Sex-Specific Role for Dopamine Receptor D2 in Dorsal Raphe Serotonergic Neuron Modulation of Defensive Acoustic Startle and Dominance Behavior

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Visual Abstract
A subtype of dorsal raphe (DR) serotonergic neuron, denoted Drd2-Pet1, is poised for regulation by dopamine (DA) via type-2 DA receptor (DRD2) expression. Functional removal of DRD2 in these cells through a conditional knockout (CKO) mouse strategy resulted in sex-specific behavioral abnormalities: Drd2-Pet1-CKO females exhibited reduced acoustic startle while males showed increased social dominance. Drd2-Pet1 neurons were similar in number and distribution in males versus females but exhibited sex-specific differences in neurotransmission-related mRNAs, action potential (AP) duration, and relative distribution of collaterals. Abnormalities in sensory processing and social behaviors akin to those reported here manifest in autism, schizophrenia, and posttraumatic stress disorder, in sex-specific ways. Our findings, thus, may point to novel circuits and modulatory pathways relevant to human neuropsychiatric conditions.

Key words: acoustic startle; dominance; dopamine receptor; raphe; serotonin; sex differences

Introduction

The serotonergic and dopaminergic neurotransmitter systems are known for their influence on and maladaptation in neuropsychiatric disorders, including posttraumatic stress disorder, autism spectrum disorder, and schizophrenia. Clinical and animal studies implicate serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA) in modulation of endophenotypes common to neuropsychiatric disorders, such as altered social interaction and sensory processing (Geyer and Braff, 1987; Meinecke et al., 2004; Takahashi and Kamio, 2018). Transcriptome data coupled with structure-function maps in mice show that the serotonergic and dopaminergic neuronal systems are themselves heterogeneous, comprised of functionally specialized neuronal subtypes, manifesting distinct mRNA profiles, effenter projections, electrophysiological properties, and functions (Jensen et al., 2008; Kim et al., 2009; Crawford et al., 2013; Lammel et al., 2014; Spaethling et al., 2014; Okaty et al., 2015; Deneris and Gaspar, 2018; Poulin et al., 2018, 2020; Huang et al., 2019; Ren et al., 2019; Okaty et al., 2020). An important subtype of serotonergic neuron as relates to social and defensive behaviors is denoted Drd2-Pet1 (Niederkofler et al., 2016), identified by expression of the type-2 DA receptor (Drd2) gene and the serotonergic transcription factor gene Pet1 (aka Fov). DRD2 agnosm in slice preservation droved outward (inhibitory) currents cell-autonomously in Drd2-Pet1 neurons, suppressing their excitability; and when these cells were constitutively silenced in male mice, i.e., exocytic neurotransmitter release was cell autonomously blocked, defensive, aggressive, and exploratory behaviors increased (Niederkofler et al., 2016). Here, we query whether Drd2 expression in Drd2-Pet1 cells contributes to the modulation of defensive, exploratory behaviors.

While Drd2 is expressed in many cell types throughout the midbrain and basal forebrain, expression in serotonergic neurons is restricted to a small subset of cells resident in the dorsal raphe (DR) nucleus. In these serotonergic neurons, Drd2 expression initiates around adolescence...
and continues through adulthood, at which point, Drd2 transcripts are the major DA receptor mRNA detected (Niederkofler et al., 2016). Thus, Drd2-Pet1 neurons come under DRD2 and presumably DA regulation during the developmental transition to sexual maturity. Drd2-Pet1 neurons project to brain regions involved in sensory processing, defensive, and mating behaviors including auditory brainstem regions and the sexually dimorphic medial preoptic area (mPOA; Niederkofler et al., 2016). These findings led us to hypothesize that DRD2 signaling in Drd2-Pet1 neurons contributes to social and sensory alertness and defensive behavior in a sex-specific manner.

Indeed, serotonergic and dopaminergic perturbations affect social and defensive behaviors differently in male versus female rodents. Decreases in serotonergic tone associate with increased levels of aggression in males (Brown et al., 1982; Hendricks et al., 2003; Yu et al., 2014; Niederkofler et al., 2016). By contrast, lesions of the serotonergic DR in female rats decreased maternal aggression (Holschbach et al., 2018), while DR serotonergic neuron activity in female, but not male, hamsters associates with social dominance (Terranova et al., 2016). The acoustic startle reflex (ASR), an evolutionarily-conserved, defensive reflex to loud, potentially threatening stimuli (Davis et al., 1982), also shows sex-specific differences within the context of altered 5-HT levels. Reduction in 5-HT levels enhanced ASR in female but not male rats (Pettersson et al., 2011; Jax #020631) females. From these crosses, ePet-Cre;Drd2loxP/loxP females were then bred to homozygous Drd2loxP/loxP males for ePet-Cre;Drd2loxP/loxP male and female offspring used for experiments. Experimental controls were littermates with the Drd2loxP/loxP genotype thus negative for Cre but of comparable genetic background (C57BL/6J, Jax #000664). For Drd2-Pet1 neuron cell counts, triple transgenic Drd2-Cre;Pet1-Flpe;RC-FrePe (Gong et al., 2007; Jensen et al., 2008; Brust et al., 2014; RC-FrePe Jax #029486) were generated by crossing Drd2-Cre females to Pet1-Flpe;RC-FrePe double transgenic males. Likewise for axonal projection mapping, Drd2-Cre;Pet1-Flpe;RC-FPSit (RC-FPSit Jax #030206) triple transgenic mice were generated by crossing Drd2-Cre females to Pet1-Flpe;RC-FPSit double transgenic males. For both RC-FrePe and RC-FPSit crosses, all animals of each sex were from separate litters, though males and females from the same litter were used when possible. Genotypes were determined as previously described (Brust et al., 2014). Number of animals used for each assay is listed under the description for each assay.

**Immunohistochemistry**

Mice were briefly anesthetized with isoflurane and immediately perfused intracardially with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were extracted, postfixed in 4% PFA overnight at 4°C, cryoprotected in 30% sucrose/PBS for 48 h, and embedded in OCT compound (Tissue-Tek). Coronal sections were cryosectioned as 30-μm free-floating sections then rinsed three times with PBS for 10 min, blocked in 5% normal donkey serum (NDS; Jackson Immunoresearch) and permeabilized with 0.1% Triton X-100 in PBS for 1 h at room temperature. Sections were incubated for 24-48 h in primary antibodies in the same blocking buffer at 4°C. Primary antibodies used were goat polyclonal anti-5-HT (1:1000, catalog #ab66047; Abcam), chicken polyclonal anti-GFP (1:2000, RRID:AB_2307313; AVES), rabbit polyclonal anti-DsRed (1:1000; catalog #632496; Clontech), and rabbit anti-GABA (1:500, catalog #A2052; Sigma). Following primary antibody incubation, sections were rinsed three times with PBS for 10 min and incubated in secondary antibody (Alexa Fluor 488 donkey anti-chicken IgY, 703-545-155, Jackson Immunoresearch; Alexa Fluor 564 donkey anti-rabbit IgG, A10040, Invitrogen; Alexa Fluor 647 donkey anti-goat IgG, A-21447, Invitrogen) for 1 h at room temperature, rinsed three times with PBS for 10 min, then mounted using ProLong Gold Antifade Mountant (P36930, Life Technologies). For Drd2-Pet1 neuron cell counts, GFP+ cells were counted in every sixth section. The

**Materials and Methods**

**Ethical approval**

All experimental protocols were approved by Harvard University Institutional Animal Care and Use Committees (IACUC) and were in accordance with the animal care guidelines of the National Institutes of Health.

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**Experimental animals**

Mice were housed in a temperature-controlled environment on a 12/12 h light/dark cycle with ad libitum access to standard mouse chow and water. All experimental animals were virgins. For conditional knockout of Drd2, double transgenic mice of the genotype ePet-Cre;Drd2loxP/loxP (referred to as Drd2Pet1-CKO) were generated by crossing BAC transgenic ePet-Cre (Scott et al., 2005; Jax #012712) males to homozygous Drd2loxP/loxP females (Bello et al., 2011; Jax #020631) females. From these crosses, ePet-Cre;Drd2loxP/loxP/ePet-Cre;Drd2loxP/loxP males were then bred to homozygous Drd2loxP/loxP females for ePet-Cre;Drd2loxP/loxP male and female offspring used for experiments. Experimental controls were littermates with the Drd2loxP/loxP genotype thus negative for Cre but of comparable genetic background (C57BL/6J, Jax #000664). For Drd2-Pet1 neuron cell counts, triple transgenic Drd2-Cre;Pet1-Flpe;RC-FrePe (Gong et al., 2007; Jensen et al., 2008; Brust et al., 2014; RC-FrePe Jax #029486) were generated by crossing Drd2-Cre females to Pet1-Flpe;RC-FrePe double transgenic males. Likewise for axonal projection mapping, Drd2-Cre;Pet1-Flpe;RC-FPSit (RC-FPSit Jax #030206) triple transgenic mice were generated by crossing Drd2-Cre females to Pet1-Flpe;RC-FPSit double transgenic males. For both RC-FrePe and RC-FPSit crosses, all animals of each sex were from separate litters, though males and females from the same litter were used when possible. Genotypes were determined as previously described (Brust et al., 2014). Number of animals used for each assay is listed under the description for each assay.
resulting number was multiplied by 6 to obtain the number of Drd2-Pet1 cells per animal.

**Dual immunohistochemistry and fluorescent in situ hybridization (FISH)**

For dual in situ hybridization with immunostaining for GFP + Drd2-Pet1 neuron cell bodies, PFA-perfused brain tissue from adult Drd2-Cre;Pet1-Flpe;RC-FrePe mice was collected as described above but cryosectioned at 20 μm onto slides (Superfrost Plus, catalog #48311-703, WWR), slides were warmed on a slide warmer set to 45°C for 30 min, and processed with RNAscope Multiplex Fluorescent Assay kit (Advanced Cell Diagnostics) following manufacturer’s protocol with the exception that at the end of the protocol, tissue was stained for anti-GFP, as described above, similar to Shrestha et al. (2018). The following probes were used for the dual protocol: Dmd (catalog #561551-C3), Drd2-E2 (catalog #486571-C2), Gad2 (catalog #439371-C2), and Serpin1 (catalog #501441). Cell nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI).

