Title
Effects of Prenatal Fluoxetine Use on Maternal Microbiome and Fetal Dorsal Raphe Nucleus Development

Author
Kazantsev, Maria

Publication Date
2019

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Effects of Prenatal Fluoxetine Use on Maternal Microbiome and Fetal Dorsal Raphe Nucleus Development

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Science

by

Maria Kazantsev

2019

ABSTRACT OF THE THESIS

Effects of Prenatal Fluoxetine Use on Maternal Microbiome

and Fetal Dorsal Raphe Nucleus Development

by

Maria Kazantsev

Master of Science in Physiological Science

University of California, Los Angeles 2019

Professor Elaine Y. Hsiao, Chair

Selective serotonin reuptake inhibitors (SSRIs) are used as first-line treatments by up to 10% of pregnant women suffering from major depression. However, the effects of prenatal SSRI use on fetal development are still largely unknown. Serotonin plays crucial roles in the early development of the fetal brain, and the fetus depends on a maternal source of serotonin. In humans, 90% of serotonin synthesis occurs in the gut, promoted by specific microbiota. In the present study, we used a pregnant mouse model to assess for changes in the maternal microbiome, after early prenatal fluoxetine gavage. We examined the fetal brain for changes in the development of the dorsal raphe nucleus, as well as its axonal projections to the prefrontal cortex. Our results suggest that prenatal SSRI use does not result in a significant shift in maternal microbiome, and has no direct effect on the development of the endogenous fetal serotonergic system.

The thesis of Maria Kazantsev is approved.

Patricia E. Phelps

Yvette Taché

Elaine Y. Hsiao, Committee Chair

University of California, Los Angeles

2019

Table of Contents

List of Figures

Figure **Page**

Introduction

Major depression is one of the most prevalent health issues affecting the global human population today. It is the leading cause of disability worldwide, with the World Health Organization (WHO) reporting over 322 million people affected (World Health Organization, 2017). In the United States alone, approximately 16.2 million adults are affected by major depression (Substance Abuse and Mental Health Services Administration, 2017), and this rate has continued to rise significantly since 2005 (Weinberger et al., 2018). The consequences of this illness are pervasive, impairing a person's ability to perform at school or work, putting strain on relationships with friends and family, and in many cases, resulting in suicide (World Health Organization, 2017).

Selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed as first-line treatments for depression. However, while they generally have milder side effects compared to other classes of antidepressants such as monoamine oxidase inhibitors (MAOIs) or tricyclic antidepressants (TCAs) (Ferguson, 2001), SSRIs are still far from an ideal solution. Several side effects are associated with SSRI use, including nausea and gastrointestinal issues, drowsiness, disturbed sleep rhythms, and sexual dysfunction (Ferguson, 2001; Kostev et al., 2014). Furthermore, the mechanism of action for SSRIs is not fully understood. Although SSRIs are known to inhibit the serotonin transporter (SERT), the direct impact of increased extracellular serotonin on areas implicated in depression is still unclear. As a result, there is currently no defined protocol for treatment of depression. The efficacies of different drugs vary from person to person, and a number of large-scale meta-analyses have been unable to adequately assess which products work best, or run the smallest risk of side effects (Jia et al., 2016; Magni et al., 2013; Cipriani et al.,

2016). Thus, although the use of SSRIs is extremely common (Pratt et al., 2017), there are still many gaps in our understanding about the biological effects of these drugs.

SSRIs in Pregnancy and Fetal Brain Development

This gap in knowledge for how SSRIs work mechanistically is a particular concern for pregnant women, 10-20% of whom suffer from major depression at some point throughout their pregnancy (Dubovicky et al., 2017; O'Keane & Marsh, 2007). Of these women, approximately 10% take antidepressants during pregnancy (Furu et al., 2015). Unfortunately, while the treatment of perinatal depression eliminates certain risk factors associated with mental illness, studies show that SSRIs also reach the fetus in pregnant mothers (Hendrick et al., 2003), with both the drug and its catabolic derivatives diffusing passively across the placenta, and making their way into the fetal bloodstream (Heikkinen et al., 2002). These molecules also reach the developing fetal brain, at first due to the absence of a fully developed blood-brain barrier, and later crossing it with the help of transport proteins (Zhao et al., 2015; Rochat et al., 1999). During embryogenesis, well before the birth of serotonergic neurons in the dorsal raphe nucleus (Bonnin et al., 2011; Daubert & Condron, 2010), serotonin from the mother plays crucial roles in early development of the offspring, impacting stages for cleavage, gastrulation and neurulation (Moiseiwitsch, 2000). 5-HT receptors, transporters, and degrading enzymes appear throughout the fetal brain early during gestation, implicating a role for serotonin in early neurodevelopment (Bonnin et al., 2011). However, studies that specifically test this hypothesis are lacking. We therefore examine as Aim 2 of my thesis project whether maternal SSRI treatment during pregnancy alters fetal neurodevelopment of the offspring.

SSRIs and the Microbiome

Despite the importance of the serotonergic system during fetal development, and findings that SSRIs taken by pregnant mothers can cross transplacentally into the fetus, the risks of maternal SSRI use on fetal development in humans are still unclear. While some studies report birth defects such as spontaneous abortions, preterm births, decreased intrauterine growth rates, and decreased birth weights as a result of maternal SSRI use, others find no statistically significant effects (Dubovicky et al., 2017; Zwink & Jenetzky, 2018). Furthermore, several meta-analyses of the literature have concluded that the data is too limited, the methods are problematic, and there is a strong need for more precise examination of the relationship between maternal SSRI use and fetal development (Furu et al., 2015; Zwink & Jenetzky, 2018; Gentile, 2011). This variability parallels the finding that non-pregnant patients with major depression vary in their responsiveness to SSRIs. As such, many studies are examining physiological factors that contribute to differences in the efficacy and effects of SSRIs across individuals. The gut microbiota is increasingly implicated as an important factor that modulates human responses to various xenobiotics, including SSRIs (Spanogiannopoulos et al., 2016). The microbiome interacts with drugs that pass through the GI tract, often transforming molecules from active to inactive states, or vice versa, before they even reach their target tissues. The microbiome also modulates host metabolism and transport, and may therefore have an indirect impact on the efficacy of drugs (Spanogiannopoulos et al., 2016). Moreover, 90% of the body's serotonin production occurs in enterochromaffin cells, which line the intestinal tract. Within these cells, serotonin synthesis is stimulated by metabolites from the gut microbiome (Yano et al., 2015). Therefore, the profile of gut bacteria may also be impacted by SSRIs in turn. In fact, a recent study tested over 1000 drugs against 40 representative strains from the microbiome, concluding

that 24% of these drugs, including the common SSRI fluoxetine, inhibited the growth of at least one bacterial strain (Maier et al., 2018). Other studies have also found that SSRI use is associated with alterations in the gut microbiome (Jackson et al., 2018), raising the question of whether microbial interactions with SSRIs may modify their effects on host physiology. Therefore, we examine as Aims 1 and 3 of my thesis project the question of whether the maternal gut microbiome is altered in response to maternal SSRI treatment, and whether the gut microbiome is necessary for the effects of maternal SSRI use on fetal neurodevelopment.

Experimental Aims

My thesis project aims to uncover answers to these fundamental questions regarding maternal SSRI use, interactions between the microbiome and SSRIs, and effects of maternal SSRI use on fetal neurodevelopment:

1. Does SSRI use during pregnancy change the maternal microbiome?
2. Does perinatal SSRI use influence developmental outcomes in the fetus?
3. Are developmental changes in fluoxetine-exposed embryos mediated by the maternal microbiome?

To tackle these questions, we used a pregnant mouse model (**Figure 1**). Mice were randomly divided into three groups: a vehicle/control group treated with saline during the second trimester of pregnancy (SAL), an experimental group treated with fluoxetine (FLX) during pregnancy, and a third group in which the microbiome was first depleted with antibiotics, then treated with fluoxetine during pregnancy (AF). The latter is a control for the FLX group, to discern whether developmental consequences are due to changes in microbiome, or other effects of SSRI treatment. We chose to work with fluoxetine because it is among the most commonly prescribed SSRIs on the market, as the active ingredient in brand-name drugs such as Prozac (Cipriani et al, 2016).

Materials and Methods

Mice and antibiotic treatment

Specific pathogen free (SPF) C57BL/6J mice from the Jackson laboratory were group housed in ventilated cages, with free access to standard rodent chow and water *ad libitum*. The holding room maintains a controlled temperature (22-25°C) and humidity, as well as a 12-hour light/dark cycle. Prior to breeding, bedding from all cages was mixed every 3 days to ensure similar exposure to bacteria, and thus homogenize the gut microbiota across all mice. At approximately four weeks of age, all male and female mice in the antibiotic-fluoxetine (AF) group began an oral gavage with an antibiotic cocktail of vancomycin (50 mg/kg), neomycin (100 mg/kg), and metronidazole (100 mg/kg). 200 µl of the cocktail was administered twice daily by oral gavage, at 8:00 and 17:00, for 7 days. Throughout the gavage and thereafter, the antibiotic group was maintained by adding 600 µl each of ampicillin (1g/3mL), neomycin (1mg/mL) and vancomycin (1g/6mL) to 200 mL of sterile drinking water. During this period the saline and fluoxetine groups were also gavaged twice daily with 200 µl of saline solution, for 7 days.

Timed mating and fluoxetine treatment

All mice were then paired for breeding, and females were checked daily for vaginal plugs. The day of post-coital vaginal plug formation was considered embryonic day 0.5 (E0.5), after which dams were separated and monitored for weight gain over 7 days. On day E7.5 (approximately the end of the first trimester), dams that gained at least 1.5 grams began an 8-day gavage with either a saline or fluoxetine hydrochloride solution (4 mg/ml, Santa Cruz). A dosage of 10 mg/kg was administered daily at 8:00, as used in previous studies (Rodríguez-Gaztelumendi et al., 2009; Machado et al., 2012). At E14.5 mouse dams were sacrificed by cervical dislocation.

The E14.5 time point was chosen to align with a few key developmental events. Serotonergic neurons appear in the dorsal raphe nucleus (DRN) around E10.5, at which time the embryo also begins to generate its own tryptophan hydroxylase (TPH2), an enzyme essential for serotonin synthesis in the brain (Daubert et al., 2011; Côté et al., 2006). By E12.5 the axons of serotonergic neurons begin projecting from the DRN towards targets such as the hypothalamus, amygdala, ventral tegmental area (VTA), hippocampus, and prefrontal cortex (Michelsen et al., 2007). At this time the embryo also develops a large portion of its serotonin receptors and produces substantially more TPH2. However, it is still dependent on maternal sources of serotonin until approximately E16.5 (Bonnin et al., 2006; Côté et al., 2006). Therefore, examining fetal development at embryonic day 14.5 ensures that we can visualize dorsal raphe neurons as they project towards targets in the brain, but also that the fetus remains vulnerable to the effects of fluctuating maternal serotonin levels.

16S rDNA sequencing

Fecal samples were collected for the saline and fluoxetine groups on gestational days E3, E6, E8, E11 and E14.5, and kept frozen at -80°C until DNA extraction. We collected a total sample size of N = 7 for each treatment group, and AF samples were not sequenced because the microbiome is depleted in these animals. To prepare the 16S library for sequencing, bacterial genomic DNA was extracted from mouse fecal samples using the MoBio PowerSoil® DNA Isolation Kit, and purified using the QIAquick PCR Purification Kit (Qiagen). Next, the sequencing library was generated according to methods adapted from Caporaso et al. (2012). The V4 regions of the 16S rDNA gene were PCR amplified using universal primers barcoded with unique oligonucleotides, Illumina adaptors, and 30 ng of the extracted genomic DNA. The PCR reaction was set up in

triplicate, and the product was then purified again using the Qiaquick PCR purification kit (Qiagen). DNA concentration was quantified using a BioTek Synergy H1 Multi-Mode Microplate Reader, and approximately 250 ng of purified PCR product from each sample was pooled and sequenced by Laragen, Inc. Sequencing was performed using the Illumina MiSeq platform and 2 x 250bp reagent kit for paired-end sequencing. Operational taxonomic units (OTUs) were chosen by open reference OTU picking based on 99% sequence similarity to the most recent Greengenes 13_8 database. Taxonomy assignment and rarefaction were performed using QIIME2-2019.1 (Caporaso et al., 2018).

Immunohistochemistry

To analyze axon development in the fetus, we used embryos collected from pregnant dams at embryonic day 14.5 (see *Materials and Methods*). Embryos were quickly collected and fixed in a 4% paraformaldehyde solution for 1 day, after which they are transferred to a 30% sucrose solution for cryoprotection. After a week in sucrose, embryos are frozen in OCT (Tissue-Tek, VWR) and preserved at -80°C. Using a cryo-microtome, embryos were cut sagittally at 10 µm and mounted on glass slides (Superfrost Ultra Plus, ThermoFisher Scientific), with 10 serial sections per embryo. These were stored at -20°C. In preparation for immunohistochemistry, slides were incubated in DAKO antigen retrieval solution (Agilent) at 90°C for two minutes, washed, and then incubated for 1 hour at room temperature with a 10% natural donkey serum solution. After another wash cycle, slides were incubated with primary antibodies for 30 hours at 4°C. The following antisera were used: anti-5-HT (rat monoclonal, Abcam, ab6336, 1:100), anti-SERT (rabbit polyclonal, Abcam, ab9726, 1:500), and anti-TPH2 (goat polyclonal, US Biological 208476, 1:500). Slides were incubated with secondary antibodies for 1 hour at room

temperature, using antisera from ThermoFisher Scientific (donkey anti-rat, Alexa Fluor 488, 1:1000; donkey anti-goat, Alexa Fluor 568, 1:1000; donkey anti-rabbit, Alexa Fluor 647, 1:1000). Sections were covered with glass cover slips (@@) and Prolong Gold antifade reagent with DAPI (ThermoFisher Scientific), air-dried for one hour, and maintained at 4°C. The final sample size for each group was N = 8 embryos for the saline (SAL) group, N = 6 embryos for the fluoxetine (FLX) group, and N = 5 embryos for the antibiotic-fluoxetine (AF) group.

