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Loss of adult 5-HT1A autoreceptors results in a paradoxical anxiogenic response to antidepressant treatment

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Loss of adult 5-HT1A autoreceptors results in a paradoxical anxiogenic response to antidepressant treatment

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Running Title: 5-HT1A autoreceptors in antidepressant-induced anxiety

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Abstract

Selective serotonin (5-HT) reuptake inhibitors (SSRIs) are first-line antidepressants, but require several weeks to elicit their actions. Chronic SSRI treatment induces desensitization of 5-HT1A autoreceptors to enhance 5-HT neurotransmission. Mice (both sexes) with gene deletion of 5-HT1A autoreceptors in adult 5-HT neurons (1AcKO) were tested for response to SSRIs. Tamoxifen-induced recombination in adult 1AcKO mice specifically reduced 5-HT1A autoreceptor levels. The 1AcKO mice showed a loss of 5-HT1A autoreceptor-mediated hypothermia and electrophysiological responses, but no changes in anxiety- or depression-like behavior. Sub-chronic fluoxetine (FLX) treatment induced an unexpected anxiogenic effect in 1AcKO mice in the novelty suppressed feeding (NSF) and elevated plus maze tests, as did escitalopram in the NSF test. No effect was seen in wild-type (WT) mice. Sub-chronic FLX increased 5-HT metabolism in prefrontal cortex, hippocampus and raphe of 1AcKO but not WT mice, suggesting hyper-activation of 5-HT release. To detect chronic cellular activation, FosB⁺ cells were quantified. FosB⁺ cells were reduced in entorhinal cortex and hippocampus (CA2/3) and increased in dorsal raphe 5-HT cells of 1AcKO mice, suggesting increased raphe activation. In WT but not 1AcKO mice, FLX reduced FosB⁺ cells in the median raphe, hippocampus, entorhinal cortex, and median septum, which receive rich 5-HT projections. Thus, in the absence of 5-HT1A autoreceptors, SSRIs induce a paradoxical anxiogenic response. This may involve imbalance in activation of dorsal and median raphe to regulate septo-hippocampal or fimbria-fornix pathways. These results suggest that markedly reduced 5-HT1A autoreceptors may provide a marker for aberrant response to SSRI treatment.
SIGNIFICANCE STATEMENT

Serotonin-selective reuptake inhibitors are effective in treating anxiety and depression in humans and mouse models. However, in some cases SSRI’s can increase anxiety, but the mechanisms involved are unclear. Here we show that rather than enhancing SSRI benefits, adulthood knockout of the 5-HT1A autoreceptor, a critical negative regulator of 5-HT activity, results in an SSRI-induced anxiety effect that appears to involve a hyper-activation of the 5-HT system in certain brain areas. Thus, subjects with very low levels of 5-HT1A autoreceptors, such as during childhood or adolescence, may be at risk for an SSRI-induced anxiety response.
**Introduction**

Major depression is a prevalent and multifactorial mental illness involving genetic and environmental stressors that negatively affect mood and emotion (Krishnan and Nestler, 2008; aan het Rot et al., 2009; Northoff, 2013). Depressive symptoms are correlated with reduced 5-HT neurotransmission, and can be precipitated in recovered depressed subject by acute tryptophan depletion, which lowers 5-HT levels (Young and Leyton, 2002; Jans et al., 2007).

The 5-HT1A receptor is widely expressed in the brain to regulate mood and emotion, both as a post-synaptic heteroreceptor, and as a major presynaptic autoreceptor in 5-HT neurons of the raphe nuclei. The 5-HT1A receptor is an inhibitory Gi/Go-coupled receptor (Albert et al., 1996) that negatively regulates 5-HT neuronal firing activity via negative feedback upon release of 5-HT (Albert, 2012; Blier and El Mansari, 2013). An increase in raphe 5-HT1A autoreceptor level is observed in depressed subjects by PET imaging using the 5-HT1A antagonist [\(^{11}\text{C}\)]-WAY100635 (Hesselgrave and Parsey, 2013). Similarly, a specific increase in 5-HT1A binding in the rostral dorsal raphe was seen by autoradiography studies of post-mortem brains of depressed suicide victims, using the 5-HT1A-selective agonist [\(^{3}\text{H}\)]-DPAT (Stockmeier et al., 1998; Boldrini et al., 2008). Thus, an elevated level of 5-HT1A autoreceptors and reduced 5-HT neurotransmission are associated with human depression, and in mouse depression models (Albert et al., 2014; Garcia-Garcia et al., 2014).

Selective serotonin reuptake inhibitors (SSRIs) are first-line antidepressants due to their specificity and tolerability (Cipriani et al., 2009; Rush et al., 2009). However, antidepressant treatments have a delay of several weeks before an appreciable antidepressant effect is seen and this can lead to patients discontinuing prescription use and increased suicide attempts. Increased levels of 5-HT1A autoreceptors could account for the delay in SSRI action (Albert, 2012; Coplan...
et al., 2014). Acute SSRI treatment induces a transient increase in 5-HT in target areas and in the raphe. Negative feedback from raphe 5-HT1A autoreceptors reduces 5-HT neuronal firing. Following chronic SSRI treatment, 5-HT1A autoreceptor desensitize, leading to dis-inhibition of 5-HT neurons to enhance their firing rate and increase in 5-HT release (Pineyro and Blier, 1999; Albert and Lemonde, 2004). Accordingly, mice with a partial suppression of raphe 5-HT1A autoreceptor levels displayed chronic stress resilience and a robust and rapid (within 8 days) response to chronic SSRI fluoxetine (FLX), while WT mice showed no response even after 26 d of treatment (Richardson-Jones et al., 2010). Similarly, using siRNA to reduce the expression of 5-HT1A autoreceptors acutely during adulthood has an antidepressant-like effect and does not affect the anxiety levels in rats (Bortolozzi et al., 2012). Conversely, up-regulation of 5-HT1A autoreceptors by 5-HT neuron-specific knockout of the HTR1A repressor Freud-1 results in FLX-resistant anxiety and depression in mice (Vahid-Ansari et al., 2017). These results suggest that SSRI action is enhanced when 5-HT1A autoreceptors are partially depleted, but is attenuated when they are up-regulated.

