



NEUROSCIENCE

An AAV-CRISPR/Cas9 strategy for gene editing across divergent rodent species: Targeting neural oxytocin receptors as a proof of concept

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A major issue in neuroscience is the poor translatability of research results from preclinical studies in animals to clinical outcomes. Comparative neuroscience can overcome this barrier by studying multiple species to differentiate between species-specific and general mechanisms of neural circuit functioning. Targeted manipulation of neural circuits often depends on genetic dissection, and use of this technique has been restricted to only a few model species, limiting its application in comparative research. However, ongoing advances in genomics make genetic dissection attainable in a growing number of species. To demonstrate the potential of comparative gene editing approaches, we developed a viral-mediated CRISPR/Cas9 strategy that is predicted to target the oxytocin receptor (*Oxtr*) gene in >80 rodent species. This strategy specifically reduced OXTR levels in all evaluated species ($n = 6$) without causing gross neuronal toxicity. Thus, we show that CRISPR/Cas9-based tools can function in multiple species simultaneously. Thereby, we hope to encourage comparative gene editing and improve the translatability of neuroscientific research.

INTRODUCTION

Nature provides an abundant variety of organisms using neural systems to interact with their environment to ensure survival and maximize fitness in diverse ways (1). Comparative neuroscience takes advantage of this diversity among species to find general principles of neural circuit functioning as well as mechanisms giving rise to variation in neural function and behavior (2). One efficient method to investigate the functional role of neural circuits is genetic dissection, a family of molecular approaches that allows for the manipulation of targeted genes (e.g., gene knockdown or exogenous expression) in specific cell populations (3–5). Traditionally, genetic dissection has been tractable in only a handful of model species, most commonly in laboratory mice, and this has limited our ability to leverage this powerful comparative approach to help improve treatments for neurobehavioral psychiatric disorders (6). However, ongoing efforts to construct whole-genome assemblies of nontraditional model species (7), along with the recent development of efficient genome editing tools (8, 9), which can be used in conjunction with versatile viral vectors (10), have made it increasingly feasible to use genetic dissection strategies in a wide range of

species (11–15). We believe that the application of genetic dissection approaches across a range of species has great fundamental and translational value, as it will greatly enhance our ability to take advantage of nature's remarkable diversity to bolster our understanding of species-specific and general mechanisms of complex brain-behavior relationships. Here, we demonstrate the feasibility of designing viral-mediated CRISPR/Cas9 tools for targeted gene editing across a wide range of model organisms. While we focus on neural oxytocin receptors (OXTRs) in rodents, this strategy should be effective for targeting any gene in any tissue across model organisms.

As a proof of concept, we developed a strategy for disrupting OXTR signaling in a wide range of rodents, as diverse members of this order are used in OXT research, particularly in relation to social behavior (16–22). OXT is a highly conserved peptide that has been studied in many species (14, 23–27), and this research has highlighted its role in the regulation of numerous social behaviors, including parental care, social bonding, mate preference, social recognition, and social vigilance (28, 29). The OXT system also has translational potential as a target for psychiatric disorders with disruptions in the social domain (30–32). A remarkable feature of the oxytocinergic system is that *Oxt* expression patterns are highly conserved across species, with *Oxt* being expressed primarily in paraventricular and supraoptic hypothalamic nuclei across vertebrates (33). This is in sharp contrast to its receptor (*Oxtr*), which shows strong intra- and interspecific variation even among closely related species (28, 29, 34, 35). Variation in OXTR density in various brain regions affects the processing of social information, which is thought to contribute to diversity in social behaviors (20, 28, 36). To be able to explore OXTR function from a comparative perspective, tools are needed to enable the genetic dissection of OXTR signaling in multiple species. While systemic *Oxtr* knockout lines exist for a few species (13, 14, 23, 37), a single, cross-species

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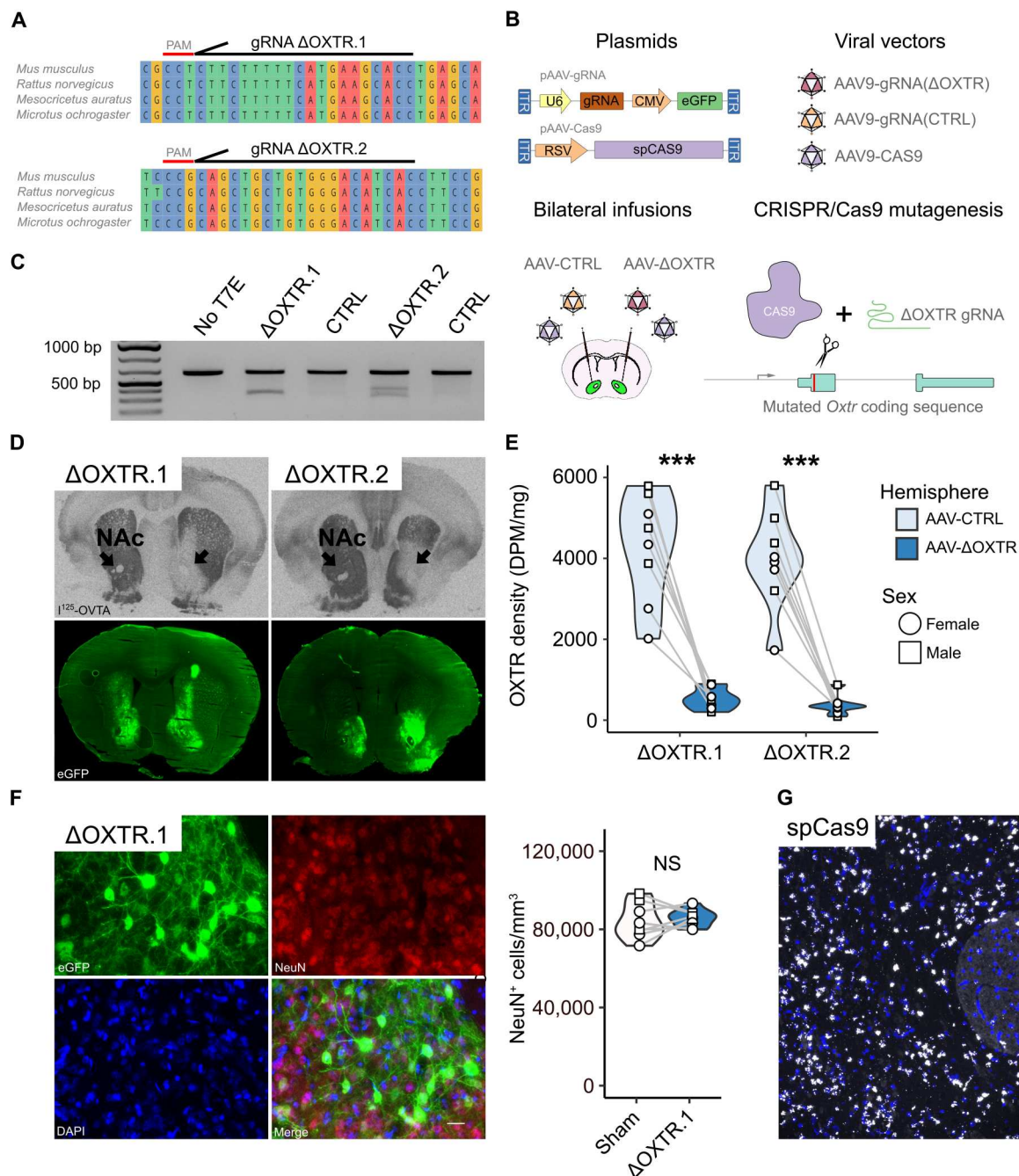