**FISH**

For FISH validation of Drd2 conditional knockout and Gad2 expression analysis, adult Drd2<sup>Pet1-CKO</sup> or control brain tissue was fresh frozen in OCT (TissueTek) and cryosectioned at 16 μm onto slides (Superfrost Plus, catalog #48311-703, WWR) and then processed with RNAscope Multiplex Fluorescent Assay kit (Advanced Cell Diagnostics) following manufacturer’s protocol for fresh frozen tissue. The following probes were used: Drd2-E2 (catalog #486571-C2), Drd2-O4 (Exon7/8; catalog #534241), Tph2 (catalog #413241-C3), Gad2 (catalog #439371-C2), and cre (catalog #312281). Cell nuclei were visualized with DAPI.

**Image collection**

All images were acquired on a Nikon Ti inverted spinning disk confocal microscope with 488-, 561-, 647-nm laser lines and Andor Zyla 4.2 Plus sCMOS monochrome camera. Images were acquired with Nikon Elements Acquisition software AR 5.02. For RNA quantification and Drd2<sup>Pet1-CKO</sup> validation experiments, four images were taken of brain slices containing the DR: the first directly ventral to the aqueduct then one field of view below and to the left and right to capture each lateral wing.

**FISH quantification**

Quantification was conducted blind to sex and genotype. For Drd2<sup>Pet1-CKO</sup> validation, all Pet1+ (serotonergic) neurons within each image were identified, then the viewer outlined the DAPI-stained nuclei of each Pet1+ neuron and scored the presence of Drd2 puncta as “positive” (having puncta) or “negative” (no puncta). The total number of Drd2+ Pet1+ neurons was then divided by the total number of Pet1+ neurons to yield the “% Drd2+Pet1+ neurons.”

For quantification of Dmd, Drd2, Gad2, and Serpin1 manual counting of each mRNA punctum per cell was conducted by a trained viewer. All cells counted fit the criteria of GFP+ with a DAPI+ nucleus. The viewer outlined the GFP+ cell body in FIJI (https://Fiji.sc/; Schindelin et al., 2012) while only viewing that channel and then counted the number of distinct RNA puncta within that cell outline. Brain sections sampled were from five males and five female animals.

For quantification of Drd2-Exon7/8 and Gad2 puncta in Drd2<sup>Pet1-CKO</sup> tissue, DR sections corresponded to inter-aural −0.80 to 1.04 mm and bregma −4.60 to −4.84 mm based on DAPI staining and anatomic landmarks (Franklin and Paxinos, 2008), where Drd2-Pet1 neurons are most enriched. A series of custom FIJI scripts and a CellProfiler (McQuin et al., 2018) pipeline were used to process and analyze confocal images of RNAscope FISH signal in a semi-automatic manner. Analysis was performed in 2D on maximum intensity projections of 6–μm-thick z-stacks. First, a (step 1) preprocessing FIJI script separated channels and preprocessed them for (step 2) CellProfiler to use as input to segment nuclei. The DAPI-stained channel was preprocessed by a Gaussian blur with a diameter of 18 before segmenting with the IdentifyPrimaryObjects module with a diameter range 30–100 pixels using a minimum cross entropy global thresholding strategy. Objects outside of the diameter range or those on the edges were excluded. A threshold smoothing scale of 1.3488 was used and the image was automatically declumped based on intensity values. Finally, holes were filled in the resulting label map image, which was exported for use in FIJI (step 3). In FIJI, the user manually excluded misidentified objects or added additional nuclei that were missed by the automatic detection pipeline. A highly similar script was recently published (Okaty et al., 2020), though this current script performs additional difference of Gaussian (Marr and Hildreth, 1980) based filtering for each FISH channel. For each FISH probe, after background subtraction with a rolling ball radius of 50 pixels, the image was duplicated and a Gaussian blur was performed at two different σ levels, one which obscured small background pixels but preserved mRNA puncta, and a more extreme blur that only retained larger diffuse background puncta. The difference of these two images was then calculated and puncta localized using the Find Maxima function. To find appropriate settings for each FISH channel, we compared the performance of several sets of parameters to automatically detect puncta versus a hand count of puncta. We were able to achieve excellent concordance between the hand count and automatic puncta detection. Table 1 summarizes our settings and performance in a linear regression against the hand count for each FISH probe (statistics calculated in GraphPad Prism v8.4.3 and Microsoft Excel v2002).

**Behavioral assays**

All assays, except the resident-intruder assay, were conducted in an initial cohort of 15 control (eight males, seven females) and 11 Drd2<sup>Pet1-CKO</sup> (six males, five females) mice. All behavioral assays were conducted at postnatal day (P)90 or later. The run order for the initial cohort was open field, elevated plus maze, tail suspension test, forced swim test, social interaction, acoustic startle response, prepulse inhibition of acoustic startle, water T-
Table 1: Settings for Gad2 quantification in Drd2<sup>Pet1-CKO</sup> tissue

| Probe | S1 | S2 | Prominence | $R^2$ | RMSE | MAE |
|-------|----|----|-------------|-------|------|-----|
| E2    | 0.25 | 1 | 175 | 0.8696 | 0.5957 | 0.2458 |
| E7/8  | 0.5 | 1 | 100 | 0.9421 | 0.8054 | 0.35 |
| Cre   | 0.5 | 1 | 100 | 0.9679 | 3.829 | 2.2244 |
| Fev   | 0.25 | 2 | 75  | 0.9555 | 4.414 | 3.7047 |
| Gad2  | 0.25 | 16 | 150 | 0.8568 | 2.804 | 1.7973 |

Summary of settings and performance in a linear regression for semi-automated protocol versus hand counts for each FISH probe.

Acoustic startle response
Mice were placed in a perforated holder (acrylic cylinder with 3.2-cm internal diameter) that allowed movement to be monitored. Animal holders were placed on top of a transducer platform, measuring the active response to both weak and startle stimuli, adjacent to a speaker, within an individual acoustic chamber (Med Associates). Each session consisted of a 5 min acclimation period followed by 10 blocks of 11 trials each with white noise acoustic stimuli (20–120 dB). Each startle stimulus (20–120 dB, in 10-dB increments) was played once per block, in a quasi-random order with a variable intertrial interval of 10–20 s (average of 15 s). The duration of the stimulus was 40 ms. Responses were recorded for 150 ms from startle onset and are sampled every ms. Mice were placed back into the home cage immediately after testing. Males and female were run on different days. This assay was conducted in 30 control mice (14 males, 16 females) and 28 Drd2<sup>Pet1-CKO</sup> (13 males, 15 females), as two separate cohorts per sex.

Tube test of social dominance
Two age-matched (~P90), weight-matched mice of the same sex are introduced into opposite ends of a clear PVC tube (30.5 cm in length with an internal diameter of 2.5 cm) allowing them to interact in the middle but not pass each other within the tube. The subordinate mouse will back out allowing the dominant mouse to pass through (Lindzey et al., 1961). For each pair, five consecutive trials were run with a maximum time of 2 min per trial. Trials ended when one mouse backed out of the tube such that all four limbs are outside of the tube which was then recorded as a “backout” for that mouse. Matches lasting >2 min were excluded from analysis and scored as a draw. A rise of introduction to the tube were alternated between trials and the tube was cleaned with ethanol between each trial. Opponents were from different litters and had never been housed together. This assay was conducted in 24 Drd2<sup>Pet1-CKO</sup> males versus 24 control males and 23 Drd2<sup>Pet1-CKO</sup> females versus 23 control females, conducted across three cohorts of animals.

Resident-intruder assay
Drd2<sup>Pet1-CKO</sup> or control mice were group-housed with male siblings until adulthood (P90) when they were single-housed for one night in the test cage to establish territorial residency. On day 1, a five-week-old Swiss Webster (Charles River) male, the “intruder,” was introduced to the cage divided with a clear perforated divider for 5 min. After 5 min, the perforated divider was removed, and the mice could interact for 5 min, in which the encounter was video recorded. Number of attack bites were counted by a trained, blinded viewer. The intruder mouse was introduced for 3 d to obtain an average number of attack bites per day. The intruder mouse had a lower body weight than the resident male. This assay was conducted only in males, as female laboratory-reared mice do not display territorial aggression (Palanza, 2001; Lonstein and Gammie, 2002) This assay was conducted in 26 Drd2<sup>Pet1-CKO</sup> and 24 controls.

Auditory brainstem response (ABR)
ABRs were recorded in a separate cohort of adult mice (males: 10 control and seven Drd2<sup>Pet1-CKO</sup>; females: eight control and seven Drd2<sup>Pet1-CKO</sup>) aged P71–P102 to correspond to the age of animals in other assays. ABRs were conducted similar to (Maison et al., 2013). Mice were...
anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (7.5 mg/kg) and placed in a soundproof chamber on a heating pad. Acoustic stimuli were delivered using EPL Cochlear Function Test Suite (CFTS) software and analyzed using ABR peak analysis software [1.1.1.9, Massachusetts Eye and Ear (MEE)]. All ABR thresholds, amplitudes, and latencies were read by an investigator blind to mouse genotype.

**Electrophysiology**

Slice preparation and whole-cell patch-clamp recordings were conducted as previously described (Rood et al., 2014; Niederkofler et al., 2016). Briefly, to assess membrane and action potential (AP) characteristics a protocol of repeated sweeps of 500-ms current injections stepping in 20-pA steps from −80 to 180 pA was administered to cells in current clamp. Data were analyzed using Clampfit (Molecular Devices). Some cells included in cell property analyses were also used to generate data on the function of DRD2 receptors in the DR (Niederkofler et al., 2016). However, the intrinsic cell properties data we present in this article have not been previously published and include cells not part of the Niederkofler et al. (2016) dataset.

**Projection mapping**

Brain tissue from six females and five males from different litters, but with a female and male from the same litter where possible, were collected at P90 and processed as previously described (Niederkofler et al., 2016). Target region identification was based on anatomic landmarks identified by DAPI staining, anti-choline acetyltransferase (goat polyclonal anti-ChAT, 1:500, AB144P; EMD234 Millipore) staining, and/or anti-tyrosine hydroxylase (rabbit anti-TH, 1:1000, AB152, Millipore) staining. Staining and imaging protocols were identical among the eleven samples analyzed.

**Quantification of target innervation**

Target innervation was quantified in a similar manner to (Niederkofler et al., 2016). Briefly, image stacks were acquired bilaterally per brain region analyzed for each animal using a Nikon Ti inverted spinning disk microscope with a Plan Fluor 40×/1.3 Oil DIC H/N2 objective, 488-, 561-, and 647-nm laser lines, and Andor Zyla 4.2 Plus sCMOS monochrome camera. Images were acquired with Nikon Elements Acquisition software AR 5.02. Image stacks (.nd2 files) were imported to FIJI for analysis of axon projection area. Each stack contained 21 optical slices of 0.3 μm. Innervation density was quantified by a FIJI macro, such that all images were treated identically, including background subtraction, thresholding and particle counting as described in (Niederkofler et al., 2016). We then divided the total area occupied by the projection signal by the total area of the 21 optical slices to obtain the percent area occupied by projection signal. This was then averaged within images of the same brain region across male or female samples. Brain regions analyzed were either those previously described to be innervated by Drd2-Pet1 neurons in males only (Niederkofler et al., 2016) or those involved in auditory processing and ASR.

