bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 # Male-specific vasotocin expression in the medaka tuberal
2 # hypothalamus: androgen dependence and probable role in
3 # aggression
4
5 Yukika Kawabata-Sakata [a, b], Shinji Kanda [c], Kataaki Okubo [a, *]
6
7 [a] Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University
8 of Tokyo, Bunkyo, Tokyo 113-8657, Japan
9 [b] Department of Pathophysiology, Tokyo Medical University, Shinjuku, Tokyo 160-8402, Japan
10 [c] Laboratory of Physiology, Atmosphere and Ocean Research Institute, The University of Tokyo,
11 Kashiwa, Chiba 277-8564, Japan
12
13 ***Corresponding author:** Kataaki Okubo; Department of Aquatic Bioscience, Graduate School of
14 Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan. Email: a-
15 okubo@g.ecc.u-tokyo.ac.jp.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

16 # Abstract
17
18 Terrestrial vertebrates have a population of androgen-dependent vasotocin (VT)-expressing neurons in
19 the extended amygdala that are more abundant in males and mediate male-typical social behaviors,
20 including aggression. Teleosts lack these neurons but instead have novel male-specific VT-expressing
21 neurons in the tuberal hypothalamus. Here we found in medaka that *vt* expression in these neurons is
22 dependent on post-pubertal gonadal androgens and that androgens can act on these neurons to directly
23 stimulate *vt* transcription via the androgen receptor subtype Ara. Furthermore, administration of
24 exogenous VT induced aggression in females and alterations in the androgen milieu led to correlated
25 changes in the levels of tuberal hypothalamic *vt* expression and aggression in both sexes. However,
26 genetic ablation of *vt* failed to prevent androgen-induced aggression in females. Collectively, our results
27 demonstrate a marked androgen dependence of male-specific *vt* expression in the teleost tuberal
28 hypothalamus, although its relevance to male-typical aggression needs to be further validated.
29
30 **Keywords:** aggression, androgen, hypothalamus, teleost, vasotocin

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

31 # Introduction
32
33 Aggression is an adaptive behavioral trait that is crucial for competition for territories, food, and
34 mating partners and the establishment of social hierarchies. Although the regulation of aggression in
35 vertebrates involves many different neural mechanisms, particular attention has been given to two
36 hormonal systems in the brain: sex steroids and nonapeptides (Kelly and Wilson, 2020).
37 Sex steroids are peripherally derived or produced in the brain and act on neural circuits to modulate
38 behavior, primarily through binding to specific nuclear receptors that serve as ligand-gated transcription
39 factors (Yang and Shah, 2014). Androgens, among other sex steroids, play a central role in facilitating
40 aggression, and males are typically more aggressive than females due to their androgen-dominated
41 steroid milieu (Hashikawa *et al*., 2018; Lischinsky and Lin, 2020). In rodents, the stimulatory effects of
42 androgens on aggression are largely mediated by the activation of estrogen receptors (ESRs) after their
43 conversion to estrogens in the brain (Yang and Shah, 2014). Recent studies have revealed that activation
44 of the ESR subtype ESR1 in the ventromedial hypothalamus (VMH) is particularly important for the
45 expression of aggressive behavior (Chen and Hong, 2018; Hashikawa *et al*., 2018; Lischinsky and Lin,
46 2020). However, the transcriptional targets of ESR1 that mediate aggressive behavior remain elusive,
47 and the specific role of sex steroids in the regulation of aggression is unclear. Furthermore, and
48 importantly, it is unlikely that the findings in rodents apply to other vertebrates including primates and
49 teleost fish, where androgens act directly on behaviorally relevant neural circuits via the androgen
50 receptor (AR) without conversion to estrogens (Okubo *et al*., 2019; 2022).
51 Nonapeptides, namely, vasotocin (VT, also called vasopressin in mammals) and oxytocin (OT),
52 are evolutionarily conserved neuropeptides that have been associated with a wide range of social
53 behaviors, including aggression (Theofanopoulou *et al*., 2021; Mennigen *et al*., 2022). The largest
54 population of neurons expressing VT and that expressing OT both lie in the paraventricular nucleus
55 (PVN), where they project throughout the brain, as well as to the pituitary, to modulate behavior (Rigney
56 *et al*., 2022). In many terrestrial vertebrates, additional neuronal populations expressing VT in an
57 androgen-dependent, and hence male-biased, manner have been identified in the extended amygdala,
58 specifically in the bed nucleus of the stria terminalis (BNST) and the medial amygdala (MeA) (Kelly
59 and Goodson, 2014; Aspesi and Choleris, 2022; Rigney *et al*., 2022; 2023). These neurons serve as the
60 main regulators of male-typical social behaviors, and elicit pro- or anti-aggressive behavioral responses
61 in males, depending on species and social context (Aspesi and Choleris, 2022).
62 Notably, however, teleost fish lack VT-expressing neurons in the extended amygdala, even though
63 their aggression—like that of terrestrial vertebrates—seems to depend on VT and is generally more
64 prevalent in males (Godwin and Thompson, 2012; Rigney *et al*., 2023). This suggests that, in teleosts,
65 VT may function within another neural circuit to elicit high levels of aggression in males. In line with
66 this idea, teleosts have several populations of VT-expressing neurons in the tuberal hypothalamus, in
67 addition to the major population that spans the brain nucleus homologous to the PVN and its immediate
68 surroundings (Godwin and Thompson, 2012; Oldfield *et al*., 2015). Our previous findings further

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

69 revealed that, in medaka fish (*Oryzias latipes*), the populations in the posterior tuberal nucleus (NPT)
70 and the posterior part of the ventral tuberal nucleus (pNVT) are confined to males (Kawabata *et al*.,
71 2012). The NPT and pNVT are considered homologous to the ventral tegmental area/substantia nigra
72 and the anterior hypothalamus, respectively (Forlano and Bass, 2011; Loveland and Hu, 2018), both of
73 which have been implicated in nonapeptide-regulated social behavior (Rigney *et al*., 2022).

74 To our knowledge, no information is available on the regulation or role of VT-expressing neuronal
75 populations in the NPT and pNVT, but it has been reported in pupfish (*Cyprinodon nevadensis*
76 *amargosae*) that VT expression in the hypothalamus is higher in socially dominant, highly aggressive
77 males (Lema *et al*., 2015). Taken together, the above observations led us to hypothesize that VT
78 expression in either or both of these neuronal populations is induced exclusively in males in an
79 androgen-dependent manner and contributes to the high levels of aggression typical of males. Here we
80 tested this hypothesis by investigating the regulatory mechanisms and physiological roles of male-
81 specific VT expression in the tuberal hypothalamus of medaka.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

82 # Materials and Methods

83

84 ## Animals

85 All experimental procedures involving animals were performed in accordance with the University
86 of Tokyo Institutional Animal Care and Use Committee guidelines. The committee requests the
87 submission of an animal-use protocol only for use of mammals, birds, and reptiles, in accordance with
88 the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in
89 Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports,
90 Science and Technology of Japan (Ministry of Education, Culture, Sports, Science and Technology,
91 Notice No. 71; June 1, 2006). Accordingly, we did not submit an animal-use protocol for this study,
92 which used only teleost fish and thus did not require approval by the committee.

93 Wild-type medaka of the d-rR strain and *vt* knockout medaka produced in this study were kept
94 under controlled conditions at 28 °C and a photoperiod of 14:10 light/dark, and were fed with live
95 *Artemia nauplii* and dry food (Otohime; Marubeni Nisshin Feed, Tokyo, Japan) 3–4 times a day.
96 Sexually mature fish between 3 and 6 months of age were used in all experiments except for the analysis
97 of age-dependent changes in *vt* expression, for which fish of 1, 2, 3, and 7 months of age were employed.
98 To control for genetic diversity and environmental variation, siblings raised in the same conditions were
99 assigned as the comparison group in all experiments, including those with knockout fish. All sampling
100 was done 1–2.5 hours after initiation of the light period.

101

102 ## Gonadectomy and drug treatment

103 A small incision was made in the ventrolateral abdominal wall of anesthetized fish (0.02% tricaine
104 methane sulfonate). The gonad was removed through the incision, which was then closed with nylon
105 thread. Following a 3-day recovery period in saline (0.9% sodium chloride), gonadectomized fish were
106 reared for 6 days in water containing 100 ng/ml of 11-ketotestosterone (KT; the primary androgen in
107 teleosts that cannot be converted to estrogens) (Cosmo Bio, Tokyo, Japan) or estradiol-17β (E2; the
108 primary estrogen in teleosts and other vertebrates) (Fujifilm Wako Pure Chemical, Osaka, Japan), or
109 vehicle alone (ethanol) and then sampled. Sham-operated control fish were subjected to the same
110 surgical procedure as gonadectomized fish but without removing the gonad and then treated with vehicle
111 alone.

