Oxytocin facilitates allo-parental behavior under stress in laboratory mice

<https://doi.org/10.1523/ENEURO.0405-21.2022>

Cite as: eNeuro 2022; 10.1523/ENEURO.0405-21.2022

Received: 28 September 2021

Revised: 6 December 2021

Accepted: 4 January 2022

This Early Release article has been peer-reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

Alerts: Sign up at www.eneuro.org/alerts to receive customized email alerts when the fully formatted version of this article is published.

1 **Title Page**

2

3 **1. Manuscript Title (50 word maximum)**

4 Oxytocin facilitates allo-parental behavior under stress in laboratory mice

5

6 **2. Abbreviated Title (50 character maximum)**

7 Oxytocin, vasopressin and parental care

8

9 **3. List all Author Names and Affiliations in order as they would appear in the published article**

10 Yousuke Tsuneoka^{12#}, Chihiro Yoshihara^{1#}, Ryuko Ohnishi^{13#}, Sachine Yoshida^{12#}, Eri Miyazawa¹,
11 Masanobu Yamada⁴, Kazuhiko Horiguchi⁴, W. Scott Young⁵, Katsuhiko Nishimori⁶, Tadafumi Kato⁷
12 and Kumi O. Kuroda^{1*}

13 ¹ Laboratory for Affiliative Social Behavior, RIKEN Brain Science Institute, 351-0198 Japan

14 ² Current address: Department of Anatomy, School of Medicine, Toho University, 143-8540, Japan

15 ³ Current address: Department of Bioscience and Biotechnology, Faculty of Agriculture, University
16 of the Ryukyus, 903-0213, Japan

17 ⁴ Department of Internal Medicine, Division of Endocrinology and Metabolism, Gunma University
18 Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan.

19 ⁵ Section on Neural Gene Expression, National Institute of Mental Health, National Institutes of
20 Health, Bethesda, MD, United States

21 ⁷ Department of Obesity and Internal Inflammation, Fukushima Medical University, Fukushima
22 960-1295, Japan

23 ⁸ Department of Psychiatry and Behavioral Science, Graduate School of Medicine, Juntendo
24 University, Tokyo, Japan.

25

26 [#]: These authors contributed equally to this work.

27

28 **4. Author Contributions:**

29 YT, CY, RO, SY, KK designed and performed research, and analyzed data, with help from WSY, MY,
30 KH, KN, TK. EM analyzed data. KK organized the study. WSY, MY, KH, KN, TK, YT, CY, RO, SY,
31 EM, KK wrote the paper.

32

33 **5. Correspondence should be addressed to (include email address)**

* Contact: Kumi O. Kuroda, Laboratory for Affiliative Social Behavior, RIKEN Center for Brain Science, 2-1 Hirosawa, Wako-shi, Saitama, 351-0198 Japan.
e-mail: kumi.kuroda@a.riken.jp
Tel: +81-48-467-6949, Fax: +81-48-467-6947

6. Number of Figures

10

7. Number of Tables

2

8. Number of Multimedia

0

9. Number of words for Abstract

249

10. Number of words for Significance Statement

91

11. Number of words for Introduction

652

12. Number of words for Discussion

967

13. Acknowledgements

This investigation was supported in part by RIKEN Brain Science Institute (2004-2017), RIKEN Center for Brain Science (2018-21 to KK), a Long-Term Fellowship of the Human Frontier Science Program (2002-2004, KK), DRI Research Grant from RIKEN (2005-2006, KK), Research Facilitation Grant of Uehara Memorial Foundation, Japan (2008 to KK) and by JSPS KAKENHI Grant Numbers JP17790832, JP26282220, JP18H02710 to KK, JP21890307 to YT, and JP20890287 to SY. WSY research was supported by the intramural research program of the NIMH

67 (ZIAMH002498). We wish to thank Drs. Valery Grinevich, Geert de Vries, William Armstrong, and
68 Michael Numan for helpful discussion, Kashiko Tachikawa for initial breeding and phenotyping,
69 Sayaka Komatsu, Teppo Maruyama, Lana Ohkuma for technical assistance, and the RIKEN Center
70 for Brain Science, Research Resources Division for animal husbandry.

71

72 **14. Conflict of Interest**

73 The authors declare no competing financial interests.

74

75 **15. Funding sources**

76 This investigation was supported in part by RIKEN Brain Science Institute (2004-2017), RIKEN
77 Center for Brain Science (2018-21 to KK), a Long-Term Fellowship of the Human Frontier Science
78 Program (2002-2004, KK), DRI Research Grant from RIKEN (2005-2006, KK), Research
79 Facilitation Grant of Uehara Memorial Foundation, Japan (2008 to KK) and by JSPS KAKENHI
80 Grant Numbers JP17790832, JP26282220, JP18H02710 to KK, JP21890307 to YT, and JP20890287
81 to SY. WSY research was supported by the intramural research program of the NIMH
82 (ZIAMH002498).

83

84 **Abstract (max 250 words incl citations)**

85 Oxytocin (Oxt) controls reproductive physiology and various kinds of social behaviors, but
86 the exact contribution of Oxt to different components of parental care still needs to be determined.
87 Here we illustrate the neuroanatomical relations of the parental nurturing-induced neuronal activation
88 with magnocellular oxytocin neurons and fibers in the medial preoptic area (MPOA), the brain
89 region critical for parental and alloparental behaviors. We utilized genetically-targeted mouse lines
90 for *Oxt*, *oxytocin receptor (Oxtr)*, *vasopressin receptor 1a (Avpr1a)*, *vasopressin receptor 1b*
91 (*Avpr1b*), and *thyrotropin-releasing hormone (Trh)* to systematically examine the role of Oxt-related
92 signaling in pup-directed behaviors. The *Oxtr-Avpr1a-Avpr1b* triple knockout (TKO), and
93 *Oxt-Trh-Avpr1a-Avpr1b* quadruple KO (QKO) mice were grossly healthy and fertile, except for their
94 complete deficiency in milk ejection and modest deficiency in parturition secondary to maternal loss
95 of the *Oxt* or *Oxtr* genes. In our minimal stress conditions, pup-directed behaviors in TKO and QKO
96 mothers and fathers, virgin females and males were essentially indistinguishable from those of their
97 littermates with other genotypes. However, *Oxtr* KO virgin females did show decreased pup retrieval
98 in the pup-exposure assay performed right after restraint stress. This stress vulnerability in the *Oxtr*
99 KO was abolished by the additional *Avpr1b* KO. The general stress sensitivity, as measured by
100 plasma cortisol elevation after restraint stress or by the behavioral performance in the open field and
101 elevated plus maze, were not altered in the *Oxtr* KO but were reduced in the *Avpr1b* KO females,
102 indicating that the balance of neurohypophysial hormones affects the outcome of pup-directed
103 behaviors.

104
105 **Significance Statement (max 120 words)**

106 Parental care without suckling induces the most significant transcriptional activation in the
107 caregivers' anterior commissural nucleus, the third-largest population of oxytocin neurons in the
108 medial preoptic area, but not in its non-oxytocinergic neurons. The pup-directed behaviors in
109 postpartum mothers, fathers and virgin males and females of *oxytocin receptor (Oxtr)*, *vasopressin*
110 *receptor (Avpr) 1a* and *1b* triple knockout mice were essentially normal in our standard experimental
111 conditions with minimal stress. Under stressful conditions, however, *Oxtr* KO mice showed
112 decreased parental nurturing behaviors, which was compensated for by combining with the *Avpr1b*
113 KO.

114

115

116 **Introduction (max 750 words incl citations)**

117 Oxytocin (Oxt) and vasopressin (Avp) are nonapeptide hormones with a common ancestor
 118 gene. Avp is critically involved in osmoregulation and Oxt stimulates uterine contraction during
 119 parturition and milk-ejection during nursing (Robinson and Verbalis, 2003; Wakerley, 2005). In
 120 addition to these peripheral functions, Oxt and Avp have direct actions onto neurons expressing their
 121 receptors, and participate in the control of anxiety, pain and stress responses, social recognition, pair
 122 bonding, and aggression (Insel, 2010; Neumann and Landgraf, 2012; Onaka et al., 2012; Hurlemann
 123 and Grinevich, 2017; Grinevich and Neumann, 2020).

124 For maternal and allomaternal behaviors, there are numerous reports supporting the
 125 positive effects of oxytocin (Takayanagi et al., 2005; Caldwell and Young, 2006; Marlin et al., 2015).
 126 The facilitatory roles of Oxt in parental care are reported most frequently during the high-stress
 127 conditions, such as the onset/initial learning phase of parenting or in a non-home cage environment
 128 (for example, (Pedersen et al., 1982; Marlin et al., 2015; Carcea et al., 2021)). In these cases,
 129 however, Oxt facilitation of maternal behavior could be due to OT's anxiolytic / anti-stress effect in
 130 general, rather than via its primary role in maternal behavior *per se* (McCarthy et al., 1992;
 131 McCarthy, 1995; Yoshihara et al., 2017). Moreover, there are several studies reporting that multiple
 132 components of parental behavior are intact in mice harboring genetic mutations of the Oxt-Oxtr
 133 system (Nishimori et al., 1996; Young et al., 1996; Gross et al., 1998; Macbeth et al., 2010),
 134 impeding a coherent explanation of the exact role of oxytocin in different components of parental
 135 care. One of the confounding factors is the possible cross-activation/compensatory mechanisms of
 136 the Oxt and Avp systems via their receptors. Specifically, more than 80 % structural homology
 137 between Oxtr and Avpr1a causes significant cross-activation (Manning et al., 2012; Jurek and
 138 Neumann, 2018), which is not negligible as both Oxt and Avp are implicated in maternal behavior
 139 (Bosch and Neumann, 2008).

140 From the viewpoint of functional neuroanatomy, we have previously studied the pattern of
 141 neuronal activation during maternal, paternal and allomaternal care in mice (Tsuneoka et al., 2013;
 142 Tsuneoka et al., 2015). We focused on the medial preoptic area (MPOA), the critical brain area for
 143 parental care (Numan, 1974; Numan and Numan, 1994; Numan, 2020), and investigated expression
 144 of c-Fos protein, the component of AP-1 transcription factor as a reliable readout of neuronal
 145 activation (Herdegen and Leah, 1998). The most pronounced c-Fos expression after two hours of
 146 nurturing behavior is observed at the anterior commissural nucleus (AC, previously abbreviated as
 147 ACN) of the MPOA. The AC contains the third largest population of magnocellular Oxt neurons, Oxt
 148 fibers as well as Avp fibers, and thyrotropin-releasing hormone (Trh)-producing neurons (Peterson,

149 1966; Armstrong et al., 1980; Rhodes et al., 1981; Sofroniew, 1985; Castel and Morris, 1988;
150 Grinevich and Akmayev, 1997). Both oxytocinergic and non-oxytocinergic AC neurons are c-Fos
151 positive after two hours of pup exposure in postpartum mothers. In virgin females, however, the
152 transcriptionally-activated AC neurons during allomaternal behavior are essentially
153 non-oxytocinergic, while roughly 40 % of these activated AC neurons expressed calcitonin-receptor
154 (Calcr) (Yoshihara et al., 2021). Moreover, while Calcr neurons in the central part of the MPOA
155 (cMPOA), which lies ventrally to the AC, are functionally critical for parental nurturing (Yoshihara
156 et al., 2021), all non-oxytocinergic AC neurons including Calcr neurons are not. Still, the close
157 spatial correlation of the parenting-induced activation pattern and the distribution of oxytocin
158 neurons and fibers in the MPOA (see Fig. 1, 2 and (Tsuneoka et al., 2013)) is remarkable, and
159 prompted us to further investigate this issue. Here, to elucidate the exact role of Oxt system in
160 parental care, we used seven lines of genetic mutant mice in combination, covering genes for *Oxt*,
161 *Trh*, *Oxtr*, and *Avp receptors 1a* (*Avpr1a*), and *1b* (*Avpr1b*), and performed a systematic investigation
162 of the role of Oxt-Avp system in postpartum-maternal, -paternal and allomaternal behaviors, as well
163 as infanticide of virgin male mice (collectively called "pup-directed behaviors").
164

