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Investigating Changes in Monoamine Transmission Induced by Stress

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Investigating Changes in Monoamine Transmission Induced by Stress

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular Toxicology

by

Merel Dagher

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ABSTRACT OF THE DISSERTATION

Investigating Changes in Monoamine Transmission Induced by Stress

by

Merel Dagher

Doctor of Philosophy in Molecular Toxicology

University of California, Los Angeles, 2022

Professor Anne M. Andrews, Chair

The serotonin system, among other neurotransmitter systems, plays an important role in mood and anxiety disorders. Stress, which is the biggest risk factor for the development of mood and anxiety disorders, leads to many lifelong changes that affect the overall function of monoamine transmission and behavior. My dissertation work set out to investigate (1) the effects of *in utero* stress exposure on neurochemistry and behavior during adulthood and whether concomitant maternal citalopram treatment rescued adverse stress effects, (2) the selectivity of optogenetically stimulating dopamine neurons and the interplay between monoamine systems, (3) SERT genotypes on freezing behaviors after ambiguous cue presentation, and (4) neuroinflammation produced by biosensors to monitor neurotransmitters *in vivo*.

The primary investigation of my graduate work was focused on effects of *in utero* stress exposure and whether treatment with citalopram could mitigate the adverse effects of stress. To investigate this question, I used a mouse model in which pregnant dams underwent a chronic stress paradigm during their pregnancy. Some dams received concomitant

treatment with citalopram, an SSRI. I then examined developmental neurochemistry, and adult neurochemical and behavioral changes. I found that pups from the stressed group had elevated neurochemical and amino acid tissue levels. Moreover, I found that pups born to stressed dams had increases in anxiety- and depressive- like behavior and that these effects were rescued in pups that received concomitant *in utero* citalopram exposure. Finally, stressed pups had increased serotonin concentrations after SERT blockade in the vHPC and after a systemic injection of a kappa opioid receptor agonist. My findings suggest beneficial outcomes when treating stress during pregnancy on overall offspring health.

The dissertation of Merel Dagher is approved.

Michael D. Collins

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Anne M. Andrews, Committee Chair

University of California, Los Angeles

2022

For my parents

Table of Contents

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Vita

I received my Bachelor of Arts in Cognitive Science and Public health at the University of California, Berkeley (UCB). At UCB, I was actively involved in the chemistry department as an undergraduate teaching assistant. I was also a peer educator for the Department of Student Affairs, where I gave workshops on the importance of being an active bystander and a peer advocate.

I began my graduate studies in 2017 at the University of California, Los Angeles (UCLA), initially as a master's student in the department of Environmental Health Science in the School of Public Health. I joined the lab of Dr. Anne Andrews in January of 2018 and switched into the Molecular Toxicology IDP to pursue my doctoral degree. In Dr. Andrews' group, my research has focused on investigating the role of serotonin transmission in mood and anxiety disorders, elucidating brain pathways involved in anxiety responses, and assessing *in utero* stress exposure on offspring health. I was awarded the T32 NIDA fellowship for the 2021-2022 academic year.

CHAPTER I

Safety of Pharmacotherapeutics for the Treatment of Mood and Anxiety Disorders During Pregnancy: From Classical Antidepressants to new Therapeutic Avenues

Introduction: Safety of using antidepressants and anxiolytics during pregnancy

Use of antidepressants during pregnancy: Prevalence and combination therapies

According to the Center for Disease Control (CDC), one in seven women of reproductive age is prescribed medication for anxiety or mood disorders. Estimates of perinatal depression or anxiety range from 5-25% and are underreported due to social stigma.[1] Psychiatric disorders, which are not physically obvious and viewed by many as character flaws or personal weakness, are often met with doubt, scrutiny, and a lack of empathy. Woody *et al.* found that the incidence of depression during pregnancy is 15% and ~15% of women experience a new episode of depression within the first three months postpartum.[2] Furthermore, suicide accounts for 20% of postpartum deaths.

Approximately 5-8% of pregnant women with mood or anxiety disorders will be prescribed medication for the treatment of pathology during pregnancy.[3,4] While being prescribed an antidepressant or filling a prescription often is used as a proxy for SSRI exposure, factors such as actually taking the medication, severity of depressive symptomology, and timeframe of use during pregnancy, are not always reported.[5] The safety of antidepressant use during pregnancy remains controversial due to an incomplete understanding of how exposure affects fetal development and long-term effects on offspring. By contrast, maternal mood and anxiety disorders have known adverse health outcomes on fetal development and long-term effects.[6] A holistic understanding of the safety of antidepressants with respect to fetal outcomes *and* maternal health will improve clinical guidelines and recommendations. Additionally, knowledge about antidepressant safety

during pregnancy will promote maternal autonomy, thus informing decisions to continue (or discontinue) pharmacotherapy during pregnancy.

Mild to moderate mood and anxiety disorders are treated with nonmedication therapies such as cognitive-behavioral therapy.[7] Patients report the highest positive outcomes when "talk therapy" is used in combination with medication.[8,9] Thus, tapering medication treatment with simultaneous substitution of behavioral interventions may be an effective option for treating mood and anxiety disorders during pregnancy that avoids exposing fetuses to the possible teratogenic effects of antidepressants (see below). Yet, for many, particularly individuals with severe mood and anxiety disorders, behavioral therapies do not provide sufficiently effective treatment. According to an international review on Clinical Practice Guidelines regarding treatment for perinatal use of antidepressants, guidelines converge that mild-to-moderate depression during pregnancy should be treated first with psychotherapy, before moving to pharmacotherapy.[10] Integrated approaches using pharmaco- and behavioral therapies are warranted, particularly for more severe or refractory perinatal mood and anxiety disorders in light of evidence showing the efficacy of combined treatments.[11]

This review will focus on perinatal pharmacotherapy for treating mood and anxiety disorders, particularly, three broad medication categories—SSRIs, SNRIs, and atypical antidepressants. I will discuss the prevalence of their use, pharmacology, and potential side effects for mother and fetus. Additionally, new therapeutic targets will be discussed, *e.g.*, 5HT2A and kappa opioid receptors.

The DSM criteria and prevalence of mood and anxiety disorders

The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) defines depressive disorders as characterized by "five or more symptoms during the same two-week period that are a change from previous functioning." These symptoms include depressed mood, loss of interest or pleasure known as anhedonia, weight gain or loss, slowing down of thought and reduction of physical movement, fatigue, feelings of worthlessness, diminished ability to concentrate, and suicidal ideation. The DSM-V defines anxiety disorders as characterized by "excessive anxiety and worry (apprehensive expectation), occurring more days than not for at least 6 months."[12] Anxiety disorders include generalized anxiety disorder (GAD), social anxiety disorder (SAD), panic disorders, and specific phobias.

The World Health Organization (WHO) ranks depression as the leading cause of disability worldwide, as measured by the number of years lived with disability (YLDs).[13] Depressive disorders contribute ~4.4% to all YLDs. Anxiety disorders are ranked 6th, contributing to ~3.4% of YLDs. Over 300 million people worldwide suffer from depression, and greater than 250 million people suffer from an anxiety disorder. Mood and anxiety disorders are often comorbid[14] as concurrent diagnoses occur in upwards of 70% of patients.[15] According to the Anxiety and Depression Association of America (ADAA), anxiety disorders are the most common mental illness in the United States, affecting 40 million individuals annually. Limited progress has been made in the diagnosis and management of mood and anxiety disorders, largely because their etiologies remain fundamentally unknown.

FDA category classification for medication use during pregnancy

Classifying drugs for use during pregnancy emerged in 1979, after the thalidomide tragedy.[16] Thalidomide was introduced to the European market in 1957 as a safer alternative to barbiturates for treating insomnia.[17] Thalidomide was also marketed as a sedative for children and to pregnant women for vomiting, nausea, and morning sickness.[17] Thalidomide was widely used during the 1950s and 60s in European countries but was never introduced to the American market due to the efforts of the FDA medical officer Frances Oldham Kelsey.[17] Concerned about a lack of safety data , Kelsey refused to authorize thalidomide for use in the United States. Kelsey had read a letter published in the British Medical Journal by Alexander Leslie Florence linking thalidomide to nerve damage, something that was never disclosed to Kelsey by the United States licensing representative. As Kelsey correctly suspected, thalidomide use during pregnancy was subsequently shown to cause birth defects, predominantly phocomelia, *i.e.*, severe malformations of the extremities, in thousands of exposed babies.[17]

In response to thalidomide, the FDA implemented labeling requirements for medications used during pregnancy in 1979. Drugs fall into one of five categories—A, B, C, D, or X. Drugs in categories A and B are associated with well-controlled studies in pregnant women or animal studies wherein minimal or no fetal risks have been detected.[16] Drugs in categories C and D have some documented fetal risk associated with their use, but their benefits may outweigh their risks. Drugs in category X should not be used during pregnancy, as possible benefits are greatly outweighed by the risks of use.[16] Approximately 60% of all drugs fall into category C, highlighting the lack of research surrounding the safety of drug use during pregnancy.[18] Most antidepressants fall into category C.[19] As will be discussed in

the next section, many physiological, pharmacokinetic, hormonal, and behavioral changes occur during pregnancy. As a result, studying the safety of medications specifically in pregnant subjects is crucial.

Pregnancy-induced changes that affect drug action

Global changes occur in all body systems during pregnancy, including the cardiovascular, gastrointestinal, and endocrine systems.[20,21] These changes are particularly relevant for drug metabolism, which can change significantly in gravid individuals. Moreover, the mammalian placenta produces new hormones and receptors, which change drug pharmacokinetics and internal homeostasis.

Pharmacokinetics

Absorption

Absorption is the process of transporting a drug from its site of administration to the systemic circulation.[22,23] Many mechanisms are involved in absorption, including passive diffusion, carrier-mediated membrane transport, including active and facilitated diffusion, and uptake by nonspecific drug transporters, *e.g.*, P-glycoprotein.[23,24] Factors such as route of administration, gastric pH, lipophilicity, and molecular size affect the bioavailability of drugs, *i.e.*, how much of a drug is available in the circulation non-intravenous administration, *e.g.*, oral, intranasal, transdermal, *vs.* intravenous administration.[23,25] Drugs taken orally undergo the first-pass effect, in which they are metabolized by gastrointestinal organs, particularly the liver, reducing their overall bioavailability.[25]

Pregnancy results in decreased gastrointestinal motility and increased gastric pH, which affect drug absorption after oral administration.[24] Decreased gastrointestinal motility slows absorption of drugs, while increased gastric pH deprotonates some drugs, which

reduces absorption. Vomiting is a common symptom during pregnancy, particularly in the first trimester. Emesis may reduce drug concentrations, especially with the oral route of administration.[25]

Distribution

Distribution is the process by which drugs move from the bloodstream to the tissues.[22] Factors such as blood plasma protein binding and membrane permeability affect distribution. For instance, drugs that are tightly bound by plasma proteins have reduced tissue availability. The blood-brain and blood-placenta barriers actively reduce drug entry to the brain and fetus, respectively. The volume of distribution (Vd) is a theoretical volume used to indicate how extensively a drug will distribute in the body.[25] High Vd indicates high distribution and low plasma protein binding.

During late pregnancy, fasting glucose levels decrease while hepatic production of glucose increases.[26] Approximately one-third of the increased glucose is utilized by uterine, fetal, and placental tissue. Increases in adipose tissue result in average gains of 3.5 kg of fat.[26] While adipose tissue increases, little is known about how this factor affects drug distribution.[25] Maternal plasma volume increases throughout pregnancy. Cardiac output increases and drug-plasma protein binding decreases.[27,28] Taken together, these changes result in a higher Vd for lipophilic drugs, yet reduced plasma concentrations of drugs.[29] Blood flow to the uterus also increases 10-fold, and drugs that are highly lipophilic and small readily cross the fetal-placental barrier.[25] This leads to a build-up of drug concentration in the amniotic fluid, providing an additional source of fetal exposure to drugs.[30,31]

Metabolism

Metabolism is the process by which drugs are modified by enzymatic processes, typically in the gastrointestinal tract or liver.[22] Metabolism of certain medications is greatly affected by enzyme isoforms, particularly enzymes belonging to the CYP450 family, during Phase I metabolism.[29] Phase I reactions are oxidation, reduction, or hydrolysis reactions, which make drugs more hydrophilic. In Phase II metabolism, conjugation reactions occur, such as sulfation and glucuronidation, which increase the size and hydrophilicity of drugs to facilitate excretion.[24]

The study of pharmacogenomics, *i.e.*, how genetic variations affect drug response, is useful in understanding maternal-fetal dynamics with respect to drug exposure during pregnancy.[32] For example, key findings have identified ultrafast metabolizers *vs.* intermediate or poor metabolizers of SSRIs and how these differences may be exaggerated during pregnancy.[33] Progesterone levels, which increase during pregnancy, induce greater CYP3A4 activity accelerating the metabolism of drugs metabolized by CYP34A, *e.g.*, fluoxetine, paroxetine, venlafaxine, and bupropion.[29] Contrasting with the increased activity of CYP3A4, CYP2C19 activity is reduced by almost half, which is particularly important for the metabolism of citalopram and escitalopram.[29] Reduced activity of CYP2C19 results in reduced metabolism and elevated drug plasma concentrations..

Excretion

Excretion eliminates drugs and their metabolites from the body, typically through urine or feces, but also through exhalation or sweat.[22] Renal, cardiac, and hepatic actions affect overall clearance rates. Steady-state concentration is determined by drug dose and clearance rate. Clearance from plasma circulation changes overall drug concentrations and

half-life. During pregnancy, glomerular filtration rates increase, thereby increasing renal clearance. Increased renal clearance leads to decreased drug concentrations. In one study, increased renal clearance translated to increased depression scores, necessitating dose adjustments.[34] Pharmacokinetic changes during pregnancy are summarized in **Table I.1**.

Neuroendocrine changes

Pharmacokinetic changes are influenced by pregnancy-related hormonal changes.[27] The hormones important in this regard are hypothalamus-pituitary-adrenal (HPA) hormones, as well as hormones secreted by the placenta.

The HPA axis is an important modulator of stress responses .[35] Activation of the HPA axis results in cortisol release from the adrenal glands *via* synthesis and release of hypothalamic and pituitary hormones. During pregnancy, corticotropin-releasing hormone (CRH) increases from concentrations below 200 pg/mL to concentrations well above 1000 pg/mL in the plasma.[36] The main function of CRH, which is produced in the hypothalamus, is to stimulate the synthesis of adrenocorticotropic hormone (ACTH). The release of ACTH stimulates the synthesis of cortisol, glucocorticoids, mineralocorticoids, and androsterone.

While cortisol release by the adrenal glands normally inhibits hypothalamic and pituitary production of their respective hormones, maternal cortisol leads to stimulation, synthesis, and release of cortisol, ACTH and CRH in the fetus.[36] The placenta expresses genes for CRH and placental CRH and hypothalamic CRH genes have near opposite responses due to differences in transcription factors. Thus, maternal stress has poor effects on fetal health. The placenta can "sense" stress-related changes leading to adverse effects, *e.g.*, preterm birth or preeclampsia.[37] Increased cortisol indicates increased stress levels and can have

behavioral and physiological effects on mothers. Furthermore, postpartum depression, which is associated with responses to stress, may affect childcare, leading to reduced maternal engagement with the baby, greater unpredictability in routines, and reduced breastfeeding duration.[38]

Other hormones influence maternal and fetal physiology. For example, progesterone downregulates maternal gastrointestinal motility, which delays the absorption of orally administered drugs.[27] Maternal estrogen, progesterone, aldosterone, and cortisol levels increase during pregnancy. Peptide and protein hormones such as prolactin, neuropeptide Y, and human chorionic gonadotropin also increase. [27] Estrogen and progesterone upregulate or downregulate CYP enzyme expression, thereby influencing drug metabolism. Native growth hormone (GH) levels decrease, but other growth hormones produced by the placenta increase, as discussed in the next section.[27]

The placenta

During pregnancy, an entirely new organ is formed—the placenta. The placenta plays important roles in protecting the fetus from maternal immune responses, supplying nutrients, and providing gas exchange between fetal and maternal circulations.[39] The placenta prevents maternal immune system activation to paternal antigens expressed by fetal cells.[40] The placenta promotes several regulatory mechanisms, *e.g.*, altered antigen presentation and T-cell differentiation, which create "distractions" for the maternal immune system, thereby leading to fetal viability.[41] Maternal IgG antibodies cross the placental barrier and provide passive immunity to the fetus.[39]

The placenta is a highly active endocrine organ.[40] The placenta produces and secretes human placental hormone (hPL) and placental growth hormone (PGH), which regulate

maternal metabolism, *e.g.*, lipolysis promotion in adipose tissue. The hPL and PGH levels increase 30-fold (~1 g/day) and 100-fold (~14 ng/mL after week 28), respectively, during pregnancy, yet little is known about their overarching effects, especially on drug metabolism and other pharmacokinetic parameters.[27]

The placenta is the sole link between the fetus and mother. Drugs that cross the maternal blood-placental barrier will reach the fetus.[39] Three types of drug transfer occur.[42] Type 1 drugs rapidly cross the placenta and significant concentrations are observed in maternal and fetal plasma. Type 2 drugs reach greater concentrations in the fetus than the mother, *e.g.*, ketamine. Type 3 drugs have incomplete transfer, resulting in higher concentrations in the maternal plasma compared to fetal plasma. Many factors affect drug transfer, including placental surface area and thickness, drug molecular weight and lipid solubility, and the pH of maternal and fetal blood.[39] Drug transfer occurs through placental passive or facilitated diffusion, active transport, or pinocytosis.[39] Antidepressants like SSRIs inhibit some placental drug transporters, *e.g.*, P-glycoprotein, which binds numerous endogenous ligands, including cortisol and aldosterone. As such, inhibition of P-glycoprotein induces alterations in fetal exposure to maternal hormones.[25]

Drug-placental interactions have not fully been investigated. Deeper understanding about interactions between drugs and the placenta and subsequent effects on fetal drug exposure will influence prescription recommendations, particularly for women who decide to remain on antidepressant treatment during pregnancy.

In sum, trimester-specific pharmacokinetic, hormonal, and anatomical changes occur during pregnancy. Factors such as the onset of antidepressant administration, onset of maternal depression or anxiety, drug dose, and duration of treatment will need rigorous

investigation, as they may explain discrepancies, *i.e.*, conflicting evidence between studies about physiological, psychomotor, behavioral, and cognitive risks, associated with current knowledge about *in utero* antidepressant exposure on fetal and infant health.

Second Generation Antidepressants: SSRIs and SNRIs

Today, the most commonly prescribed medications used to treat depression and anxiety are the selective serotonin reuptake inhibitors (SSRIs). Rodent models point to the serotonin transporter (SERT), which is the primary site for SSRI action, as a key modulator of serotonin transmission and anxiety-related behavior.[43-46] Clinical trials[47] and studies of animal models suggest that serotonin signaling plays an important role in the manifestation of and vulnerability to mood and anxiety disorders.[48-51] Nonetheless, SSRIs generally only improve mood after weeks of consistent administration. Still, all SSRIs are more effective than placebos, and 40-60% of patients see mood improvement upon sustained administration of SSRIs.[47]

Origins of the monoamine hypothesis of depression

In the 1940s and 50s, depressive disorders were treated by invasive brain procedures leading to side effects and permanent disability. The history of treating psychiatric disorders is one of inhumane treatment, lack of informed consent, and luck.[52] While electroconvulsive therapy (ECT) had been the primary treatment for depressive disorders, in the 1950s, lobotomies were proposed as a more effective alternative, particularly for patients with severe depression.[53] Pharmacotherapies took a center stage for treating mood and anxiety disorders after the downfall of Walter Freeman, a neuroscientist who developed and used transorbital lobotomies.[54] Transorbital lobotomies did not require surgery, so Freeman operated alone after his partner, neurosurgeon James W. Watts refused to perform lobotomies due to their lack of safety.