Fig. 1. Design of AAV-CRISPR/Cas9 strategy to functionally perturb OXTR signaling in multiple species and its validation in the prairie vole. (A) Schematic depicting the two conserved regions in the *Oxtr* coding sequence in four rodent species that are amenable to CRISPR/Cas9-mediated mutagenesis. (B) Schematics illustrating the major elements within the AAV plasmids, the AAV particles and how they are infused in the brain, as well as how the CRISPR/Cas9 technique functions. (C) Image of agarose gel evidencing gene editing after infusion of AAV- Δ OXTR, but not AAV-CTRL. (D) Top images are representative 125 I-OVTA autoradiograms of brain sections of AAV-CRISPR/Cas9-injected prairie voles, and bottom images are adjacent brain sections that show viral-induced eGFP fluorescence. Black arrows indicate the target area. (E) Quantification of OXTR levels in AAV- Δ OXTR-injected hemispheres and AAV-CTRL-injected hemispheres. Paired *t* tests, Δ OXTR.1: $N = 8$, $***P = 0.0001$; Δ OXTR.2: $N = 8$, $***P = 4.36 \times 10^{-5}$. (F) Images of native eGFP fluorescence, immunofluorescence of NeuN protein, and 4',6-diamidino-2-phenylindole (DAPI) staining in AAV- Δ OXTR-infected tissue on the left and quantification of NeuN⁺ cells in AAV- and sham-injected tissue. Paired *t* test: $N = 8$, $P = 0.6$. Scale bar, 20 μ m. NS, nonsignificance. (G) Representative image of *spCas9* mRNA expression in the prairie vole. Scale bar, 100 μ m.

tool to robustly disrupt functional OXTR signaling using targeted gene disruption in a spatially and temporally controlled manner in multiple rodent species has not been available. The development of such a tool is valuable to the comparative neuroscience field as it negates the need for extensive validation in each individual species. It will facilitate the applicability of gene editing in species that are studied for their interesting behavior, but for which genetic tools are not readily available, and thereby diversify the investigation of behaviors that are known to be modulated by OXT, but are not easily studied in mice (e.g., biparental care, pair bonding, partner loss, and female aggression) (15, 20, 35, 38).

Viral-mediated CRISPR/Cas9 genome editing has emerged as an efficient and versatile strategy to induce indels (insertions/deletions) in protein-coding sequences and perturb associated functional protein levels (11). We adopted an adeno-associated virus (AAV)-based strategy to deliver the CRISPR/Cas9 components to adult neural tissue (10) and reduce OXTR protein levels in vivo. The system consists of a Cas9 endonuclease from *Streptococcus pyogenes* (spCas9) that induces double-strand breaks in the DNA, and a guide RNA (gRNA), whose specificity determines the genomic location of Cas9-mediated mutagenesis. By targeting regions in the *Oxtr* coding sequence that are conserved across rodent species, we demonstrated the efficacy of this AAV-CRISPR/Cas9 vector at reducing OXTR density in six rodent species used in behavioral neuroendocrine research, and we predict that this approach will be effective in more than 80 rodent species. Furthermore, we demonstrated selectivity of the AAV-CRISPR/Cas9 approach by assessing its effect on the expression of a close match in the rodent genome, the arginine vasopressin receptor 1A (*Avpr1a*). Thereby, this tool will facilitate the comparative study of OXT signaling and advance our understanding of general principles of this ancient neuropeptide's function as well its role in modulating a diverse range of social behaviors in a species-specific manner. These results also provide a proof of principle for a strategy that can be widely applied to other genes in comparative neuroscience research.

RESULTS

Design of viral strategy to target *Oxtr* in multiple rodents

Our goal was to develop a viral vector-mediated tool to reduce OXTR density in brains across a wide range of rodent model species. We aimed to validate the efficacy of our tool using six model species that are used in OXT research: *Acomys cahirinus* (spiny mouse), *Mesocricetus auratus* (golden hamster), *Microtus ochrogaster* (prairie vole), *Mus musculus* (house mouse), *Peromyscus californicus* (California deer mouse), and *Rattus norvegicus* (Norway rat). We previously developed an AAV-CRISPR/Cas9 strategy using a gRNA targeting the prairie vole *Oxtr* to efficiently reduce functional OXTR in the brain (11, 39); however, the gRNA target sequence was not conserved across rodent species. Here, we used a similar approach using gRNAs targeting *Oxtr* sequences that are conserved across rodent species. To identify conserved *Oxtr* coding sequences that were amenable for mutagenesis by AAV-CRISPR/Cas9, we used the ClustalW algorithm of the msa package in R/Bioconductor to align the *Oxtr* coding sequences of species with available RefSeq sequence data (golden hamster, house mouse, Norway rat, and prairie vole) (40). Two conserved regions were found to be of sufficient length for

gRNA targeting (>19 nucleotides) and to contain a permissive protospacer adjacent motif (PAM) sequence (5'-NGG-3') (Fig. 1A). The gRNA(Δ OXTR)s target sequences in or just before the second transmembrane domain.

Validation of the viral strategy in the prairie vole

We produced AAV-gRNA(Δ OXTR) vectors to target these two regions and first assessed their effectiveness and specificity in the prairie vole. AAV-gRNA(Δ OXTR) vectors were combined with AAV-Cas9 vectors and injected unilaterally in the nucleus accumbens (NAc), while the contralateral side received an AAV-CTRL mix (Fig. 1B). The gRNA(CTRL) targets a sequence of the bacterial *LacZ* gene, which is not expressed in vertebrates. The NAc expresses high levels of *Oxtr* in prairie voles and is thought to mediate monogamous pair bonding behaviors (41). Two weeks after surgery, AAV-CRISPR/Cas9-mediated genomic editing was validated through a T7 endonuclease assay (Fig. 1C). DNA was isolated from enhanced green fluorescent protein (eGFP)-positive tissue (Fig. 1D), and the AAV-CRISPR/Cas9-targeted *Oxtr* coding regions were polymerase chain reaction (PCR)-amplified. T7 endonuclease restriction was observed in PCR amplicons, which were of the expected length from AAV- Δ OXTR-infected tissue, but not in AAV-CTRL-infected tissue, indicating that specific mutagenesis had occurred in the *Oxtr* coding sequence. The digested fragments were of the expected size based on the position of the gRNA target sequence. To test whether these mutations translated into reduced OXTR protein levels, we performed I¹²⁵-OVTA autoradiography on brain tissue (Fig. 1, D and E). Both AAV-gRNA(Δ OXTR) vectors strongly reduced I¹²⁵-OVTA binding in the NAc, indicating that the generated *Oxtr* indels disrupted functional OXTR production. Reduction in OXTR levels was not caused by neuronal cell death because AAV- Δ OXTR-injected and sham-injected hemispheres contained a similar number of cells that were positive for the neuronal marker NeuN. In addition, we performed RNAscope in situ hybridization and found faithful expression of *spCas9* in AAV-injected hemispheres ($N = 2$) (Fig. 1G).