112 In separate experiments, males and females (including *vt* knockout females) with intact gonads
113 were reared for 9 days in water containing 250 ng/ml of the AR antagonist cyproterone acetate (CA)
114 (LKT Laboratories, St. Paul, MN) and 100 ng/ml of KT, respectively. These fish were observed daily
115 for changes in aggressive behavior and, when necessary, were sampled on day 9. The concentration of
116 sex steroids used was determined based on steroid levels in medaka serum (Tilton *et al*., 2003). Ovarian-
117 intact females were also treated intraperitoneally with 0.02 ng/mg body weight of Vt peptide (Fujifilm
118 Wako Pure Chemical) or vehicle alone (saline) and observed for changes in aggressive behavior 2 hours
119 after treatment.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

120

121 **Single-label *in situ* hybridization**

122 A digoxigenin (DIG)-labeled cRNA probe for *vt* was generated by PCR amplification of a DNA
123 fragment corresponding to nucleotides 1–845 (845 bp) of the medaka *vt* cDNA followed by *in vitro*
124 transcription using T7 RNA polymerase and DIG RNA Labeling Mix (Roche Diagnostics, Basel,
125 Switzerland). The *in situ* hybridization procedure has been outlined in detail elsewhere (Hiraki-
126 Kajiyama *et al*., 2019). Briefly, brains were fixed in 4% paraformaldehyde (PFA), paraffin embedded,
127 and coronally sectioned at 10-μm thickness. Hybridization signal was visualized with an anti-DIG
128 antibody conjugated to alkaline phosphatase (RRID: AB_514497; Roche Diagnostics) and nitro blue
129 tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Roche Diagnostics). The
130 color was allowed to develop for 5 hours. All sections to be compared were processed in the same batch.
131 To obtain quantitative data, all relevant sections were photographed and converted to black and white
132 binary images by thresholding using Adobe Photoshop (Adobe, San Jose, CA), and the total area of *vt*
133 expression signal across all the relevant sections was calculated for each brain nuclei using ImageJ
134 (https://imagej.nih.gov/ij/).

135

136 **Double-label *in situ* hybridization**

137 The double-label *in situ* hybridization procedure has been described in detail elsewhere (Kawabata-
138 Sakata *et al*., 2020). Briefly, brains were fixed in 4% PFA, embedded in 20% sucrose/5% agarose, and
139 cryosectioned at 20-μm thickness in the coronal plane. Sections were simultaneously hybridized with
140 the *vt* probe, which was labeled with fluorescein using T7 RNA polymerase and Fluorescein RNA
141 Labeling Mix (Roche Diagnostics), and a DIG-labeled AR (*ara*, NM_001122911; *arb*, NM_001104681)
142 probe (Hiraki *et al*., 2012). Fluorescein was detected with an anti-fluorescein antibody conjugated to
143 horseradish peroxidase (RRID: AB_2737388; PerkinElmer, Waltham, MA) and visualized using the
144 TSA Plus Fluorescein System (PerkinElmer); DIG was detected with an anti-DIG antibody conjugated
145 to alkaline phosphatase (RRID: AB_514497; Roche Diagnostics) and visualized with Fast Red (Roche
146 Diagnostics). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to identify cell
147 nuclei. Fluorescent images were captured using a TCS SP8 confocal laser-scanning microscope (Leica
148 Microsystems, Wetzlar, Germany) with the following excitation/emission wavelengths: 405/410–480
149 nm (DAPI), 488/495–545 nm (fluorescein), and 552/565–700 nm (Fast Red).

150

151 **Transcriptional activity assay**

152 A bacterial artificial chromosome (BAC) clone containing the medaka *vt* locus (clone ID: ola1-
153 127B15) was obtained from National BioResource Project (NBRP) Medaka
154 (http://www.shigen.nig.ac.jp/medaka/). The *vt* gene and flanking regions were sequenced and analyzed
155 for the presence of potential canonical bipartite androgen-responsive element (ARE)-like sequences
156 using Jaspar (version 5.0_alpha; http://jaspar.genereg.net/). cDNA fragments encoding full-length
157 medaka Ara and Arb (NM_001104681 and NM_001170833, respectively) were amplified by PCR and

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

158 inserted into the expression vector pcDNA3.1/V5-His-TOPO (Thermo Fisher Scientific, Waltham, MA).
159 Fragments of genomic DNA upstream of the first methionine codon (2677 bp) and downstream of the
160 stop codon (1469 bp) of *vt* were amplified by PCR from the BAC clone and inserted into the respective
161 NheI and XbaI sites of the luciferase reporter vector pGL4.10 (Promega, Madison, WI). The resultant
162 reporter construct was transiently transfected into COS-7 cells using Lipofectamine LTX (Thermo
163 Fisher Scientific), together with an internal control vector pGL4.74 (Promega) and either the Ara or Arb
164 expression construct. Six-hour post-transfected cells were treated with 0, 10^{-10}, 10^{-8}, or 10^{-6} M KT for
165 18 hours in Dulbecco's modified Eagle's medium (phenol red-free) containing 5% charcoal-treated fetal
166 bovine serum (Thermo Fisher Scientific). After cell lysis, luciferase activity was determined using the
167 Dual-Luciferase Reporter Assay System (Promega) on the GloMax 20/20n Luminometer (Promega).
168 All assays were conducted in duplicate and repeated independently three times.
169 To determine the ARE responsible for androgen induction of *vt* transcription, assays were also
170 conducted with luciferase reporter constructs containing only the fragment upstream of the first
171 methionine codon or carrying point mutations in the identified ARE-like sequences. A construct
172 containing the upstream fragment was prepared as described above. Constructs containing point-
173 mutated ARE-like sequences were prepared using the PrimeSTAR Mutagenesis Basal Kit (Takara Bio,
174 Shiga, Japan). Because an ARE half-site can function alone to confer androgen inducibility (Pihlajamaa
175 *et al*., 2015), both half-sites of each ARE-like sequence were mutated (into a HindIII recognition site
176 sequence [AAGCTT] to facilitate confirmation of the mutation). The procedures for cell transfection,
177 KT treatment, and luciferase activity measurements were the same as above, except that a single dose
178 (10^{-6} M) of KT was used.
179 Additional assays were performed with luciferase reporter constructs containing truncated versions
180 of the downstream fragment to see the effects of simultaneous loss of multiple ARE-like sequences in
181 this fragment. Constructs containing the full-length (1469 bp) or serially 3′-truncated (970 bp and 485
182 bp) downstream fragment were generated as described above and used in these assays. Cell transfection,
183 KT treatment (10^{-6} M), and luciferase activity measurements were performed as described above.
184

185 **Aggressive behavior test**

186 The aggressive behavior test was conducted as previously described (Yamashita *et al*., 2020).
187 Briefly, four fish of the same genotype and sex that were not familiar with one another were housed in
188 a 2-liter rectangular tank and separated from each other by opaque partitions. After 10-min acclimation
189 to the tank, the partitions were removed to allow the fish to interact. Their behavior was recorded for 30
190 min with a digital video camera (Everio GZ-G5; Jvckenwood, Kanagawa, Japan, or iVIS HF S11/S21;
191 Canon, Tokyo, Japan). All tests were done 1–5 hours after initiation of the light period. Video recordings
192 were manually analyzed for the total number of each aggressive act (chase, fin display, circle, strike,
193 and bite).
194