165 **Materials and Methods**

166 **Animals**

167 All animal experimentation was approved by and conducted in accordance with regulations
 168 of the Animal Experiment Judging Committee of RIKEN, which was based on the National Institutes
 169 of Health guide “Principles of Laboratory Animal Care” (NIH publication no. 85-23, revised 1985).
 170 The male C57BL/6J mice used for this study were obtained originally from the Jackson Laboratory.
 171 The oxytocin (B6;129S-*Oxt*^{tm1Wsy}/J; stock number 2713) (Young et al., 1996), *Avpr1a*
 172 (B6.129P2-*Avpr1a*^{tm1Dgen}/J; stock number 5776) (Deltagen, 2005) and *Avpr1b* KO mouse strains
 173 (B6;129X1-*Avpr1b*^{tm1Wsy}/J; stock number 6160) (Wersinger et al., 2002) were obtained from the
 174 Jackson Laboratory (Bar Harbor, ME). A *Oxt* KO mouse strain described in (Takayanagi et al.,
 175 2005) and a *Trh* KO mouse strain described in (Yamada et al., 1997) were developed by co-authors
 176 and available from their laboratories. Another *Avpr1a* and *1b* double KO mouse strain
 177 (B6;129Sv-*V1a*^{tm1Gzt}*V1b*^{tm1Gzt}; stock number 559) (Tanoue et al., 2004; Koshimizu et al., 2006) was
 178 obtained from the Center for Animal Resources and Development, Kumamoto University
 179 (Kumamoto, Japan). All the mice, except for the *Avpr1b* KO mouse in the quadruple KO (QKO)
 180 study, were backcrossed more than 7 times to C57BL/6J mouse. The *Avpr1a* (JAX 5776), *Avpr1b*
 181 (JAX 6160), *Oxt* and *Trh* quadruple KO mice line was named QKO; *Avpr1a* (JAX 5776), *Avpr1b*
 182 (JAX 6160), and *Oxt* triple KO mice line was named TKO; and *Avpr1a*, *Avpr1b* (*V1a*^{tm1Gzt}*V1b*^{tm1Gzt};
 183 Kumamoto Univ. stock number 559), and *Oxt* triple KO mice line was named HIR in this study.

184 Mice were raised in our breeding colony under controlled conditions (12 h light/dark cycle;
 185 lights on at 8:00 A.M.; 23 ± 2°C; 55 ± 10% humidity; and ad libitum access to water and food). Mice
 186 were weaned at 4 weeks of age and were housed in groups of four or five in ventilated shoebox cages
 187 (267 × 483 × 152 mm) with TEK-Fresh bedding (Harlan, Indianapolis, IN, USA). All mice were 12–
 188 24 weeks old at the start of experiments.

189 For generating QKO mice, the *Avpr1b* mouse line were a C57BL/6J × 129 mixed
 190 background. For generating TKO mice, we used the *Avpr1b* mouse line which were backcrossed 5
 191 times to C57BL/6J background. Breeding of genetic mutant mice were performed as described
 192 (Kuroda et al., 2011); briefly, first, double-KO mice of approximate combinations were produced by
 193 crossbreeding of single-KO mice. Then, we crossbred different combination of double-KO mice to
 194 produce QKO or TKO mice. The TKO male mice were crossbred to triple-heterozygous or
 195 single-KO/double-heterozygous female mice, and their littermate offspring were used for the
 196 behavioral testing. After the QKO male mice were obtained, they were crossbred to

double-KO/double-heterozygous or single-KO/triple-heterozygous female mice, and the offspring were used for the behavioral testing.

The genotypes of mice were determined by electrophoresis of the product from polymerase chain reaction using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) or Quick Taq[®] HS DyeMix (TOYOBO, Japan) with the specific primer sets for each mouse strain as described [Table 1]. The genomic DNA was obtained from last 2mm of tail. Samples were put into 100ul of proteinase K solution (100mM Tris-HCl (pH 8.0), 5mM EDTA, 200mM NaCl, 0.2% (w/v) SDS, 2% (v/v) 15mg/ml proteinase K (Takara, Japan)) and heated at 56 °C for 2 hours, 95°C for 2min, and then diluted by 300 ul distilled water. The genotyping were doubly confirmed after the end of each experiment.

Assessment of pup-directed behaviors in standard condition

For pup-exposure assays, two days before the first behavioral testing, mice were housed individually in clean cages with purified paper bedding (Alpha-Dri, Shepherd Specialty Papers, Watertown, TN, USA) and a piece of cotton square (Nestlet, Ancare, Bellmore, NY, USA) as nest material.

Pup-directed behaviors of mice were examined as described (Kuroda et al., 2011). Briefly, either three of their own pups or three unfamiliar pups 1-5 days old (donor pups) were gently introduced to the corner of the home cage of the subject mice avoiding the nest. Behavioral responses toward pups were observed as follows;

Latency to the first sniffing: the latency to when the subject sniffed a pup for the first time in a trial.

Latency to the first pup retrieval: the latency to when the subject retrieved the first pup to the nest.

Latency to the last pup retrieval: the latency to when the subject retrieved the last pup to the nest. If the subject retrieved all 3 pups to the nest, the retrieval of the 3rd pup was considered as the last pup retrieval. If the subject retrieved 2 pups to where another pup was located and made a new nest there, the retrieval of the 2nd pup was considered as the last pup retrieval. If the subject did not retrieve any pup, or retrieved only 1 or 2 pups and failed to retrieve the rest, the last pup retrieval time was regarded as a missing value.

Total time in nest for 3 pups: the sum of the duration of each pup being in the nest. If the subject retrieved 2 pups to where another pup was located and made a new nest there, the pup that was already in the new nest site was regarded as being in the nest since when the first pup was retrieved to the new nest site.

Pup grouping: collecting all three pups in the nest so that the pups contact each other.

230 **Full parental behavior:** when the subject exhibited all of the following behaviors within a trial, it
 231 was regarded as “full parental behavior”: sniffing, retrieving and grouping all the pups, and
 232 crouching over them in the nest for more than 1 minute.

233 Also, in the QKO, TKO, and HIR analyses, the subjects' behaviors were coded at 15 sec
 234 intervals for pup sniffing, pup licking, nest building, retrieval, crouching over pups, and non-pup
 235 directed behaviors (such as feeding, resting, running in the cage). In addition, pups' audible distress
 236 calls induced by the subjects' contacts were noted. If the subject mice started to attack and bite the
 237 pups, all pups were immediately removed from the cage, and the subjects were deemed as
 238 “infanticidal”. The attacked pups were immediately euthanized. For the QKO and TKO mothers (Fig.
 239 4 and 5), the observation period was separated into two periods. The mother mice were observed for
 240 15 min just after introduction of pups (first observation of the day); and then after a 15 min resting
 241 period with the introduced pups, they were again observed for 15 min (second observation of the
 242 day). The duration data from the first 15-min observation periods was used for analyses.

243 For the *Oxt-Trh* double KO (DKO) mice, four of the females used for the analyses of
 244 pup-directed behaviors were tested twice as a virgin, but only the results from the first trials were
 245 analyzed (Fig. 3A). These four females were not used for the further analyses. Other virgin females
 246 were tested only once as a virgin. Of the DKO females used for the analyses of postpartum
 247 pup-directed behaviors, only four were experienced the pup-exposure test as a virgin (whose results
 248 were included in Fig. 3A). The postpartum tests were performed either on the day of delivery or one
 249 day later. All the *Oxt-Trh* DKO male mice were tested once as virgins (Fig. 3I). Only males who
 250 exhibited infanticidal behavior as virgins were used for further analysis. Males were then cohabited
 251 with females. Some of these males were separated from their mates immediately after copulation
 252 (assessed by vaginal plugs), and tested on the day the paired females delivered (Fig. 3J). The other
 253 males were separated from their female mates after 7 days of cohabitation, and tested on the day the
 254 paired females delivered (Fig. 3L).

255 For the QKO and TKO female mice (Fig 4, 5), the responses to pups were first examined
 256 once per day for 4 straight days. Each female was subsequently paired with a sexually-experienced
 257 male C57BL/6J mouse to allow mating and delivery. The male mouse was changed to another in the
 258 case that the female did not become pregnant by 3 weeks cohabitation. On the day of delivery, the
 259 newborn pups were removed, and their location in the cage, general health, milk band and remaining
 260 fetal tissues were briefly examined according to the previous study (Kuroda et al., 2011). Then using
 261 their own 3 pups, the subject mothers were observed for their behavior as described above. In the
 262 case that the newborn pups were not cleaned properly, unhealthy or dead, donor pups were used

263 instead. Use of donor pups does not significantly affect pup exposure assay (Kuroda et al., 2011). If
264 the mother showed any apparent abnormality of their movement caused by their parturition
265 (observed in *Oxt* and *Oxtr* KO mice), we did not conduct the behavioral test on the day of delivery.
266 On the next morning (PPD1), the resident pups in the home cage were gently removed and their
267 conditions were checked again as they had been the day before (day of delivery). Then, the female
268 mice were totally isolated from the pups. After the isolation, they were subjected to behavioral
269 testing once again 13 days later.

270 For the TKO male mice and their littermates (Fig 7), the responses to pups were examined
271 once before mating. Each male was subsequently paired with two parous C57BL/6J mice to allow
272 mating. They were checked for vaginal plugs daily, and the cohabitation with females continued for
273 17 days after confirmation of the plugs to assure pregnancy as indicated by enlargement of abdomen.
274 In the case that a female become pregnant without a plug, both females were removed from the
275 mating cage. If there were no sign of pregnancy after a two-month cohabitation, the males were not
276 used for the subsequent tests. One to two days after one of the paired females delivered, the male
277 behavior was examined using their biological offspring once per day for 2 straight days, and once
278 again 12-14 days later.

279 For the HIR female mice (Fig. 8A, B), different individuals were used for virgin female
280 and mother mice assays. For virgin females, the responses to pups were first examined once per day
281 for 3 straight days. For PPD0 mothers, they were subjected to behavioral testing on the day of
282 delivery as described above, but with 3 donor pups instead of their own. After the testing, mother
283 mice were totally isolated from the pups. Then they were subjected to behavioral testing for 2
284 straight days. For the HIR male mice (Fig 8C, D), different individuals were used for virgin male and
285 father mice assays. For virgin males, the responses to pups were first examined once per day for 3
286 straight days. For father mice, they were cohabitated with female and own pups for 2 days after the
287 delivery. Then, the father mice were totally isolated from the pups. They were subjected to
288 behavioral testing once per day for 3 straight days, using 3 donor pups.

289

290 **Assessment of pup-directed behaviors after restraint stress**

291 Pup-directed behaviors immediately after acute restraint stress were first examined once
292 per day for 4 straight days using virgin female TKO mice (Fig. 9). Restraint stress was given for 30
293 min using well-ventilated 50 ml conical tubes. The mice were released into their own home cages
294 after three unfamiliar pups of 1-5 days old were gently introduced to the corner of the cage of the
295 subject mice avoiding the nest. Behavioral responses toward pups were observed in the same way as

in the TKO virgin female assays under standard conditions as described in the previous section.

Assessment of stress sensitivity

Phenotypic behavioral differences between *Oxt*^{+/-} and ^{-/-}, *Avpr1a*^{+/-} and ^{-/-}, and *Avpr1b*^{+/-} and ^{-/-} mice were assessed with a specific behavioral and physiological test battery consisting of open-field (OF) and elevated plus maze (EPM) test using virgin male and female TKO mice for Figure 10. The cohort of mice were different from those in Figure 5-8. The OF and EPM tests were conducted basically as previously described (Kuroda et al., 2008) with minor modifications as described below. The effects of restraint stress on plasma corticosterone (CORT) levels were also examined.

1. Open-field test

The subjects were individually housed 2 days prior to the experiment, and were given white noise on the day before the experiment. An open-field monitoring system equipped with four monitoring channels was used (O'Hara & Co., Ltd., Tokyo, Japan). Mice were placed in the center of the open field (50 cm x 50 cm, 40 cm high gray acrylic walls, bright-light condition of 70 lux) and allowed to explore for 15 min. The distance traveled and percent of time at the center area of the field (size is 36% of the field) were measured using an automatic monitoring system Time OFCR4 (O'Hara & Co., Ltd.).