The viral strategy does not affect AVPR1A levels in the prairie vole

Then, we sought to assess potential off-target effects of the gRNA(Δ OXTR)s. The vasopressin receptor 1A (*Avpr1a*) and vasopressin receptor 1B (*Avpr1b*) genes are closely related to *Oxtr* and share high sequence homology (42). While both gRNA target sequences do not align to the prairie vole *Avpr1b* sequence [>10-base pair (bp) mismatches], the gRNA(Δ OXTR.2) target sequence has the highest sequence homology to *Avpr1a* (3-bp mismatches) of all genes, which makes it the most likely off-target gene in the prairie vole genome for this gRNA. The gRNA(Δ OXTR.1) target sequence differs from the prairie vole *Avpr1a* sequence by 8 bp. To functionally assess the specificity of the gRNAs, we used the injection strategy as described above and targeted a region in the prairie vole in which *Avpr1a* is expressed: the ventral pallidum. AVPR1A autoradiography revealed no disruption in AVPR1A levels in the targeted area, demonstrating the selectivity of our gRNA(Δ OXTR)s (Fig. 2).

Efficacy of the viral strategy in five more rodent species

Next, we tested the efficacy of AAV-gRNA(Δ OXTR.1) in five more rodent species (Fig. 3). As OXTR expression varies extensively

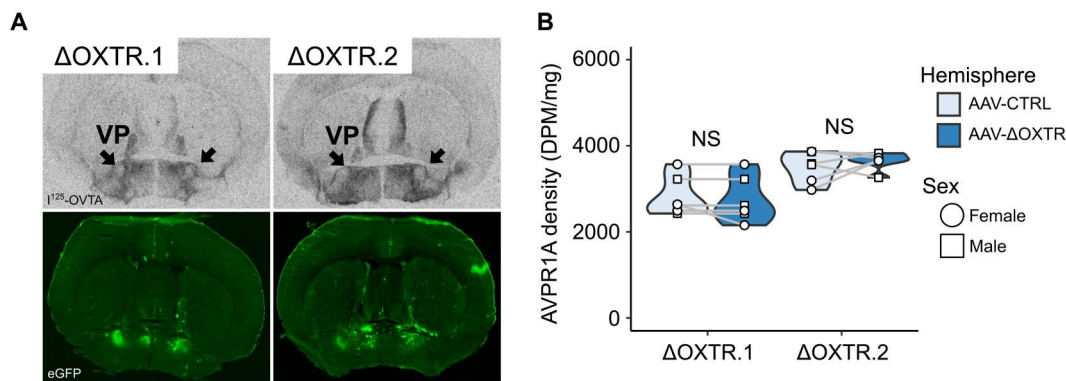


Fig. 2. AAV- ΔOXTR does not affect AVPR1A levels in the prairie vole ventral pallidum. (A) Top images are representative ^{125}I -AVP autoradiograms of brain sections of AAV-CRISPR/Cas9-injected prairie voles, and bottom images are adjacent brain sections that show viral-induced eGFP fluorescence. Black arrows indicate the target area (VP, ventral pallidum). (B) Quantification of OXTR levels in AAV- ΔOXTR -injected hemispheres and AAV-CTRL-injected hemispheres. Paired t tests, $\Delta\text{OXTR.1}$: $N = 6$, $P = 0.37$; $\Delta\text{OXTR.2}$: $N = 6$, $P = 0.38$.

among species, a range of brain areas was selected for injection based on the distribution of OXTR in each species (43–45). The NAc was targeted in spiny mice, the endopiriform cortex in golden hamsters, the ventromedial hypothalamus (VMH) in house mice, the lateral septum in California deer mice, and the central amygdala (CeA) in Norway rats. AAV-gRNA($\Delta\text{OXTR.1}$) significantly reduced OXTR levels across species and areas. In spiny mice, however, the efficacy was lower than in other species (fig. S1). During the preparation of this manuscript, the genome assembly of spiny mice became available, and we found no PAM sequence next to the target sequence of gRNA($\Delta\text{OXTR.1}$), which could explain the decreased effectiveness of gRNA($\Delta\text{OXTR.1}$) in spiny mice. However, the gRNA($\Delta\text{OXTR.2}$) target sequence is located next to a PAM site, so we injected a second batch of animals with this vector and observed strongly reduced OXTR levels. In sum, for all species, we achieved a significant reduction in OXTR levels.

The viral strategy targets a wide range of rodent species

We subsequently used the blastn algorithm from the National Center for Biotechnology Information (NCBI) BLAST+ software suite to search available whole-genome data and identify species in which either of the two gRNA(ΔOXTR) target sequences aligns perfectly and is thus predicted to work (46). We first aligned all available rodent RefSeq *Oxtr* sequences ($N = 30$) and found gRNA($\Delta\text{OXTR.1}$) to be functional in 15 species and gRNA($\Delta\text{OXTR.2}$) to be functional in 23 species (Fig. 4). Next, we searched for perfect alignment of the gRNA target sequences in all publicly available rodent genomes ($N = 231$). In this search, we found gRNA($\Delta\text{OXTR.1}$) to perfectly align in 43 species, and gRNA($\Delta\text{OXTR.2}$) in 80 species. Together, these viral vectors are likely to function in at least 81 rodent species (Table 1). Of note is that most of the rodent genomes are not annotated (e.g., spiny mouse and California deer mouse), so a perfect alignment does not necessarily indicate that the *Oxtr* coding sequence is targeted, and could indicate genes that closely resemble *Oxtr*, such as the vasopressin receptor genes. However, if interested in using this tool in a species for which no annotated genome is available, one could determine the sequence homology of the regions surrounding the

gRNA target sequence with other *Oxtr* sequences to ensure specific targeting of the *Oxtr* gene.

The viral strategy targets a wide range of nonrodent mammalian species

Last, we performed a BLAST search on all available RefSeq sequences in mammals and found gRNA target sequences in the *Oxtr* gene in many more mammalian species (Table 2). This search predicted that gRNA($\Delta\text{OXTR.1}$) specifically targets the *Oxtr* gene in five nonrodent mammalian species and that gRNA($\Delta\text{OXTR.2}$) is effective in 67 nonrodent mammals. This search further indicated that gRNA($\Delta\text{OXTR.2}$) targets vasopressin 1b (*Avpr1b*) in 127 nonrodent mammalian species (Table 3).

DISCUSSION

We here developed a specific, highly efficient tool to reduce functional OXTR densities in multiple rodent species used in socio-behavioral studies. We targeted the *Oxtr* coding sequence in five different brain regions across six rodent species and reduced OXTR levels in all cases. Injection of AAV- ΔOXTR did not result in gross neurotoxicity, as demonstrated by unaffected NeuN expression, nor did it reduce the protein density of the most probable off-target gene product, AVPR1A. Thereby, we validated this method for use in comparative OXT research.

