
Figures and figure supplements

Male-predominant galanin mediates androgen-dependent aggressive chases in medaka

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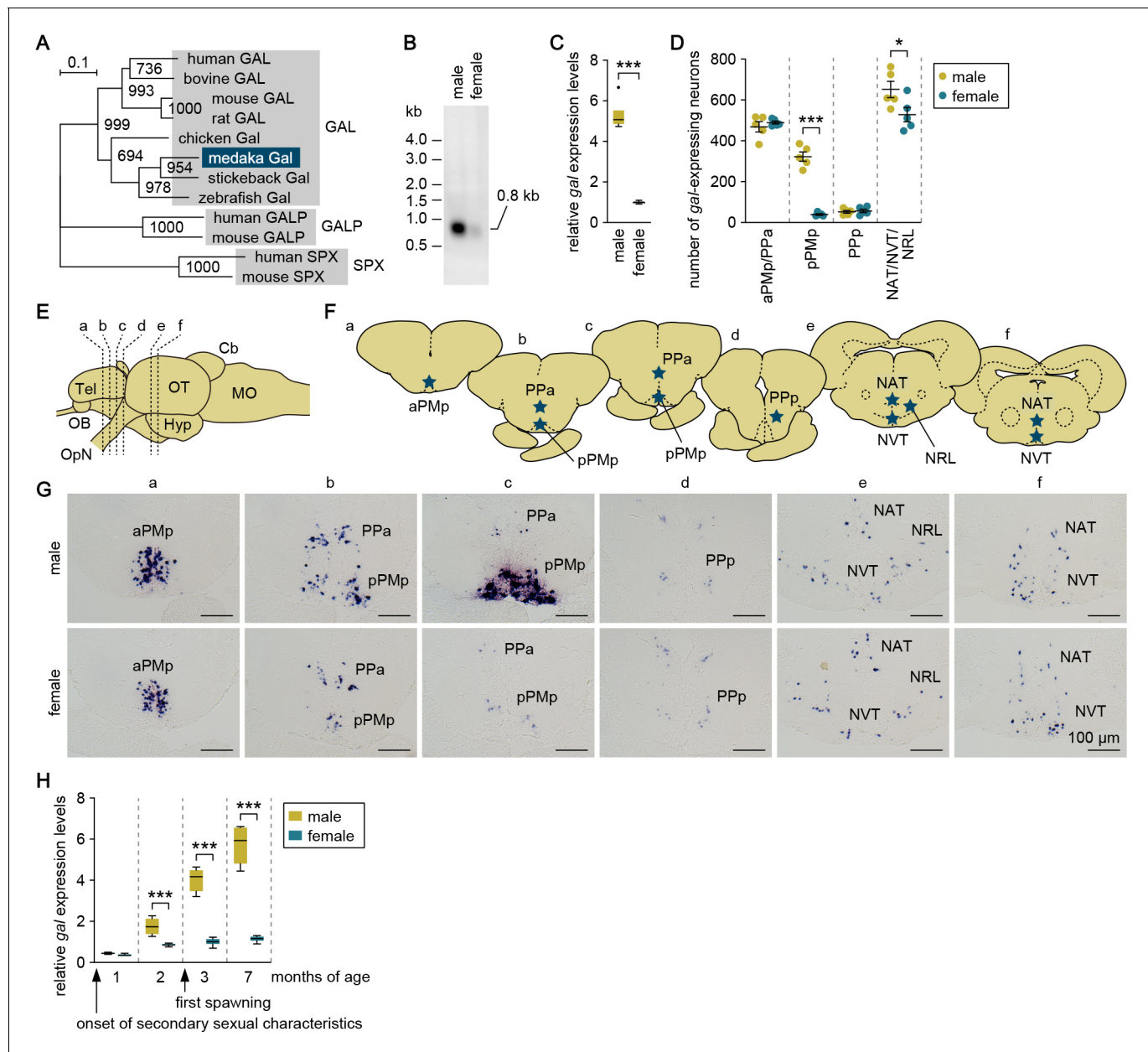


Figure 1. Male-biased sexual dimorphism exists in *gal* expression in the MPOA. (A) Phylogenetic tree showing the relationship of medaka Gal to other known GAL family proteins. The number at each node indicates bootstrap values for 1000 replicates. Scale bar represents 0.1 substitutions per site. GALP, galanin-like peptide; SPX, spexin hormone. For species names and GenBank accession numbers, see **Supplementary file 2**. (B) Detection of *gal* transcript in the whole brain of adult males and females by Northern blot analysis. Sizes (in kb) of RNA markers and the detected band are indicated on the left and right, respectively. (C) Confirmation of male-biased expression of *gal* in adult whole brain by real-time PCR ($n = 8$ per sex). *** $p < 0.001$ (unpaired *t*-test with Welch's correction). (D) Number of *gal*-expressing neurons in each brain nucleus of adult males and females ($n = 5$ per sex). * $p < 0.05$; *** $p < 0.001$ (unpaired *t*-test). (E) Line drawing of a lateral view (anterior to the left) of the medaka brain showing the approximate levels of sections in panel G. (F) Line drawing of coronal sections showing the location of brain nuclei containing *gal*-expressing neurons (stars). (G) Representative micrographs showing *gal*-expressing neurons in each brain nucleus of adult males (upper panels) and females (lower panels). Scale bars represent 100 μm . (H) Levels of *gal* expression in the whole brain of males and females during growth and sexual maturation ($n = 8$ per sex and stage). *** $p < 0.001$ (Bonferroni's post hoc test). For abbreviations of brain regions and nuclei, see **Supplementary file 1**. See also **Figure 1—figure supplement 1**.

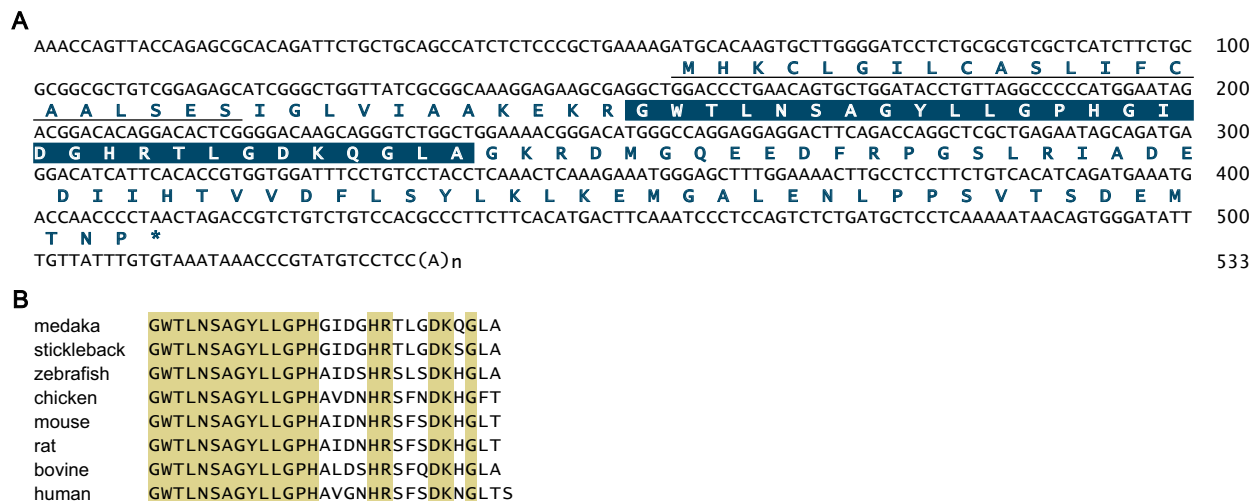


Figure 1—figure supplement 1. Sequence information for medaka *gal*. (A) Nucleotide and deduced amino acid sequences of the medaka *gal* cDNA. The predicted signal peptide is underlined and the mature Gal polypeptide is boxed. Asterisk indicates stop codon. Nucleotide numbers are shown at the right of each sequence line. (B) Comparison of mature GAL polypeptide sequences from medaka and other vertebrate species. Amino acids identical in all sequences are shaded in beige. For species names and GenBank accession numbers, see **Supplementary file 2**.

