Targeting neurons with functional oxytocin receptors: A novel set of simple knock-in mouse lines for oxytocin receptor visualization and manipulation

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Targeting neurons with functional oxytocin receptors:

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2. Abbreviated title

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4. Author contributions

Y.U.I., H.M., and R.K. designed the experiments. Y.U.I., H.M., K.H., R.K., Y.M., E.K., J.A. and S.K. performed the experiments. Y.U.I., H.M., K.H., R.K., M.Y., M.H., and T.I. analyzed and discussed the results. Y.U.I., H.M., and T.I. wrote the manuscript. All authors have read and agreed to the final version of the manuscript.

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14. Conflict of interest

50 Authors report no conflict of interest.

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54 **16. Material Availability**

55 Four knock-in mouse lines generated in this study will be made available with a materials transfer
56 agreement.

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87 **Targeting neurons with functional oxytocin receptors:**
 88 **A novel set of simple knock-in mouse lines for oxytocin receptor visualization and**
 89 **manipulation**

90
 91
 92 **Abstract**

93 The neuropeptide oxytocin (Oxt) plays important roles in modulating social behaviors.
 94 Oxytocin receptor (Oxtr) is abundantly expressed in the brain and its relationship to
 95 socio-behavioral controls has been extensively studied using mouse brains. Several genetic
 96 tools to visualize and/or manipulate Oxtr-expressing cells, such as fluorescent reporters and
 97 Cre recombinase drivers, have been generated by ES-cell based gene targeting or bacterial
 98 artificial chromosome (BAC) transgenesis. However, these mouse lines displayed some
 99 differences in their Oxtr expression profiles probably due to the complex context and
 100 integrity of their genomic configurations in each line. Here we apply our sophisticated
 101 genome-editing techniques to the *Oxtr* locus, systematically generating a series of knock-in
 102 mouse lines, in which its endogenous transcriptional regulations are intactly preserved and
 103 evaluate their expression profiles to ensure the reliability of our new tools. We employ the
 104 epitope tagging strategy, with which C-terminally fused tags can be detected by highly
 105 specific antibodies, to successfully visualize the Oxtr protein distribution on the neural
 106 membrane with super-resolution imaging for the first time. By utilizing T2A self-cleaving
 107 peptide sequences, we also induce proper expressions of tdTomato reporter, codon-improved
 108 Cre recombinase, and spatiotemporally inducible Cre-ERT2 in Oxtr-expressing neurons.
 109 Electrophysiological recordings from tdTomato-positive cells in the reporter mice support the
 110 validity of our tool design. Retro-orbital injections of AAV-PHP.eB vector into the Cre line
 111 further enabled visualization of recombinase activities in the appropriate brain regions.
 112 Moreover, the first-time Cre-ERT2 line drives Cre-mediated recombination in a
 113 spatiotemporally controlled manner upon tamoxifen administration. These tools thus provide
 114 an excellent resource for future functional studies in Oxt-responsive neurons and should
 115 prove of broad interest in the field.

116
 117
 118 **Significance Statements**

119 Here we develop a novel series of genome-edited mouse lines to help understand the circuit
 120 mechanisms underlying Oxt actions, by enabling the visualization and manipulation of
 121 Oxtr-expressing neurons in a manner precisely reflecting their endogenous expression

profiles. The epitope tagging strategy allows super-resolution imaging to decipher the 3-D distributions of Oxtr protein on neural membranes for the first time. The red fluorescent tdTomato reporter serves as a reliable visualization tool for Oxtr-expressing cells, whose expression profiles are validated using electrophysiological recordings. Both iCre and iCre-ERT2 drivers, which properly induce recombinase activities in Oxtr-expressing cells, would largely contribute to viral vector-dependent functional analyses and fate mapping studies. These tools thus offer a new option for the research communities to improve our knowledge of Oxt-Oxtr circuitry.

Introduction

The neuropeptide oxytocin (Oxt) has attracted great attention not only from researchers but also from the general public because of its profound social (Froemke and Young, 2021), pro-social (Marsh et al., 2020), anxiolytic (Neumann and Slattery, 2016), and anti-stress (Onaka and Takayanagi, 2019) behavioral effects in animals. Thanks to the advanced neuro-technologies, including optogenetics (Deisseroth, 2011; Grinevich and Neumann, 2021), chemogenetics (Grinevich and Neumann, 2021; Grund et al., 2019), and calcium imaging (Hung et al., 2017; Maldonado et al., 2021; Resendez et al., 2020), the complex neurobiology of the Oxt system has been gradually revealed over the last decade. The oxytocin receptor (Oxtr), a member of the G-protein-coupled receptor (GPCR) family, processes and transfers signals into the cytoplasm to modulate downstream circuitries (Grinevich and Neumann, 2021). Although a growing number of studies have shed light on Oxt-Oxtr signaling in various brain regions such as the prefrontal cortex (PFC) (Nakajima et al., 2014; Tan et al., 2019), lateral septum (Horiai et al., 2020; Menon et al., 2018), nucleus accumbens (Dölen et al., 2013; Nardou et al., 2019), hippocampus (Owen et al., 2013; Raam et al., 2017), ventral tegmental area (Hung et al., 2017), and others, each study has used different tools to visualize and manipulate Oxtr-expressing neurons.

Some studies have used an antibody (called OXTR-2) generated in a research laboratory to detect Oxtr proteins (Marlin et al., 2015; Mitre et al., 2016). Unfortunately, this antibody is not available for the public to replicate the experimental findings. In addition, because of the general difficulty in generating antibodies to lipophilic GPCRs with low epitope exposures, no anti-Oxtr antibody suitable for immunohistochemistry is commercially available. From these reasons, the *Oxtr-Venus* knock-in mouse line was generated (Yoshida et al., 2009) and used for receptor visualization (Dölen et al., 2013; Hung et al., 2017; Newmaster et al., 2020). However, contrary to our expectations, expressions of the Venus

reporter in the brain was reported to largely decrease during development (Newmaster et al., 2020). In this reporter line, the 1st coding exon had been replaced with Venus sequences (Extended Data Fig.1-1b). The resulting knock-in allele lacks its endogenous configurations of intron and 3' UTR sequences, both of which likely contain transcriptional regulatory elements essential for the spatiotemporally coordinated *Oxtr* expressions. This might considerably affect the Venus reporter expression profiles.

Cre recombinase driver mice also play pivotal roles in both conditional knockout experiments and viral vector-dependent functional analyses. Although two *Oxtr-Cre* knock-in lines utilizing internal ribosome entry site (IRES) sequences for their bicistronic expressions have been generated (Hidema et al., 2016; Ryan et al., 2017) (Extended Data Fig.1-1c, 1-1d), those expression profiles reported were relatively sparse and did not well overlap with each other, probably due to the different context and integrity of their genomic configurations. In addition, the *Oxtr-T2A-Cre-D* line recently became available from The Jackson Laboratory (B6.Cg-*Oxtr*^{tm1.1(Cre)Hze}/J, Stock No. 031303), generated as a part of project in Allen Institute for Brain Science (Daigle et al., 2018) (Extended Data Fig.1-1e), yet its detailed expression profile has not been described.

Endogenously, the *Oxtr* gene locus consists of two coding exons divided by a long intron (Extended Data Fig.1-1a). In prairie vole and human, some single-nucleotide polymorphisms (SNPs) in this long intron have been reported to be associated with *Oxtr* expression densities or social behavioral phenotypes (Jurek and Neumann, 2018; King et al., 2016). The authors suggested that these SNPs might overlap with cis-regulatory elements in the intron (King et al., 2016). The 3' UTR of the *Oxtr* gene has also been reported to contain nucleotide variations that are related to social behavioral phenotypes in humans (Jurek and Neumann, 2018). Considering these reports, it must be critical to design knock-in lines by maximally preserving the original transcriptional regulations, so as to precisely recapitulate endogenous *Oxtr*-expression profiles.

For proteins with no antibodies available for immunohistological detection, the epitope tagging strategy could be a promising breakthrough (Brizzard, 2008). Although the previously reported *Oxtr-IRES-Cre* line was designed to harbor an HA-tag at the C-terminus of the receptor protein (Extended Data Fig.1-1c), the epitope tag could not be detected in the brain tissues probably because of its relatively lower expression levels, caused by the non-native exon/intron configuration (Hidema et al., 2016). In this study, we first employed the novel PA-tag system in which human podoplanin-derived epitope tag is recognized by its highly specific monoclonal antibody (Fujii et al., 2014). Since this system has been successfully applied to many *in vitro* procedures, such as protein purification and

192 visualization, we anticipated that it would also work *in vivo* to determine the subcellular
193 distributions of endogenous Oxtr protein in neurons. We next utilized the HA-tag system,
194 because its commercially available antibody (Cell Signaling Technology, C29F4, #3724) has
195 been successfully adopted by *in vivo* genome editing technique, SLENDR, to clearly map the
196 subcellular localization of endogenous proteins in brain tissues with low background staining
197 (Mikuni et al., 2016).