**Statistical analyses**

Data are presented as mean ± SEM. Statistical analyses were conducted in GraphPad Prism version 8.1. Statistical significance was determined by unpaired $t$ test between control versus Drd2Pet1-CKO groups or male versus female groups except where noted: open field, forced swim test, acoustic startle response, and ABR statistical significance was determined using two-way ANOVA. For the resident-intruder assay, the tube test of social dominance, and rotarod, statistical significance was determined using the non-parametric Mann–Whitney $U$ test. A result was considered significant if $p < 0.05$. Detailed statistical results are reported in Table 2.

**Results**

**Visualization of Drd2-Pet1 serotonergic neurons and the loss of Drd2 gene expression in Drd2Pet1-CKO mice**

As our first step, we confirmed the anatomic distribution of Drd2-Pet1 neurons in the mouse brainstem, observing cell soma distributed across the rostral and lateral regions of the DR nucleus (Fig. 1A) as previously reported (Niederkofler et al., 2016). Drd2-Pet1 neurons were marked by GFP expression in triple transgenic Drd2-Cre;Pet1-Flpe;RC-Frepe (Gong et al., 2007; Jensen et al., 2008; Brust et al., 2014) mice in which cells positive for both Cre and Flpe activity—here those cells having expressed Drd2 and Pet1—have recombined the RC-Frepe intersectional reporter allowing GFP expression; Flpe recombination alone configured RC-Frepe to drive mCherry expression, thus marking the remaining Pet1+ (Drd2-negative) serotonergic neurons (Fig. 1A,B). As expected (Niederkofler et al., 2016), GFP+ Drd2-Pet1 neurons showed detectable 5-HT by immunostaining and Drd2 mRNA by fluorescent in situ hybridization (FISH) (Fig. 1C).

To query the behavioral requirement for Drd2 gene expression in Drd2-Pet1 neurons, we deployed the ePet1-cre driver (Scott et al., 2005) to delete floxed Drd2 gene sequences (Bello et al., 2011), creating a functional null Drd2 allele selectively in Pet1 neurons (Fig. 1D), and then subjected these Drd2Pet1-CKO mice to behavioral phenotyping. Cre-negative, Drd2flox/flox littermates served as controls. To confirm loss of Drd2 gene expression in Pet1 neurons, we analyzed Drd2Pet1-CKO and control Drd2flox/flox brain tissue sections by mRNA in situ hybridization using a probe designed to detect exon 2-containing Drd2 mRNA, as exon 2 was the floxed gene portion to be excised by Cre recombination; concomitant identification of serotonergic neurons was by detection of Pet1 transcripts (Fig. 1F,G). Robust loss of Drd2 expression was observed in serotonergic neurons in both male and female mice (15.23 ± 2.41% of Pet1+ neurons in the DR express Drd2 transcripts in controls ($n = 6$), consistent with prior findings, compared with 3.87 ± 0.73% in Drd2Pet1-CKO ($n = 6$), $p = 0.0011$, unpaired $t$ test; Fig. 1F). The few residual Pet1+ cells harboring Drd2 transcripts likely reflects a limitation in cell capture by the
| Behavior/experiment | Line | Data structure (normality) | Type of test | Comparison | Power | F/df | p   |
|--------------------|------|---------------------------|--------------|------------|-------|------|-----|
| Validation of Drd2 CKO | 1E   | Yes                       | Unpaired t test | Control vs Drd2Pet1+/−-CKO | t = 4.514, df = 10 | p = 0.0011 |
| Open field distance | 2A   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, time (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 8.816 | p < 0.0001 |
| Open field % distance traveled | 2B   | Yes                       | Unpaired t test | Control vs Drd2Pet1+/−-CKO | t = 1.781, df = 24 | p = 0.0876 |
| Rotarod | 2C   | No                        | Mann-Whitney, two-tailed | Control vs Drd2Pet1+/−-CKO | M-W U = 142 | p = 0.1899 |
| Elevated plus maze (% time in open arm) | 2D   | Yes                       | Unpaired t test | Control vs Drd2Pet1+/−-CKO | t = 1.250, df = 24 | p = 0.2234 |
| Tail suspension test | 2E   | Yes                       | Unpaired t test | Control vs Drd2Pet1+/−-CKO | t = 0.3485, df = 24 | p = 0.7305 |
| Forced swim test | 2F   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, time (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 0.3090 | p = 0.9067 |
| Water T maze (%correct during acquisition) | 2H   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, time (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 1.631 | p = 0.0689 |
| Water T maze (%correct during reversal) | 3C   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, time (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 1.72 | p = 0.0001 |
| Water T maze (%correct during reversal) | 3D   | Yes                       | Pearson r correlation | Control trial number × startle response Drd2Pet1+/−-CKO trial number × startle response | r = -0.195 | p = 0.5893 |
| ASR (M) | 3E   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, dB (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 2.425 | p = 0.1319 |
| ASR latency (M) | 3F   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, dB (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 21.67 | p = 0.0001 |
| ASR (F) | 3G   | Yes                       | Pearson r correlation | Control trial number × startle response Drd2Pet1+/−-CKO trial number × startle response | r = 0.1171 | p = 0.7473 |
| ASR latency (F) | 3H   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, dB (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 7.475 | p = 0.0001 |
| PPI (M) | 4I   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, prepulse dB (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 31.34 | p = 0.0001 |
| PPI (F) | 3J   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, prepulse dB (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 1.059 | p = 0.2249 |
| ABR amplitude (M) | 4C   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, peak (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 5.09 | p = 0.0011 |
| ABR latency (M) | 4D   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, peak (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 11.71 | p = 0.0001 |
| ABR amplitude (F) | 4F   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, peak (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 1.211 | p = 0.2249 |
| ABR latency (F) | 4G   | Yes                       | Repeated-measures ANOVA | F1, genotype, F2, peak (F<sub>F1</sub> × F<sub>F2</sub>) | F<sub>F1</sub>,<sub>F2</sub>,<sub>df</sub> = 4.60 | p = 0.0001 |

(Continued)
Table 2: Continued

| Behavior/experiment | Line | Data structure (normality) | Type of test | Comparison | Power | F/df | p   |
|---------------------|------|---------------------------|--------------|------------|-------|------|-----|
| ABR threshold (F)   | 4H   | Yes                       | Unpaired t test | Control vs Drd2Pet1-CKO | t = 0.1566, df = 13 | p = 0.8770 |
| 5.6                 |      |                           |              |            |       |      |     |
| 8                   |      |                           |              |            |       |      |     |
| 16                  |      |                           |              |            |       |      |     |
| 32                  |      |                           |              |            |       |      |     |
| Social interaction (M, % time with stranger) | 5A | Yes                       | Unpaired t test | Control vs Drd2Pet1-CKO | t = 0.6283, df = 12 | p = 0.5415 |
| Social interaction (F, %time with stranger) | 5B | Yes                       | Unpaired t test | Control vs Drd2Pet1-CKO | t = 0.9598, df = 10 | p = 0.3598 |
| Resident-intruder assay | 5C | No                        | Mann-Whitney, two-tailed | Control vs Drd2Pet1-CKO | M-W U = 289.5 | p = 0.6649 |
| Tube test of social dominance | 5E |                           |              |            |       |      |     |

| Male                | No    | Mann-Whitney, two-tailed | Control vs Drd2Pet1-CKO | M-W U = 166 | p = 0.0065 |
| Female              | No    | Mann-Whitney, two-tailed | Control vs Drd2Pet1-CKO | M-W U = 253 | p = 0.8123 |
| Drd2-Pet1 neuron count | 6A | Yes                       | Unpaired t test | Male vs female | t = 0.8160, df = 12 | p = 0.4304 |
| Soma size           | 6C    | Yes                       | Unpaired t test | Male vs female | t = 1.021, df = 8 | p = 0.3372 |
| Gene expression     | 6D    |                           |              |            |       |      |     |
| Dmd                 | Yes   | Unpaired t test | Male vs female | t = 0.9581, df = 7 | p = 0.3899 |
| Drd2                | Yes   | Unpaired t test | Male vs female | t = 1.514, df = 8 | p = 0.1686 |
| Gad2                | Yes   | Unpaired t test | Male vs female | t = 2.498, df = 8 | p = 0.0370 |
| Sepin1              | Yes   | Unpaired t test | Male vs female | t = 1.459, df = 7 | p = 0.1879 |
| %Gad2+ Drd2-Pet1 neurons | 6E | Yes                       | Unpaired t test | Male vs female | t = 1.876, df = 8 | p = 0.0975 |
| % Drd2-Exon7/8+     | 7B    | Yes                       | Unpaired t test | Male vs female | t = 1.291, df = 10 | p = 0.8998 |
|                     |       |                           | One-way ANOVA | Control/Pet1 probe vs Drd2Pet1+CKO | F<sub>1, 15</sub> = 0.1003 | p = 0.9051 |
|                     |       |                           |                | Pet1 probe vs Drd2Pet1-WT+CKO | F<sub>1, 15</sub> = 0.1003 | p = 0.9051 |
| % GAD65 in Cre + neurons | 7D | Yes                       | Unpaired t test | Male vs female | t = 3.057, df = 8 | p = 0.0157 |
| Gad2 punctate per cell | 7E | Yes                       | Unpaired t test | Male vs female | t = 1.768, df = 8 | p = 0.1151 |
| Nucleus area        | 7F    | Yes                       | Unpaired t test | Male vs female | t = 0.9931, df = 8 | p = 0.3487 |
| Resting membrane potential | 8A | Yes                       | Unpaired t test | Male vs female | t = 0.2113, df = 61 | p = 0.8334 |
| Membrane resistance | 8B    | Yes                       | Unpaired t test | Male vs female | t = 0.4084, df = 61 | p = 0.6844 |
| AP threshold        | 8B    | Yes                       | Unpaired t test | Male vs female | t = 1.8197, df = 61 | p = 0.0737 |
| AP amplitude        | 8D    | Yes                       | Unpaired t test | Male vs female | t = 1.0474, df = 61 | p = 0.2990 |
| AP duration         | 8E    | Yes                       | Unpaired t test | Male vs female | t = 2.2583, df = 61 | p = 0.0275 |
| AHP amplitude       | 8F    | Yes                       | Unpaired t test | Male vs female | t = 1.350, df = 61 | p = 0.1821 |
| Innervation densities | 9C | Yes                       | Unpaired t test | Male vs female | t = 1.285, df = 9 | p = 0.2308 |
| DP5i                | Yes   | Unpaired t test | Male vs female | t = 2.3989, df = 9 | p = 0.8158 |
| PAG                 | Yes   | Unpaired t test | Male vs female | t = 0.1978, df = 9 | p = 0.8476 |
| mPOA                | Yes   | Unpaired t test | Male vs female | t = 0.07798, df = 9 | p = 0.9398 |
| DLG                 | Yes   | Unpaired t test | Male vs female | t = 0.6732, df = 9 | p = 0.5178 |
| nHb                 | Yes   | Unpaired t test | Male vs female | t = 0.7901, df = 9 | p = 0.4498 |
| PrnC                | Yes   | Unpaired t test | Male vs female | t = 1.6949, df = 9 | p = 0.1592 |
| IC                  | Yes   | Unpaired t test | Male vs female | t = 1.514, df = 8 | p = 0.1686 |
| LL                  | Yes   | Unpaired t test | Male vs female | t = 0.9100, df = 9 | p = 0.3865 |
| SOC                 | Yes   | Unpaired t test | Male vs female | t = 0.9282, df = 9 | p = 0.3775 |
| CNC                 | Yes   | Unpaired t test | Male vs female | t = 0.2997, df = 9 | p = 0.7712 |