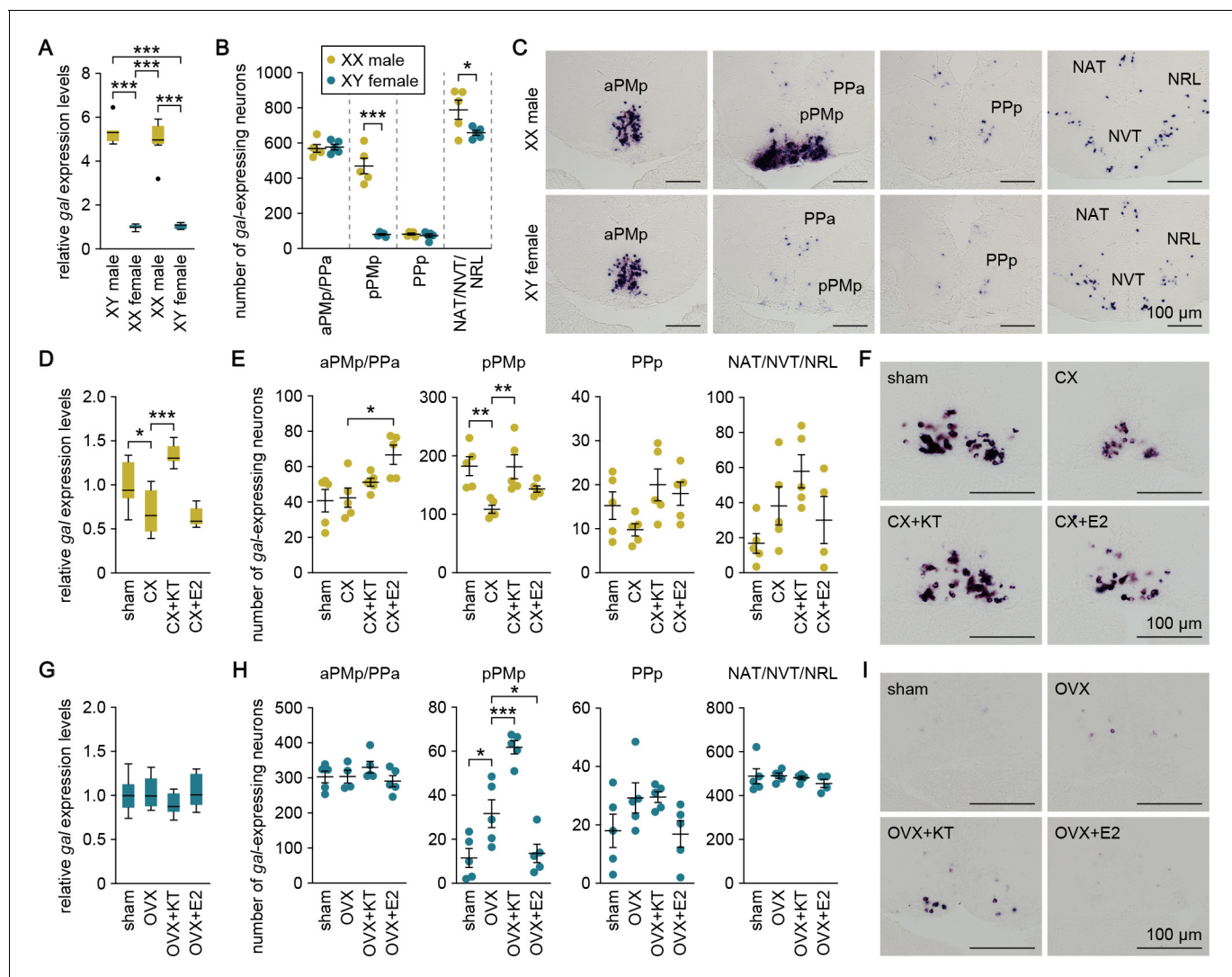


Figure 2. Sexually dimorphic *gal* expression is dependent on adult sex steroids. (A) Levels of *gal* expression in the whole brain of sex-reversed adult XX males and XY females versus typical adult XY males and XX females (n = 8 per group). ***p<0.001 (Bonferroni's post hoc test). (B) Number of *gal*-expressing neurons in each brain nucleus of sex-reversed adult XX male and XY females (n = 5 per group). *p<0.05; ***p<0.001 (unpaired t-test). (C) Representative micrographs showing *gal*-expressing neurons in each brain nucleus of sex-reversed adult XX males (upper panels) and XY females (lower panels). (D) Levels of *gal* expression in the whole brain of sham-operated males (sham; n = 8) and castrated males exposed to vehicle alone (CX; n = 8), KT (CX+KT; n = 9), or E2 (CX+E2; n = 6). *p<0.05; ***p<0.001 (Bonferroni's post hoc test). (E) Number of *gal*-expressing neurons in each brain nucleus of sham, CX, CX+KT, and CX+E2 males (n = 5 per group). *p<0.05; **p<0.01 (Bonferroni's post hoc test). (F) Representative micrographs showing *gal* expression in pMPp of sham, CX, CX+KT, and CX+E2 males. (G) Levels of *gal* expression in the whole brain of sham females and ovariectomized females exposed to vehicle alone (OVX), KT (OVX+KT), or E2 (OVX+E2) (n = 12 per group). (H) Number of *gal*-expressing neurons in each brain nucleus of sham, OVX, OVX+KT, and OVX+E2 females (n = 5 per group). *p<0.05; ***p<0.001 (Bonferroni's post hoc test). (I) Representative micrographs showing *gal* expression in pMPp of sham, OVX, OVX+KT, and OVX+E2 females. Scale bars represent 100 μ m. For abbreviations of brain nuclei, see **Supplementary file 1**. See also **Figure 2—figure supplement 1**.