198 To achieve these concepts, here we apply our refined genome-editing skills to the
199 *Oxtr* locus to generate four novel knock-in mouse lines, and evaluate their expression profiles
200 to ensure the reliability *in vivo*.

201

202

203 **Materials and Methods**

204 **Animals**

205 All animal procedures were performed in accordance with the Animal Care Committee's
206 regulations. B6C3F1 female mice for fertilized eggs collection and ICR female mice as
207 surrogate mothers were purchased from SLC Japan. One-cell stage zygotes were obtained by
208 mating B6C3F1 stud males with super-ovulated females. Pronuclear injections and mouse
209 transgenesis experiments were performed by standard protocols (Nagy et al., 2003).
210 C57BL/6J mice for back-crossing were purchased from Charles River Japan.
211 B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze}/J (also referred to as Ai9, Stock No. 007909) were
212 introduced from The Jackson Laboratory.

213

214 **Preparation of CRISPR components**

215 To insert epitope-tag, reporter, and recombinase sequences into the *Oxtr* locus via the
216 CRISPR/Cas9 system, the guide RNA sequences closest to the stop codon TGA in the 2nd
217 exon (depicted in Fig.1a) were selected. Two parts of the CRISPR guide RNA, crRNA and
218 tracrRNA, were chemically synthesized (FASMAC, Japan) and recombinant Cas9 protein
219 (EnGen Cas9 NLS S. pyogenes) was purchased from New England Biolabs. For pronuclear
220 injections, equimolar crRNA and tracrRNA were mixed and annealed (94°C, 2 min, then at
221 room temperature for 10 min).

222

223 **Generation of the *Oxtr*^{1×PA-tdTom} mouse line**

224 To insert 1×PA-tag, T2A self-cleaving peptide, and tdTomato sequences just upstream from
225 the stop codon (Fig.1b), the targeting vector containing those sequences flanked by 2.9 kb
226 homology arms on both sides was constructed by the In-Fusion cloning system (Takara Bio).

227 The mixture of annealed crRNA/tracrRNA, Cas9 protein, and the targeting vector was
 228 injected into mouse fertilized eggs at the concentrations of 38, 50, and 25 ng/μl, and the eggs
 229 were transferred into the oviduct of surrogate mothers to raise pups.

230

231 **Generation of the *Oxtr*^{3×PA} mouse line**

232 To insert three tandem copies of PA-tag sequences just upstream from the stop codon (Fig.1b),
 233 3×PA-tag sequences flanked by 129 and 118 bp homology arms were artificially synthesized
 234 (Integrated DNA Technologies). To avoid unwanted excision of PA-tag sequences by the
 235 internal recombination among three copies, synonymous substitutions were introduced into
 236 each tag sequences. By using this synthesized DNA as the template, long single-stranded
 237 DNA (ssDNA) donors were prepared with the Phospho-PCR method (Guide-it Long ssDNA
 238 Production System, Takara Bio) (Inoue et al., 2021). The mixture of annealed
 239 crRNA/tracrRNA, Cas9 protein, and long ssDNA donor was injected into mouse zygotes at
 240 the concentrations of 38, 50, and 25 ng/μl, and the zygotes were transferred into the oviduct
 241 of surrogate mothers to raise pups.

242

243 **Generation of the *Oxtr*^{1×PA-iCre} mouse line**

244 To insert 1×PA-tag sequences, T2A self-cleaving peptide sequences, and codon-improved Cre
 245 recombinase (iCre) (Shimshek et al., 2002) sequences just upstream from the stop codon
 246 (Fig.1b), the targeting vector containing those sequences flanked by 245 and 294 bp
 247 homology arms was constructed via the In-Fusion cloning system. By using this vector as the
 248 template, long ssDNA donors were synthesized by the Phospho-PCR method. The mixture of
 249 annealed crRNA/tracrRNA, Cas9 protein, and long ssDNA donor was injected into mouse
 250 zygotes at the concentrations of 38, 50, and 18.5 ng/μl, and the zygotes were transferred into
 251 the oviduct of surrogate mothers to raise pups.

252

253 **Generation of the *Oxtr*^{3×HA-iCreERT2} mouse line**

254 To insert three copies of HA-tag, T2A self-cleaving peptide, iCre, and mutated ligand binding
 255 domain of the human estrogen receptor (ERT2) sequences (Indra et al., 1999) just upstream
 256 from the stop codon (Fig.1b), the targeting vector containing those sequences flanked by 2 kb
 257 homology arms on both sides was constructed by combining artificial gene synthesis
 258 (Integrated DNA technologies) and In-Fusion cloning system. Synonymous base substitutions
 259 were introduced into each HA-tag sequence to obviate unwanted internal recombination
 260 among them. The mixture of crRNA/tracrRNA complex, Cas9 protein, and the targeting

vector was injected into mouse 1-cell embryos at the concentrations of 50, 100, and 15 ng/ μ l, and the embryos were transferred into the oviduct of surrogate mothers to raise pups.

Validation of the founder knock-in mice and their backcrossing

Genomic DNAs were prepared from newborns' tail by proteinase K treatment in Lysis Buffer (Takara Bio). Knock-in mice were screened by PCR using Tks Gflex DNA polymerase (Takara Bio) or PrimeSTAR GXL DNA polymerase (Takara Bio). To exclude the possibilities that the donor DNAs had been randomly integrated into the genome, a subset of genotyping primers was designed external to the donor DNAs' homology arms. For knock-in candidates, PCR products were analyzed by Sanger sequencing to confirm carrying correct insertions. Obtained founders were backcrossed with C57BL/6J to get F1 heterozygous progenies, which were again sequence-verified to harbor the designed knock-in allele. F1 heterozygous mice were then intercrossed to generate homozygous knock-in mice. For histological, electrophysiological, and viral analyses, homozygous knock-in mice after F3 generations were used.

Antibodies and viral vectors

Primary antibodies and dilutions used were Guinea Pig anti-tdTomato (1:2000, Frontier Institute AB_2631185), Rat anti-PA-tag (1:2000, FUJIFILM Wako Chemicals NZ-1 012-25861), Rabbit anti-HA-tag (1:2000, Cell Signaling Technology C29F4 3724S), Goat anti-Somatostatin (1:2000, Santa Cruz Biotechnology sc-7819), Guinea Pig anti-GFP (1:1000, Frontier Institute AB_2571575), Rabbit anti-Cux1 (CDP, 1:2000, Santa Cruz Biotechnology sc-13024), and Rabbit anti-Oxytocin (1:2000, IMMUNOSTAR 20068). Appropriate Alexa Fluor-conjugated secondary antibodies were from Thermo Fisher Scientific, abcam, or Jackson ImmunoResearch and used at dilutions of 1:600. AAV-PHP.eB CBh_FLEX-GFP WPRE was kindly gifted by Dr. Hirokazu Hirai (the program for Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS), AMED).

Immunohistochemistry

Brain samples were prepared from 2- to 11-week-old male homozygous knock-in mice and immunohistochemically analyzed by standard protocols. Briefly, brains were intracardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.4), post-fixed with the same fixative for 16 hr, and cryo-protected with 30% sucrose in PBS, then embedded in OCT compound (Sakura Finetek Japan) to be frozen. Brains were sliced into 40

296 μm coronal sections using a Leica cryostat. Sections were treated in blocking buffer (10%
 297 normal donkey serum, 0.3% Triton X-100 in PBS) for 1 hr at room temperature with gentle
 298 agitation. Sections were then incubated with primary antibodies in blocking solution (3%
 299 normal donkey serum, 0.3% Triton X-100 in PBS) overnight at 4°C. After being washed with
 300 PBS, sections were treated with appropriate Alexa Fluor-conjugated secondary antibodies
 301 (Thermo Fisher Scientific, abcam, or Jackson ImmunoResearch) and DAPI for 1 hr at room
 302 temperature. After washing, sections were mounted onto glass slides with Permafluor
 303 (Thermo Fisher scientific) or VECTASHIELD Vibrance antifade mounting medium (Vector
 304 Laboratories). Fluorescent images were obtained on a KEYENCE BZ-X710. For confocal
 305 and super-resolution imaging, an OLYMPUS SpinSR10 was used to take z-stacked images.
 306 Movies were constructed by using an OLYMPUS cellSens software.