2. Elevated plus maze test

A week after the OF test, the mice were tested in the EPM for 15 min. The maze was set at a height of 50 cm above the floor and consisted of four arms (25 cm x 5 cm), and a platform made of gray acrylic: two opposite arms were open, and the other two arms were enclosed by 15-cm-high transparent walls (room was illuminated at 70 lux). A mouse was placed in the center platform, positioned to face one of the open arms, and allowed to explore the maze for 15 min. The time spent in the different arms and the numbers of arm entries were automatically analyzed using Time EP2 Two Maze System (O'Hara & Co., Ltd.).

3. Restraint stress and plasma CORT

A week after the EPM assessments, half the subject mice were given acute restraint stress for 30 min using well-ventilated 50 ml conical tubes. The rest of mice were left without stress for 30 min. Then the mice were decapitated and the trunk blood immediately collected was assayed for CORT by ELISA (Corticosterone, ELISA Kit, AssayMax; ASSAYPRO, MO, USA). Blood collection was performed in the morning, between 10:00-12:00.

329 **Preparation of brain sections**

330 For histological analyses in Figure 6, TKO mice used for the Figure 5 experiments were
331 deeply anesthetized with sodium pentobarbital (50mg/kg, i.p.) and then perfused transcardially with
332 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4). The brains were
333 removed, immersed in the same fixative at 4°C overnight, followed by cryoprotection in the series of
334 20% and 30% (w/v) sucrose in PBS for two days, embedded in O.C.T. Compound (Sakura Finetek
335 Japan, Tokyo, Japan), and stored at -80°C until cryosectioning. Brains were cryosectioned coronally
336 at a thickness of 40µm and evaluated using the mouse brain atlas (Franklin and Paxinos, 2007).
337 Every third section from the serial sections was processed for immunohistochemistry (IHC).

338 **Immunohistochemistry**

340 IHC on free-floating sections was performed essentially as described (Tsuneoka et al.,
341 2013). Single and double labelling was performed for immunohistochemical detection of
342 neuropeptide Y (NPY), Avp, c-Fos and NeuN. To label Oxt neurons, anti-NPY was used, as Oxt and NPY
343 are cleaved from the same precursor, preprooxyphysin. The anti-NPY antibody is more sensitive than
344 an anti-Oxt antibody. The sections were washed with PBS containing 0.2% Triton-100 (PBST),
345 incubated with 0.3% H₂O₂ in methanol for 5 minutes, washed with PBST, blocked with 0.8% Block
346 Ace (Dainihon-Seiyaku, Osaka, Japan) in PBST, and incubated at 4°C overnight with goat primary
347 antibody against NPY (1:6000, sc-7810, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The
348 following morning, the sections were washed and incubated with biotin-conjugated horse anti-goat
349 secondary antibody (1:2000, BA-9500, Vector Laboratories, Inc., Burlingame, CA, USA) for 2 hours
350 and then in ABC peroxidase reagent (Vectastain ABC Elite kit; Vector Laboratories) for 1 hour
351 according to the manufacturer's instructions. The labelling was visualized by incubation in 3,3'-
352 diaminobenzidine (DAB) solution with nickel intensification (DAB peroxidase substrate kit, Vector
353 Laboratories) for 5 minutes. For c-Fos-NPY and Avp-NeuN double staining, the first staining was
354 processed similarly except the staining procedure used rabbit primary antibody against c-Fos (1:5000,
355 sc-52, RRID:AB_2106783, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or Avp (1:5000,
356 AB1565, Millipore, CA, USA), and biotin-conjugated horse anti-rabbit secondary antibody (1:2000,
357 BA-1100, Vector Laboratories, Inc., Burlingame, CA, USA). The sections underwent the second
358 staining, which used anti-NPY antibody, mouse anti-NeuN antibody (1:6000, MAB377, Millipore
359 Corporation, Temecula, USA). For anti-NeuN double staining, horse anti-mouse secondary antibody
360 (1:2000, BA-2000, Vector Laboratories, Inc., Burlingame, CA, USA), and the ABC alkaline
361 phosphatase reagent (Vectastain ABC-AP kit, Vector Laboratories) were used. The brown signals

were developed by 5 minutes of immersion in DAB solution without nickel, and pink color was developed by 5 minutes of immersion in Vector Red substrate (Vector red alkaline phosphatase substrate kit, Vector Laboratories). Subsequently, they were washed with PBS and then mounted on gelatin-coated slides using mounting medium (Vectashield; Vector Laboratories).

Histological analysis

For labeling of NPI-ir and c-Fos-ir cells, three rostral-to-caudal sections where the NPI and c-Fos expression were prominent were examined. Double-labeled sections of c-Fos and NPI were viewed under a brightfield microscope (Leica DM6000B; Leica Microsystems, Germany). The labeling protocol was described (Tsuneoka et al., 2013).

For analysis of NPI-ir and Avp-ir cells of TKO female mice (Fig. 6), brightfield photomicrographs were obtained using a digital slide scanner (NanoZoomer Digital Pathology; Hamamatsu Photonics, Shizuoka, Japan) with a 20-40x objective. The contrast and brightness of the all photographs were adjusted only linearly and uniformly for all the micrographs used in one experiment, using software (Image J (Rasband, 1997-2018)). Captured images were manually labelled by Image J. PVH and SO was identified by counterstaining using NeuN-ir. Anatomical nomenclature and classification were used according for reference (Watson et al., 2011).

Statistical analysis

All statistical analyses were conducted using R v. 3.6.3 (R Development Core Team, 2018). Welch's t-test and Fisher's exact test were used to compare continuous and categorical data, respectively. Multiple comparisons were conducted with P-values adjusted by Holm's method. Error bars are mean $\pm$ standard error of mean unless otherwise specified. Datapoints of "censored", incomplete retrieval observations were replaced by the maximum observation time (30min for virgin females, virgin males, and fathers in Figure 4, 5, 7, 9, and mothers in Figure 8; 15 min for mothers in Figure 4, 5) (e.g., if a subject mouse retrieves only one pup within the session, the latencies for the second and third retrieval are regarded as 30 min, rather than as missing datapoints. We also tried survival analyses, but comparing the effects of three genetic loci became complicated in the survival analysis. Please see also the Extended data Fig. 9-1 for another strategy, and the main conclusion is the same). The biological replicate number is the same as the number of samples, and the technical replicates are not applicable or taken in this study. All the statistical details and the numbers of animals used can be found either in the figure legends or results.

396 Results

397 Neuroanatomical relations of the activated neurons during alloparenting of Oxt neurons and 398 fibers in the preoptic and adjoining areas

399 Our initial motivation to study the relations between Oxt and parental behavior derived
400 from the remarkable spatial correlation of neuronal activation after pup nurturing with the
401 distribution of oxytocinergic neurons and fibers in the MPOA (Fig. 1, 2). We have already reported
402 the quantification of each MPOA subregions for nurturing-induced activation (Tsuneoka et al., 2013),
403 and here we illustrate anatomical and spatial relations between these neuronal activation with Oxt
404 neurons and fibers. Observing c-Fos expression pattern, we found that parasagittal and horizontal
405 sections consistently depicted the anatomical structures of the paraventricular nucleus of the
406 hypothalamus (PVH) (arrowheads), AC (Box in Fig. 1A, 1B), and the c-Fos neuronal distributions
407 after 2 hours (Fig1A, 1B) or 6 hours (Fig. 2) of pup nurturing (arrows) in C57BL/6 virgin female
408 mice. c-Fos-ir neurons were found most densely in the AC, compared to the PVH, SO, and other
409 MPOA subregions. Within the AC, Oxt-ir thick dendrites with a characteristic corkscrew-like
410 morphology and irregular varicosities (Fig. 1B, arrows) were numerous (Castel and Morris, 1988).
411 Even outside of the AC, the regions with dense c-Fos-ir neurons were colocalized with Oxt fibers,
412 particularly in the preoptic area but not in the hypothalamus (for example, areas indicated by arrows
413 in the ventral MPOA, Fig. 1, left, second; Fig. 2, left, first and second panels), indicating the spatial
414 correlation between the non-Oxt MPOA neurons involved in nurturing behaviors and the Oxt
415 neurons involved in parturition and lactation. In contrast, there were several spherical tissue masses
416 (numbered as 1-4 in the Fig. 1A, 2) devoid of both c-Fos and Oxt fibers, suggesting that the
417 developmental formation of the parenting-relevant anatomical structure was intermixed with other
418 cell masses in the MPOA. Specifically, the tissue mass 1 roughly corresponds with the ventral part of
419 the septohypothalamic area, mass 2 to the medial preoptic nucleus, mass 3 overlaps with the
420 striohypothalamic area, and mass 4 overlaps with the posteroventral medial preoptic area and the
421 ventral part of anterior hypothalamic nucleus. This unique organization might be formed by the
422 embryonic preoptic-strial migration stream (Bayer and Altman, 2004).

423 In these virgin females, that were not suckled during brief pup nurturing, the induced
424 c-Fos-ir were limited to non-oxytocinergic neurons. There were scarce c-Fos-ir Oxt neurons in the
425 analyzed area that included the AC, PVH, PVPOA and SO (Fig. 1A, 1B, 2) of the pup-exposed virgin
426 females ($\text{c-Fos-ir cells} / \text{Fos} + \text{Oxt double-ir cells} = 0.067\%$, $\text{Oxt-ir cells} / \text{c-Fos} + \text{Oxt double-ir cells}$
427 $= 0.915\%$), in harmony with previous studies (Tsuneoka et al., 2013; Okabe et al., 2017). (Sheehan et
428 al. found significant c-Fos induction in PVH by pup exposure (Sheehan et al., 2000), although they

included the AC as the anterior magnocellular part of PVH). It should be noted, however, that Fos expression itself does not imply the function of Oxt neurons. For example, while Oxt neurons are shown to be functional in facilitating social contacts among females, only about 1% of Oxt neurons (30% of parvocellular Oxt neurons, which are about ~3% of total Oxt neurons) in the PVH become c-Fos positive during social contacts (Tang et al., 2020).

Delayed delivery of *Oxt* KO mice, and grossly-normal pup-directed behaviors of *Trh-Oxt* double mutant mice

The above observations prompted us to directly address the role of Oxt and Avp systems in pup nurturing behaviors in mutant mice. Because normal maternal care was shown previously in three independent genetically targeted mouse lines of *Oxt* (Nishimori et al., 1996; Young et al., 1996; Gross et al., 1998), we hypothesized that there is functional redundancy of Oxt neurons in the AC. Neurons expressing *Trh* are distributed in the AC (Simerly et al., 1986), and the orthopaedia-expressing developmental progenitor cells are the same for Oxt and *Trh* neurons (Acampora et al., 1999). Moreover, *Trh* receptor (*Trhr*) was one of the transcripts upregulated in the dorsolateral MPOA of parenting mice in our previous DNA microarray study (Kuroda et al., 2007; Kuroda et al., 2008). These data prompted us to examine the maternal and alloparental behaviors of *Oxt-Trh* double KO (DKO) mice (Fig. 3).

Oxt-Trh DKO mice were born at a Mendelian ratio, and appeared healthy and normal as their littermates with other genotypes, with a minor retardation of body growth of *Trh* KO mice (Yamada et al., 1997). The majority of DKO virgin females also showed normal allomaternal behaviors (Fig. 3A). DKO female mice mated and became pregnant similarly to their non-KO (heterozygous or wildtype) littermates (Fig. 3B). However, a significant proportion (18 out of 35, 51.4 %) of *Oxt* KO mothers showed severe delay of parturition, such that the labor continued for more than 24 hrs after the delivery of the first pup, there was a dead pup found in the vaginal entry, or the maternal health deteriorated visibly, leading to spontaneous maternal death or euthanasia for the animal's welfare (5 out of 35, 14.3 %) (Fig. 3C). The delayed-labor phenotype of *Oxt* KO mothers was not found in the initial studies in the mixed background (Nishimori et al., 1996; Young et al., 1996) but was suggested in a later study (Roizen et al., 2007), and significant in the present study in the C57BL/6 genetic background. These mothers that experienced abnormal labor were excluded from further analyses for maternal behaviors. The rate of abnormal labor was not significantly affected by the *Trh* genotype.