Freeman performed lobotomies on more than 4,000 patients. He performed transorbital lobotomies in 2,500 patients despite no formal surgical training. Antipsychotic

medications were discovered in the 1930s, in parallel with the rise of ECT and lobotomies. In 1955, chlorpromazine, a first-generation antipsychotic, was approved for use[55], and turned the tide away from invasive procedures to drugs as therapeutics for mood and anxiety disorders.[54]

In the 1950s, major breakthroughs for antidepressants arose.[52] Physicians noted that tuberculosis medications, namely isoniazid and iproniazid, improved the mood of hospitalized tuberculosis patients. Isoniazid and iproniazid were discovered to be monoamine oxidase inhibitors (MAOIs).[56] Monoamine oxidase is responsible for the metabolism of monoamine neurotransmitters, *i.e.*, dopamine, norepinephrine, and serotonin.[57] The MAOIs prevent degradation of the monoamine neurotransmitters, thus increasing their concentrations in brain tissue[58] and prolonging their duration of action in the extracellular space. The MAOIs are now used to treat psychiatric disorders, such as anxiety and depression. Tricyclic antidepressants (TCAs) were also serendipitously discovered to inhibit the reuptake of monoamine neurotransmitters. The MAOIs and TCAs are referred to as first-generation antidepressants.

The fact that MAOIs and TCAs improved mood and reduced anxiety led to the catecholamine hypothesis of depression proposed by Schildkraut, Bunney, and Davis in 1965.[59,60] A role for serotonin came later when the TCA imipramine was discovered to inhibit serotonin reuptake, in addition to norepinephrine reuptake. Because TCAs were shown to inhibit serotonin reuptake and MAOIs were shown to affect serotonin synthesis, Coppen proposed that serotonin was important in the mood-improving properties of MAOIs and TCAs.[61] The serotonergic hypothesis of depression suggests that decreased serotonin levels are a cause of depression. Based on the evolving serotonin hypothesis and the fact that

MAOIs and TCAs had many adverse side effects, *e.g.*, seizures, cardiac dysfunction, pharmaceutical companies set out to discover drugs that selectively impacted the serotonin system.

In 1972, the pharmaceutical company Eli Lily reported on the properties of fluoxetine, which was designated the most powerful and selective serotonin reuptake inhibitor at the time.[62] In 1987, fluoxetine was approved by the FDA for clinical use. Fluoxetine is a second-generation antidepressant, along with all other SSRIs, SNRIs, and bupropion. Fluoxetine, like all SSRIs, has fewer side effects compared to MAOIs and TCAs but can be ineffective for many patients despite the rapid rise in prescriptions. More recently, atypical antidepressants have emerged. In 2019, ketamine was FDA-approved for treatment-resistant depression. The use of ketamine implicates neurotransmitter systems in addition to the monoamines in the etiology and/or treatment of depression and anxiety.[63]

Statistics on use

In the United States, 13% of adults aged 18 and older use antidepressants, mainly SSRIs. Of those who are pregnant, 1-5% use SSRIs.[64] Effects of exposure to SSRIs are different depending on trimester, duration and consistency of use, and metabolic profiles, which will be discussed below. Serotonin-norepinephrine reuptake inhibitors (SNRIs) are another treatment option for depression. In addition to their use in the treatment of mood and anxiety disorders, SNRIs are also prescribed for chronic pain.[65-67] Specific use statistics are difficult to obtain because SNRIs are generally lumped together with SSRIs and categorized under the umbrella term of 'antidepressant'. Nonetheless, trends suggest that prescriptions of SNRIs, along with atypical antidepressants, are on the rise.[68] The SNRIs are prescribed

during pregnancy, though at lower rates than SSRIs, and are also designated under category C by the FDA.[18]

Pharmacokinetics

The SSRIs, administered orally *via* pills or liquid capsules, include fluoxetine, citalopram, escitalopram, sertraline, paroxetine, and fluvoxamine. In 2011 and 2013, vilazodone and vortioxetine, respectively, were approved by the FDA for major depressive disorder, though they are the least prescribed SSRIs due to their short time on the market. Pharmacokinetic properties of the SSRIs are summarized in **Table I.2**.

All SSRIs undergo first-pass metabolism in the liver.[69] The SSRIs are metabolized by the CYP enzymes, primarily CYP2D6. Fluoxetine has the highest volume of distribution and longest half-life compared to other SSRIs and has an active metabolite, norfluoxetine. Both fluoxetine and norfluoxetine have high affinity for SERT, as well as 5HT2A and D2 receptors. Citalopram has the highest selectivity for SERT compared to other SSRIs. Almost all of the pharmacological effects of citalopram are attributed to the (S)-enantiomer or escitalopram[70], which is sold under the brand name Lexapro®.[71] Sertraline is unique among SSRIs as it also binds to dopamine transporters. Some studies have investigated sertraline as a treatment for stimulant use disorders, as sertraline delays relapse rates compared to placebo.[72,73] Vilazodone and vortioxetine, while potent SERT inhibitors, also show partial agonist activity at serotonin receptors.[74,75]

The pharmacokinetic properties of SSRIs change during pregnancy.[76] For example, drug metabolism by some CYP isoenzymes increases.[77] For paroxetine, which is exclusively metabolized by CYP2D6, women who are extensive or ultrarapid metabolizers showed decreased serum levels of paroxetine and significantly increased depression symptoms.[33]

Intermediate or poor metabolizers showed increased paroxetine serum levels during pregnancy but had no change in depressive symptoms. An ongoing study by the National Institute of Child Health and Development (NICHD) is examining how antidepressant concentrations change with respect to physiological changes during pregnancy and in the postpartum period (Clinical Trail #: NCT02519790).

Like SSRIs, SNRIs are administered orally *via* pills or liquid capsules. The family of SNRIs consists of duloxetine (Cymbalta®), desvenlafaxine (Pristiq®), levomilnacipran (Fetzima®), milnacipran (Savella®), and venlafaxine (Effexor®, discontinued). Most of the SNRIs were approved for use by the FDA in the early 2000s, *sans* venlafaxine, which was approved in 1993 (immediate-release) and 1997 (extended-release), and levomilnacipran, which was approved in 2013. Milnacipran is the only SNRI not used for the treatment of major depression or anxiety disorders; rather, it is almost exclusively prescribed for the treatment of fibromyalgia.[65] The half-life of the SNRIs is around 10 hours.[78] All SNRIs except milnacipran require metabolism by liver CYP enzymes. Milnacipran bypasses CYP metabolism and is metabolized by Phase II conjugation. The bioavailability and volume of distribution of SNRIs are similar to the SSRIs.[78] Pharmacokinetic properties of SNRIs are summarized in **Table I.3**.

Pharmacodynamics

The SSRIs act on serotonin transporters (SERT), blocking the reuptake of serotonin into presynaptic neurons. While some SSRIs, such as paroxetine and sertraline, have affinity for other monoamine transporters (MATs), the SSRIs are mostly selective for SERT. The SNRIs target both serotonin and norepinephrine transporters with high affinity, thereby blocking the reuptake of both monoamines into presynaptic neurons.[78] An indirect target of

SNRIs is the dopamine transporter (DAT). In the prefrontal cortex, dopamine is predominately taken up by NET, so SNRIs work as triple agents, to some extent, *via* indirect inhibition of dopamine reuptake by NET in the prefrontal cortex.[79]

Some SNRIs are used to treat chronic pain conditions, such as chronic musculoskeletal pain, diabetic peripheral neuropathic pain, and fibromyalgia.[80] Both duloxetine and milnacipran are prescribed for fibromyalgia. Effectiveness in treating chronic pain implicates roles for norepinephrine and serotonin transmission.[81] The raphe nuclei, the sites of serotonergic cell bodies, and the locus coeruleus, the site of most norepinephrine cell bodies, send their projections to the dorsal horn of the spinal cord *via* the dorsolateral funiculus (DLF).[82] Serotonin and norepinephrine descending fibers suppress pain transmission presumably by hyperpolarization of afferent sensory neurons, preventing the relay of nociception to the thalamus, and eventually, cortical regions.[80] Increased extracellular norepinephrine and serotonin increase inhibition of ascending pathways. Serotonin- and norepinephrine-mediated inhibition of ascending pathways lead to their therapeutic effects for chronic pain conditions.

Microdialysis and voltammetry studies have confirmed that serotonin reuptake inhibitors produce elevated extracellular concentrations of serotonin in a matter of minutes.[83,84] Yet, for most patients, SSRIs take 1-6 weeks to improve mood, if improvements occur at all.[85] Thus, the mechanism by which SSRIs improve mood must involve effects beyond their immediate action at SERT. Potential therapeutic mechanisms include prolonged increases in extracellular serotonin, desensitization/downregulation of serotonin1A autoreceptors, and increased brain-derived neurotrophic factor (BDNF), synaptogenesis, and neurogenesis, which will be discussed in a subsequent section.[86-89]

Fetal exposure and risk associated with SSRI use

The SSRIs (and SNRIs) cross the fetal-placental barrier, though not in similar ways.[90] A 2003 report of umbilical cord SSRI concentrations from 38 women found that maternal doses of sertraline and fluoxetine were significantly correlated with umbilical cord serum drug concentrations.[90] This correlation, however, is not observed for citalopram The SSRIs and SNRIs have also been detected in the amniotic fluid, which the fetus swallows, providing another source of exposure to the fetus.[30,91] Finally, babies are exposed to SSRIs *via* breast milk, though concentrations are low (often undetectable) and may not be of clinical relevance.[92-94] Three potential fetal risks discussed below are persistent pulmonary hypertension (PPHN), neonatal adaptation syndrome (NAS), and congenital malformations.

In 2006, the FDA issued a health advisory warning against the use of SSRIs during pregnancy due to an increased risk of PPHN. When adjusting for confounding factors, such as the severity of maternal mood or anxiety disorder, the risk of PPHN was minimal to none leading the FDA to rescind this warning in 2011.[95] Risk of teratogenesis is low and there are no specific patterns of major malformations.[19] Paroxetine is the only SSRI in category D (*vs.* C) due to reports of an increased occurrence of cardiac malformations in infants exposed to paroxetine during the first trimester.[96] Cardiac malformations induced by paroxetine appear to be dose- and trimester-specific.[97]

A 2020 retrospective cohort study examined SSRI exposure in the context of prenatal and placental outcomes. This study found decreased birth weights, increased adverse neonatal outcomes, *e.g.*, hypoglycemia, seizures, *etc.*, and reduced placental weights.[98] Like most studies on SSRI exposure during pregnancy, there was limited information about the type of SSRI, duration, or dose.[98] In most studies, increased spontaneous abortion rates were

reported in conjunction with maternal antidepressant treatment, whether SSRIs, SNRIs, or atypical antidepressants were at issue.[99] As discussed below, these studies were confounded, however, by the occurrence of maternal psychiatric disorders, which by themselves produce adverse neonatal outcomes.[6]

Infants exposed to SSRIs *in utero* during late pregnancy experience withdrawal at birth.[99] Withdrawal leads to poor neonatal adaptation syndrome (NAS).[100] Up to 30% of infants develop a spectrum of NAS symptoms, including jitteriness, motor hyperactivity, irritability, and a weak cry.[101] Some hypothesize that NAS is the result of withdrawal from maternal medication. Others hypothesize that NAS results from overstimulation of the serotonergic system, leading to toxicity from increased serotonin concentrations. Regardless of the cause of NAS, its symptoms are self-limiting, normally dissipating in as little as hours. The NAS syndrome does not seem to have prolonged effects on infant health outcomes.[101]

Risks associated with SNRI use are similar to those of SSRIs. Effects of SNRIs are dose-dependent with higher doses posing increased risks.[102] In the case of venlafaxine, no morphological or biochemical changes were seen in rat pups exposed *in utero.*[103] Yet, at higher doses (70 mg/kg), pregnant dams given venlafaxine had reduced body weights and their pups showed increased locomotor activity.[103] No adverse pregnancy outcomes were observed between groups, although rates of spontaneous abortions were higher in the venlafaxine exposed group.[99]

All SSRIs and SNRIs can cause serotonin syndrome and are contraindicated with monoamine oxidase inhibitors (MAOIs).[104] Serotonin syndrome is a potentially fatal side effect of increased serotonin concentrations, though it rarely occurs with SSRI use during pregnancy.[105,106]

Longitudinal cohort studies

Most studies examining the effects of antidepressants during pregnancy are observational and useful for descriptive information. The most common observational studies on the association between antidepressants and adverse fetal outcomes are either case-control or cohort studies.[107,108] While observational studies provide important insights about how exposures, *e.g.,* to SSRIs, affect offspring outcomes, *e.g.,* birth defects, these types of studies are associated with confounding factors that prevent clear study conclusions.[109] Broadly, confounding occurs when the presence of a variable other than the exposure of interest influences the estimated effect of exposure on a given outcome.[109] A confounding variable is one that is associated with both the exposure and the outcome.[109]

Lack of strong evidence surrounding antidepressant safety risks, particularly SSRIs, advises against discontinuing antidepressant use during pregnancy.[10] Many studies have found no adverse effects on infant neurobehavioral outcomes upon perinatal exposure to antidepressants.[110-115] Other studies report adverse effects, particularly when looking at outcomes involving motor inhibitory control and birth weight.[113,116-119] In a 2014 study by Santucci *et al.,* infant psychomotor development was significantly different in infants exposed to perinatal antidepressants at 26 and 52 weeks after birth. Motor differences were no longer observed at 78 weeks, suggesting transient self-correcting changes.[118] In a 2020 study, motor dysfunction in children exposed to antidepressants was no longer significantly different from children in the unexposed group when adjusting for the severity of maternal anxiety.[116] Risk for preterm birth is increased in individuals taking SNRIs compared to SSRIs.[120] However, preterm birth has also repeatedly been associated with maternal depression.[19]

Another 2020 study found that exposure to *in utero* antidepressants increased the odds of poor developmental health, measured by the Early Development Instrument survey, in kindergarteners.[121] Developmental vulnerability was seen in ~20% of exposed children *vs.* 16% of children born to depressed mothers who did not take SSRIs or SNRIs. Limitations of this study include a lack of control for factors such as disease severity, specific antidepressant medications, and time of gestational exposure.[121] Notably, many of the complications observed in children of depressed mothers are often reported with antidepressant exposure, pointing at the confounding nature of underlying maternal pathology.[122] For example, a population-based cohort study of children born from 2006-to 2007 in Sweden found that intellectual disability reported in infants exposed to antidepressants *in utero* was likely attributed to underlying maternal depression.[123]

Maternal depression during pregnancy is associated with many fetal complications, including preeclampsia, low birth weight, and premature birth.[38] A recent longitudinal study found that exposure to maternal depressive symptoms adversely affects children's developing executive function at 3 and 6 years of age.[124] Moreover, antenatal depression and anxiety directly impact postpartum parenting stress, which can negatively impact parent-child relationships.[8] In a 2020 systematic review, Rommel *et al.* found that underlying maternal disorder drove reported associations of neurodevelopmental, physical, and psychiatric fetal and infant outcomes.[125] The effects of maternal depression or anxiety are exacerbated in non-White families, particularly those with low-income status.[126] Thus, when examining the risk of antidepressants in offspring, conflating risks posed by maternal mood and anxiety disorders often falsely attributes risks to antidepressants.

Another factor that significantly alters epidemiological findings is control group selection.[127] Control groups are often comprised of healthy women with no psychiatric diagnoses. Some controls have included women receiving psychotherapy instead of pharmacotherapy, women in remission from depression and anxiety disorders at the time of their pregnancy, or matched siblings with no psychiatric disorders to account for genetic and environmental variability.[127] In a 2014 study, McDonagh *et al.* concluded that more specific treatment comparisons, *e.g.*, specific SSRIs, severity of depression, and factors such as timing and dose of exposure and outcomes assessments by blinded evaluators are needed to draw concrete conclusions.[107] These authors advocate for including pregnant women in randomized controlled trials.

While longitudinal studies provide meaningful information, risk due to antidepressant exposure during pregnancy would be better assessed using randomized controlled trials (RCTs). Nonetheless, RCTs pose ethical concerns when conducted during pregnancy. Neonatal safety is a major issue and often pits maternal health against fetal exposure. Only recently (1993) did the FDA lift the ban on pregnant women participating in RCTs. Lack of pregnant women in RCTs has resulted in a general lack of established precedent for medication safety during pregnancy.[128] However, RCTs may not be able to be used in the investigation of antidepressant use during pregnancy for ethical reasons.[127] Withholding treatment from patients is considered unethical.

Prospective cohort studies can address limitations by incorporating better control groups, specific inclusion and exclusion criteria, and by controlling for relevant variables that influence infant outcomes, *e.g.*, socioeconomic status, race and ethnicity, parity, severity of maternal illness, *etc.* Since participants cannot be truly randomized and treatment length,

medication type, or dose cannot be fully controlled, preclinical (animal) studies are warranted. To understand the causal effects of exposure to antidepressants, at least those common to mammals, studies using animal models are of utmost importance.

Animal models for antidepressant use during pregnancy

Animal models have several advantages for understanding the biological and behavioral effects of maternal SSRIs on offspring. Simply, they enable specific temporal manipulations without the difficulties of having to try to determine precisely how SSRIs were used in individual women. Treatment administration and dose are controlled by investigators in preclinical studies. Moreover, genetic and environmental variability are more highly controlled (though not nonexistent) in animal studies.[129] Thus, while not perfect models for encompassing human psychiatric pathologies, animal models benefit from controlled manipulations to attribute causality in ways that longitudinal human studies cannot.[130]

Stress is one of the biggest risk factors that predispose women to develop and maintain mood and anxiety disorders.[6] In animals, stress induces anxiety- and depressive-like symptoms during or after pregnancy.[131] Paradigms that produce stress in laboratory animals include chronic unpredictable stress (CUS) and social defeat stress (SDS).[131] The former utilizes ethologically relevant stressors that include predator odor, overnight light exposure (circadian rhythm disruption), and wet bedding or cage-tilt (nest insecurity) to elicit transient, unpredictable, and chronic stressful events.[132,133] Maternal separation is used to assess the effects of poor caregiving during the postnatal period on preweaning pups.[134]

Changes in behavior in mothers and offspring are assessed through an array of behavior tests to assess anxiety- and depressive-like behaviors.[135] Tests such as the elevated plus maze (EPM), open field test (OFT), and novelty suppressed feeding test (NSF) are used to quantify anxiety-related behavior.[136] The EPM, OFT, and NSF place animals in an approach-avoidance conflict, *i.e.*, a brightly lit arena when an animal has been food-deprived for 12-24 h in the NSF test.[137] The forced swim test (FST), tail suspension test (TST), and sucrose preference test (SPT) are used to assess depressive-like behaviors, though these tests are generally less robust in their translational value.[138,139]

The FST and TST place animals in highly stressful situations for short periods of time, *i.e.*, mice or rats are briefly forced to swim in a cylinder of water and or are suspended by their tails.[140] While the FST, TST, and SPT tests, and particularly the FST, have been extensively used to predict antidepressant efficacy, the interpretation of their behavioral outputs is hotly contested.[139,141] Still, when taken together, these tests provide information about behavioral changes between animal control and treatment groups even if the interpretation of the behavioral changes is open to interpretation.