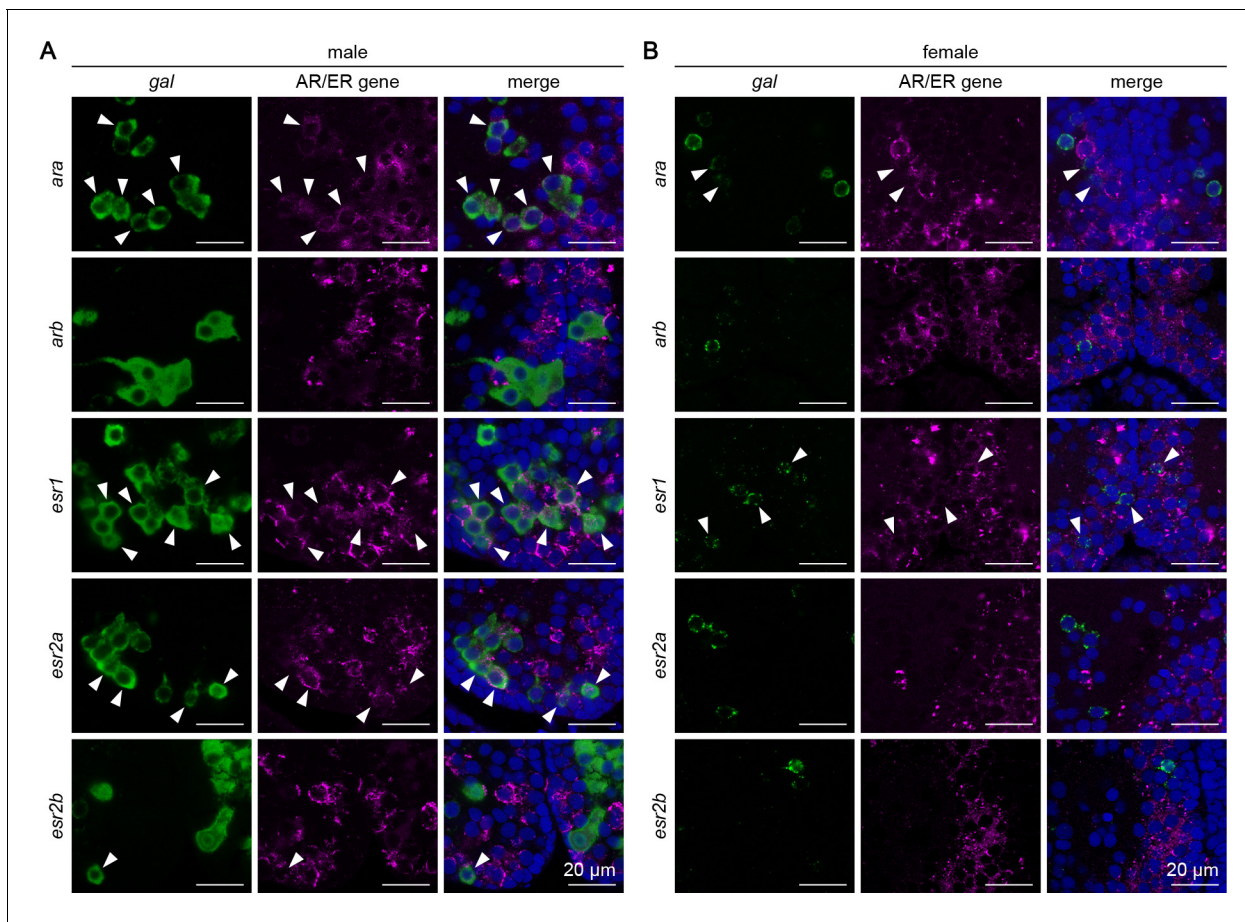


Figure 2—figure supplement 1. Expression of sex steroid receptors in sexually dimorphic *gal* neurons in pMP. Representative micrographs showing the expression of androgen receptor (AR) and estrogen receptor (ER) genes in pMP, where sexually dimorphic *gal*-expressing neurons reside, in males (A) and females (B). In each row, left and middle panels show images of, respectively, *gal* (green) and ER/AR gene (magenta) expression in the same section; right panels show the merged images with nuclear counterstaining (blue). Arrowheads indicate representative neurons coexpressing *gal* and ER/AR. Scale bars represent 20 μm.