The home-cage postpartum maternal behaviors of *Oxt* KO and DKO mothers, including

nest building, placentophagia, and pup grouping were indistinguishable from those of their wild-type or heterozygous littermates (Fig. 3D-F). However, the pups born to *Oxt* KO mothers were weak, devoid of milk, and died within 2 days with 100 % genetic penetrance (Fig. 3G; the 3 mothers with pups surviving through PPD1 lost them by PPD2), in concordance with previous literature (Nishimori et al., 1996; Young et al., 1996). At this time, we did not formally examine actual suckling behaviors; yet, the elongated nipples were found in all four *Oxt* KO mothers examined, with no clear milk bands observed in their litters, suggesting that the nursing behaviors per se were performed by *Oxt* KO mothers. Among six *Oxt*^{+/+} mothers examined, five mothers had elongated nipples, and four of them had the pups with a clear milk band. One *Oxt* heterozygous mother which did not have clearly elongated nipples had only one pup survive on the day of delivery.

Because unfed or unhealthy pups do not properly induce maternal retrieval, and because mouse mothers show maternal behaviors toward donor pups as well as their own pups, we performed pup retrieval assays with fed donor pups for all the subject mothers for consistency (see (Kuroda et al., 2011)). The *Oxt* KO mothers with or without *Trh* KO exhibited normal pup retrieval toward donor pups (Fig. 3H).

We next examined pup-directed behaviors in *Oxt-Trh* DKO male mice. DKO virgin and post-mating (separated from females after copulation within a day) males performed infanticide as much as their littermates with other genotypes (Fig. 3I, J). After cohabitation with a female for 7 days, the proportion of males that successfully fertilized females was not significantly different among the genotypes (Fig. 3K). At the time of the females' deliveries, no differences in pup-directed behaviors were found between DKO and their littermates (Fig. 3L). As previously reported, C57BL/6 fathers need to stay with the pregnant mate female until late gestation to become mostly paternal (Tachikawa et al., 2013). Two *Oxt* KO males stayed with their pregnant mates until late gestation, as well as 3 males that stayed with their mates until delivery, turned paternal. Overall, these results indicate no abnormalities in pup-directed behaviors in DKO male and female mice.

Pup-directed behaviors of *Oxt-Trh-Avpr1a-Avpr1b* quadruple KO (QKO) female mice

Oxytocin's role in pup-directed behaviors might be obscured by interactions with the Avp system, possibly at the receptor level. Two kinds of Avp receptors, *Avpr1a* and *Avpr1b*, are expressed in the brain (Koshimizu et al., 2012). Therefore, we created *Oxt-Trh-Avpr1a-Avpr1b* quadruple KO (QKO) mice, after crossing each line into the C57BL/6 genetic background at least four times.

During the breeding procedure, QKO male and female mice were born at a mendelian ratio, and appeared healthy. Moreover, they were grossly as fertile as their littermates with other genotypes,

although we did not specifically examine their sexual behaviors in detail. The complete lack of milk transfer to the pups and the modest labor delay in *Oxt* KO mothers were consistently found throughout this study as shown in Fig. 3. To focus on maternal behaviors, we excluded from further analyses the mothers that experienced a severely disturbed labor.

The breeding procedure was inevitably complicated and laborious, because there are 3⁴ (81) genotypes for the four gene loci. In this circumstance, testing all these genotypes separately was not practically feasible. Therefore, we categorized the genotypes into the following groups as in the Fig. 4: one comparison between QKO vs. all the other genotypes combined, and four comparisons for each gene locus as heterozygous vs. KO (e.g., QKO vs. *Oxt*^{-/-}; *Trh*^{-/-}; *Avpr1a*^{-/-}; *Avpr1b*^{-/-}; QKO vs. *Oxt*^{-/-}; *Trh*^{-/-}; *Avpr1a*^{-/-}; *Avpr1b*^{-/-}, etc.).

Pup exposure assays were performed on four consecutive days in virgin females, on the day of delivery in the postpartum mothers and 13 days after the separation of pups at PPD1 (post-isolation, Fig. 4A). In essence, QKO females showed grossly indistinguishable nurturing behaviors, including latencies for first sniffing, first and last pup retrieval, and durations of crouching over pups, licking, and nest building (Fig. 4B-G). The consistent changes of pup-directed behaviors between genotypes in Fig. 4 and Fig. 5 were the increased licking behavior in *Avpr1a* KO (thus also QKO) mothers at PPD0. Anecdotally, we noted a mild tendency of polyurea by the soiled bedding of *Avpr1a* KO mice, even though normal basal urine output was reported for *Avpr1a* KO in a previous report in a genetically-mixed background (Koshimizu et al., 2006). The possible craving for water in *Avpr1a* KO and QKO postpartum mothers could have caused increased anogenital licking, and compensatory decrease of the crouching behavior. Other differences among genotypes were sporadic and not consistent (Fig. 5), suggesting the scarcity of robust deficits in maternal or allomaternal care of the QKO mutant females.

Pup-directed behaviors of *Oxtr-Avpr1a-Avpr1b* triple knockout (TKO) and *Oxt-Trh-Avpr1a-Avpr1b* quadruple KO (QKO) female mice

When the *Oxtr* KO mouse line (Takayanagi et al., 2005) became available to us, we created *Oxtr-Avpr1a-Avpr1b* triple knockout (TKO) mice, after crossing each line into C57BL/6 genetic background at least four times, aiming at complete abolition of the Oxt-Avp system in the brain. First, we have examined if the lack of *Oxtr* or *Avpr* genes affected the expression of Oxt or Avp though compensatory mechanisms, as suggested previously (Vaidyanathan and Hammock, 2020). In our experimental conditions, no significant differences of the expression levels of Oxt and Avp between *Oxtr* KO and heterozygous females were detected in the AC, SO, or PVH (Fig. 6).

As in *Oxt* KO mothers, complete lack of milk transfer to the pups (Takayanagi et al., 2005) and the mild labor delay in *Oxtr* KO mothers were also observed throughout this study. Again, we examined allomaternal and postpartum maternal behaviors in TKO females, excluding the mothers with a severely disturbed labor.

Similar to the results from QKO, we did not find any robust defects in maternal and allomaternal care in TKO females. The only consistent statistical significance (Fig. 4) was found in the increased licking duration in *Avpr1a* KO and TKO PPD0 females. Nipple elongation, indicating suckling, was found in all nine *Oxtr* KO mothers examined on PPD0, and no clear milk band was observed in the pups' stomachs. In particular, pup-retrieval latencies, the reliably measurable index for parental motivation in mice (Kuroda et al., 2011), did not differ among genotypes for the first, second (data not shown) or the third pup (Fig. 4, 5) in either QKO, TKO or any single-locus KO females, indicating that there were no gross defects in pup retrieval in *Oxt*, *Oxtr*, *Avpr1a*, *Avpr1b*, *Trh* single KOs or in TKO or QKO females.

Pup-directed behaviors in TKO male mice

Next, we examined the pup-directed behaviors of male mice, focusing on TKO mutant lines. In virgin *Oxtr* KO males, a significant *increase* of parental behavior in virgin males and decrease of infanticide in fathers on PPD1 were observed (Fig. 7F). The frequency of infanticide is affected by various environmental stimuli and internal factors (Parmigiani and vom Saal, 1994), and it is possible that an anxiolytic effect of *Oxt* may cause this difference. Paternal behavior at two different timepoints, however, was not significantly affected by any of the single KOs or by the TKO (Fig. 7B-F), as seen in our female mice.

Confirmation of the results in another *Avpr1a* and *Avpr1b* mutant strains

The paucity of parental care-related phenotypes in the genetic mutants for the *Oxt*-*Avp* system so far identified was surprising. One concern about the genetic mutant line of *Avpr1a* used in the Figs. 3-7 was that it was not a null mutation, but an insertion mutation, and there was no obvious phenotype. This means that we have to rely solely on the PCR genotyping to segregate the mutant from the wild-type allele, unlike the *Oxt* and *Oxtr* mutants where lack of milk is 100% observable in mutant mothers. We therefore obtained another targeted-mutant line of *Avpr1a* which has been confirmed for the phenotype in the cardiovascular system (Egashira et al., 2004; Koshimizu et al., 2006), along with the *Avpr1b* mutant line created in the same laboratory (Tanoue et al., 2004), and bred for the new TKO mice (i.e., the same mutant line for *Oxtr*, and independent lines for *Avpr1a*

and *Ib* from Fig. 5 and 7), termed HIR.

Examination of virgin female, postpartum, virgin male and paternal HIR TKO mice (Fig. 8) did not show any significant effects of any genotype on any pup-directed behaviors, except for the increased pup licking in one of three timepoints each in virgin and postpartum females, and decreased nest building behavior in one and two of the three timepoints each in virgin and postpartum females in the standard pup-exposure assay (Fig. 8A, B).

Parental care of *Oxtr* KO is susceptible for physical stress

So far, our experiments did not elucidate the significant role of Oxt-Avp system in parental care, even in combination with mutations of 8 related genetic loci, indicating that the genetic redundancy among these genes was unlikely the cause of unsuccessful detection of phenotypes in pup-directed behaviors. However, it was still possible that the role of Oxt-Avp system in parental nurturing was not readily detectable in the minimal-stress laboratory environment. Actually, parental nurturing is always in trade-off with other drives, such as hunger and self-security (Li et al., 2019; Yoshihara et al., 2021), but these competing needs do not occur in laboratory environments. Therefore, we next examined the parental care in more stressful conditions, and found a significant decrease of nurturing behaviors if the virgin females were tested right after 30 min of restraint stress (Fig. 9, Extended data Fig. 9-1). Three of the *Oxtr* KO virgin females exhibited infanticide toward donor pups after the restraint stress (Fig. 9A), although this effect did not reach statistical significance. The *Oxtr* KO virgin females also showed decreased pup retrieval, and thus the total nesting time summed for all the pups was reduced (Fig. 9D, E) in the pup-exposure assay performed right after restraint stress. On the other hand, this stress vulnerability caused by *Oxtr* KO was abolished by additional *Avpr1b* KO (Fig. 9E). Rather opposite effects of *Oxtr* and *Avpr1b* were found on nurturing behavior under stress. Such distinct roles of *Oxtr* and *Avpr1b* has been demonstrated in fear control in the central amygdala (Huber et al., 2005) and with the Bruce effect (Wersinger et al., 2008), suggesting the importance of fine balance of neurohypophysial hormones on behavioral outcome in these complex contexts.

***Oxtr* KO females exhibit normal stress reactivity in non-maternal context**

To determine whether the effect of *Oxtr* KO in disturbing alloparental care is secondary to the well-known general anxiolytic effect of Oxt or not, the general stress sensitivities of the mutants were examined. Plasma cortisol levels after restraint stress, or the behavioral performances in the open field and elevated plus maze were not altered in *Oxtr* KO females (Fig. 10A-C). On the other

594 hand, there was a significant increase of general anxiety in *Avpr1b* KO females, as evidenced
595 consistently by the three measurements (Fig. 10A-C). This anxiogenic effect of *Avpr1b* was limited
596 to females and not found in males, except for one measurement on the elevated plus maze (Fig.
597 10D-F). These results suggest that while the mild increase of alloparental nurturing after restraint
598 stress in *Avpr1b* KO may be secondary to the general stress resistance, the decreased alloparenting
599 after stress in *Oxtr* KO is rather specific to a pup-nurturing context.

600

601

602 Discussion

603 This study has shown that the genetic mutation in *Oxtr* causes deficits in alloparental
 604 nurturing specifically under a stressful condition. This finding is in harmony with the previous
 605 literature, which unequivocally demonstrated anti-stress and anxiolytic effects of Oxt in various
 606 conditions, including in a semi-natural environment (Ragnauth et al., 2005; Brunton and Russell,
 607 2008; Neumann, 2008; Yoshida et al., 2009; Viviani et al., 2011; Knobloch et al., 2012; Sabihi et al.,
 608 2014; Menon et al., 2018). Environmental stress and risk factors should be inevitable in the feral life,
 609 thus this role of Oxt should be important for maintaining motivation to nurture. The differences
 610 between our findings with *Oxtr* KO mice and those of Takayanagi et al. (2005) may have been due to
 611 the different stress levels involved in pup exposure assays (see (Yoshihara et al., 2017) for details).