Atypical antidepressants: Bupropion and ketamine

While serotonin and norepinephrine play key roles in anxiety responses and depressive mood states, these neurochemical pathways are connected with other transmitter systems. In mood disorders, reward and learning systems are impaired and many patients experience depression characterized by the common symptom of anhedonia.[142,143] Anhedonia refers to the inability to experience pleasure.[142] From neuroimaging studies, the role of dopamine and glutamate have emerged, particularly in the context of dysfunctional reward and deficits in learning.[142]

Dopamine plays a role in neuropsychiatric pathologies, as the reward system in those who are depressed or anxious is impaired.[144] Glutamate, which is the most abundant neurotransmitter, is important for synaptogenesis—the birth of new synaptic connections and neuroplasticity—the overall 'flexibility' or plasticity of neural connections.[145] Synaptogenesis and neuroplasticity are key factors in the therapeutic effects of antidepressants, as well as being a part of healthy cognitive processes.[146] Thus, new avenues of research are targeting other neurotransmitter systems, including the glutamatergic, dopaminergic , and cholinergic systems.[147-149]

The dopaminergic system and its targets

Dopamine is a monoamine neurotransmitter, similar to serotonin and norepinephrine. Dopamine transmission is implicated in mood and anxiety states. Evidence arises from studies on monoamine oxidase inhibitors (MAOIs), tricyclics (TCAs), mild stimulants, *e.g.,* Wellbutrin®, Adderall®, Ritalin®, and drugs of abuse that lead to improved mood, energy, focus, and reduced negative states, *e.g.*, methamphetamine, cocaine, which primarily target DAT.[150] Dopamine cell bodies are located in the substantia nigra (SN) and

ventral tegmental area (VTA), two neighboring brain nuclei that have different but overlapping projection profiles.[151] Dopaminergic projections target a number of brain regions, including the striatum, amygdala, prefrontal cortex, and hippocampus, and affect processes, including reinforcement learning, reward, and mood.[151]

Bupropion history and statistics on use

Bupropion use has steadily increased over the last decade. Initially synthesized as an antidepressant, bupropion was also found to aid in smoking cessation and became FDA approved as a therapy for nicotine use disorder. While bupropion initially gained FDA approval in 1985, it was removed from the market due to fears of increased seizure risks. With more careful dosing guidelines, bupropion was reintroduced and is now used by millions, primarily as an antidepressant.[152]

Pharmacokinetics

Bupropion is extensively metabolized by liver CYP2B6 to its active metabolite hydroxybupropion.[29,153] By contrast, bupropion inhibits CYP2D6 resulting in potential drug interactions. To a lesser extent, bupropion is metabolized by CYP2B6 enzymes located in the brain. The distribution of brain CYP2B6 is heterogeneous leading to brain region-specific effects. For example, CYP2B6 is highly expressed in astrocytes in layer I of the frontal cortex and at the blood-brain interface, suggesting an important role of this enzyme in brain drug action and penetration.[154]

Bupropion has a high lipid solubility and a low molecular weight, leading to almost 100% absorption when taken orally.[153] However, bioavailability is only 5-20%. Low bioavailability has little impact on effectiveness, however, because the active metabolite, hydroxybupropion, has equal antidepressant effects to that of bupropion. The half-lives of

bupropion and hydroxybupropion are ~18 and ~20 hours, respectively. Steady-state concentrations are achieved within 5-7 days of continuous dosing.[153]

Bupropion is available as immediate-release (IR), sustained-release (SR), and extended-release (XL) formulations.[153] Details of bupropion formulations are summarized in **Table I.4**.Differences in individual responses to bupropion, typically the IR formulation, influence which formulation is prescribed, as the different bupropion formulations have differing half-lives, durations of onset, and onset of action.[155]

Pharmacodynamics

Bupropion, whose tradename is Wellbutrin, acts as a reuptake inhibitor at dopamine and norepinephrine transporters.[152] Additionally, bupropion is a partial antagonist at nicotinic acetylcholine receptors, specifically alpha-3 beta-4 subunit-containing receptors.[147] Bupropion is mechanistically distinct from SSRIs and SNRIs, and importantly, does not have direct effects on the serotonin system. A 2006 study compared the efficacy of sertraline (SSRI), venlafaxine (SNRI), and bupropion in patients who were treatment-resistant to citalopram (SSRI).[156] The study concluded that there were no differences in the rates of remission between these three groups.[156] Importantly, this study suggests that intolerance or lack of efficacy of one SSRI does not imply intolerance or lack of efficacy to all SSRIs and that within-class, *e.g.*, SSRIs, and out-of-class, *e.g.*, SNRIs, bupropion, medication switches are reasonable choices.

Bupropion is commonly prescribed in addition to an SSRI, which seems to reduce sexual dysfunction associated with SSRI use and helps to improve remission rates.[157,158] Discontinuation of bupropion generally stems from stimulatory effects, although its discontinuation rate is no different from other second-generation antidepressants, *e.g.*,

SSRIs.[153] A clinical study examined the use of bupropion sustained-release for the treatment of postpartum depression and found bupropion to be well-tolerated (no patients discontinued treatment) and more than half of the patients had improved mood scores.[159] This study only had a small sample size (N=8), which limits its findings.[159] Currently, only allopregnanolone, a neurosteroid, is specifically FDA-approved (2019) for postpartum depression.[160,161]

Fetal exposure and risk associated with perinatal bupropion exposure

Bupropion and its metabolite hydrobupropion cross the blood-placenta barrier and are retained in placental tissue.[162] Exposure to bupropion did not affect placental viability. Infant exposure *via* breastmilk is minimal for bupropion, only amounting to ~2% of the maternal dose.[99]

Bupropion is prescribed during pregnancy and is a category C medication under the FDA classification system.[96] This medication is indicated for antidepressant treatment and to assist with smoking cessation.[162] A prospective cohort study compared pregnant women exposed to bupropion *vs.* other antidepressants during the first trimester of pregnancy.[163] Bupropion was not associated with increases in congenital malformations, gestational age at birth, or birth weight compared to other antidepressants. A higher rate of spontaneous abortions in the bupropion-exposed group compared to those not exposed to antidepressants was observed. However, like other studies, a limitation is separating whether increases in spontaneous abortions are a result of antidepressant use or underlying affective disorders.[163] Overall, bupropion does not seem to produce teratogenic effects, but more research is needed.[99]

Glutamatergic system and its targets

Glutamate is the most abundant neurotransmitter in the CNS. Glutamate transmission is important in synaptogenesis, functional connectivity between brain regions, and homeostasis.[149] Key glutamatergic receptors include the NMDA and AMPA receptors, which are inotropic ligand-gated receptors, and metabotropic receptors, *e.g.*, mGLUR5 receptors, which are emerging as new therapeutic targets.[164]

Glutamate is a nonessential amino acid and is required for the synthesis of the oppositional inhibitory neurotransmitter GABA. Termination of glutamate transmission requires glial reuptake, as opposed to reuptake *via* presynaptic neurons. These properties distinguish the glutamatergic system from previously discussed monoamine systems.[145,149]

Ketamine history and statistics on use

Ketamine was synthesized in 1962 by Calvin Stevens and gained FDA approval for human use as an anesthetic in 1970.[165] Soon after, ketamine appeared on the illicit drug market and became widely abused. By the mid-1980s, ketamine became linked to "dance culture" and was used in a variety of settings.[166] In 2013 and 2016, esketamine, the (S)-enantiomer of ketamine, received the status of Breakthrough Therapy Designation for treatment-resistant depression (TRD) and major depressive disorder (MDD). In 2018, many studies were published showcasing the ability of ketamine to improve mood rapidly in patients with treatment-resistant depression and to reduce rates of suicidality significantly.[167-169] These findings resulted in a 2019 decision by the FDA to approve ketamine for treatment-resistant depression.[63]

Pharmacokinetics

The effects of ketamine are almost instantaneous. With intravenous injection, the onset of action is about 30 seconds. With intramuscular or intranasal administration, onset of action is still well below 10 minutes. Ketamine is metabolized into its active and major metabolite norketamine in the liver by the enzyme CYP2B6.[170] Approximately 80% of ketamine is demethylated to norketamine, and norketamine is measured in blood plasma within minutes of intravenous ketamine administration.[170]

At subanesthetic doses, ketamine produces rapid antidepressant effects, within 4 hours of administration.[171] Doses of ketamine that produced antidepressant effects included 0.2 mg/kg intravenously, 0.25-0.5 mg/kg intramuscularly, and 50 mg intranasally.[172] While single doses of ketamine *via* intravenous infusion produce rapid antidepressant effects, fear that these effects would not last prompted research into multiple infusions over longer timeframes.[173] Now, the typical procedure is to receive six ketamine infusions over a span of several weeks.[174] In addition to the intravenous route of administration, esketamine (Spravato®) has been developed by Janssen Pharmaceuticals as an intranasal formulation.[167,168] A current clinical trial is examining optimal intranasal dose for sustained antidepressant effects (Clinical Trial# NCT04599855).

Pharmacodynamics

Ketamine is a noncompetitive antagonist at NMDA receptors, which are glutamatergic receptors.[170,175] The NMDA receptors are coincidence detectors, requiring intracellular depolarization to remove the magnesium ions that block the channel pore and prevent ligand binding.[175] Ketamine blocks the channel, thereby inhibiting receptor activation even in the presence of both events. The NMDA receptors are on GABAergic neurons; inhibition leads to

disinhibition of dopaminergic neurons and subsequent dopamine and glutamatergic release and AMPA receptor activation.[149]

The antidepressant mechanism of action of ketamine is an area of active research. Hypotheses for the mechanism of action of ketamine involve increased neuroplasticity, increased glutamatergic transmission *via* AMPA receptors, and increased brain derived neurotropic factor (BDNF).[176-178] The general hypothesis is that (1) ketamine blocks NMDA receptors on GABAergic neurons leading to (2) a glutamate surge that activates AMPA receptors, resulting in (3) increased BDNF release and mTOR signaling, which increases protein synthesis and AMPA receptor cycling.[172,176,179,180]

Fetal exposure and risks associated with perinatal drug exposure

Ketamine crosses the blood-placenta barrier, as shown by both animal and human studies.[181,182] Ketamine use is not advised during pregnancy and has been shown to produce adverse effects in offspring in animal studies.[182] In many animal species, ketamine exposure during pregnancy led to neurodegeneration in fetal brains.[183] Ketamine effects are both dose- and time-dependent, as fetal exposure and development are key mediators of overall ketamine effects.[182] While ketamine during pregnancy is not advised, a study examined the effects of using ketamine to induce anesthesia in women receiving a caesarian section.[184] The authors found that a ketamine dose of 0.5 mg/kg protected against postpartum depression and had no effects on baby health as measured by the Apgar scale.[184]

Rebranding old drugs for new uses: Psychedelics and kappa antagonists

Two other classes of therapeutics that have not yet extensively been studied in the context of their use as antidepressants are psychedelics and kappa receptor antagonists. Many studies highlight their potential therapeutic value, which when coupled with their low abuse potential, make them highly desirable medications. Psychedelics bring the focus back on the serotonin system, however instead of working as indirect agonists, *e.g.*, SSRIs, these drugs are agonists at serotonin receptors, particularly 5HT2A receptors. Kappa opioid antagonists, as their name suggests, block kappa opioid receptors (KORs), potentially reducing dysphoric symptoms.

Psychedelics

Psychedelics, often referred to as hallucinogens, are a class of drugs that induce feelings of euphoria, connectedness, and perceptual alterations, with little-to-no abuse potential.[185] In the 1960s and 70s, psychedelics were criminalized and still have Drug Enforcement Agency (DEA) Schedule I designations, making them difficult to study.[186,187] Criminalizing psychedelics occurred in response to the anti-war, anti-establishment, hippie movement of the 1970s.[188] Psychedelics include psilocin, the active compound in psilocybin, *N,N*-dimethyltryptamine (DMT), lysergic acid diethylamide (LSD), and mescaline.[189]

While the mechanisms of action of psychedelics differ from SSRIs, both drug classes are agonists at 5HT2A receptors.[187] They target other receptors as well, including most of the serotonin receptors, all dopamine receptors, and norepinephrine receptors.[189] Importantly, the psychedelic properties of these drugs are the result of biased agonism and

they display brain region specificity[186,189], as not all drugs that target 5HT2A receptors produce hallucinations.[190]

Limited clinical studies have been carried out on psychedelics due to their DEA scheduling. Yet, promising evidence from studies on psilocybin and LSD in treating psychiatric disorders such as anorexia nervosa and depression have recently emerged.[191] Long-term effects of microdosing psychedelics are not yet known, nor has their safety during pregnancy been systematically assessed.[189] Thus, pushes for revised scheduling from the DEA and increased research regarding the long-term effects of these drugs are expected to impact their use as antidepressants and anxiolytics. The hypothesized mechanisms of the therapeutic effects of psychedelics are that they enhance synaptic plasticity, which will be discussed subsequently.[189]

Kappa antagonists

The KORs are important in the stress system, and stress is one of the biggest risk factors for the development of mood and anxiety disorders.[148] *In vivo* rodent studies highlight the antidepressant effects of KOR antagonists.[192] In the 1980s, U50,488, a KOR agonist, was used in clinical trials as a potential therapeutic, but these trials were quickly terminated due to the dose-dependent dysphoria induced by U50,488. Because KOR agonists induce dysphoric effects and KOR antagonists improve mood, KORs are of particular interest in depressive disorders, particularly, in cases where dysphoria is a core symptom.

Use of KOR antagonists as antidepressants is also highlighted in the success of buprenorphine in managing treatment-resistant depression.[193,194] Buprenorphine, a drug in the treatment of opioid use disorder, is a partial agonist at mu opioid receptors and an antagonist at kappa opioid receptors.[195,196] This dual mechanism of action pointed to kappa

receptors as being important in relieving aversive states associated with withdrawal.[196] Based on the success of buprenorphine, the biopharmaceutical company Alkermes developed ALKS 5461, a 1:1 combination of buprenorphine and samidorphan (a potent mu opioid receptor antagonist), for TRD.[148] The FDA gave Fast Track Designation to ALKS 5461 in 2013. Unfortunately, ALKS 5461 failed to meet primary efficacy endpoints in 2016 and again in 2018.[197]. Thus, while KOR antagonists appear promising, further research is needed regarding specific mechanisms of action, including an extensive study of affinity and off-target effects. All medication classes discussed are summarized in **Figure I.2** and **Table I.5**.

Beyond molecular targets: Shared neurobiological mechanisms of antidepressants

Many of the classical antidepressants, including SSRIs, SNRIs, and TCAs, work at the level of blocking the reuptake of monoamines. As previously described, reuptake inhibition happens rapidly, yet the therapeutic effects of the SSRIs and SNRIs take weeks to months to develop. Thus, while identifying proximal molecular targets are important for understanding antidepressant mechanisms of action, the delayed onset implicates downstream mechanisms of action, *i.e.*, compensatory or homeostatic processes, which must be considered to determine fully how these therapeutics work.[86]

Antidepressant treatments come in many forms. Pharmacotherapeutics, psychotherapies, and stimulation techniques, *e.g.*, ECT, transcranial magnetic stimulation, vagus nerve stimulation, *etc.*, all provide therapeutic effects. Importantly, while their proximal mechanisms differ significantly, each causes structural and functional neuroadaptation, processes that underlie neuroplasticity.[89,198] Important factors that contribute to neuroplasticity include brain-derived neurotrophic factor (and possibly other trophic factors, *e.g.*, vascular endothelial growth factor, and synaptogenesis.

(1) *Brain-derived neurotrophic factor*

Brain-derived neurotrophic factor (BDNF) is a key mediator of the effects of SSRIs and even ketamine, a newly approved antidepressant medication.[199] This growth factor has many roles in the central nervous system, including the regulation of neuronal maturation and synaptic plasticity. Many studies have shown that chronic stress, a key factors for the development of neuropsychiatric disorders, reduces BDNF production in

specific brain regions, *e.g.*, hippocampus.[89,200-203] Reduced BDNF also leads to reduced serotonergic innervation of the hippocampus.[204] In contrast, the therapeutic effects of SSRIs depend on increases in hippocampal and cortical expression of BDNF.[199] Furthermore, ketamine, which rapidly produces therapeutic effects, transiently increases BDNF in the hippocampus.[172] Thus, BDNF appears to be a key mediator in both the dysfunction produced by stress and the therapeutic effects produced by antidepressants. The effects of BDNF are not limited to pharmacotherapeutics. Both ETC and other stimulation techniques also result in increases in BDNF production.[205,206]

One of the downstream effects of increased BDNF signaling is increased hippocampal neurogenesis. Postmortem and brain imaging studies have found atrophy and neuronal loss in the prefrontal cortex and hippocampus of depressed or anxious patients.[207] Studies also show that stress decreases the rates of hippocampal neurogenesis, whereas chronic SSRI use increases neurogenesis.[208,209] Taken together, these data suggest that increased BDNF signaling is needed for SSRI efficacy, wherein hippocampal neurogenesis is facilitated.[210] In fact, a current clinical trial is examining the use of BDNF gene therapy for early Alzheimer's disease and mild cognitive impairment (Clinical Trial# NCT05040217).

(2) *Synaptogenesis and synaptic strengthening*

The therapeutic effects of antidepressants may either be neurogenesis dependent or independent.[211] While many studies have shown that neurogenesis is important for SSRI efficacy, the birth of new neurons in adult mammals only occurs in the subventricular zone of the rostral migratory stream and in the subgranular zone of the hippocampal dentate gyrus. Moreover, adult neurogenesis occurs only at low rates in primates and

decreases with age. The process by which existing neurons form new synaptic connections is, known as synaptogenesis. Neuroplasticity is the subsequent strengthening (or weakening) of existing connections. The number of dendritic spines and synaptic connections is downregulated by stress, which is mediated, in part, by brain glucocorticoids.[203] In a study by Bessa *et al.,* the authors showed that the therapeutic effects of antidepressants were mediated *via* neuronal remodeling even when neurogenesis was blocked.[212] Thus, the downstream effects of BDNF on synaptogenesis and synaptic plasticity in regions beyond (and including) the hippocampus, *e.g.,* prefrontal cortex, amygdala, are needed for the therapeutic effects of antidepressant pharmacotherapies and other modalities.[213]

Other factors are involved in the neurobiology of mood and anxiety disorders and developments of therapeutics that directly affect them may be promising. Two global changes that occur in mood and anxiety disorders are increased neuroinflammation and dysfunctional HPA axis signaling.[87,214] Increased neuroinflammation and dysfunctional HPA axis signaling could be used as biomarkers, which would allow for tangible monitoring of mood and anxiety disorders, though more research is needed.[215] During pregnancy, levels of cortisol, BDNF, and neuroinflammatory markers all change, leading to complexities in terms of using these biomarkers during pregnancy and postpartum.[216-218]

Conclusions

Pregnancy induces a number of physiological, metabolic, hormonal, and psychological changes. Pregnancy is also a stressful experience for many individuals, with stress being the biggest known risk factor for developing a mood or anxiety disorder. Treatment options for women who are experiencing a neuropsychiatric disorder are of utmost importance, especially as the prevalence and incidence of neuropsychiatric disorders continue to climb. Both SSRIs and SNRIs seem to have limited adverse effects on overall fetal health, yet as discussed above, SSRIs and SNRIs are not effective for everyone and have a delayed therapeutic onset. With recent advances in psychiatry come novel antidepressants, namely ketamine, which while not recommended for use during pregnancy, may inspire more efficacious and safe medications in the future.

Depression and anxiety disorders cause global changes in behavior, and behavioral changes are largely attributed to dysfunction of the nervous system. Pharmacotherapy for mood and anxiety disorders is limited by an incomplete understanding of the underlying neural circuits and neurochemistry. Monoamine systems, as well as the glutamatergic, GABAergic, and opioid systems, all work together to produce behavior. Thus, as we learn more about the anatomical and functional interplay between these neurotransmitter systems, therapeutic targets may be identified to account for individual variability and improve personalized medicine.