Based on the findings above, we generated an inducible conditional HTR1A knockout mouse (1AcKO) to excise the 5-HT1A gene specifically in 5-HT neurons during adulthood. We find that the 1AcKO mice had no significant change in baseline anxiety- or depression-like behaviors. Upon sub-chronic treatment with SSRIs, we however observed an unexpected anxiogenic effect. In 1AcKO mice, we found increased FLX-induced 5-HT metabolism, consistent with a hyperactive 5-HT system. We also found FLX-induced changes in FosB+ cells specific to 1AcKO in raphe, hippocampal and entorhinal cortex. These findings may provide a model for the pro-anxiety effects of a hyper-activated 5-HT system on response to SSRI treatment.
Materials and methods

Animals

TPH2-CreERT2 mice (stock#016584, C57BL/6N background, Jackson Labs, https://www.jax.org/strain/016584; RRID:IMSR_JAX:016584) were bred to flx-5HT1A mice (Samuels et al., 2015) to create an inducible, conditional 5-HT1A knockout (designated 1AcKO mice). To maintain the mouse line, heterozygous mice for the flx-5-HT1A gene and hemizygous for the TPH2-CreERT2 gene were mated to obtain TPH2-CreERT2/5-HT1A-WT and 1AcKO littermates. Mice were single-housed in standard Plexiglas cages on a 12-hour light/dark cycle with ad libitum access to food and water. All animal studies and experimental procedures were approved by the University of Ottawa Animal Care Committee in accordance with guidelines established by the Canadian Council of Animal Care.

TPH2-CreERT2-flx5HT1A genotype

DNA was extracted (REDExtract-N-AMP Tissue PCR kit, Sigma) from tissue samples and amplified using two sets of PCR primers. The primers (2 μM) for the flx-5HT1A gene were: 1A-5, 5’-GGG CGT CCT CTT CAC GTA G-3’ and 1A-7, 5’-CAG GGA CGT TGT GGT GTT GT-3’, and PCR was done using ONE-Taq Mastermix (New England Biolabs) yielding 254-bp products. The PCR cycle was: 94°C, 2 min; 15 cycles, 94°C, 30 sec; 68°C, 30 sec/-0.5°C cycle; 68°C, 20 sec; 20 cycles, 94°C, 30 sec; 68°C, 30 sec; 68°C for 20 sec; 68°C for 5 min. The primers (2 μM) for TPH2-CreERT2 were: 5’-GCT GAG AAA GAA AAT TAC ATC G-3’, 5’-TGG CTT GCA GGT ACA GGA GG-3’, 5’-CAA ATG TTG CTT GTC TGG TG-3’ and 5’-GCT AGT CGA GTG CAC AGT TT-3’ and PCR was done using EasyTaq buffer and polymerase (Transgen Biotech) yielding a 200-bp product. The PCR cycle
was: 94°C, 1 min; 35 cycles, 94°C, 15 sec, 57°C, 20 sec, 72°C, 10 sec; 72°C, 2 min. Each mouse was genotyped both at weaning and at sacrifice.

Drugs and Treatment

Tamoxifen (cat#T5648) and escitalopram (cat#E4786Z) were obtained from Sigma-Aldrich and fluoxetine was obtained from Enzo Biosciences (cat#BML-NS140-0250, Farmingdale, NY).

Both escitalopram (30 mg/kg/day) and fluoxetine (18 mg/kg/day) were dissolved in water and administered in the drinking water (vehicle). Drug consumed was determined by measuring the amount of drinking water consumed every 3 days (~3 ml/day) and did not differ between groups.

In order to accurately measure treatment consumption, for behavioral assays mice were single housed at the start of treatment until perfusion. The dose for both drugs was chosen based on previous studies showing that they are clinically relevant doses (Santarelli et al., 2003; Berger et al., 2012). Tamoxifen (180 mg/kg, i.p.) was dissolved in ethanol, diluted in sunflower oil (30% ethanol) and administered to both WT and 1AcKO mice on three consecutive days.

Immunofluorescence

Mice were anesthetized (Euthanyl; 0.01 ml/g), perfused by cardiac infusion of PBS followed by 4% paraformaldehyde via the left ventricle. Whole brains were extracted, post-fixed in 4% paraformaldehyde for 1h. Brains were kept in 30% sucrose solution, changed daily for 4 days and frozen. Coronal brain slices (n=4 slices, 25-μm) were taken using a cryostat with respect to Bregma to match sections across groups. Bregma values (mm) and templates (μm) for cell counting were: entorhinal cortex (EC, -1.94; 210 x 305), nucleus accumbens (NAc, +0.98, 321 x 428), medial (MSN, +0.26, 85 x 210) and lateral septum (LSN, +0.26, 80 x 100), rostral hippocampus (-1.22), CA1, (138 x 210), CA2/3 (321 x 428), DG (321 x 428), amygdala (Amy, -1.94, 80 x 128), lateral habenula (LHb, -1.94, 80 x 100) and raphe nuclei (DR, Bregma -4.16, -1.94, 80 x 128), lateral habenula (LHb, -1.94, 80 x 100) and raphe nuclei (DR, Bregma -4.16,
4.41, -4.66, -4.91, comprising four 25-μm slices/brain), median (MR, 150 x 875) and dorsal raphe (DR, 321 x 650). Slices were thaw-mounted on Superfrost Slides (Thermo-Fisher) and stored at -80°C. Staining was done using antibodies to YFP (1:500 chicken anti-GFP, Abcam ab13970, RRID:AB_300798; secondary, 1:250 CY3-goat anti-chicken Jackson, 103-545-155, RRID:AB_2337390); TPH (1:100 sheep anti-TPH, Millipore ab1541, RRID:AB_90754; secondary, 1:200 CY2-donkey anti-sheep, Jackson 713-165-003; RRID:AB_2340727); 5-HT1A receptor (purified anti-5-HT1A 1:50 (Czesak et al., 2012), secondary, AlexaFluor-donkey anti-rabbit 1:1000; ThermoFisher, A-21206, RRID:AB_2535792; or FosB (1:500 rabbit anti-FosB, Santa Cruz sc-48, RRID:AB_631515; secondary, 1:1000 AlexaFluor-donkey anti-rabbit), with conditions as previously described (Vahid-Ansari et al., 2017; Vahid-Ansari and Albert, 2018). Images were acquired on a Zeiss Axio Observer D1 microscope under 20X magnification and analyzed using Axiovision imaging software (RRID:SCR_002677). Positively-stained cells co-localized with DAPI were manually counted in a blinded procedure, within standardized templates (see Results) drawn by ImageJ 1.48v software (RRID:SCR_003070). Cell counts for quantification of FosB+ cells was done as described previously (Vialou et al., 2015; Vahid-Ansari and Albert, 2018) using 1 field/brain region, 1 section/area except raphe (4 sections), with the number and sex of animals shown as data points in figures.