Image Acquisition

Slides were imaged using a 20X objective on a Zeiss LSM780 confocal microscope, equipped with an Argon laser (488 nm), a Diode 561 nm and HeNe 633 nm. Images were acquired across eight Z-sections, scanning a total of 5.31 µm at a 1024 x 1024 pixel resolution. Scans were tiled in the Zen Black Edition software and stitched using the Zen 2.1 (Blue Edition) software.

Quantification for IHC Staining

To compare 5HT, SERT and TPH2 levels in each group, sagittal E13.5 and E15.5 brain sections from the Allen Developing Mouse Brain Atlas were used as a reference to locate the midpoint of each embryo brain (2008). Beginning at the midpoint, five consecutive brain sections were analyzed for each embryo, as a means of consistently quantifying both DRN neurons and axon projections in a medial-to-lateral progression, spaced 100 µm apart (**Figure 2**). DRN neurons were counted using the ImageJ Puncta Analyzer plugin developed by the Eroglu Lab at Duke University, using methods adapted from Ippolito & Eroglu, 2010. A single region of interest (ROI) was generated to designate the DRN region and used for all sections, recording colocalized puncta for 5HT and SERT, as well as 5HT and TPH2 (**Figure 3A**). Axon projections

were quantified in ImageJ using three separate, but consistent, ROIs for all sections (**Figure 3B**). Integrated Density readings for these ROIs were used to assess signal intensity, with additional readings recorded in non-staining regions, normalized to the same area and subtracted from the original measures to account for background noise. Axon projections were also quantified after setting an intensity threshold for each channel, thus providing a count of individual puncta in addition to staining intensity.

Statistical Analysis

Imaging data was summed for all five sections in each embryo. All imaging data was analyzed using Prism 8 software. Comparisons between SAL and FLX groups were then performed using a two-tailed unpaired t-test, whereas comparisons between SAL, FLX and AF groups were analyzed using a one-way ANOVA. Post-hoc analysis for differences between individual gestational time points was performed using Tukey's Multiple Comparisons Test.

Results

Aim 1: Determine the effects of maternal fluoxetine treatment on the maternal gut microbiome

To determine whether maternal microbiome is altered in response to maternal treatment with the SSRI fluoxetine, we sequenced 16S ribosomal DNA of fecal microbiota samples collected from pregnant mouse dams. First, we evaluated the alpha diversity of the microbiota, or the number of unique bacterial species within each sample. To do this we used the Faith Phylogenetic Diversity (PD) metric, calculated for each of the five gestational time points within each treatment group (**Figure 4**). The Faith PD metric is the sum of phylogenetic branches that span a given set of taxa on a phylogenetic tree, thereby describing the quantity of phylogenetic differences in a sample, or species richness (Faith, 1992; Faith & Baker, 2007). Our results showed no significant difference between the saline and fluoxetine groups, when analyzed both as a Kruskal-Wallis one-way ANOVA, as well as Kruskal-Wallis pairwise comparisons between each group (**Figure 5A**). This indicates no difference in alpha diversity of the microbiota between pregnant dams in the saline and fluoxetine groups, regardless of gestational day.

Next, we analyzed beta diversity, or the relative distribution of different types of bacteria between groups. We first used principal coordinates analysis (PCoA) to assess similarities and dissimilarities between the two groups, plotting each data point at a distance that is relative to its dissimilarity from others, and basing the axes on the 2 most significant dimensions of analysis. The UniFrac distance used for assembling the PCoA plots can be either unweighted or weighted with regards to species abundance. The unweighted calculation is a metric describing quantitative differences between groups, as it only takes into account the presence or absence of

species within each sample. The weighted calculation, on the other hand, describes qualitative differences as well. This is because it takes into account the abundance of each unique species and compares it between samples, in addition to the number of species present. Our analysis of unweighted results revealed no differences in beta diversity across different timepoints for either saline or fluoxetine groups (**Figure 6A**). Neither group showed clear shifts in clustering as gestation progressed, which suggests that there is no change in maternal microbiome composition as the fetus develops. Furthermore, there is no separation between saline and fluoxetine clusters for any of the gestational timepoints, indicating no significant difference in microbiome composition in response to prenatal fluoxetine treatment. Thus, our next step was to generate weighted PCoA plots, to assess whether fluoxetine had any effect on species abundance (**Figure 6B**). For the qualitative analysis we again saw a lack of initial clustering for either treatment group. Similarly, the saline group shows no clear shift over time. Some samples in the fluoxetine group do seem to shift slightly in gestational days E11 and E14, however there is no clear clustering for the treatment group as a whole. Additionally, there is still no clear separation between saline and fluoxetine groups, suggesting again that prenatal fluoxetine treatment does not affect the maternal microbiome. Analyses including the third PCoA axis yielded similarly insignificant results.

However, despite seeing no global changes by our PCoA plots, we proceeded to generate a microbial taxonomy chart to determine whether select microbial taxa were altered by fluoxetine experiment (**Figure 7**). Using the Qiime 2 software, we were able to assign phylogenetic classifications for unique species present in each sample, down to the species level. Results were calculated for each treatment group and organized by embryonic day. As seen in **Figure 7**, the

saline group exhibits a relatively constant distribution of the microbiota throughout the pregnancy, without any significant changes following the beginning of saline gavage on E7. The fluoxetine group, while relatively consistent as well, does suggest some decrease in abundance of the *Bacteroidia* class, while seeing a simultaneous enrichment of the *Clostridia* class, moving from the E3 to E14 time points. However, further statistical analysis showed that these shifts were not significant. A test for changes in microbial abundance, using a permutational multivariate analysis of variance, or PERMANOVA, revealed no significant difference between treatments, across all five days of gestation, for any of the bacterial species identified in the original phylogenetic classification.

Taken together, these results indicate that prenatal fluoxetine exposures during the second trimester of pregnancy do not have an effect on the bacterial composition of the maternal gut microbiome in mice.

Aim 2: Examine effects of maternal fluoxetine treatment fetal neurodevelopment

Having assessed for changes in microbiome of fluoxetine-treated dams, we continued to investigate our second aim: determining whether maternal fluoxetine exposure alters neurodevelopment in the fetus itself. Although these effects may not be mediated by changes in the maternal microbiome, our experiment may still provide valuable information about SSRI use during pregnancy. Therefore, we addressed this question by investigating the developing serotonergic system in the fetal brain.

The DRN is a bilateral and heterogeneous brainstem nucleus located in the periaqueductal gray region between the midbrain and hindbrain (Michelsen et al., 2007; Alonso et al., 2013). Although the nucleus contains neurons that utilize a variety of neurotransmitters, including dopamine, GABA, and glutamate, serotonin is the primary neurotransmitter in about 70% of these neurons (Michelsen et al., 2007). Thus, the DRN is well known as the origin of extensive serotonergic connections in the brain, projecting axons to targets including the substantia nigra, caudate putamen, hippocampus and entorhinal cortex, PVN of the thalamus, central amygdala, lateral hypothalamus, and importantly, the prefrontal cortex (Michelsen et al., 2007). Because of its extensive participation in serotonergic signaling, the DRN is a potential target for developmental changes as a result of maternal SSRI use, and could have downstream effects in a variety of brain regions. For example, a recent study investigated the effects of early postnatal SERT manipulation (P2-P10) on the development of axons that originate in the prefrontal cortex and innervate the DRN (Soiza-Reilly et al., 2018). Both ablation of SERT and postnatal fluoxetine treatment resulted in significant changes in axon development, including hyperinnervation of the DRN by axons from the prefrontal cortex. Therefore, we hypothesized that maternal SSRI exposure may alter the reciprocal innervation from DRN to prefrontal cortex. The outgrowth of these projections, of which more than 80% are serotonergic, begins during prenatal brain development - around E12.5 in mice (Michelsen et al., 2007). Here we examined the effects of oral maternal fluoxetine administration on fetal DRN neurons and their axons at the E14.5 time point, because it is ideal for visualizing the early development of axonal projections to the prefrontal cortex.

Serotonergic Markers

To assess for the presence and outgrowth of axons originating in the DRN, we used three different markers that are essential to the serotonergic system: 5-HT itself, the serotonin transporter SERT, and the enzyme tryptophan hydroxylase 2 (TPH2), which is crucial for serotonin synthesis in the brain. Because serotonin is produced by serotonergic neurons, it is the most direct way to label and track them as they extend to various areas in the brain. However, we decided to use additional markers for several reasons. First, maternal serotonin is present throughout the fetal brain in addition to serotonergic cells and may therefore lead to nonspecific labeling in areas where it acts as a developmental guidance cue (Daubert et al., 2010). Additionally, while the serotonin antibody can reliably mark cells in the DRN itself, the axons that project specifically from the DRN to the prefrontal cortex appear as very fine varicosities in the sagittal plane (Michelsen et al., 2007). These projections can sometimes be difficult to distinguish from some levels of background staining, so the use of additional markers allows us to locate and quantify these axons with greater certainty.

We decided to use SERT as a second marker. Much like serotonin itself, SERT occurs in all serotonergic neurons (Daws & Gould, 2011), and has been used to label serotonergic axons reliably in previous work (Bonnin et al., 2011). Furthermore, it is a crucial regulator of extracellular serotonin levels and the functional target of SSRIs. However, in the brain, SERT is transiently expressed in a non-monoaminergic population of neurons in the internal capsule (Verney et al., 2002). Therefore, it is also best paired with another stain, such as 5-HT.

Finally, the enzyme TPH2 was used as a third marker, because it is essential to 5-HT production in the brain. TPH2 is present in all raphe neurons and begins to appear as early as E10.5, ensuring that it will be present, at least to some extent, in our brain sections at E14.5. As it is not present elsewhere in the brain, otherwise occurring only in the myenteric plexus of the gastrointestinal tract (Côté et al., 2006), TPH2 is a highly specific marker for serotonergic neurons.

Prenatal fluoxetine does not affect DRN neuron development or navigation of serotonergic axons to the prefrontal cortex

Quantification of neurons in the DRN was performed by assessing colocalization of puncta between the 5-HT and SERT channels, and also between the 5-HT and TPH2 channels (**Figure 8**). The use of colocalization for quantifying staining ensured that the analyzed cells were truly part of the serotonergic population within the DRN. However, there was no visible difference in DRN staining between SAL and FLX embryos (**Figure 8A-D**), and indeed, our results did not differ between treatment groups (**Figure 8E, 8F**). One thing we noticed, however, was that there was a significant disparity in the number of $SERT^+$ and $TPH2^+$ puncta labeled in the DRN. This is not entirely unexpected because endogenous production of serotonin, in which the TPH2 enzyme plays a crucial role, is not fully developed until approximately E16.5 in fetal mouse brain. In addition, unlike 5-HT and SERT, TPH2 has not been implicated as a guidance cue in early brain development and is therefore not expected to appear as robustly as other markers at the E14.5 time point. However, to rule out the possibility that prenatal fluoxetine exposure has an impact on the production of TPH2 in serotonergic neurons, we assessed the ratio of $TPH2^+$ to $SERT^+$ puncta for each treatment group (**Figure 8G**). A two-tailed test showed no significant

difference in TPH2/SERT puncta ratio between SAL and FLX embryos, suggesting that TPH2 development in the DRN is not adversely affected by prenatal fluoxetine exposure ($p = 0.6382$).

Next, we looked at serotonergic axon projecting from the DRN to the prefrontal cortex (**Figure 9**). Two-tailed t-test analysis with Welch's correction showed no statistically significant differences in integrated density readings for 5-HT, SERT, or TPH2 channels ($p = 0.3755$, 0.6101, and 0.5375, respectively), between SAL and FLX embryos (**Figure 9B, 9E, 9H**). Similarly, no significant differences were found for quantitative analysis of puncta analyzed using a visual threshold, between SAL and FLX embryos in the 5-HT, SERT, and TPH2 channels ($p = 0.1813$, 0.7875, and 0.8854, respectively, **Figure 9C, 9F, 9I**). We also assessed staining differences independently for each of the three axon regions, across all three channels. Two-way ANOVA analyses and pairwise comparisons for both integrated density readings and puncta counts showed no significant difference between saline and fluoxetine groups in all three channels (**Figure 10**).

Taken together, these results suggest that the development of serotonergic neurons in the DRN, as well as the navigation of serotonergic axon projections to the forebrain, are unaffected by prenatal fluoxetine use during the second trimester of gestation in mice.