Statistical values are provided for behavioral analyses of Drd2<sup>Pet1-CKO</sup> mice and comparison of Drd2-Pet1 neuron properties in male versus female mice. Figure numbers are included to reference corresponding graphs. Statistical analyses were conducted in GraphPad Prism version 8.1.

ePet-cre driver. Reliable immunodetection to confirm the expected parallel loss of DRD2 protein in PET1 cells remains unavailable.

Behavioral assessments in Drd2<sup>Pet1-CKO</sup> mice and the detection of sex-specific sensory, defensive, and social behaviors

Having validated effective loss of Drd2 expression specific to Pet1 neurons in Drd2<sup>Pet1-CKO</sup> mice, next, we screened these mice for behavioral alterations in comparison to sibling control Drd2<sup>lox/lox</sup> (Cre-negative) mice. Locomotor behaviors were explored first because they are known to be influenced by serotonergic and dopaminergic manipulations (Baik et al., 1995; Gainetdinov et al., 1999; Holmes et al., 2003; Seo et al., 2019), and because motor alterations can affect performance in and interpretation of subsequent behavioral assays. Notably, we found no differences between Drd2<sup>Pet1-CKO</sup> versus control mice (males or females) in the locomotor behaviors reflected in the open field and rotarod tests, such as distance traversed (Fig. 2A) and location within the field (Fig. 2B), vertical rearing, length of time on the rotating rod (Fig. 2C), which reflects balance, coordination, physical conditioning, and motor-planning. Next, we explored measures of depression-like and anxiety-like behaviors, as they are altered in various 5-HT-pathway or DA-pathway mouse models and pharmacological manipulation of these neurotransmitter systems show positive clinical effect. (Lucki, 1998; Hendricks et al., 2003; Holmes et al., 2009).
Figure 1. Visualization of Drd2-Pet1 serotonergic neurons and the loss of Drd2 gene expression in Drd2-Pet1-CKO mice. A, Drd2-Pet1 neurons are intersectionally labeled with GFP (green) and Pet1-only positive cell bodies labeled with mCherry (magenta) in a coronal brain section of the DR from a P90 triple transgenic Drd2-Cre;Pet1-Flpe;RC:FrePe mouse. Scale bars: 200 \( \mu \)m. B, Intersectional genetic strategy: expression of Drd2-Cre and Pet1-Flpe transgenes results in dual recombination of intersectional allele, RC:FrePe, labeling cells expressing Drd2 and Pet1 with GFP. C, Dual immunohistochemistry for GFP (green) and 5-HT (serotonin, magenta) coupled with FISH detection of Drd2 mRNA, which shows co-localization of intersectionally labeled Drd2-Pet1 neuron cell bodies with 5-HT and Drd2 mRNA. Scale bars: 10 \( \mu \)m. D, Strategy for conditional deletion of Drd2 in serotonergic neurons (referred to throughout as Drd2-Pet1-CKO). Cre recombination excises Drd2 exon 2 (magenta) producing serotoninergic-specific (boxed in green) deletion of Drd2 gene sequences. E, Percentage (mean \( \pm \) SEM) of Pet1+ serotonergic neurons that express Drd2 in control (\( n = 6 \)) versus Drd2-Pet1-CKO (\( n = 6 \)) shows reduction of Drd2 expression in Pet1+ neurons (controls: 15.23 \( \pm \) 2.41 Drd2-Pet1-CKO: 3.87 \( \pm \) 0.73 Drd2-Pet1-CKO) dual positive neurons per brain, Drd2-Pet1-CKO: 3.87 \( \pm \) 0.73 Drd2-Pet1 dual positive neurons per brain, \( p = 0.0011 \), unpaired t test). Filled black diamonds represent male mice, open gray circles represent female mice. F, G, FISH on (F) control and (G) Drd2-Pet1-CKO tissue. Drd2 transcripts detected in Pet1+ cells in control sections, but not in Drd2-Pet1-CKO mice, indicative of loss of Drd2. cre transcript is not present in control (F, far right) but is present in Drd2-Pet1-CKO Pet1 cells, as expected (G, far right). Pet1, Drd2, and cre transcript are shown separately in grayscale. Note Drd2 expression remains in non-Pet1 cells (arrow). Dotted lines drawn to encircle DAPI nuclei. Scale bars: 25 \( \mu \)m.
We observed no differences in performance in the elevated plus maze (Fig. 2D), tail suspension test (Fig. 2E), or forced swim test (Fig. 2F) in Drd2Pet1-CKO males and females compared with littermate controls. Additionally, contextual fear conditioning (Fig. 2G) and water T-maze acquisition and reversal (Fig. 2H) were not affected, suggesting no impairment of memory and learning in Drd2Pet1-CKO mice.

Because the serotonergic and dopaminergic systems are implicated in modulating the ASR (Davis and Aghajanian, 1976; Davis et al., 1980; Meloni and Davis, 1999, 2000a,b), we explored that next. The ASR is an evolutionarily conserved reflex involving rapid contraction of facial and skeletal muscles into a protective posture in response to a loud, threatening stimulus. We hypothesized that Drd2-Pet1 neurons modulate this response, given their dense projections to auditory brain regions (Niederkofler et al., 2016) and the observation that following acoustic startle, the activity of certain serotonergic neurons increases in the lateral wings of the DR (Spannuth et al., 2011), a location in which we find Drd2-Pet1 neurons. We measured startle responses to weak and startling stimuli ranging from 20 to 120 dB presented in a randomized order (Fig. 3A, B), or forced swim test (Fig. 2F) in Drd2Pet1-CKO males and females compared with littermate controls. Additionally, contextual fear conditioning (Fig. 2G) and water T-maze acquisition and reversal (Fig. 2H) were not affected, suggesting no impairment of memory and learning in Drd2Pet1-CKO mice.

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Figure 3. Drd2<sup>Pert1-CKO</sup> females, but not males, display attenuated acoustic startle responses (ASR). A, Schematic of ASR experimental design. After an initial 5-min acclimation, mice are exposed to 10 blocks of 11 trials of auditory stimuli ranging from 20 to 120 dB in quasi-randomized order with a 10- to 20-s intertrial interval (ITI). B, Schematic of ASR measurement apparatus, mouse is placed in a perforated holding chamber atop transducer platform adjacent to speaker (for detailed description, see Materials and Methods). C, F, Averaged ASR magnitudes (mean ± SEM) across increasing stimulus intensities in (C) male Drd2<sup>Pert1-CKO</sup> (blue, n = 13) and controls (black, n = 14), no significant difference, p = 0.7745, two-way ANOVA and (F) female Drd2<sup>Pert1-CKO</sup> (blue, n = 15) and controls (black, n = 16), Drd2<sup>Pert1-CKO</sup> females display significantly attenuated ASR, p = 0.0011, two-way ANOVA. D, G, Group averaged ASR for 10 trials at 110-dB stimulus in (D) males and (G) females, demonstrates no habituation to the startle stimulus; x-axis numbers refer to trial number out of 110 total trials. E, H, No significant differences in latency to startle are observed in (E) males, p = 0.1319, two-way ANOVA and (H) females, p = 0.5452, two-way ANOVA. I, J, No significant differences in prepulse inhibition of acoustic startle are observed in (I) males (n = 8 control, 6 Drd2<sup>Pert1-CKO</sup>), p = 0.4325, two-way ANOVA or (J) females (n = 7 control, 5 Drd2<sup>Pert1-CKO</sup>, p = 0.4380, two-way ANOVA).
and Drd2<sup>Pet1-CKO</sup>, <i>r</i> = 0.0517, Pearson correlation; Fig. 3D,G). Further, we observed no differences in latency to startle in either females or males (Fig. 3E,H). Females were of similar mass (controls: 32.117 ± 3.15 g vs Drd2<sup>Pet1-CKO</sup>: 37.2 ± 2.427 g, unpaired t test, <i>p</i> = 0.2031) regardless of genotype, thus differences in weight and its relative impact on transduction of the startle response via the piezoelectric platform were not a confound.

While Drd2<sup>Pet1-CKO</sup> females showed diminished response magnitudes to startling acoustic stimuli, they nevertheless expressed normal acoustic prepulse inhibition (PPI) whereby even the diminished response to startling acoustic stimuli (e.g., 120-dB stimuli) was further blunted proportionately when immediately preceded by a weak, non-startling stimulus (e.g., 65-, 75-, or 85-dB stimuli; Fig. 3I,J). Thus, sensorimotor gating, as measured by acoustic PPI, appeared relatively intact; the acoustic dysfunction instead centered on the ASR itself.