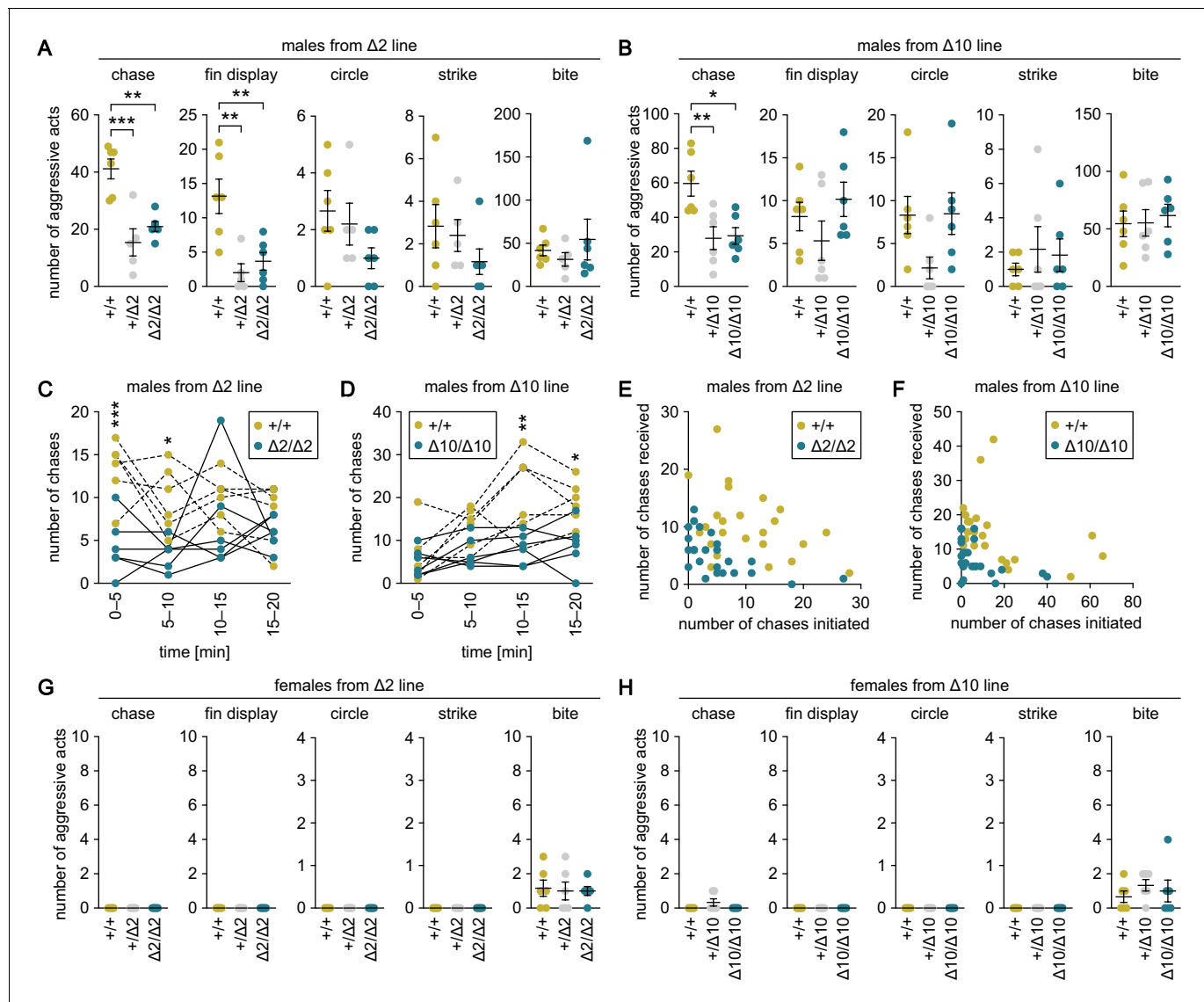


Figure 3. Genetic ablation of *gal* specifically suppresses male–male chases. (A, B) Sum of each aggressive behavioral act (chase, fin display, circle, strike, and bite) performed by wild-type ($+/+$), heterozygous ($+/ \Delta 2$ and $+/ \Delta 10$), and homozygous ($\Delta 2 / \Delta 2$ and $\Delta 10 / \Delta 10$) males from $\Delta 2$ (A) and $\Delta 10$ (B) *gal* knockout lines. $n = 6$ per group, except $+/ \Delta 2$ males, where $n = 5$. $**p < 0.01$; $***p < 0.001$ (Bonferroni's post hoc test). (C, D) Number of chases performed by wild-type and homozygous males from $\Delta 2$ (C) and $\Delta 10$ (D) *gal* knockout lines for each 5-min interval. $n = 6$ per genotype. Asterisks indicate significant differences between the two genotypes in the same time interval. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Bonferroni's post hoc test). (E, F) Number of chases initiated (x-axis) and received (y-axis) by each wild-type and homozygous male from $\Delta 2$ (E) and $\Delta 10$ (F) *gal* knockout lines. (G, H) Sum of each aggressive behavioral act (chase, fin display, circle, strike, and bite) performed by wild-type, heterozygous, and homozygous females from $\Delta 2$ (G) and $\Delta 10$ (H) *gal* knockout lines. $n = 6$ per group. See also **Figure 3—figure supplement 1** and **Figure 3—figure supplement 2**.

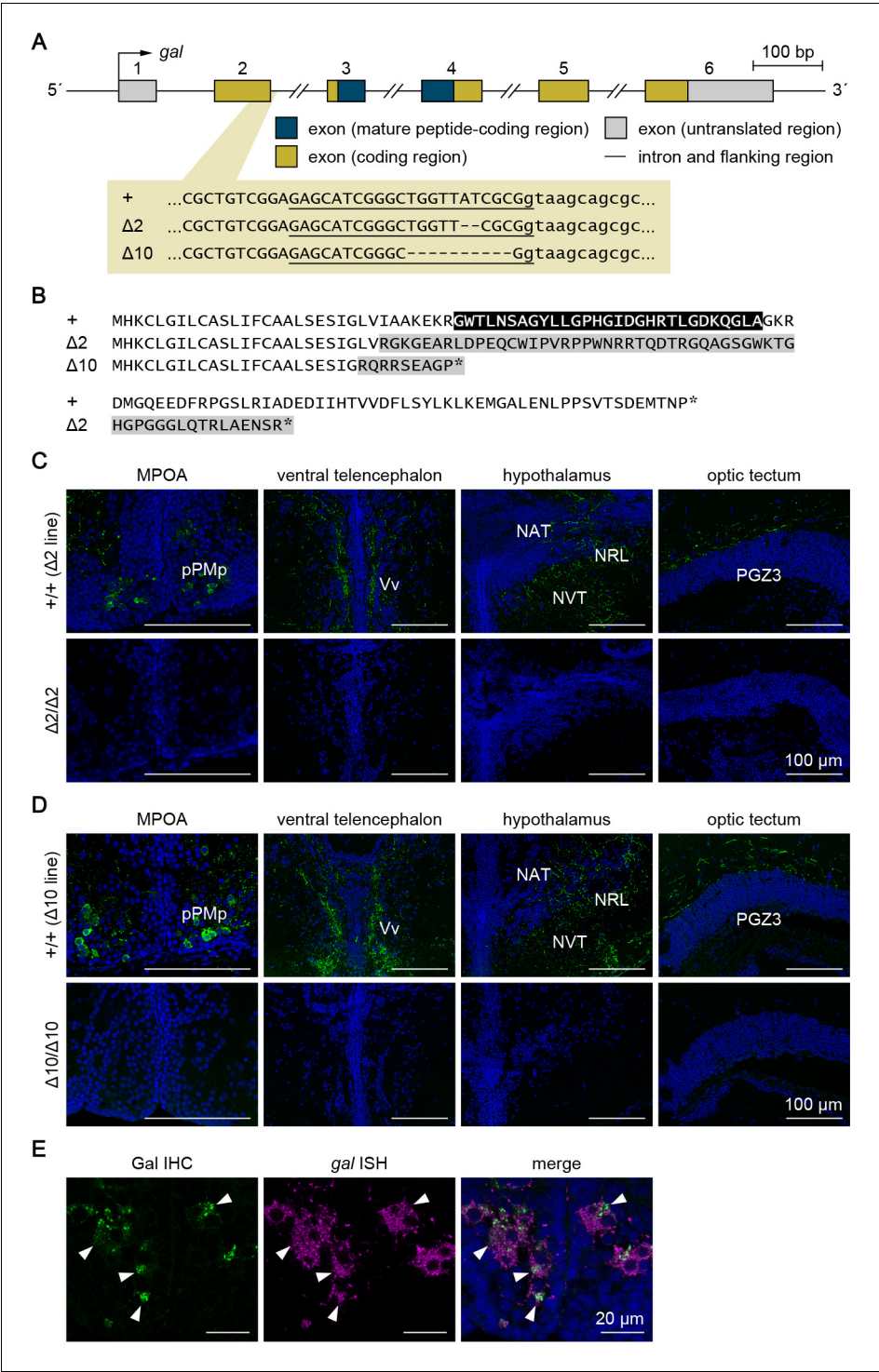


Figure 3—figure supplement 1. Generation and verification of *gal* knockout medaka. Two different lines of *gal* knockout medaka (Δ2 and Δ10) were generated by CRISPR/Cas9-mediated genome editing. **(A)** Gene structure of *gal* showing the location of the CRISPR target site, which is enlarged to show the nucleotide sequences of the wild-type (+) and targeted (Δ2 and Δ10) alleles. Exon numbers are indicated above each exon. Exon and intron sequences are given in uppercase and lowercase letters, respectively. The target sequence complementary to the CRISPR RNA is underlined, and deleted nucleotides are indicated by dashes. **(B)** Comparison of the deduced Gal precursor protein sequences of the +, Δ2, and Δ10 alleles. The mature Gal polypeptide is indicated in white letters on a black background. The altered sequence caused by a frameshift is shaded in gray. Asterisks indicate stop