Aim 3: Assessing the role of the maternal microbiome in mediating effects of fluoxetine treatment on fetal brain development

In addition to looking at the development of DRN neurons and axon projections in saline- and fluoxetine-treated dams, we also assessed whether these structures may be mediated by the

maternal microbiome. For this purpose, we introduced the antibiotic-fluoxetine (AF) group, where mothers are depleted of their microbiome by antibiotic treatment, and subsequently treated with fluoxetine throughout gestation. By comparing the effects of fluoxetine treatment between pregnant mice with full microbiomes and those with virtually absent microbiomes, we initially aimed to assess whether changes in fetal brain development are due to interactions with the microbiome (if changes observed in the fluoxetine group are no longer seen in the antibiotic-fluoxetine group), or due to some other mechanism, elsewhere in the system (if the fluoxetine and antibiotic-fluoxetine groups were to exhibit the same phenotype). Although we did not see significant differences in maternal microbiome composition between saline and fluoxetine groups, nor in the development of DRN neurons and axon projections to the cortex, this third comparison was still useful to assess whether fluoxetine can be harmful to the fetus after maternal microbiome depletion. To quantify serotonergic cells in the DRN and axon projections to the PFC, we used the same staining and imaging techniques as those outlined in Aim 2.

Depleted maternal microbiome does not affect the impact of prenatal fluoxetine use on DRN neuron development or navigation of serotonergic axons to the prefrontal cortex

As in Aim 2, quantification of neurons in the DRN was performed by assessing colocalization of puncta between 5-HT and SERT channels, as well as 5-HT and TPH2 channels (**Figure 11**). Similarly, our results showed no statistically significant differences between SAL, FLX and AF groups (**Figure 11E, 11F**). In addition, calculation of $TPH2^+$ to $SERT^+$ puncta ratio showed no significant difference between SAL, FLX and AF embryos, confirming once more that TPH2 development in the DRN is not adversely affected by prenatal fluoxetine exposure (**Figure 11G**).

Next, we looked again at serotonergic axon projections from the DRN to the prefrontal cortex (**Figure 12**). Analysis by one-way ANOVA showed no statistically significant differences in integrated density readings for 5-HT, SERT, or TPH2 channels ($p = 0.8058$, 0.9135, and 0.8642, respectively), between SAL, FLX and AF embryos (**Figure 12B, 12E, 12H**). Similarly, no significant differences were found for quantitative analysis of puncta reaching threshold levels between SAL, FLX and AF embryos in 5-HT, SERT, and TPH2 channels ($p = 0.3745$, 0.7263, and 0.2753, respectively, **Figure 12C, 12F, 12I**). Once again, we assessed staining differences independently for each of the three axon regions as well, across all three channels. Two-way ANOVA analyses and pairwise comparisons, for both integrated density readings and puncta counts, showed no significant difference between SAL, FLX and AF groups in all three channels (**Figure 13**).

Discussion

In summary, we have found no evidence to suggest differences in maternal microbiomes between pregnant mouse dams treated with fluoxetine during the second week of gestation, and those treated with saline. A thorough analysis of 16S rDNA sequences between the two groups indicated no differences in alpha diversity (species richness), unweighted beta diversity (difference in unique species between groups), or weighted beta diversity (difference in species type and abundance between groups). Even pairwise comparisons yielded no significant differences between groups for specific gestational time points. This suggests that the maternal microbiome, though known to fluctuate throughout gestation for many women (Santacruz et al., 2010), is not impacted by the SSRI fluoxetine when administered over the course of the second trimester in mice.

Furthermore, our results did not show any significant changes in the number of serotonergic cells located in the DRN, when examined across saline, fluoxetine, and antibiotic-fluoxetine groups. This was confirmed by the use of three different serotonergic markers, assessed for colocalized puncta in the DRN region. Likewise, we failed to see changes in the development of axons that project from cell bodies in the DRN towards the prefrontal cortex in the forebrain. Quantification from three axon regions, for all three experimental groups (SAL, FLX and AF), yielded no significant results, whether compared individually for each axon region or as a sum representing the entire area stained, for developing mouse brain at gestational day E14.5.

Altogether, these results support the idea that fluoxetine use during pregnancy does not have an effect on bacterial composition in the maternal microbiome. Furthermore, prenatal fluoxetine use

during the second week of gestation in mice does not appear to affect the development of the brain's main serotonergic system, whether by means of the microbiome (as verified by saline-fluoxetine comparisons) or possible off-target effects of prenatal fluoxetine exposure (as seen by the lack of differences between saline, fluoxetine and antibiotic-fluoxetine groups). In general, this finding provides a positive outlook for those who seek to control major depression during pregnancy, without causing harm to the developing fetus.

Thus, despite the fact that the DRN and its projections comprise the main serotonergic system in the brain, our results suggest that its development is not dependent on feedback from circulating 5-HT levels in the fetal brain, or elsewhere in the developing embryo. This is supported by recent research investigating the development of serotonergic neurons in the brain, where 5-HT was not implicated as a key signaling factor (Deneris & Gaspar, 2018). Furthermore, while studies have shown that cell cultures of raphe neurons respond to 5-HT and increase axon growth, *in vivo* genetic models fail to show deficits in the development of the central serotonergic system, regardless of whether brain 5-HT was depleted or increased (Gaspar et al., 2003). Nevertheless, in addition to the general role of serotonin in modulating neuronal proliferation, differentiation, and apoptosis, several neuronal circuits in the brain are specifically influenced by serotonergic signaling in early development. This is evidenced by early transient expression of the serotonin transporter, SERT, and accumulation of 5-HT in neuronal populations that do not synthesize it themselves, including the thalamus, hypothalamus, and limbic system (Gaspar et al., 2003). However, while the specific function of 5-HT has been studied extensively as a guidance cue for thalamocortical axons projecting to the somatosensory cortex (van Kleef et al., 2012), a process in which the hypothalamus also plays a role, its participation in the early development of regions

such as the hippocampus and amygdala has not yet been determined (Shimogori et al., 2010; Bocchio et al., 2016).

Current literature addressing these concerns is largely focused on early postnatal administration of fluoxetine or other SSRIs (Ansorge et al., 2004; Gur et al., 2013). But while these studies do often show behavioral deficits in rodent offspring, the varied timing between experiments makes it difficult to determine with certainty whether SSRI exposure is actually responsible. On the one hand, the first 20 days of postnatal brain development in rodents generally coincides with the third trimester of human gestation; on the other, this time point is well past the developmental timelines for multiple neuronal circuits, including the serotonergic system described here, as well as other systems influenced by serotonergic signaling such as the hippocampus (Semple et al., 2013).

A more clinically applicable perspective has been pursued in countless studies investigating behavioral outcomes in children who were exposed to SSRIs prenatally. Many of these studies have suggested associations between prenatal fluoxetine exposure and the development of psychiatric diseases such as depression, anxiety disorders, drug addiction and autism in children (Gur et al., 2013). However, these studies vary greatly in timing of SSRI exposure, the types of SSRIs administered, and the way that subjects are assessed for such behaviors in early childhood. The ultimate result of such varied conditions is a large number of outcomes that do not seem to agree with each other in any reliable fashion. A recent abstract presented by Dr. Sarah Hutchinson and senior author, Dr. Tim Oberlander at the 2018 Pediatric Academic Societies meeting even suggests that long-term outcomes for children with prenatal SSRI exposure may

confer improved executive function skills by 12 years of age (Pediatric Academic Societies, 2018).

Therefore, the development of a standard model and procedure is key to future studies investigating the impact of prenatal SSRI exposure on developmental outcomes, so as to elucidate greater clarity on the subject. Even within our own model, subsequent testing of behavioral outcomes in the offspring would be beneficial for determining whether prenatal fluoxetine exposure truly results in adverse behavioral outcomes. Additional groups of pregnant dams could also be assessed under stressed and non-stressed conditions, to simulate the maternal stress typically associated with major depression during pregnancy, and which is often thought to be a confounding factor with SSRI use that is difficult to distinguish in clinical studies (Gur et al., 2013). Once an effective model is developed, with confirmed behavioral outcomes, further testing of the neuronal circuits outlined above will be essential.

Despite the growing number of studies investigating prenatal SSRI exposure in rodent and human models alike, there is still an overwhelming lack of inquiry regarding the mechanisms that are potentially modulating the variety of behavioral outcomes we have seen in the literature. Here we have shown significant support against any modifications to maternal microbiome as a result of prenatal fluoxetine exposure during the second trimester in mice, as well as lack of changes in the developing serotonergic DRN system in fetal mouse brain. Future research in the field should investigate the effect of early gestational exposure to fluoxetine on other key neuronal circuits, particularly those influenced by 5-HT in early development.

Figures

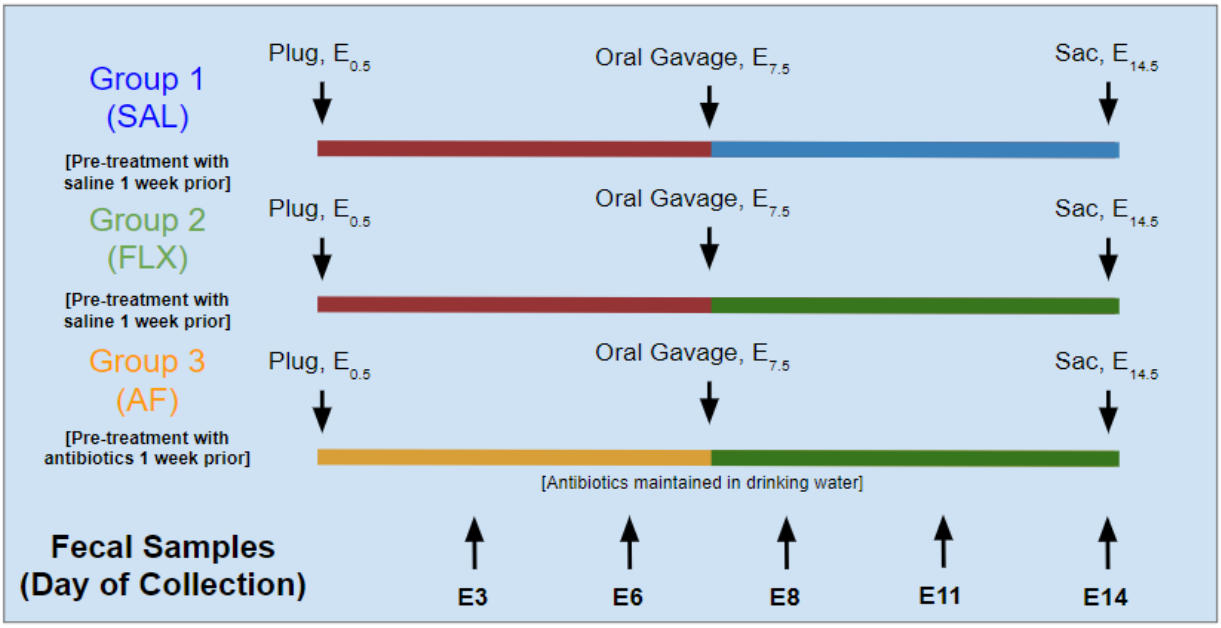

Figure 1: Experimental timeline for pregnant mouse model for control (SAL) and treatment (FLX, AF) groups. Mice were randomly divided into one of three groups, after which they all underwent one week of either saline (SAL and FLX groups) or antibiotic (AF) gavage, administered twice daily. Thereafter the AF group was maintained by adding antibiotics to drinking water. After a week of oral gavage mice were paired for breeding and checked for pregnancy daily. Vaginal plug dates were noted as embryonic day 0.5 (E0.5). Fecal samples were collected and weights measured for all mice on E3 and E6. All dams who gained at least 1.5g in the first week began oral gavage with either saline (SAL group) or fluoxetine (FLX and AF groups). Pregnant mice were weighed and gavaged daily, with fecal samples collected on E8, E11, and E14.5. All mice were sacrificed, and embryos collected, on E14.5.