Having observed attenuation of the ASR in female Drd2<sup>Pet1-CKO</sup> mice, we assessed whether hearing was broadly disrupted as revealed by ABRs evoked by sound stimuli (Zhou et al., 2006). ABRs were recorded in response to pure tone stimuli at 5.6, 8, 16, and 32 kHz (n = 8 control females, 7 Drd2<sup>Pet1-CKO</sup> females, and 10 control males, 7 Drd2<sup>Pet1-CKO</sup> males). Across all these frequencies, the measured ABR waveforms (averaged ABR waveforms shown at 16 kHz at 80-dB SPL; Fig. 4A,B), peak amplitudes [shown for peaks 1–3 at 16 kHz at 80-dB SPL for males (<i>p</i> = 0.2032, two-way ANOVA) and females (<i>p</i> = 0.1387, two-way ANOVA); Fig. 4C,F], and latencies to peaks [shown for peaks 1–3 at 16 kHz at 80-dB SPL for males (<i>p</i> = 0.0804, two-way ANOVA) and females (<i>p</i> = 0.9430, two-way ANOVA); Fig. 4D,G] were indistinguishable between Drd2<sup>Pet1-CKO</sup> mice and littermate controls. As well, the ABR threshold to elicit a waveform was not significantly different between Drd2<sup>Pet1-CKO</sup> and control mice at 5.6, 8, 16, or 32 kHz (<i>p</i> > 0.05 at all frequencies, unpaired t test) in males (Fig. 4E) or females (Fig. 4H). Thus, hearing overall, as measured by ABR, appeared largely unaffected in Drd2<sup>Pet1-CKO</sup> mice.

ABRs were conducted in adult mice (ages P71–P102) to align with the age at which the other behavioral assays were performed. However at such ages, C57BL/6J mice, the strain background here, exhibit some age-related hearing loss at higher frequencies (Kane et al., 2012), which we saw here at 32 kHz with two control and three Drd2<sup>Pet1-CKO</sup> females and five control and three Drd2<sup>Pet1-CKO</sup> males. At all other tested frequencies, the ABRs were effectively normal for both genotypes, with one exception being a Drd2<sup>Pet1-CKO</sup> female that exhibited undetectable ABRs at 5.6 kHz, but otherwise normal responses at all other frequencies tested including 32 kHz. These findings at 32 and 5.6 kHz are likely independent of the ASR phenotype observed in females because all animals had normal hearing at 8 and 16 kHz, frequencies included in the white noise startle stimulus of the ASR test.

Next, we examined social behavior in Drd2<sup>Pet1-CKO</sup> mice using the three-chambered test of sociability (Moy et al., 2004) that measures preference to investigate a social stimulus (a novel “stranger” mouse inside a holder) as compared with an object (an empty holder). Drd2<sup>Pet1-CKO</sup> mice showed no alterations in sociability compared with controls and both control and Drd2<sup>Pet1-CKO</sup> spent significantly more time investigating the stranger than the object (Fig. 5A,B). Females of both genotypes displayed preference toward the social stimuli only for the first 5 min of the assay (Fig. 5B, white bars), while males displayed this preference throughout the 10-min assay. Similar sex differences in sustained preference for the social stimulus have been described in C57BL/6J mice (Netser et al., 2017).

We assayed intermale, territorial aggression in a separate cohort of mice using a resident-intruder assay. Females were not tested, as they have been shown to display low or no aggression in most forms of this assay (Palanza, 2001; Lonstein and Gammie, 2002). We observed no statistically significant difference in number of attack bites delivered to the intruder mouse by Drd2<sup>Pet1-CKO</sup> males (n = 26) compared with number of attack bites delivered to the intruder by controls (n = 24; Drd2<sup>Pet1-CKO</sup>: 4.07 ± 1.50 bites, controls: 1.77 ± 0.39 bites, <i>p</i> = 0.6649, Mann–Whitney test; Fig. 5C) noting, however, that four Drd2<sup>Pet1-CKO</sup> males displayed high levels of aggression.

To assay social dominance, we performed the tube test, which has relevance in females as well as males (Lindzey et al., 1961; van den Berg et al., 2015; Zhou et al., 2017). Two mice are simultaneously released into opposite ends of a clear tube of sufficiently narrow diameter that prevents mice from passing by each other and instead requires that one back out for the other, more dominant “winning” mouse, to move forward (Fig. 5D). Drd2<sup>Pet1-CKO</sup> males won a higher percentage of trials against non-sibling, weight-matched, and genetic background-matched opponent males (shown as percent of trials won, Drd2<sup>Pet1-CKO</sup>: 65.83 ± 9%, n = 24; controls: 34.17 ± 9%, n = 24; <i>p</i> = 0.0065, Mann–Whitney test; Fig. 5E). By contrast, we observed no difference in percent of trials won by female Drd2<sup>Pet1-CKO</sup> mice as compared with female sibling controls (Drd2<sup>Pet1-CKO</sup>: 48.7 ± 8%, n = 23; controls 51.3 ± 8%, n = 23; <i>p</i> = 0.8123, Mann–Whitney test; Fig. 5E).

Drd2-Pet1 neurons in males versus females exhibit differences in candidate molecular and biophysical properties but not in cell number

Given these sex-specific differences in behaviors observed in Drd2<sup>Pet1-CKO</sup> mice, next we looked for sex-specific differences in Drd2-Pet1 cellular properties, beginning with cell number. Analyzing triple transgenic Drd2-Cre; Pet1-Flpe; RC-FrePe males versus females, we found no difference in number of GFP<sup>+</sup> Drd2-Pet1 neurons per brain (males: 410.40 ± 55.30 cells/brain, females: 313 ± 87.52 cells/brain, <i>p</i> = 0.4304, unpaired t test; Fig. 6A). Further, in both males and females, Drd2-Pet1 neurons distributed as expected across the rostral-caudal and medial-lateral axis of the DR.

To understand whether gene expression might differ between male and female Drd2-Pet1 neurons, we examined single-cell RNA sequencing data previously analyzed for expression of serotonergic pathway genes as validation that Drd2-Pet1 cells were indeed serotonergic (Niederkofler et al., 2016). Comparison across sex, albeit...
lacking statistical significance given the small sample size, highlighted four genes for further evaluation, Drd2, Dmd (encoding Dystrophin, a component of protein scaffolds in the CNS; Perronnet and Vaillend, 2010), Gad2 (encoding glutamate decarboxylase 2 involved in catalyzing the production of the neurotransmitter GABA), and Serpini1 (encoding the serine protease Neuroserpin, important for synapse formation and plasticity; Galliciotti and Sonderegger, 2006). Quantitative in situ mRNA detection using dual FISH with immunodetection on tissue sections from Drd2-Cre;Pet1-Flpe;RC-FrePe mice revealed greater abundance of average Gad2 transcripts (puncta) per cell in males versus females [Gad2: 20.46 ± 2.243 in males (n = 5) vs 12.20 ± 2.427 in females (n = 5), p = 0.0370, unpaired t test; Fig. 6D]. There was no difference in the percentage of Drd2-Pet1 neurons expressing Gad2 in male versus female mice (Fig. 6E). No difference in soma size (GFP-stained cell body) was observed between males and females suggesting that transcript differences were not because of larger soma volume measured (Fig. 6C). No significant differences in mRNA abundance were observed between males and females for Dmd, Drd2, or Serpini1 (see Table 2).

As a first step toward understanding whether sex-specific gene expression differences observed in wild-type
mice persist or are altered in Drd2\textsuperscript{Pet1-CKO} mice, we assessed Gad2 transcript levels in Drd2\textsuperscript{Pet1-CKO} cells. In these cells, the floxed exon 2 of Drd2 is excised by Cre recombination. Therefore, to identify mutant Drd2 mRNA and thus the mutant Drd2\textsuperscript{Pet1-CKO} cells, we used a multi-probe strategy involving one probe to intact downstream exons 7 and 8 (referred to here as Drd2-E7/8), another to exon 2 (referred to as Drd2-E2), and another to either cre or Pet1. We examined expression in the DR region most enriched with Drd2-Pet1 neurons. We found Drd2-E7/8 \textsuperscript{+} puncta in Pet1\textsuperscript{+} cells in both controls and Drd2\textsuperscript{Pet1-CKO} mice, whereas Drd2-E2 \textsuperscript{+} puncta were detectable in control tissue but greatly reduced in Drd2\textsuperscript{Pet1-CKO} as expected given the efficiency of Cre-mediated gene deletion (Fig. 7A; see Drd2-E2 quantification in Fig. 1E). Drd2-E7/8 puncta were detected in 35.97 ± 2.403% of Pet1\textsuperscript{+} cells in control mice (n = 6) compared with 36.53 ± 3.621% of Pet1\textsuperscript{+} cells in Drd2\textsuperscript{Pet1-CKO} mice (n = 6; p = 0.8998, unpaired t test; Fig. 7B). Similarly, in a separate experiment using an in situ probe to cre mRNA, 34.91 ± 2.238% of cre\textsuperscript{+} cells expressed Drd2-E7/8 (n = 10 mice, one-way ANOVA compared with Pet1 control and Drd2\textsuperscript{Pet1-CKO} cell expression, p = 0.9051; Fig. 7B). Next, we analyzed Gad2 mRNA transcript levels in Drd2\textsuperscript{Pet1-CKO} cells (dual Drd2-E7/8 and cre\textsuperscript{+} cells) in the DR (Fig. 7C). In males, we observed 87.44 ± 3.034% of Drd2\textsuperscript{Pet1-CKO} cells were Gad2\textsuperscript{+}, while this percentage was 75.76 ± 0.5862% in females (p = 0.0157, unpaired t test; Fig. 7D). In these Drd2\textsuperscript{Pet1-CKO} cells, there were 14.25 ± 1.325 transcripts per cell in males and 10.13 ± 2.074 transcripts per cell in females (p = 0.1151, unpaired t test; Fig. 7E). Because of the tightly packed distribution of cells in the DR, puncta were measured only within cre\textsuperscript{+} DAPI-stained nuclei to ensure puncta were not assigned to more than one cell. The area of nuclei did not differ between males (114.9 ± 3.030 \(\mu\)m\(^2\)) and females (110.9 ± 1.768 \(\mu\)m\(^2\); p = 0.3497, unpaired t test; Fig. 7F). Thus, in Drd2\textsuperscript{Pet1-CKO} males as compared with Drd2\textsuperscript{Pet1-CKO} females, a greater percentage of the Drd2-Pet1 cells harbored Gad2 transcripts; of these Gad2-expressing cells, however, transcript levels were not significantly different between males versus female Drd2\textsuperscript{Pet1-CKO} mice.