Figures

Figure I.1

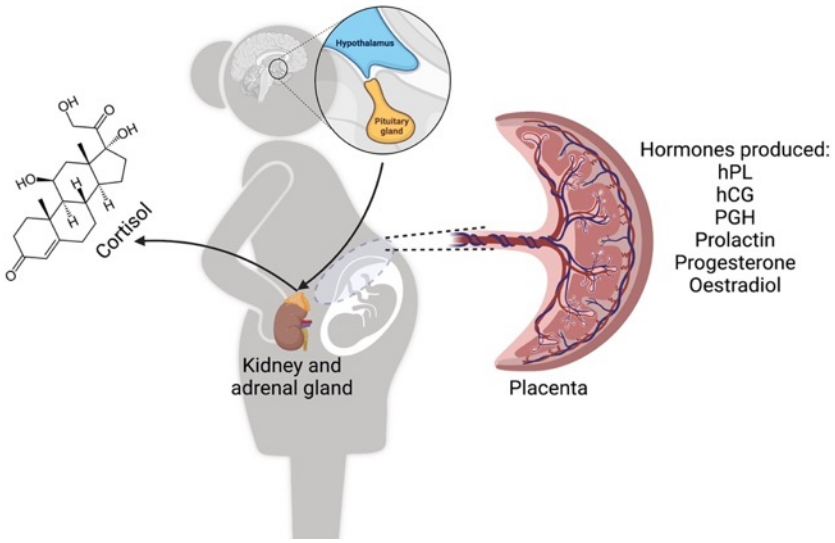

Figure I.1: The HPA axis and placental action in pregnancy. Figure was created with BioRender.

Figure I.2

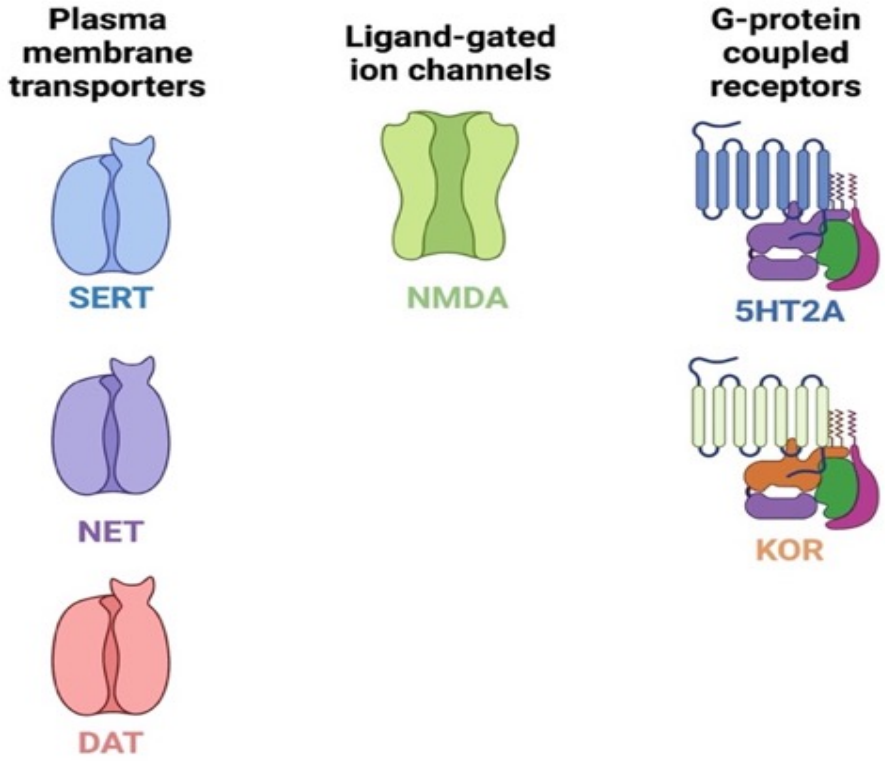

Figure I.2: Endogenous targets for pharmacotherapeutics. The second-generation antidepressants, SSRIs, SNRIs, and bupropion target SERT, SERT and NET, and NET and DAT, respectively. The atypical antidepressants ketamine targets NMDA receptors. Promising therapeutics, *i.e.*, psychedelics and KOR antagonists, target 5HT2A and KORs, respectively. All medications described have other molecular targets that may contribute to their efficacy. Figure was created with BioRender.

Table II.1

Pharmacokinetic Property	Change during pregnancy	Consequence
Absorption	• Increased gastric pH • Decreased gastrointestinal motility • Altered CYP450 activity	• Reduced systemic absorption • Altered bioavailability
Distribution	• Increased plasma volume and cardiac output • Reduced drug-binding plasma proteins, such as albumin • Increased adipose tissue	• Reduced drug concentrations • Increased volume of distribution for hydrophilic and lipophilic drugs
Metabolism	• Phase I and II enzymatic changes	• Altered drug metabolism, esp. of drugs that use CYP450 enzymes
Elimination	• Increased renal clearance	• Reduced steady-state concentration • Increased elimination

Table I.1: Pharmacokinetic changes during pregnancy.

Table II.2

Medication	Bioavailability (F)	Volume of distribution (V_d) (L/kg)	Half-life ($t_{1/2}$)	Enzymes in metabolism	Time to reach steady-state
Fluoxetine	<90%	20-45	1-4 days	CYP2D6	>3 weeks
Fluvoxamine	~50%	~5	8-28 hrs	CYP2D6	10 days
Citalopram	~80%	14-16	~36 hrs	CYP2C19	6-10 days
Escitalopram	~80%		27-32 hrs	CYP3A4	7-10 days
Sertraline	~44%	20	22-37 hrs	CYP3A4	5-7 days
Paroxetine	<50%	3-12	16-19 hrs	CYP2D6	7-14 days
Vilazodone	~72%	8	25 hrs	CYP3A4	~5 days
Vortioxetine	~75%	37	66 hrs	CYP2D6	2 weeks

Table I.2: Pharmacokinetic properties of SSRIs.

- Bioavailability: the amount of unmetabolized drug that enters systemic circulation compared to that of intravenous administration.
- Volume of distribution: propensity of the drug either to remain in the plasma or redistribute to other organs.
- Half-life: the amount of time required for the drug concentration to be reduced by half.
- Steady-state: half-life of the drug multiplied by 4.5

Table II.3

Medication	Half-life ($t_{1/2}$)	Enzymes in metabolism	Preferential affinity for NET or SERT
Venlafaxine	11-14 hrs	CYP2D6	30x higher affinity for SERT vs. NET
Desvenlafaxine	11 hrs	CYP3A4	10x higher affinity for SERT vs. NET
Levomilnacipran	12 hrs	CYP3A4	3x higher affinity for NET vs. SERT
Milnacipran	8-10 hrs	Phase II conjugation	No preference for NET vs. SERT
Duloxetine	12 hrs	CYP2D6	10x higher affinity for SERT vs. NET

Table I.3: Pharmacokinetic properties of SNRIs.

Table II.4

Bupropion formulation	Dose (mg)	Frequency of intake	Maximum recommended dose
Immediate release	75 and 100	2x daily	450 mg
Sustained release	100, 150, and 200	1-2x daily	400 mg
Extended release	150 and 300	1x daily	450 mg

Table I.4: Bupropion formulations

Table II.5

Medication	Mechanism of action	FDA pregnancy category
SSRIs	Block serotonin reuptake *via* SERT inhibition; indirect agonists at serotonin receptors	C for all except D for paroxetine
SNRIs	Block serotonin and norepinephrine reuptake *via* SERT and NET inhibition; indirect agonists at serotonin and norepinephrine receptors	C for all
Bupropion	Indirect agonist at NET and DAT; antagonist at nicotinic acetylcholine receptors	C
Ketamine	Competitive antagonist at NMDA receptors	NA
Hallucinogens	Agonists at 5HT2A receptors	NA
Kappa Antagonists	Antagonists at kappa opioid receptors	NA

Table I.5: Pharmacodynamic summary of drug classes.

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CHAPTER II

Prenatal citalopram exposure promotes resilience in male offspring exposed to maternal stress

Abstract

Mood and anxiety disorders are highly prevalent during pregnancy and can lead to adverse maternal and offspring outcomes. Selective serotonin reuptake inhibitors are the most commonly used medications to treat mood and anxiety disorders. Both human and animal studies suggest that serotonin signaling plays an important role in the vulnerability to and manifestation of stress-associated affective disorders. Moreover, the serotonin system is an early orchestrator of brain development. In this study, timed-pregnant mice underwent chronic, unpredictable stress during the latter half of their pregnancies using ethologically relevant and/or mild stressors. Some of the mice received the serotonin-selective reuptake inhibitor antidepressant citalopram (Celexa) concomitantly in their drinking water. After birth, tissue serotonin levels at three developmentally relevant timepoints for serotonin system maturation were assayed in the offspring. A subset of the adult offspring was tested using behavioral assays to assess longterm behavior effects of *in utero* exposures. Finally, male adult offspring underwent microdialysis in the ventral hippocampus to investigate longterm neurochemical effects. Offspring of stressed mothers had higher serotonin tissue levels and protein concentrations in the forebrain at postnatal day seven compared to control animals. Male adult offspring displayed greater anxiety-like behavior and stress responsiveness than sex-matched control animals. These effects were rescued in male mice whose mothers were exposed to concomitant citalopram. No changes were observed in basal or stimulated serotonin concentrations during adulthood. Yet, male adults exposed to *in utero* stress had increased kappa opioid receptor agonist-induced serotonin release in the presence of serotonin transporter blockade, which was attenuated by *in utero* exposure to

citalopram. These findings suggest long-term benefits of treating maternal depression or anxiety during pregnancy.

Introduction

According to the Center for Disease Control (CDC), one in seven women of reproductive age are prescribed medication for anxiety or mood disorders. Estimates of perinatal depression or anxiety range from 5-25%.[1] Approximately 3% of pregnant women with mood or anxiety disorders will take medication for the treatment of their mood or anxiety disorder.[2] The safety of antidepressant use during pregnancy remains contested due to incomplete knowledge of how exposure can affect fetal development and long-term effects. Yet, maternal mood and anxiety disorders have known adverse health outcomes on fetal development and long-term effects.[3-5] Epidemiological studies have shown that untreated mood or anxiety disorders during pregnancy increase offspring risk for similar disorders.[6,7] In fact, this risk doubles that of offspring who were not exposed to maternal depressive or anxiety disorders during pregnancy.[8]

The most commonly prescribed medications to treat depression and anxiety are the selective serotonin reuptake inhibitors (SSRIs).[9] Rodent models point to the serotonin transporter (SERT), which is the primary site for SSRI action, as a key modulator of serotonin transmission and anxiety-related behavior.[10-13] Both clinical trials[14] and studies of animal models suggest that serotonin signaling plays an important role in the manifestation of and vulnerability to mood and anxiety disorders.[15-18]

While SSRIs improve maternal mood and relieve symptoms of mood and anxiety disorders, the long-term safety of these medications have not been elucidated.[19] The SSRIs are placed in category C or D of pregnancy medications, as animal models have shown potential risks of SSRI use, however possible benefits may outweigh the risks. Some epidemiological studies have shown associations between prenatal SSRI exposure and

physiological and cognitive dysfunction.[20-22] However, due to lack of randomized control trials, risk-benefit profiles of the SSRIs have not been investigated in the absence of confounding variables, *e.g.*, maternal disorder severity.[23-25] The benefit of minimizing side effects of untreated maternal mood and anxiety disorders may outweigh potential side effects of SSRIs.[11,26] Choosing the correct treatment option is crucial for the health of future offspring.

Behavioral changes in adulthood due to maternal stress exposure have also been recorded. For example, mice exposed to prenatal stress exhibited increased anxiety-like responses and increased serotonin neurons in the DRN than control animals.[27] *In utero* stress exposure with concomitant SSRI treatment has been less studied. One study showed SSRI treatment during the early, but not late, postnatal period attenuated changes induced by prenatal stress in HPA axis response, hippocampal serotonin levels, and synaptic density.[28] Another study showed that fluoxetine treatment of dams on postnatal day one attenuated prenatal stress effects in their offspring.[29]

The aforementioned studies examined the effects of direct and indirect, *e.g., in utero*, postnatal SSRI exposure in prenatally stressed offspring. Also important is to examine SSRI exposure during maternal stress paradigms throughout during phases of the gestation period and how it effects postnatal, as well as adult, offspring neurochemistry and behavior. Velasquez *et al.* previously showed administration of citalopram during an *in utero* maternal stress paradigm reversed the effects of stress on serotonin levels in fetuses at embryonic day 17.[30] We hypothesized that effects of citalopram in mitigating adverse stress effects would carry into postnatal development and have prolonged protective effects throughout development and into adulthood.

Along with the serotonin system, the kappa opioid receptor (KOR) system has gained increased interest within stress, anxiety, and depression research.[31] While a preponderance of evidence has shown that KOR activation decreases dopamine, the effects of KOR activation on serotonin neurotransmission are varied. Tao *et al.* found decreases in serotonin efflux after kappa agonist infusion into rat DRN.[32] However, two other studies saw no changes in serotonin levels.[33] Land et. al utilized a phosphor-selective antibody directed against the activated form of KOR to determine sites of dynorphin release and subsequent KOR activation.[34] Stress also induced dynorphin release and activated KORs in the dorsal raphe, nucleus accumbens, hippocampus, and ventral tegmental area.

Repeated forced swim stress and KOR agonist-induced stress increase ventral striatum SERT expression, which is attenuated by microinjection of the antagonist, norBNI, into the DRN but not nucleus accumbens.[35] This suggests that KOR activation within the dorsal raphe mediates SERT expression during stressful events. Additionally, constitutive knockout of one or both SERT alleles rescues KOR-agonist induced conditioned place aversion phenotype. Our work further investigates the role of the kappa system in response to *in utero* stress and how it may modulates serotonin transmission.

Further research into long term effects of offspring born to stressed dams and stress dams given citalopram need to be conducted to determine if the embryonic changes persist into adulthood. Our study aimed to examine effects of maternal stress and SSRI administration in offspring during early postnatal period as well as into adulthood. We hypothesized that increased serotonin levels in early postnatal forebrain tissue of offspring born to stressed dams would be attenuated in concomitantly treated animals with citalopram. Moreover, we hypothesized that adult offspring born to stressed dams would

display increased anxiety-related behavior in adulthood, which would be rescued by maternal SSRI administration. Finally, we hypothesized adult offspring born to stress mothers would have increased responsiveness to the aversive effects of KOR activation.

Methods

Animals

A total of *N*=75 timed-pregnant CD-1 dams were purchased from Charles River. These dams were 12-24 weeks old at time of pregnancy. Dams were either single-housed (stressed groups) or housed in groups of 2-4 same-sex dams per cage until embryonic day 17 (E17). All dams were single-housed post E17 and until their offspring were weaned at postnatal day 21 (P21). Food and water were available ad libitum throughout the experiment, with the exception of E16, in which dams were food-deprived for less than 24 hours for the novelty suppressed feeding (NSF) behavior test. After weaning, offspring were housed 2-5 mice per cage with sex-matched siblings. The light–dark cycle (12/12 h) was set to lights on at 0730 h (ZT0). The same light schedule was strictly maintained in the room where behavior tests were carried out. The Association for Assessment and Accreditation of Laboratory Animal Care International has fully accredited UCLA. All animal care and use met the requirements of the NIH Guide for the Care and Use of Laboratory Animals, revised 2011. The UCLA Chancellor's Animal Research Committee (Institutional Animal Care and Use Committee) preapproved all procedures.

Animals (*N*=25) undergoing microdialysis were implanted with guide cannulas at 3-6 months of age. Surgeries were carried out under aseptic conditions with isoflurane anesthesia on a KOPF Model 1900 Stereotaxic Alignment System (KOPF, Tujunga, CA). A CMA/7 guide cannula (Harvard Apparatus # CMAP000138) for a microdialysis probe was aimed at the vHPC (AP-3.6 mm, ML±3.2 mm, DV-1.5 mm from Bregma). Each guide cannula was secured to the skull with Bosworth Trim II (Henry Schein #2509679). Animals recovered from the surgery for at least three days before microdialysis. Following each

surgery, mice were given twice daily carprofen injections (5 mg/kg, 1 mg/mL, subcutaneously) for the first three days.

Chronic unpredictable stress paradigm during pregnancy

Dams underwent chronic unpredictable stress during E8 through E14. On E8, mice were restrained, ears clipped for identification, and given an i.p. saline injection. Mice in the stressed groups were single housed through the duration of the stress paradigm. During E9, dams were exposed to chemical found in fox urine, which was placed on a nestlet on their wire lid of cage for 15-minutes. On E10, dams were exposed to constant light overnight from ZT12-24. On E11, dams were put in a restraint tube for 30 min. On E12, dams were exposed to constant white noise overnight from ZT12-24. On E13, each cage was tilted 45-degrees for the duration of the light cycle, from ZT0-12. On E14, the bedding of each cage was saturated with water and left overnight from ZT12-24. On days E15-17, all animals, irrespective of group, underwent the following behavior tests, which served as additional stressors: open field test (OFT), forced swim test (FST), and novelty suppressed feeding (NSF).

Postnatal dissections and tissue collection

Brain removal for neurochemical analysis was done on P7, P14, and P21. At each developmental timepoint, 1-3 pups were selected from a litter at random. The pups were weighed, their sex was identified, if possible, and they were transported into the dissection room. During the dissection procedure, pups were sacrificed *via* decapitation. Their brains were rapidly removed and placed in deionized water for no longer than one minute. Afterwards, brains were sectioned, and the following brain regions were collected: at P7, forebrain, midbrain, and hindbrain; at P14 and P21, frontal cortex, hypothalamus, left

hippocampus, brainstem, left striatum, and the right hemisphere. Samples were placed in Eppendorf tubes and immediately placed on dry ice before storage in a -80° C freezer.

High-Performance Liquid Chromatography (HPLC).

Tissue Samples

All analysis was performed using an Amuza HTEC-500 integrated HPLC system (Amuza Corporation, San Diego, CA) containing an Amuza Insight autosampler for measuring standard samples and brain homogenate tissue samples. Chromatographic separation was achieved using an Agilent Poroshell 120 (SB-C18, 3.0x100mm, 2.7μm) column and a neurotransmitter mobile phase with 0.1M Monochloroacetic Acid (Sigma #402923), 0.2-0.4g/L Octanesulfonic Acid (Acros #41636), pH 2.6, 50 mg/L EDTA·Na_2 (Sigma #03682), 0.01% Triethylamine (EMD TX1200) and 5-10% Acetonitrile (EMD AX0145) in water purified via a Milli-Q Synthesis A10 system (EMD Millipore Corporation, Billerica, MA). Amino acid analysis uses a Phenomenex Kinetex LC Column (C18, 100x3mm, 2.6 um, #00D-4462-Y0) column and a differing mobile phase which consists of 0.05-0.2M sodium phosphate monobasic (Fluka #17844), 50 mg/L EDTA·Na_2 (Sigma #03682), pH 7.3-7.5, and 20-25% MeOH (EMD #MMX04751) in water purified via a Milli-Q Synthesis A10 system (EMD Millipore Corporation, Billerica, MA).