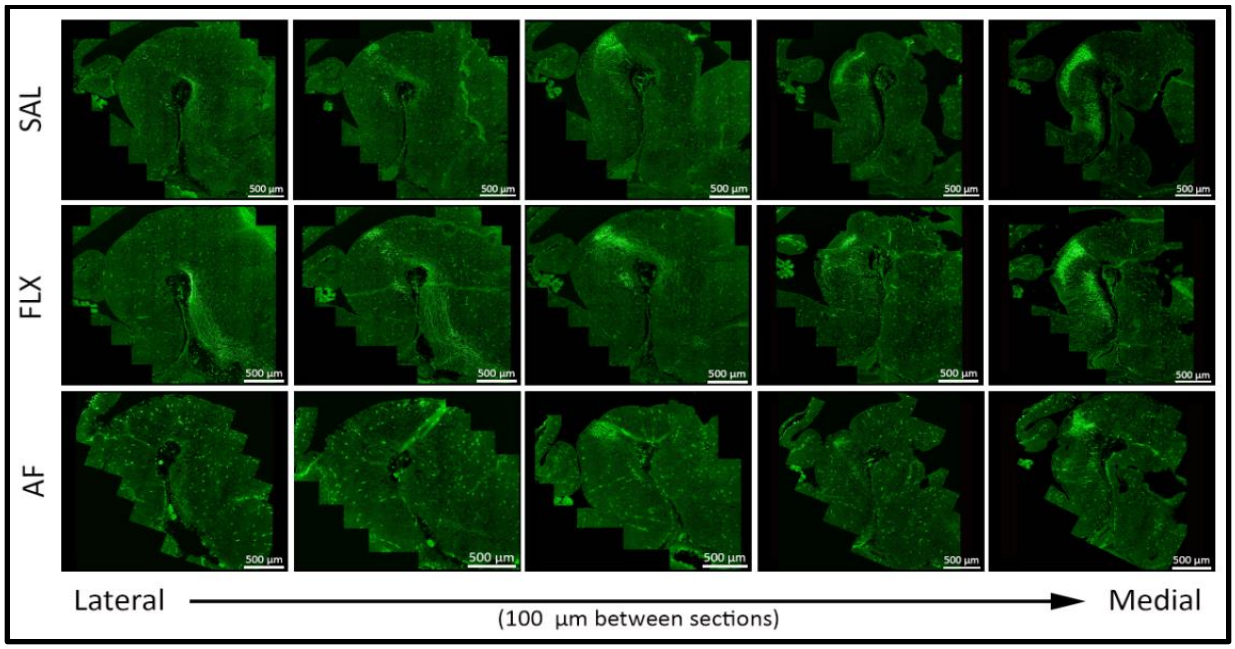

Figure 2: Matched sagittal brain sections moving from lateral to medial brain in E14.5 mouse embryo. Serial sections were cut on a cryo-microtome in 10-slide sequences, generating a distance of 100 µm between each section. Each brain was cut sagittally from the left lateral side of the brain, across to the right lateral side, generating 10 total sections for each brain. The Allen Developing Mouse Brain Atlas (2008) was used as a reference to locate the midpoint of each brain, and five sections projecting laterally from this midpoint were selected for quantifying changes in DRN and axon development. Matched representative sections for saline- (SAL), fluoxetine- (FLX) and antibiotic/fluoxetine-treated (AF) embryos are presented here. It should be noted that the AF group has slightly smaller sections and a faster progression, due to the nature of AF mouse embryos, which are typically smaller than wild-type embryos with non-depleted maternal microbiomes. No such deficits are known, nor were identified in this experiment, for fluoxetine-treated embryos, which matched very well to saline-treated fetal brain sections.

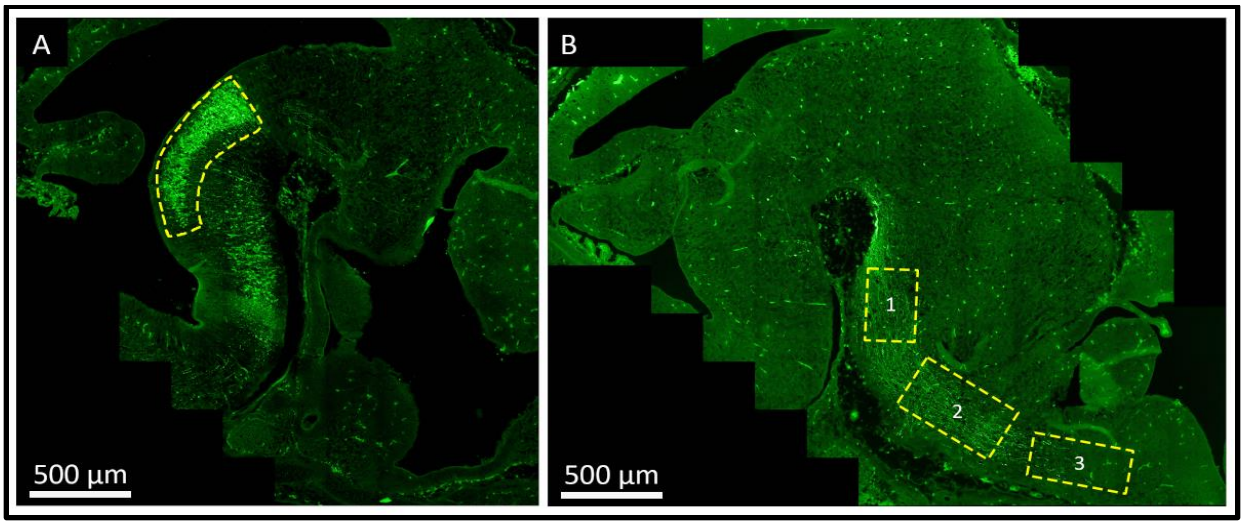

Figure 3: Regions of interest (ROIs) used for quantification in DRN and axon projection regions. (A) Region of interest drawn for DRN quantification. The same ROI was superimposed onto all images analyzed for DRN staining and used to designate the region to be quantified by the Puncta Analyzer plugin for ImageJ. (B) For axon quantification, three different regions of interest were drawn to span the entirety of the axon progression. The same three ROIs were superimposed onto all images analyzed for axon projection staining and used to designate the region to be quantified by integrated density analysis and particle analysis with thresholds in ImageJ. Region 1 designates the area where axons first project from the midbrain into the forebrain. Region 2 contains the bulk of axon projections at this stage in development and passes by the developing hypothalamus. Region 2 was designated with care to avoid quantifying axon projections moving towards the hypothalamus. Finally, Region 3 collected data for axon projections that are near the basal forebrain at E14.5.

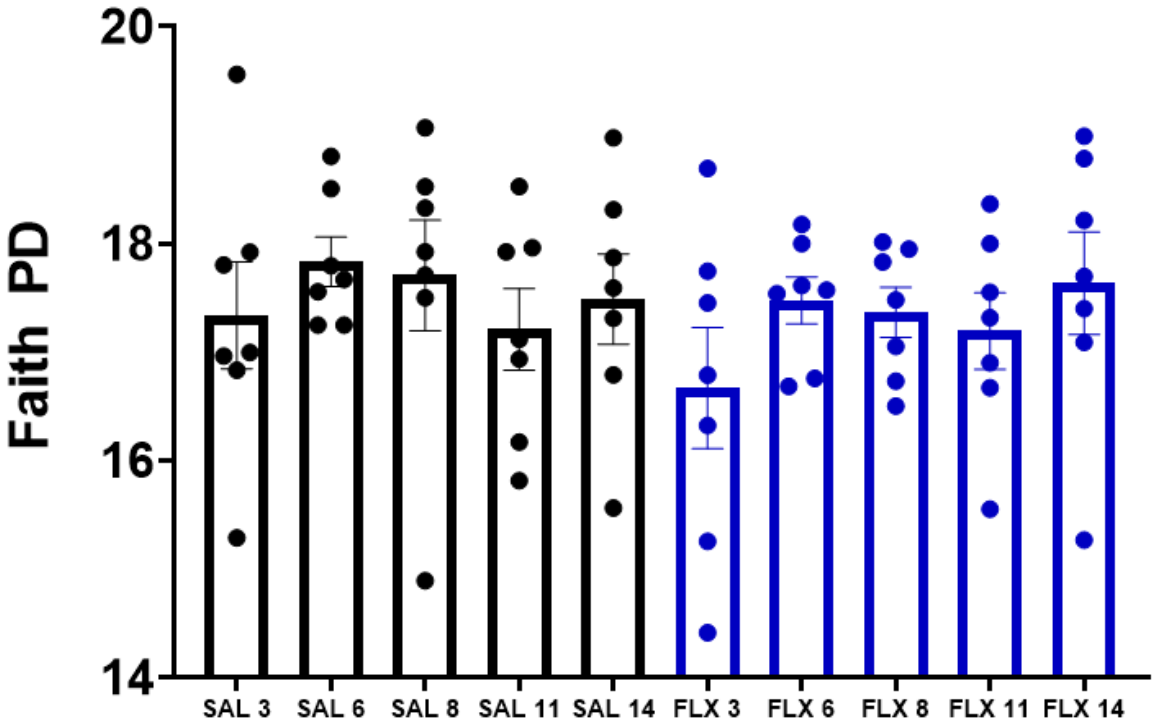

Figure 4: Faith Phylogenetic Diversity (PD) measurements used to assess alpha diversity in pregnant mouse microbiota. The Faith PD metric is used to assess species richness within a sample, or how many unique types of bacteria are present in a microbiome. This metric was calculated for all gestational timepoints in each treatment group. There was no significant difference across groups when compared using a Kruskal-Wallis one-way ANOVA ($p = 0.7190$).

Pairwise Comparison	(A) Alpha Diversity p-values	(B) Beta Diversity p-values
SAL 3 vs SAL 6	0.337904	0.412
SAL 3 vs SAL 8	0.337904	0.604
SAL 3 vs SAL 11	0.848006	0.192
SAL 3 vs SAL 14	0.749394	0.506
SAL 3 vs FLX 3	0.277439	0.197
SAL 6 vs SAL 8	0.565299	0.954
SAL 6 vs SAL 11	0.277439	0.603
SAL 6 vs SAL 14	0.749394	0.393
SAL 6 vs FLX 6	0.406234	0.703
SAL 8 vs SAL 11	0.337904	0.312
SAL 8 vs SAL 14	0.406234	0.794
SAL 8 vs FLX 8	0.224799	0.584
SAL 11 vs SAL 14	0.749394	0.072
SAL 11 vs FLX 11	0.94906	0.213
SAL 14 vs FLX 14	0.749394	0.29
FLX 3 vs FLX 6	0.277439	0.433
FLX 3 vs FLX 8	0.277439	0.328
FLX 3 vs FLX 11	0.482203	0.242
FLX 3 vs FLX 14	0.179712	0.063
FLX 6 vs FLX 8	0.654721	0.469
FLX 6 vs FLX 11	0.482203	0.412
FLX 6 vs FLX 14	0.482203	0.142
FLX 8 vs FLX 11	0.848006	0.857
FLX 8 vs FLX 14	0.406234	0.353
FLX 11 vs FLX 14	0.337904	0.44

Figure 5: P-values from pairwise comparisons across treatment groups and gestational days, for alpha and beta diversity analyses. (A) Post-hoc pairwise comparisons were performed for SAL and FLX dams across all 5 gestational timepoints, with p-values from each two-tailed t-test shown above. These were assessed in addition to a one-way ANOVA that was calculated for all groups (**Figure 4**). None of the comparisons showed statistically significant differences. (B) Pairwise comparisons of p-values generated by the Qiime 2 program for post-hoc analysis of beta diversity, analyzed in addition to an original PERMANOVA test. None of these comparisons showed statistically significant differences between groups.

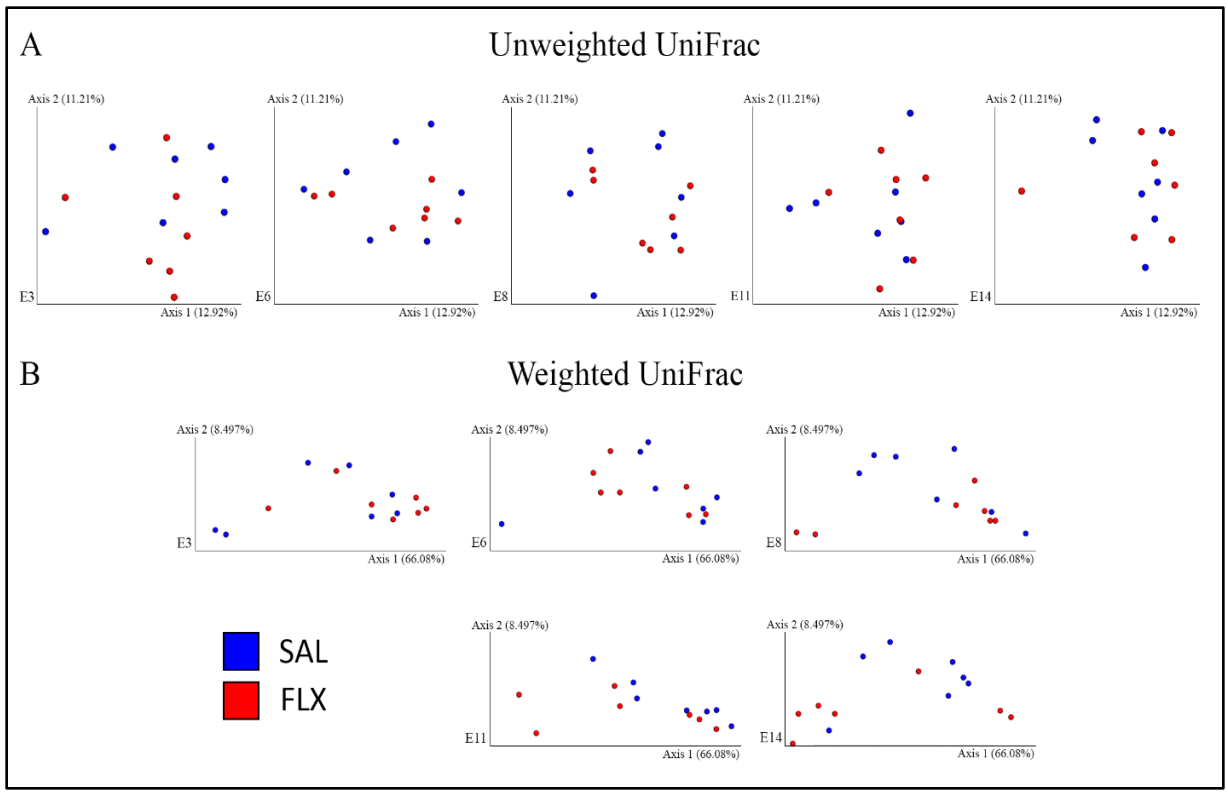

Figure 6: Unweighted and weighted PCoA plots comparing beta diversity for saline and fluoxetine groups across five gestational timepoints. (A) Unweighted UniFrac measurements were calculated for quantitative analysis of beta diversity, between saline- and fluoxetine-treated pregnant dam microbiota. The PCoA plots display five different gestational time points, with samples plotted against the two principal coordinate axes. Neither the saline nor the fluoxetine group showed any clear shifts in clustering across the five time points. (B) Weighted UniFrac measurements are shown for qualitative analysis of beta diversity between saline- and fluoxetine-treated pregnant dam microbiota. These PCoA plots take into account the abundance of unique species in each sample, in addition to their presence or absence. While there were no clear shifts in clustering for the saline group, the fluoxetine group seems to have shifted from a general cluster on gestational day E3, which spreads out across the plot by gestational day E14.5.