5-HT1A receptor autoradiography

WT and 1AcKO littermate mice (14 weeks old, n=3) were sacrificed by cervical dislocation and decapitation. Extracted brains were immediately frozen on dry ice and stored at -80°C until sectioning. Brains were cryosectioned (25-μm), mounted on Superfrost Slides and maintained at -80°C until processing. Sections were processed for [125I]MPPI (Perkin Elmer, Boston, MA) autoradiography as described (Vahid-Ansari et al., 2017), and exposed to Kodak BioMax MR
film (VWR) for 7h. Films were scanned at 1200-dpi resolution using an Epson Perfection V500 Photo Scanner. Signal density was quantified using the mean luminosity function in ImageJ. The level of 5-HT1A binding (μCi) was quantified by standardizing the signal density to an adjacent background with non-specific binding. Sections between Bregma -4.36 mm and -4.60 mm were averaged for the DR and MR and Bregma -1.82 mm was used for the HPC. Signals fell within the linear range of the film and were quantified following the standard curve using ARC146-F 14C standard (American Radiochemicals Inc, St. Louis, MO).

Whole-cell electrophysiology

Brainstem slices (300-μm) were prepared from 12-15 week old mice as previously described (Geddes et al., 2016). Slices were incubated in a chamber bubbled with 95% O2 and 5% CO2 and containing standard Ringer’s solution. DRN neurons were visualized using an upright microscope equipped with differential interference contrast (DIC) optics (Olympus BX51W1; 40×/0.80 N.A. objective) and 5-HT cells were identified using morphological and biophysical characteristics previously established (Calizo et al., 2011; Geddes et al., 2016). Whole-cell recordings were acquired using an Axon Multiclamp 700B amplifier and digitized with an Axon Digidata 1440A (or 1550) digitizer. The borosilicate glass electrodes were filled with an internal solution containing 115 mM potassium gluconate, 20 mM KCl, 10 mM sodium phosphocreatine, 10 mM Hepes, 4 mM ATP(Mg2+), and 0.5 mM GTP (pH 7.25) (adjusted with KOH; osmolarity, 280–290 mOsmol/L). To determine the function of 5-HT1A autoreceptor, we monitored the effects of the non-selective 5-HT1A agonist 5-CT (100 nM). For all voltage-clamp recordings, access resistance was continuously monitored by applying a 125 ms, 2 mV hyperpolarizing pulse every 10 s. Recordings were discarded if the access resistance changed by >30% during the course of the recordings.
High Performance Liquid Chromatography (HPLC) Analysis

Levels of 5-HT and 5-HIAA were quantified in extracts of dissected tissues by HPLC (Vahid-Ansari et al., 2017). For HPLC, mice were sacrificed by cervical dislocation and decapitation and dorsal raphe, hippocampus and prefrontal cortex were dissected, pooled, frozen immediately on dry ice and maintained at -80°C until homogenization. In brief, 300 μL of homogenization solution (0.3 M monochloroacetic acid, 0.1 mM EDTA, 10% methanol and internal standard) was added to each sample followed by sonication. Following sonication, 100 μL was aliquoted and frozen for protein concentration determination (Pierce™ Coomassie (Bradford) Protein Assay). The remaining 200 μL was centrifuged and the supernatant analyzed for 5-HT and 5-HIAA content using HPLC (Agilent Technologies, Waldbronn, Germany). A 10-μL volume of supernatant was injected into the HPLC system reverse phase analytical column (Phenomenex Kinetex® 2.6 μm C-18, 100 x 4.6 mm) and electrochemical detector (VT-03 flow cell, Intro detector; Antec Leyden, Montreal, Quebec, Canada), with an applied potential of 500 mV, a filter of 1 s, and a range of 100 nA/V. The flow rate was 0.5 ml/min with mobile phase consisting of (in mM): 90 NaH₂PO₄, 1.7 1-octane sulfonic acid (sodium salt), 50 citric acid (monohydrate), 5 KCl, 50 EDTA, and 14% acetonitrile, final pH 3.0. Quantification of analytes was performed by comparing area under the curve with known external standards using computerized Agilent ChemStation chromatography data acquisition system (Agilent).

8OH-DPAT-Induced Hypothermia

The hypothermia procedure was performed between 11 AM and 4 PM. Mice were weighed and internal temperature was taken using a rectal thermometer every 10 minutes for 40 minutes (4 baseline measurements). Animals were administered 8OH-DPAT (0.75 mg/kg, i.p., Sigma) or vehicle (0.9% saline) followed by six measurements of post-treatment body temperature at 10-
min intervals. Each mouse received saline injection on the first day and 8OH-DPAT injection on
the third day. For analysis purposes, the first baseline temperature was discarded. The remaining
three baseline values were averaged and the difference between the average baseline and
recorded temperature was plotted across time.

Behavioral testing
All behavior tests were performed in the University of Ottawa Behavioral Core Facility using 11
week-old 1AcKO and WT littermate mice between 9 AM and 4 PM. One test was done per day
and experimental apparati were cleaned between each trial (Vahid-Ansari et al., 2017). The
behavioral phenotype of 1AcKO vs. WT mice was investigated in several cohorts by 2 different
experimenters who were blind to each mouse condition (genotype and treatment). At the start of
treatment, mice were single housed and maintained on treatment until perfusion. In each cohort,
the proportion of males: females was close to equal, plotted as blue and pink symbols,
respectively. For females, estrous cycle staining was done at the end of behavioral testing.
Vaginal smears were taken from 20 female WT and 1AcKO mice every day at 2 pm for four
consecutive days, the length of the mouse estrous cycle. Smears were dried overnight on
Superfrost slides (Fisher) and stained with 0.1% cresyl violet (Sigma) and staged (McLean et al.,
2012). Estrus cycle staging revealed a relatively similar distribution of each cycle (proestrus,
estrus, metestrus/diestrus) between each genotype across the four days of testing, as reported in
normal mice (McLean et al., 2012). Mice were kept under their respective antidepressant
treatment during all the tests until they were sacrificed.

Novelty Suppressed Feeding (NSF) test
Mice were food restricted for 24 h and placed in a corner of a 45 x 45 x 45 cm open box with
bedding, overhead illumination (100 lux) and a pellet in the center. Latency for the mouse to first
approach to the food within 10 min was recorded. Mice were returned to the home cage with a weighed food pellet and the latency to feed and total food consumption after 5 min was recorded.