The column temperature was maintained at 21-35 °C. The volumetric flow rate was 300−525 μL/min. Electrochemical detection was performed using an Amuza pure graphite (PG) working electrode with an applied potential of +600 mV vs an Ag/AgCl reference electrode. 5-HT (Sigma #H9523), 5-HIAA (Sigma # H8876), NE (Sigma # A9512), DA (Sigma # H8502), DOPAC (Sigma #850217), HVA (Sigma # H1252), Phenylalanine (Sigma #78019), Tryptophan (Sigma # 51145), Tyrosine (Sigma # 93829), Valine (Sigma # 94619),

Isoleucine (Sigma # 12752), and Leucine (Sigma # L8000) standards were prepared in ice-cold sonication solution (0.1M Glacial Acetic Acid, and 1 mg/mL EDTA-2NA). Standard curves, which were verified with each group of samples, encompassed physiological concentration ranges (30−500 nM). The limit of detection was ≤160 amol, and the practical limit of quantification was ≤320 amol. All experiments were collected at 12-18 min intervals for neurotransmitters and 20-25 min for amino acids.

Microdialysis

Male offspring (*N*=25) underwent microdialysis at 3-6 months of age. On the night before the first testing day (ZT10-12), each mouse was transferred to the testing room in its home cage where a CMA/7 microdialysis probe (2 mm length, 6 kDa cutoff, Metal Free, CMA8010772) was inserted into the guide cannula. Subjects were placed in a new smaller cage that had their own bedding from the home cage, and artificial cerebrospinal fluid (aCSF) (147 mM NaCl (Fluka #73575), 3.5 mM KCl (Fluka #05257), 1.0 mM $CaCl_2$ (Aldrich #499609), 1.0 mM NaH_2PO_4, 2.5 mM $NaHCO_3$ (Fluka #88208), 1.2 mM $MgCl_2$ (Aldrich #449172), pH 7.3 ± 0.03 was continuously perfused through the probe *via* a liquid swivel (375/D/22QM, Instech Laboratories Inc., Plymouth Meeting, PA) at 0.3 µL/min flow rate for 14-16 h. Subjects were tethered to the liquid swivel but otherwise could move freely in their home cages during the entirety of testing.

Microdialysis experiments were carried out between ZT1-8. Basal samples were collected from ZT1-2, followed by three high K^+ stimulations each lasting 5 minutes, separated by an hour of aCSF infusion from ZT2-5. After the last high K^+ stimulation, animals were either (1) infused with 10 µM citalopram in the vHPC for 2 hours, (2) systemically injected with 10 mg/kg of U50,588H (Sigma-Aldrich #D8040) or U69,593 (Sigma-Aldrich

#U103), then infused with 10 μM citalopram in the vHPC for 2 hours, or (3) infused with 10 μM citalopram in the vHPC for 2 hours, then systemically with an injection of 10 mg/kg U50,588 or U69,593. Doses for citalopram and U50,588 and U69,593 were determined empirically through previous experiments. For citalopram, an initial dose of 20 μM was used for *N*=6 animals however led to SERT saturation. The dose was brought down to 10 μM for subsequent animals. The U50,588 dose was determined in a conditioned place aversion assay, where 10 mg/kg induced the greatest aversion to the drug-paired side in male mice. Due to U50, 588 manufacturer unavailability, U69, 593 was used in place at 10 mg/kg in the last *N*=7 mice.

All analysis was performed using an Amuza HTEC-500 integrated HPLC system (Amuza Corporation, San Diego, CA) containing an Amuza Insight autosampler for measuring standard samples and an Amuza EAS-20s online autoinjector for quantifying microdialysis dialysates. Chromatographic separation was achieved using an Amuza PP-ODS III column (4.6 mm ID x 30 mm length, 2 μm particle diameter) and a phosphate buffered mobile phase with 14.886 g/L NaH_2PO_4 (Fluka #17844), 1.02 g/L Na_2HPO_4 (Thermo Scientific AC448160050), 2% MeOH (EMD #MX0475), 50 mg/L EDTA·Na_2 (Sigma #03682), and 600 mg/L sodium decanesulfonate (TCI #I0348) in Optima LC/MS grade water (Fisher Scientific CAS# 7732-18-5).

The column temperature was maintained at 25 °C. The volumetric flow rate was 500 μL/min. Electrochemical detection was performed using an Amuza WE-3G graphite working electrode with an applied potential of +450 mV vs an Ag/AgCl reference electrode. Dopamine (Sigma #H8502) and serotonin (Sigma #H9523) standards were prepared in a 1:1 mixture of mobile phase and regular aCSF . Standard curves, which were verified weekly,

encompassed physiological concentration ranges (0–10 nM). The limit of detection was ≤160 amol, and the practical limit of quantification was ≤320 amol. All online dialysate sample experiments were collected at 5 min intervals at a dialysate flow rate of 2.5 µL/min and injected immediately onto the HPLC system for analysis by using the online EAS-20s autoinjector. ES280 PowerChrom Chromatography Data System (CDS) Software (eDAQ, Australia) was used to collect, display, and analyze all chromatographic signals.

Behavior tests

Elevated Plus Maze

The elevated plus maze (EPM) was used to assess avoidance of anxiogenic environments.[36,37] A single maze was used throughout the study having two opposing open arms (30 cm length x 5 cm width) with a 0.5-cm lip around the edges of the open arms to prevent animals from falling. The maze also had two opposing closed arms (30 cm length x 5 cm width x 15 cm height) and a center platform (5 cm x 5 cm) and was raised 38.5 cm from the floor. The walls of the closed arms were constructed of clear Plexiglas to ensure even light levels across all arms. The floor of the maze was constructed of a continuous piece of black opaque Plexiglas. Mice were placed on the center platform facing a closed arm and allowed to explore the maze freely for 5 min. The maze was cleaned with Accel Solution and dried between same-sex mice. When switching between male and female mice, the apparatus was cleaned with 70% ethanol solution followed by Accel Solution.

Behavior was videotaped and visually scored using the ANYmaze behavioral tracking system (Stoelting Co., Wood Dale, IL). Parameters quantified included latency to first open arm entry, open arm distance, %open-arm time, and %open arm distance. Time in the open arms was analyzed as a percentage of total arm time excluding time spent in the center zone

of the maze. Likewise, distance traveled in the open arms was analyzed as a percentage of total arm distance excluding the distance traveled in the center zone of the maze.

Open Field Test

Locomotor activity in an open field test (OFT) was assessed using a digital video tracking system ANYmaze. Open field arenas were 50 cm x 50 cm x 40 cm height. The center area was defined by a 22 cm x 22 cm central square. Animals were considered to be in the center after the midpoint of the body entered the center area. Mice were placed in a corner of the open field and allowed to freely explore for 30 min. Total distance traveled (m) and the ratio of center distance to total distance was measured. Additionally, time spent exploring the center region was analyzed. Testing environments were thoroughly cleaned with Accel Solution and 70% ethanol and allowed to dry between mice.

Forced Swim Test

The forced swim test (FST) was carried out as previously described with minor modifications.[30] Briefly, each mouse was placed in a clear glass cylinder containing autoclaved room temperature water (22±2°C) at a depth of 15 cm. Behavior was videotaped during 6-min swim sessions. Afterwards, mice were transferred to a warm environment while their fur dried before being returned to their home cages. Cylinders were cleaned with Accel Solution and refilled with fresh water between mice. Data were collected and analyzed using ANY-maze behavioral tracking software. Time spent immobile during the last 4 min of each 6 min trial was summed.

Novelty Suppressed Feeding

The novelty suppressed feeding (NSF) test measures latency to eat in a novel environment and is performed by adapting previously used procedures.[38] Mice were pre-

weighed, singly housed, then fasted and habituated in the behavioral testing room for up to 24 h prior to NSF testing. After fasting for up to 24 h, mice were weighed before testing began. Any mouse that lost more than 13% body weight within the 24 h, were excluded from testing and automatically given free access to food and water. Individually, food pellets were placed in the home cage. The 13% cut-off was determined empirically through previous cohorts for the CD-1 mouse strain.

We timed the latency for mice bite the pellet in the home cage that they were fasted in. Once the mice took a bite of the pellet or 5 min passed, whichever occurs first, the mice were then transferred to the novel arena. Mice were placed at the edge of a novel arena with a food pellet on a brightly lit white platform. The rest of the arena was dark and covered. We timed the latency to bite the pellet. After the mice bit the pellet or 10 min elapsed, the mice were transferred back to a clean cage with free access to food and water. In a subset of animals, mice were transferred back into their fasting cage with one pellet of food. Pellets were weighed prior to and 10 min after mice were given full access to the food pellet. Mice were then transferred back to a clean cage with free access to food and water.

Statistical analysis

Statistical analyses were carried out using Prism, v.9.3.0 (GraphPad Inc., La Jolla, CA). Data are expressed as group means ± SEMs, with $P<0.05$ considered to be statistically significant.

Results

Maternal physiological and behavioral outcomes

All statistical analysis can be found in **Table II.S2**. A general experimental overview is shown in **Figure II.1**. Timed-pregnant dams arrived on embryonic day 7 (E7), where they were randomly assigned to one of four groups. Dams in the stressed groups (CUS, CUS+CIT) underwent a chronic unpredictable stress paradigm from E8 through E14 (**Fig. II.1A, Table II.S1**). All dams then underwent three behavior tests, open field test, forced swim test, and novelty suppressed feeding, from E14-E18. Stress increased speed and distance travelled in dams in the open field test (**Fig. II.S3**).

Although no group differences were seen in the NSF, overall pregnancy decreased the latency to feed in the home cage and novelty arena compared to sex matched non-pregnant adult offspring (**Fig. II.S4A, B, D**). Dams in the CUS group took significantly less time to take a bite in the novelty arena compared to sex matched non-pregnant adult offspring also in the CUS group (**Fig. II.S4C**). No physiological differences were observed in dams belonging to different treatment groups nor in litter size (**Fig. II.S1**). Significant correlations between liter size and pup weights at postnatal days 7 and 14 were observed (**Fig. II.S2**) and were similar across treatments.

In a subset of cohorts, brains of pups were removed and dissected at three different early postnatal timepoints. Pups were left to age to three months old where they underwent behavior tests and microdialysis. The adult offspring that were used for microdialysis experiments were separated into three paradigms, in which the order of pharmacological agents administered were varied to test differing hypotheses (**Fig. II.1B**).

Postnatal tissue analysis

Brains from postnatal day 7, 14, and 21 (P7, P14, and P21, respectively) pups from each group were removed and dissected into individual brain regions. At P7, brains were sectioned into three regions, forebrain, midbrain and hindbrain. At P14 and P21, specific brain regions were differentiated and allowed for us to dissect individual regions such as frontal cortex, hippocampus, and striatum. High performance liquid chromatography with electrochemical detection (HPLC-ECD) identified and quantified neurotransmitter and amino acid levels in brain sections.

Normalized neurotransmitter levels for forebrain, midbrain, and hindbrain showed no significant difference in serotonin or dopamine levels at P7 (**Fig. II.2A-C**). In midbrain, a significant decrease of HIAA in pups exposed to prenatal stress was observed. In hindbrain, increased HVA was observed in pups exposed to prenatal stress. However, significant differences were seen in protein concentrations between control and CUS groups within the forebrain at P7 (**Fig. II.2D**). No differences in protein concentrations were observed in midbrain or hindbrain, indicating differences due to cut size was unlikely (**Fig. II.2E, F**). Analysis of raw nanomolar concentration show pups exposed to prenatal stress had increased serotonin, HIAA, and norepinephrine in the forebrain, but not midbrain nor hindbrain (**Fig. II.2G-I**). No differences were observed in P14 or P21 brains.

Adult offspring behavior

At three months of age, offspring were assessed for anxiety- and depressive-like phenotypes using four widely used behavior tests; EPM, OFT, FST, and NSF. Behavior tests were completed in the order from least to most stressful. No group differences were observed in the EPM (**Fig. II.S5**). *In utero* citalopram treatment altered OFT behavior in male

adult offspring (**Fig. II.3**). Distance traveled in the arena was significantly reduced in the CIT group compared to all other groups for male offspring, but not in females (**Fig. II.3A-C**). For the entire duration of testing, exposure to *in utero* CIT significantly decreased center entries, center distance compared to entire arena, and center time in males compared to controls (**Fig. II.3D-F**). The first five minutes was analyzed alone since this is when the arena is most novel and anxiogenic. Within the first 5 min of testing, *in utero* CIT exposure irrespective of stress decreased center entries and time spent in the center zone in males, but not females (**Fig. II.3G,I**).

In the FST, prenatal stress significantly decreased immobility time in males, but not females (**Fig. II.4B**), compared to controls. Concomitant treatment of CIT rescued the stress induced decreases in immobility in males. In females, immobility time was increased in the CUS+CIT group compared to control and CUS groups. Irrespective of sex or group, immobility time increased across the test duration. Group differences were seen during the last four minutes of the test (**Fig. II.4C, D**).

The last behavior test performed was the NSF. Adult offspring were fasted for 24 h before undergoing the NSF test. Mice that lost more than 13% body weight were excluded from the study. Interestingly, male offspring born to stressed mothers had the highest percentage of exclusion, with almost 50% of the group excluded (**Fig. II.5A**). No significant pre-fast weight differences were seen between groups of each sex (**Fig. II.5B**). Prenatal stress significantly increased the ratio between latency to feed in novelty arena compared to the home cage in male adult offspring but not females (**Fig. II.5C**). Treatment with CIT attenuated the effects of stress on the ratio between latency to feed in novelty arena compared to home cage. To determine if these effects were due to decreased appetite, a

subset of mice were given a pre-weighed pellet of food and allowed to eat for 10 min post NSF testing in their home cage. At the end of the 10 min period, food pellets were weighed again to determine amount eaten. No differences were observed between groups for amount of food eaten after 10 min (**Fig. II.5D**).

Adult offspring neurochemistry

To assess the effects of prenatal stress and SSRI treatment on adult offspring neurochemistry, male adult offspring underwent microdialysis. Only males were used for microdialysis, as behavior data pointed to males being more susceptible to the effects of prenatal stress than females. The ventral hippocampus (vHPC) was targeted due to its involvement in anxiety responses and dense innervation from serotonin neurons (**Fig. II.S7A**). Peaks were identified and quantified based on standard curves (**Fig. II.S7B**) and peaks were confirmed *in vivo* with infusion of serotonin and dopamine (**Fig. II.S7C, D**). No differences in basal or stimulated extracellular serotonin levels were observed (**Fig. II.6A-C**). No differences in basal extracellular dopamine levels were observed (**Fig. II.6D, E**), however the CUS group had higher stimulated dopamine levels compared to control and CUS+CIT groups (**Fig. II.6F**).

To probe potential mechanisms of stress-induced behavioral changes, 8-OH-DPAT injections were given to male and female adult offspring to determine differences in 5HT1A receptor activity. No differences were seen and thus not probed further in microdialysis experiments (**Fig. II.S6**). Citalopram was perfused into the vHPC to determine differences in SERT activity. Moreover, KOR agonists were injected to assess a link between the serotonin system and kappa system. After basal and stimulated levels of both neurotransmitter, 10 uM citalopram was infused for 2 h then a KOR-agonist was injected (U50,488 or U69,593, 10

mg/kg, i.p.) and dialysate samples were collected for additional 1.5 h while CIT was perfused (**Fig. II.7 A, D**).

A significant effect of CIT infusion and KOR agonist was observed, but not for *in utero* treatment on dopamine concentrations (**Fig. II.7E, F**). As expected, KOR activation decreased dopamine levels, however unexpectedly, CIT perfusion also decreased dopamine levels. A significant effect of treatment and drug on serotonin concentrations were observed (**Fig. II.7B, C**). To ensure that CIT was not causing increased serotonin release over longer durations, we infused CIT for more than 3 hr (**Fig. II.S9**). While we did observe a significant correlation between serotonin concentration over the longer perfusion period, the increase was less than 1 nM, confirming that larger increases in serotonin levels were the result of KOR agonist injection. Stress potentiated the increase in serotonin levels induced by KOR activation, which is rescued in the CUS+CIT group (**Fig. II.7B**). To test if these results were dependent on SERT blockade, the KOR agonist was injected prior to CIT perfusion. Decreases in dopamine levels were seen, however, no differences in serotonin levels were observed post KOR injection without SERT blockade (**Fig. II.S8**).

Discussion

Our study points to the long-term benefits of treating dams experiencing stressful pregnancies with SSRIs as a way to mitigate adverse outcomes in offspring health. We found that stress induced developmental changes in offspring protein, neurotransmitter, and amino acid tissue levels in early postnatal development. While these effects were no longer observed by postnatal day 14 or 21, their impacts on behavior were seen in adulthood. We found that male, but not female, animals born to stressed mothers displayed increased anxiety and depressive-like behavior. Moreover, we found that stressed males were more susceptible to stress challenges, *i.e.*, overnight fasting, than females. These adverse behavioral phenotypes were rescued in animals whose mothers were concomitantly treated with citalopram *in utero*.

Lastly, we assayed neurochemical changes in adult male mice. While we did not see changes in basal or stimulated serotonin release, we did find pharmacological-induced changes in neurochemistry. Male mice born to stressed mothers showed higher serotonin concentrations in the vHPC in response to citalopram administration with and without the presence of kappa opioid receptor activation. These effects were rescued in the male mice whose mothers had been treated with citalopram *in utero*.

Our results add to the existing expansive literature on the adverse effects of maternal stress during pregnancy. Importantly, our results also highlight the long-term benefits of pharmacological treatments of stress experienced by pregnant mothers. The decision to take medication during pregnancy is influenced by many factors, which include severity of maternal disorder, type of medication, and other complications. However, contrary to social perception that all medications during pregnancy are harmful to the developing fetus, our

results show that SSRIs do not cause changes litter size, fetal birth weight, or viability, and are efficacious in mitigating the effects of maternal stress in a mouse model.

While we probed the vHPC, many other brain regions are implicated in the adverse effects of stress, particularly cortical regions such as the prefrontal cortex (PFC). Moreover, KOR receptor expression and localization on cell populations, *i.e.*, dopaminergic neurons, and involvement of the kappa system in stress responses point to an expansive system with much to be uncovered. Future experiments should focus on specifically probing neurochemical pathways, *i.e.*, MRN → vHPC and MRN → PFC, to understand how the brain adapts to prenatal stress exposure. Moreover, future experiments may isolate the effects of prenatal stress from postnatal maternal care, as maternal care is an important influencer of offspring development.

In sum, we showed that untreated maternal stress during pregnancy has prolonged developmental effects that impacts long-term offspring health. This study fills an important gap in previous literature in answering how long-term behavioral and neurochemical effects are impacted given adverse *in utero* exposures. Importantly, our study strongly points to the safety and efficacy of citalopram, a commonly prescribed SSRI, in attenuating adverse neurochemical and behavioral effects induced by stress. Finally, we show an important link between stress-reactivity and the integral role of the serotonin and kappa systems in mediating responses to stress.

Figures

Figure II.1

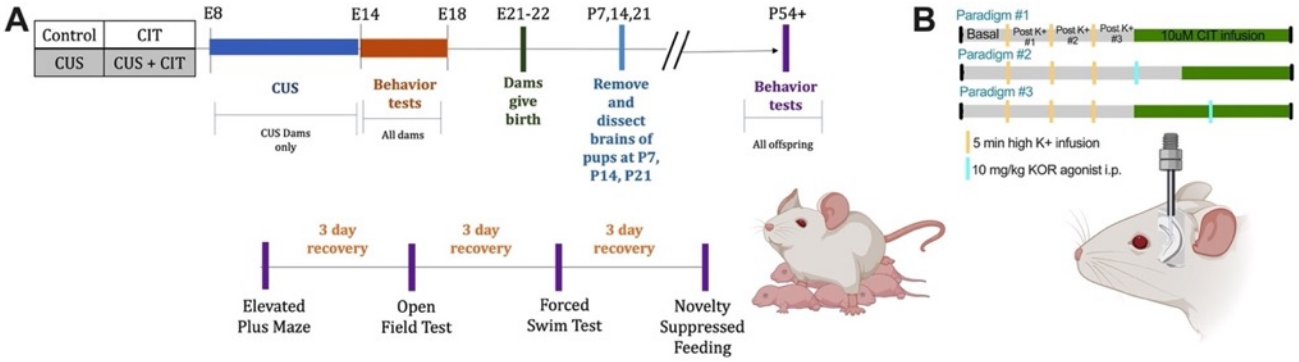

Figure II.1: Overview of experimental paradigms. A. Timeline of *in utero* exposure and subsequent postnatal and behavior testing schedule. **B.** Timeline for different microdialysis paradigms.