612 We also observed the modest deficits in parturition by either the *Oxt* or *Oxtr* mutation, a
 613 phenotype that was not observed in the original KO studies (Nishimori et al., 1996; Young et al.,
 614 1996; Gross et al., 1998; Takayanagi et al., 2005). However, this phenotype was observed partly in
 615 later studies (Roizen et al., 2007; Yoshida et al., 2019), possibly because of the difference in genetic
 616 backgrounds used. On the other hand, the complete lack of milk ejection despite vigorous suckling
 617 by starving pups was confirmed as originally reported (Nishimori et al., 1996; Young et al., 1996;
 618 Gross et al., 1998; Takayanagi et al., 2005).

619 Except for these phenotypes, however, this study has revealed the relative paucity of
 620 effects of *Oxt-Avp* genetic targeting on pup-directed behaviors in laboratory mice. In particular, we
 621 have found almost no robust facilitatory role of Oxt on maternal or allomaternal nurturing behaviors
 622 under our standard laboratory condition. And the TKO and QKO phenotypes indicate that the normal
 623 parental care is not due to the compensation for the congenital lack of Oxt or *Oxtr* by *Avp* or *Trh*
 624 molecular signaling. Of course, the compensation may occur via non-*Avpr1a*, non-*Avpr1b*, non-*Trh*
 625 mechanism. In contrast to the inability to compensate for the deficit in milk ejection due to the lack
 626 of *Oxt* or *Oxtr*, these data provide no evidence for *Oxt-Avp-Trh*'s role in parental care, at least after
 627 chronic loss. Whether acute loss of one, two or all three neuropeptide signalling systems would affect
 628 parental care can be tested by acute molecular knockdown through AAV-mediated RNA interference,
 629 for example.

630 This study has several limitations other than the use of unnaturally, relatively stress-free
 631 laboratory conditions. For example, unlike laboratory mouse strains, virgin female wild mice are
 632 often not spontaneously maternal, and even unresponsive or infanticidal toward unfamiliar pups
 633 (McCarthy and vom Saal, 1985; Soroker and Terkel, 1988; Chalfin et al., 2014). Moreover,
 634 domesticated mice show differences in Oxt and *Avp* expression in brain regions known to regulate

635 social behavior and emotion (Ruan and Zhang, 2016). Therefore, inbreeding and domestication may
 636 have rendered lab mice even less dependent upon the facilitatory effects of Oxt for the initiation of
 637 maternal behavior in inexperienced (first-time) mothers under standard laboratory conditions.

638 Second, the studied functions of Oxt neurons may be mediated by co-expressing
 639 neurotransmitters or neuropeptides, such as glutamate (Xu et al., 2020), corticotropin-releasing factor,
 640 cholecystokinin and dynorphin (Levin and Sawchenko, 1993). If this is the case, while Oxt *molecules*
 641 are not important, Oxt *neurons* may be important, and the function of Oxt neurons on pup-directed
 642 behaviors should be detected by manipulation of Oxt expressing neuronal activity by using
 643 AAV-mediated tetanus toxin or Gi-DREADD, for example, as performed in previous work (Tang et
 644 al., 2020).

645 Lastly, Oxt function may be more visible in nursing behavior and/or in the later lactation
 646 period. We observed nipple elongation, evidence for nursing on the day of delivery, consistent with
 647 the previous finding with a forebrain-specific *Oxtr* KO (Macbeth et al., 2010). However we did not
 648 formally exclude other possible problems in nursing-related behaviors. In particular, the Oxt neurons
 649 could be involved in the phenomenon called "Pavlovian milk conditioning". It has been known
 650 anecdotally that experienced lactating human and dairy animal mothers let down milk with
 651 conditional stimuli, such as a baby cry or smell, and bells signaling milking time in a dairy farm, all
 652 prior to physical suckling (Grosvenor and Mena, 1972; McNeilly et al., 1983; Fuchs et al., 1987;
 653 Tancin et al., 2001; Domjan, 2005). Once this conditioning is formed, the mothers may have milk
 654 letdown just by approaching and receiving infant sensory cues, or in human cases, just to think about
 655 the infants, prior to actual suckling by the infants, thus facilitating milk transfer to the infants. We
 656 recently identified a key player in maternal care, calcitonin receptor (Calcr)-expressing neurons in
 657 the cMPOA and the AC (Yoshihara et al., 2021). Calcr-expressing neurons are significantly activated
 658 during and required for maternal and allomaternal care. The close spatial relationship of Oxt neurons
 659 and Calcr neurons in the AC suggests that simultaneous firing of these neurons should occur
 660 repeatedly in the AC during maternal care, and may form the synaptic connections between these
 661 neuronal populations, which may be responsible for the Pavlovian milk conditioning. If this is the
 662 case, it may best explain why there is a striking co-distribution of the abundant activated
 663 non-oxytocinergic neurons among Oxt neurons in the AC and Oxt-fibers in the MPOA.

664 Even positive reports for Oxt's facilitation of parental care show relatively mild effects on
 665 specific components of parental care (Pedersen et al., 1994; Takayanagi et al., 2005; Marlin et al.,
 666 2015; Carcea et al., 2021), rather than abolishing many aspects of complex parental behaviors.
 667 Further investigations are needed to examine these possibilities and determine the exact role of Oxt

668 system in pup-directed behaviors. However, at least the present findings provide a caution for the
669 pervasive, yet not-well substantiated, claim of an indispensable role of Oxt in all kinds of parental
670 care.
671

References

- Acampora D, Postiglione MP, Avantaggiato V, Di Bonito M, Vaccarino FM, Michaud J, Simeone A (1999) Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the *Orthopedia* gene. *Genes Dev* 13:2787-2800.
- Armstrong WE, Warach S, Hatton GI, McNeill TH (1980) Subnuclei in the rat hypothalamic paraventricular nucleus: a cytoarchitectural, horseradish peroxidase and immunocytochemical analysis. *Neuroscience* 5:1931-1958.
- Bayer SA, Altman J (2004) Development of the telencephalon: neural stem cells, neurogenesis, and neuronal migration. In: *The rat nervous system*, 3 Edition (Paxinos G, ed), pp 27-73. San Diego: Elsevier.
- Bosch OJ, Neumann ID (2008) Brain vasopressin is an important regulator of maternal behavior independent of dams' trait anxiety. *Proc Natl Acad Sci U S A* 105:17139-17144.
- Brunton PJ, Russell JA (2008) Keeping oxytocin neurons under control during stress in pregnancy. *Prog Brain Res* 170:365-377.
- Caldwell HK, Young WS (2006) Oxytocin and Vasopressin: Genetics and Behavioral Implications. In: *Handbook of neurochemistry and molecular neurobiology* (Abel L, Lim R, eds), pp 573-607. Berlin: Springer-Verlag.
- Carcea I et al. (2021) Oxytocin neurons enable social transmission of maternal behaviour. *Nature*.
- Castel M, Morris JF (1988) The neurophysin-containing innervation of the forebrain of the mouse. *Neuroscience* 24:937-966.
- Chalfin L, Dayan M, Levy DR, Austad SN, Miller RA, Iraqi FA, Dulac C, Kimchi T (2014) Mapping ecologically relevant social behaviours by gene knockout in wild mice. *Nat Commun* 5:4569.
- Deltagen I (2005) NIH initiative supporting placement of Deltagen, Inc. mice into public repositories. MGI Direct Data Submission. In.
- Domjan M (2005) Pavlovian conditioning: a functional perspective. *Annu Rev Psychol* 56:179-206.
- Egashira N, Tanoue A, Higashihara F, Mishima K, Fukue Y, Takano Y, Tsujimoto G, Iwasaki K, Fujiwara M (2004) V1a receptor knockout mice exhibit impairment of spatial memory in an eight-arm radial maze. *Neurosci Lett* 356:195-198.
- Franklin KBJ, Paxinos G (2007) *The mouse brain in stereotaxic coordinates*, 3rd Edition. San Diego: Academic Press.
- Fuchs AR, Ayromloo J, Rasmussen AB (1987) Oxytocin response to conditioned and nonconditioned stimuli in lactating ewes. *Biol Reprod* 37:301-305.
- Grinevich V, Akmayev I (1997) An accessory magnocellular nucleus, anterior commissural nucleus, in the rat hypothalamus: immunohistochemical, tract-tracing, in situ hybridization, and experimental studies. *Biogenic Amines* 13:333-348.
- Grinevich V, Neumann ID (2020) Brain oxytocin: how puzzle stones from animal studies translate into psychiatry. *Mol Psychiatr*.

- 710 Gross GA, Imamura T, Luedke C, Vogt SK, Olson LM, Nelson DM, Sadovsky Y, Muglia LJ (1998)
- 711 Opposing actions of prostaglandins and oxytocin determine the onset of murine labor. *Proc Natl*
- 712 *Acad Sci U S A* 95:11875-11879.
- 713 Grosvenor CE, Mena F (1972) Evidence that the litter exerts an inhibitory influence on milk secretion in
- 714 the rat during late lactatio. *Fed Proc (Fed Am Stud Exp Biol)* 31:275.
- 715 Herdegen T, Leah JD (1998) Inducible and constitutive transcription factors in the mammalian nervous
- 716 system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. *Brain Res*
- 717 *Brain Res Rev* 28:370-490.
- 718 Huber D, Veinante P, Stoop R (2005) Vasopressin and oxytocin excite distinct neuronal populations in the
- 719 central amygdala. *Science* 308:245-248.
- 720 Hurlemann R, Grinevich V (2017) Behavioral pharmacology of neuropeptides : oxytocin, 1st edition.
- 721 Edition. New York, NY: Springer Berlin Heidelberg.
- 722 Insel TR (2010) The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and
- 723 affiliative behavior. *Neuron* 65:768-779.
- 724 Jurek B, Neumann ID (2018) The Oxytocin Receptor: From Intracellular Signaling to Behavior. *Physiol*
- 725 *Rev* 98:1805-1908.
- 726 Knobloch HS, Charlet A, Hoffmann LC, Eliava M, Khrulev S, Cetin AH, Osten P, Schwarz MK, Seeburg
- 727 PH, Stoop R, Grinevich V (2012) Evoked axonal oxytocin release in the central amygdala
- 728 attenuates fear response. *Neuron* 73:553-566.
- 729 Koshimizu TA, Nakamura K, Egashira N, Hiroyama M, Nonoguchi H, Tanoue A (2012) Vasopressin V1a
- 730 and V1b receptors: from molecules to physiological systems. *Physiol Rev* 92:1813-1864.
- 731 Koshimizu Ta, Nasa Y, Tanoue A, Oikawa R, Kawahara Y, Kiyono Y, Adachi T, Tanaka T, Kuwaki T, Mori
- 732 T, Takeo S, Okamura H, Tsujimoto G (2006) V1a vasopressin receptors maintain normal blood
- 733 pressure by regulating circulating blood volume and baroreflex sensitivity. *Proceedings of the*
- 734 *National Academy of Sciences* 103:7807-7812.
- 735 Kuroda KO, Meaney MJ, Uetani N, Kato T (2008) Neurobehavioral basis of the impaired nurturing in
- 736 mice lacking the immediate early gene FosB. *Brain Res* 1211:57-71.
- 737 Kuroda KO, Tachikawa K, Yoshida S, Tsuneoka Y, Numan M (2011) Neuromolecular basis of parental
- 738 behavior in laboratory mice and rats: with special emphasis on technical issues of using mouse
- 739 genetics. *Prog Neuropsychopharmacol Biol Psychiatry* 35:1205-1231.
- 740 Kuroda KO, Meaney MJ, Uetani N, Fortin Y, Ponton A, Kato T (2007) ERK-FosB signaling in dorsal
- 741 MPOA neurons plays a major role in the initiation of parental behavior in mice. *Mol Cel Neurosci*
- 742 36:121-131.
- 743 Levin MC, Sawchenko PE (1993) Neuropeptide co-expression in the magnocellular neurosecretory system
- 744 of the female rat: Evidence for differential modulation by estrogen. *Neuroscience* 54:1001-1018.
- 745 Li XY, Han Y, Zhang W, Wang SR, Wei YC, Li SS, Lin JK, Yan JJ, Chen AX, Zhang X, Zhao ZD, Shen WL,
- 746 Xu XH (2019) AGRP Neurons Project to the Medial Preoptic Area and Modulate Maternal
- 747 Nest-Building. *J Neurosci* 39:456-471.