To explore potential sex differences in electrophysiological properties characterizing Drd2-Pet1 neurons, we...
conducted whole-cell recordings from GFP-labeled Drd2-Pet1 neurons in brain slices from triple transgenic Drd2-Cre;Pet1-Flpe;RC-FrePe males and females. Examination of cell membrane characteristics revealed no sex differences in resting membrane potential (AP; Fig. 8A) or resistance (Fig. 8B). Analyses of AP characteristics revealed an increase in AP duration (Fig. 8E) in male Drd2-Pet1 cells as compared with female (2.847 ± 0.155 ms, n = 19 cells vs 2.54 ± 0.094 ms, n = 44 cells, respectively, p = 0.0275, unpaired t test), but no differences in AP threshold (Fig. 8C), amplitude (Fig. 8D), or afterhyperpolarization (AHP) amplitude (Fig. 8F).

Differing covariance in axonal collateral densities from Drd2-Pet1 neurons directed to auditory targets in males versus females

As a first step in exploring sex differences in Drd2-Pet1 neuron circuitry that may underlie the sex-specific behavioral phenotypes in Drd2-Pet1-CKO mice, we compared relative innervation density to brain regions involved in sensory processing and social behavior in male and female mice. Boutons from Drd2-Pet1 neurons were selectively marked with a Synaptophysin-GFP fusion protein using triple transgenic Drd2-Cre;Pet1-Flpe;RC-FPsit mice (Fig. 9A,B; Niederkofler et al., 2016). At P90, the same age

Figure 6. Sex-specific transcript level differences in Drd2-Pet1 neurons. A, Dual immunohistochemistry and FISH depicting green GFP+ Drd2-Pet1 neurons along with transcript puncta in male (top) and female (bottom) brain sections from Drd2-Cre;Pet1-Flpe;RC-FrePe mice. Drd2 (cyan) and Gad2 (magenta) expression shown together and separately in gray scale. Scale bar: 10 μm. B, Number of Drd2-Pet1 neurons (GFP-positive cells in Drd2-Cre;Pet1-Flpe;RC-FrePe mice) per animal in males (black diamonds, n = 7) and females (open gray circles, n = 7) is not significantly different. Males: 410.40 ± 55.30 cells/brain, females: 313 ± 87.52 cells/brain, p = 0.4336, unpaired t test. C, Drd2-Pet1 neuron soma size (GFP+ cell body) does not differ in males (n = 5 males) versus females (n = 5 females), p = 0.3372, unpaired t test. D, Number of FISH mRNA puncta per cell in males versus females. Male cells have significantly more Gad2 puncta than female cells [20.46 ± 2.243 in males (n = 5) vs 12.20 ± 2.427 in females (n = 5), p = 0.0370, unpaired t test]. E, 86.47 ± 4.181% of male Drd2-Pet1 cells express Gad2 versus female 74.00 ± 5.168% in female cells, p = 0.0975, unpaired t test. Error bars indicate SEM throughout. For C, D, larger symbols outlined in black represent animal averages used for statistical analysis, smaller symbols represent individual cells, matched in color to the average.
at which the behavioral assays were conducted, we collected brain tissue and quantified projections to the cochlear nucleus complex (CNC), superior olivary complex (SOC), lateral lemniscus (LL), inferior colliculus (IC), caudal pontine reticular nucleus (PNC; critical for ASR; Davis et al., 1982), dorsal lateral geniculate nucleus (dLGN), mPOA, medial habenula (mHb), periaqueductal gray (PAG), and dorsal paragigantocellular nucleus (DPGi; shown as percentage of target area occupied by projections; Fig. 9C). We observed no significant sex differences in the cohort average for absolute innervation density to each of these 10 brain regions. However, because we observed considerable interanimal variability in bouton densities at targets, we next explored correlation of innervation density across brain regions.

Figure 7. Gad2 expression in Drd2Pet1-CKO cells. A, FISH with probes to Drd2 exon 7/8 (D2-E7/8, green) and Drd2 exon 2 (D2-E2, magenta) in Pet1 (white) cells in control (top) and Drd2Pet1-CKO (bottom) DR tissue. D2-E7/8, D2-E2, and Pet1 expression shown together and separately in gray scale. B, Percent of Pet1+ cells (left and middle) with Drd2-Exon7/8 expression in control (35.97 ± 2.403%, n = 6) and Drd2Pet1-CKO (36.53 ± 3.621%, n = 6), p = 0.8998, unpaired t test. Data also shown for percent of cre cells (right) with Drd2-Exon7/8, 34.91 ± 2.238%, compared with Pet1 probe control and Drd2Pet1-CKO p = 0.9051, one-way ANOVA. Males, black diamonds, females, open gray circles. C, FISH showing cre+ Drd2Pet1-CKO cells (white) with Drd2-Exon7/8 (green) and Gad2 (red) in male (top) and female (bottom) in the DR nucleus. Drd2-Exon7/8, Gad2, and cre are shown together and separately in gray scale. Scale bar: 10 μm. D, A larger percentage of male Drd2Pet1-CKO cells (87.44 ± 3.034%) express Gad2 versus female Drd2Pet1-CKO cells (75.76 ± 0.5862%), *p = 0.0157, unpaired t test. E, Number of Gad2 mRNA puncta per cell in Drd2Pet1-CKO cells in males (n = 6) versus females (n = 4). Male cells have 14.25 ± 1.325 Gad2 puncta per cell compared with 10.13 ± 2.074 in female cells, p = 0.1151, unpaired t test. F, Drd2Pet1-CKO nucleus size (area used to quantify puncta levels) does not differ in males (n = 6 males) versus females (n = 4 females), p = 0.3497, unpaired t test. Error bars indicate SEM throughout. For E, F, larger symbols outlined in black represent animal averages used for statistical analysis, smaller symbols represent individual cells, matched in color to the average.
Using pairwise correlations between auditory brain regions (Fig. 9D), we constructed a correlation matrix that shows positively correlated regions in green and negatively correlated regions in black (Fig. 9E). This visualization reveals that most auditory brain regions are positively correlated in males (SOC and LL, Pearson’s $r = 0.89$) with only the LL and cochlear nucleus being slightly negatively correlated (Pearson’s $r = -0.28$). Interestingly, a greater number of innervated regions were negatively correlated in females, including the CNC with both the SOC and the IC ($r = 0.68$ and $r = 0.75$, respectively), as well as PNC and IC ($r = -0.67$). The innervation of the PNC and SOC was significantly negatively correlated ($r = -0.85$, $p = 0.033$, two-tailed test). Further, we expanded analyses to include the dLGN, a region critical for visually-cued potentiation of the acoustic startle (Tischler and Davis, 1983), and found that in females innervation of the dLGN was not strongly correlated with innervation of auditory brain regions, while in males this dLGN innervation was highly negatively correlated with both the SOC ($r = -0.91$, $p = 0.033$, two-tailed test) and the IC ($r = -0.91$, $p = 0.034$, two-tailed test), indicating that Drd2-Pet1 neuron circuitry may be set up to modulate multisensory information differently in males compared with females.

GABA and 5-HT in Drd2-Pet1 neurons

Given detection of Gad2 mRNA in Drd2-Pet1 neurons, we probed for GABA versus 5-HT immunopositivity in cell soma versus axonal boutons in males versus females. Punctate GABA immunostaining was indeed detectable in some Drd2-Pet1 neuron soma (Fig. 10A) in both males and females. Yet, in all target brain regions examined, GABA was undetectable in the GFP-marked Drd2-Pet1 boutons. Shown are representative images from the SOC (Fig. 10B) and IC (Fig. 10C), noting a GABA-positive cell body in the IC (boxed) and GABA-positive staining in the corpus callosum serving as a positive control for GABA immunodetection (Fig. 10D). By contrast, 5-HT immunostaining in Drd2-Pet1 boutons was readily detectable (representative images from the SOC and IC; Fig. 9B).

**Discussion**

**Strategy**

We hypothesized that loss of Drd2 gene expression and associated DRD2 signaling normally observed in certain DR Pet1<sup>+</sup> serotonergic neurons (Drd2-Pet1 neurons) could impair sensory, social, and/or defensive behaviors. We used the transgenic driver ePet-cre to delete functionally critical Drd2 gene sequences selectively in serotonergic neurons, thereby abolishing transcript and DRD2 protein function, which would normally initiate in Pet1 cells during adolescence. We validated these Drd2Pet1-CKO mice and examined behavioral responses. Further, we explored Drd2-Pet1 neurons themselves.

**Main findings**

Key findings include the following. (1) Sex-specific behavioral alterations were observed in Drd2<sup>Pet1-CKO</sup> mice. Females showed a dramatic diminution in the protective, defensive ASR as compared with Drd2<sup>flox/flox</sup> controls, while no differences were observed in males. (2) Drd2<sup>Pet1-CKO</sup> males, but not females, showed increased winning in the...
tube test of social dominance against sex-matched and age-matched controls. (3) No differences were observed in ABRs, in PPI of acoustic startle, locomotion, cognition, nor various affective behaviors. (4) No sex-specific differences were found in **Drd2-Pet1** neuron number, soma distribution, nor in the set of efferent targets; however, within-animal correlations between efferent densities across target brain regions suggest differences by sex, thus hinting at sex-specific structural differences in **Drd2-Pet1** neuronal circuitry. (5) **Drd2-Pet1** cells in males as compared with females showed longer AP durations and higher levels of **Gad2** transcripts (important for GABA synthesis); **Drd2-Pet1-CKO** cells did not show a sex specific difference in **Gad2** transcript levels, but the percentage of **Drd2-Pet1** cells that were **Gad2**⁺ in **Drd2-Pet1-CKO** males was slightly higher than in **Drd2-Pet1-CKO** females. These findings, coupled with our prior work (Niederkofler et al., 2016) implicating **Drd2-Pet1** neurons in setting levels of defensive aggressive and exploratory behaviors in male mice, suggest that **Drd2-Pet1** neurons may serve as a specialized neuromodulatory interface whereby DRD2...
signaling alters serotonergic neuronal activity to shape defensive, protective, and dominance behaviors in a sex-specific manner.

Protective ASR diminished in Drd2-Pet1-CKO females

Defensive posturing in millisecond response to abrupt noise, be it a predator or other potential hazard, is a crucial evolutionarily conserved protective mechanism. Loss or blunting of this reflex can result in life-threatening exposure, while excessive enhancement can drive unnecessary, debilitating responses that preclude normal functioning. Thus, “tuning” of the ASR setpoint to social and environmental circumstances is likely critical for species survival and well-being. The observed ASR attenuation in female Drd2-Pet1-CKO mice suggests that Drd2-Pet1 neurons and the regulation of their activity cell autonomously by DRD2 comprises a critical
modulatory node for ASR in females. Further, this node appears separate functionally from that involved in acoustic sensorimotor gating, given that acoustic PPI appeared intact in Drd2Pet1-CKO females, and from hearing, given that ABRs were indistinguishable from controls. Thus, DRD2 signaling in Drd2-Pet1 neurons forms a functional circuit node specialized in female mice to influence startling to acoustic stimuli.