308 **Electrophysiological recordings from tdTomato-positive neurons in the *Oxtr*^{I $\times$ PA-tdTom}** 309 **line**

310 Coronal prefrontal cortex slices (300 μm thickness) were prepared from 4- to 6-week-old
 311 male homozygous *Oxtr*^{I $\times$ PA-tdTom} mice, using standard procedures (Miwa et al., 2008). Slices
 312 were perfused with artificial cerebrospinal fluid (ACSF) that was saturated with 95% O₂ and
 313 5% CO₂ at 30 $\pm$ 1°C and contained (in mM) 119 NaCl, 3.5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1.0
 314 NaH₂PO₄, 26.2 NaHCO₃, and 11 D-glucose. Spontaneous spiking in tdTomato-expressing
 315 neurons in layer II and V of the prefrontal cortex (PFC) was acquired in voltage clamp
 316 cell-attached configuration with a MultiClamp 700B patch-clamp amplifier (Molecular
 317 Devices, Union City, CA, USA). The pipette solution contained ACSF, and the patch was held
 318 at 0 mV. Baseline firing was acquired for 5 min, followed by bath application of 1 μM [Thr⁴,
 319 Gly⁷]-oxytocin (TGOT) or 1 μM (d(CH₂)₅¹, Tyr(Me)², Thr⁴, Orn⁸, des-Gly-NH₂⁹)-vasotocin
 320 (OTA) for 10 min. The signal was filtered at 1 kHz and digitized at 20 kHz with National
 321 Instruments DAQ board (USB-6341) and Igor Pro (Wavemetrics) with the NeuroMatic
 322 software package (Rothman and Silver, 2018). TGOT and OTA were obtained from Bachem
 323 (Torrance, CA).

325 **Intravenous injection of AAV-PHP.eB vector to the *Oxtr*^{I $\times$ PA-iCre} line**

326 After anesthesia with isoflurane (4%), 100 μl of viral solution (AAV-PHP.eB CBh_FLEX-GFP
 327 WPRE; 2.5×10^{12} vg/ml) was intravenously injected into the retro-orbital sinus of *Oxtr*
 328 ^{I $\times$ PA-iCre/+} mice using a 1.0 ml syringe with a 27-gauge needle. Four weeks after the injection,
 329 the mice were anesthetized with sodium pentobarbital (50 mg/kg body weight,
 330 intraperitoneal) and transcardially perfused with PBS followed by 4% PFA in 0.1 M

phosphate buffer (pH 7.4). The brains were removed, postfixed in the same fixative overnight at 4°C, and immersed in 15% and 30% sucrose in PBS overnight for cryoprotection. Free-floating sections (40 µm) were cut using a sliding microtome (REM-710; Yamato Kohki Industrial Co., Ltd., Japan) and incubated in PBS with 0.3% Triton X-100 and 1% bovine serum albumin (BSA) for 1 hr at room temperature. Then, the sections were incubated for 12 hr at room temperature with primary guinea pig anti-GFP antibody (1:1,000; Frontier Institute) diluted in PBS with 0.3% Triton X-100, 2% BSA. The sections were washed in PBS and incubated for 2 hr in Alexa Fluor 488-conjugated species-specific secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:500 in PBS with 0.3% Triton X-100 and 2% BSA. Fluorescent images of GFP were captured using a fluorescent microscope (BZ-X710, KEYENCE, Osaka, Japan).

Induction of iCre activity in the *Oxtr*^{3×HA-iCreERT2} line by oral administration of TMX
Oxtr^{3×HA-iCreERT2} :: *Gt(ROSA)Sor*^{Ai9} double heterozygous males at 4 weeks were fed with powdered diet supplemented with tamoxifen (TMX, Sigma-Aldrich T5648) at the concentration of 2 mg/g for 5 consecutive days by using feeding jars (Yoshinobu et al., 2021) (n=5). For the control experiments, double heterozygous males at 4 weeks were fed with normal diet for 5 consecutive days (n=3). After 5 more days, brains were sampled at 5 weeks and immunostained as described above. To analyze effects of chronic TMX treatment on *Oxtr* and *Oxtr* expressions, 5-week-old homozygous *Oxtr*^{1×PA-tdTom} males were fed with the same protocol (n=3). Normal diet was fed to mice with the same genotype as the control (n=3). Brains were sampled at 6 weeks for immunostaining.

Results

Seamless insertions of epitope tag and functional gene cassettes into the *Oxtr* locus via CRISPR/Cas9 genome editing

To insert the epitope tag or reporter/recombinase sequences in-frame and 5' to the stop codon of the *Oxtr* gene 2nd exon, we selected the guide RNA sequences depicted in Fig.1a. The targeting strategies were basically the same for four knock-in lines; one or three copies of epitope tag sequences were directly fused 3' to the *Oxtr* coding sequences. For bicistronic expression, we inserted T2A self-cleaving peptide sequences followed by functional gene cassettes (tdTomato reporter or Cre recombinase coding sequences). We used the T2A sequences which had been successfully utilized for knock-in mouse generations, to

simultaneously express two separate proteins at nearly equimolar amounts from the same mRNA (Tang et al., 2009; Trichas et al., 2008).

We next employed the cloning-free CRISPR/Cas9 system in which chemically synthesized guide RNAs (crRNA and tracrRNA) were combined with recombinant Cas9 nuclease protein to induce highly efficient genome editing in mouse fertilized eggs (Aida et al., 2015; Inoue et al., 2021), and successfully generated one founder mouse for each knock-in design. We crossed these knock-in founders with C57BL/6J mice to obtain heterozygous F1 generations, which were sequence-verified to ensure that they correctly harbored the designed knock-in cassettes. We then intercrossed F1 heterozygous mice to obtain homozygous F2 generations. For histological, electrophysiological, and viral analyses, we prepared homozygous knock-in mice after F3 generations. Complete genomic DNA sequences and genotyping primer sequences for the four knock-in lines are supplied as .dna files (Extended Data DNA sequence Files). Detailed genotyping PCR conditions are listed in Extended Data Figure 1-2.

The epitope tagging strategy precisely visualized the endogenous Oxtr protein expression profiles and ensured the reliability of the knock-in strategy.

As depicted in Fig.1b and 2a, we first designed a dual-purpose *Oxtr*^{1×PA-tdTom} allele, in which one copy of PA-tag can monitor endogenous Oxtr protein localization and the portion of the tdTomato cassette serves as a bright fluorescent reporter. To evaluate PA-tag and tdTomato expression profiles in this line, we used homozygous male brains at 2 weeks (postnatal day 14; P14) because Oxtr expression levels in mouse brains have been reported to peak at around 2-3 weeks (Newmaster et al., 2020). In the figures, we mainly presented the section equivalent to Bregma -1.82 mm, as this level contains several well-known regions that have been reported to express Oxtr. Although tdTomato fluorescence could be detected without immunostaining as described later (Extended Data Fig.3-1a, 3-1b), we double-stained the sections with anti-tdTomato and anti-PA-tag antibodies to validate their colocalization. As a result, we observed abundant tdTomato reporter signals in the cortical region (Newmaster et al., 2020), hippocampal dentate gyrus (DG) and anterior CA2 (aCA2)/CA3 (Raam et al., 2017), paraventricular thalamus (PVT) (Newmaster et al., 2020; Watarai et al., 2020), central nucleus of amygdala (CeA) (Mitre et al., 2016; Yoshida et al., 2009), dorsal endopiriform nucleus (DEn) (Sharma et al., 2019), and posteromedial cortical amygdaloid area (PMCo) (Newmaster et al., 2020) (Fig.2d), where considerable level of Oxtr expression has already been reported. Consistently, we also detected the presence of endogenous Oxtr protein by the C-terminally fused PA-tag in the same regions (Fig.2e); however, the signal intensity was not

strong enough for higher magnification, as described below. These matched results indicated that the epitope tagging and T2A-dependent bicistronic strategies we employed properly functioned as expected. We therefore applied the same design for the *Oxtr*^{1×PA-iCre} allele (Fig 1b).

In the magnified confocal image of the primary somatosensory cortex barrel field (S1BF) in the *Oxtr*^{1×PA-tdTom} brain (Fig.2h), tdTomato and PA-tag signals showed significant colocalization. However, we noticed that the use of anti-PA-tag antibody resulted in high back-ground staining within brain tissue. To increase the signal-to-noise (S/N) ratio, we next generated the *Oxtr*^{3×PA} allele in which three tandem copies of PA-tags were simply fused to the C-terminus of Oxtr (Fig.1b and 2b). Consequently, the PA-tag signals from this 3×PA allele (Fig.2f) perfectly coincided with those from 1×PA in the *Oxtr*^{1×PA-tdTom} allele (Fig.2e), and its S/N ratio was improved. On comparing the magnified confocal images of the S1BF, the PA-tag signals in the *Oxtr*^{3×PA} line (Fig.2i) were much clearer than those in the *Oxtr*^{1×PA-tdTom} allele (Fig.2h), along with relatively lower background staining. In Fig.2i-2l, we focused on somatostatin (SST) expressing interneurons in layer IV, since Oxtr-expressing interneurons have been found in some brain areas to recruit local inhibitory circuits, which might be a major mode of oxytocinergic modulation in the brain (Froemke and Young, 2021), as described in the Discussion section below.

We further designed a dual-purpose *Oxtr*^{3×HA-iCreERT2} allele (Fig.1b and 2c). We newly employed three copies of HA-tags for endogenous Oxtr protein visualization, because a good antibody with lower background staining in the brain tissue was commercially available. To induce spatiotemporally controlled Cre recombinase expressions in mouse brains, we fused a mutated ligand-binding domain of the human estrogen receptor (ERT2) (Indra et al., 1999) with iCre recombinase. The detailed results for iCre inductions are described later in Fig.5. As shown in Fig.2g, the HA-tag signals from this 3×HA allele nicely coincided with those from the 1×PA and 3×PA alleles (Fig.2e and 2f). On the magnified confocal image of *Oxtr*^{3×HA-iCreERT2} brain shown in Fig.2k, we observed clear HA-tag signals, with relatively low background staining. The fact that three independently generated epitope-tagged lines resulted in exactly the same expression profiles confirms the reliability of our knock-in strategies.

