxtr*$^{4-/-}$ (C), *Oxtr*$^{5-/-}$ (D), and *Oxtr*$^{1-/-}$ (E) voles spent more time with their partner than a stranger of the opposite sex.

F-H. WT, *Oxtr*$^{4-/-}$ (F), and *Oxtr*$^{5-/-}$ (G) voles attacked the stranger more frequently than their partner. WT *Oxtr*^{1} males attacked the stranger more frequently (H).

Mean ± SEM; n = 25 WT and 21 mutant males, 19 WT and 21 mutant females (C); 27 WT and mutant males each, 19 WT and 21 mutant females (D); 8 WT and 8 mutant males, 8 WT and 8 mutant females each (E); 15 WT and 14 mutant males, 9 WT and 11 mutant females (F); 18 WT and mutant males each, 9 WT and 12 mutant females 15 (G); 8 WT and mutant males and females each (H); *p<0.05, **p<0.01, ***p<0.001; N.S., not significant.

See also Figure S3.

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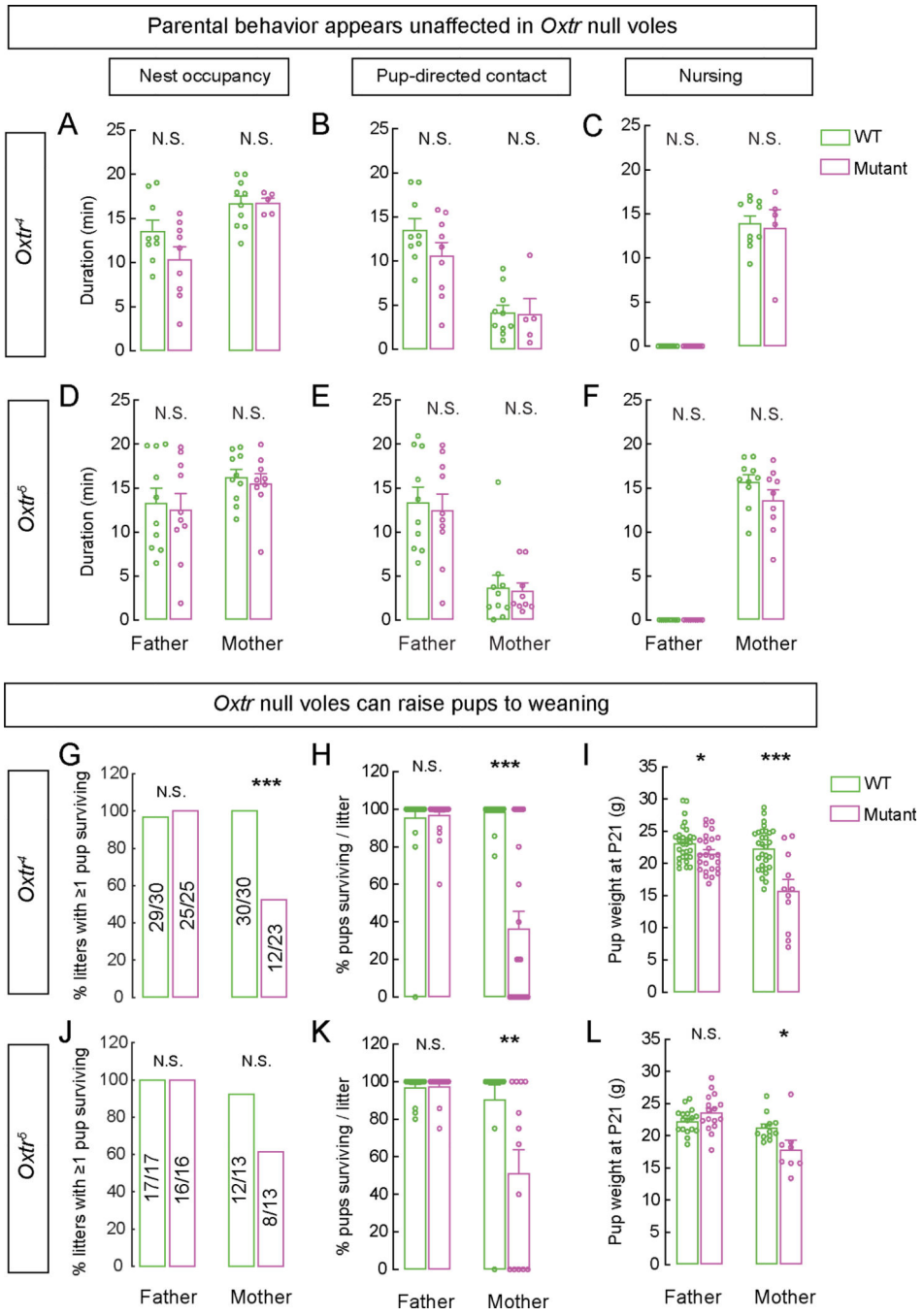