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Figure 3—figure supplement 1 continued

codons. (C, D) Successful ablation of *gal* in the $\Delta 2$ (C) and $\Delta 10$ (D) knockout lines was confirmed by the observation of Gal immunoreactivity (green) in $+/+$ males but not in $\Delta 2/\Delta 2$ or $\Delta 10/\Delta 10$ males ($n = 4$ per group). Shown are representative micrographs of the MPOA containing pPMP, where the cell bodies of Gal-expressing neurons reside, and the ventral telencephalon, hypothalamus, and optic tectum, where Gal-containing axons are densely distributed. Blue color indicates nuclear counterstaining. Scale bars represent 100 μm . For abbreviations of brain nuclei, see **Supplementary file 1**. (E) The specificity of the anti-GAL antibody was verified by a pattern of labeling consistent with *gal*-expressing neurons detected by in situ hybridization (ISH). Left and middle panels show images of, respectively, immunohistochemistry (IHC) using anti-GAL antibody (green) and ISH detecting *gal* expression (magenta) in the same pPMP section; right panel shows the merged image with nuclear counterstaining (blue). Arrowheads indicate representative neuronal cell bodies labeled by both IHC and ISH. Scale bars represent 20 μm .

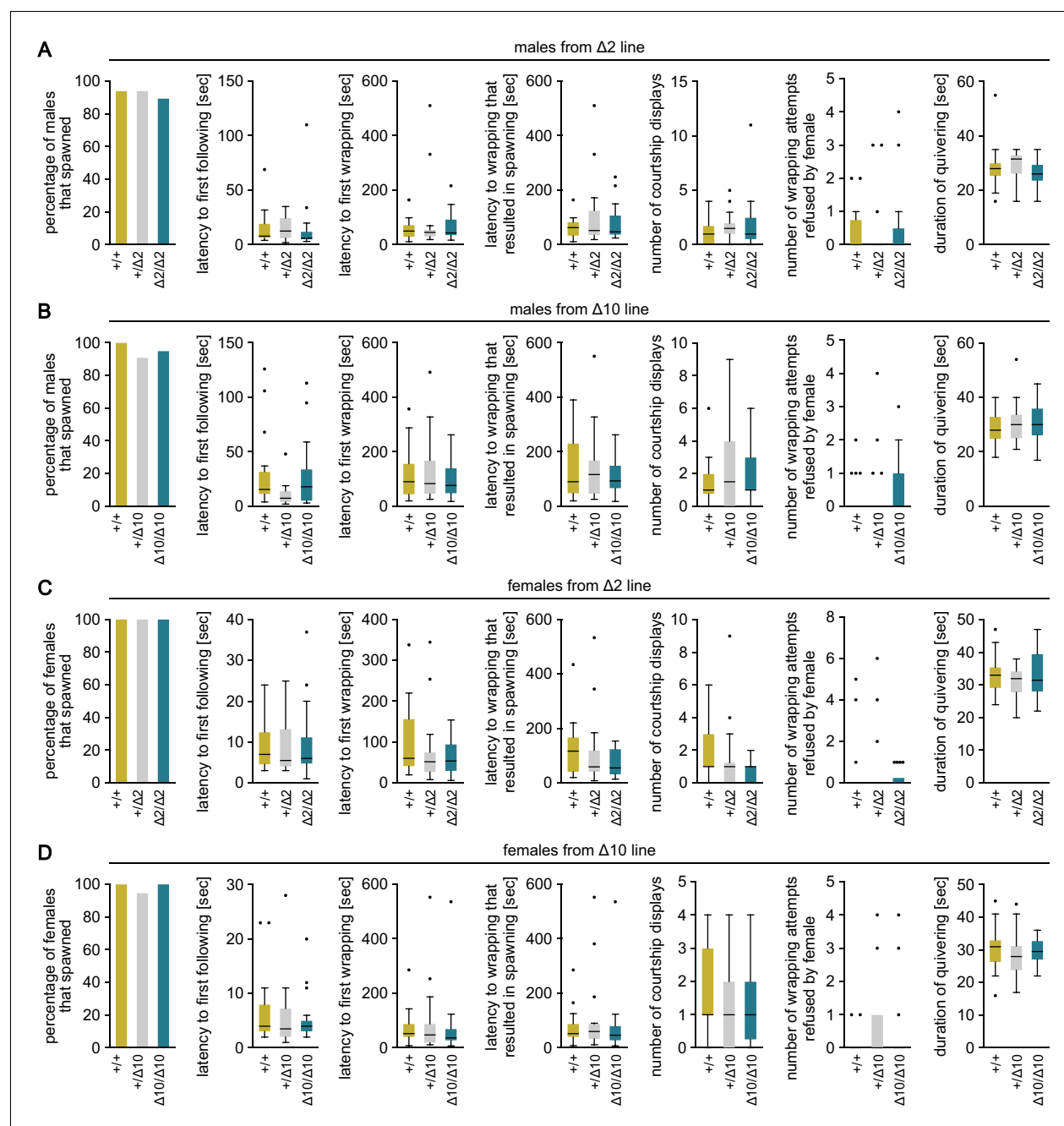


Figure 3—figure supplement 2. Mating behavior of *gal* knockout medaka. Various parameters in the mating behavior of males (A, B) and females (C, D) from $\Delta 2$ (A, C) and $\Delta 10$ (B, D) *gal* knockout lines. Results were compared among wild-type (+/+), heterozygous (+/ $\Delta 2$ and +/ $\Delta 10$), and homozygous ($\Delta 2/\Delta 2$ and $\Delta 10/\Delta 10$) genotypes. $n = 17$ – 22 per group.