***Oxtr*^{3×PA} and *Oxtr*^{3×HA-iCreERT2} mouse lines enabled visualization of endogenous Oxtr protein at the subcellular level**

We next employed spinning-disk confocal microscopy technology to observe epitope tagged Oxtr-expressing neurons at super-resolution. First, we obtained the Z-stacked

3-D image of a 3×PA-tagged neuron in the S1BF, which was identified as an SST-positive interneuron (Fig.2j and Fig.2 Movie 1). We successfully detected the distribution of PA-tagged endogenous Oxtr proteins on the plasma membrane, as expected as a member of GPCRs, whereas SST was localized in the cytoplasm. This is the first evidence showing that Oxtr proteins reside both on the cell body and on nerve fibers. The super-resolution image from a 3×HA-tagged neuron further replicated the above observation, supporting the reliability of this approach (Fig.2l and Fig.2 Movie 2).

C-terminally fused epitope tags did not disrupt the Oxtr protein function *in vivo*.

Although our experiments have confirmed the advantage of epitope tagging in visualizing endogenous protein localization, the concern remained that C-terminally fused tags may alter Oxtr protein conformation and/or stability, affecting its functions. Since Oxtr plays essential roles in lactation, pups from *Oxtr* knock-out mothers (*Oxtr*^{-/-}) have been reported to die soon after birth, due to the mother's defects in breastfeeding (Hidema et al., 2016; Takayanagi et al., 2005) (Extended Data Fig.2-1a). Therefore, we confirmed that all the homozygous mothers of our four knock-in lines had successfully nursed their pups. As shown in Extended Data Fig.2-1, the pups' digestive tracts were filled with milk (Fig.2-1b, 2-1c) and the mother's mammary glands were well developed (Fig.2-1d), indicating that the peripheral Oxtr signaling pathway was intact in the homozygous females. In addition, we performed electrophysiological recordings in tdTomato positive neurons from the homozygous *Oxtr*^{1×PA-tdTom} male, to confirm their responsiveness to agonist administration (details are shown in Fig.3 and described later), supporting that 1×PA tagged Oxtr proteins could sufficiently mediate Oxtr signals in the brain. These results indicate that fusion of one or three copies of the PA-tag or HA-tag to the C-terminus of Oxtr did not affect receptor functions *in vivo*. It has already been reported that a much larger tdTomato protein fused C-terminally to the κ opioid receptor, which is also a member of the GPCR family, did not disrupt neuronal circuitries in the knock-in mouse brain (Chen et al., 2020). Therefore, we did not expect that the relatively smaller epitope tags we had used in the present study would affect Oxtr functions. Consistent with this assumption, all the knock-in lines we generated could be maintained by crossing homozygous males with homozygous females (Extended Data Fig.2-1e, 2-1f), without the need for laborious genotyping procedures. This is a notable advantage of our new lines.

The tdTomato reporter in the *Oxtr*^{1×PA-tdTom} line precisely labeled Oxtr-expressing neurons.

Since tdTomato has been developed as a non-aggregating, extremely bright red fluorescent protein suitable for live imaging (Shaner et al., 2004), we were able to detect tdTomato signals in our homozygous *Oxtr*^{1×PA-tdTom} brains without immunostaining (Extended Data Fig.3-1a, 3-1b), allowing us to perform patch-clamp recordings on these cells (Fig.3a, 3b). We hypothesized that tdTomato-positive neurons should correctly respond to an oxytocin receptor agonist, TGOT, if the tdTomato expressions were faithfully regulated parallel to endogenous *Oxtr* expressions.

A subset of both glutamatergic and GABAergic neurons in the PFC has been reported to express *Oxtr*, which modulate female sociosexual behavior (Nakajima et al., 2014) and social memory (Tan et al., 2019). We first investigated the distribution of tdTomato-positive neurons in the PFC using immunohistochemistry, particularly in the prelimbic cortex (PrL) of *Oxtr*^{1×PA-tdTom} homozygous brain (Extended Data Fig.3-1). We found that tdTomato-positive neurons mainly resided in the layer II and V of the PrL (Extended Data Fig.3-1c, 3-1d). Consistent with a previous report (Tan et al., 2019), we identified the neurons in layer II by *Cux1* expression as glutamatergic neurons (Extended Data Fig.3-1e, 3-1f), while the neurons in layer V were confirmed by *SST* expression as GABAergic interneurons (Extended Data Fig.3-1g, 3-1h).

To test whether these tdTomato-positive neurons in *Oxtr*^{1×PA-tdTom} mice faithfully respond to TGOT application, we used 4- to 6-week-old homozygous *Oxtr*^{1×PA-tdTom} males and perform *in vitro* electrophysiological analyses using a cell-attached patch-clamp configuration (Fig.3a, 3b). We tested the neurons in layer II and V separately (Fig.3a), to test the possibility that they might have distinct responsiveness to Oxt ligands to modulate synaptic sites or membrane potentials. As the results, in tdTomato-positive layer II neurons, TGOT significantly increased the spontaneous firing rate from 2.7 ± 1.0 Hz to 7.6 ± 2.6 Hz ($n = 6$; $P = 0.0312$, Wilcoxon test, Figure 3c, 3d). In tdTomato-positive layer V neurons, TGOT also considerably increased the spontaneous firing rate from 0.99 ± 0.7 Hz to 7.3 ± 3.3 Hz ($n = 6$; $P = 0.0312$, Wilcoxon test, Figure 3e, 3f). Additionally, we confirmed that these TGOT-induced increases in spontaneous firing rates in both layer II and V neurons were inhibited by the presence of an *Oxtr* antagonist, OTA (Extended Data Fig.3-2a, 3-2b). These results clearly demonstrate that tdTomato-positive cells in *Oxtr*^{1×PA-tdTom} mice express "functional" *Oxtr* and appropriately respond to its ligand, even in the presence of a C-terminally fused PA-tag. Hence, tdTomato reporter signals in our *Oxtr*^{1×PA-tdTom} line are a reliable marker for *Oxtr*-expressing cells.

504 **The *Oxtr*^{1×PA-iCre} line sufficiently drove recombinase activities in the *Oxtr*-expressing**
 505 **neurons.**

506 To newly generate an effective Cre driver, we employed the 2A peptide-based
 507 bicistronic expression system (Trichas et al., 2008) and codon-improved Cre recombinase
 508 (iCre) sequences (Shimshek et al., 2002). For the evaluation of Cre activities in the brain, we
 509 first crossed our *Oxtr*^{1×PA-iCre} line with the well-known Cre reporter, Ai9 (Madisen et al.,
 510 2010) to obtain double heterozygous mice. As depicted in Fig.4a, Cre-dependent stop signal
 511 excision induces tdTomato expression from the Ai9 allele. We analyzed tdTomato expression
 512 profiles in *Oxtr*^{1×PA-iCre/+::Gt(ROSA)Sor^{Ai9/+}} male brains at P7, P14, and P21. We observed
 513 tdTomato signals already at P7 (Fig.4b) and found that the reporter signals became more
 514 abundant at P14 and P21 (Fig.4c, 4d). Once recombination occurs, the reporter gene remains
 515 recombined thereafter, and consequently, the reporter is expressed even in the absence of
 516 ongoing recombinase expressions. In other words, a significant proportion of tdTomato
 517 expression in adult stages does not reflect real-time Cre activity but instead reflects a
 518 cumulative effect of earlier events. Therefore, we concluded that the Ai9 reporter was
 519 unsuitable for our purpose of evaluating Cre expressions in adult brains.

520 We next tried to immunostain Cre proteins in the brain tissue, using a commercially
 521 available anti-Cre recombinase antibody (clone 2D8, MAB3120, MERCK). This antibody
 522 has been reported to be successful in staining Cre proteins in transgenic lines carrying
 523 multiple copies of transgene (Mizuguchi et al., 2012). However, we could not detect Cre in
 524 the brain tissues of our iCre knock-in mouse line using this antibody.

525 To circumvent these issues, we employed AAV-PHP.eB vector harboring
 526 CBh-FLEX-GFP (kindly gifted by Dr. Hirokazu Hirai, Brain/MINDS project). AAV-PHP.eB
 527 has been developed to achieve efficient transport across the blood-brain barrier in adult
 528 mammalian brains for broad gene delivery (Chan et al., 2017). As depicted in Fig.4e, GFP
 529 expressions can be induced by Cre-dependent inversion. We therefore retro-orbitally injected
 530 AAV-PHP.eB vector into 6-week-old heterozygous males and analyzed their brains after 4
 531 weeks. As the results, we observed abundant GFP signals in the cortical region, hippocampal
 532 DG and aCA2/CA3, PVT, CeA, DEn, and so forth, where *Oxtr* expression has previously
 533 been reported (Figure.4f-4j, Bregma -1.82 mm). Other section levels are shown in Extended
 534 Data Fig.4-1.