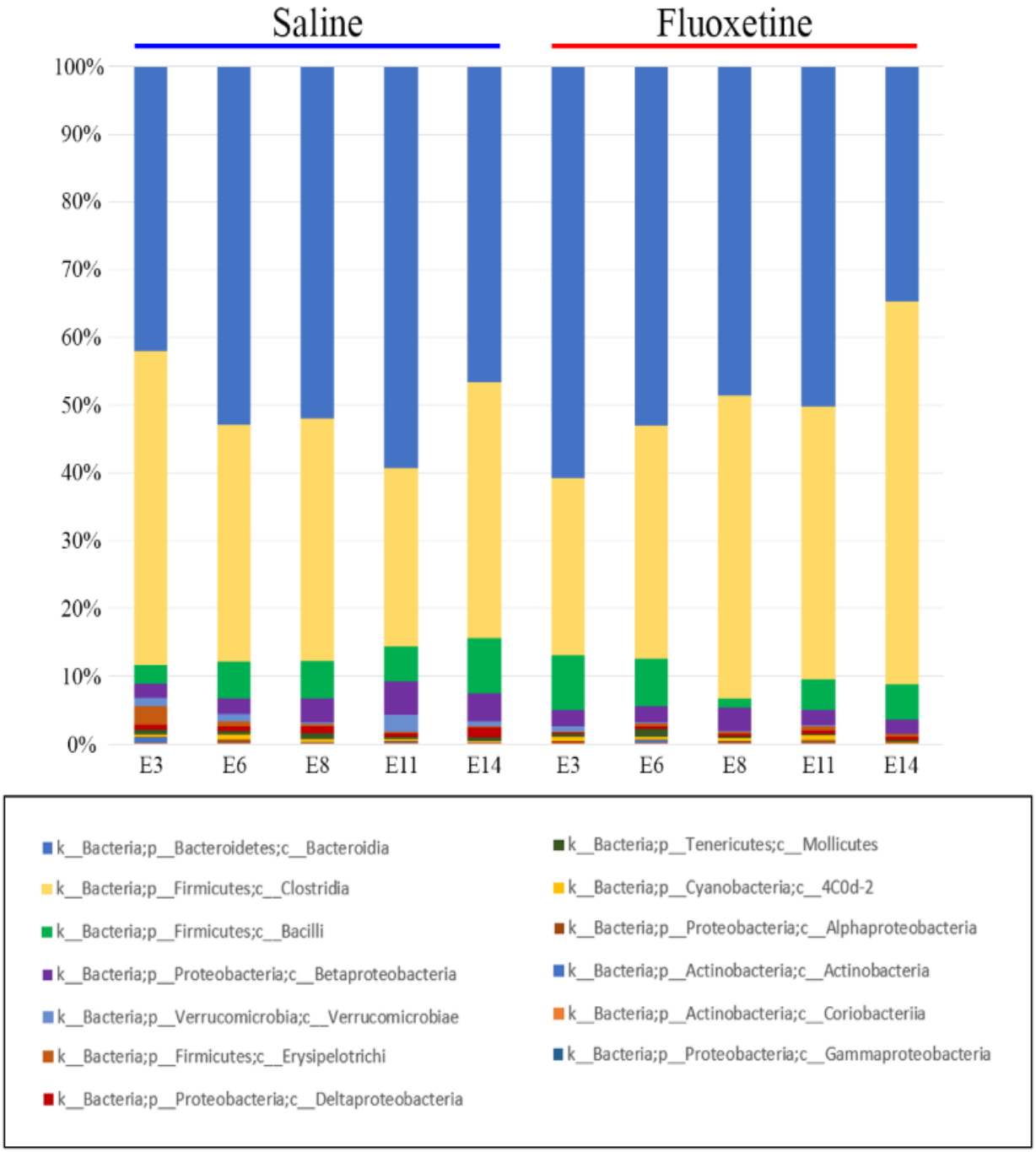
Saline
Fluoxetine
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
E3
E6
E8
E11
E14
E3
E6
E8
E11
E14
k__Bacteria;p__Bacteroidetes;c__Bacteroidia
k__Bacteria;p__Firmicutes;c__Clostridia
k__Bacteria;p__Firmicutes;c__Bacilli
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria
k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae
k__Bacteria;p__Firmicutes;c__Erysipelotrichi
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria
k__Bacteria;p__Tenericutes;c__Mollicutes
k__Bacteria;p__Cyanobacteria;c__4C0d-2
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria
k__Bacteria;p__Actinobacteria;c__Actinobacteria
k__Bacteria;p__Actinobacteria;c__Coriobacteriia
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria

Figure 7

Figure 7: Taxonomy chart with relative distribution of bacterial taxa in saline and fluoxetine groups. Bacterial taxa for each sample were analyzed by treatment and embryonic day. The relative frequency for the top 13 species present across all samples are shown. There does not seem to be any fluctuation in the top bacterial species for the saline group. The fluoxetine group, however, appears slightly less stable over time, with the taxonomy plot seeming to show enrichment in the *Clostridia* class of bacteria as gestational day increases. However, our PERMANOVA calculation across all groups did not identify any bacterial species that varied significantly between treatments or gestational days.

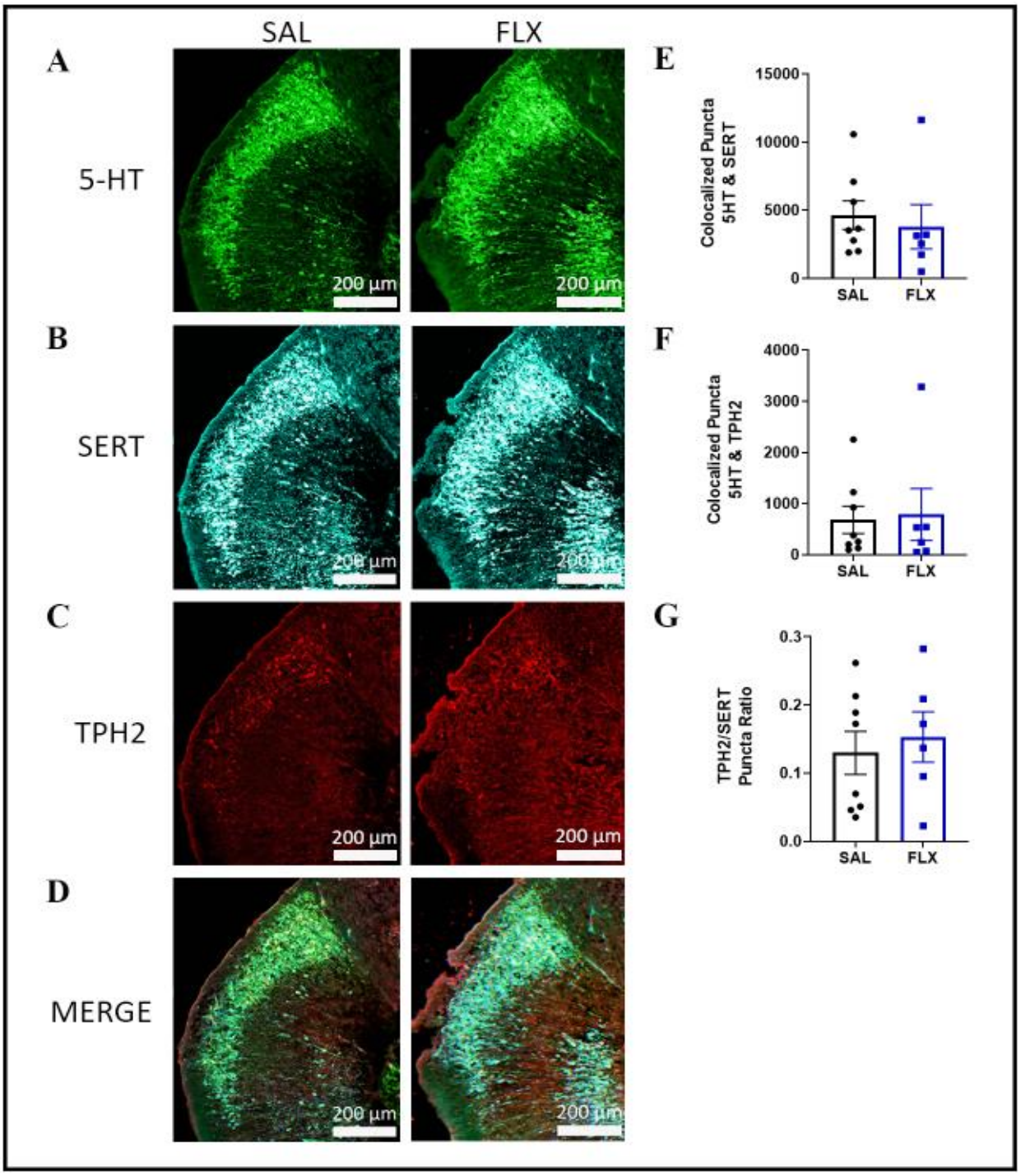
SAL
FLX
A
5-HT
B
SERT
C
TPH2
D
MERGE
200 µm
E
Colocalized Puncta
5HT & SERT
15000
10000
5000
0
SAL
FLX
F
Colocalized Puncta
5HT & TPH2
4000
3000
2000
1000
0
SAL
FLX
G
TPH2/SERT
Puncta Ratio
0.3
0.2
0.1
0.0
SAL
FLX

Figure 8

Figure 8: Imaging and quantification of cell bodies in the DRN for saline (SAL) and fluoxetine (FLX) groups. (A) SAL and FLX sections showing 5-HT staining for cells in the DRN. (B) SAL and FLX sections showing SERT staining for cells in the DRN. (C) SAL and FLX sections showing TPH2 staining for cells in the DRN. (D) SAL and FLX sections showing overlaid DRN staining for all channels (5-HT, SERT and TPH2). (E) Comparison of DRN cell counts between SAL and FLX embryos, quantified by measuring colocalized 5-HT^{+} and SERT^{+} puncta in the DRN region. A two-tailed t-test between groups showed no significant difference between saline and fluoxetine groups ($p = 0.6546$). (F) Comparison of DRN cell counts between SAL and FLX embryos, quantified by measuring colocalized 5-HT^{+} and TPH2^{+} puncta in the DRN region. A two-tailed t-test between groups showed no significant difference between saline and fluoxetine groups ($p = 0.8483$). (G) The ratio of TPH2^{+} counts to SERT^{+} counts was quantified to assess whether there was any difference in staining for the two markers, between saline and fluoxetine groups. A two-tailed t-test showed no significant difference between treatments ($p = 0.6382$).