*Light Dark Box (LD)*

Each mouse was placed in a sound-proof square chamber equally divided into two chambers measuring $27 \times 13.5$ cm for 10 min (Vahid-Ansari et al., 2016). The dark compartment was covered in black plastic and the light compartment is transparent, uncovered and lit with two bulbs at 390 lux (Med Associates, St Albans, VT, USA) with an opening allowing movement between chambers. Movement was detected by infrared transmitters and receivers placed around the chamber periphery.

*Elevated Plus Maze (EPM)*

The mouse was placed in the center of an elevated track with two 6-cm wide and 75 cm long arms crossed in the center at a perpendicular angle (Noldus, Wageningen, The Netherlands) for 10 min. The closed arm was enclosed by walls 20 cm tall while the open arm had no walls. An overhead illumination (100 lux) and a camera linked to the Ethovision tracking program (Ethovision 11.5, Noldus Information Technologies, Leesburg, VA, USA) recorded mouse movement.

*Tail Suspension (TS)*

Each mouse’s tail was taped horizontally on an aluminum bar attached to a transducer detecting the force of movement for 6 min. Immobility time was quantified as movement below a set threshold (Med Associates, Fairfax, VT, USA).

*Forced Swim Test (FST)*
Each mouse was placed in a clear Plexiglas cylinder filled with 23-25°C tap water up to 5-10 cm from the top for 6 minutes under red light. A camera placed in front of the cylinder recorded the movement and the immobility time was quantified using Ethovision software (Noldus).

**Statistical Analysis**

All statistical analyses and graphs were done using Graphpad Prism 6 (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA, RRID:SCR_002798). Data are shown as mean ± standard error of the mean (S.E.M). For behavioral and immunofluorescence studies, outliers were identified using the ROUT method in Graphpad Prism 6 and were excluded from the analysis. Data comparing the WT versus 1AcKO mice on one outcome measure were analyzed using an unpaired t-test with p <0.05 considered significant. For data comparing across genotype and treatment, two-way analysis of variance (ANOVA) was used and all post-hoc comparisons were made with Tukey’s multiple comparisons test.
Results

Specific loss of 5-HT1A autoreceptor protein and function in 1AcKO mice

To address the role of 5-HT1A autoreceptors in adulthood, we generated mice with conditional, inducible knockout of the HTR1A gene by mating TPH2-CreER\textsuperscript{T2} and flx-5HT1A mice to generate the 1AcKO mice (Fig. 1A). Since the flx-5HT1A mice have a YFP cassette inserted downstream of the 5-HT1A coding sequence that is induced upon recombination, YFP can be used as a marker of recombination. In adult 1AcKO but not WT littermates treated from postnatal days 53-55 with tamoxifen to induce recombination, two weeks later YFP expression was seen in TPH+ (5-HT) cells of the dorsal raphe nucleus (Fig. 1B, D), but not in non-serotonergic brain regions, such as prefrontal cortex or hippocampus (Fig. 1-1). In 1AcKO vs. WT mice, there was a 90% reduction in 5-HT1A-stained TPH+ cells in the dorsal raphe (Fig. 1C, E), indicating strong reduction in 5-HT1A autoreceptors. This reduction in 5-HT1A receptors was confirmed using [\textsuperscript{125}I]-MPPI to detect specific 5-HT1A receptor binding. In the raphe nuclei, a ~60% reduction in specific binding was detected (Fig. 2A, C), while no change was seen in the hippocampus (Fig. 2B, D).

The function of 5-HT1A autoreceptors in vivo was addressed by measuring DPAT-induced hypothermia, because in mice this response is dependent on 5-HT1A autoreceptors (Richardson-Jones et al., 2011). In contrast to WT littermates, in both male and female 1AcKO mice, the DPAT-induced hypothermia response was abolished (Fig. 2E). We next assessed the function of 5-HT1A autoreceptors by whole-cell electrophysiological recordings of dorsal raphe 5-HT neurons in acute midbrain slices (Fig. 2F). Bath administration of the non-selective 5-HT1A agonist 5-CT, induced a robust outward current that was evident in every recording from 5-HT neurons in WT slices. In contrast, the magnitude of this current was much reduced in
recordings from 5-HT 1AcKO cells, being noticeable in only one recording. Altogether, these results outline the effective abolishment of functional 5-HT1A autoreceptors in 5-HT neurons from 1AcKO mice.

**Anxiogenic actions of sub-chronic SSRI treatment in 1AcKO mice**

Next, we addressed the behavioral phenotype of the 1AcKO mice. Since preliminary studies had shown no difference in baseline anxiety and depression behavior between WT and 1AcKO mice in males and females (Luckhart, 2015), we pooled data from both sexes shown as blue and pink symbols. Previously, mice with a 30% suppression of 5-HT1A autoreceptors showed a more rapid and robust FLX-induced reduction in anxiety in the NSF test (Richardson-Jones et al., 2010). Hence, we examined whether sub-chronic treatment with SSRIs affected behavior in the 1AcKO, with a 90% reduction in 5-HT1A autoreceptors. As a first step, we investigated the effects of two SSRI treatments in the NSF anxiety test. Whereas sub-chronic FLX or ESC treatment had no effect in WT mice, we observed a significant increase in latency to feed in novel cage in 1AcKO, indicative of increased anxiety (Fig. 3A, B). As controls for hunger/motivation, we found no difference in latency to feed or food consumption in the home cage (Fig. 3-1A). To further establish the development of an anxiogenic effect of SSRI treatment in the 1AcKO, these mice were subjected to the LD and EPM tests. In the LD test (Fig. 3-1B), we failed to see any difference between the groups of mice in time or latency to enter the light side, suggesting no effect in this mild anxiety test. In the EPM, FLX induced a significant reduction in open arm time in 1AcKO but not WT, while ESC had no significant effect. No effect was seen on closed arm time or on total distance travelled in the maze (Fig. 3-1C). The effect of FLX on depression-related behavior was addressed by measuring the immobility time in the TS and FST (Fig. 3C, D). In both tests FLX had no effect on the
immobility time in WT or 1AcKO mice. These data indicate that sub-chronic treatment with SSRIs is anxiogenic in 1AcKO mice.