In rats, reduction of 5-HT through synthesis inhibition increases ASR in females, but not males (Pettersson et al., 2016). Predicted reciprocally is that elevated 5-HT levels might blunt ASR in females. Relating this to our findings, it is possible that Drd2-Pet1 neurons are more excitable in the absence of DRD2-mediated inhibition, resulting in increased 5-HT release, perhaps explaining the observed ASR blunting. In wild-type mice, this would predict that under conditions of DA elevation, for example through local DR DA neuron activity associated with arousal and vigilance (Cho et al., 2017), Drd2-Pet1 neuron activity would be inhibited, reducing 5-HT release and thereby tuning a more sensitive ASR, conferring a protective advantage.

The ASR circuit follows from cochlea to CNC to PNC to spiral motoneurons (Davis et al., 1982; Koch et al., 1992), and receives inputs from auditory centers such as the SOC, IC, and SC (Lauer et al., 2017). Drd2-Pet1 neurons innervate each of these areas and the PNC, and thus may impart modulation at multiple levels.

**Tube test wins increased in Drd2Pet1-CKO males**

The increased winning by Drd2Pet1-CKO males in the tube test suggests that loss of DRD2 results in an increase in or favoring of dominance behaviors, at least under these forced, one-on-one interaction conditions. We did not observe significant differences in levels of aggressive attack behaviors by Drd2Pet1-CKO males in a resident-intruder assay. Together, these findings suggest that in wild-type mice, DRD2 signaling in Drd2-Pet1 neurons contributes to tempering certain dominance behaviors under particular conditions.

Understanding how the present results align with our prior work remains a pursuit. In earlier studies using a resident-intruder assay, we observed an increase in various aggressive behaviors in mice in which Drd2-Pet1 neurons were constitutively silenced, which suggested to us that Drd2-Pet1 neuron excitation and neurotransmitter release would normally temper such behaviors (i.e., favor non-confrontational, even submissive behaviors). Because canonical DRD2 signaling is inhibitory and, as well, appears largely inhibitory in Drd2-Pet1 neurons in slice, we predicted that loss of DRD2 signaling would enhance Drd2-Pet1 cell excitability and neurotransmitter release probability, and thus would suppress or at least not enhance dominance behaviors. Yet Drd2Pet1-CKO males exhibited enhanced winning in the tube test. Perhaps DRD2 signaling in Drd2-Pet1 neurons results in cellular activity changes that ultimately lead to a tempering of one-on-one social dominance under some conditions, while extreme, constitutive Drd2-Pet1 neuron silencing is required to prompt the opposite, in the form of aggression escalation to an intruder. Indeed, other findings also support this notion that dominance by tube test does not necessarily correlate with aggression in a resident-intruder assay (Tamminäki et al., 2010). Differences might also be explained by whether the input conditions trigger Drd2-Pet1 neurons to release 5-HT versus GABA, should the latter prove a capability, noting that Drd2-Pet1 cells express Gad2, albeit we were unable to show GABA in Drd2-Pet1 boutons, only their soma.

Interestingly, a subset of Drd2Pet1-CKO males (four out of 26) did display increased levels of aggressive behaviors as compared with other Drd2Pet1-CKO mice and controls, suggesting there may be other influencing variables, yet unknown. This is plausible given that mice deficient for the long isoform of DRD2 (D2L) are reported to show anxiety-like and depressive-like behaviors only following a stress-exposure paradigm (Shioda et al., 2019). Moreover, these stress-induced affective phenotypes in D2L knock-out mice were abrogated by driving D2L expression in DR Pet1+ serotonergic neurons (Shioda et al., 2019). Together these findings suggest that the behavioral role of Drd2 expression in Drd2-Pet1 neurons may be influenced by environmental factors.

**Sex-specific differences in Drd2-Pet1 neuron properties**

The observed sex-specific differences in Gad2 transcript levels in Drd2-Pet1 neurons may contribute to the sex-specific behavioral alterations exhibited by Drd2Pet1-CKO mice. Gad2 expression in Drd2-Pet1 neurons is in line with prior reports showing Gad2 expression more generally in the serotonergic DR (Nanolopoulos et al., 1982; Calizo et al., 2011; Shikanai et al., 2012). It may be that Drd2-Pet1 neurons can release GABA as well as or instead of 5-HT under certain conditions or at particular targets. This capacity may differ in males versus females, given our observation that in males, Drd2-Pet1 neurons harbor higher levels of Gad2 mRNA. Interestingly, Drd2Pet1-CKO cells did not display this sex specific difference in Gad2 transcript, suggesting that Drd2 expression, or more broadly dopaminergic signaling in Drd2-Pet1 neurons, may affect Gad2 transcript levels. One potential model to be tested is if DRD2 signaling, in turn, alters levels of Gad2 expression to allow for neuronal release of GABA in addition to or instead of serotonin when behavioral or environmental conditions necessitate. Indeed, there is precedent for the differential usage of serotonin and glutamate by raphe serotonergic neurons (Liu et al., 2014; Kapoor et al., 2016; Sengupta et al., 2017; Wang et al., 2019), although GABAergic and serotonergic co-release has not been reported.

AP duration measured ex vivo was longer in male versus female Drd2-Pet1 cells; this may also confer neurotransmitter release properties that could contribute to behavioral differences. Additional studies are needed to determine how Drd2Pet1-CKO affects Drd2-Pet1 neuron electrophysiology, gene expression, or efferent targets. Such experiments may be achieved through crossing Drd2Pet1-CKO (ePet1-Cre;Drd2fl/fl) mice to Drd2-Flopo mice (The Jackson Laboratory strain #034419 provided by Bernardo Sabatini) along with an intersectional reporter transgene which would
allow for dual Cre-mediated and Flp-mediated fluorescent labeling of mutant Drd2-Pet1-CreKO cells. While complex genetics, this strategy would enable mutant cell visualization for electrophysiology, single-cell RNA sequencing, and analysis of axonal projections.

In both males and females, Drd2-Pet1 neurons densely innervate auditory brainstem regions, likely modulating auditory-related processes at one or multiple of these sites. In examining Drd2-Pet1 efferents, we observed inter-animal variability in regional innervation density. We speculate this may arise from subgroups within the Drd2-Pet1 neuron population that target different downstream structures. For example, some Drd2-Pet1 neurons may project specifically to the SOC while others might project specifically to the LL. If some animals have more of one subgroup than the other, averaging absolute innervation densities for each target region across all males and females may hide meaningful circuit structure. Covariance analysis of projection targets in each animal thus might hint at which brain regions come under shared regulation by Drd2-Pet1 neurons. In males, the high correlation between auditory region efferent densities suggests shared input from the same Drd2-Pet1 neurons. In females, the CNC/SOC, CNC/IC, SOC/PNC, LL/PNC, and IC/PNC combinations were more negatively correlated, suggesting there might exist a subgroup of Drd2-Pet1 neurons that targets the PNC and a different subgroup, the SOC. We speculate that in males, Drd2-Pet1 neurons contribute to a general level of serotonergic tone across the auditory brainstem, while in females, certain Drd2-Pet1 neurons selectively target and modulate specific nuclei.

In conclusion, we found that Drd2 gene expression in a specialized subset of Pet1 serotonergic neurons is required for certain defensive, dominance, and protective behaviors, involving auditory processing in a sex-specific manner. Deficits in sensory processing such as altered acoustic startle and impaired social communication and dominance behaviors manifest in human disorders including autism spectrum disorder, schizophrenia, and post-traumatic stress disorder, often in sex-specific ways (King et al., 2013; Steel et al., 2014; Matsuo et al., 2016; Thye et al., 2018) and with sex-specific differences in therapeutic outcomes (Franconi et al., 2007). The presented findings, thus, may point to novel circuit nodes of relevance to human neuropsychiatric disease.

References

Baik JH, Picetti R, Saiardi A, Thiriet G, Dierich A, Depaulis A, Le Meur M, Borrelli E (1995) Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. Nature 377:424–428.

Bello EP, Mateo Y, Gelman DM, Noain D, Shin JH, Low MJ, Alvarez VA, Lovinger DM, Rubinstein M (2011) Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors. Nat Neurosci 14:1033–1038.

Brown GL, Ebert MH, Goyer PF, Jimerson DC, Klein WJ, Bunney WE, Goodwin FK (1982) Aggression, suicide, and serotonin: relationships to CSF amine metabolites. Am J Psychiatry 139:741–746.

Brust RD, Corcoran AE, Richerson GB, Nattie E, Dymecki SM (2014) Functional and developmental identification of a molecular subtype of brain serotonergic neuron specialized to regulate breathing dynamics. Cell Rep 9:2152–2165.

Calizo LH, Akanwa A, Ma X, Pan YZ, Lemos JC, Craige C, Heemstra LA, Beck SG (2011) Raphe serotonin neurons are not homogeneous: electrophysiological, morphological and neurochemical evidence. Neuropharmacology 61:524–543.

Choi JH, Trewick JB, Robinson JE, Xiao C, Bremner LR, Greenbaum A, Gradinaru V (2017) Dorsal raphe dopamine neurons modulate arousal and promote wakefulness by salient stimuli. Neuron 94:1205–1219.e8.

Crawford LK, Rahman SF, Beck SG (2013) Social stress alters inhibitory synaptic input to distinct subpopulations of raphe serotonin neurons. ACS Chem Neurosci 4:200–209.

Davis M, Aghajanian GK (1976) Effects of apomorphine and haloperidol on the acoustic startle response in rats. Psychopharmacology (Berl) 47:217–223.

Davis M, Strachan DI, Kass E (1980) Excitatory and inhibitory effects of serotonin on sensorimotor reactivity measured with acoustic startle. Science 209:521–523.

Davis M, Gendelman DS, Tischler MD, Gendelman PM (1982) A primary acoustic startle circuit: lesion and stimulation studies. J Neurosci 2:791–805.

Deneris E, Gaspar P (2018) Serotonin neuron development: shaping molecular and structural identities. Wiley Interdiscip Rev Dev Biol 7:10.1002.

Franconi F, Brunelleschi S, Stardo L, Cuomo V (2007) Gender differences in drug responses. Pharmacol Res 55:81–95.

Franklin KBJ, Paxinos G (2008) The mouse brain in stereotaxic coordinates, Vol 3. New York: Elsevier.

Gainetdinov RR, Wetsel WC, Jones SR, Levin ED, Jaber M, Caron MG (1999) Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. Science 283:397–401.