195 **Production of knockout medaka**

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

196 The vt knockout medaka line was produced using transcription activator-like effector nuclease
197 (TALEN) technology, targeting the sequence corresponding to the mature Vt peptide (Supplementary
198 Fig. 1A), essentially as described (Takahashi et al., 2016). In brief, TALE repeat arrays were assembled
199 using the Joung Lab REAL Assembly TALEN kit (Addgene 1000000017). Synthesized TALEN
200 mRNAs were injected into the cytoplasm of one-cell stage embryos. Once these fish reached adulthood,
201 they were outcrossed to wild-type fish and the resulting progeny were tested for target site mutations by
202 T7 endonuclease I assay (Kim et al., 2009) and direct sequencing. A founder fish was identified that
203 reproducibly produced progeny carrying a 10-bp deletion that introduced a frameshift into the mature
204 Vt peptide. The progeny were intercrossed to obtain homozygous, heterozygous, and wild-type siblings.
205 Both male and female homozygous fish were viable and displayed no obvious morphological or
206 developmental defects. Each fish was genotyped by direct sequencing of the targeted locus.
207 Only female knockout fish were used in this study because we considered that the most direct
208 approach to test our hypothesis would be to confirm that exogenous androgens induce aggression in
209 wild-type females but not in *vt* knockout females. We considered males to be unsuitable for this
210 experiment because they have high levels of endogenous androgens and would be less susceptible to
211 exogenous androgens.
212
213 **Statistical analysis**
214 All quantitative data were presented as mean ± standard error of the mean. On graphs, individual
215 data points were also plotted to indicate the underlying distribution. All statistics were analyzed using
216 GraphPad Prism (GraphPad Software, San Diego, CA). Data between two groups were compared using
217 unpaired two-tailed Student's *t*-test. When the F-test indicated that the variances between groups were
218 significantly different, Welch's correction was applied. To compare data among more than two groups,
219 one-way analysis of variance (ANOVA) was performed, followed by either Dunnett's (comparisons
220 between control and experimental groups) or Bonferroni's (comparisons among experimental groups)
221 *post hoc* test. In cases where the Bartlett's and Brown-Forsythe tests revealed significant differences in
222 variances among groups, the data were subjected to log transformation to correct for heterogeneity of
223 variance. When variances remained heterogeneous following transformation, Kruskal-Wallis test was
224 utilized, followed by Dunn's *post hoc* test. For analyses of age-dependent changes in *vt* expression and
225 aggressive behavior in *vt*-deficient females treated with KT, two-way ANOVA was used to determine
226 the effects of and interactions between age and sex and between genotype and KT treatment,
227 respectively. If a significant interaction was detected, differences between groups were further analyzed
228 by Bonferroni's *post hoc* test. All data points were included in the analyses and no outliers were defined.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

229 # Results

230

231 ## Male-specific *vt* expression in the tuberal hypothalamic nucleus is dependent on post-
232 pubertal gonadal androgens

233 First, we determined the spatiotemporal pattern of *vt* expression and related sex differences in the
234 medaka brain. *In situ* hybridization analysis of brains from different ages showed that *vt* was expressed
235 in the PMp/PPa/PMm/PMg (the latter two brain nuclei are homologous to the PVN) in the preoptic area
236 and the SC/aNVT, NAT, NPT, and pNVT in the tuberal hypothalamus at all ages examined (Fig. 1A
237 and B; see Supplementary Table 1 for abbreviations of medaka brain nuclei). Signal quantification
238 revealed that expression in the PMp/PPa/PMm/PMg, shared by males and females, increased with age
239 similarly in both sexes, but levels were slightly higher in males overall (main effect of age, $p < 0.0001$;
240 main effect of sex, $p = 0.0020$; interaction between age and sex, $p = 0.5837$) (Fig. 1C). Expression in
241 both the SC/aNVT and NAT peaked at 2 months of age, when secondary sexual characters were well-
242 developed but no spawning had yet occurred (spawning began at 3 months); there were no significant
243 sex differences except for a slight female bias in the SC/aNVT at 2 months of age ($p = 0.016$ between
244 sexes) (Fig. 1C). Expression in the NPT and pNVT was male-specific at all ages examined, with
245 significant sex differences detected after 2 months of age ($p = 0.0005$, 0.0003, and 0.0078 for NPT at 2,
246 3, and 7 months, respectively; $p < 0.0001$ for pNVT at 2, 3, and 7 months) (Fig. 1C).

247 These results led us to speculate that sex steroid hormones produced by the gonads after puberty
248 onset influence the pattern of *vt* expression in the medaka brain. We tested this idea by quantitative *in*
249 *situ* hybridization of *vt* expression in fish that were gonadectomized in adulthood and treated with KT
250 or E2. In the male pNVT, castration significantly reduced *vt* expression ($p < 0.0001$), which was restored
251 by KT treatment ($p = 0.0014$) but not by E2 treatment (Fig. 1D and E). In the female pNVT, *vt* expression
252 was not detected under normal conditions but was induced by KT treatment following ovariectomy (p
253 < 0.0001) (Fig. 1F and G). In other brain nuclei, no significant differences between treatments were
254 observed (Fig. 1D and F). Collectively, these results suggest that high circulating levels of androgens
255 released from the testis stimulate *vt* expression in the pNVT in post-pubertal males, whereas in females,
256 the lack of androgen stimulation prevents its induction. In addition, the effect of androgens on *vt*
257 expression in the pNVT is transient and reversible; thus, *vt* expression is attenuated in androgen-depleted
258 adult males and, conversely, induced in estrogen-depleted and androgen-supplemented adult females.

259

260 ## Androgens can directly activate the transcription of *vt* through Ara

261 To investigate the possible direct action of androgens on *vt*-expressing neurons in the pNVT, we
262 first determined if these neurons coexpress ARs. Most teleosts, including medaka, possess two subtypes
263 of AR, designated Ara and Arb (Okubo *et al*., 2022). Double-label *in situ* hybridization for *vt* and each
264 AR subtype revealed that *ara*, but not *arb*, was abundantly expressed in the pNVT of both sexes, and
265 virtually all of the *vt*-expressing neurons in the male pNVT were positive for *ara* expression (Fig. 2A
266 and b). Note that the names of *ara* and *arb* used in the present study follow the nomenclature of Ogino

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

267 *et al*. (2016; 2023), which reflects the orthology/paralogy relationships of teleost ARs. Thus, the gene
268 referred to as *ara* in our previous publications (*e.g*., Hiraki *et al*., 2012; 2014; Kawabata-Sakata *et al*.,
269 2020; Yamashita *et al*., 2017; 2020) is denoted as *arb* in this study, and the gene referred to as *arb* as
270 *ara*.
271 Next, we tested the ability of androgens to directly activate the transcription of *vt*. Our search for
272 potential AREs in the *vt* locus of medaka identified six bipartite ARE-like sequences within the upstream
273 flanking region (positions −2600, −2225, −2168, −1620, −817, and −242 relative to the first methionine
274 codon) and nine within the downstream flanking region (positions +179, +336, +340, +448, +682, +883,
275 +1250, +1411, and +1435 relative to the stop codon) (Supplementary Fig. 2). No ARE-like sequences
276 were found in the gene body of *vt*. A transcriptional activity assay using a luciferase-based reporter
277 construct containing the upstream and downstream flanking fragments of *vt* that carried these ARE-like
278 sequences revealed that luciferase activity was dose-dependently induced by KT in the presence of either
279 Ara (p = 0.0015 at 10^{-8} M and 0.0006 at 10^{-6} M) or Arb (p = 0.0437 at 10^{-8} M and 0.0201 at 10^{-6} M),
280 with higher induction observed for Ara (Fig. 2C). An additional assay using only the upstream fragment
281 (the downstream fragment was removed from the reporter construct) resulted in loss of KT-induced
282 luciferase activity in the presence of Ara (p = 0.0379 and 0.6724 with and without the downstream
283 fragment, respectively), suggesting that the *cis*-element responsible for KT induction is present in the
284 downstream region (Fig. 2D). To further explore this finding, we introduced point mutations in each of
285 the nine ARE-like sequences located in the downstream fragment in the reporter construct and studied
286 the resulting changes in luciferase activity. However, none of the mutations had a significant impact on
287 KT-induced luciferase activity in the presence of either Ara or Arb (Fig. 2E).
288 We therefore performed additional assays using reporter constructs containing serially 3′-truncated
289 downstream fragments to examine the effects of simultaneous loss of multiple ARE-like sequences.
290 However, even after truncating the downstream fragment from 1469 bp to 485 bp, KT-induced luciferase
291 activity was not abolished in the presence of either Ara ($p < 0.0001$, = 0.0015, and 0.0178 for 1469, 970,
292 and 485 bp fragments, respectively) or Arb (p = 0.0136, 0.0198, and 0.0487, respectively). These results
293 suggest that the 485-bp region downstream of the stop codon of *vt* contains the *cis*-element responsible
294 for KT induction. Considering that none of the mutations in the ARE-like sequences found in this region
295 abolished KT induction, it may be that AR interacts with *cis*-elements other than the canonical ARE to
296 activate the transcription of *vt*. Overall, these findings indicate that androgens can act on *vt*-expressing
297 neurons in the pNVT to directly stimulate the transcription of *vt* via Ara, although the *cis*-element
298 responsible for this process remains to be identified.
299
300 **Administration of exogenous Vt elicits aggression in females**
301 If the high levels of aggression typically observed in male medaka result from male-specific
302 production of Vt in the pNVT, then female medaka should also exhibit aggression when given
303 exogenous Vt. We tested this idea by treating females with Vt and analyzing the resulting changes in
304 intrasexual aggression. Aggression in medaka and many other teleosts includes five behavioral acts:

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

chase, fin display, circle, strike, and bite (Oliveira *et al*., 2011; Kagawa, 2013). Vt treatment led to a significant increase in the number of chases (p = 0.0086), fin displays (p = 0.0104), and bites (p = 0.0011), but no strikes were induced (Fig. 3). Although not statistically significant, the treatment also evoked circles, which are not typically observed in females (Fig. 3). From these findings, it was evident that exogenous Vt elicits aggression in female medaka.