- Macbeth AH, Stepp JE, Lee HJ, Young WS, 3rd, Caldwell HK (2010) Normal maternal behavior, but increased pup mortality, in conditional oxytocin receptor knockout females. *Behav Neurosci* 124:677-685.
- Manning M, Misicka A, Olma A, Bankowski K, Stoev S, Chini B, Durroux T, Mouillac B, Corbani M, Guillon G (2012) Oxytocin and vasopressin agonists and antagonists as research tools and potential therapeutics. *J Neuroendocrinol* 24:609-628.
- Marlin BJ, Mitre M, D'Amour J A, Chao MV, Froemke RC (2015) Oxytocin enables maternal behaviour by balancing cortical inhibition. *Nature* 520:499-504.
- McCarthy MM (1995) Estrogen modulation of oxytocin and its relation to behavior. *Adv Exp Med Biol* 395:235-245.
- McCarthy MM, vom Saal FS (1985) The influence of reproductive state on infanticide by wild female house mice (*Mus musculus*). *Physiol Behav* 35:843-849.
- McCarthy MM, Kow LM, Pfaff DW (1992) Speculations concerning the physiological significance of central oxytocin in maternal behavior. *Ann N Y Acad Sci* 652:70-82.
- McNeilly AS, Robinson IC, Houston MJ, Howie PW (1983) Release of oxytocin and prolactin in response to suckling. *Br Med J (Clin Res Ed)* 286:257-259.
- Menon R, Grund T, Zoicas I, Althammer F, Fiedler D, Biermeier V, Bosch OJ, Hiraoka Y, Nishimori K, Eliava M, Grinevich V, Neumann ID (2018) Oxytocin Signaling in the Lateral Septum Prevents Social Fear during Lactation. *Curr Biol* 28:1066-1078 e1066.
- Neumann ID (2008) Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. *J Neuroendocrinol* 20:858-865.
- Neumann ID, Landgraf R (2012) Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors. *Trends Neurosci* 35:649-659.
- Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, Matzuk MM (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. *Proc Natl Acad Sci U S A* 93:11699-11704.
- Numan M (1974) Medial preoptic area and maternal behavior in the female rat. *J Comp Physiol Psychol* 87:746-759.
- Numan M (2020) *The Parental Brain: Mechanisms, Development, and Evolution*: Oxford University Press.
- Numan M, Numan MJ (1994) Expression of Fos-like immunoreactivity in the preoptic area of maternally behaving virgin and postpartum rats. *Behav Neurosci* 108:379-394.
- Okabe S, Tsuneoka Y, Takahashi A, Ooyama R, Watarai A, Maeda S, Honda Y, Nagasawa M, Mogi K, Nishimori K, Kuroda M, Koide T, Kikusui T (2017) Pup exposure facilitates retrieving behavior via the oxytocin neural system in female mice. *Psychoneuroendocrinology* 79:20-30.
- Onaka T, Takayanagi Y, Yoshida M (2012) Roles of oxytocin neurones in the control of stress, energy metabolism, and social behaviour. *J Neuroendocrinol* 24:587-598.
- Parmigiani S, vom Saal FS, eds (1994) *Infanticide and parental care*: Harwood academic publishers.
- Pedersen CA, Ascher JA, Monroe YL, Prange AJ (1982) Oxytocin Induces Maternal-Behavior in Virgin

- Female Rats. *Science* 216:648-650.
- Pedersen CA, Caldwell JD, Walker C, Ayers G, Mason GA (1994) Oxytocin activates the postpartum onset of rat maternal behavior in the ventral tegmental and medial preoptic areas. *Behav Neurosci* 108:1163-1171.
- Peterson RP (1966) Magnocellular neurosecretory centers in the rat hypothalamus. *J Comp Neurol* 128:181-190.
- Pohlert, T. (2014). The Pairwise Multiple Comparison of Mean Ranks Package (*PMCMR*). <https://CRAN.R-project.org/package=PMCMR>
- R Development Core Team (2018) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.
- Ragnauth AK, Devidze N, Moy V, Finley K, Goodwillie A, Kow LM, Muglia LJ, Pfaff DW (2005) Female oxytocin gene-knockout mice, in a semi-natural environment, display exaggerated aggressive behavior. *Genes Brain Behav* 4:229-239.
- Rasband WS (1997-2018) ImageJ. In: U. S. National Institutes of Health, Bethesda, Maryland, USA.
- Rhodes CH, Morrell JI, Pfaff DW (1981) Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin. *J Comp Neurol* 198:45-64.
- Robinson AG, Verbalis JG (2003) Posterior pituitary gland. In: Williams textbook of endocrinology, 10th Edition (Williams RH, Larsen PR, eds), pp 281-329. Philadelphia, Pa.: Saunders.
- Roizen J, Luedke CE, Herzog ED, Muglia LJ (2007) Oxytocin in the Circadian Timing of Birth. *PLoS ONE* 2.
- Ruan C, Zhang Z (2016) Laboratory domestication changed the expression patterns of oxytocin and vasopressin in brains of rats and mice. *Anatomical Science International* 91:358-370.
- Sabihi S, Durosko NE, Dong SM, Leuner B (2014) Oxytocin in the prelimbic medial prefrontal cortex reduces anxiety-like behavior in female and male rats. *Psychoneuroendocrinology* 45:31-42.
- Sheehan TP, Cirrito J, Numan MJ, Numan M (2000) Using c-Fos immunocytochemistry to identify forebrain regions that may inhibit maternal behavior in rats. *Behav Neurosci* 114:337-352.
- Simerly RB, Gorski RA, Swanson LW (1986) Neurotransmitter specificity of cells and fibers in the medial preoptic nucleus: an immunohistochemical study in the rat. *J Comp Neurol* 246:343-363.
- Sofroniew MV (1985) Vasopressin- and neurophysin-immunoreactive neurons in the septal region, medial amygdala and locus coeruleus in colchicine-treated rats. *Neuroscience* 15:347-358.
- Soroker V, Terkel J (1988) Changes in incidence of infanticidal and parental responses during the reproductive cycle in male and female wild mice *Mus musculus*. *Anim Behav* 36:1275-1281.
- Tachikawa KS, Yoshihara Y, Kuroda KO (2013) Behavioral transition from attack to parenting in male mice: a crucial role of the vomeronasal system. *J Neurosci* 33:5120-5126.
- Takayanagi Y, Yoshida M, Bielsky IF, Ross HE, Kawamata M, Onaka T, Yanagisawa T, Kimura T, Matzuk MM, Young LJ, Nishimori K (2005) Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proc Natl Acad Sci U S A* 102:16096-16101.

- 824 Tancin V, Kraetzel W-D, Schams D, Bruckmaier RM (2001) The effects of conditioning to suckling, milking
825 and of calf presence on the release of oxytocin in dairy cows. *Applied Animal Behaviour Science*
826 72:235-246.
- 827 Tang Y et al. (2020) Social touch promotes interfemale communication via activation of parvocellular
828 oxytocin neurons. *Nat Neurosci* 23:1125-1137.
- 829 Tanoue A, Ito S, Honda K, Oshikawa S, Kitagawa Y, Koshimizu TA, Mori T, Tsujimoto G (2004) The
830 vasopressin V1b receptor critically regulates hypothalamic-pituitary-adrenal axis activity under
831 both stress and resting conditions. *J Clin Invest* 113:302-309.
- 832 Tsuneoka Y, Maruyama T, Yoshida S, Nishimori K, Kato T, Numan M, Kuroda KO (2013) Functional,
833 anatomical, and neurochemical differentiation of medial preoptic area subregions in relation to
834 maternal behavior in the mouse. *J Comp Neurol* 521:1633-1663.
- 835 Tsuneoka Y, Tokita K, Yoshihara C, Amano T, Esposito G, Huang AJ, Yu LM, Odaka Y, Shinozuka K,
836 McHugh TJ, Kuroda KO (2015) Distinct preoptic-BST nuclei dissociate paternal and infanticidal
837 behavior in mice. *EMBO J* 34:2652-2670.
- 838 Vaidyanathan R, Hammock EAD (2020) Oxytocin receptor gene loss influences expression of the oxytocin
839 gene in C57BL/6J mice in a sex- and age-dependent manner. *J Neuroendocrinol* 32:e12821.
- 840 Viviani D, Charlet A, van den Burg E, Robinet C, Hurni N, Abatis M, Magara F, Stoop R (2011) Oxytocin
841 selectively gates fear responses through distinct outputs from the central amygdala. *Science*
842 333:104-107.
- 843 Wakerley JB (2005) Milk ejection and its control. In: Knobil and Neill's Physiology of reproduction, 3
844 Edition (Neill JD, ed), pp 3129-3190: Elsevier.
- 845 Watson C, Paxinos G, Puelles E (2011) The Mouse Nervous System: Academic press.
- 846 Wersinger SR, Temple JL, Caldwell HK, Young WS, 3rd (2008) Inactivation of the oxytocin and the
847 vasopressin (Avp) 1b receptor genes, but not the Avp 1a receptor gene, differentially impairs the
848 Bruce effect in laboratory mice (*Mus musculus*). *Endocrinology* 149:116-121.
- 849 Wersinger SR, Ginns EI, O'Carroll AM, Lolait SJ, Young WS, 3rd (2002) Vasopressin V1b receptor
850 knockout reduces aggressive behavior in male mice. *Mol Psychiatry* 7:975-984.
- 851 Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.
- 852 Xu S, Yang H, Menon V, Lemire AL, Wang L, Henry FE, Turaga SC, Sternson SM (2020) Behavioral state
853 coding by molecularly defined paraventricular hypothalamic cell type ensembles. *Science* 370.
- 854 Yamada M, Saga Y, Shibusawa N, Hirato J, Murakami M, Iwasaki T, Hashimoto K, Satoh T, Wakabayashi
855 K, Taketo MM, Mori M (1997) Tertiary hypothyroidism and hyperglycemia in mice with targeted
856 disruption of the thyrotropin-releasing hormone gene. *Proc Natl Acad Sci U S A* 94:10862-10867.
- 857 Yoshida M, Takayanagi Y, Inoue K, Kimura T, Young LJ, Onaka T, Nishimori K (2009) Evidence that
858 oxytocin exerts anxiolytic effects via oxytocin receptor expressed in serotonergic neurons in mice.
859 *J Neurosci* 29:2259-2271.
- 860 Yoshida M, Takayanagi Y, Ichino-Yamashita A, Sato K, Sugimoto Y, Kimura T, Nishimori K (2019)
861 Functional Hierarchy of Uterotonics Required for Successful Parturition in Mice. *Endocrinology*

160:2800-2810.

Yoshihara C, Numan M, Kuroda KO (2017) Oxytocin and Parental Behaviors. In: Behavioral pharmacology of neuropeptides : oxytocin (Hurlemann R, Grinevich V, eds), pp 119-153: Curr Top Behav Neurosci.

Yoshihara C, Tokita K, Maruyama T, Kaneko M, Tsuneoka Y, Fukumitsu K, Miyazawa E, Shinozuka K, Huang AJ, Nishimori K, McHugh TJ, Tanaka M, Itohara S, Touhara K, Miyamichi K, Kuroda KO (2021) Calcitonin receptor signaling in the medial preoptic area enables risk-taking maternal care. Cell Rep 35:109204.

Young WS, 3rd, Shepard E, Amico J, Hennighausen L, Wagner KU, LaMarca ME, McKinney C, Ginns EI (1996) Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. J Neuroendocrinol 8:847-853.

875 **Figure/Table/Extended Data Legends**

876

877 **Figure 1. Sagittal view of anatomical distribution of oxytocin neurons, fibers and**
878 **parenting-induced c-Fos expression**

879 Distribution of neurophysin I (NPI) (brown)-ir and c-Fos (black)-ir cell in and around of MPOA of
880 virgin females after pup exposure (parasagittal section).

881 **A:** Left and right panels show representative photographs and their diagrammatic drawings,
882 respectively. These sections were stained by IHC. Black squares and plus (+) symbols respectively
883 represent strongly and weakly expressed c-Fos-ir neurons without NPI-ir. Filled and open red circles
884 respectively represent strongly and weakly NPI-ir cell bodies without c-Fos signals. Green squares
885 respectively represent NPI-ir cell bodies with c-Fos-ir. Red lines represent NPI-ir fibers. Numbers
886 1-4 show the areas relatively devoid of c-Fos-ir, NPI-ir neurons and fibers. Arrows indicate Oxt-ir
887 thick dendrites with a corkscrew-like morphology and irregular varicosities. Panels are arranged in
888 the medial-lateral order, from the left top to left bottom, and the right top to right bottom.