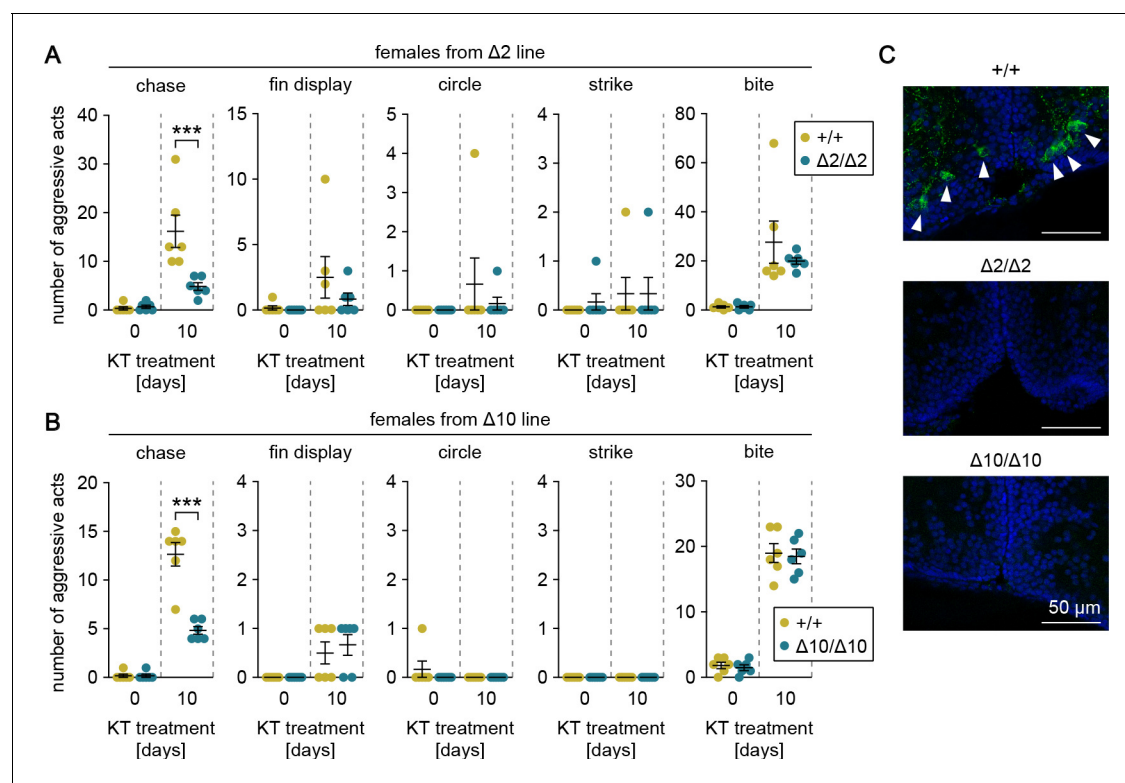


Figure 4. Genetic ablation of *gal* attenuates androgen-induced chases in females. (**A**, **B**) Sum of each aggressive behavioral act (chase, fin display, circle, strike, and bite) performed by KT-treated females from $\Delta 2$ (**A**) and $\Delta 10$ (**B**) *gal* knockout lines. Asterisks indicate significant differences between wild-type (+/+) and homozygous ($\Delta 2/\Delta 2$ and $\Delta 10/\Delta 10$) genotypes on the same day. $n = 6$ per group. *** $p < 0.001$ (Bonferroni's post hoc test). (**C**) Representative micrographs of coronal pMPp sections from KT-treated females of $\Delta 2$ and $\Delta 10$ *gal* knockout lines showing the induction of Gal-expressing neurons in wild-type but not homozygous knockout females. Arrowheads indicate Gal-immunoreactive (green) neuronal cell bodies. Blue color indicates nuclear counterstaining. Scale bars represent 50 μ m.

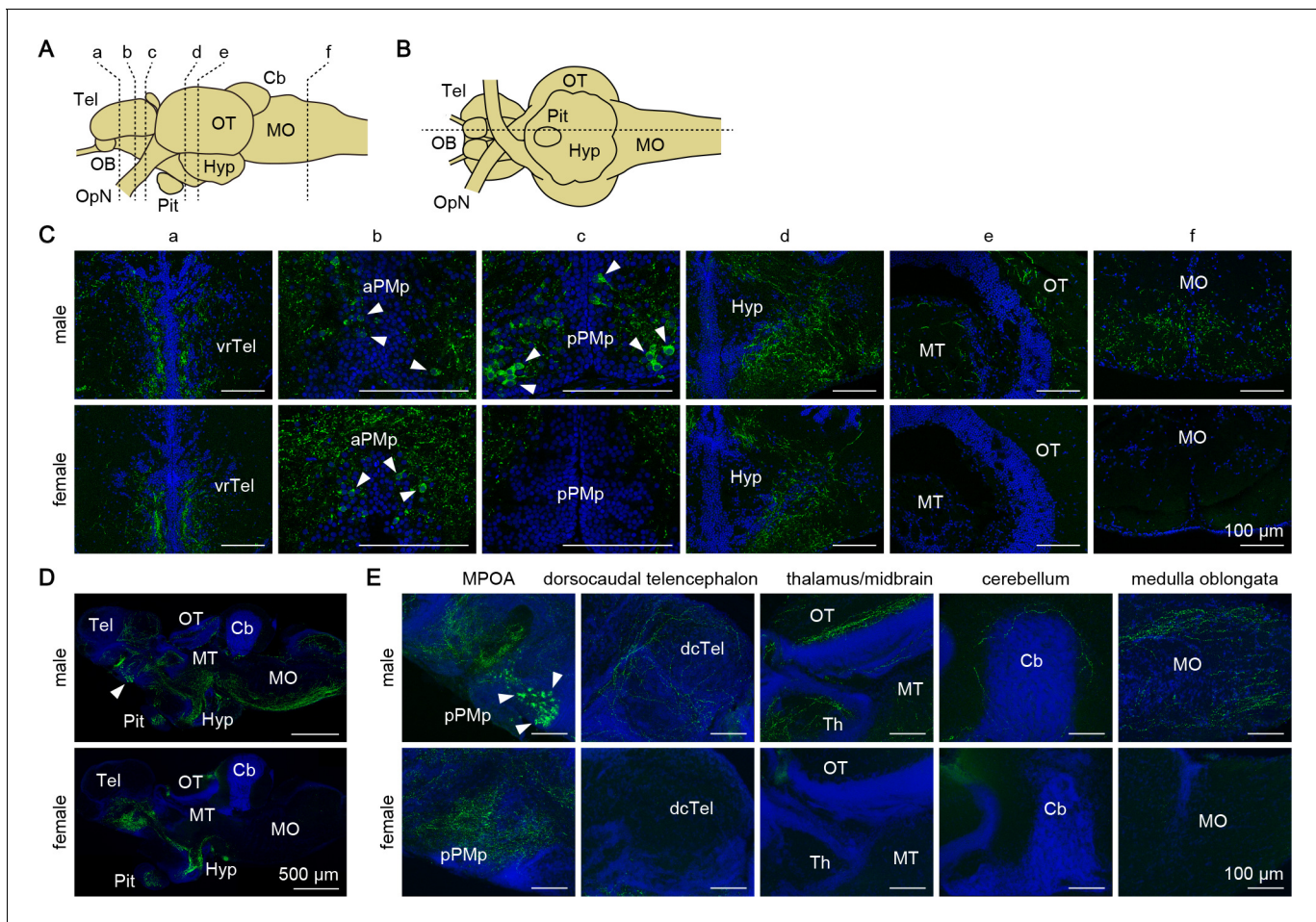


Figure 5. Gal peptide produced male-predominantly is transported to various brain regions. (A, B) Line drawings of lateral (A) and ventral (B) views (anterior to the left) of the medaka brain showing the approximate levels of sections in panels C and D, respectively. (C) Representative micrographs of coronal brain sections from adult males (upper panels) and females (lower panels) showing the distribution of Gal-immunoreactive cell bodies and axons. Arrowheads indicate Gal-immunoreactive neuronal cell bodies. Scale bars represent 100 μm . (D, E) Representative low (D) and high (E) magnification micrographs of sagittal brain sections (anterior to the left) from adult males (upper panels) and females (lower panels) showing the distribution of Gal-immunoreactive cell bodies and axons. Arrowheads indicate Gal-immunoreactive neuronal cell bodies in pPMp. Scale bars represent 500 μm (D) and 100 μm (E). For abbreviations of brain regions and nuclei, see **Supplementary file 1**. See also **Figure 5—figure supplement 1**.