**FLX-induced 5-HT metabolism and cellular activity in 1AcKO mice.**

Because 5-HT1A autoreceptors negatively regulate 5-HT neuronal activity, we hypothesized that 1AcKO mice treated with FLX may show increased release of 5-HT, resulting in increased levels of the metabolite, 5-HIAA. To test this, we measured both 5-HT and 5-HIAA levels in prefrontal cortex, hippocampus and raphe tissues (Fig. 4, statistics Fig. 4-1). There was a significant decrease in basal 5-HT and 5-HIAA levels only in the hippocampus of vehicle-treated 1AcKO compared to WT mice. In hippocampus, FLX treatment reduced 5-HT levels in both WT and 1AcKO, but increased 5-HIAA only in the 1AcKO, resulting in increased 5-HIAA/5-HT ratio. In all tissues, sub-chronic FLX treatment reduced 5-HT levels more strongly in 1AcKO than in WT mice. This led to significant increase in 5-HIAA/5-HT ratio in all tissues of 1AcKO mice with no change in WT mice. Thus, loss of 5-HT1A autoreceptors results in increased 5-HT metabolism in prefrontal cortex, hippocampus and raphe in response to sub-chronic FLX treatment.

We next addressed whether neuronal activity of the raphe or other brain regions was altered in 1AcKO mice treated with FLX (Fig. 3, timeline), using FosB staining after sacrifice as a marker for chronic neuronal activation (Figs. 5-6). In the raphe, there were no changes in the number of 5-HT neurons (TPH+ cells, Fig. 5B, C; statistics, Fig. 5-1). However, the number of FosB/TPH+ neurons was increased in 1AcKO dorsal raphe, suggesting a higher level of chronic neuronal activation (Fig. 5B). In 1AcKO mice, treatment with FLX induced a reduction in FosB/TPH+ dorsal raphe cells, but had no effect in WT. Oppositely in the median raphe (Fig. 5C), FLX reduced FosB/TPH+ cells in WT, but no change was seen in 1AcKO sections. Thus
dorsal and median raphe showed differential regulation upon depletion of 5-HT1A autoreceptors and chronic treatment with FLX.

FosB positive cells were found in several brain regions, and were quantified to correlate with FLX-induced anxiety (Fig. 6). Comparing vehicle-treated 1AcKO to WT, significant reductions in FosB+ cells were seen in EC, CA2/3, MSN and Amy, and an increase in NAc. FLX treatment reduced FosB+ cell counts in several regions of WT but not 1AcKO mice (CA1, DG, MSN), and more strongly in EC of WT compared to 1AcKO mice. FLX-treated 1AcKO showed the most robust reductions in FosB in CA1, DG, Amy and LHb, compared to WT. Thus, there was a reduced effect of FLX treatment in several brain regions in 1AcKO compared to WT, but regions in which FLX response was more robust in 1AcKO mice may contribute to the altered behavioral response.
Discussion

Role of reduced 5-HT1A autoreceptors in SSRI-induced anxiety

In this study we have generated the 1AcKO mice with 5-HT neuron-specific knockout of the HTR1A gene to probe the role of 5-HT1A autoreceptors in behavior and response to sub-chronic SSRI treatment. Previously, early life suppression of 5-HT1A autoreceptor expression was shown to induce a developmental anxiety phenotype in the LD and open field tests that was not seen upon 5-HT1A autoreceptor suppression in adulthood (Richardson-Jones et al., 2010; Richardson-Jones et al., 2011). In order to avoid developmental effects, we addressed whether inducible knockout of the 5-HT1A autoreceptor in adulthood would affect behavior. We found that the 1AcKO have a 90% reduction in 5-HT1A receptor-positive 5-HT neurons, although total binding of 5-HT1A autoreceptors in the raphe was reduced by only about 60%. This may reflect background from 5-HT1A receptors in non-serotonergic neurons (Fig. 1C), such as GABAergic interneurons (Bonnavion et al., 2010). Using electrophysiology, we found that 5-HT1A-mediated currents in 5-HT neurons were largely absent in 1AcKO slices. Similarly, DPAT-induced hypothermia was abolished in 1AcKO mice (Fig. 2E), indicating impaired 5-HT1A autoreceptor function in vivo.

In the 1AcKO mice, baseline (or vehicle) anxiety and depression behaviors did not differ from the WT littermates in the tests used, consistent with our findings in 1AcKO mice on a different genetic background with 80% loss of binding (Vahid-Ansari et al., 2017), and with findings following adulthood suppression of 5-HT1A autoreceptors by 30% (Richardson-Jones et al., 2010). However, transient siRNA-mediated suppression of 5-HT1A autoreceptors by 30-40% for up to 3 days in C57BL6 mice resulted in an anti-depressed effect in the TS and FST, with no change in anxiety in the EPM test (Bortolozzi et al., 2012). In our studies, depletion of
5-HT1A autoreceptors was more extensive and chronic (FST and TS were done >32 d post-
tamoxifen). In this time, compensatory mechanisms, such as recurrent inhibition from prefrontal
cortical projections to raphe GABAergic neurons (Geddes et al., 2016), may have normalized
behavior. Conversely, increasing 5-HT1A autoreceptor levels by 50% in adulthood upon
deletion of the repressor Freud-1/CC2D1A in 5-HT neurons results in anxiety and depression
phenotypes that are resistant to chronic FLX treatment (Vahid-Ansari et al., 2017). Sex-
dependent anxiety and depression phenotypes have been reported in other mouse models with
similar up-regulation of 5-HT1A autoreceptors (Luckhart et al., 2016; Philippe et al., 2018).
Although we tested both sexes here, our ability to detect sex differences was underpowered. In
contrast, transgenic over-expression of adult 5-HT1A autoreceptors by several-fold results in an
aggression phenotype (Audero et al., 2013). However, our results suggest that deletion of adult
5-HT1A autoreceptors does not alter baseline anxiety/depression-like behaviors, although we did
not test for effects on stress resiliency. The lack of behavioral response to sub-chronic FLX in
the WT mice may indicate their insensitivity to the antidepressant effects of enhancing serotonin.

The anxiogenic response to sub-chronic SSRI treatment of 1AcKO mice was unexpected.
In previous studies, a striking anxiolytic effect of sub-chronic FLX treatment was seen in the
NSF test in mice with a 30% suppression of 5-HT1A autoreceptors, but not in normal mice
(Richardson-Jones et al., 2010) similar to our WT mice. The main difference is the more
extensive reduction in 5-HT1A autoreceptors in our 1AcKO mice. Upon SSRI treatment of
1AcKO mice, serotonin-induced inhibition of 5-HT1A-positive GABAergic raphe neurons
(Bonnavion et al., 2010) could further reduce local inhibition of 5-HT neurons, in addition to loss
of 5-HT1A autoreceptors. This synergistic effect upon SSRI-induced blockade of reuptake could
drive the anxiogenic response. A likely candidate for this effect is the ratio of median to dorsal
raphe activity, which has been shown to correlate with anxiety in the postnatal FLX model of anxiety-depression (Teissier et al., 2015). The 1AcKO mice appeared resistant to median raphe inactivation upon chronic SSRI treatment, but displayed reduced activity of 5-HT neurons in the dorsal raphe (Fig. 5C).