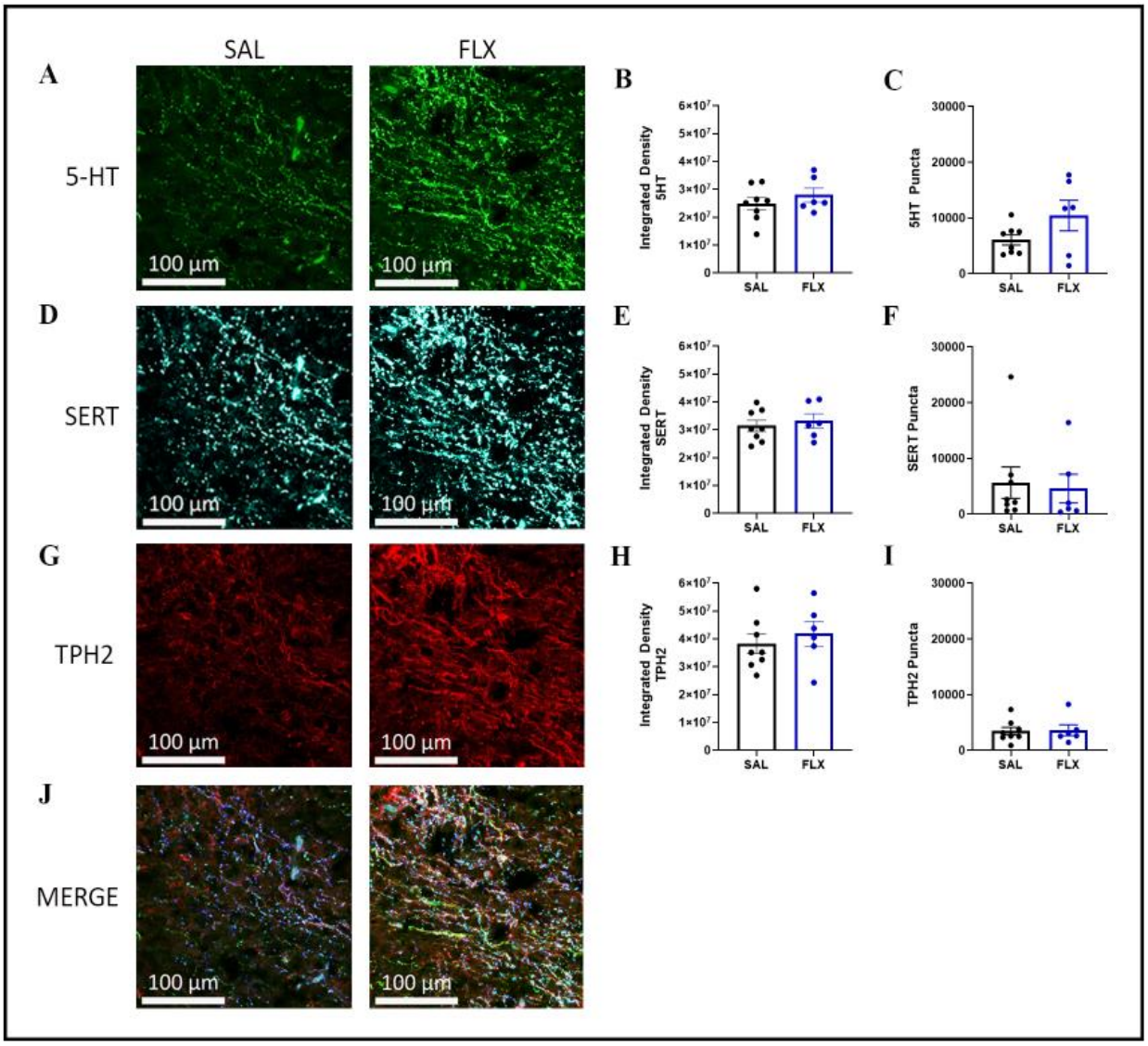
SAL
FLX
A
5-HT
100 µm
B
Integrated Density 5HT
C
5HT Puncta
D
SERT
E
Integrated Density SERT
F
SERT Puncta
G
TPH2
H
Integrated Density TPH2
I
TPH2 Puncta
J
MERGE
SAL
FLX

Figure 9

Figure 9: Imaging and quantification of axon projections moving from DRN to PFC in saline (SAL) and fluoxetine (FLX) groups. (A) SAL and FLX sections showing 5-HT staining for serotonergic axon projections in axon region 2, as depicted by the ROI in **Figure 3B**. (B) Integrated density readings were summed across five brain sections for all SAL and FLX embryos, to analyze for 5-HT staining intensity. A two-tailed t-test with Welch's correction showed no significant difference between SAL and FLX embryos ($p = 0.3755$). (C) 5-HT^+ puncta were quantified across five brain sections for all SAL and FLX embryos to analyze number of axons present. A two-tailed t-test with Welch's correction showed no significant difference between SAL and FLX embryos ($p = 0.1813$). **(**D) SAL and FLX sections showing SERT staining for serotonergic axon projections in axon region 2. (E) Integrated density readings for SERT^+ axon projections in SAL and FLX embryos. A two-tailed t-test with Welch's correction showed no significant difference between treatment groups ($p = 0.6101$). (F) SERT^+ puncta quantified for axon projections in SAL and FLX embryos. A two-tailed t-test with Welch's correction showed no significant difference between treatment groups ($p = 0.7875$). (G) SAL and FLX sections showing TPH2 staining for serotonergic axon projections in axon region 2. (H) Integrated density readings for TPH2^+ axon projections in SAL and FLX embryos. A two-tailed t-test with Welch's correction showed no significant difference between treatment groups ($p = 0.5375$). (I) TPH2^+ puncta quantified for axon projections in SAL and FLX embryos. A two-tailed t-test with Welch's correction showed no significant difference between treatment groups ($p = 0.8854$). (J) SAL and FLX sections showing overlaid serotonergic axon staining for all channels (5-HT, SERT and TPH2) in axon region 2.

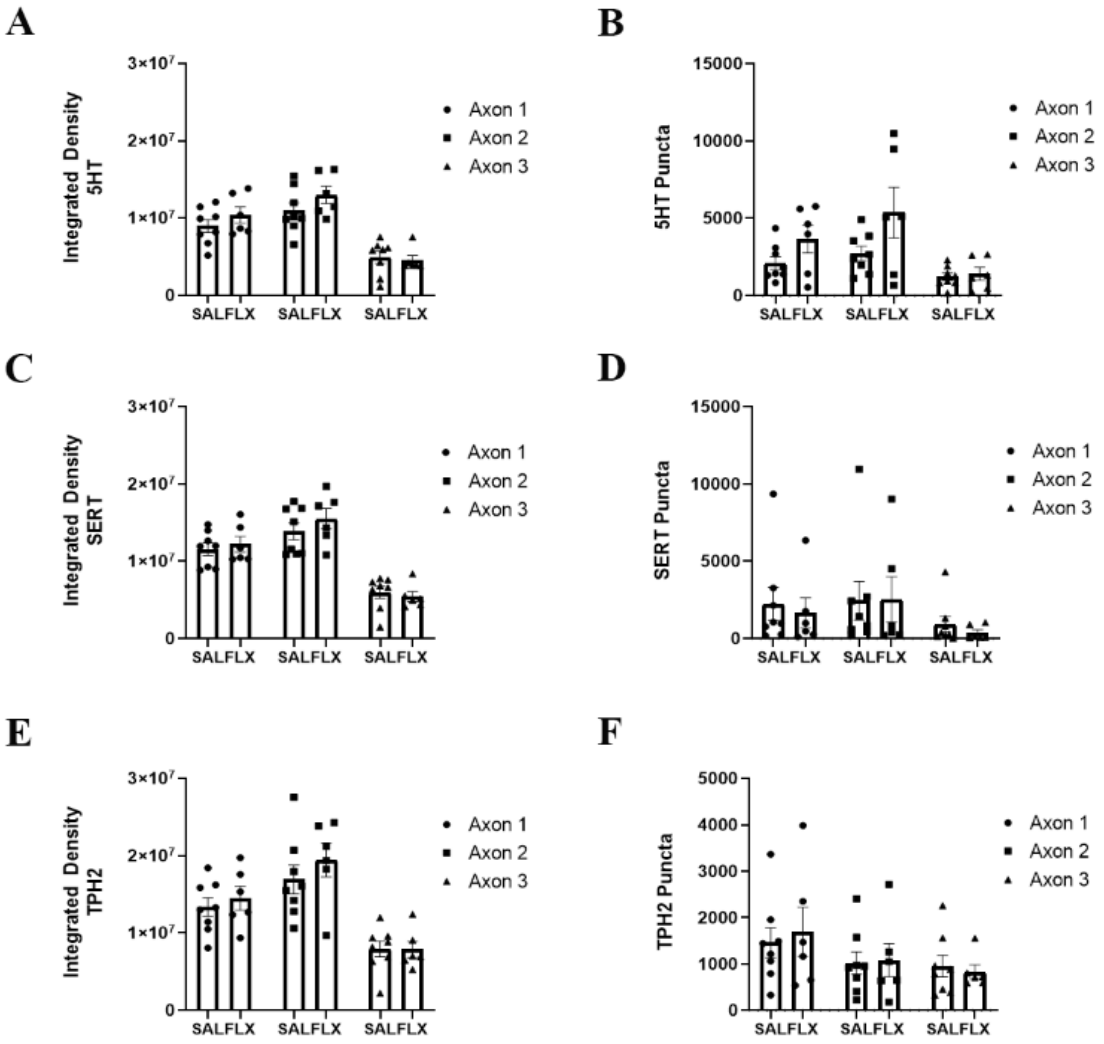
A
Integrated Density 5HT
3×10⁷
2×10⁷
1×10⁷
0
SALFLX
SALFLX
SALFLX
Axon 1
Axon 2
Axon 3
B
5HT Puncta
15000
10000
5000
0
SALFLX
SALFLX
SALFLX
Axon 1
Axon 2
Axon 3
C
Integrated Density SERT
3×10⁷
2×10⁷
1×10⁷
0
SALFLX
SALFLX
SALFLX
Axon 1
Axon 2
Axon 3
D
SERT Puncta
15000
10000
5000
0
SALFLX
SALFLX
SALFLX
Axon 1
Axon 2
Axon 3
E
Integrated Density TPH2
3×10⁷
2×10⁷
1×10⁷
0
SALFLX
SALFLX
SALFLX
Axon 1
Axon 2
Axon 3
F
TPH2 Puncta
5000
4000
3000
2000
1000
0
SALFLX
SALFLX
SALFLX
Axon 1
Axon 2
Axon 3

Figure 10

Figure 10: Region-specific quantification of axon projections moving from DRN to PFC, for saline (SAL) and fluoxetine (FLX) groups. Axon projections were compared between groups using both integrated density, to assess the intensity of axon labeling, as well as thresholded counts for puncta within each channel, to evaluate presence of axons quantitatively. No significant difference was found between saline and fluoxetine groups for any of the three regions examined: Axon ROIs 1, 2 and 3 as shown in **Figure 3**. (A) 5-HT integrated density comparison between saline and fluoxetine groups for Axon Region 1 ($p = 0.6894$), Axon Region 2 ($p = 0.4702$), and Axon Region 3 ($p = 0.9735$). (B) 5-HT puncta comparison between saline and fluoxetine groups for Axon Region 1 ($p = 0.5901$), Axon Region 2 ($p = 0.2228$), and Axon Region 3 ($p = 0.9918$). (C) SERT integrated density comparison between saline and fluoxetine groups for Axon Region 1 ($p = 0.9203$), Axon Region 2 ($p = 0.5876$), and Axon Region 3 ($p = 0.9403$). (D) SERT puncta comparison between saline and fluoxetine groups for Axon Region 1 ($p = 0.8874$), Axon Region 2 ($p = 0.9975$), and Axon Region 3 ($p = 0.9032$). (E) TPH2 integrated density comparison between saline and fluoxetine groups for Axon Region 1 ($p = 0.8980$), Axon Region 2 ($p = 0.6077$), and Axon Region 3 ($p = 0.9998$). (D) TPH2 puncta comparison between saline and fluoxetine groups for Axon Region 1 ($p = 0.9480$), Axon Region 2 ($p = 0.9963$), and Axon Region 3 ($p = 0.9859$).

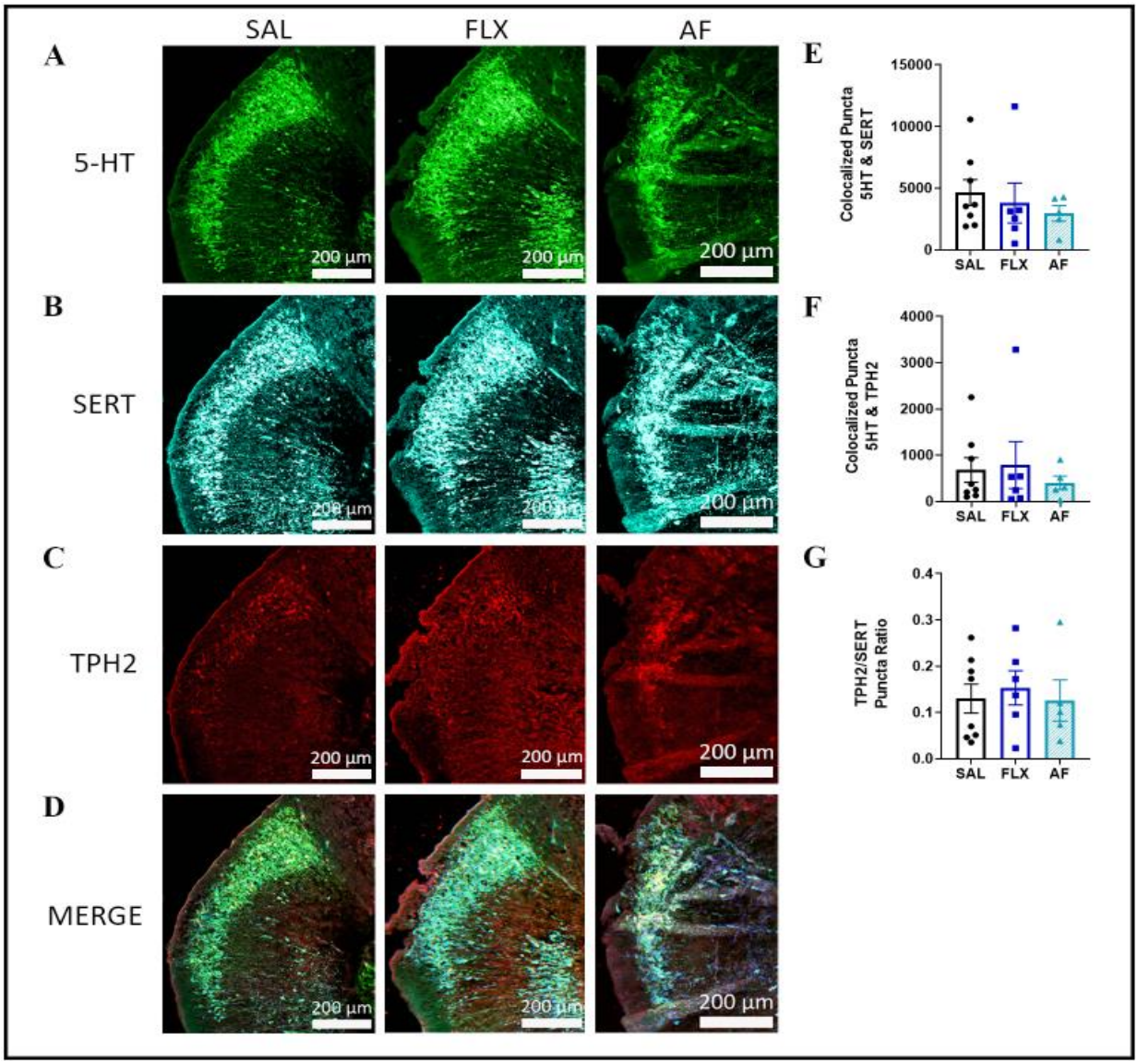

Figure 11: Imaging and quantification of cell bodies in the DRN, with AF group added. (A) SAL, FLX and AF sections showing 5-HT staining for cells in the DRN. (B) SAL, FLX and AF sections showing SERT staining for cells in the DRN. (C) SAL, FLX and AF sections showing TPH2 staining for cells in the DRN. (D) SAL, FLX and AF sections showing overlaid DRN staining for all channels (5-HT, SERT and TPH2). (E) Comparison of DRN cell counts between SAL, FLX and AF embryos, quantified by measuring colocalized 5-HT$^+$ and SERT$^+$ puncta in the DRN region. A one-way ANOVA between groups showed no significant difference between treatments ($p = 0.6280$). (F) Comparison of DRN cell counts between SAL, FLX and AF embryos quantified by measuring colocalized 5-HT$^+$ and TPH2$^+$ puncta in the DRN region. A one-way ANOVA between groups showed no significant difference between treatments ($p = 0.7581$). (G) Ratio of TPH2$^+$ to SERT$^+$ counts between SAL, FLX and AF groups. A one-way ANOVA showed no significant difference between treatments ($p = 0.8598$).