Our tool represents a major advancement over other techniques that are used to genetically manipulate OXTR levels. It is more effective than short hairpin RNA (shRNA)-mediated knockdown (36) and more versatile and selective than systemic knockouts. The ability to reduce OXTR density in multiple species is of great value to OXT research, which has a strong comparative tradition. One of the guiding hypotheses that have emerged from comparative OXT research is that species-specific *Oxtr* expression patterns influence the regulation of social behaviors (29, 33, 47, 48) and that OXTR in different circuits can exert different effects on behavior (49). For example, socially monogamous species of voles have higher densities of OXTR in the NAc than do nonmonogamous species, and manipulating OXTR density in that region affects pair bonding behaviors (20). OXTR signaling in the NAc is critical for pair bond formation and maintenance (38), by modulating the

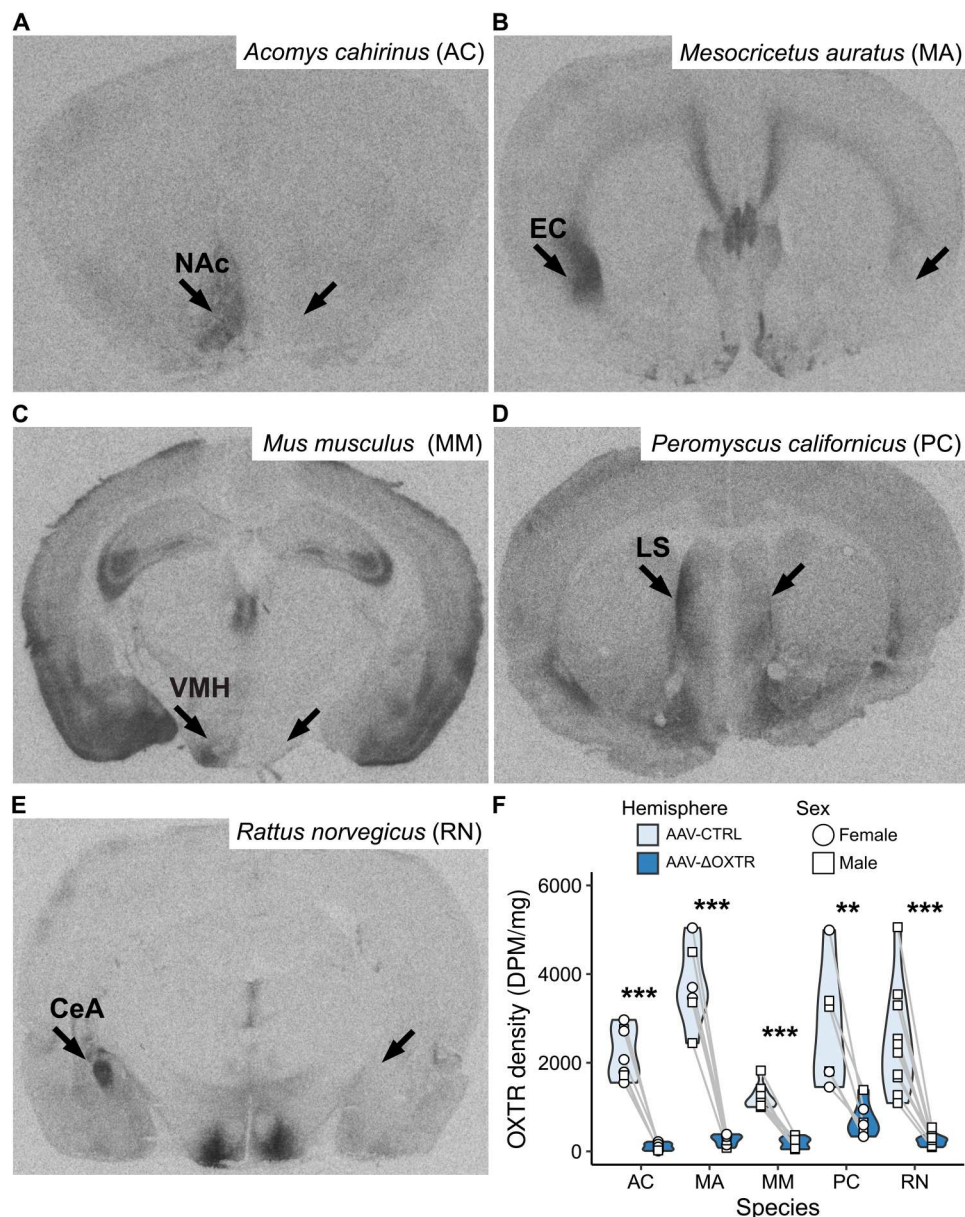


Fig. 3. AAV-ΔOXTR reduces OXTR density in five more rodent species. Representative images of ^{125}I -OVTA autoradiograms of AAV-CRISPR/Cas9-injected (A) spiny mouse (AC), (B) golden hamster (MA), (C) house mouse (MM), (D) California deer mouse (PC), and (E) Norway rat (RN). (F) Quantification of OXTR levels in AAV-ΔOXTR-injected hemispheres and AAV-CTRL-injected hemispheres per species. Paired t tests: AC: $N = 7$, $***P = 4.57 \times 10^{-5}$; MA: $N = 6$, $***P = 0.0001$; MM: $N = 7$, $***P = 4.1 \times 10^{-5}$; PC: $N = 6$, $**P = 0.006$; RN: $N = 10$, $***P = 0.0001$. Black arrows depict the target areas (NAc, nucleus accumbens; EC, endopiriform cortex; VMH, ventromedial hypothalamus; LS, lateral septum; CeA, central amygdala).

salience of social stimuli (50), while genetically mediated variation in OXTR in the NAc modulates pair bonding and the effects of early-life experience on pair bonding (34, 35, 51). In addition, OXTR levels tend to be high in brain areas that associate with the primary sensory modality of a species (52). In rodents, the olfactory system is densely populated with OXTR, while in primates the visual system has high levels of OXTR. Therefore, variation in OXTR distribution is thought to underlie differences in social strategies and contribute to diversity in social behaviors (53, 54). In theory, future comparative genome editing strategies could facilitate testing of this

hypothesis by allowing direct comparison of OXTR function across species.

A limitation of our strategy is that while it targets many species, it does not target all species. Part of this limitation stems from the evolvability of the target gene, as it will be easier to design comparative gene editing strategies to target conserved genes, rather than for genes of which many variants exist. For genes with abundant sequence diversity, only the use of multiple gRNAs can ensure the targeting of all gene variants. However, the relative ease with which gRNAs can be multiplexed in AAV vectors should make it possible to target a wide variety of species with one single strategy.