Altering the androgen milieu leads to correlated changes in the levels of aggression and *vt* expression in the tuberal hypothalamus

If androgen-dependent *vt* expression in the pNVT facilitates aggression, there is likely to be a correlation among the levels of *vt* expression in the pNVT, androgen action, and aggression. To explore this relationship, we administered KT to females with intact ovaries and evaluated changes in intrasexual aggression and *vt* expression in the pNVT. There was a significant increase in the number of chases after 4 days of KT treatment (p = 0.0421), and this increase persisted throughout the remaining treatment period (p = 0.0136 on day 5 and 0.0054 on day 7) (Fig. 4A). Although not statistically significant, the number of fin displays, circles, strikes, and bites also increased (Fig. 4A). *In situ* hybridization analysis revealed that KT treatment, even without ovariectomy, induced *vt* expression in the pNVT (p = 0.0043), along with increased aggression (Fig. 4B and C). In other brain nuclei, no significant changes in *vt* expression were noted with KT treatment (Fig. 4B).

Next, we treated males with intact testes with the AR antagonist CA to inhibit androgen/AR signaling and conducted similar behavioral and expression analyses. The number of chases, fin displays, and circles was significantly reduced after 3 days of treatment (p = 0.0094, 0.0044, 0.0289, and 0.0007 for chases on days 3, 5, 7, and 8, respectively; p = 0.0323, 0.0275, and 0.0038 for fin displays on days 3, 4, and 5 onward, respectively; p = 0.0024, 0.0024, and 0.0003 for circles on days 3, 4, and 5 onward, respectively), and the number of bites was significantly reduced after 1 day of treatment (p = 0.0075 on days 1, 4, 5, and 6) (Fig. 4D). *In situ* hybridization analysis revealed that CA treatment substantially attenuated *vt* expression in the pNVT (p = 0.0005) (Fig. 4E and F). There were no notable changes in *vt* expression in other brain nuclei, except for a slight decrease in the PMp/PPa/PMm/PMg (p = 0.0402) (Fig. 4E).

Taken together, these results demonstrate a clear correlation among *vt* expression in the pNVT, androgen action, and aggression, and strengthen our hypothesis that the high levels of aggression typical of males are attributable to androgen-dependent *vt* expression in the pNVT.

***vt* deficiency fails to prevent androgen-induced aggression in females**

Lastly, we further tested our hypothesis by producing a *vt* knockout medaka line and comparing the aggression levels of females treated with KT between genotypes. We considered that, if our hypothesis is correct, KT treatment should elicit aggressive behavior in $vt^{+/+}$ and $vt^{+/-}$ females, but not in $vt^{-/-}$ females. We employed TALEN-mediated genome editing to produce a medaka line with a deleterious frameshift mutation in *vt* (Supplementary Fig. 1A), resulting in an inability to produce the

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

343 mature Vt peptide (Supplementary Fig. 1B). We then treated $vt^{+/+}$, $vt^{+/-}$, and $vt^{-/-}$ females with KT and
344 analyzed their aggression levels on days 0 (before treatment), 5, and 9. Contrary to our expectations,
345 however, aggression was induced even in $vt^{-/-}$ females, and no significant differences between
346 genotypes were found for any aggressive act (Supplementary Fig. 1C). Therefore, this analysis did not
347 support our hypothesis.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

348 # Discussion

349

350 We previously identified male-specific populations of VT-expressing neurons in the tuberal
351 hypothalamus of medaka (Kawabata *et al*., 2012). Here we proposed and tested the hypothesis that VT
352 expression in these neuronal populations is induced exclusively in males in an androgen-dependent
353 manner and contributes to high, male-typical levels of aggression. Teleost fish, like terrestrial
354 vertebrates, have a major population of VT-expressing neurons in the brain nucleus homologous to the
355 PVN and its surrounding areas (PMp/PPa/PMm/PMg) (Godwin and Thompson, 2012). It has been
356 reported in several teleost species, including medaka, that the number of one or more subpopulations of
357 these VT-expressing neurons correlates with the level of aggression, and for this reason, male-typical
358 aggression in teleosts has been attributed to this neuronal population (Godwin and Thompson, 2012;
359 Kagawa, 2013; Silva and Pandolfi, 2019). On the other hand, no attempt has been made to assess the
360 contribution of the tuberal hypothalamic populations to aggression.

361 Here we first demonstrated that male-specific VT expression in the tuberal hypothalamus is indeed
362 dependent on androgens. More specifically, VT expression in the pNVT of the tuberal hypothalamus is
363 induced exclusively in post-pubertal males by large amounts of androgens secreted by the testes. To our
364 knowledge, this is the first report showing that teleosts, although lacking the VT-expressing neurons in
365 the extended amygdala that are androgen-dependent and thus male-biased, have VT-expressing neurons
366 with comparable properties in a distinct brain nucleus. We observed no sex steroid dependence of VT
367 expression in other brain nuclei including the PMp/PPa/PMm/PMg, as reported in other teleost species
368 such as bluehead wrasses (*Thalassoma bifasciatum*) (Semsar and Godwin, 2003). In teleosts, therefore,
369 the neuronal population in the pNVT is presumably responsible for reproductive cycle-dependent VT
370 functions, including increased aggression during the mating period.

371 Despite the similarities, there are important differences in androgen-dependent VT expression in
372 the extended amygdala of terrestrial vertebrates and the tuberal hypothalamus of teleosts. First, in the
373 rodent extended amygdala, a large fraction of testosterone is converted locally to E2 and then acts
374 through the ESR (Aspesi and Choleris, 2022), whereas in the medaka tuberal hypothalamus, KT, an
375 androgen that cannot be converted to E2, acts directly through the AR. Because the effects of androgens
376 on male behavior rely primarily on an AR-mediated pathway in many vertebrate species (Okubo *et al*.,
377 2022), our findings in medaka may be broadly applicable to other species. The second difference
378 concerns whether treating adult females with sex steroids induces VT expression above basal levels. In
379 adult female rats, VT expression in the BNST is elevated by estrogen treatment but only to levels
380 observed prior to ovariectomy (De Vries *et al*., 1994; Turano *et al*., 2019). This is because, in rodents,
381 and probably in other terrestrial vertebrates, sex steroids produced in the fetal gonads in a sex-specific
382 manner act on the developing BNST to shape irreversible and enduring sex differences in VT expression
383 (Rigney *et al*., 2022; 2023). Furthermore, in rodents, this sex difference is due in part to the direct
384 neuronal actions of sex chromosome-linked genes, which are independent of sex steroid effects (De
385 Vries *et al*., 2002). In medaka, by contrast, treating females with androgens markedly induced VT

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

386 expression in the pNVT, which was virtually absent in ovary-intact and ovariectomized conditions.
387 Taken together with the observation that castration in males severely reduced VT expression in the
388 pNVT, it seems that the sexually dimorphic pattern of VT expression in medaka can be reversed between
389 the sexes in response to changes in the adult androgen milieu. It is known that the sexual phenotypes of
390 teleosts (behavioral or otherwise), unlike those of terrestrial vertebrates, are highly labile across the
391 lifespan and can be reversed between the sexes, even as adults (Okubo *et al.*, 2019; 2022); the present
392 study now shows that manipulation of the adult androgen milieu effectively reverses sex-typical
393 aggressive behavior. The reversibility of sexually dimorphic VT expression may reflect this fact.
394 It may be relevant to note here that KT-induced *vt* expression in the pNVT of females was apparent
395 but at a relatively low level compared to that of castrated males. A possible explanation for this
396 observation is that full induction of *vt* expression by KT in females requires a relatively long period of
397 time because it involves the generation of new neurons or the activation of the counterparts of male *vt*-
398 expressing neurons that are in a quiescent, non-activated state. It would be worthwhile to determine if
399 treating females with KT for a longer period of time results in comparable levels of *vt* expression as
400 males.
401 The question then arises whether androgen/AR signaling induces VT expression in pNVT neurons
402 directly or indirectly through other target genes or cells. We found that *ara*, one of the two AR genes in
403 teleosts, is expressed in almost all VT-expressing neurons in the pNVT. We further showed by
404 transcriptional activity assays that androgens can directly stimulate the transcription of *vt* and that the
405 magnitude of this stimulation is greater through Ara than through Arb. These results suggest that
406 androgens directly activate VT expression in the pNVT through binding to Ara. This finding is
407 consistent with recent work in cichlids (*Astatotilapia burtoni*) showing that *ara* and *arb* are functionally
408 differentiated, with *ara* responsible for promoting aggression (Alward *et al.*, 2020). Although it is not
409 known whether VT is a direct transcriptional target of AR signaling (or ESR signaling) in terrestrial
410 vertebrates, as it is in medaka, androgen-induced *Vt* expression in the BNST of adult rats has been
411 reported to involve changes in the DNA methylation pattern of the *Vt* promoter (Auger *et al.*, 2011).
412 Future work will be needed to clarify whether a similar mechanism exists in the medaka pNVT and to
413 identify functional AREs in the medaka *vt* locus (which eluded detection in the present survey) in order
414 to clarify the evolutionary conservation and divergence of regulatory mechanisms for sexually
415 dimorphic VT expression.
416 We also tested the hypothesized role of male-specific VT expression on aggression. Although VT
417 has been implicated in aggressive behavior in many teleost species (Godwin and Thompson, 2012; Silva
418 and Pandolfi, 2019), Yokoi *et al.* (2015) observed no anomalies in intrasexual aggression of male
419 medaka carrying a missense mutation in *vt* (leading to replacement of the first methionine with arginine
420 and loss of the ability to produce the mature Vt peptide). While this observation may suggest that VT is
421 not involved in aggression in medaka, we showed here that administration of exogenous Vt peptide
422 elicited aggressive behavior in female medaka, which typically exhibit little or no aggression. This result
423 indicates that VT certainly serves to facilitate aggression in medaka as well as in other teleosts (although