Alterations in basal and FLX-induced brain networks upon loss of 5-HT1A autoreceptors

Although we did not detect baseline behavioral changes in 1AcKO mice in the assays used, loss of 5-HT1A autoreceptors did have functional effects on responses to acute DPAT (reduced hypothermia), sub-chronic FLX (increased 5-HT metabolism and anxiety) and on basal and FLX-induced neuronal activity. Following sub-chronic (11-day) FLX treatment at a clinically relevant dose that partially blocks 5-HT reuptake (Vahid-Ansari et al., 2017), the 5-HIAA/5-HT ratio was increased in 1AcKO PFC, hippocampus and raphe compared to WT. This increase in 5-HT metabolism is consistent with increased FLX-induced 5-HT release that could result from increased firing of 5-HT neurons upon loss of 5-HT1A autoreceptors in the 1AcKO mice. Similarly, 8-day sub-chronic but not chronic FLX treatment induced >2-fold increase in 5-HT release in hippocampus but not PFC of mice with adult suppression of 5-HT1A autoreceptors (Richardson-Jones et al., 2010). The lack of change in basal 5-HT or 5-HIAA in raphe and PFC is consistent with the lack of behavioral phenotype in untreated or vehicle-treated 1AcKO compared to WT mice.

Interestingly, FosB activity in dorsal and median raphe was differentially altered: FLX inhibited dorsal raphe FosB, but maintained FosB in median raphe (Fig. 5B). The median raphe projects mainly to the septum and hippocampus, while the rostral dorsal raphe projects to amygdala, NAc, and PFC (Commons, 2016; Muzerelle et al., 2016). In rats, microinjection of 5-
HT1A antagonist WAY100635 or neuronal activator kainate in the median raphe is anxiogenic, and this effect was blocked by intra-hippocampal injection of WAY100635, implicating a median raphe-dorsal hippocampal circuit in anxiety (Dos Santos et al., 2008). Optogenetic or chemogenetic activation of median raphe 5-HT neurons in mice elicits anxiety-like behavior (Ohmura et al., 2014; Teissier et al., 2015). These results suggest that maintained FLX-induced 5-HT release from median raphe-hippocampal projections (Muzerelle et al., 2016) may mediate increased anxiety-like behavior in the 1AcKO mice (Belzung et al., 2014). On the other hand, optogenetic stimulation of hippocampal DG granule cells has a rapid anti-anxiety effect (Kheirbek et al., 2013) and 5-HT1A heteroreceptors on these cells are required for the anti-anxiety effect of chronic FLX treatment in the NSF (Samuels et al., 2015). Hence different populations of hippocampal neurons may mediate the pro- and anti-anxiety of actions of FLX treatment.

Comparing 1AcKO to WT mice, basal FosB activity was altered in three brain areas that are linked via the fimbria-fornix pathway, with reduced FosB in EC, CA2/3 and MSN. This pathway is important for spatial information processing, however since the assays we used did not specifically test for this we did not see any difference between 1AcKO and WT behaviors. However, in 1AcKO vs. WT mice a reduced FLX-induced inhibition of FosB was seen in these regions and additional hippocampal and medial septal areas suggesting a more widespread and robust effect. The septo-hippocampal pathway is implicated in sensory gating of spatial information (Kaifosh et al., 2013), and is innervated by the median raphe (Szonyi et al., 2016). Maintenance of median raphe activity to enhance 5-HT release in these areas post-FLX in the 1AcKO mice may have contributed to the anxiety phenotype (Dos Santos et al., 2008; An et al.,
Interestingly, both NSF and EPM rely on spatial cues to elicit anxiety more than the LD box test, which may more robustly recruit hippocampal input. We have previously seen that multiple brain regions are activated in a mouse model of post-stroke anxiety and depression (Vahid-Ansari and Albert, 2018). Importantly, chronic FLX treatment reversed the anxiety and depression phenotypes in this post-stroke model, and reduced FosB in glutamatergic cells in most brain regions, including prefrontal cortex, hippocampus, and lateral septum. By contrast, the inability of FLX to reduce FosB in the 1AcKO compared to WT mice was associated with its anxiogenic effect. This suggests that the combination of genotype and FLX treatment results in hyperactivation of the 5-HT system that could contribute to FLX-induced anxiety in the 1AcKO mice, but is not the only factor.

Clinical relevance of low 5-HT1A autoreceptors

These findings may have implications for SSRI-induced anxiogenic responses seen clinically following sub-chronic treatment, particularly in adolescence (Sinclair et al., 2009; Goldsmith and Moncrieff, 2011; Olivier et al., 2011). Comparing transgenic/knockout mouse models that alter 5-HT neurotransmission, a U-shaped relation to anxiety is seen: increased anxiety occurs in models with very low 5-HT, as well as models with elevated 5-HT release (Albert et al., 2014). Depression-like behavior is associated with reduced 5-HT in mouse models treated with sub-chronic FLX increases anxiety. Because 5-HT1A autoreceptors are expressed at low levels in mice until late-adolescence (p21-p30) (Richardson-Jones et al., 2011), the reduced binding in 1AcKO mice mimics this aspect of adolescent 5-HT regulation. Transient suppression of 5-HT1A autoreceptors by 40% during this period of adolescence (p14-30) induces
baseline anxiety-like behavior in mice (Donaldson et al., 2014). Similarly, chronic FLX treatment in adolescence increases anxiety-like behavior in mice (Oh et al., 2009; Iniguez et al., 2014) and is associated with risk of suicide in human youths (Olivier et al., 2011). The region-specific activity changes that associate with anxiety in the 1AcKO mice may provide insight into aberrant clinical responses to SSRI treatment.

In summary, upon knockout of 5-HT1A autoreceptors in adulthood we did not find changes in anxiety- or depression-like behaviors in 1AcKO mice. However, we did find increased activity of 5-HT neurons, and a paradoxical anxiogenic response to sub-chronic FLX treatment that may be driven by increased median raphe 5-HT release and activation of septo-hippocampal pathways.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.