889 **B:** The high-magnification image of the blue squared region in (A), containing the AC. The
890 circle-headed arrow indicate double-labeled cells of NPI and c-Fos. White arrowheads indicate
891 single-labeled cells of c-Fos, black arrowheads indicate single-labeled cells of NPI, and small arrows
892 indicate NPI-ir fibers.

893

894

895 **Figure 2. Horizontal view of anatomical distribution of oxytocin neurons, fibers and**
896 **parenting-induced c-Fos expression**

897 Distribution of NPI (brown)-ir and c-Fos (black)-ir cell in and around of MPOA of virgin females
898 after pup exposure (horizontal section). Left and right panels show representative photographs and
899 their diagrammatic drawings, respectively. These sections were stained by IHC. Black squares and
900 plus symbols respectively represent strongly and weakly expressed c-Fos-ir neurons. Filled and open
901 red circles respectively represent NPI-ir cell bodies with or without c-Fos signals. Red lines represent
902 NPI-ir fibers. Numbers 1-4 show the areas relatively devoid of c-Fos-ir, NPI-ir neurons and fibers.
903 All panels are arranged in Ventral-Dorsal order from the left, top to bottom, and then the right, top to
904 bottom.

905

906

907 **Figure 3. Parental behavior, fertility, and abnormal delivery of the *Trh-Oxt* double KO (DKO)**

908 **mice.**

909 **A:** Responses to pup exposure in virgin females.

910 **B:** Fertility ratio of the females after mating.

911 **C:** Proportion of abnormal deliveries (prolonged for more than 24 hours, and/or maternal distress
912 with remaining pups in uterus, which sometimes caused maternal death in labor). "Abnormal (died)"
913 means maternal death by PPD3, and "Abnormal (euthanized)" means maternal health deterioration
914 necessitating euthanasia by PPD3.

915 **D:** The scores for nest quality, indicated by the use of nest material and nest shape (see Methods) at
916 PPD 0.

917 **E:** The placentophagia score (1, all the live pups were cleaned for amniotic membrane, umbilical
918 cord, and placenta; 0.5, partially; 0, none of the pups were cleaned) at PPD 0.

919 **F:** The spontaneous pup grouping score (1, all the live pups were grouped in the nest; 0.5, the live
920 pups were in the nest except one; 0, all the live pups were outside of the nest.) at PPD 0.

921 **G:** The pup survival rate (number of live pups at PPD 1 divided by the number of live pups at PPD 0
922 morning) of each mother.

923 **H:** Schematic of the experimental timeline and responses to pup exposure in mothers. Only four of
924 them (*Oxt*^{+/+}-*Trh*^{+/+}: 1, *Oxt*^{-/-}-*Trh*^{+/+}: 2, *Oxt*^{-/-}-*Trh*^{-/-}: 1) had experienced the pup-exposure assay as a
925 virgin, whose results are included in Fig. 3A.

926 **I:** Responses to pup exposure in virgin males.

927 **J:** Responses to pup exposure in males, which were separated from their female mates immediately
928 after copulation. Copulation was assessed by vaginal plugs in females. Only males whose mates were
929 later confirmed as pregnant are shown.

930 **K:** Fertility ratios of the males after mating (7 days of cohabitation with females).

931 **L:** Responses to pup exposure in males, which were separated from their female mates after 7 days
932 of cohabitation.

933 For **D-H**, data from only mothers who had normal delivery are shown. For **J-L**, data from only males
934 who exhibited infanticide as a virgin are shown.

935 (+/*) means wild-type (+/+) or heterozygous KO (+/-), and (-/-) means homozygous KO for the
936 designated genetic locus. A black vertical bar in schematic of the experimental timelines indicates the
937 day a pup-exposure assay was performed. Error bars represent the mean $\pm$ SEM. Different letters
938 denote significant differences (multiple comparisons by Fisher's exact test for categorical data, and
939 Welch's *t*-test for continuous data), $p < 0.05$. Numbers of animals used are described in the figure.

940

941

942

943 **Figure 4. Pup-directed behaviors of the virgin and postpartum *Oxt-Trh-Avpr1a-Avpr1b***
944 **quadruple KO (QKO) female mice in the standard pup-exposure assay.**

945 **A:** Schematic of the experimental timeline. “Post-isolation (PI)”: the mothers tested 13 days after the
946 isolation from pups at PPD1. A black vertical bar indicates the day a pup-exposure assay was
947 performed.

948 **B:** Gross pup-directed behaviors in each genotype, with the number of mice for each behavioral
949 category in each genotype.

950 **C-G:** Details of pup-directed behaviors compared between quadruple (-/-) vs. all other genotypes
951 (others) (C); heterozygous (+/-) versus homozygous KO (-/-) for *Avpr1b* (D), *Avpr1a* (E), *Trh* (F),
952 and *Oxt* (G), regardless of other genetic loci. The left three variables on the abscissae are latencies,
953 the right three variables are proportions of the time bins in which the indicated behaviors were
954 performed.

955 Error bars represent the mean $\pm$ SEM. Fisher’s exact test for categorical data; Welch’s *t*-test for
956 continuous data, $^{**}p < 0.01$, $^{*}p < 0.05$. Numbers of animals used are described in the figure.

957

958

959 **Figure 5. Pup-directed behaviors of the virgin and postpartum *Oxtr-Avpr1a-Avpr1b* triple KO**
960 **(TKO) female mice.**

961 **A:** Schematic of the experimental timeline. “Post-isolation (PI)”: the mothers tested 13 days after the
962 isolation from pups at PPD1. A black vertical bar indicates the day a pup-exposure assay was
963 performed.

964 **B:** Gross pup-directed behaviors in each genotype, with the number of mice for each behavioral
965 category in each genotype.

966 **C-G:** Details of pup-directed behaviors compared between triple (-/-) vs. all other genotypes
967 (others) (C); triple (-/-) vs *Oxtr*^{+/-}; *Avpr1a*^{+/-}; *Avpr1b*^{+/-} (triple (+/-)) (D), *Avpr1b* (E), *Avpr1a* (F), and
968 *Oxtr* (G), regardless of other genetic loci. The left three variables on the abscissae are latencies, the
969 right three variables are proportions of the time bins in which the indicated behaviors were
970 performed. Error bars represent the mean $\pm$ SEM. Fisher’s exact test for categorical data; Welch’s
971 *t*-test for continuous data, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. Numbers of animals used are
972 described in the figure.

973

974

Figure 6. Oxt (neurophysin I, NPI) and AVP expressions in the MPOA were not affected by *Oxtr* or *Avpr* genetic targeting.

Distribution of NPI and AVP (black)-ir cells in the MPOA of *Avpr1a-Oxtr-Avpr1b* triple mutant (TKO) female mice. triple (+/-) mean *Avpr1a*^{+/+}; *Oxtr*^{+/+}; *Avpr1b*^{+/+}, triple (-/-) mean *Avpr1a*^{-/-}; *Oxtr*^{-/-}; *Avpr1b*^{-/-} TKO virgin female mice. (A) The sections were stained by IHC. AC (B, E), SO (C, F) and PVH (D, G) was identified by counterstain using NeuN. All panels are arranged in anterior–posterior order. (B–G) High-magnification images of same-colored squares. (H–K) The numbers of NPI-ir neurons in the AC (H), SO (I) and PVH (J), and of Avp-ir neurons in the PVH (K) of triple (+/-) (N = 4) and (-/-) (N = 4) virgin females. Error bars represent the mean ± SEM.

Figure 7. Responses to pups in the standard pup-exposure assay with the virgin and copulated *Oxtr-Avpr1a-Avpr1b* triple KO (TKO) male mice.

A: Schematics of the experimental timeline. A black vertical bar indicates the day a pup-exposure assay was performed.

B–F: Gross pup-directed behaviors in each genotype, with the number of mice for each behavioral category in each genotype. *Oxtr*^{-/-}; *Avpr1a*^{-/-}; *Avpr1b*^{-/-} (triple (-/-)) vs all other genotypes (others) (B); triple (-/-) vs *Oxtr*^{+/+}; *Avpr1a*^{+/+}; *Avpr1b*^{+/+} (triple (+/+)) (C); heterozygous (+/-) vs. homozygous (-/-) KO for each four genetic loci for *Avpr1b* (D), *Avpr1a* (E), and *Oxtr* (F), regardless of other genetic loci.

Blue asterisk: Fisher’s exact test between parental (pups retrieved) vs. non-parental (infanticide or no pup retrieved); red asterisk: Fisher’s exact test between infanticidal vs. non-infanticidal; **p* < 0.05.

Figure 8. Confirmation of the results with HIR strain of *Oxtr-Avpr1a-Avpr1b* triple KO mice.

A, B: Responses to pups, schematics of the experimental timeline, latencies, and proportion of time of pup-directed behaviors in the standard pup-exposure assay in virgin (A) and postpartum (B) females. The left three variables are latencies, the right three variables are proportion of time bins performed the indicated behavior.

C, D: Responses to pups and schematic of the experimental timeline of pup-directed behaviors in the standard pup-exposure assay in virgin males (C) and father mice (D).

A black vertical bar in schematic of the experimental timelines indicates the day a pup-exposure assay was performed. Error bars represent the mean ± SEM. Fisher’s exact test for categorical data; Welch’s *t*-test for continuous data, ***p* < 0.01, **p* < 0.05. Numbers of animals used are described in

the figure.

Figure 9. Effect of restraint stress on the pup-directed behaviors of the virgin and postpartum *Oxtr-Avpr1a-Avpr1b* triple KO (TKO) female mice.

A: Gross pup-directed behaviors in each genotype, with the number of mice for each behavioral category in each genotype.

B-D: Details of pup-directed behaviors compared between heterozygous (+/-) versus homozygous KO (-/-) for *Avpr1b* (**B**), *Avpr1a* (**C**), and for *Oxtr* (**D**), disregarding other genetic loci. The left two variables are latencies, the right two variables are proportion of time bins performed the indicated behavior.

E: Total amount of pup retrieval behavior, measured by the total time the 3 pups spent in the nest. Different letters denote significant differences (multiple comparisons by Welch's *t*-test, $p < 0.05$). See also the Extended Data Figure 9-1 for the results of Kruskal-Wallis rank sum tests with post hoc multiple comparisons by Nemenyi test.

Error bars represent the mean $\pm$ SEM. Welch's *t*-test for continuous data; blue asterisk: Fisher's exact test between parental (pups retrieved) versus non-parental (infanticide or no pup retrieved), $*p < 0.05$.

Different letters denote significant differences (multiple comparisons by Welch's *t*-test for continuous data), $p < 0.05$. Numbers of animals used are described in the figure.

Figure 10. Stress sensitivity of the virgin *Oxtr-Avpr1a-Avpr1b* triple KO (TKO) female and male mice.

Behavioral characteristics in the open-field test (**A, D**) and elevated plus maize (**B, E**), and effect of restraint stress on plasma corticosterone level (**C, F**). (+/-) means heterozygous, (-/-) means homozygous, and * means (+/-) or (-/-) KO for the designated genetic locus.

Error bars represent the mean $\pm$ SEM. Welch's *t*-test, $*p < 0.05$. Numbers of animals used are described in the figure.