535 As we had designed the *Oxtr*^{1×PA-tdTom} and *Oxtr*^{1×PA-iCre} alleles based on the same
 536 concepts (Fig.1b and Fig.4e, 4k), their expression profiles should closely resemble each other.
 537 Predictably, we found that the tdTomato expression profiles from 11-week-old homozygous
 538 *Oxtr*^{1×PA-tdTom} male brains (Fig.4l-4p) were quite similar to those of iCre visualized by

AAV-PHP.eB administration (Fig.4f-4j). Other section levels, shown in Extended Data Fig.4-1, confirm the trend. These results ensured the reliability of our new Cre driver line.

A new genetic tool, *Oxtr*^{3×HA-iCreERT2} line, could be used to target Oxtr-expressing neurons in a spatiotemporally controlled manner.

We next investigated whether the *Oxtr*^{3×HA-iCreERT2} allele properly functioned as an inducible Cre driver. To detect Cre activity in the brain, we crossed the *Oxtr*^{3×HA-iCreERT2} line with Ai9 to obtain double heterozygous mice. As depicted in Fig.5a, tamoxifen (TMX) binds to iCre-ERT2 expressed in the cytoplasm, resulting in iCre translocation into the nucleus to flip out the stop signal from Ai9 allele and elicit tdTomato expressions. We orally administered TMX to 4-week-old males for 5 consecutive days by feeding them a TMX-supplemented powdered diet (Fig.5b). For the control experiments, we fed 4-week-old males with normal diet for 5 consecutive days. After 5 more days, we sampled and analyzed their brains at the level of Bregma -1.82 mm. As shown in Fig.5c, the normal diet did not induce any leaky tdTomato reporter expression. Contrarily, the TMX-containing diet successfully induced iCre recombinase activity in the cortical region, hippocampal DG and aCA2/CA3, PVT, CeA, DEN, and PMCo, where *Oxtr* expression has previously been reported (Figure.5d-5i). In the constitutive iCre line, too many cells were labeled with tdTomato to be able to clearly distinguish each neuron (Fig.4d). However, reporter expression was only sparsely induced in the inducible iCre line, allowing the identification of cell morphology at single-cell resolution (Fig.5d-5i). As a result, we observed two types of *Oxtr*-expressing neurons with different morphologies and properties in the S1BF (Fig.5i). Consistent with the previous reports (Nakajima et al., 2014; Tan et al., 2019), some tdTomato-labelled neurons in layer II turned out to be Cux1-positive glutamatergic neurons (Fig.5j-5l), while other tdTomato-labelled neurons in layer IV were identified as SST-positive interneurons (Fig.5m-5o).

These results demonstrated that the first-time inducible-Cre driver for *Oxtr*-expressing neurons efficiently drives Cre-mediated recombination in a spatiotemporally controlled manner upon TMX administration.

Since subcutaneous implantations of a pellet containing estrogen into gonadectomized rodents have been reported to affect *Oxt/Oxtr* transcript levels (Bale and Dorsa, 1995; Garcia et al., 2017; Nomura et al., 2002; Patisaul et al., 2003; Vanya et al., 1997) and radioactively labeled OTA binding to *Oxtr* (Young et al., 1998), chronic administrations of TMX, a selective estrogen receptor modulator, might change *Oxt/Oxtr* expression levels in the *Oxtr*^{3×HA-iCreERT2} line. To analyze the effect of 5-days oral TMX

574 treatment on Oxt/Oxtr expressions, we used homozygous *Oxtr*^{1×PA-tdTom} mice, in which
 575 real-time Oxtr expressions can be monitored as tdTomato signals, and examined
 576 Oxt-expressing neurons by immunostaining (Extended Data Fig.5-1a). Although long-term
 577 (12 days to 3 months) estrogen treatments have been reported to significantly increase Oxt
 578 transcript levels in the paraventricular hypothalamic nucleus (PVN) (Nomura et al., 2002;
 579 Patisaul et al., 2003) and supraoptic nucleus (SON) (Garcia et al., 2017), our relatively
 580 short-term (5 days) TMX treatment did not affect Oxt expression levels in these nuclei
 581 (Extended Data Fig5-1b). In addition, while long-term estrogen treatments have been
 582 reported to affect Oxtr transcript levels in the bed nucleus of stria terminalis (BNST) (Garcia
 583 et al., 2017), ventromedial hypothalamus (VMH) (Bale and Dorsa, 1995; Vanya et al., 1997),
 584 and medial amygdala (MeA) (Garcia 2017), our 5-days TMX treatment had no effect on Oxtr
 585 expression profiles in these brain areas (Extended Data Fig5-1c, 5-1d). Furthermore, while
 586 radioactively labeled OTA binding to Oxtr has been reported to increase in the DEN and
 587 PMCo by long-term estrogen treatment (Young et al., 1998), Oxtr expression levels itself
 588 were not affected upon our TMX administrations (Extended Data Fig5-1d). From these
 589 results, we concluded that 5-days TMX treatments have little effect on Oxt/Oxtr expression
 590 profiles in young male mice. However, we need to carefully interpret the results obtained
 591 from *Oxtr*^{3×HA-iCreERT2} line by chronic TMX administrations because estrogens play key roles
 592 in mediating social behaviors (Ervin et al., 2015; Laredo et al., 2014) as described in
 593 Discussion section.

594
 595

596 Discussion

597 Genetic tools that provide access to Oxtr-expressing cells remain limited. To
 598 improve this situation, we have newly generated a series of genome-edited mouse lines that
 599 are to make a valuable contribution to the growing research on oxytocin. We have created
 600 simple and seamless knock-in designs that maximally preserve the endogenous
 601 transcriptional regulation of the *Oxtr* gene.

602 The first achievement of the present study was the successful use of epitope tags to
 603 visualize Oxtr protein distributions at the subcellular level for the first time. Two strategies,
 604 the RNAscope-based *in situ* hybridization and the *Oxtr-Venus* knock-in mice, have previously
 605 been utilized for Oxtr visualization. However, these methods detect transcript expression, but
 606 not Oxtr protein localization *per se*. To overcome these limitations, we generated epitope tag
 607 knock-in mice by genome editing. We employed the PA-tag, a 12 amino acid epitope derived
 608 from human podoplanin, which could be detected using the rat monoclonal antibody, NZ-1

(Fujii et al., 2014). This epitope-antibody combination has been reported to show a high binding affinity of $K_D = 4.9 \times 10^{-10}$ (M), which is much stronger than those of HA, FLAG, and other affinity tag systems, and which has been successfully used to many biological procedures *in vitro* (Fujii et al., 2014). In addition, we have previously reported the applicability of the PA-tag system in mouse brains and testes in *in vivo* studies (Arimura et al., 2020; Kimura et al., 2021). Here, we triplicated the PA-tag to improve the S/N ratio of immunostaining, by which the background staining in brain tissues were reduced, allowing us to successfully carry out super-resolution imaging of Oxt protein. We also utilized the HA-tag because it has been successfully employed for SLENDR, which enabled *in vivo* protein labeling via genome editing in mammalian brains (Mikuni et al., 2016). Similarly, utilizing three copies of the HA-tag enabled us to reconfirm the result from the 3×PA tag knock-in brains.

Although we have roughly demonstrated that Oxt proteins are present not only on cell bodies but also on neurites, it remains unclear on which part of neurites (axons, dendrites, or both) they reside. To date, three types of oxytocinergic modulation on neurons have been proposed (Froemke and Young, 2021). As the first-order modulation, Oxt directly depolarizes excitatory cells that express Oxt. In the second-order modulation, Oxt acts on inhibitory interneurons expressing Oxt, to regulate synaptic inhibitions. Furthermore, in the third-order modulation, Oxt is believed to reside on axon terminals, where the presynaptic Oxt activation enhances transmitter release and indirectly impacts on other excitatory transmissions (Dölen et al., 2013; Nardou et al., 2019). However, the presence of Oxt in axon terminals has not yet been proven. Therefore, our *in vivo* super-resolution imaging could serve as a significant step toward deciphering the subcellular distribution of Oxt protein in such studies.

The second achievement of the present study was the development of an Oxt reporter line expressing the bright red fluorescent protein, tdTomato, which is suitable for live imaging. *In vitro* electrophysiological analyses were successfully carried out on tdTomato-positive neurons in our reporter line. Such analyses may not have been possible in the *Oxt-Venus* line (Yoshida et al., 2009), because expression levels of Venus-positive cells have been reported to largely decline after 3 weeks (Newmaster et al., 2020). In addition, tdTomato's red fluorescence could be advantageous when calcium indicators such as the genetically encoded GCaMPs are used, as they mainly contain GFP and emit green fluorescence which overlaps with Venus signals.