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Figure 1. Loss of 5-HT1A autoreceptors in adult 5-HT neurons of 1AcKO mice

A. Conditional Knockout Strategy. To knockout the HTR1A gene in adult 5-HT neurons, TPH2-CreERT2 and flx-5HT1A mice were bred to generate 1AcKO mice; upon treatment with tamoxifen the 5-HT1A gene is knocked out and YFP is expressed in TPH-positive serotonin neurons. B, C. Tamoxifen-induced YFP expression and 5-HT1A receptor loss in dorsal raphe 5-HT neurons. Dorsal raphe sections from tamoxifen-treated 1AcKO and WT littermates were stained for TPH (5-HT neuron marker) and YFP (marker of recombination) or 5-HT1A receptor. Scale bar is shown. D, E. Quantification of TPH/YFP and TPH/5-HT1A positive cells. Data represent mean ± S.E. (n=4), ****p < 0.0001, unpaired two-tailed Student’s t-test; D, t=152.9 df=6; E, t=90.39 df=4. No YFP signal was detected in other brain regions (Extended Data Figure 1.1).

Figure 2. Loss of raphe 5-HT1A binding and autoreceptor function in 1AcKO mice

A, B. 5-HT1A receptor autoradiography of raphe and hippocampus. Above: Shown are representative images of [125I]MPPI autoradiography of sections from 1AcKO and wild-type (WT) littermate mice showing dorsal (DR) and median raphe (MR) (A, boxes) and hippocampal (B, circumscribed) regions. C, D. Average intensity (μCi/g) was quantified for each region using templates above. Data represent mean ± SEM (n=3/group), unpaired two-tailed Student’s t test; DR: p=0.0039**, t=16.05 df=3; MR: P=0.0147*, t=5.088 df=3; Hippocampus: P=0.1999 ns, t=1.440 df=6. E. 5-HT1A-induced hypothermia. In WT, 8OH-DPAT (DPAT: 0.75 mg/kg, i.p.) induced body temperature reduction compared to saline (Sal), which was abolished in 1AcKO mice in both males and females. Data represent mean ± S.E. (n=7-12/group); two-way ANOVA,
Tukey’s post-test showed significant changes for: WT-DPAT vs. WT-Sal (black stars, below DPAT data); and WT-DPAT vs. 1AcKO-DPAT (blue stars, between DPAT data); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (see Extended Data Figs. 2.1-2.2 for statistics). The pretreatment temperatures (°C) were (Mean ± SD): M-Sal WT 38.0±0.24, KO 38.0 ±0.24; M-DPAT WT 38.6±0.50, KO 38.3±0.31; F-Sal WT 38.1±0.32, KO 38.2±0.47; F-DPAT WT 38.2±0.27, KO 38.2±0.31. F. Reduced 5-HT1A-mediated outward currents in 1AcKO mice.

Whole-cell voltage clamp recordings of DR 5-HT neurons from WT (n=6) and 1AcKO (KO, n=7) mice. At left, time course of 5-HT1A-mediated outward current in response to 5-CT (100 nM; Vm = -55 mV). At right, the average peak steady-state 5-HT1AR-mediated currents from recorded 5-HT neurons. Data are presented as mean ± S.E.; *p<0.05, unpaired two-tailed Student’s t-test.

Figure 3. Anxiogenic response to sub-chronic SSRI treatment in 1AcKO mice

Shown above is the experimental timeline. At the start of antidepressant treatment, wild-type (WT) and 1AcKO mice were single-housed (SH), treated with tamoxifen (Tam), and treated with fluoxetine (FLX), escitalopram (ESC) or vehicle (Veh). After 9 days, the mice tested using the indicated behavior tests, maintaining treatment, and sacrificed at the end testing. Data points from individual male (M, blue) and female (F, pink) mice are shown. A. SSRI-induced anxiety in 1AcKO in the novelty suppressed feeding (NSF) test. Vehicle, FLX and ESC were administered to WT (n (M/F (outlier))= 9/8 (2), 8/11, 4/4, respectively) and 1AcKO (n=6/4 (1), 5/7, 4/4, respectively) mice. Latency to feed in novel cage was recorded. FLX vs. Veh: F (3,53) = 7.941, P = 0.0002; ESC vs. Veh, F (3,38) = 3.172, P = 0.0351. B. FLX-induced anxiety in 1AcKO mice in the elevated plus maze (EPM) test. Vehicle, FLX and ESC were administered to
Flx1A WT (n=9/8, 8/11, 4/4, respectively) and KO (n=6/6, 5/8 (1), 4/4, respectively), and time spent in the open arm was measured. FLX vs. VEH: F (3, 56) = 4.128, P = 0.0103; ESC vs. VEH: F (3, 40) = 1.938, P = 0.1389. C, D. Tail suspension (TS), forced swim test (FST). For TS: WT, n (M/F (outlier))=3/3, 4/5, 4/4; KO, n=2/3, 4/3, 4/4; for FST: WT n=5/5, 7/6, 4/4; KO, n=2/3 (1), 3/3, 4/4, for Veh, FLX, ESC, respectively. No effect of FLX or ESC was seen on immobility time in these two tests of depression-like behavior. TS (vs. VEH): FLX, F (3,23) = 0.6195, P = 0.6095; ESC, F (3,23) = 0.6724, P = 0.5777; FST: FLX, F (1,30) = 0.0358, P = 0.8512; ESC, F (1,27) = 1.458, P = 0.2377. Data represent mean ± S.E., *p<0.05; **p<0.01; ***p<0.001 for drug vs. VEH, or as indicated, by two-way ANOVA, Tukey’s post-test. No differences between genotypes were observed in control measures for NSF (latency to feed, food consumption), EPM (closed arm time), or in the LD test (see Extended Data Fig. 3-1).

**Figure 4. Enhanced 5-HIAA/5-HT in FLX-treated 1AcKO mice.**

Tissue 5-HT and 5-HIAA content in WT and 1AcKO mice was quantified by HPLC in prefrontal cortex (PFC), hippocampus (Hippo) and dorsal raphe (DR) following fluoxetine (FLX) or vehicle (Veh) treatment (21-23 d), and the 5-HIAA/5-HT ratio calculated. Data points from individual male (M, blue) and female (F, pink) mice are shown. No changes were seen in DR, but significant reduction in 5-HT and increase in 5-HIAA was seen in 1AcKO PFC and hippocampus, while only partial or opposite effects were seen in WT. For WT (Veh, FLX), n (M/F)= 3/3, 3/3; for KO, n=3/3, 5/5. Data represent mean ± S.E., *p<0.05, **p<0.01, two-way ANOVA, Tukey’s post-test (for treatment × genotype interaction see Extended Data Fig. 4-1).
Figure 5. Opposite FLX-induced FosB changes in 5-HT neurons of 1AcKO vs. wild-type mice.