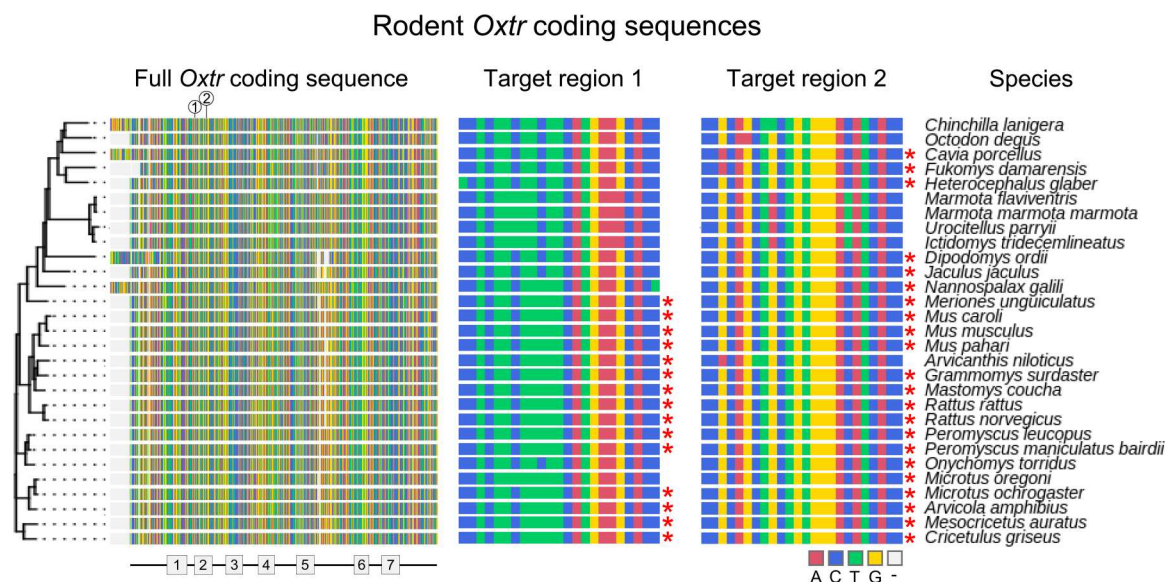


Fig. 4. Alignment of all current rodent RefSeq *Oxt* coding sequences and their match to the gRNA target sequences. Depicted on the left is a multiple sequence alignment of all available RefSeq rodent *Oxt* coding sequences ($N = 30$). The labels on top indicate the position of the gRNA target sequences, and the labels on the bottom indicate the transmembrane portions of the protein. In the middle, the gRNA target regions are shown. A red asterisk next to the sequence indicates perfect alignment with the gRNA sequence. Scientific names are shown on the right.

It is also important to consider that while two-thirds of the mutated gene products are likely to be nonfunctional because of frameshift mutations, one-third of mutated gene products might retain some functionality (e.g., receptor dimerization) (55). While our tool induces a near-complete loss of a specific functionality of the OXT receptor (i.e., ligand binding), we cannot exclude the possibility that a small part of the mutated gene products retains some form of residual activity. However, the tool is easily adapted to target other protein domains for functional characterization. Perhaps the greatest challenge to the efficient applicability of comparative genome editing is the design of gRNAs that target the widest range of species with preservation of specificity and efficiency. To simplify the design of comparative gene editing, gRNA-specific computational tools will have to be developed.

This work demonstrates the feasibility of designing genetic tools that are functional in multiple species. Although our tool was designed to target rodent *Oxt* coding sequences, it is predicted to target many nonrodent mammalian species as well. This shows that the design of genetic tools that work in multiple species is not limited to a single order but can be designed to target a much wider range of species. One of the main advantages of widely used neuroscience techniques, like chemo- and optogenetics, is that they can be used in a wide array of species (19, 56–58). This not only has given tremendous insight into the functioning of neural circuits but also has direct translational value, precisely because these techniques function in many species and thus allow the elucidation of general and species-specific principles of gene-brain-behavior relationships. Therefore, we believe that the comparative design of genetic tools will greatly enhance the translatability of future genetic techniques, both when used in research as well as in the clinic. In sum, we hope that this work will encourage the application of genetic dissection in comparative neuroscience and thereby

advance our understanding of the general and species-specific principles of neural circuit functioning.

MATERIALS AND METHODS

Design and synthesis of viral vectors

Oxt coding sequences of *M. auratus*, *M. ochrogaster*, *M. musculus*, and *R. norvegicus* were aligned with the ClustalW algorithm in the R/Bioconductor msa package to identify conserved regions (40). Within conserved regions, possible gRNA sequences were identified using Benchling. Three candidate sequences were selected on the basis of predicted efficiency and off-target effects. A control gRNA was designed to target the bacterial *lacZ* gene. gRNA sequences cloned into pAAV-U6-gRNA-CMV-eGFP were the following: gRNA(Δ OXT.1), 5'-GGTGCTTCATGAAAAAGAAG-3'; gRNA(Δ OXT.2), 5'-GTGATGTCCCACAGCAGCTG-3'; gRNA(Δ OXT.3), 5'-GCCCCACCTGCTGTGTCGTC-3'; and gRNA(CTRL), 5'-GTGAGCGAGTAACAACCCGT-3'. Oligos were cloned into pAAV-U6-gRNA-CMV-eGFP (Addgene, plasmid #85451, gift of H. Lei) after plasmid digestion with Sap I. The first batch of viral particles was synthesized using pAAV-U6-gRNA-CMV-eGFP, pAAV-RSV-spCas9 (Addgene, plasmid #85450, gifted by H. Lei), pAAV9-SPAKFA (Penn Vector Core, PA, USA), and pAAV/Ad (American Type Culture Collection, VA, USA). AAV9 particles were produced in human embryonic kidney (HEK) 293T cells, purified with AVB-affinity chromatography (59), and concentrated by centrifugal filtration (Amicon Ultra-4, Fisher Scientific, NH, USA), after which viral titer was determined using quantitative PCR targeting the inverted terminal repeats (60). Five viruses were generated: AAV9-U6-gRNA(Δ OXT.1)-CMV-eGFP, AAV9-U6-gRNA(Δ OXT.2)-CMV-eGFP, AAV9-U6-gRNA(Δ OXT.3)-CMV-eGFP, AAV9-U6-gRNA(CTRL)-CMV-eGFP, and AAV9-RSV-spCas9. AAV-

Table 1. List of rodent species that contain gRNA(Δ OXTR) target sequences in their genome.

Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Acomys cahirinus</i>	No	Yes
<i>Acomys russatus</i>	No	Yes
<i>Apodemus speciosus</i>	No	Yes
<i>Apodemus sylvaticus</i>	No	Yes
<i>Arvicanthis niloticus</i>	Yes	No
<i>Arvicola amphibius</i>	Yes	Yes
<i>Capromys pilorides</i>	No	Yes
<i>Cavia aperea</i>	No	Yes
<i>Cavia porcellus</i>	No	Yes
<i>Cavia tschudii</i>	No	Yes
<i>Cricetomys gambianus</i>	No	Yes
<i>Cricetulus griseus</i>	Yes	Yes
<i>Dasyprocta punctata</i>	No	Yes
<i>Dinomys branickii</i>	No	Yes
<i>Dipodomys ordii</i>	No	Yes
<i>Dipodomys spectabilis</i>	No	Yes
<i>Dipodomys stephensi</i>	No	Yes
<i>Dolichotis patagonum</i>	No	Yes
<i>Ellobius lutescens</i>	Yes	Yes
<i>Ellobius talpinus</i>	Yes	Yes
<i>Erethizon dorsatum</i>	No	Yes
<i>Fukomys damarensis</i>	No	Yes
<i>Glis glis</i>	No	Yes
<i>Grammomys surdaster</i>	Yes	Yes
<i>Heterocephalus glaber</i>	No	Yes
<i>Hydrochoerus hydrochaeris</i>	No	Yes
<i>Hylomyscus alleni</i>	Yes	Yes
<i>Hystrix brachyura</i>	No	Yes
<i>Hystrix cristata</i>	No	Yes
<i>Jaculus jaculus</i>	No	Yes
<i>Lophiomyes imhausi</i>	No	Yes
<i>Mastomys coucha</i>	Yes	Yes
<i>Mastomys natalensis</i>	Yes	Yes
<i>Meriones unguiculatus</i>	Yes	Yes
<i>Mesocricetus auratus</i>	Yes	Yes
<i>Microtus agrestis</i>	Yes	Yes
<i>Microtus arvalis</i>	No	Yes
<i>Microtus fortis</i>	Yes	Yes
<i>Microtus montanus</i>	Yes	Yes
<i>Microtus ochrogaster</i>	Yes	Yes
<i>Microtus oeconomus</i>	Yes	Yes
<i>Microtus oregoni</i>	No	Yes
<i>Microtus richardsoni</i>	Yes	Yes
<i>Mus caroli</i>	Yes	Yes
<i>Mus minutoides</i>	Yes	Yes
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Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Mus musculus</i>	Yes	Yes
<i>Mus pahari</i>	Yes	Yes
<i>Mus spicilegus</i>	Yes	Yes
<i>Mus spretus</i>	Yes	Yes
<i>Muscardinus avellanarius</i>	No	Yes
<i>Myocastor coypus</i>	No	Yes
<i>Myodes glareolus</i>	Yes	Yes
<i>Nannospalax galili</i>	No	Yes
<i>Neotoma lepida</i>	Yes	Yes
<i>Ondatra zibethicus</i>	Yes	Yes
<i>Onychomys torridus</i>	No	Yes
<i>Orientallactaga bullata</i>	No	Yes
<i>Pedetes capensis</i>	No	Yes
<i>Peromyscus attwateri</i>	Yes	Yes
<i>Peromyscus aztecus</i>	Yes	Yes
<i>Peromyscus californicus</i>	Yes	Yes
<i>Peromyscus eremicus</i>	Yes	Yes
<i>Peromyscus leucopus</i>	Yes	Yes
<i>Peromyscus maniculatus</i>	Yes	Yes
<i>Peromyscus melanophrys</i>	Yes	Yes
<i>Peromyscus nudipes</i>	Yes	Yes
<i>Peromyscus polionotus</i>	Yes	Yes
<i>Phodopus roborovskii</i>	Yes	Yes
<i>Phodopus sungorus</i>	No	Yes
<i>Praomys delectorum</i>	Yes	Yes
<i>Psammomys obesus</i>	Yes	Yes
<i>Rattus norvegicus</i>	Yes	Yes
<i>Rattus rattus</i>	Yes	Yes
<i>Rhabdomys dilectus</i>	Yes	Yes
<i>Rhizomys pruinosus</i>	No	Yes
<i>Rhombomys opimus</i>	Yes	Yes
<i>Rhynchomys soricoides</i>	Yes	Yes
<i>Sigmodon hispidus</i>	No	Yes
<i>Thryonomys swinderianus</i>	No	Yes
<i>Typhlomys cinereus</i>	No	Yes
<i>Zapus hudsonius</i>	No	Yes