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

424 it is possible that the induced aggression may represent a pharmacological effect rather than a
425 physiological role of VT). Furthermore, manipulation of the androgen milieu of adult male and female
426 medaka to inhibit or facilitate aggression revealed a clear correlation among *vt* expression in the pNVT,
427 androgen action, and aggression. More specifically, treatment of male medaka with an AR antagonist
428 resulted in a marked reduction in both aggression and *vt* expression in the pNVT, whereas androgen
429 treatment of females elicited both of them. All of these findings support our hypothesis that male-
430 specific VT expression contributes to male-typical high levels of aggression, thereby strengthening its
431 validity.
432 Finally, we further tested this hypothesis using *vt*-deficient female medaka but, contrary to our
433 expectations, androgen treatment induced comparable levels of aggression in *vt*-deficient females as in
434 wild-type females. A straightforward interpretation of this result would suggest that our hypothesis is
435 incorrect and androgen-induced aggression is not mediated by VT; however, the lack of detectable
436 effects of *vt* deficiency might also be due to functional compensation by other genes or pathways. A
437 good candidate for this functional compensation is OT, a nonapeptide closely related to VT that can
438 partially activate VT receptors (Mennigen *et al*., 2022; Rae *et al*., 2022). In medaka, *ot*-expressing
439 neurons are not observed in the pNVT, where male-specific *vt*-expressing neurons reside (Kawabata *et*
440 *al*., 2012), but it is possible that *ot*-expressing neurons in another brain nucleus project to the same brain
441 region as male-specific *vt*-expressing neurons and compensate for *vt* function. Contrary to male medaka
442 carrying a missense mutation in *vt*, which showed comparable levels of aggression to wild-type males,
443 as described above (Yokoi *et al*., 2015), male medaka carrying a missense mutation in the VT receptor
444 subtype *v1a2* (leading to replacement of asparagine at position 68 with isoleucine) were found to be less
445 aggressive (Yokoi *et al*., 2015). This discrepancy can also be explained if OT compensates for the loss
446 of VT function via V1A2. It would be interesting to test this idea in future studies by producing double-
447 knockout medaka for *vt* and *ot* and analyzing their behavioral phenotypes.
448 In summary, our results have demonstrated that VT expression in the tuberal hypothalamic nucleus
449 is induced exclusively in males, most likely as a result of direct transcriptional activation of VT by
450 androgen/Ara signaling, although it remains to be determined whether this VT expression is relevant to
451 the high levels of aggression typical of males. The anterior hypothalamus of rodents, which is
452 homologous to the teleost pNVT, contains no VT-expressing neurons; however, it receives heavy
453 projections from male-biased VT neurons in the BNST and abundantly expresses the behaviorally-
454 relevant V1A receptor, thus representing a primary site of action of VT for aggression (Rigney *et al*.,
455 2022; 2023). If the V1A receptor homolog is also expressed in the teleost pNVT, VT produced in this
456 brain nucleus may contribute to male-typical aggression by acting in an autocrine/paracrine manner.
457 Further studies, including analyses of receptor expression and axonal projection patterns, will be needed
458 to test this idea. It would also be worthwhile investigating whether the current findings in medaka apply
459 to other teleosts. The resulting information on species variation in the spatiotemporal expression patterns
460 and regulatory mechanisms of VT may provide some insight into the diversity of social structures and
461 behavioral patterns in teleosts.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

462
Acknowledgments

463

464 The authors would like to thank the National BioResource Project (NBRP) Medaka for providing
465 the BAC clone, Dr. Junpei Yamashita and Takayasu Tsumaki for technical assistance, and Dr. Towako
466 Hiraki-Kajiyama and Akio Takeuchi for experimental support. This work was supported by the Ministry
467 of Education, Culture, Sports, Science, and Technology (MEXT), Japan, and the Japan Society for the
468 Promotion of Science (JSPS) (MEXT/JSPS grant numbers 13J04816 (to YKS), 17H06429, and
469 23H02305 (to KO)).

470

471
Declarations of interest

472

473 The authors declare no competing interests.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

474 **References**

475

476 1. Alward BA, Laud VA, Skalnik CJ, York RA, Juntti SA, Fernald RD (2020) Modular genetic
477 control of social status in a cichlid fish. Proc Natl Acad Sci USA 117:28167–28174.
478 doi:10.1073/pnas.2008925117
479 2. Aspesi D, Choleris E (2022) Neuroendocrine underpinning of social recognition in males and
480 females. J Neuroendocrinol 34:e13070. doi:10.1111/jne.13070
481 3. Auger CJ, Coss D, Auger AP, Forbes-Lorman RM (2011) Epigenetic control of vasopressin
482 expression is maintained by steroid hormones in the adult male rat brain. Proc Natl Acad Sci USA
483 108:4242–4247. doi:10.1073/pnas.1100314108
484 4. Chen P, Hong W (2018) Neural circuit mechanisms of social behavior. Neuron 98:16–30.
485 doi:10.1016/j.neuron.2018.02.026
486 5. De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-
487 Badge R, Burgoyne PS, Arnold AP (2002) A model system for study of sex chromosome effects
488 on sexually dimorphic neural and behavioral traits. J Neurosci 22:9005–9014.
489 doi:10.1523/JNEUROSCI.22-20-09005.2002
490 6. De Vries GJ, Wang Z, Bullock NA, Numan S (1994) Sex differences in the effects of testosterone
491 and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis
492 of rats. J Neurosci 14:1789–1794. doi:10.1523/JNEUROSCI.14-03-01789.1994
493 7. Forlano PM, Bass AH (2011) Neural and hormonal mechanisms of reproductive-related arousal in
494 fishes. Horm Behav 59:616–629. doi:10.1016/j.yhbeh.2010.10.006
495 8. Godwin J, Thompson R (2012) Nonapeptides and social behavior in fishes. Horm Behav 61:230–
496 238. doi:10.1016/j.yhbeh.2011.12.016
497 9. Hashikawa K, Hashikawa Y, Lischinsky J, Lin D (2018) The neural mechanisms of sexually
498 dimorphic aggressive behaviors. Trends Genet 34:755–776. doi:10.1016/j.tig.2018.07.001
499 10. Hiraki T, Nakasone K, Hosono K, Kawabata Y, Nagahama Y, Okubo K (2014) Neuropeptide B is
500 female-specifically expressed in the telencephalic and preoptic nuclei of the medaka brain.
501 Endocrinology 155:1021–1032. doi:10.1210/en.2013-1806
502 11. Hiraki T, Takeuchi A, Tsumaki T, Zempo B, Kanda S, Oka Y, Nagahama Y, Okubo K (2012)
503 Female-specific target sites for both oestrogen and androgen in the teleost brain. Proc Royal Soc
504 B 279:5014–5023. doi:10.1098/rspb.2012.2011
505 12. Hiraki-Kajiyama T, Yamashita J, Yokoyama K, Kikuchi Y, Nakajo M, Miyazoe D, Nishiike Y,
506 Ishikawa K, Hosono K, Kawabata-Sakata Y, Ansai S, Kinoshita M, Nagahama Y, Okubo K (2019)
507 Neuropeptide B mediates female sexual receptivity in medaka fish, acting in a female-specific but
508 reversible manner. eLife 8:e39495. doi:10.7554/eLife.39495
509 13. Kagawa N (2013) Social rank-dependent expression of arginine vasotocin in distinct preoptic
510 regions in male *Oryzias latipes*. J Fish Biol 82:354–363. doi:10.1111/j.1095-8649.2012.03490.x
511 14. Kawabata Y, Hiraki T, Takeuchi A, Okubo K (2012) Sex differences in the expression of