Figure 4. Prairie voles lacking Oxtr display bi-parental care and can raise pups to weaning

A-F. WT, *Oxtr*$^{4-/-}$, and *Oxtr*$^{5-/-}$ mothers and fathers exhibit equivalent nest occupancy (A, D), pup-directed contacts (B, E), and nursing behavior (C, F) with their pups.

G, J. *Oxtr*$^{4-/-}$ mothers wean fewer litters compared to WT mothers.

H, K. Litters from *Oxtr*$^{4-/-}$ and *Oxtr*$^{5-/-}$ mothers have fewer pups surviving to weaning compared to WT mothers

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I, L. *Oxtr*$^{4-/-}$ mothers and fathers and *Oxtr*$^{5-/-}$ mothers weaned pups with significantly lower body weight compared to WT parents. Only litters with ≥1 pup surviving to weaning were analyzed.

Mean ± SEM; n = 9 WT and mutant males each, 10 WT and 5 mutant females (A-C); 10 WT and mutant males each, 10 WT and 9 mutant females (D-F); 30 WT and 25 mutant males, 30 WT and 23 mutant females (G,H); 29 WT and 25 mutant males, 30 WT and 11 mutant females (I); 17 WT and 16 mutant males, 13 WT and mutant females each (J,K); 17 WT and 16 mutant males, 12 WT and 8 mutant females (L);*p<0.05, **p<0.01, ***p<0.001; N.S., not significant.

See also Figure S4.

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Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Cas 9 protein	PNA BIO, Inc	CP02
Cas9 mRNA	TRILINK BIOTECHNOLOGIES, INC	L-7206
d(CH2)5[Tyr(Me)2,Thr4,Orn8,(125I)Tyr9-NH2] (125I-OVTA)	PerkinElmer, Inc.	NEX254010UC
[Thr4Gly7]-oxytocin	Bachem	4013837.0005
Experimental models: Organisms/strains		
Prairie vole: *Oxtr*$^{1-/-}$	This paper	
Prairie vole: *Oxtr*$^{4-/-}$	This paper	
Prairie vole: *Oxtr*$^{5-/-}$	This paper	
Oligonucleotides		
sgRNA1: GAGCATCGCTGACCTGGTGGTGGC	This paper	
sgRNA2: CAGCTGCTGTGGGACATCACCTTC	This paper	
Avp forward: TGCGTGTTTCCTGAGCC	This paper	
Avp reverse: ATGTTGGGTCCGAAGCAG	This paper	
Oxt forward: CTGCTACATCCAGAACTGTCC	This paper	
Oxt reverse: AAACAGCCCAGCTCATCG	This paper	
Gapdh forward: GGTAAAGTCATCCCAGAGCTG	This paper	
Gapdh reverse: CCTGCTTCACCACCTTCTTG	This paper	
Software and algorithms		
R (version 1.1.423)	R Core	https://www.R-project.org/
GraphPad Prism (version 7.03)	GraphPad Software	https://graphpad.com/scientificsoftware/prism/
BehaviorTracker (version 1.5)	BehaviorTracker	www.behaviortracker.com
MATLAB	MathWorks	https://www.mathworks.com/products.html RRID: SCR_001622