gRNA and AAV-Cas9 vectors were diluted to 3.0×10^{10} and 1.5×10^{10} genomic copies/ μ l, respectively, and mixed in a 1:1 ratio. In initial experiments in the prairie vole, AAV-gRNA(Δ OXTR.3) showed markedly lower efficiency and was not tested further. A second batch of AAV9-viruses was generated by VectorBuilder (Chicago, IL, USA): AAV9-U6-gRNA(Δ OXTR.1)-CMV-eGFP (2.0×10^{10} genomic copies/ μ l), AAV9-U6-gRNA(Δ OXTR.2)-CMV-eGFP, AAV9-U6-gRNA(CTRL)-CMV-eGFP (2.0×10^{10} genomic copies/ μ l), and AAV9-RSV-spCas9 (1.5×10^{10} genomic copies/ μ l). Both batches of the virus showed similar efficacy, and data from both batches have been pooled.

Animals

All experiments were performed following the guidelines and approved by the respective Institutional Animal Care and Use Committees (Emory University, Georgia State University, and University of California, Davis). The following animals were used in this study: California deer mice (B.C.T., University of California, Davis: 3 males and 3 females), golden hamsters (H.E.A., Georgia State University: 3 males and 3 females), house mice (L.J.Y., Emory University: 7 males), Norway rat (L.J.Y., Emory University: 10 males), prairie vole (L.J.Y., Emory University: 16 males and 16 females), and spiny mice (A.M.K., Emory University: 3 males and

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Table 2. List of mammalian species that contain gRNA(Δ OXTR) target sequences in their *Oxtr* gene.

Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Acomys russatus</i>	No	Yes
<i>Ailuropoda melanoleuca</i>	No	Yes
<i>Aotus nancymae</i>	No	Yes
<i>Apodemus sylvaticus</i>	No	Yes
<i>Arvicanthis niloticus</i>	Yes	No
<i>Arvicola amphibius</i>	Yes	Yes
<i>Balaenoptera acutorostrata scammoni</i>	No	Yes
<i>Balaenoptera musculus</i>	No	Yes
<i>Callithrix jacchus</i>	No	Yes
<i>Canis lupus dingo</i>	No	Yes
<i>Canis lupus familiaris</i>	No	Yes
<i>Cavia porcellus</i>	No	Yes
<i>Cebus imitator</i>	No	Yes
<i>Ceratotherium simum simum</i>	No	Yes
<i>Choloepus didactylus</i>	No	Yes
<i>Chrysochloris asiatica</i>	No	Yes
<i>Cricetulus griseus</i>	Yes	Yes
<i>Dipodomys ordii</i>	No	Yes
<i>Dipodomys spectabilis</i>	No	Yes
<i>Echinops telfairi</i>	No	Yes
<i>Elephantulus edwardii</i>	No	Yes
<i>Elephas maximus indicus</i>	No	Yes
<i>Eptesicus fuscus</i>	No	Yes
<i>Equus asinus</i>	No	Yes
<i>Equus caballus</i>	No	Yes
<i>Equus przewalskii</i>	No	Yes
<i>Equus quagga</i>	No	Yes
<i>Erinaceus europaeus</i>	No	Yes
<i>Fukomys damarensis</i>	No	Yes
<i>Galeopterus variegatus</i>	No	Yes
<i>Globicephala melas</i>	No	Yes
<i>Grammomys surdaster</i>	Yes	Yes
<i>Heterocephalus glaber</i>	No	Yes
<i>Hipposideros armiger</i>	No	Yes
<i>Jaculus jaculus</i>	No	Yes
<i>Lagenorhynchus obliquidens</i>	No	Yes
<i>Lemur catta</i>	No	Yes
<i>Lipotes vexillifer</i>	No	Yes
<i>Manis javanica</i>	No	Yes
<i>Manis pentadactyla</i>	No	Yes
<i>Mastomys coucha</i>	Yes	Yes
<i>Meles meles</i>	No	Yes
<i>Meriones unguiculatus</i>	Yes	Yes
<i>Mesocricetus auratus</i>	Yes	Yes
<i>Microcebus murinus</i>	No	Yes
continued on next page		

Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Microtus fortis</i>	Yes	Yes
<i>Microtus ochrogaster</i>	Yes	Yes
<i>Microtus oregoni</i>	No	Yes
<i>Miniopterus natalensis</i>	No	Yes
<i>Monodon monoceros</i>	No	Yes
<i>Mus caroli</i>	Yes	Yes
<i>Mus musculus</i>	Yes	Yes
<i>Mus pahari</i>	Yes	Yes
<i>Mustela erminea</i>	No	Yes
<i>Myodes glareolus</i>	Yes	Yes
<i>Myotis brandtii</i>	No	Yes
<i>Myotis davidii</i>	No	Yes
<i>Myotis lucifugus</i>	No	Yes
<i>Myotis myotis</i>	No	Yes
<i>Nannospalax galili</i>	No	Yes
<i>Neophocaena asiaeorientalis asiaeorientalis</i>	No	Yes
<i>Ochotona princeps</i>	No	Yes
<i>Onychomys torridus</i>	No	Yes
<i>Orcinus orca</i>	No	Yes
<i>Oryctolagus cuniculus</i>	No	Yes
<i>Otolemur garnettii</i>	No	Yes
<i>Peromyscus californicus insignis</i>	Yes	Yes
<i>Peromyscus leucopus</i>	Yes	Yes
<i>Peromyscus maniculatus bairdii</i>	Yes	Yes
<i>Phocoena sinus</i>	No	Yes
<i>Phodopus roborovskii</i>	Yes	Yes
<i>Pipistrellus kuhlii</i>	No	Yes
<i>Propithecus coquereli</i>	No	Yes
<i>Rattus norvegicus</i>	Yes	Yes
<i>Rattus rattus</i>	Yes	Yes
<i>Rhinolophus ferrumequinum</i>	No	Yes
<i>Rhinolophus sinicus</i>	No	Yes
<i>Saimiri boliviensis boliviensis</i>	No	Yes
<i>Sapajus apella</i>	No	Yes
<i>Talpa occidentalis</i>	No	Yes
<i>Trichechus manatus latirostris</i>	No	Yes
<i>Tupaia chinensis</i>	No	Yes
<i>Tursiops truncatus</i>	Yes	Yes
<i>Ursus americanus</i>	No	Yes
<i>Ursus arctos</i>	No	Yes
<i>Ursus maritimus</i>	No	Yes
<i>Vulpes lagopus</i>	No	Yes
<i>Vulpes vulpes</i>	No	Yes

Table 3. List of mammalian species that contain gRNA(Δ OXTR) target sequences in their *Avpr1b* gene.

Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Acinonyx jubatus</i>	No	Yes
<i>Ailuropoda melanoleuca</i>	No	Yes
<i>Aotus nancymae</i>	No	Yes
<i>Artibeus jamaicensis</i>	No	Yes
<i>Balaenoptera acutorostrata scammoni</i>	No	Yes
<i>Balaenoptera musculus</i>	No	Yes
<i>Bison bison bison</i>	No	Yes
<i>Bos indicus</i>	No	Yes
<i>Bos indicus</i> \times <i>Bos taurus</i>	No	Yes
<i>Bos mutus</i>	No	Yes
<i>Bos taurus</i>	No	Yes
<i>Bubalus bubalis</i>	No	Yes
<i>Budorcas taxicolor</i>	No	Yes
<i>Callorhinus ursinus</i>	No	Yes
<i>Camelus bactrianus</i>	No	Yes
<i>Camelus dromedarius</i>	No	Yes
<i>Camelus ferus</i>	No	Yes
<i>Capra hircus</i>	No	Yes
<i>Cebus imitator</i>	No	Yes
<i>Cercocebus atys</i>	No	Yes
<i>Cervus canadensis</i>	No	Yes
<i>Cervus elaphus</i>	No	Yes
<i>Chlorocebus sabaes</i>	No	Yes
<i>Choloepus didactylus</i>	No	Yes
<i>Colobus angolensis palliatus</i>	No	Yes
<i>Dasypus novemcinctus</i>	No	Yes
<i>Delphinapterus leucas</i>	No	Yes
<i>Echinops telfairi</i>	No	Yes
<i>Enhydra lutris kenyoni</i>	No	Yes
<i>Eptesicus fuscus</i>	No	Yes
<i>Equus asinus</i>	No	Yes
<i>Equus caballus</i>	No	Yes
<i>Equus przewalskii</i>	No	Yes
<i>Equus quagga</i>	No	Yes
<i>Erinaceus europaeus</i>	No	Yes
<i>Eumetopias jubatus</i>	No	Yes
<i>Felis catus</i>	No	Yes
<i>Globicephala melas</i>	No	Yes
<i>Gorilla gorilla gorilla</i>	No	Yes
<i>Halichoerus grypus</i>	No	Yes
<i>Homo sapiens</i>	No	Yes
<i>Hyaena hyaena</i>	No	Yes
<i>Hylobates moloch</i>	No	Yes
<i>Lagenorhynchus obliquidens</i>	No	Yes
<i>Lemur catta</i>	No	Yes
continued on next page		

Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Leopardus geoffroyi</i>	No	Yes
<i>Leptonychotes weddellii</i>	No	Yes
<i>Lipotes vexillifer</i>	No	Yes
<i>Lontra canadensis</i>	No	Yes
<i>Lutra lutra</i>	No	Yes
<i>Lynx canadensis</i>	No	Yes
<i>Lynx rufus</i>	No	Yes
<i>Macaca fascicularis</i>	No	Yes
<i>Macaca mulatta</i>	No	Yes
<i>Macaca nemestrina</i>	No	Yes
<i>Macaca thibetana thibetana</i>	No	Yes
<i>Mandrillus leucophaeus</i>	No	Yes
<i>Manis pentadactyla</i>	No	Yes
<i>Meles meles</i>	No	Yes
<i>Microcebus murinus</i>	No	Yes
<i>Miniopterus natalensis</i>	No	Yes
<i>Mirounga angustirostris</i>	No	Yes
<i>Mirounga leonina</i>	No	Yes
<i>Monodon monoceros</i>	No	Yes
<i>Mustela erminea</i>	No	Yes
<i>Mustela putorius furo</i>	No	Yes
<i>Myotis brandtii</i>	No	Yes
<i>Myotis lucifugus</i>	No	Yes
<i>Myotis myotis</i>	No	Yes
<i>Nannospalax galili</i>	No	Yes
<i>Neogale vison</i>	No	Yes
<i>Neomonachus schauinslandi</i>	No	Yes
<i>Neophocaena asiaeorientalis asiaeorientalis</i>	No	Yes
<i>Nomascus leucogenys</i>	No	Yes
<i>Odobenus rosmarus divergens</i>	No	Yes
<i>Odocoileus virginianus texanus</i>	No	Yes
<i>Orcinus orca</i>	No	Yes
<i>Oryx dammah</i>	No	Yes
<i>Ovis aries</i>	No	Yes
<i>Pan paniscus</i>	No	Yes
<i>Pan troglodytes</i>	No	Yes
<i>Panthera leo</i>	No	Yes
<i>Panthera pardus</i>	No	Yes
<i>Panthera tigris</i>	No	Yes
<i>Panthera uncia</i>	No	Yes
<i>Papio anubis</i>	No	Yes
<i>Phacochoerus africanus</i>	No	Yes
<i>Phoca vitulina</i>	No	Yes
<i>Phocoena sinus</i>	No	Yes
<i>Phyllostomus discolor</i>	No	Yes
<i>Phyllostomus hastatus</i>	No	Yes
continued on next page		

Species	gRNA(Δ OXTR.1)	gRNA(Δ OXTR.2)
<i>Physeter catodon</i>	No	Yes
<i>Ptilocolobus tephrosceles</i>	No	Yes
<i>Pipistrellus kuhlii</i>	No	Yes
<i>Pongo abelii</i>	No	Yes
<i>Prionailurus bengalensis</i>	No	Yes
<i>Prionailurus viverrinus</i>	No	Yes
<i>Propithecus coquereli</i>	No	Yes
<i>Pteropus alecto</i>	No	Yes
<i>Pteropus giganteus</i>	No	Yes
<i>Pteropus vampyrus</i>	No	Yes
<i>Puma concolor</i>	No	Yes
<i>Puma yagouaroundi</i>	No	Yes
<i>Rhinolophus ferrumequinum</i>	No	Yes
<i>Rhinolophus sinicus</i>	No	Yes
<i>Rhinopithecus bieti</i>	No	Yes
<i>Rhinopithecus roxellana</i>	No	Yes
<i>Rousettus aegyptiacus</i>	No	Yes
<i>Saimiri boliviensis boliviensis</i>	No	Yes
<i>Sapajus apella</i>	No	Yes
<i>Sturnira hondurensis</i>	No	Yes
<i>Suncus etruscus</i>	No	Yes
<i>Sus scrofa</i>	No	Yes
<i>Talpa occidentalis</i>	No	Yes
<i>Theropithecus gelada</i>	No	Yes
<i>Trachypithecus francoisi</i>	No	Yes
<i>Tupaia chinensis</i>	No	Yes
<i>Tursiops truncatus</i>	No	Yes
<i>Ursus americanus</i>	No	Yes
<i>Ursus arctos</i>	No	Yes
<i>Ursus arctos horribilis</i>	No	Yes
<i>Ursus maritimus</i>	No	Yes
<i>Vicugna pacos</i>	No	Yes
<i>Zalophus californianus</i>	No	Yes

9 females). All animals were sexually naïve adults and housed in standard laboratory conditions with ad libitum water and food provided.