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

512 vasotocin/isotocin, gonadotropin-releasing hormone, and tyrosine and tryptophan hydroxylase
513 family genes in the medaka brain. Neuroscience 218:65–77.
514 doi:10.1016/j.neuroscience.2012.05.021
515 15. Kawabata-Sakata Y, Nishiike Y, Fleming T, Kikuchi Y, Okubo K (2020) Androgen-dependent
516 sexual dimorphism in pituitary tryptophan hydroxylase expression: relevance to sex differences in
517 pituitary hormones. Proc Royal Soc B 287:20200713. doi:10.1098/rspb.2020.0713
518 16. Kelly AM, Goodson JL (2014) Social functions of individual vasopressin-oxytocin cell groups in
519 vertebrates: what do we really know? Front Neuroendocrinol 35:512–529.
520 doi:10.1016/j.yfrne.2014.04.005
521 17. Kelly AM, Wilson LC (2020) Aggression: perspectives from social and systems neuroscience.
522 Horm Behav 123:104523. doi:10.1016/j.yhbeh.2019.04.010
523 18. Kim HJ, Lee HJ, Kim H, Cho SW, Kim JS (2009) Targeted genome editing in human cells with
524 zinc finger nucleases constructed via modular assembly. Genome Res 19:1279–1288.
525 doi:10.1101/gr.089417.108
526 19. Lema SC, Sanders KE, Walti KA (2015) Arginine vasotocin, isotocin and nonapeptide receptor
527 gene expression link to social status and aggression in sex-dependent patterns. J Neuroendocrinol
528 27:142–157. doi:10.1111/jne.12239
529 20. Lischinsky JE, Lin D (2020) Neural mechanisms of aggression across species. Nat Neurosci
530 23:1317–1328. doi:10.1038/s41593-020-00715-2
531 21. Loveland JL, Hu CK (2018) Commentary: arginine vasotocin preprohormone is expressed in
532 surprising regions of the teleost forebrain. Front Endocrinol 9:63. doi:10.3389/fendo.2018.00063
533 22. Mennigen JA, Ramachandran D, Shaw K, Chaube R, Joy KP, Trudeau VL (2022) Reproductive
534 roles of the vasopressin/oxytocin neuropeptide family in teleost fishes. Front Endocrinol
535 13:1005863. doi:10.3389/fendo.2022.1005863
536 23. Ogino Y, Ansai S, Watanabe E, Yasugi M, Katayama Y, Sakamoto H, Okamoto K, Okubo K,
537 Yamamoto Y, Hara I, Yamazaki T, Kato A, Kamei Y, Naruse K, Ohta K, Ogino H, Sakamoto T,
538 Miyagawa S, Sato T, Yamada G, Baker ME, Iguchi T (2023) Evolutionary differentiation of
539 androgen receptor is responsible for sexual characteristic development in a teleost fish. Nat
540 Commun 14:1428. doi:10.1038/s41467-023-37026-6
541 24. Ogino Y, Kuraku S, Ishibashi H, Miyakawa H, Sumiya E, Miyagawa S, Matsubara H, Yamada G,
542 Baker ME, Iguchi T (2016) Neofunctionalization of androgen receptor by gain-of-function
543 mutations in teleost fish lineage. Mol Biol Evol 33:228–244. doi:10.1093/molbev/msv218
544 25. Okubo K, Miyazoe D, Nishiike Y (2019) A conceptual framework for understanding sexual
545 differentiation of the teleost brain. Gen Comp Endocrinol 284:113129.
546 doi:10.1016/j.ygcen.2019.02.020
547 26. Okubo K, Nishiike Y, Fleming T, Kikuchi Y, Hiraki-Kajiyama T (2022) Sex steroid regulation of
548 male- and female-typical mating behaviors in teleost fish. In "Spectrum of Sex" (Tanaka M,
549 Tachibana M, Eds) pp.111–133, Springer, Singapore. doi:10.1007/978-981-19-5359-0_7

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

550 27. Oldfield RG, Harris RM, Hofmann HA (2015) Integrating resource defence theory with a neural
551 nonapeptide pathway to explain territory-based mating systems. Front Zool 12:S16.
552 doi:10.1186/1742-9994-12-S1-S16
553 28. Oliveira RF, Silva JF, Simões JM (2011) Fighting zebrafish: characterization of aggressive
554 behavior and winner–loser effects. Zebrafish 8:73–81. doi:10.1089/zeb.2011.0690
555 29. Pihlajamaa P, Sahu B, Jänne OA (2015) Determinants of receptor- and tissue-specific actions in
556 androgen signaling. Endocr Rev 36:357–384. doi:10.1210/er.2015-1034
557 30. Rae M, Lemos Duarte M, Gomes I, Camarini R, Devi LA (2022) Oxytocin and vasopressin:
558 signalling, behavioural modulation and potential therapeutic effects. Br J Pharmacol 179:1544–
559 1564. doi:10.1111/bph.15481
560 31. Rigney N, de Vries GJ, Petrulis A (2023) Modulation of social behavior by distinct vasopressin
561 sources. Front Endocrinol 14:1127792. doi:10.3389/fendo.2023.1127792
562 32. Rigney N, de Vries GJ, Petrulis A, Young LJ (2022) Oxytocin, vasopressin, and social behavior:
563 from neural circuits to clinical opportunities. Endocrinology 163:bqac111.
564 doi:10.1210/endocr/bqac111
565 33. Semsar K, Godwin J (2003) Social influences on the arginine vasotocin system are independent of
566 gonads in a sex-changing fish. J Neurosci 23:4386–4393. doi:10.1523/JNEUROSCI.23-10-
567 04386.2003
568 34. Silva AC, Pandolfi M (2019) Vasotocinergic control of agonistic behavior told by neotropical
569 fishes. Gen Comp Endocrinol 273:67–72. doi:10.1016/j.ygcen.2018.04.025
570 35. Takahashi A, Kanda S, Abe T, Oka Y (2016) Evolution of the hypothalamic-pituitary-gonadal axis
571 regulation in vertebrates revealed by knockout medaka. Endocrinology 157:3994–4002.
572 doi:10.1210/en.2016-1356
573 36. Theofanopoulou C, Gedman G, Cahill JA, Boeckx C, Jarvis ED (2021) Universal nomenclature
574 for oxytocin–vasotocin ligand and receptor families. Nature 592:747–755. doi:10.1038/s41586-
575 020-03040-7
576 37. Tilton SC, Foran CM, Benson WH (2003) Effects of cadmium on the reproductive axis of
577 Japanese medaka (*Oryzias latipes*). Comp Biochem Physiol C Toxicol Pharmacol 136:265–276.
578 doi:10.1016/j.cca.2003.09.009
579 38. Turano A, Osborne BF, Schwarz JM (2019) Sexual differentiation and sex differences in neural
580 development. Curr Top Behav Neurosci 43:69–110. doi:10.1007/7854_2018_56
581 39. Yamashita J, Kawabata Y, Okubo K (2017) Expression of isotocin is male-specifically up-
582 regulated by gonadal androgen in the medaka brain. J Neuroendocrinol 29:e12545.
583 doi:10.1111/jne.12545
584 40. Yamashita J, Takeuchi A, Hosono K, Fleming T, Nagahama Y, Okubo K (2020) Male-
585 predominant galanin mediates androgen-dependent aggressive chases in medaka. eLife 9:e59470.
586 doi:10.7554/eLife.59470
587 41. Yang CF, Shah NM (2014) Representing sex in the brain, one module at a time. Neuron 82:261–-