Vehicle (Veh) or fluoxetine (FLX) was administered to WT and 1AcKO mice for 24 days. A. Merge of immunofluorescence staining of dorsal (DR) and median (MR) raphe nuclei using DAPI (nuclei), anti-TPH (5-HT marker) and anti-FosB (chronic activity marker) from WT (left) or 1AcKO (right) mice. Carat symbols show examples of FosB/TPH+ cells. For images of single staining see Extended Data Fig. 5-2. B. Quantification of total TPH+, FosB+ and FosB/TPH+ cells in dorsal and median raphe for male (M, blue) and female (F, pink) mice. In dorsal raphe, FLX reduced TPH/FosB+ cells in 1AcKO but not WT; in median raphe, FLX reduced TPH/FosB+ cells in WT but not 1AcKO. For DR (TPH+, FosB+, TPH/FosB+), n (M/F (outliers))= 3/4, 3/4, 4/4 (WT-Veh); 4/5, 5/4, 6/5 (WT-FLX); 5/6, 5/5, 8/8 (1) (KO-Veh); 7/5, 8/5, 11/9 (1) (KO-FLX); for MR, n=5/5, 5/5, 5/5 (1) (WT-Veh); 4/4, 3/3, 4/5 (WT-FLX); 5/6, 5/5 (1) (KO-Veh); 6/6, 5/6, 6/5 (1) (KO-FLX). Significant treatment x genotype interaction was detected for FosB/TPH+ cells in both DR and MR (Fig. 5.1). Data represent mean ± S.E., *p< 0.05; ***p< 0.001, two-way ANOVA, Tukey’s post-test.

Figure 6. Brain regional differences in FLX-induced FosB changes in 1AcKO vs. wild-type mice.

Shown above are figurative images of coronal sections at Bregma levels (Paxinos and Franklin, 2001) and including regions of interest and templates (μm x μm) used for cell counting. Vehicle (Veh) or fluoxetine (FLX) was administered to mice for 24 days. Immunofluorescence staining for FosB was compared in 1AcKO and WT brain sections showing detectable FosB, and FosB+ cells were quantified and plotted for male (M, blue) and female (F, pink) mice. For WT-Veh,

Significant treatment x genotype interaction was detected for EC, CA2/3, MSN, with trend for LSN (Fig. 6-1). Data represent mean ± S.E., *p< 0.05; **p<0.01; ***p<0.001; ****p<0.0001, two-way ANOVA, Tukey’s post-test.
Extended Data Figure Legends

Figure 1-1. Lack of recombination in prefrontal cortex and nucleus accumbens.

Immunofluorescence of representative brain sections of prefrontal cortex (PFC) and nucleus accumbens (NAc) from wild-type (WT) and 1AcKO mice treated with tamoxifen. DAPI was used to detect nuclei and anti-GFP to detect YFP as a marker of recombination. No YFP staining was detectable in contrast to raphe (Fig. 1). Scale bar is shown.

Figure 2-1. Extended Statistical Data for Figure 2E.

Data analysis of agonist-induced hypothermia in male 1AcKO mice (Figure 2E) is presented. Statistical results of a two-way ANOVA comparing genotype and time are shown for male WT or 1AcKO mice. Bold font indicates statistical significance.

Figure 2-2. Extended Statistical Data for Figure 2E.

Data analysis of agonist-induced hypothermia in female 1AcKO mice (Figure 2E) is presented. Statistical results of a two-way ANOVA comparing genotype and time are shown for female WT or 1AcKO mice. Bold font indicates statistical significance.

Figure 3-1. Control measures in anxiety tests and LD anxiety are unchanged.

Wild-type (WT) and 1AcKO mice treated with Vehicle (Veh), fluoxetine (FLX, left panels) or escitalopram (ESC, right panels) were subjected to NSF (A) LD (B) and EPM (C), for the groups described in Fig. 3. Data points from individual male (blue) and female (pink) mice are shown.

A. NSF test. The control measures of latency to feed in the home cage (above) and food consumed in home cage (below) did not differ. B. LD test. Neither FLX nor ESC altered time...
spent or entries into the light zone, although there was a trend for fewer entries in FLX-treated WT mice. N values (M/F): WT-Veh 3/3; FLX 5/5; ESC 4/4; 1AcKO-Veh 2/2; FLX 4/4; ESC 4/4. C. EPM. Time in closed arms and total distance travelled did not differ between groups. Data represent mean ± S.E., analyzed by two-way ANOVA, Tukey’s post-test.

**Figure 4-1. Extended Statistical Data for Figure 4.**
Statistical analysis of tissue 5-HT metabolite data following fluoxetine (FLX) treatment (Figure 4). Data were analyzed by 2-way ANOVA for treatment × genotype interaction; post hoc Tukey was done comparing Vehicle vs. FLX treatment. Bold, statistically significant results; PFC, prefrontal cortex; Hippo, hippocampus; DR, dorsal raphe.

**Figure 5-1. Extended Statistical data for Figure 5.**
Statistical analysis of TPH+, FosB+, and FosB/TPH+ cells in raphe of cells in 1AcKO vs. W.T. mice following fluoxetine (FLX) treatment (Figure 5B, C). Data were analyzed by 2-way ANOVA for treatment × genotype interaction; post hoc Tukey test was done comparing Vehicle vs. FLX treatment. Bold, statistically significant results; DR, dorsal raphe; MR, median raphe.

**Figure 5-2. Images of TPH/FosB raphe staining.**
Vehicle (Veh) or fluoxetine (FLX) was administered to mice for 24 days. Immunofluorescence staining of dorsal (DR) and median (MR) raphe sections using DAPI (nuclei), anti-TPH (5-HT marker) and anti-FosB (chronic activity marker) from WT (left) or 1AcKO (right) mice. The merged version of these images is shown in Fig. 5A.
Figure 6-1. Extended Statistical Data for Figure 6.

Statistical analysis of FosB+ cells in brain regions in 1AcKO vs. W.T. mice following fluoxetine (FLX) treatment. Data from Fig. 6 were analyzed by 2-way ANOVA for treatment × genotype interaction; post hoc Tukey was done comparing Vehicle vs. FLX treatment. Bold, statistically significant results; bold italic indicates a non-significant trend. EC, entorhinal cortex; NAc, nucleus accumbens; LSN, lateral septal nucleus; MSN, medial septal nucleus; hippocampal CA1, CA2/3, and dentate gyrus (DG); Amy, amygdala; LHb, lateral habenula.