Thirdly, we generated both constitutive and inducible Cre drivers for Oxt-expressing neurons. IRES elements, utilized in the existing two *Oxt-IRES-Cre* knock-in

lines, have been reported to occasionally cause lower expression of the downstream cistron due to factors such as experimental cell types or cloned gene cassettes (Ibrahimi et al., 2009). Accordingly, we employed the 2A peptide system, which allowed nearly equimolar expression of two cistrons (Tang et al., 2009; Trichas et al., 2008). As we paid much attention to maintain the endogenous *Oxtr* gene configuration in our knock-in allele, the design of our *Oxtr-T2A-iCre* is indeed different from that of *Oxtr-T2A-Cre-D*, available from the Jackson Laboratory (Daigle et al., 2018). While the latter inserted an exogenous bovine growth hormone polyA signal and ignored the *Oxtr*'s own endogenous polyA sequence (Extended Data Fig. 1-1e), our knock-in mice simply made use of the endogenous polyA signal instead (Fig. 1b). Unlike the systematically generated Cre allele as a part of a big project, our iCre knock-in allele was carefully customized for the *Oxtr* locus, which is a promising development for future oxytocin studies. In addition, our inducible Cre line was suitably responsive to TMX administration. Although intraperitoneal injection is the most popular TMX administration protocol, it may sometimes be toxic to cause the death of test animals. Therefore, we employed an oral administration protocol (Andersson et al., 2010; Yoshinobu et al., 2021) instead, to ensure the safe and sufficient induction of Cre activity.

TMX is a potent and clinically used selective estrogen receptor modulator that blocks effects of estrogens in breast tissues but facilitates estrogen actions in others such as uterus tissues (Osborne, 1998). Thus, chronic TMX treatment has the possibility to interfere with estrogen function in multiple tissues including the brain. Estrogens are involved in social behavior at multiple levels, from the detection and integration of social information to more complex behavior such as social preference, aggression, and social learning and memory (Ervin et al., 2015). As an example, in the hippocampus, estrogens are locally synthesized and modulate memory-related synaptic plasticity by the rapid non-classical indirect pathway via synapse-localized estrogen receptors (Laredo et al., 2014; Mukai et al., 2010). We hence need to carefully consider the potential interference by TMX to the behavioral outcomes from *Oxtr* manipulations in using *Oxtr*^{3×HA-iCreERT2} mice.

Oxt and arginine vasopressin (Avp) are evolutionally related molecules that differ only in two of the nine amino acids. Since their receptors also show significant structural homology to one another, the selectivity of Oxt and Avp for their own receptor is not absolute. Considerable receptor crosstalk has been reported to occur among Oxt, Avp and their receptors (Carter, 2017; Song and Albers, 2018). While exogenously administrated Avp can act on *Oxtr* in higher concentration and so can do Oxt on Avp receptor 1a (Avpr1a), limited lines of evidence indicate that endogenously released Oxt and Avp can produce functionally significant responses by activating each other's receptors (Song and Albers, 2018). Based on

receptor autoradiography approach with relatively low resolution, Oxtr and Avpr1a appear to show distinct and non-overlapping expression in the rodent brain (Dumais and Veenema, 2016). However, it remains still unknown whether the localization of Oxtr and Avpr1a is mutually exclusive at the synaptic levels, and thus the possibility of crosstalk following synaptic release of these peptides should rigorously be examined. In this context, our new genetic tools could play a part in clarifying these receptors' distribution with much higher resolution, deepening our understanding of ligand-receptor interactions for Oxt and Avp. Generation of Avpr1a genome-edited mouse lines might further help precisely visualize its endogenous expression profiles.

Oxt is expected as a potential therapeutic for social deficits represented by autism spectrum disorders. Managing socio-emotional behavior in health and disease to increase resilience to chronic stress has become much more important, especially given the ongoing COVID-19 pandemic (Grinevich and Neumann, 2021). Although the preclinical trials of exogenous Oxt administration via intranasal delivery are currently in progress (Quintana et al., 2021), second-generation strategies to potentiate endogenous Oxt signaling should be preferred in the future (Ford and Young, 2021). Moreover, a fundamental shift from the traditional brain-wide chronic pharmacological intervention to a circuit-specific approach is now anticipated, owing to significant advances in our understanding of the neural circuit mechanisms underlying social behavior in animal models (Ford and Young, 2021). Hence, to establish dependable and effective Oxt-based therapeutic interventions, we need reliable animal models to help uncover the mechanisms underlying Oxt-Oxtr circuitry. Our new genetic tools would play a significant role in meeting this demand and we are going to make the four knock-in lines generated in this study quickly and widely available. This will eventually help deepen our knowledge for the elaborated molecular machinery and functional regions of Oxt-Oxtr actions in our own brain.

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904 **Figure legends**

905 **Figure 1. A series of *Oxtr* knock-in mouse lines generated by CRISPR/Cas9 genome** 906 **editing**

907 **a**, Targeting strategy to insert a functional cassette just upstream from the translational stop
 908 codon (TGA) of mouse *Oxtr* gene locus is schematically outlined. *Oxtr* gene has two coding
 909 regions shown as solid black bars that are divided by a long intron. Its 3' UTR shown as an
 910 open bar is also relatively long. PAM and guide sequences to recruit CRISPR components
 911 onto the *Oxtr* stop codon are depicted at the top. Solid grey bar indicates a knock-in cassette.
 912 Note that the endogenous *Oxtr* gene configuration including the long intron and 3' UTR are
 913 intactly preserved in the resulting knock-in allele even after the genome editing. For
 914 comparison with already-existing knock-in lines, see Extended Data Fig.1-1.

915 **b**, Four knock-in alleles generated in this study are schematically depicted. Epitope tag
 916 sequences (1×PA, 3×PA, or 3×HA) are fused 3' to the *Oxtr* coding sequences. T2A
 917 self-cleaving peptide sequences are employed for bicistronic functional cassette expression
 918 (tdTomato, iCre, and ERT2) in *Oxtr*-expressing cells. Complete genomic DNA sequences and
 919 genotyping primer sequences for four knock-in lines are supplied as .dna files (Extended
 920 Data DNA sequence Files 1-1, 1-2, 1-3, and 1-4). Detailed genotyping PCR conditions are
 921 listed in Extended Data Figure 1-2.

922

923 **Extended Data Figure 1-1. Already-existing reporter/Cre lines for *Oxtr***
 924 **visualization/manipulation**

- 925 **a.** Endogenous *Oxtr* gene configuration is schematically depicted. *Oxtr* gene has two coding
 926 regions shown as solid black bars that are divided by a long intron (11.5 kb). Its 3' UTR
 927 shown as an open bar is also relatively long (3.3 kb).
- 928 **b.** *Oxtr-Venus* knock-in allele (Yoshida et al., 2009). As the 1st coding exon is replaced with
 929 Venus sequences followed by bovine growth hormone (GH) polyA signal, this allele lacks the
 930 endogenous configuration of intron and 3'UTR sequences.
- 931 **c.** *Oxtr cDNA^{HA}-IRES-Cre* knock-in allele (Hidema et al., 2016). As is the case with
 932 *Oxtr-Venus* in **b**, the 1st coding exon is replaced with *Oxtr cDNA-HA-IRES-Cre* cassette.
 933 Although the C-terminally fused HA-tag is detected in uterus tissues, it cannot be detected in
 934 brain tissues.
- 935 **d.** *Oxtr^{Cre:GFP}* knock-in allele (Ryan et al., 2017). While the long intron is preserved, the
 936 exogenously integrated polyA signal is prioritized over the endogenous *Oxtr*'s polyA.
 937 Exogenous polyA signals might affect stabilities of mRNAs.
- 938 **e.** *Oxtr-T2A-Cre-D* knock-in allele (Daigle et al., 2018). As is the case in **d**, the exogenously
 939 introduced polyA signal is prioritized over the endogenous *Oxtr*'s polyA.
- 940 **f.** *Oxtr-Cre BAC transgenic* allele (Gerfen et al., 2013). Although BAC transgenesis is
 941 undoubtedly a useful technique, a non-native genomic context via random integration might
 942 unexpectedly modify endogenous expression profiles.

944 **Extended Data DNA sequence Files (supplied as a zip file)**

945 Complete genomic DNA sequences and genotyping primer sequences for four knock-in lines
 946 generated in this study are supplied as .dna files that can be opened by using SnapGene
 947 software.

949 **Extended Data Figure 1-2. Genotyping primers and PCR conditions used in this study**

- 950 **a.** Names of four knock-in lines generated in this study are listed.
- 951 **b.** Genotyping primer names are listed.
- 952 **c.** Primer sequences (5' to 3') are listed.
- 953 **d.** PCR enzymes used for genotyping are listed.
- 954 **e.** PCR products' size for knock-in alleles and wild type allele are listed.
- 955 **f.** PCR conditions for genotyping are listed.
- 956 **g.** Electrophoresis conditions are listed.