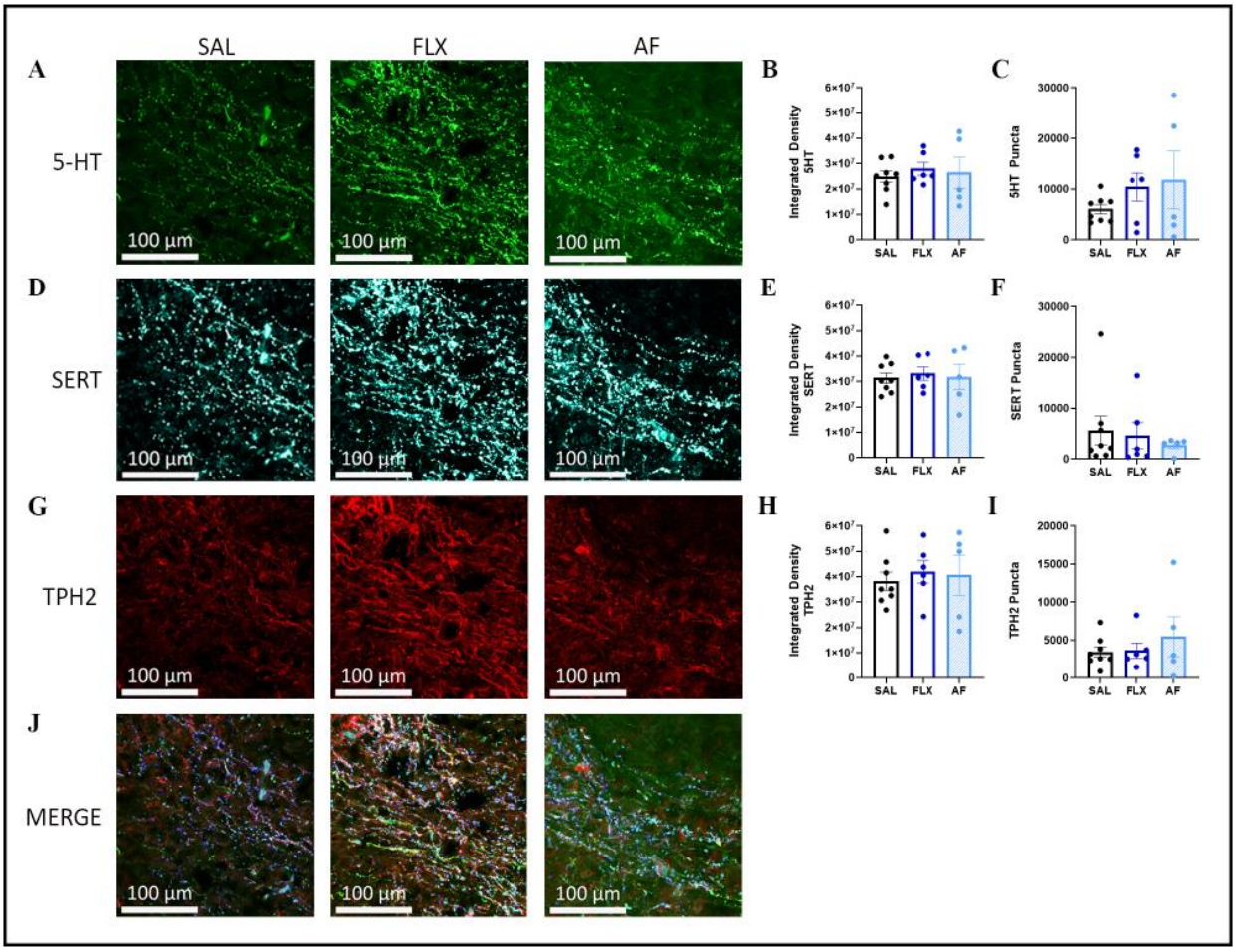

Figure 12: Imaging and quantification of axon projections moving from DRN to PFC, in with AF group added. (A) SAL, FLX and AF sections showing 5-HT staining for serotonergic axon projections in axon region 2. (B) Integrated density readings for 5-HT$^+$ axon projections in SAL, FLX and AF embryos. A one-way ANOVA showed no significant difference between treatment groups ($p = 0.8058$). (C) 5-HT$^+$ puncta quantified for axon projections in SAL, FLX and AF embryos. A one-way ANOVA showed no significant difference between treatment groups ($p = 0.3745$). **(**D) SAL, FLX and AF sections showing SERT staining for serotonergic axon projections in axon region 2. (E) Integrated density readings for SERT$^+$ axon projections in SAL, FLX and AF embryos. A one-way ANOVA showed no significant difference between treatment groups ($p = 0.9135$). (F) SERT$^+$ puncta quantified for axon projections in SAL, FLX and AF embryos. A one-way ANOVA showed no significant difference between treatment groups ($p = 0.7263$). (G) SAL, FLX and AF sections showing TPH2 staining for serotonergic axon projections in axon region 2. (H) Integrated density readings for TPH2$^+$ axon projections in SAL, FLX and AF embryos. A one-way ANOVA showed no significant difference between treatment groups ($p = 0.8642$). (I) TPH2+ puncta quantified for axon projections in SAL, FLX and AF embryos. A one-way ANOVA showed no significant difference between treatment groups ($p = 0.2753$). (J) SAL, FLX and AF sections showing overlaid serotonergic axon staining for all channels (5-HT, SERT and TPH2) in axon region.

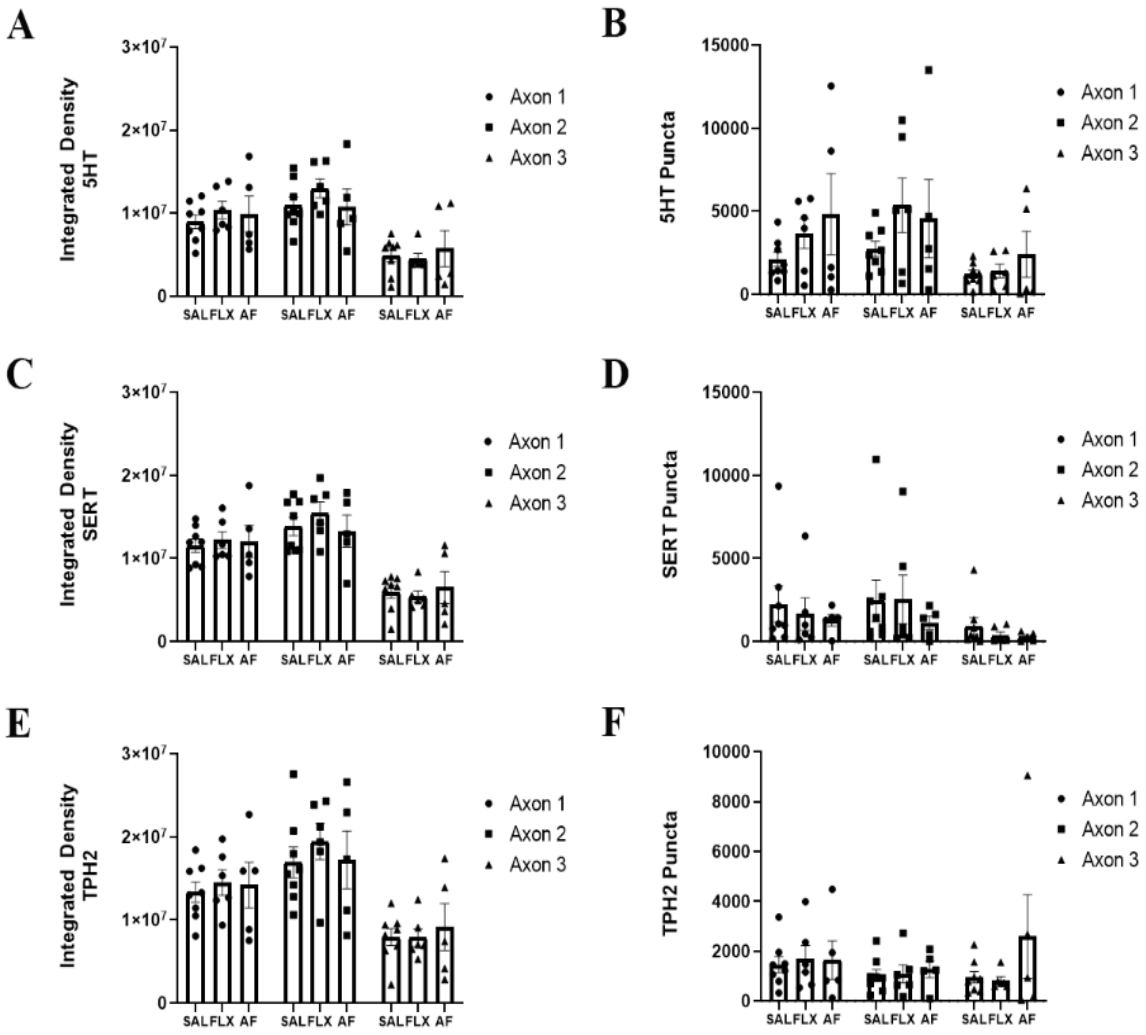
A
Integrated Density 5HT
3×10^7
2×10^7
1×10^7
0
SAL FLX AF SAL FLX AF SAL FLX AF
Axon 1
Axon 2
Axon 3
B
5HT Puncta
15000
10000
5000
0
SAL FLX AF SAL FLX AF SAL FLX AF
Axon 1
Axon 2
Axon 3
C
Integrated Density SERT
3×10^7
2×10^7
1×10^7
0
SAL FLX AF SAL FLX AF SAL FLX AF
Axon 1
Axon 2
Axon 3
D
SERT Puncta
15000
10000
5000
0
SAL FLX AF SAL FLX AF SAL FLX AF
Axon 1
Axon 2
Axon 3
E
Integrated Density TPH2
3×10^7
2×10^7
1×10^7
0
SAL FLX AF SAL FLX AF SAL FLX AF
Axon 1
Axon 2
Axon 3
F
TPH2 Puncta
10000
8000
6000
4000
2000
0
SAL FLX AF SAL FLX AF SAL FLX AF
Axon 1
Axon 2
Axon 3

Figure 13

Figure 13: Region-specific quantification of axon projections moving from DRN to PFC, with AF group added. Tukey's multiple comparisons test was used to perform pairwise analyses across the three treatment groups. The p-values reported here correspond to SAL vs. AF and FLX vs. AF comparisons, respectively. (A) 5-HT integrated density comparison between SAL, FLX, and AF groups for Axon Region 1 (p = 0.8656, 0.9652), Axon Region 2 (p = 0.9952, 0.4936), and Axon Region 3 (p = 0.8980, 0.8135). (B) 5-HT puncta comparison between SAL, FLX, and AF groups for Axon Region 1 (p = 0.2379, 0.7828), Axon Region 2 (p = 0.5185, 0.8887), and Axon Region 3 (p = 0.7506, 0.8354). (C) SERT integrated density comparison between SAL, FLX, and AF groups for Axon Region 1 (p = 0.9614, 0.9950), Axon Region 2 (p = 0.9460, 0.4703), and Axon Region 3 (p = 0.9546, 0.8357). (D) SERT puncta comparison between SAL, FLX, and AF groups for Axon Region 1 (p = 0.7495, 0.9613), Axon Region 2 (p = 0.5917, 0.5903), and Axon Region 3 (p = 0.8780, 0.9970). (E) TPH2 integrated density comparison between SAL, FLX, and AF groups for Axon Region 1 (p = 0.9518, 0.9931), Axon Region 2 (p = 0.9931, 0.7366), and Axon Region 3 (p = 0.8986, 0.9016). (D) TPH2 puncta comparison between SAL, FLX, and AF groups for Axon Region 1 (p = 0.9721, 0.9977), Axon Region 2 (p = 0.9521, 0.9765), and Axon Region 3 (p = 0.1185, 0.1127).