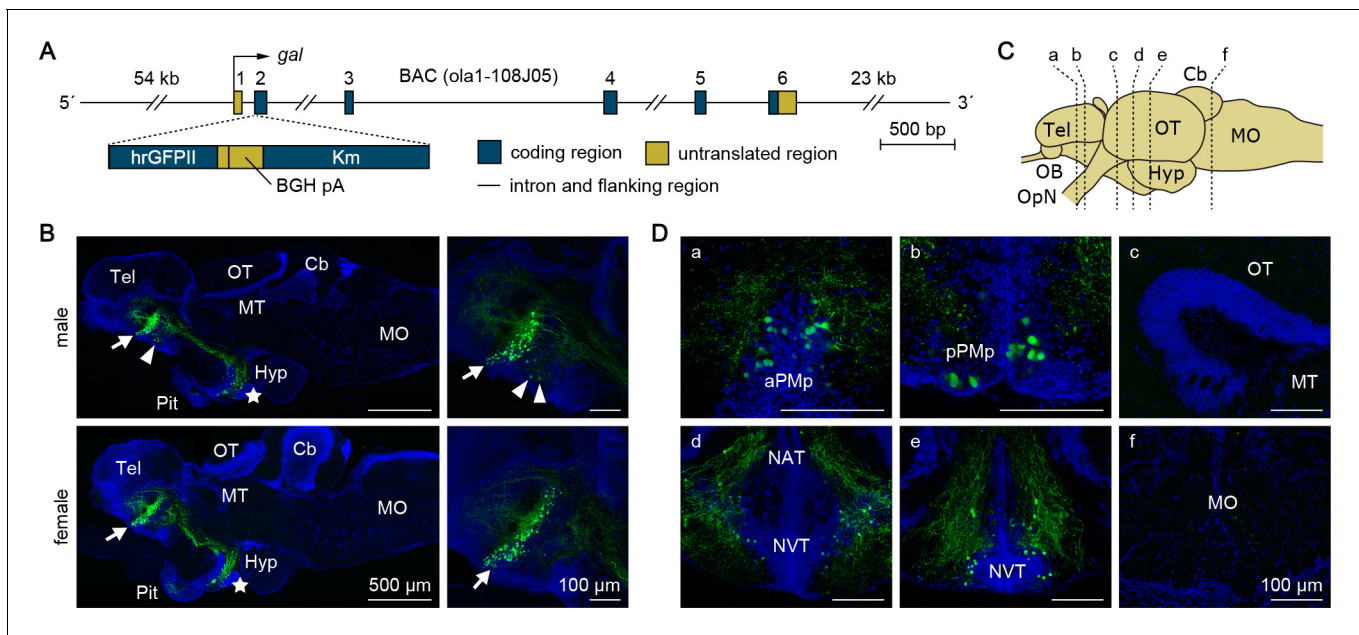


Figure 5—figure supplement 1. Generation and fluorescence imaging of *gal*-GFP transgenic medaka. (A) Structure of the transgene in *gal*-GFP transgenic medaka. A 7 bp sequence containing the translation initiation site of *gal* in a medaka BAC clone (clone ID: ola1-108J05) was replaced by a 2136 bp DNA cassette containing the humanized *Renilla reniformis* GFP II (hrGFP II)-coding sequence, bovine growth hormone polyadenylation signal (BGH pA), and kanamycin resistance gene (Km). This BAC clone contains the whole transcriptional unit of *gal* together with 54 kb of 5'-flanking and 23 kb of 3'-flanking sequences. (B) Representative micrographs of sagittal brain sections (anterior to the left) from adult male (upper panels) and female (lower panels) *gal*-GFP transgenic medaka showing the distribution of GFP expression. Left panels show low-magnification images of the whole brain; right panels show high-magnification images of the MPOA. Arrows and arrowheads indicate GFP-labeled neuronal cell bodies in aPMp/PPa and pPMp, respectively; stars indicate those in NAT/NVT/NRL. Scale bars represent 500 μ m (left panels) and 100 μ m (right panels). (C) Line drawing of a lateral view (anterior to the left) of the medaka brain showing the approximate levels of sections in panel D. (D) Representative micrographs of coronal brain sections from *gal*-GFP transgenic medaka showing the distribution of GFP expression. Only images of males are presented because there were no obvious sex differences in the distribution of GFP expression. Scale bars represent 100 μ m. For abbreviations of brain regions and nuclei, see **Supplementary file 1**.