958

959 **Figure 2. Epitope-tagging strategy precisely visualizes the endogenous Oxt protein**
960 **expression profiles and ensures the reliability of knock-in strategies.**

961 **a-c**, Schematic diagram of *Oxt*^{1×PA-tdTom} allele, *Oxt*^{3×PA} allele, and *Oxt*^{3×HA-iCreERT2} allele are
962 arranged on the top. Resulting Oxt-expressing neurons are depicted below them. To visualize
963 endogenous Oxt protein localization, PA-tag or HA-tag are fused to the C-terminus of
964 receptor protein and detected by anti-tag antibodies. To enhance the detection, three copies of
965 PA-tag or HA-tag are tandemly connected. C-terminally fused PA-tag/HA-tag do not disrupt
966 the receptor function *in vivo* as shown in Extended Data Fig.2-1. tdTomato reporter and
967 iCre-ERT2 recombinase are bicistronically expressed in the cytoplasm by employing the T2A
968 self-cleaving peptide system.

969 **d,e**, Expression profiles of tdTomato reporter and 1×PA-tag in the *Oxt*^{1×PA-tdTom} homozygous
970 brain from 2-week-old male are arranged (equivalent to Bregma -1.82 mm). tdTomato
971 expressions are observed in the areas that have been reported to express Oxt, such as the
972 primary somatosensory cortex barrel field (S1BF), hippocampal dentate gyrus (DG) and
973 anterior CA2 (aCA2)/CA3, paraventricular thalamus (PVT), central nucleus of amygdala
974 (CeA), dorsal endopiriform nucleus (DEn), and posteromedial cortical amygdaloid area
975 (PMCo). Notably, PA-tag signals are also observed in the same regions, indicating the
976 T2A-dependent bicistronic expression system works well.

977 **f**, Expression profiles of 3×PA-tag in the *Oxt*^{3×PA} homozygous brain from 2-week-old male
978 nicely match up with those of tdTomato in **d** and 1×PA-tag in **e**.

979 **g**, Expression profiles of 3×HA-tag in the *Oxt*^{3×HA-iCreERT2} homozygous brain from
980 2-week-old male well coincide with those of 1×PA-tag in **e** and 3×PA-tag in **f**. Note that
981 independently generated three epitope-tagged lines result in the same expression profiles,
982 ensuring the reliability of knock-in strategies.

983 **h**. Magnified confocal image of the boxed area (S1BF, layer IV) in **d-e** indicates tdTomato
984 signals nicely coincide with PA-tag staining. However, this line is not suitable for higher
985 magnification due to its unwanted back-ground staining of PA-tag antibody.

986 **i**. Enlarged confocal image of the boxed area (S1BF, layer IV) in **f** shows clearer signals
987 derived from 3×PA-tag than those from 1×PA-tag in **h**.

988 **j**. Super resolution image of a somatostatin (SST)-positive PA-tagged Oxt-expressing neuron
989 indicates PA-tag signals are observed both on the surface of cell body and on the neurites,
990 whereas SST is localized in the cytoplasm. 3-D movie of this interneuron is supplied as Fig.2
991 Movie 1.

992 **k.** Magnified confocal image of the boxed area (S1BF, layer IV) in **g** shows much clearer
 993 signals derived from 3×HA-tag than those from 1×PA-tag in **h**.
 994 **l.** Super resolution image of an SST-positive HA-tagged *Oxtr*-expressing neuron indicates
 995 HA-tag signals are observed on the surface of cell body and on the neurites, reconfirming the
 996 result from 3xPA-tag knock-in line in **j**. 3-D movie of this interneuron is supplied as Fig.2
 997 Movie 2.

998
 999 **Movie 1. Super resolution image of an SST-positive PA-tagged *Oxtr*-expressing neuron**
 1000 PA-tag signals shown in green are observed both on the surface of cell body and on the
 1001 neurites, whereas SST signals shown in magenta are localized in the cytoplasm. DAPI
 1002 nuclear staining signals are shown in blue.

1003
 1004 **Movie 2. Super resolution image of an SST-positive HA-tagged *Oxtr*-expressing neuron**
 1005 HA-tag signals shown in magenta are observed both on the surface of cell body and on the
 1006 neurites, whereas SST signals shown in green are localized in the cytoplasm. DAPI nuclear
 1007 staining signals are shown in blue.

1008
 1009 **Extended Data Figure 2-1. Homozygous knock-in males and females do not have any**
 1010 **problems in their reproductive functions.**

1011 **a.** Pups from *Oxtr* knock-out mothers (*Oxtr*^{-/-}) has been reported to die soon after birth due to
 1012 mother's defects in breast-feeding since *Oxt* plays essential roles in lactation (Hidema et al.,
 1013 2016). Pups' digestive tracts are not filled with milk.

1014 **b.** Digestive tract of a pup from homozygous *Oxtr*^{1×PA-tdTom} mother is filled with milk,
 1015 indicating homozygous mothers have no problems in lactation.

1016 **c.** Digestive tract of a pup from homozygous *Oxtr*^{1×PA-iCre} mother is filled with milk,
 1017 indicating the peripheral *Oxt* signaling pathway is intact.

1018 **d.** Mammary glands of homozygous *Oxtr*^{1×PA-tdTom} mother are well-developed.

1019 **e.** A homozygous *Oxtr*^{1×PA-iCre} mother can nurse her pups by lactating.

1020 **f.** A pair of homozygous *Oxtr*^{1×PA-iCre} mother and father, and their homozygous progenies.
 1021 Homozygous knock-in males are fertile.

1022
 1023
 1024 **Figure 3. Functional verification of tdTomato-positive neurons in *Oxtr*^{1×PA-tdTom} mice by**
 1025 ***in vitro* electrophysiological analyses**

1026 **a**, Schematic diagram of a mouse brain coronal section which shows placement of recording
 1027 electrodes in layer II and layer V of the prelimbic cortex (PrL). IL; infralimbic cortex.
 1028 Distribution of tdTomato-positive neurons in the PrL of *Oxtr*^{I×PA-tdTom} homozygous brain are
 1029 shown in Extended Data Fig.3-1.

1030 **b**, Representative differential interference contrast (DIC; left), fluorescent (red; middle), and
 1031 merged (right) images of a layer V neuron in the PrL from 4-week-old *Oxtr*^{I×PA-tdTom}
 1032 homozygous male are arranged.

1033 **c**, Representative cell-attached recording traces from a layer II tdTomato-positive cell in the
 1034 PrL. Left panel shows the baseline firing rate. Upon treatment of oxytocin receptor agonist,
 1035 TGOT, the firing rates are significantly increased as shown in right panel. Scale bar, 10 pA, 1
 1036 sec.

1037 **d**, Quantification of spontaneous firing activities from layer II tdTomato-positive cells in the
 1038 PrL ($n = 6$ cells from 6 mice). Light red lines represent individual neurons' responses. Red
 1039 circles with line represent average response. Presence of 1 μ M TGOT significantly increases
 1040 the firing rate. $*P < 0.05$ by Wilcoxon's test. As shown in Extended Data Fig.3-2a, this
 1041 TGOT-induced increase in firing rates is inhibited by presence of Oxtr antagonist, OTA.

1042 **e**, Representative cell-attached recording traces from a layer V tdTomato-positive cell in the
 1043 PrL. Left panel indicates the baseline firing rate. On treatment of TGOT, the firing rates are
 1044 considerably increased as shown in right panel. Scale bar, 50 pA, 1 sec.

1045 **d**, Quantification of firing activities from layer V tdTomato-positive cells in the PrL ($n = 6$
 1046 cells from 6 mice). Administration of 1 μ M TGOT considerably increases the spontaneous
 1047 firing rate. $*P < 0.05$ by Wilcoxon's test. As shown in Extended Data Fig.3-2b, this
 1048 TGOT-induced increase in firing rates is inhibited by presence of OTA.

1049

1050 **Extended Data Figure 3-1. Characteristics of tdTomato-positive neurons in the**
 1051 **prelimbic cortex (PrL)**

1052 Serial coronal sections of 5-week-old homozygous *Oxtr*^{I×PA-tdTom} male brain at Bregma 1.94
 1053 mm are arranged.

1054 **a**, Endogenous tdTomato fluorescence can be detected without immunostaining.

1055 **b**, Enlarged image of the boxed area in **a**.

1056 **c**, Enhanced tdTomato fluorescent signals are detected with anti-tdTomato antibody.

1057 **d**, Enlarged image of the boxed area in **c** indicates tdTomato-positive cells reside in both
 1058 layer II/III and in layer V of the PrL.

1059 **e, f**, tdTomato-expressing neurons in layer II express Cux1, one of the glutamatergic neuron
 1060 markers.

g, h, tdTomato-expressing neurons in layer V express somatostatin (SST), one of the interneuron markers.