References

Allen Institute. (2008) Allen Developing Mouse Brain Atlas. Retrieved from http://developingmouse.brain-map.org/static/atlas

Alonso, A., Merchan, P., Sandoval, J. E., Sánchez-Arrones, L., Garcia-Cazorla, A., Artuch, R., Ferrán, J. L., MartÍnez-de-la-Torre, M., Puelles, L. (2013). Development of the serotonergic cells in murine raphe nuclei and their relations with rhombomeric domains. *Brain Struct. Funct. 218:* 1229–1277. doi: 10.1007/s00429-012-0456-8

Ansorge, M. S., Zhou, M., Lira, A., Hen, R., Gingrich, J. A. (2004). Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science, 306(5697); 879-881*. doi: 10.1126/science.1101678

Bocchio, M., McHugh, S. B., Bannerman, D. M., Sharp, T., & Capogna, M. (2016). Serotonin, Amygdala and Fear: Assembling the Puzzle. *Frontiers in Neural Circuits*, *10, 24*. doi:10.3389/fncir.2016.00024

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., ...Caporaso, J.G. (2018). QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Preprints 6:e27295v2.* doi: 10.7287/peerj.preprints.27295v2

Bonnin, A., Goeden, N., Chen, K., Wilson, M. L., King, J., Shih, J. C., Blakely, R. D., Deneris, E. S., … Levitt, P. (2011). A transient placental source of serotonin for the fetal forebrain. *Nature*, *472*(7343), 347-50. doi: 10.1038/nature09972

Bonnin, A., Peng, W., Hewlett, W., Levitt, P. (2006). Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. *Neuroscience 141*:781–794. doi: 10.1016/j.neuroscience.2006.04.036

Cipriani, A., Zhou, X., Del Giovane, C., Hetrick, S. E., Qin, B., Whittington, C., Coghill, D., Zhang, Y., Hazell, P., Leucht, S., Cuijpers, P., Pu, J., Cohen, D., Ravindran, A. V., Liu, Y., Michael, K. D., Yang, L., Liu, L., Xie, P. (2016) Comparative efficacy and tolerability of antidepressants for major depressive disorder in children and adolescents: a network meta-analysis. *Lancet, 388*, pp. 881-890. doi: 10.1016/S0140-6736(16)30385-3

Côté, F., Fligny, C., Bayard, E., Launay, J. M., Gershon, M. D., Mallet, J., & Vodjdani, G. (2006). Maternal serotonin is crucial for murine embryonic development. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(1), 329-34. doi: 10.1073/pnas.0606722104

Daubert, E. A., & Condron, B. G. (2010). Serotonin: a regulator of neuronal morphology and circuitry. *Trends in Neurosciences*, *33*(9), 424-34. doi: 10.1016/j.tins.2010.05.005

Daws, L. C., & Gould, G. G. (2011). Ontogeny and regulation of the serotonin transporter: providing insights into human disorders. *Pharmacology & Therapeutics, 131*(1), 61-79. doi: 10.1016/j.pharmthera.2011.03.013

Deneris, E., & Gaspar, P. (2018). Serotonin neuron development: shaping molecular and structural identities. *Wiley interdisciplinary reviews. Developmental Biology*, *7(1).* doi:10.1002/wdev.301

Dubovicky, M., Belovicova, K., Csatlosova, K., Bogi, E. (2017). Risks of using SSRI / SNRI antidepressants during pregnancy and lactation. *Interdisciplinary Toxicology*, *10*(1), 30–34. http://doi.org/10.1515/intox-2017-0004

Faith, D.P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.;61:1–10.* doi: 10.1016/0006-3207(92)91201-3

Faith, D. P., & Baker, A. M. (2007). Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evolutionary Bioinformatics Online, 2, 121–128.*

Ferguson, J. M. (2001). SSRI Antidepressant Medications: Adverse Effects and Tolerability. *Primary Care Companion to The Journal of Clinical Psychiatry*, *3*(1), 22–27.

Furu, K., Kieler, H., Haglund, B., Engeland, A., Selmer, R., Stephansson, O., Valdimarsdottir, U. A., Zoega, H., Artama, M., Gissler, M., Malm, H., … Nørgaard, M. (2015). Selective serotonin reuptake inhibitors and venlafaxine in early pregnancy and risk of birth defects: population based cohort study and sibling design. *BMJ (Clinical Research ed.)*, *350*, h1798. doi:10.1136/bmj.h1798

Gaspar, P., Cases, O., & Maroteaux, L. (2003). The developmental role of serotonin: news from mouse molecular genetics. *Nature Reviews Neuroscience 4: 1002-1012*. doi: 10.1038/nrn1256

Gentile, S. (2011). Selective serotonin reuptake inhibitor exposure during early pregnancy and the risk of birth defects. *Acta Psychiatr Scand. 123*(4):266–75. doi: 10.1111/j.1600-0447.2011.01673.x

Gur, T. L., Kim, D. R., & Epperson, C. N. (2013). Central nervous system effects of prenatal selective serotonin reuptake inhibitors: sensing the signal through the noise. *Psychopharmacology*, *227*(4), 567–582. doi:10.1007/s00213-013-3115-8

Heikkinen, T., Ekblad, U., Laine, K. (2002). Transplacental transfer of citalopram, fluoxetine and their primary demethylated metabolites in isolated perfused human placenta. *BJOG, 109,* pp. 1003-1008. doi: 10.1111/j.1471-0528.2002.01467.x

Hendrick, V., Stowe, Z. N., Altshuler, L. L., Hwang, S., Lee, E., Haynes, D. (2003) Placental passage of antidepressant medications. *Am J Psychiatry. 160*: 993–6. doi:10.1176/appi.ajp.160.5.993

Ippolito, D. M., & Eroglu, C. (2010). Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. *Journal of Visualized Experiments : JoVE, (45), 2270*. doi:10.3791/2270

Jackson, M. A., Verdi, S., Maxan, M. E., *et al.* (2018). Gut microbiota associations with common diseases and prescription medications in a population-based cohort. *Nature Communications.* Volume 9, Article number: 2655. doi.org/10.1038/s41467-018-05184-7

Jia, Y., Zhu, H., & Leung, S. W. (2016). Comparative efficacy of selective serotonin reuptake inhibitors (SSRI) in treating major depressive disorder: a protocol for network meta-analysis of randomised controlled trials. *BMJ Open, 6*(6), e010142. doi: 10.1136/bmjopen-2015-010142

Kostev, K., Rex, J., Eith, T., Heilmaier, C. (2014). Which adverse effects influence the dropout rate in selective serotonin reuptake inhibitor (SSRI) treatment? Results for 50,824 patients. *GMS German Medical Science, 12,* Doc 15. doi: 10.3205/000200

Machado, D. G., Cunha, M. P., Neis, V. B., Balen, G. O., Colla, A., Brocardo, P. S., Bettio, L. E., Capra, J. C., Rodrigues, A. L. (2012). Fluoxetine reverses depressive-like behaviors and increases hippocampal acetylcholinesterase activity induced by olfactory bulbectomy. *Pharmacol. Biochem. Behav. 103*:220–229. doi: 10.1016/j.pbb.2012.08.024

Magni, L. R., Purgato, M., Gastaldon, C., Papola, D., Furukawa, T. A., Cipriani, A., Barbui, C. (2013). Fluoxetine versus other types of pharmacotherapy for depression. *Cochrane Database of Systematic Reviews 2013, Issue 7. Art. No.: CD004185.* doi: 10.1002/14651858.CD004185.pub3

Maier, L., Pruteanu, M., Kuhn, M., Zeller, G., Telzerow, A., Anderson, E. E., Brochado, A. R., Fernandez, K. C., Dose, H., Mori, H., Patil, K. R., Bork, P., … Typas, A. (2018). Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature*, *555*(7698), 623-628. doi: 10.1038/nature25979

Michelsen, K.A., Schmitz, C., Steinbusch, H. W. M. (2007). The dorsal raphe nucleus--from silver stainings to a role in depression. *Brain Res. Rev., 55*, pp. 329-342. doi: 10.1016/j.brainresrev.2007.01.002

Moiseiwitsch, J. R. (2000). The role of serotonin and neurotransmitters during craniofacial development. *Crit Rev Oral Biol Med. 11*(2):230–9.

O'Keane, V., Marsh, M.S. (2007). Depression during pregnancy. *BMJ (Clinical research ed.), 334* (7601), 1003-5. doi: 10.1136/bmj.39189.662581.55

Pediatric Academic Societies. (2018). Long-term effects of pre-birth exposure to anti-depressants 12 years later. *ScienceDaily*. Retrieved May 17, 2019 from www.sciencedaily.com/releases/2018/05/180505091822.htm

Pratt, L. A., Brody, D. J., Gu, Q. (2017). Antidepressant use among persons aged 12 and over: United States, 2011–2014. *NCHS Data Brief, no 283.* Hyattsville, MD: National Center for Health Statistics.

Rochat, B., Baumann, P., Audus, K. L. (1999). Transport mechanisms for the antidepressant citalopram in brain microvessel endothelium. *Brain Res., 831,* pp. 229-236. doi: 10.1016/S0006-8993(99)01461-4

Rodríguez-Gaztelumendi, A., Rojo, M. L., Pazos, A., Díaz, A. (2009). Altered CB receptor-signaling in prefrontal cortex from an animal model of depression is reversed by chronic fluoxetine. *J Neurochem, 108*, pp. 1423-1433. doi: 10.1111/j.1471-4159.2009.05898.x

Santacruz, A., Collado, M.C., Garcia-Valdes, L., Segura, M.T., Martin-Lagos, J.A., Anjos, T., Marti-Romero, M., Lopez, R.M., Florido, J., Campy, C., Sanz, Y. (2010). Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. *The British Journal of Nutrition. 104:83–92*. doi: 10.1017/s0007114510000176

Semple, B. D., Blomgren, K., Gimlin, K., Ferriero, D. M., & Noble-Haeusslein, L. J. (2013). Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology, 106-107, 1–16.* doi:10.1016/j.pneurobio.2013.04.001

Shimogori, T., Lee, D. A., Miranda-Angulo, A., Yang, Y., Wang, H., Jiang, L., … Blackshaw, S. (2010). A genomic atlas of mouse hypothalamic development. *Nature Neuroscience, 13(6), 767–775*. doi:10.1038/nn.2545

Soiza-Reilly, M., Meye, F. J., Olusakin, J., Telley, L., Petit, E., Chen, X., Mameli, M., Jabaudon, D., Sze, J. Y., Gaspar, P. (2018). SSRIs target prefrontal to raphe circuits during development modulating synaptic connectivity and emotional behavior. *Molecular Psychiatry* (published online). doi: 10.1038/s41380-018-0260-9

Spanogiannopoulos, P., Bess, E. N., Carmody, R. N., Turnbaugh, P. J. (2016). The microbial pharmacists within us: a metagenomic view of xenobiotic metabolism. *Nature Reviews. Microbiology, 14*(5), 273–287. doi.org/10.1038/nrmicro.2016.17

Substance Abuse and Mental Health Services Administration. (2017). Key substance use and mental health indicators in the United States: Results from the 2016 National Survey on Drug Use and Health *(HHS Publication No. SMA 17-5044, NSDUH Series H-52). Rockville, MD: Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration.*

van Kleef, E.S., Gaspar, P., & Bonnin, A. (2012). Insights into the complex influence of 5-HT signaling on thalamocortical axonal system development. *The European Journal of Neuroscience, 35*(10), 1563–1572. doi:10.1111/j.1460-9568.2012.8096.x

Verney, C., Lebrand, C., Gaspar, P. (2002). Changing distribution of monoaminergic markers in the developing human cerebral cortex with special emphasis on the serotonin transporter. *Anat Rec. 267:87–93*. doi: 10.1002/ar.10089

Weinberger, A. H., Gbedemah, M., Martinez, A. M., Nash, D., Galea, S., Goodwin, R. D. (2018). Trends in depression prevalence in the USA from 2005 to 2015: widening disparities in vulnerable groups. *Psychological Medicine 48,* pp. 1308–1315. doi:10.1017/S0033291717002781

World Health Organization (2017). Depression and Other Common Mental Disorders: Global Health Estimates. *Rep. CC BY-NC-SA 3.0 IGO.Geneva: World Health Organization.*

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K., … Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell, 161*(2), 264-76. doi: 10.1016/j.cell.2015.02.047

Zhao, Z., Nelson, A. R., Betsholtz, C., Zlokovic, B. V. (2015). Establishment and dysfunction of the blood-brain barrier. *Cell, 163,* pp. 1064-1078. doi: 10.1016/j.cell.2015.10.067

Zwink, N., & Jenetzky, E. (2018). Maternal drug use and the risk of anorectal malformations: systematic review and meta-analysis. *Orphanet Journal of Rare Diseases, 13*(1), 75. doi:10.1186/s13023-018-0789-3