Figure II.2

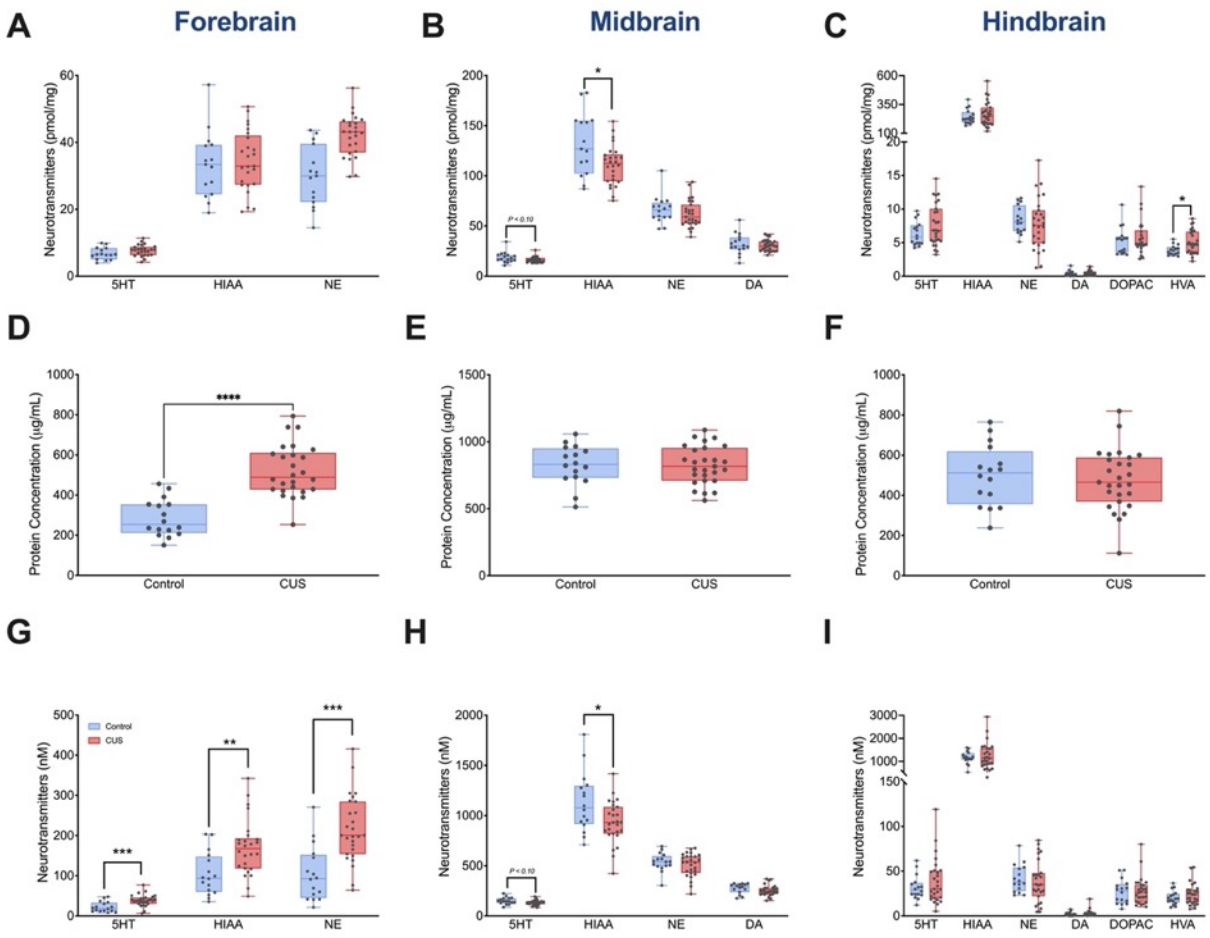

Figure II.2: Postnatal day 7 tissue analysis shows increased neurotransmitters and protein concentration for stressed pups. Normalized **A.** forebrain, **B.** midbrain, and **C.** hindbrain neurotransmitter tissue levels. **D**. Protein concentrations are significantly increased at P7 in the forebrain of stressed pups, but not the **E.** midbrain or **F.** hindbrain. Non-normalized neurotransmitter tissue levels show significant increases in pups born to stressed mothers in **G.** forebrain, but not **H.** midbrain or **I.** hindbrain. *P<0.05, **P<0.01, ***P<0.001

Abbreviations: Serotonin (5HT), 5-Hydroxyindolacetic acid (HIAA), norepinephrine (NE), dopamine (DA), 3,4-Dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)

Figure II.3

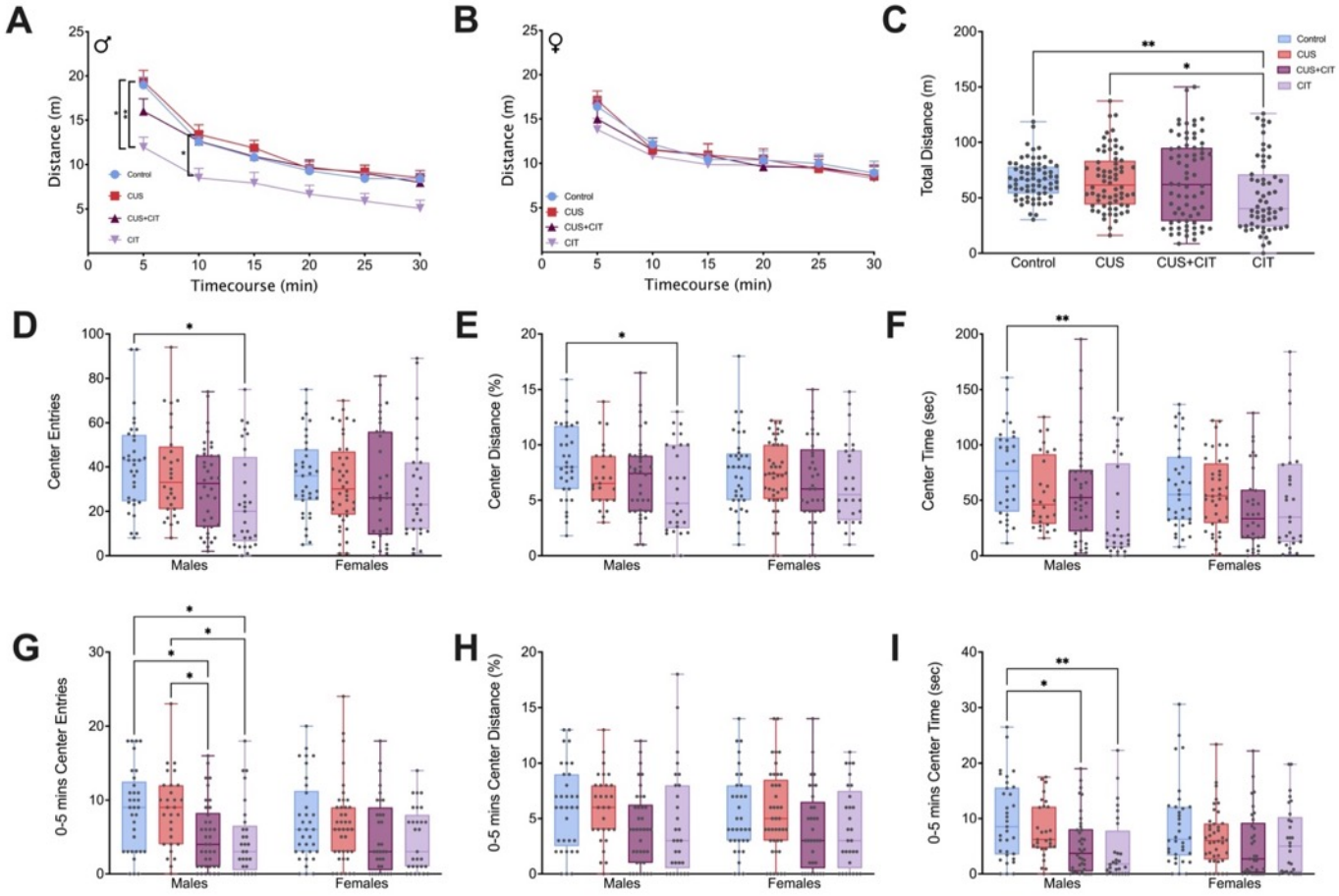

Figure II.3: *In utero* citalopram exposure changes OFT behavior in male adult offspring. *In utero* treatment differences in distance traveled in first five and ten minutes in **A.** males, but not **B.** females. Significant effect of **C.** *in utero* treatment on total distance traveled between control and CIT groups and CUS and CIT groups. **D-F.** Center zone parameters for full 30 min OFT time course show significant changes in CIT group on **D.** number of entries into the center zone, **E.** distance travelled in the center zone compared to the entire arena, and **F.** time spent in the center zone. No changes were observed in females. **G-**I. Significant decreases of *in utero* CIT exposure on **G.** number of center entries, and **I.** time spent in the center zone, but not **H.** percent center distance during first 5 min of test. No changes were observed in females. *P<0.05, **P<0.01, ***P<0.001

Figure II.4

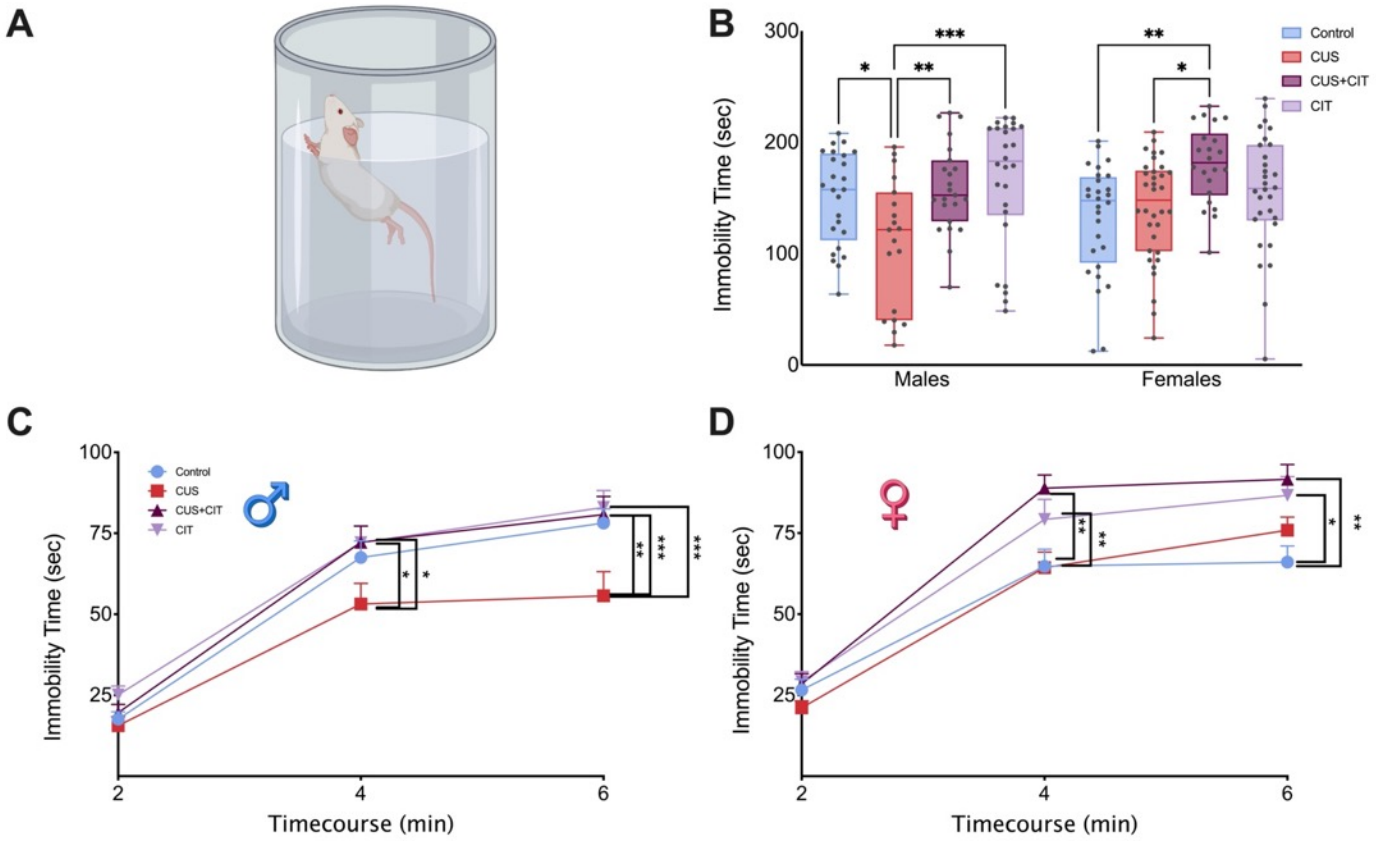

Figure II.4: Adult offspring force swim test A. Cartoon of FST behavioral apparatus. **B.** *In utero* CUS exposure significantly reduces immobility time of male adult offspring; these reductions are rescued in the CUS+CIT male group. *In utero* CUS+CIT significantly increases immobility time in female adult offspring, compared to control and CUS groups. Immobility time is summed over the last 4 minutes of the FST. **C.** Male and **D.** female immobility time course shows significant effects of treatment and time. *P<0.05, **P<0.01, ***P<0.001

Figure II.5

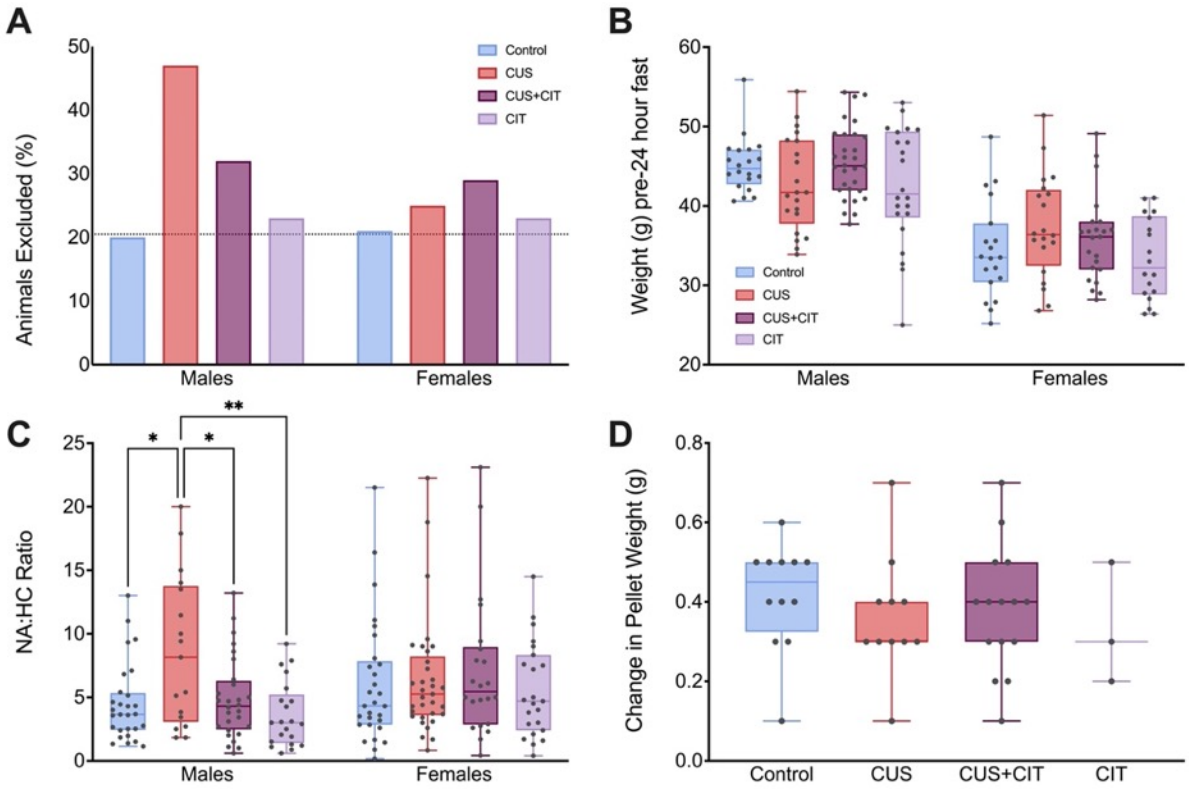

Figure II.5: Adult offspring novelty suppressed feeding test A. Percentages of animals excluded based on percent body weight loss. Adult male CUS animals were excluded at twice the rate of other groups. **B.** No differences in body weights between treatment groups of the same sex pre-24 hour fast. **C.** Significant effect of treatment in male adult offspring on ratio between latency to feed in novelty area compared to the home cage. No difference in **D.** change in pellet weight 10 min after testing corresponding to amount of food eaten. *P<0.05, **P<0.01

Figure II.6

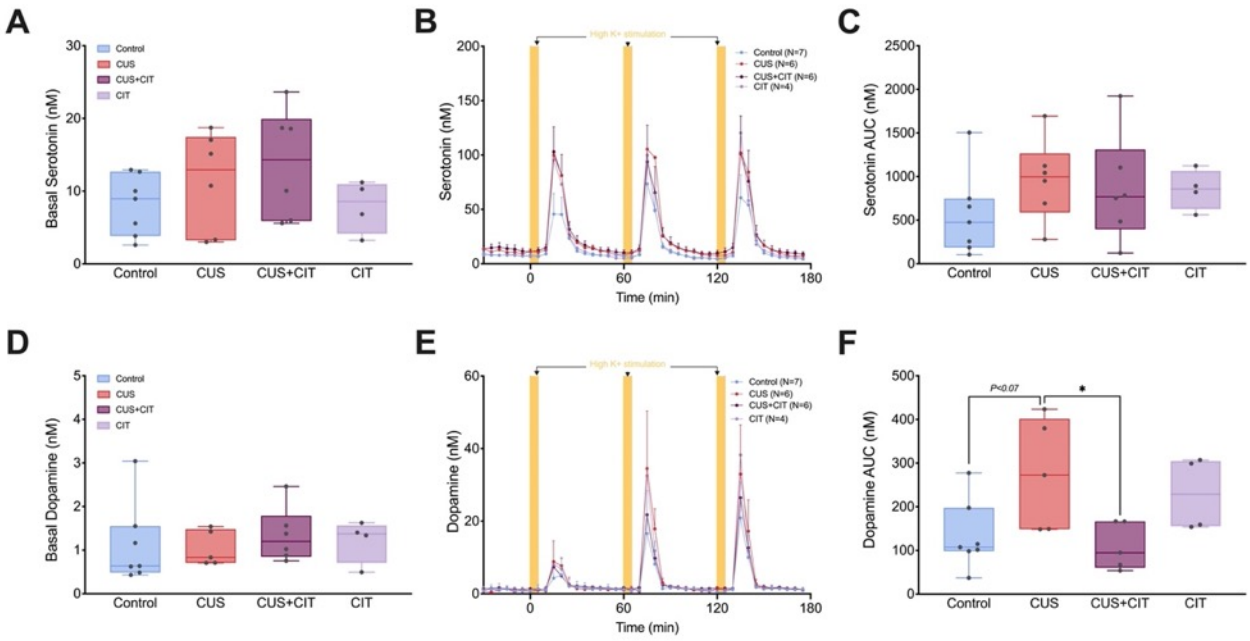

Figure II.6: No basal extracellular serotonin or dopamine differences in adult offspring exposed to prenatal stress. Basal serotonin (**A**) and dopamine (**D**) levels in adult offspring. Microdialysis time course of extracellular serotonin (**B**) and dopamine (**E**). The yellow bars indicate 120 µM potassium stimulation (5 min). Serotonin (**C**) and dopamine high potassium (**F**) stimulated release quantified by area under the curve. *P<0.05,