Extended Data Figure 3-2. Blocking effects of oxytocin receptor antagonist, OTA on TGOT-induced increase of spontaneous firing activities in tdTomato-positive neurons

a, Mean spontaneous firing activities of layer II tdTomato-positive cells in the prelimbic cortex (PrL) from *Oxtr*^{I×PA-tdTom} homozygous males. Light red lines represent individual neurons' responses. Red circles with line represent average response. Firing activities remain unchanged in the presence of 1 μM OTA (left panel, *n* = 6 cells from 4 mice). 1 μM TGOT application in the presence of 1 μM OTA do not increase firing activities of tdTomato-positive cells (right panel, *n* = 3 cells from 3 mice). ns, not significant.

b, Mean spontaneous firing activities of layer V tdTomato-positive cells in the PrL from *Oxtr*^{I×PA-tdTom} homozygous males are arranged. Firing activities remain unchanged the in presence of 1 μM OTA (left panel, *n* = 7 cells from 6 mice). 1 μM TGOT application in the presence of 1 μM OTA do not affect firing rates of tdTomato-positive cells (right panel, *n* = 5 cells from 3 mice)

Figure 4. *Oxtr*^{I×PA-iCre} mice reliably drive recombinase activities in *Oxtr*-expressing cells.

a, Schematics of *Oxtr*^{I×PA-iCre} allele and *Gt(ROSA)Sor*^{Ai9} allele are depicted.

b-d, tdTomato expression profiles in *Oxtr*^{I×PA-iCre/+}::*Gt(ROSA)Sor*^{Ai9/+} male brains at P7, P14, and P21 are arranged (equivalent to Bregma -1.82 mm). Constitutive tdTomato expressions from the *Gt(ROSA)Sor* locus after Cre-dependent stop signal excision gradually accumulate over time. tdTomato signals are observed already at P7 and then become more abundant at P14 and P21.

e, Schematics of *Oxtr*^{I×PA-iCre} allele and AAV-PHP.eB vector harboring CBh_FLEX-GFP. GFP expression can be induced by Cre-dependent inversion.

f, Expression profiles of GFP in the *Oxtr*^{I×PA-iCre} heterozygous brain (Bregma -1.82 mm) from 11-week-old male to which AAV-PHP.eB vector has been retro-orbitally injected at 6 weeks. Other section levels are shown in Extended Data Fig.4-1.

g-j, Enlarged images of the boxed areas in f are arranged. GFP expressions are observed in the areas that have been reported to express *Oxtr*, such as the S1BF, hippocampal DG and aCA2/CA3, PVT, CeA, and DEn, indicating this mouse line sufficiently drives Cre activities in *Oxtr*-expressing cells.

k. Schematic diagram of *Oxtr*^{I×PA-tdTom} allele is depicted.

1096 **l.** tdTomato expression profiles in the *Oxtr*^{1×PA-tdTom} homozygous brain (Bregma -1.82 mm)
 1097 from 11-week-old male are arranged. Other section levels are shown in Extended Data
 1098 Fig.4-1.

1099 **m-p,** Enlarged images of the boxed areas in **l** are arranged. These tdTomato expression
 1100 profiles correspond to the GFP expressions shown in **g-j** induced by iCre upon AAV-PHPeB
 1101 administration.

1102

1103 **Extended Data Figure 4-1. Atlas of iCre/tdTomato expression profiles**

1104 **Left side;** Expression profiles of GFP in *Oxtr*^{1×PA-iCre} heterozygous brain from 11-week-old
 1105 male to which AAV-PHP.eB CBh_FLEX-GFP vector has been retro-orbitally injected at 6
 1106 weeks. **Right side;** tdTomato expression profiles in *Oxtr*^{1×PA-tdTom} homozygous brain from
 1107 11-week-old male.

1108 **a,b,** Bregma 1.78 mm. M1; primary motor cortex, S1; primary somatosensory cortex, PrL;
 1109 prelimbic cortex, DEn; dorsal endopiriform claustrum, IEn; intermediate endopiriform
 1110 claustrum.

1111 **c,d,** Bregma 1.42 mm. Cg; cingulate cortex, S1; primary somatosensory cortex, AcbC;
 1112 accumbens nucleus, core, AcbSh; accumbens nucleus, shell.

1113 **e,f,** Bregma 0.62 mm.

1114 **g,h,** Bregma -0.10 mm. LS; lateral septum.

1115 **i,j,** Bregma -0.58 mm. DEn; dorsal endopiriform nucleus.

1116 **k, l,** Bregma -2.30 mm. V2M; secondary visual cortex medial area, V1; primary visual cortex,
 1117 V2L; secondary visual cortex lateral area, Au; auditory cortex, PMCo; posteromedial cortical
 1118 amygdaloid area.

1119 **m,n,** Bregma -2.92 mm.

1120

1121

1122 **Figure 5. Cre recombinase activities can be reliably induced in Oxtr-expressing neurons** 1123 **upon tamoxifen administrations to the *Oxtr*^{3×HA-iCreERT2} line.**

1124 **a,** Schematics of *Oxtr*^{3×HA-iCreERT2} allele and *Gt(ROSA)Sor*^{Ai9} allele are depicted. Tamoxifen
 1125 (TMX) binds to iCre-ERT2 expressed in the cytoplasm, resulting in iCre translocation into
 1126 the nucleus to flip out stop signal from Ai9 allele and induce tdTomato expression.

1127 **b.** Schematic diagram of experimental protocol. *Oxtr*^{3×HA-iCreERT2}::*Gt(ROSA)Sor*^{Ai9} double
 1128 heterozygous mice can freely access to TMX-supplemented diet from Day 0 to Day 5. At Day
 1129 10, brains are sampled and immunostained. For control experiments, TMX-free normal diet is

1130 fed to same genotype mice. As shown in Extended Data Fig5-1, 5-days TMX treatment has
 1131 no effects on Oxt/Oxtr expression profiles in 5-week-old males.
 1132 **c.** TMX-free normal diet does not induce any leaky tdTomato signals in the brain from
 1133 5-week-old male (Bregma -1.82 mm).
 1134 **d.** TMX-supplemented diet induces tdTomato expressions in the cortical region, hippocampal
 1135 DG and aCA2/CA3, PVT, CeA, DEn, and PMCo, where Oxtr expressions has been reported.
 1136 The density of the tdTomato positive cells is much sparser than that induced by the
 1137 constitutive iCre line shown in Fig 4d.
 1138 **e-h,** Enlarged images of the boxed areas in **d** are arranged.
 1139 **i.** Enlarged image of the S1BF in **d**.
 1140 **j-l,** Confocal image of the boxed area in **i**, labeled with Cux1 immunostaining, reveals the
 1141 morphology of Oxtr-expressing glutamatergic neuron in layer II. White arrowhead in **k**
 1142 indicates the colocalization of Cux1 and tdTomato.
 1143 **m-o,** Confocal images of the boxed area in **i**, labeled with Cux1 and somatostatin (SST)
 1144 immunostaining, reveals the morphologies of Oxtr-expressing SST-positive interneurons in
 1145 layer IV. White arrows in **n** indicate the colocalization of SST and tdTomato.
 1146
 1147 **Extended Data Figure 5-1. 5-days TMX treatment has no effect on Oxt/Oxtr**
 1148 **expression profiles in male $Oxtr^{1 \times PA-tdTom/1 \times PA-tdTom}$ mice.**
 1149 **a.** Schematic diagram of experimental protocol. 5-week-old homozygous $Oxtr^{1 \times PA-tdTom}$ males
 1150 can freely access to TMX-supplemented diet from Day 0 to Day 5 (n=3). At Day 10, brains
 1151 are sampled for immunostaining. For control experiments, TMX-free normal diet is fed to
 1152 mice with the same genotype (n=3).
 1153 **b.** Oxt expression profiles in respective $Oxtr^{1 \times PA-tdTom/1 \times PA-tdTom}$ male brain are arranged
 1154 (Bregma -0.94 mm). Left column shows Oxt-expressing neurons in the paraventricular
 1155 hypothalamic nucleus (PVN) and supraoptic nucleus (SON) from normal diet conditions. In
 1156 right column, 5-days TMX treatment has no effect on Oxt expression profiles in these nuclei.
 1157 **c.** tdTomato expression profiles in respective $Oxtr^{1 \times PA-tdTom/1 \times PA-tdTom}$ male brain are arranged
 1158 (Bregma 0.62 mm). In this knock-in line, tdTomato monitors real-time Oxtr expressions. Left
 1159 column shows tdTomato-expressing cells in the bed nucleus of stria terminalis (BNST) from
 1160 normal diet conditions. In right column, 5-days TMX treatment has no effect on tdTomato
 1161 expressions in the BNST.
 1162 **d.** tdTomato expression profiles in respective $Oxtr^{1 \times PA-tdTom/1 \times PA-tdTom}$ male brain are arranged
 1163 (Bregma -1.82 mm). Left column shows tdTomato-expressing cells in the ventromedial
 1164 hypothalamus (VMH), medial amygdala (MeA), PMCo, and DEn from normal diet

1165 conditions. In right column, 5-days TMX treatment has no effect on tdTomato expressions in
1166 these brain areas.

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1168 **Materials Availability**

1169 Four knock-in mouse lines generated in this study will be made available with a materials
1170 transfer agreement.

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