1038 **Table 1. Primers used for genotyping.**

1039

strain	gene loci	primer sequence (5' to 3')	Tm
B6;129S- <i>Oxt</i> ^{mi/Wsy} /J;	<i>Oxt</i>	gtgctggacctggatatgcgcaag	68
		agcgtcctttgcccggggccgaggggagacactgtggctgtgg	
		ctgctaagcgcatgctccagactgc	
<i>Trh</i> KO	<i>Trh</i>	tctcgctcgtgacccatggcgatg	64
		tctcgctcgtgacccatggcgatg	
		ttactcctccagaggttcctcgac	
B6.129P2-Avpr1a ^{miDgen} /J	<i>Avpr 1a</i>	cgcaacgaggagctggcgaagctgg	64
		gcggtaggtgatgtcccagcacagc	
		gggccagctcattcctcccactcat	
<i>Oxtr</i> KO	<i>Oxtr</i>	gttgggaacagcggtgatta	64
		ccttggaagcaggaggtgaag	
		gctgcgagtggtggtgacttc	
B6;129X1-Avpr1b ^{mi/Wsy} /J	<i>Avpr 1b</i>	acccttcccagcctctgagcccagaaagcgaagg	64
		gaaacggctactctctccgattccaaaagaag	
		acctgtagatatattgacagccgg	
B6;129Sv-V1a ^{mi/Gst} V1b ^{mi/Gst}	<i>Avpr 1a</i>	acaagtgttttgtaactagtactcta	60
		aggggcttctggtcacgccttgt	
		acatagcgttggtaccctgtgat	
	<i>Avpr 1b</i>	gaaacggctactctctcgattccaaaagaag	60
		gcgaattcgatatcaagcttatcga	
		acctgtagatatattgacagccgg	

1040

1041 **Table 2. Mouse genetic mutant lines and control genotypes used in each Figure.**
1042

Figure	Abbreviation	KO mice	Source	Genotype of control groups	Comment
Fig. 3	DKO	<i>Trh</i> KO mice	Yamada et al.	<i>Trh</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} or <i>-/-</i>	^{+/-} means ^{+/+} or ^{+/-}
		<i>Oxtr</i> KO mice	JAX (#2713)		
Fig. 4	QKO	<i>Avpr1a</i> KO mice	JAX (#5776)	(Fig. 4C) All other genotypes besides <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Trh</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
		<i>Avpr1b</i> KO mice	JAX (#6160)	(Fig. 4D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Trh</i> ^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-} or <i>-/-</i></i>	
		<i>Trh</i> KO mice	Yamada et al.	(Fig. 4E) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} or <i>-/-; <i>Trh</i>^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-} or <i>-/-</i></i></i>	
		<i>Oxtr</i> KO mice	JAX (#2713)	(Fig. 4F) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} or <i>-/-; <i>Trh</i>^{+/-}; <i>Oxtr</i>^{+/-} or <i>-/-</i></i>	
				(Fig. 4G) <i>Avpr1a</i> ^{+/-} or <i>-/-; <i>Avpr1b</i>^{+/-} or <i>-/-; <i>Trh</i>^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-}</i></i></i>	
Fig. 5	TKO	<i>Avpr1a</i> KO mice	JAX (#5776)	(Fig. 5C) All other genotypes besides <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
		<i>Avpr1b</i> KO mice	JAX (#6160)	(Fig. 5D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
		<i>Oxtr</i> KO mice	Takayanagi et al.	(Fig. 5E) <i>Avpr1a</i> ^{+/-} or <i>-/-; <i>Avpr1b</i>^{+/-}; <i>Oxtr</i>^{+/-} or <i>-/-</i></i>	
				(Fig. 5F) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-} or <i>-/-</i></i>	
				(Fig. 5G) <i>Avpr1a</i> ^{+/-} or <i>-/-; <i>Avpr1b</i>^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-}</i></i>	
Fig. 6	TKO	<i>Avpr1a</i> KO mice	JAX (#5776)	<i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
		<i>Avpr1b</i> KO mice	JAX (#6160)		
		<i>Oxtr</i> KO mice	Takayanagi et al.		
Fig. 7	TKO	<i>Avpr1a</i> KO mice	JAX (#5776)	(Fig. 7B) All other genotypes besides <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
		<i>Avpr1b</i> KO mice	JAX (#6160)	(Fig. 7C) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
		<i>Oxtr</i> KO mice	Takayanagi et al.	(Fig. 7D) <i>Avpr1a</i> ^{+/-} or <i>-/-; <i>Avpr1b</i>^{+/-}; <i>Oxtr</i>^{+/-} or <i>-/-</i></i>	
				(Fig. 7E) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-} or <i>-/-</i></i>	
				(Fig. 7F) <i>Avpr1a</i> ^{+/-} or <i>-/-; <i>Avpr1b</i>^{+/-} or <i>-/-; <i>Oxtr</i>^{+/-}</i></i>	

Fig. 8	HIR	<i>Avpr1a</i> and <i>1b</i> double KO mice <i>Oxtr</i> KO mice	Kumamoto university (#559) Takayanagi et al.	<i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	JAX (#5776) <i>Avpr1a</i> KO line was not a null mutation, Kumamoto (#599) <i>Avpr1a</i> KO line has been confirmed for the phenotype in cardiovascular system.
Fig. 9	TKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Oxtr</i> KO mice	JAX (#5776) JAX (#6160) Takayanagi et al.	(Fig. 9B) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 9C) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 9D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
Fig. 10	TKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Oxtr</i> KO mice	JAX (#5776) JAX (#6160) Takayanagi et al.	(Large panels) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Small left panels) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Small middle panels) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Small right panels) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	

1043
1044

Figure 9-1. Effects of restraint stress on the pup-directed behaviors of the virgin *Oxtr-Avpr1a-Avpr1b* (TKO) triple mutant female mice with non-parametric statistical analysis.

Parental behavior, measured by the total time the 3 pups spent in nest, of *Oxtr-Avpr1a-Avpr1b* triple mutant virgin female mice in standard condition (a) and after restraint stress (b).

(+/-) means heterozygous, (-/-) means homozygous, and * means (+/-) or (-/-) for the designated genetic locus. *HH means (*Avpr1a*^{*}, *Oxtr*^{+/-}, *Avpr1b*^{+/-}; gray with white stripe), *KH means (*Avpr1a*^{*}, *Oxtr*^{-/-}, *Avpr1b*^{+/-}; black with white stripe), *HK means (*Avpr1a*^{*}, *Oxtr*^{+/-}, *Avpr1b*^{-/-}; gray), and *KK means (*Avpr1a*^{*}, *Oxtr*^{-/-}, *Avpr1b*^{-/-}; black).

Results of non-parametric Kruskal-Wallis rank sum tests are shown above the graphs. # and ## denote significant differences between two groups in *post hoc* multiple comparisons (Nemenyi test; # $p < 0.05$, ## $p < 0.01$).

Nemenyi tests were performed by the R package *PMCMR* ver. 4.3 (Pohlert, 2014). The box plots were generated by *geom_boxplot* function with default settings in R package *ggplot2* ver. 3.1.0 (Wickham, 2016). The medians are represented by the horizontal bar in the middle of each box. The lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The upper whisker extends from the hinge to the largest value no further than 1.5 * IQR from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 * IQR of the hinge. Data beyond the end of the whiskers are called "outlying" points and are plotted individually (Wickham, 2016).

A Virgin female: triple (+/-) vs (-/-)

B Mother: triple (+/-) vs (-/-)

C Virgin male: triple (+/-) vs (-/-)

D Father: triple (+/-) vs (-/-)

A Pup-ex assay after restraint stress

A Female: Open Field Test

B Female: Elevated Plus Maze

C Female: plasma cortisol level

D Male: Open Field Test

E Male: Elevated Plus Maze

F Male: plasma cortisol level

Table 1. Primers used for genotyping.

strain	gene loci	primer sequence (5' to 3')	Tm
B6;129S- <i>Oxt</i> ^{tm1Wsy} /J;	<i>Oxt</i>	gtgctggacctggatatgcgcaag	68
		agcgtcctttgcccggggccgaggggagacactgtggctgtgg	
		ctgctaagcgcatgctccagactgc	
<i>Trh</i> KO	<i>Trh</i>	tctcgtcgtgacccatggcgatg	64
		tctcgtcgtgacccatggcgatg	
		ttactcctccagaggttcctgac	
B6.129P2- <i>Avpr1a</i> ^{tm1Dgen} /J	<i>Avpr 1a</i>	cgcaacgaggagctggcgaagctgg	64
		gcggtaggtgatgtcccagcacagc	
		gggccagctcattcctcccactcat	
<i>Oxtr</i> KO	<i>Oxtr</i>	gttgggaacagcggtgatta	64
		ccttggaagcaggaggtgaag	
		gctgcgcagtggtggtgacttc	
B6;129X1- <i>Avpr1b</i> ^{tm1Wsy} /J	<i>Avpr 1b</i>	accccttcccagcctctgagcccagaaagcgaagg	64
		gaaacggctactctctccgattccaaaagaaag	
		acctgtagatatttgacagcccg	
B6;129Sv-V1a ^{tm1G23} V1b ^{tm1G23}	<i>Avpr 1a</i>	acaagtgttttgtaactagtgtactcta	60
		aggggcttctgggtcacgccttgt	
		acatagcgttggtaccctgtgat	
	<i>Avpr 1b</i>	gaaacggctactctctcgattccaaaagaag	60
		gcgaattcgatatcaagcttatcga	
		acctgtagatatttgacagcccg	

Table 2. Mouse genetic mutant lines and control genotypes used in each Figure.

Figure	Abbreviation	KO mice	Source	Genotype of control groups	Comment
Fig. 3	DKO	<i>Trh</i> KO mice <i>Oxt</i> KO mice	Yamada et al. JAX (#2713)	<i>Trh</i> ^{+/-} ; <i>Oxt</i> ^{+/-}	^{+/-} means ^{+/+} or ^{-/-}
Fig. 4	QKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Trh</i> KO mice <i>Oxt</i> KO mice	JAX (#5776) JAX (#6160) Yamada et al. JAX (#2713)	(Fig. 4C) All other genotypes besides <i>Avpr1a</i> ^{-/-} ; <i>Avpr1b</i> ^{-/-} ; <i>Trh</i> ^{-/-} ; <i>Oxt</i> ^{-/-} (Fig. 4D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Trh</i> ^{+/-} ; <i>Oxt</i> ^{+/-} (Fig. 4E) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Trh</i> ^{+/-} ; <i>Oxt</i> ^{+/-} (Fig. 4F) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Trh</i> ^{+/-} ; <i>Oxt</i> ^{+/-} (Fig. 4G) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Trh</i> ^{+/-} ; <i>Oxt</i> ^{+/-}	
Fig. 5	TKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Oxtr</i> KO mice	JAX (#5776) JAX (#6160) Takayanagi et al.	(Fig. 5C) All other genotypes besides <i>Avpr1a</i> ^{-/-} ; <i>Avpr1b</i> ^{-/-} ; <i>Oxtr</i> ^{-/-} (Fig. 5D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 5E) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 5F) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 5G) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
Fig. 6	TKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Oxtr</i> KO mice	JAX (#5776) JAX (#6160) Takayanagi et al.	<i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
Fig. 7	TKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Oxtr</i> KO mice	JAX (#5776) JAX (#6160) Takayanagi et al.	(Fig. 7B) All other genotypes besides <i>Avpr1a</i> ^{-/-} ; <i>Avpr1b</i> ^{-/-} ; <i>Oxtr</i> ^{-/-} (Fig. 7C) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 7D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 7E) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 7F) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
Fig. 8	HIR	<i>Avpr1a</i> and <i>1b</i> double KO mice <i>Oxtr</i> KO mice	Kumamoto university (#559) Takayanagi et al.	<i>Avpr1a</i> ^{-/-} ; <i>Avpr1b</i> ^{-/-} ; <i>Oxtr</i> ^{+/-}	JAX (#5776) <i>Avpr1a</i> KO line was not a null mutation, Kumamoto (#599) <i>Avpr1a</i> KO line has been confirmed for the phenotype in cardiovascular system.
Fig. 9	TKO	<i>Avpr 1a</i> KO mice <i>Avpr 1b</i> KO mice <i>Oxtr</i> KO mice	JAX (#5776) JAX (#6160) Takayanagi et al.	(Fig. 9B) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 9C) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-} (Fig. 9D) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	
Fig. 10	TKO	<i>Avpr 1a</i> KO mice	JAX (#5776)	(Large panels) <i>Avpr1a</i> ^{+/-} ; <i>Avpr1b</i> ^{+/-} ; <i>Oxtr</i> ^{+/-}	

Avpr1b KO mice JAX (#6160)
Oxtr KO mice Takayanagi et al.

(Small left panels) *Avpr1a*^{+/-}; *Oxtr*^{-/-}; *Avpr1b*^{+/-}; *Oxtr*^{-/-}; *Oxtr*^{+/-}
 (Small middle panels) *Avpr1a*^{+/-}; *Avpr1b*^{+/-}; *Oxtr*^{+/-}; *Oxtr*^{-/-}
 (Small right panels) *Avpr1a*^{+/-}; *Avpr1b*^{+/-}; *Oxtr*^{+/-}; *Oxtr*^{-/-}