Figure II.7

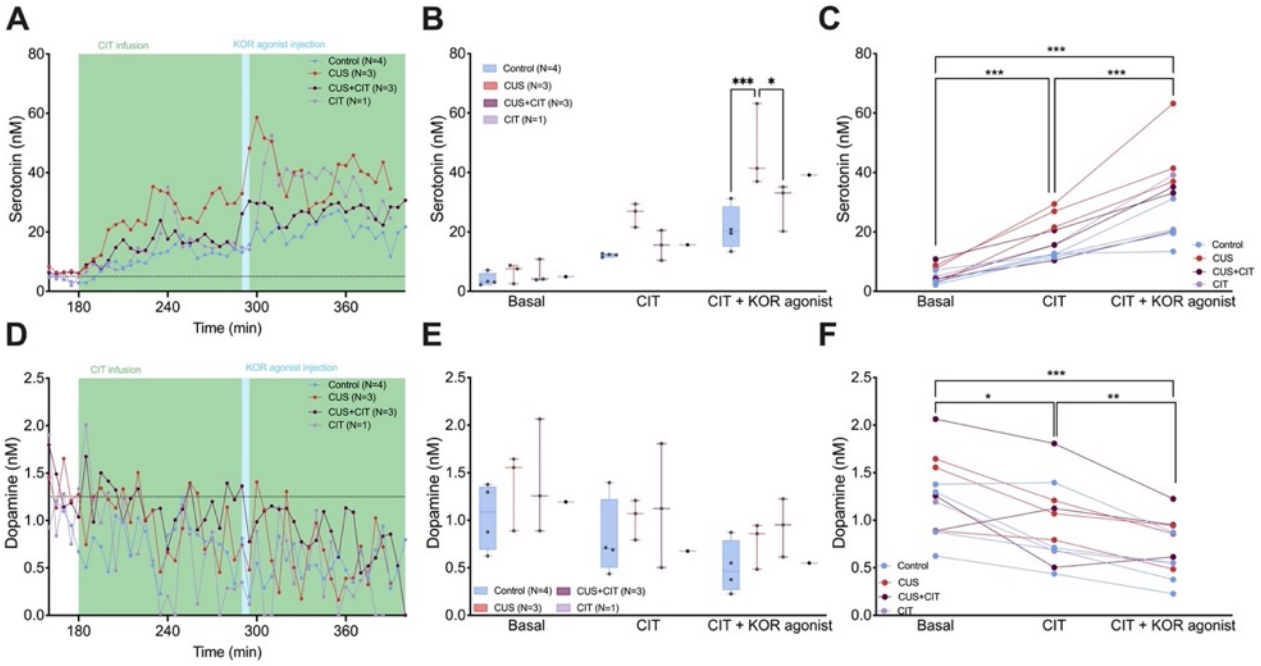

Figure II.7: Decreases in dopamine and increases in serotonin, post concomitant CIT and KOR agonist. A. Time course before and after CIT infusion and KOR agonist injection for serotonin concentrations. Significant effect of **B.** treatment, and **C.** drug on serotonin concentrations. **D.** Time course before and after CIT infusion and KOR agonist injection for dopamine concentrations. Significant effect of **F.** CIT infusion and agonist injection, but not **E.** *in utero* treatment on dopamine concentrations. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Table II.S1

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
	E7: timed-pregnant dams arrive; CUS groups split into single-housed cages	**E8 @10am:** ear tags, five-minute restraint, saline injection, and brief transportation stress	**E9 @ 12pm**: fox urine	**E10 4pm – 7am:** overnight light	**E11 10am - 12pm:** 30-minute restraint in restraint tubes	**E12 4pm-7am:** overnight static noise
E13 8am – 4pm: 45-degree cage tilt	**E14 4pm-7am:** overnight wet bedding	**E15 6pm-10pm:** OFT test	**E16 12-2:30pm:** FST test; @7pm fast for NSF	**E17 5-7pm:** NSF test		

Table II.S1: Chronic unpredictable stress paradigm

Figure II.S1

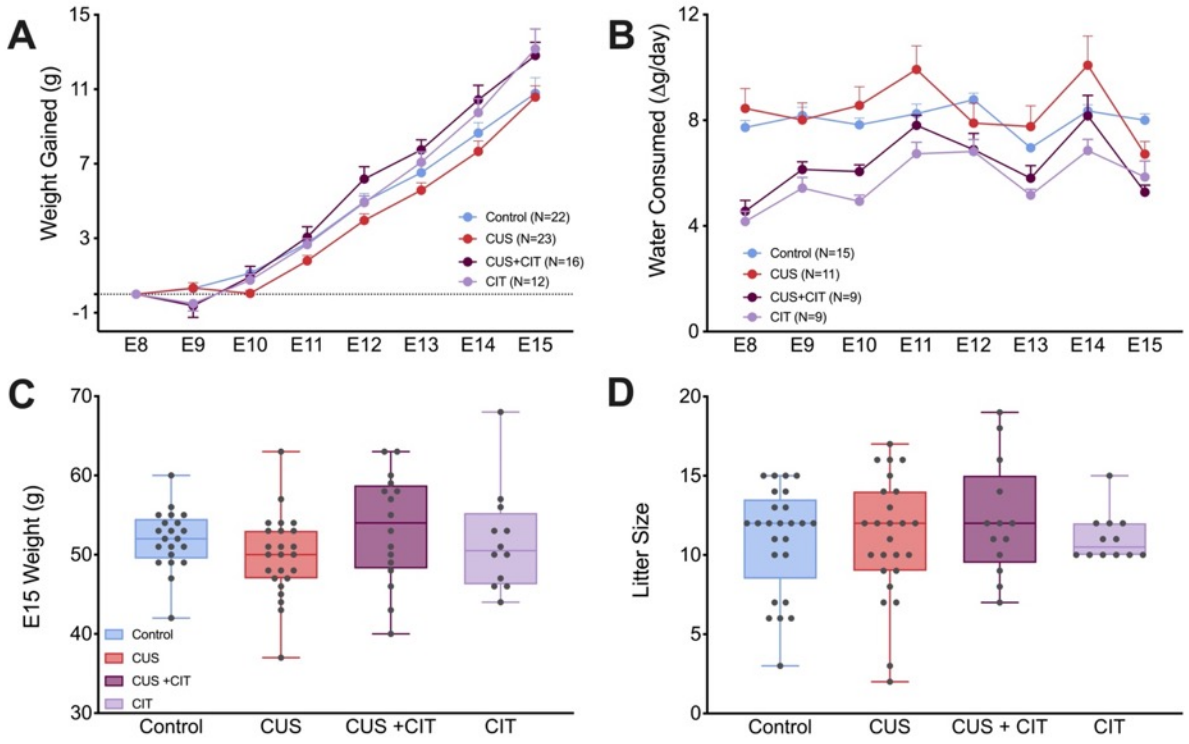

Figure II.S1: No changes in maternal measures or outcomes. A. Weight increases over the course of pregnancy. **B.** Water consumption between groups by day. The groups that received CIT in their drinking water had 1.5% sucrose to mask the taste. **C.** No changes in dam weights at E15 between groups. **D.** No changes in litter size between groups.

Figure II.S2

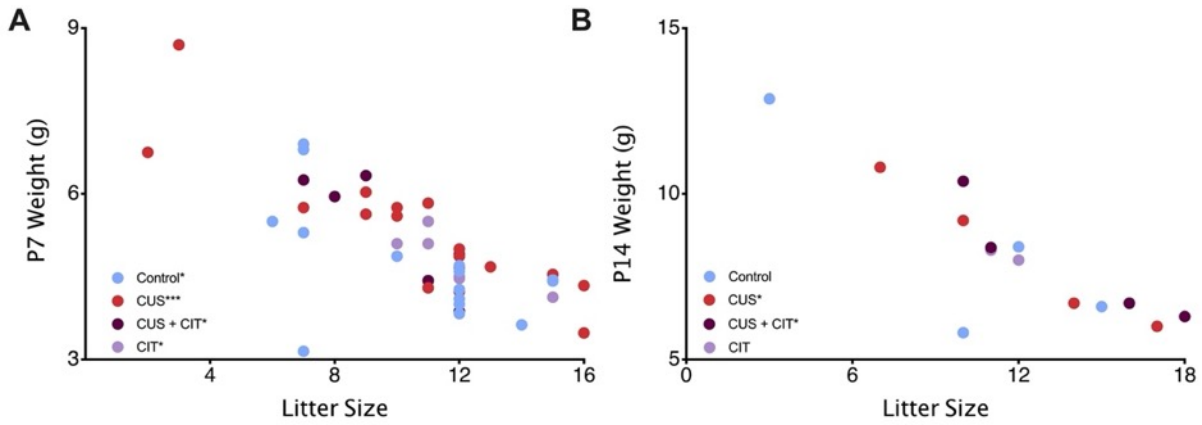

Figure II.S2: Correlation between liter size and postnatal day weights across treatments. Significant correlation between **A.** postnatal day 7 (P7) and **B.** postnatal day 14 (P14) weight of pups and maternal literal size.

Figure II.S3

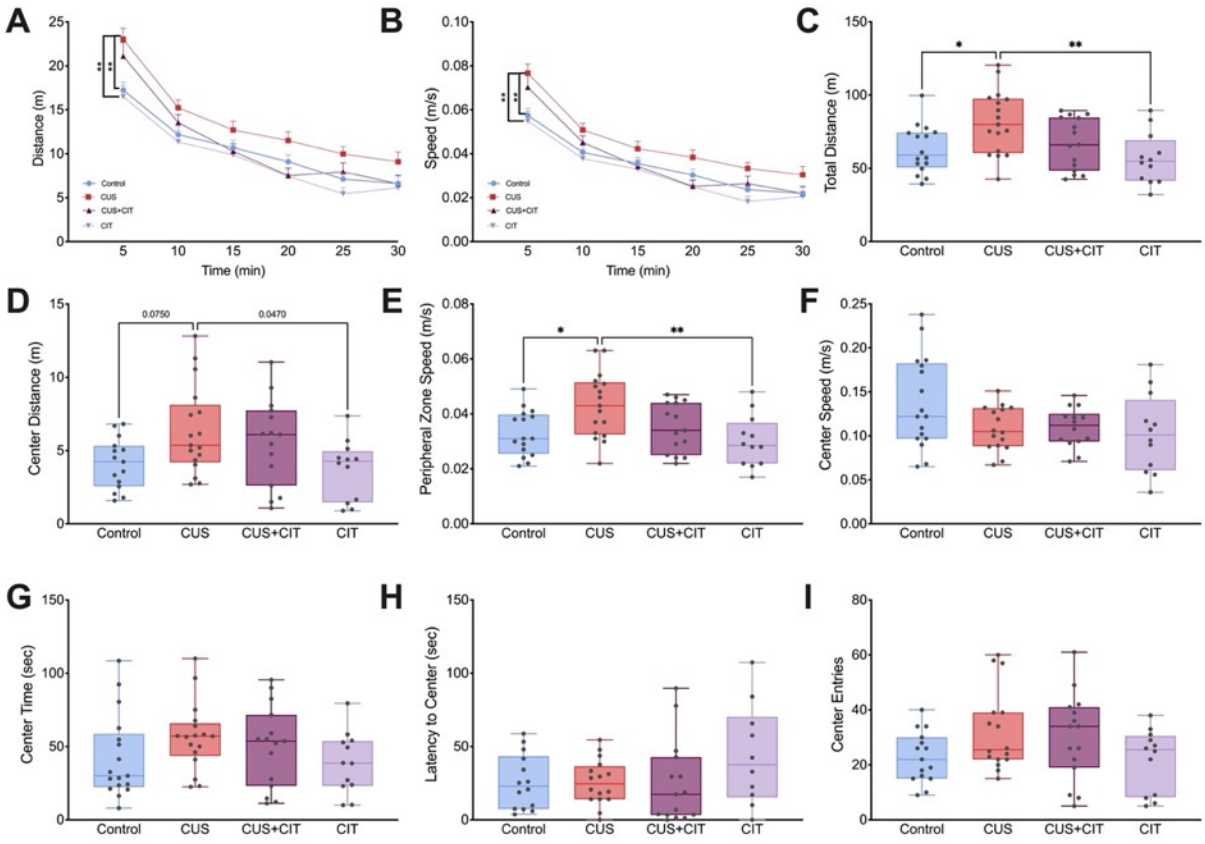

Figure II.S3: Increased speed and distance travelled in the OFT of dams exposure to CUS. A. Distance travelled and. **B.** speed during the 30 minute OFT. **C.** Increased total arena distance travelled in the CUS group. **D.** Trending increased center distance travelled in the CUS group. **E.** Increased peripheral zone speed, but not **F.** center speed in the CUS group. **G-H**. No changes in center zone parameters between groups. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Figure II.S4

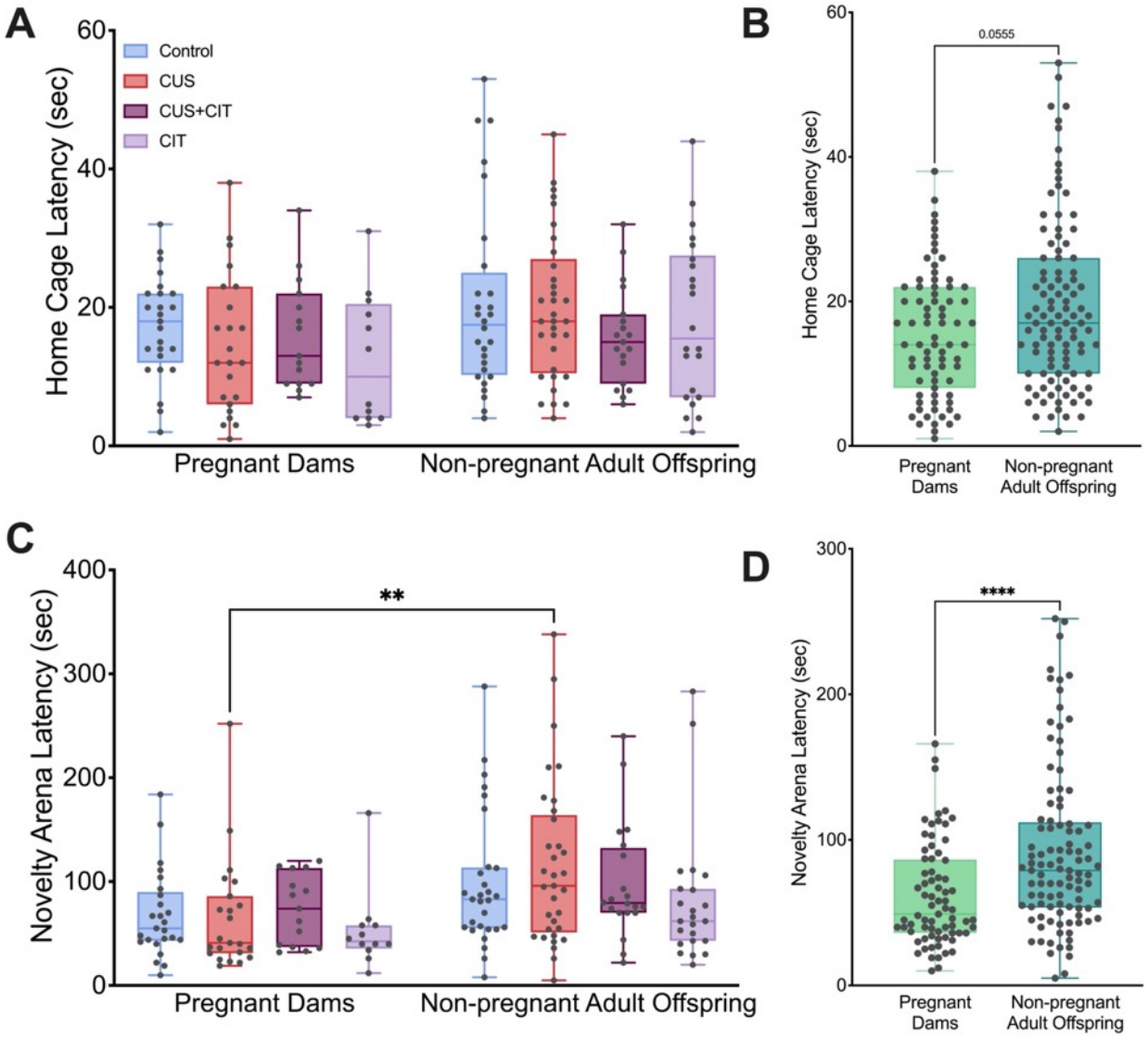

Figure II.S4: Pregnancy-induced changes in the NSF. A. No changes in home cage latency to feed between groups. **B.** Trending decrease in home cage latency collapsed on treatment group between pregnant and non-pregnant female mice. **C.** Significant effect of treatment and **D.** pregnancy on novelty arena latency feeding time. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Figure II.S5

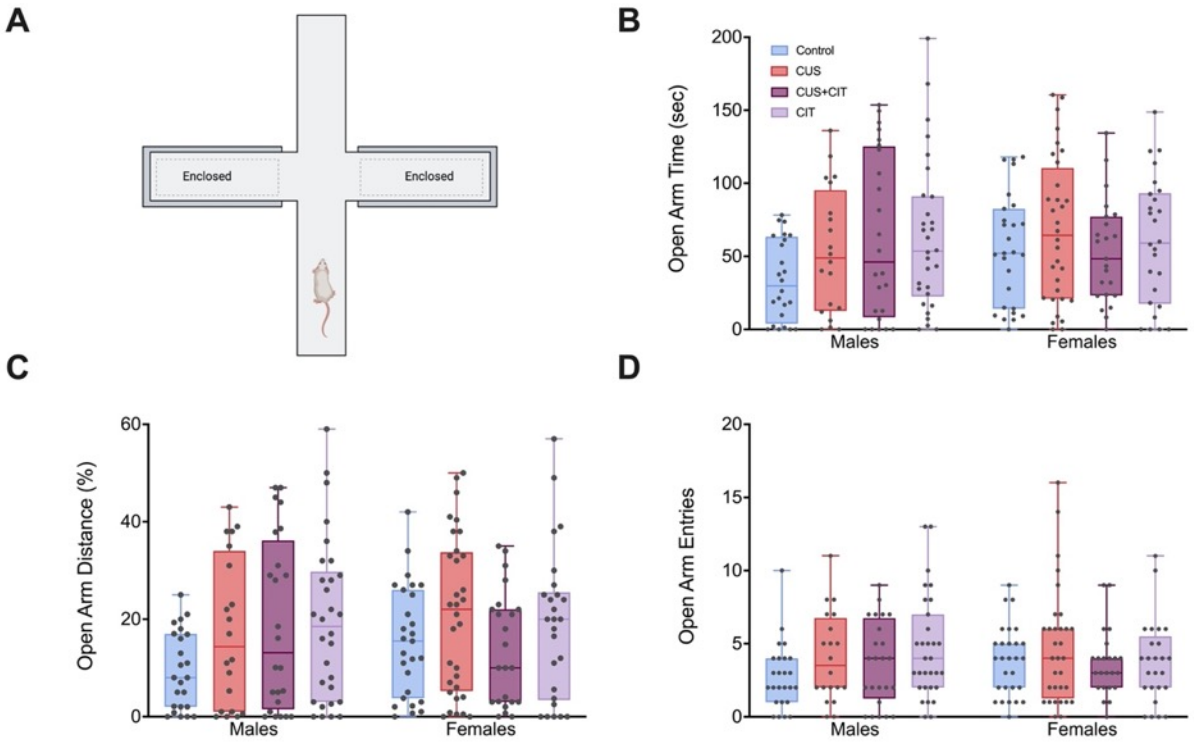

Figure II.S5: No changes in the EPM of 3-month-old adult offspring. A. Cartoon of EPM behavioral apparatus **B-D.** No changes in open arm parameters between groups or sex-specific differences.