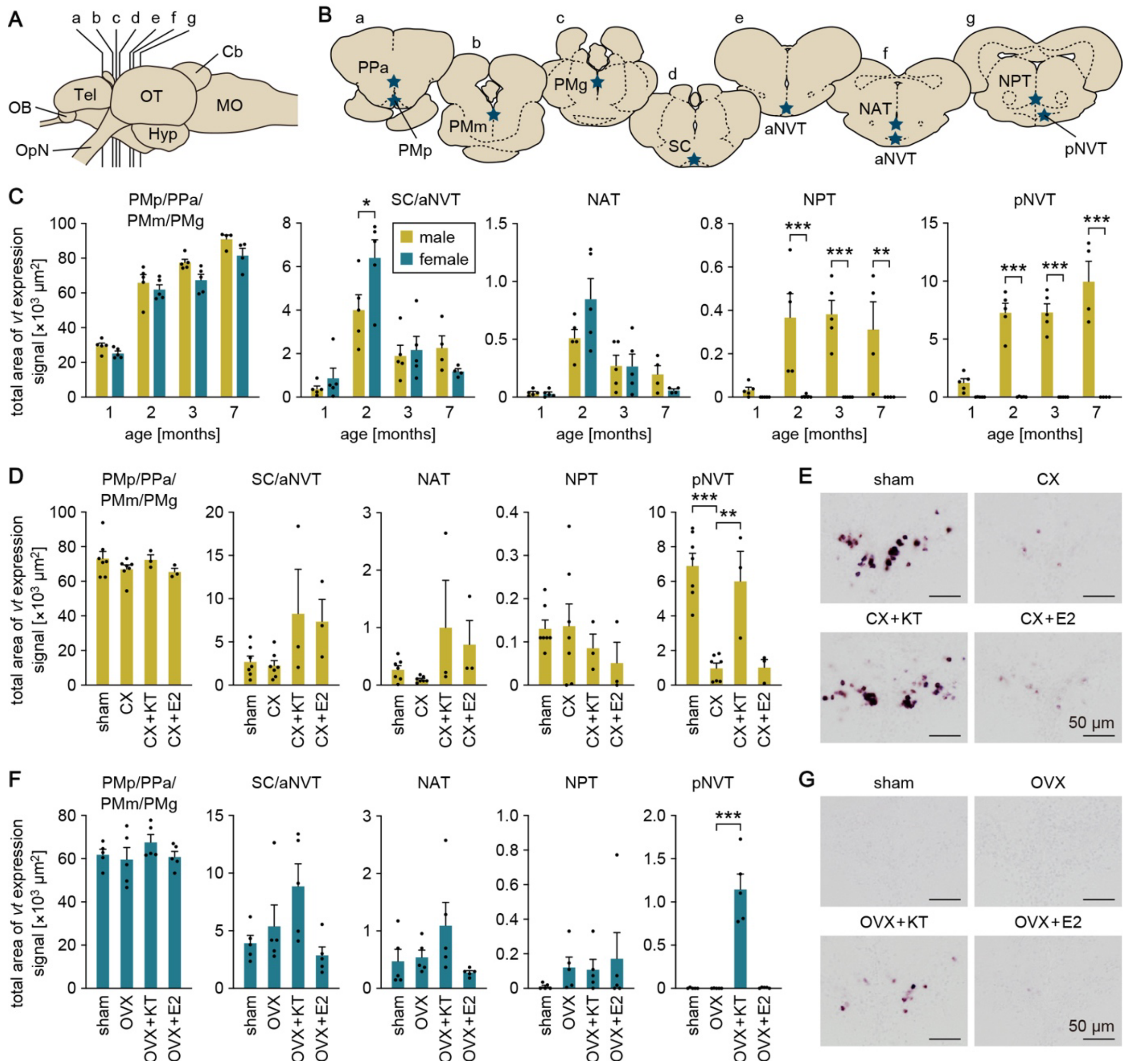
bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

588 278. doi:10.1016/j.neuron.2014.03.029
589 42. Yokoi S, Okuyama T, Kamei Y, Naruse K, Taniguchi Y, Ansai S, Kinoshita M, Young LJ,
590 Takemori N, Kubo T, Takeuchi H (2015) An essential role of the arginine vasotocin system in
591 mate-guarding behaviors in triadic relationships of medaka fish (*Oryzias latipes*). PLoS Genet
592 11:e1005009. doi:10.1371/journal.pgen.1005009

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

593 # Figures

594



595

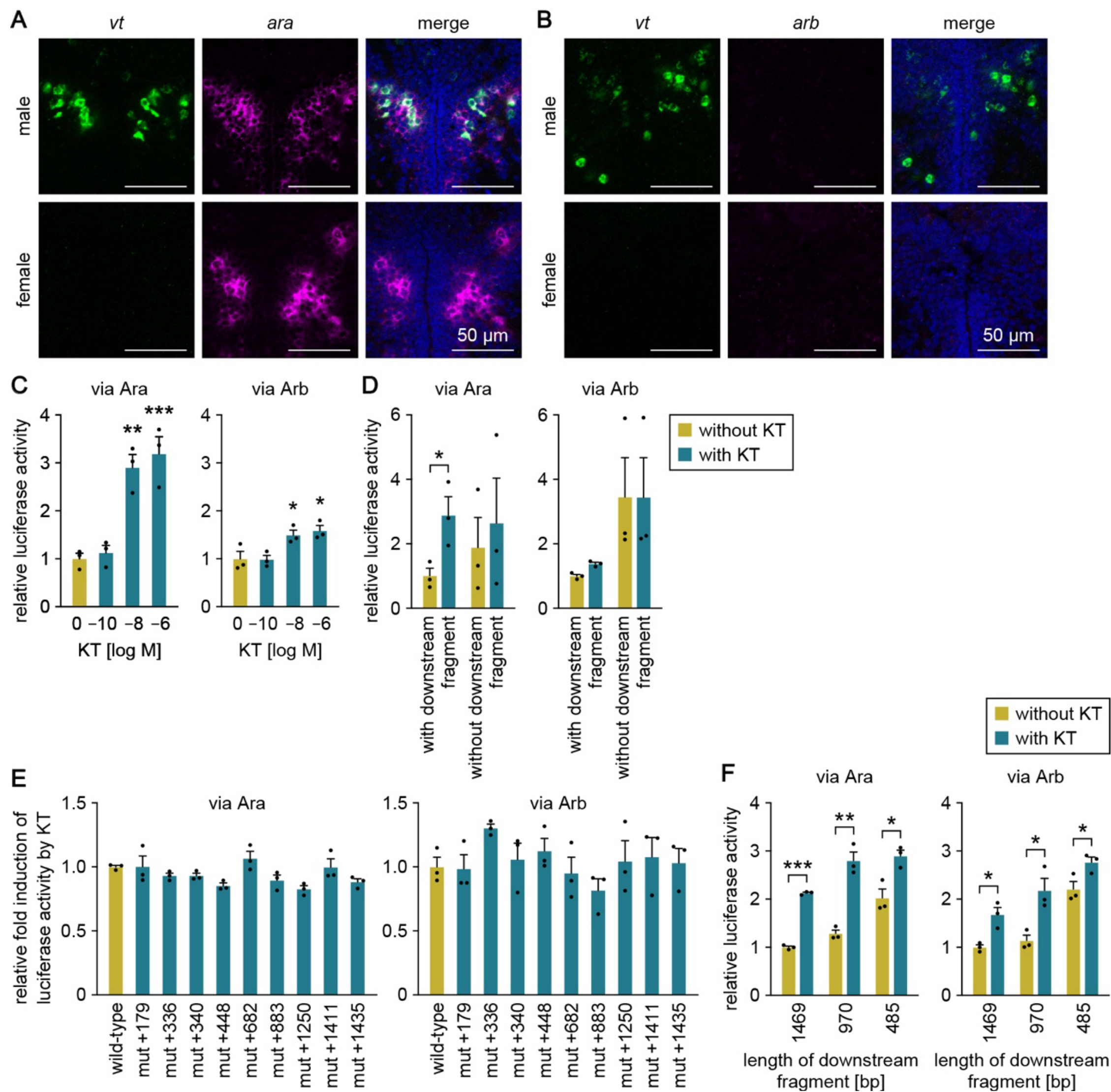
596

597 **Fig. 1. Male-specific *vt* expression in the tuberal hypothalamic nucleus is dependent on**
598 **post-pubertal gonadal androgens.** (A) Schematic drawing of the medaka brain (lateral view,
599 anterior to left) depicting the approximate levels of sections shown in panel B. (B) Coronal brain sections
600 containing nuclei in which *vt* is expressed (stars). See Supplementary Table 1 for abbreviations of brain
601 regions and nuclei. (C) Total area of *vt* expression signal in each brain nucleus of males (yellow columns)
602 and females (blue columns) at different ages (n = 5 per sex and age, except n = 4 at 7 months). (D) Total
603 area of *vt* expression signal in each brain nucleus of sham-operated males (sham) and castrated males
604 treated with vehicle alone (CX), KT (CX+KT), or E2 (CX+E2) (n = 7 for sham and CX; n = 3 for
605 CX+KT and CX+E2). (E) Representative images of *vt* expression in the pNVT of sham, CX, CX+KT,
606 and CX+E2 males. Scale bars are all 50 µm. (F) Total area of *vt* expression signal in each brain nucleus
607 of sham females and ovariectomized females treated with vehicle alone (OVX), KT (OVX+KT), or E2

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

608 (OVX+E2) (n = 5 for each group). (G) Representative images of *vt* expression in the pNVT of sham,
609 OVX, OVX+KT, and OVX+E2 females. Scale bars are all 50 μm. Statistical differences were
610 determined by Bonferroni's *post hoc* test (C, D, F). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