Intracranial surgeries

For all species, anesthesia was induced by exposure to a 2 to 4% iso-flurane/oxygen mix and maintained at 1 to 3%. Three daily doses of meloxicam or carprofen (2 to 5 mg/kg, depending on species) were administered after surgery. Using a stereotaxic apparatus, animals were unilaterally injected with a 1:1 mix of AAV9-RSV-Cas9 and AAV9-gRNA(Δ OXTR), while the contralateral side received a 1:1 mix of AAV9-RSV-Cas9 and AAV9-gRNA(CTRL). Stereotaxic co-ordinates and injected volumes are summarized in Table 4.

T7 endonuclease I assay

eGFP-infected tissue was collected from fresh-frozen brain sections, and DNA was isolated using the Blood & Tissue DNA kit (Qiagen, Germany). Fragments surrounding the gRNA target sides were PCR-amplified using Q5-polymerase (New England Biolabs, MA, USA) and the primer set 5'-AGCAGTCAAAAACACCGTCC-3' (forward) and 5'-GACACCTGGACAACATCATCGG-3' (reverse) under these cycling conditions: 98°C for 2 min, 34 cycles of 98°C for 15 s, 63°C for 15 s, and 72°C for 30 s, and 2 min of final elongation. Next, PCR fragments were used for the T7 endonuclease I assay according to the manufacturer's instructions (Integrated DNA Technologies, IA, USA).

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Table 4. Stereotaxic coordinates and injected volume. AP, anterior-posterior; ML, medial-lateral; DV, dorsal-ventral; EC, endopiriform cortex; VP, ventral pallidum; LS, lateral septum; CeA, central amygdala.

Common name	Area	AP	ML	DV	Angle	Volume (nl)
Spiny mouse	NAC	+2.9	±1	−5	0	300
Golden hamster	EC	+3.6	±3.2	−6	0	200
House mouse	VMH	−1.22	±2.0	−5.3	0	300
Prairie vole	NAC	+1.7	±2.1	−4.7	10	300
Prairie vole	VP	+0.2	±1.8	−5.1	10	300
California deer mouse	LS	−0.36	±0.7	−3.5	0	300
Norway rat	CeA	−1.8	±4.2	−8	0	300

OXTR and AVPR1A autoradiography

Fresh-frozen brains were sectioned on a cryostat (Eprelia Cryostat NX-70, Thermo Fisher Scientific, MA, USA) at 20 μm, mounted on Superfrost Plus slides (Fisher Scientific), and stored at −80°C until use. Autoradiography was performed as previously described (41). Briefly, slides were thawed and fixed for 2 min in 0.1% paraformaldehyde in phosphate-buffered saline (PBS) for 2 min, washed in 50 mM tris in PBS (pH 7.4, 2 × 10 min), and incubated in 50 mM tris buffer, supplemented with 0.1% bovine serum albumin and 50 pM I¹²⁵-OVTA (2200 Ci/mmol, ornithine vasotocin analog, #NEX254010UC, PerkinElmer, MA, USA) or 50 pM I¹²⁵-AVP (2200 Ci/mmol, linear arginine vasopressin, #NEX310010UC, PerkinElmer) at room temperature (RT) for 1 hour. Unbound ligand was removed by washing in 50 mM tris with 0.2% MgCl₂ at 4°C (4 × 5 min) and 30 min at RT. Slides were dipped in Milli-Q, dried, and placed in a cassette with BioMax MR film (Sigma-Aldrich, MO, USA). After 7 days, films were developed and imaged using an MCID core system (Interfocus Co., UK). Mean gray values of viral-targeted regions, corrected for background, were determined in ImageJ. I¹²⁵-activity (disintegrations per minute) was calculated using an I¹²⁵-standard and taken as a proxy for OXTR or AVPR1A density. Differences in OXTR or AVPR1A density were determined by comparing protein density levels in AAV-ΔOXTR-injected regions to protein density levels in contralateral AAV-CTRL-injected regions.

Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused with PBS, followed by PBS supplemented with 4% paraformaldehyde. Brains were sectioned on a cryostat (Cryostat NX-70) at 40 μm and stored in cryoprotectant buffer at −20°C until use. Sections were thawed, washed in PBS, and blocked and permeabilized in PBS supplemented with 0.1% Tween (PBST) and 5% normal donkey serum for 1 hour at RT. Next, sections were incubated in PBST supplemented with 0.5% rabbit anti-NeuN (1:1000, AB104225, Abcam, UK) at 4°C overnight. After PBS washes, sections were incubated in anti-rabbit Alexa Fluor 568 antibodies (1:500, Molecular Probes, OR, USA) for 1 hour. Sections were mounted and coverslipped in Fluoromount-G containing 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). Z-stacks

with ×60 magnification of eGFP-injected regions and the corresponding sham-injected contralateral region were imaged using a Keyence microscope. Z-stacks were projected with maximal intensity, and NeuN-positive cells were manually counted in ImageJ by a blinded observer.

In situ hybridization

Fresh-frozen brains (N = 2 × 2 hemispheres) were sectioned on a cryostat (Cryostat NX-70) at 20 μm, mounted on Superfrost Plus slides (Fisher Scientific), and stored at −80°C until use. Slides were thawed, and RNAscope in situ hybridization was done according to the manufacturer’s protocol (#320293, ACD Inc., MN, USA). Briefly, slides were pretreated with Protease (#320842, ACD Inc.) and incubated with spCas9 probes (#519411, ACD Inc.) for 2 hours at 40°C. Sections were washed, and signal amplification was performed using the kit’s reagents. Last, sections were coverslipped in Fluoromount-G containing DAPI (Thermo Fisher Scientific). The 20× images were made on a Keyence BZ-X700 microscope (Keyence, Japan).

Statistical analyses

All statistical analyses were performed in RStudio, using paired t tests (Figs. 1, E and F, 2B, and 3F and fig. S1B), with α = 0.05.

BLAST

We used the blastn algorithm in the NCBI BLAST+ software suite to search for perfect alignment of the target RNA sequences plus the permissive PAM sequence (5'-NGG-3') to rodent (taxid 9989) and mammalian (taxid 40674) genomes to identify species in which our tool is predicted to be functional.

Supplementary Materials

This PDF file includes:
Fig. S1

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An AAV-CRISPR/Cas9 strategy for gene editing across divergent rodent species: Targeting neural oxytocin receptors as a proof of concept

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