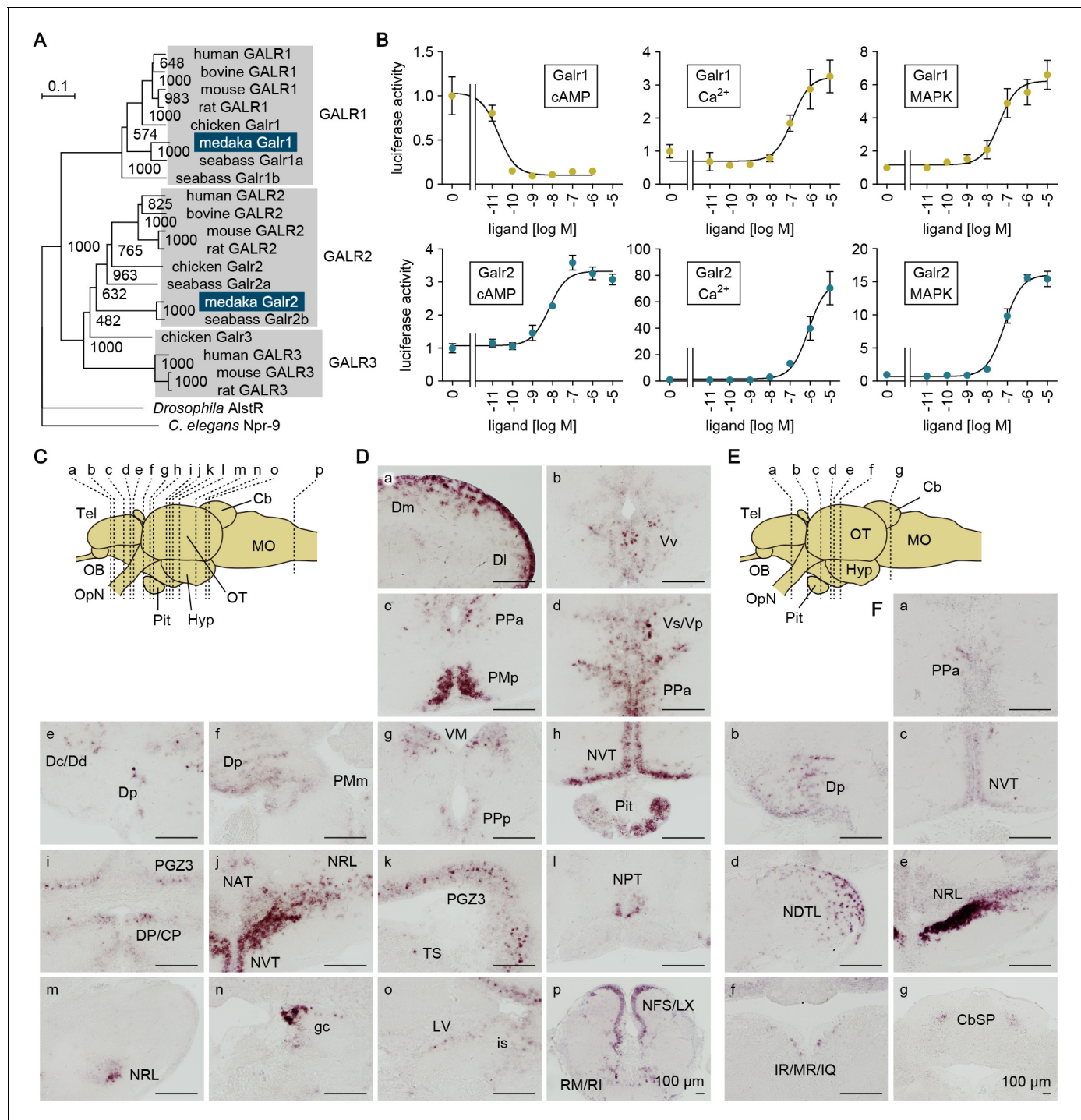


Figure 6. Gal receptors coupled to different signaling pathways are expressed widely in the brain. (A) Phylogenetic tree showing the relationship of medaka Galr1 and Galr2 to other known GAL receptors. The number at each node indicates bootstrap values for 1000 replicates. Scale bar represents 0.1 substitution per site. AlstR, allatostatin receptor. For species names and GenBank accession numbers, see **Supplementary file 2**. (B) Intracellular signaling pathways initiated by the activation of medaka Galr1 (upper graphs) and Galr2 (lower graphs). Cells transfected with Galr1 or Galr2 were stimulated with increasing concentrations of Gal and assayed for luciferase activity indicative of intracellular cAMP levels (left panels), Ca^{2+} levels (middle panels), and MAPK (right panels) ($n = 3$). x-axis shows the concentration of Gal; y-axis shows the fold change in luciferase activity relative to the basal level, which was measured in the absence of Gal. (C) Line drawing of a lateral view (anterior to the left) of the medaka brain showing the approximate levels of sections in panel D. (D) Distribution of *galr1* expression in the brain and pituitary. (E) Line drawing of a lateral view (anterior to the left) of the medaka brain showing the approximate levels of sections in panel F. (F) Distribution of *galr2* expression in the brain and pituitary. All images Figure 6 continued on next page

Figure 6 continued

are coronal sections. Only images of males are shown because there were no obvious sex differences in the distribution of expression ($n = 5$ per sex). Scale bars represent 100 μm . For abbreviations of brain regions and nuclei, see **Supplementary file 1**. See also **Figure 6—figure supplement 1**.

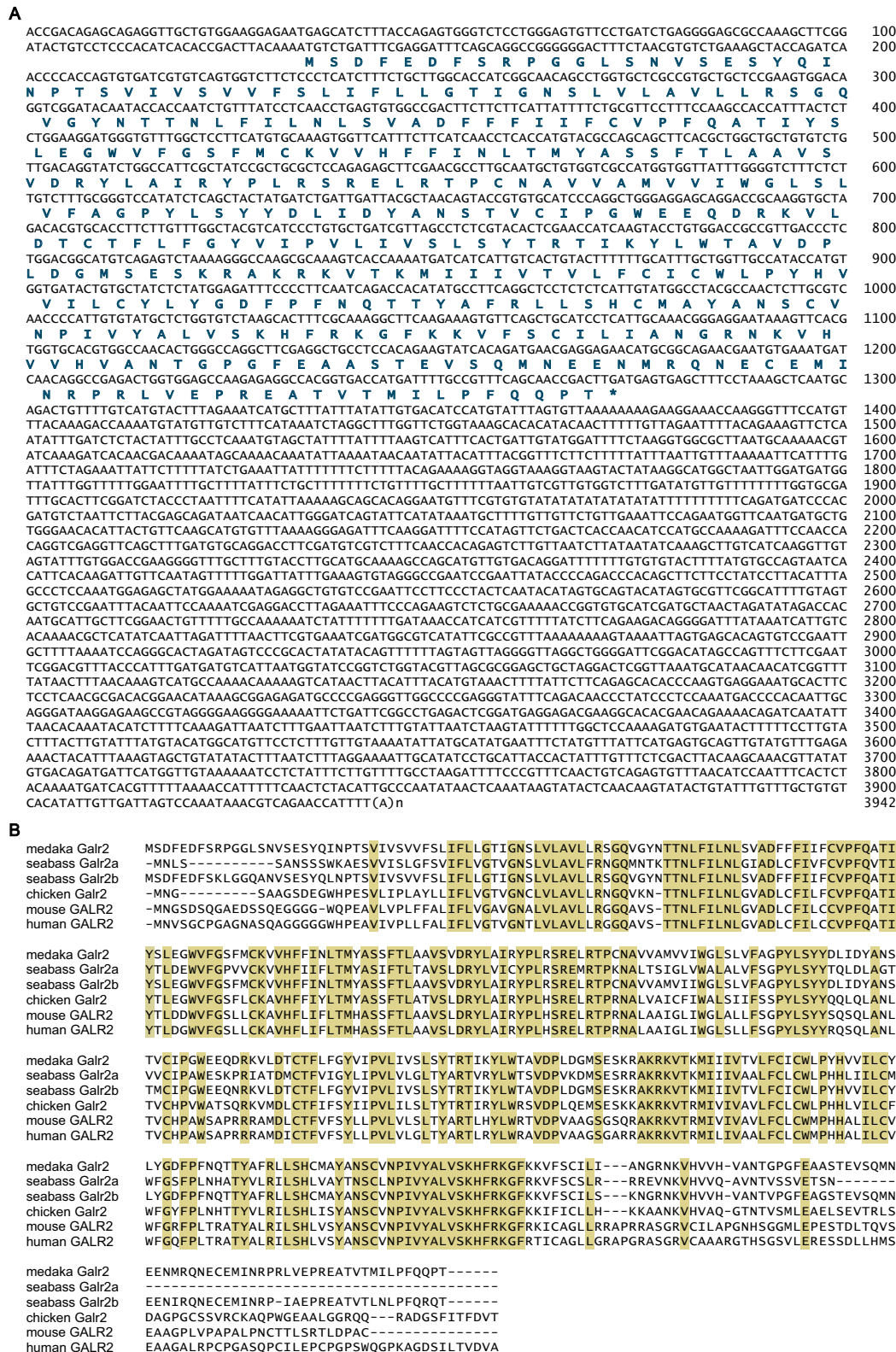


Figure 6—figure supplement 1. Sequence information for medaka *galr2*. (A) Nucleotide and deduced amino acid sequences of the medaka *galr2* cDNA. Asterisk indicates stop codon. Nucleotide numbers are shown at the right of each sequence line. (B) Sequence comparison of medaka Galr2 and Figure 6—figure supplement 1 continued on next page

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its orthologs in other vertebrate species. Amino acids identical in all sequences are shaded in beige. For species names and GenBank accession numbers, see **Supplementary file 2**. Supplementary Data List.