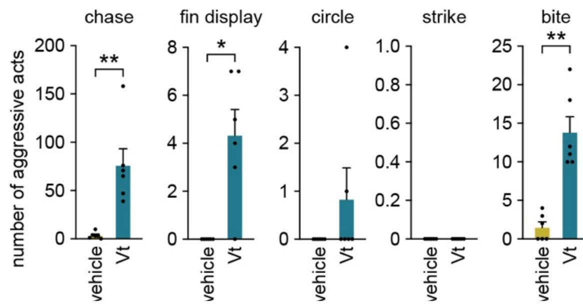
611
612

613 **Fig. 2. Androgens can directly activate the transcription of *vt* through Ara.** (A, B)
614 Representative images of the expression of *ara* (A) and *arb* (B) in the pNVT, where *vt* exhibits sexually
615 dimorphic expression. In each row, left panels show *vt* expression (green), middle panels show *ara*/*arb*
616 expression (magenta), and right panels show the merged images with DAPI staining (blue). Scale bars
617 are all 50 µm. (C) Stimulation of *vt* transcriptional activity by KT. A luciferase-based reporter construct
618 containing genomic fragments upstream of the first methionine codon and downstream of the stop codon
619 of *vt* was transfected into COS-7 cells, in conjunction with an Ara or Arb expression construct. Cells
620 were stimulated to varying concentrations of KT, and luciferase activity was measured. Fold induction
621 was calculated relative to unstimulated cells. (D) Effects of removing the downstream fragment on KT-
622 induced luciferase activity. A luciferase reporter construct with or without the downstream fragment
623 was transfected into cells, along with the Ara or Arb expression construct. Cells were stimulated with or
624 without KT (blue and yellow columns, respectively), and luciferase activity was determined. Fold
625 induction was calculated relative to the construct with the downstream fragment without KT stimulation.
626 (E) Effects of mutations in ARE-like sequences on KT-induced luciferase activity. A wild-type

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

627 luciferase reporter construct (wild-type) or a construct carrying a mutation in the ARE-like sequence at
628 position +179 (mut+179), +336 (mut+336), +340 (mut+340), +448 (mut+448), +682 (mut+682), +883
629 (mut+883), +1250 (mut+1250), +1411 (mut+1411), or +1435 (mut+1435) was transfected into cells,
630 along with the Ara or Arb expression construct. Cells were stimulated with or without KT, and fold
631 induction of luciferase activity was calculated relative to the wild-type construct. (F) Effects of 3′-
632 truncation of the downstream fragment on KT-induced luciferase activity. Cells were transfected with a
633 reporter construct containing 1469-bp, 970-bp, or 485-bp downstream fragment and an Ara or Arb
634 expression construct. Cells were stimulated with or without KT (blue and yellow columns, respectively),
635 and fold induction of luciferase activity was calculated relative to the construct containing the 1469 bp
636 fragment without KT stimulation. Statistical differences were determined by Dunnett's *post hoc* test
637 (versus unstimulated control (C) or wild-type construct (E)) and unpaired t-test (D and F). *$p < 0.05$;
638 **$p < 0.01$; ***$p < 0.001$.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.



639
640
641 **Fig. 3. Administration of exogenous Vt elicits aggression in females.** Shown is the sum of
642 each aggressive act (chase, fin display, circle, strike, and bite) exhibited by females receiving vehicle
643 only or Vt (n = 6 for each treatment). Statistical differences were determined by unpaired *t*-test with
644 Welch's correction. *$p < 0.05$; **$p < 0.01$.

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

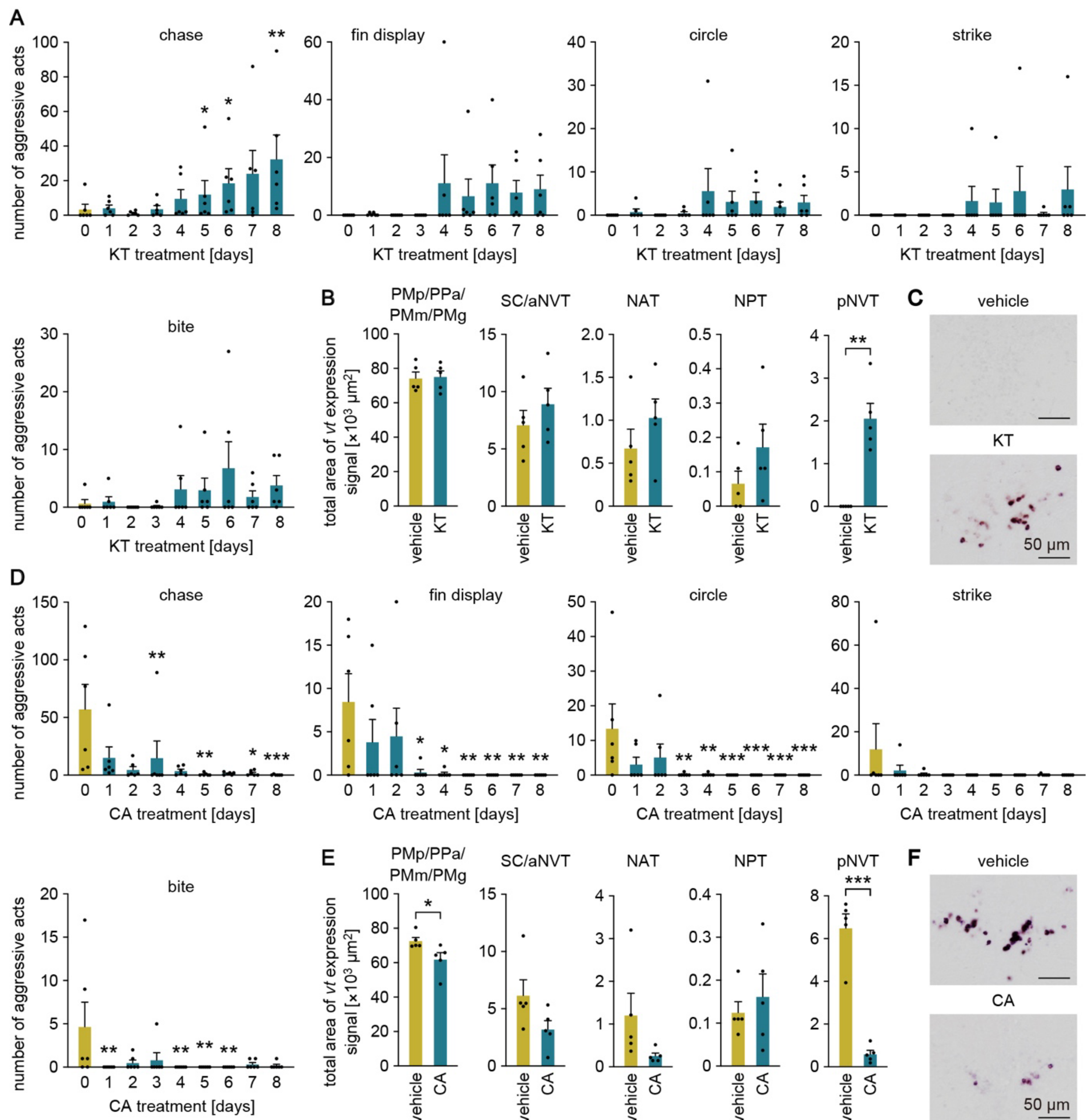


Fig. 4. Altering the androgen milieu leads to correlated changes in the levels of aggression and *vt* expression in the tuberal hypothalamus. (A) Daily changes in each aggressive act (chase, fin display, circle, strike, and bite) exhibited by KT-treated females (n = 6). (B) Total area of *vt* expression signal in each brain nucleus of vehicle- or KT-treated females (n = 5 per treatment). (C) Representative images of *vt* expression in the pNVT of vehicle- or KT-treated females. Scale bars are 50 µm. (D) Daily changes in each aggressive act (chase, fin display, circle, strike, and bite) exhibited by males treated with the androgen receptor antagonist CA (n = 6). (E) Total area of *vt* expression signal in each brain nucleus of vehicle- or CA-treated males (n = 5 per treatment). (F) Representative images of *vt* expression in the pNVT of vehicle- or CA-treated males. Scale bars are 50 µm. Statistical differences were determined by Dunnett's *post hoc* test (versus day 0) (A, D), except for the fin display data in panel D, which was determined by Dunn's *post hoc* test, and unpaired *t*-test with

bioRxiv preprint doi: https://doi.org/10.1101/2023.09.13.557517; this version posted September 13, 2023. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

658 or without Welch's correction (B, E). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.