Gallicciotti G, Sonderegger P (2006) Neuroserpin. Front Biosci 11:33–45.

Geyer MA, Braff DL (1987) Startle habituation and sensorimotor gating in schizophrenia and related animal models. Schizophrenia Bull 13:643–666.

Gong S, Doughty M, Haarbaugh CR, Cummins A, Hatten ME, Heintz N, Tischler MD, Gendelman PM (1982) A primary acoustic startle circuit: lesion and stimulation studies. J Neurosci 2:791–805.

Gregor G, Ebert MH, Goodwin FK (1982) Aggression, suicide, and serotonin: relationships to CSF amine metabolites. Am J Psychiatry 139:741–746.

Hendricks TJ, fyodorov DV, Wegman LJ, Lelutiu NB, Pehek EA, Yamamoto B, Silver J, Weeber EJ, Sweatt JD, Deneris ES (2003) Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. Neuron 37:233–247.

Holmes A, Yang RJ, Lesch KP, Crawley JN, Murphy DL (2003) Mice lacking the serotonin transporter exhibit 5-HT(1A) receptor-mediated abnormalities in tests for anxiety-like behavior. Neuropsychopharmacol 28:2077–2088.

Holschbach MA, Vitale EM, Lonstein JS (2018) Serotonin-specific lesions of the dorsal raphe disrupt maternal aggression and caregiving in postpartum rats. Behav Brain Res 348:53–64.

Huang KW, Ochadarena NE, Philson AC, Hyun M, Binbaum JE, Cicconet M, Sabatini BL (2019) Molecular and anatomical organization of the dorsal raphe nucleus. Elife 8:e46464.

Jensen P, Farago AF, Awatramani RB, Scott MM, Deneris ES, Dymecki SM (2008) Redefining the serotonergic system by genetic lineage. Nat Neurosci 11:417–419.

Kane KL, Longo-Guess CM, Gagnon LH, Ding D, Salvi RJ, Johnson KR (2012) Genetic background effects on age-related hearing loss associated with Cdh23 variants in mice. Hear Res 283:80–88.

Kapoor V, Provost AC, Agarwal P, Murthy VN (2016) Activation of raphe nuclei triggers rapid and distinct effects on parallel olfactory bulb output channels. Nat Neurosci 19:271–282.
Kim JC, Cook MN, Carey MR, Shen C, Regehr WG, Dynecki SM (2009) Linking genetically defined neurons to behavior through a broadly applicable silencing allele. Neuron 63:305–315.

King MW, Street AE, Gradus JL, Vogt DS, Resick PA (2013) Gender differences in posttraumatic stress symptoms among OEF/OIF veterans: an item response theory analysis. J Trauma Stress 26:175–183.

Koch M, Lingenhöhl K, Pilz PK (1992) Loss of the acoustic startle response following neurotoxic lesions of the caudal pontine reticular formation: possible role of giant neurons. Neuroscience 49:617–625.

Lammel S, Lim BK, Malenka RC (2014) Reward and aversion in a heterogeneous midbrain dopamine system. Neuropharmacology 76 [Pt B]:351–359.

Lauer AM, Behrens D, Klump G (2017) Acoustic startle modification as a tool for evaluating auditory function of the mouse: progress, pitfalls, and potential. Neurosci Biobehav Rev 77:194–208.

Lindzey G, Winston H, Manosevitz M (1961) Social dominance in inbred mouse strains. Nature 191:474–476.

Liu Z, Zhou J, Li Y, Hu F, Lu Y, Ma M, Feng Q, Zhang JE, Wang D, Zeng J, Bao J, Kim JY, Chen ZF, El Mestikawy S, Luo M (2014) Dorsal raphe neurons signal reward through 5-HT and glutamate. Neuron 81:1360–1374.

Lonstein JS, Gammie SC (2002) Sensory, hormonal, and neural control of maternal aggression in laboratory rodents. Neurosci Biobehav Rev 26:869–888.

Lucki I (1998) The spectrum of behaviors influenced by serotonin. Biol Psychiatry 44:151–162.

Maison SF, Usubuchi H, Liberman MC (2013) Efferent feedback minimizes cochlear neuropathy from moderate noise exposure. J Neurosci 33:5542–5552.

Marr D, Hildreth E (1980) Theory of edge detection. Proc R Soc Lond B Biol Sci 207:187–217.

Matsuo J, Ota M, Hori H, Hidese S, Teraishi T, Ishida I, Hiraishi M, Owa M, Schilling W, Conlon RA, Strowbridge BW, Deneris ES (2005) A genetic approach to access serotonin neurons for in vivo and in vitro studies. J Neurosci 25:1260–1267.

McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, Doan M, Ding L, Rafelski SM, Thirstrup D, Luzi N, Tran T, Rood BD, Brust RD, Hennessy ML, deBairos Okaty BW, Freret ME, Rood BD, Brust RD, Hennessy ML, deBairos D, Kim JC, Cook MN, Dynecki SM (2015) Multi-scale molecular deconstruction of the serotonin. Neuron 88:774–791.

Meloni EG, Davis M (1999) Enhancement of the acoustic startle response by dopamine D1 and 5-HT1A agonists and corresponding changes in c-fos expression in the dorsal raphe of rats. Psychopharmacology (Berl) 144:373–380.

Meloni EG, Davis M (2000a) Enhancement of the acoustic startle response by dopamine D1 receptor agonist SKF 82958. Psychopharmacology (Berl) 144:373–380.

Meyer RA, Warden BM, Capogna M (2012) Dorsal raphe serotonin neurons in mice: immature hyperexcitability transitions to adult state during first three postnatal weeks suggesting sensitive period for environmental perturbation. J Neurosci 32:16201–16213.

Meyers JA, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Schindl B, Tinevez JY, White DJ, Hartenstein V, Elciceri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682.

Miyata A, Suzuki M, Koyama T, Takeuchi K, Saito M, Miyasaka K, Iwasaki S, Saitoh K, Murase S, Ichinose Y, Fujisawa H, Iwahara Y, Okada Y, Kano H, Saito K, Shiozaki K, Ohno S, Shimizu T, Ito K, Koyama T, Saito M, Miyasaka K, Iwasaki S, Saitoh K, Murase S, Ichinose Y, Fujisawa H, Iwahara Y, Okada Y, Kano H, Saito K, Shiozaki K, Ohno S, Shimizu T, Ito K (2018) Sensory neuron diversity in the inner ear is shaped by activator and suppressor gene expression profiling approaches. Trends Neurosci 43:155–169.

Niemeyer KM, Bocchio M, Caron MG, Geyer MA (2001) Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. J Neurosci 21:305–315.

Nouwen A, Sengupta A, Bocchio M, Caron MG, Geyer MA (2007) Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. J Neurosci 27:1270–1276.

Niemeyer KM, Bocchio M, Caron MG, Geyer MA (2001) Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. J Neurosci 21:305–315.

Nouwen A, Sengupta A, Bocchio M, Caron MG, Geyer MA (2007) Prepulse inhibition deficits and perseverative motor patterns in dopamine transporter knock-out mice: differential effects of D1 and D2 receptor antagonists. J Neurosci 27:1270–1276.
Spaethling JM, Piel D, Dueck H, Buckley PT, Morris JF, Fisher SA, Lee J, Sul JY, Kim J, Bartfai T, Beck SG, Eberwine JH (2014) Serotonergic neuron regulation informed by in vivo single-cell transcriptomics. FASEB J 28:771–780.

Spannuth BM, Hale MW, Evans AK, Lukkes JL, Campeau S, Lowry CA (2011) Investigation of a central nucleus of the amygdala/dorsal raphe nucleus serotonergic circuit implicated in fear-potentiated startle. Neuroscience 179:104–119.

Steel Z, Marnane C, Iranpour C, Chey T, Jackson JW, Patel V, Silove D (2014) The global prevalence of common mental disorders: a systematic review and meta-analysis 1980-2013. Int J Epidemiol 43:476–493.

Takahashi H, Kamio Y (2018) Acoustic startle response and its modulation in schizophrenia and autism spectrum disorder in Asian subjects. Schizophr Res 198:16–20.

Tammimäki A, Käenmäki M, Kambur O, Kulesskaya N, Keisala T, Karvonen E, García-Horsman JA, Rauvala H, Männistö PT (2010) Effect of S-COMT deficiency on behavior and extracellular brain dopamine concentrations in mice. Psychopharmacology (Berl) 211:389–401.

Terranova JI, Song Z, Larkin TE 2nd, Hardcastle N, Norvelle A, Riaz A, Albers HE (2016) Serotonin and arginine-vasopressin mediate sex differences in the regulation of dominance and aggression by the social brain. Proc Natl Acad Sci USA 113:13233–13238.

Thye MD, Bednarz HM, Herringshaw AJ, Sartin EB, Kana RK (2018) The impact of atypical sensory processing on social impairments in autism spectrum disorder. Dev Cogn Neurosci 29:151–167.

Tischler MD, Davis M (1983) A visual pathway that mediates fear-conditioned enhancement of acoustic startle. Brain Res 276:55–71.

van den Berg WE, Lambalais S, Kushner SA (2015) Sex-specific mechanism of social hierarchy in mice. Neuropsycho-pharmacology 40:1364–1372.

Wang HL, Zhang S, Qi J, Wang H, Cachope R, Mejias-Aponte CA, Gomez JA, Mateo-Semidey GE, Beaudoin GMJ, Paladin CA, Cheer JF, Morales M (2019) Dorsal raphe dual serotonin-glutamate neurons drive reward by establishing excitatory synapses on VTA mesoaccumbens dopamine neurons. Cell Rep 26:1128–1142.e7.

Weissbourd B, Ren J, DeLoach KE, Guenthner CJ, Miyamichi K, Luo L (2014) Presynaptic partners of dorsal raphe serotonergic and GABAergic neurons. Neuron 83:645–662.

Yu Q, Teixeira CM, Mahadevia D, Huang Y, Balsam D, Mann JJ, Gingrich JA, Ansorge MS (2014) Dopamine and serotonin signaling during two sensitive developmental periods differentially impact adult aggressive and affective behaviors in mice. Mol Psychiatry 19:688–698.

Zhou T, Zhu H, Fan Z, Wang F, Chen Y, Liang H, Yang Z, Zhang L, Lin L, Zhan Y, Wang Z, Hu H (2017) History of winning remodels thalamo-PFC circuit to reinforce social dominance. Science 357:162–168.

Zhou X, Jen PH, Seburn KL, Frankel WN, Zheng QY (2006) Auditory brainstem responses in 10 inbred strains of mice. Brain Res 1091:16–26.