Figure II.S6

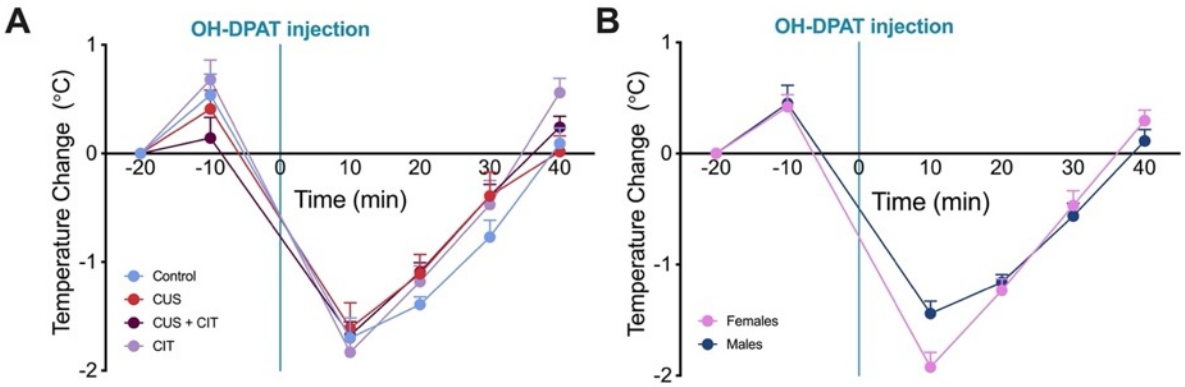

Figure II.S6: No changes in the temperature after 5HT1A agonist. No temperature changes by **A.** treatment or by **B**. sex after 8-OH-DPAT i.p. injection.

Figure II.S7

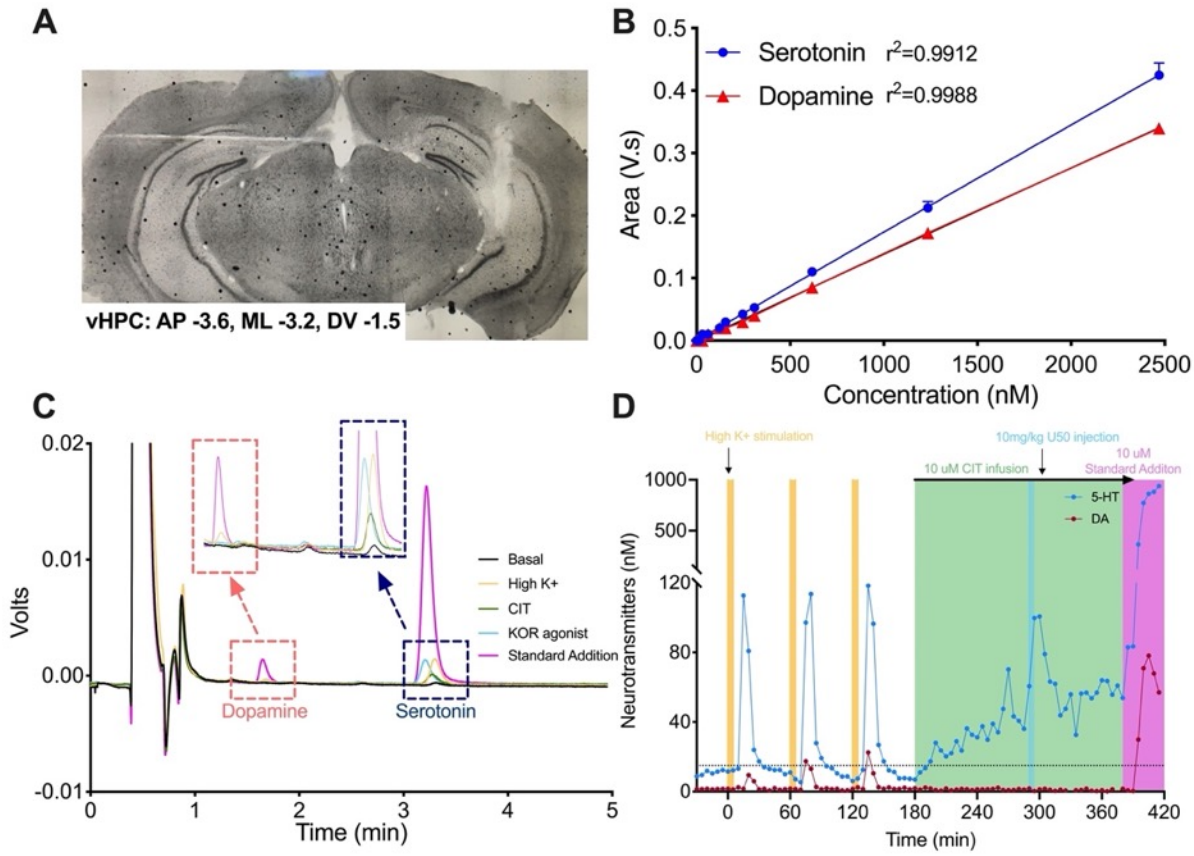

Figure II.S7: Microdialysis probe and peak verification. A. Cresyl violet image showing microdialysis cannula and probe localization to the ventral hippocampus. **B.** Standard curves for dopamine and serotonin used to quantify analyte concentrations. A standard curve was run each week prior to microdialysis experiments N=6/analyte. **C.** Overlay of five chromatograms from a representative mouse showing dopamine and serotonin peaks before (basal) and after pharmacological manipulations to verify peak identities. **D.** Time course from the same mouse in C.

Figure II.S8

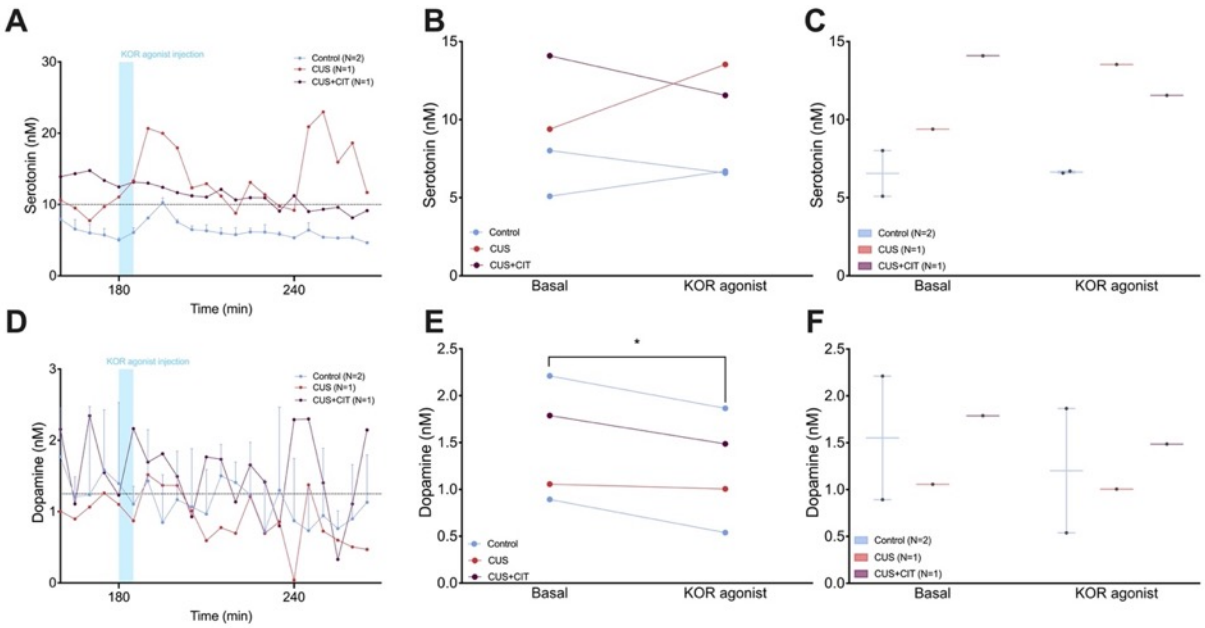

Figure II.S8: Decreases in dopamine, but not serotonin, post KOR agonist i.p. injection. A. Time course before and after KOR agonist injection for serotonin concentrations. No effect of **B.** KOR injection or **C.** *in utero* treatment on serotonin concentrations. **D.** Time course before and after KOR agonist injection for dopamine concentrations. Significant effect of **E.** KOR injection, but not **C.** *in utero* treatment on dopamine concentrations. *P<0.05

Figure II.S9

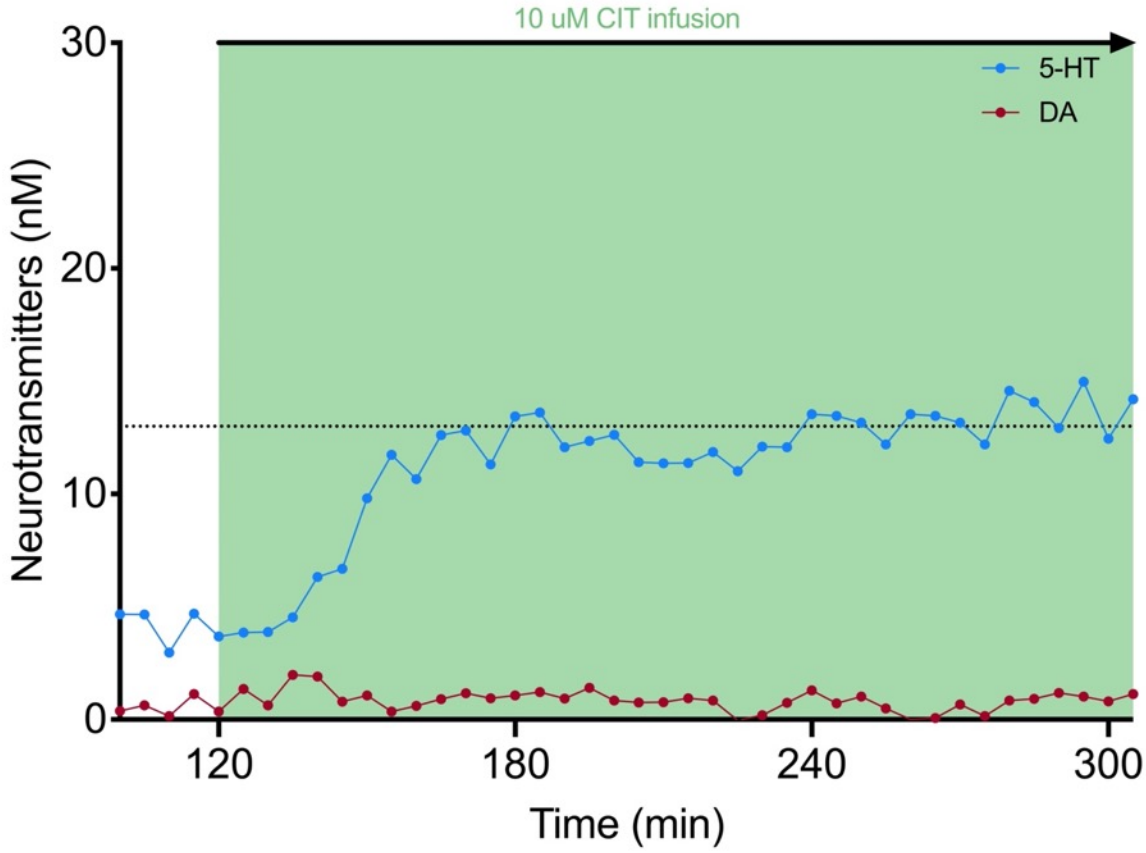

Figure II.S9: Stable serotonin levels after three hr of CIT infusion. Serotonin concentrations remained stable during longer infusion of CIT. Time course is taken from N=1 animal that underwent basal collection and two high K^+ potassium stimulations. Each point represents a 5 min sample. Points pre-120 min are neurotransmitter concentrations post-high K^+ #2 and pre-CIT infusion. Infusion of CIT began at 120 min (or 2 hours into microdialysis testing day) and continued until 310 min, for a duration of three hr and 10 min.

Table II.S2

<table>
<tr><th>Figure</th><th>Comparison</th><th>Test and result</th></tr>
<tr><td>Figure 2A</td><td>Pup forebrain neurotransmitters (pmol/mg) between treatment</td><td>Serotonin: Unpaired t-test; P<0.08
HIAA: Unpaired t-test; P>0.05
Norepinephrine: Unpaired t-test; P<0.001</td></tr>
<tr><td>Figure 2B</td><td>Pup midbrain neurotransmitters (pmol/mg) between treatment</td><td>Serotonin: Mann-Whitney test; P>0.05
HIAA: Unpaired t-test; P<0.05
Norepinephrine: Mann-Whitney test; P>0.05
Dopamine: Unpaired t-test; P>0.05</td></tr>
<tr><td>Figure 2C</td><td>Pup hindbrain neurotransmitters (pmol/mg) between treatment</td><td>Serotonin: Unpaired t-test; P>0.05
HIAA: Unpaired t-test; P>0.05
Norepinephrine: Unpaired t-test; P>0.05
Dopamine: Mann-Whitney test; P>0.05
DOPAC: Mann-Whitney test; P>0.05
HVA: Unpaired t-test; P<0.05</td></tr>
<tr><td>Figure 2D</td><td>Pup forebrain protein concentrations between treatment</td><td>Unpaired t-test; P>0.001</td></tr>
<tr><td>Figure 2E</td><td>Pup midbrain protein concentrations between treatment</td><td>Unpaired t-test; P>0.05</td></tr>
<tr><td>Figure 2F</td><td>Pup hindbrain protein concentrations between treatment</td><td>Unpaired t-test; P>0.05</td></tr>
<tr><td>Figure 2G</td><td>Pup forebrain neurotransmitters (nM) between treatment</td><td>Serotonin: Unpaired t-test; P<0.001
HIAA: Unpaired t-test; P<0.01
Norepinephrine: Unpaired t-test; P<0.001</td></tr>
<tr><td>Figure 2H</td><td>Pup midbrain neurotransmitters (nM) between treatment</td><td>Serotonin: Unpaired t-test; P<0.08
HIAA: Unpaired t-test; P<0.05
Norepinephrine: Unpaired t-test; P>0.05
Dopamine: Mann-Whitney test; P>0.05</td></tr>
<tr><td>Figure 2I</td><td>Pup hindbrain neurotransmitters (nM) between treatment</td><td>Serotonin: Unpaired t-test; P>0.05
HIAA: Unpaired t-test; P>0.05
Norepinephrine: Unpaired t-test; P>0.05
Dopamine: Unpaired t-test; P>0.05
DOPAC: Unpaired t-test; P>0.05
HVA: Unpaired t-test; P>0.05</td></tr>
<tr><td>Figure 3A</td><td>Male distance traveled across time</td><td>Two-way ANOVA<table><tr><td>Time x treatment</td><td>F (15, 605) = 3.81; P<0.001</td></tr><tr><td>Time</td><td>F (2.6, 315) = 221; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 121) = 4.89; P<0.01</td></tr><tr><td>Subject</td><td>F (121, 605) = 21.1.; P<0.001</td></tr></table></td></tr>
</table>

Figure 3B	***Female distance traveled across time***	***Two-way ANOVA*** Time x treatment: F (15, 675) = 1.15; P>0.05 ***Time: F (5, 675) = 88.4; P<0.001*** Treatment: F (3, 135) = 4.89; P>0.05 ***Subject: F (135, 675) = 24.0.; P<0.001***
Figure 3C	***Total distance traveled between treatments***	***Kruskal-Wallis test; P<0.001***
Figure 3D	***Entries into center zone***	***Two-way ANOVA*** Interaction: F (3, 256) = 0.68; P>0.05 Sex: F (1, 256) = 0.06; P>0.05 ***Treatment: F (3,256) = 6.33; P<0.001***
Figure 3E	***% center distance in OFT***	***Two-way ANOVA*** Interaction: F (3, 258) = 0.50; P>0.05 Sex: F (1, 258) = 0.08; P>0.05 ***Treatment: F (3,258) = 2.96; P<0.05***
Figure 3F	***Time spent in the center zone***	***Two-way ANOVA*** Interaction: F (3, 256) = 2.26; P>0.05 Sex: F (1, 256) = 0.80; P>0.05 ***Treatment: F (3,256) = 3.85; P<0.05***
Figure 3G	***Entries into center zone in first 5 min***	***Two-way ANOVA*** Interaction: F (3, 257) = 0.62; P>0.05 Sex: F (1, 257) = 1.67; P>0.05 ***Treatment: F (3,257) = 7.04; P<0.001***
Figure 3H	***% center distance in first 5 min***	***Two-way ANOVA*** Interaction: F (3, 258) = 0.06; P>0.05 Sex: F (1, 258) = 0.79; P>0.05 ***Treatment: F (3,258) = 3.52; P<0.05***
Figure 3I	***Time spent in the center zone in first 5 min***	***Two-way ANOVA*** Interaction: F (3, 256) = 0.68; P>0.05 Sex: F (1, 256) = 0.06; P>0.05 ***Treatment: F (3,256) = 6.33; P<0.001***
Figure 4B	***2-6 min immobility time in FST***	***Two-way ANOVA*** ***Interaction: F (3, 199) = 3.23; P<0.05 ** Sex: F (1, 199) = 0.70; P>0.05 ***Treatment: F (3,199) = 8.43; P<0.001***
Figure 4C	***Male immobility time across time***	***Two-way ANOVA*** ***Time x treatment: F (6, 190) = 2.42; P<0.05*** ***Time: F (1.78, 169) = 378; P<0.001*** ***Treatment: F (3, 95) = 3.68; P<0.05*** ***Subject: F (95, 190) = 5.32; P<0.001***

<table>
<tr><td>Figure 4D</td><td>Female immobility time across time</td><td>Two-way ANOVA<table><tr><td>Time x treatment</td><td>F (6, 212) = 3.71; P<0.01</td></tr><tr><td>Time</td><td>F (1.90, 202) = 371; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 106) = 4.96; P<0.01</td></tr><tr><td>Subject</td><td>F (106, 212) = 4.44; P<0.001</td></tr></table></td></tr>
<tr><td>Figure 5B</td><td>Animal weights pre-NSF fast</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 165) = 1.81; P>0.14</td></tr><tr><td>Sex</td><td>F (1, 165) = 101; P<0.001</td></tr><tr><td>Treatment</td><td>F (3,165) = 1.97; P>0.12</td></tr></table></td></tr>
<tr><td>Figure 5C</td><td>Ratio of latency to feed in the novelty arena compared to the home cage</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 193) = 2.70; P<0.05</td></tr><tr><td>Sex</td><td>F (1, 193) = 2.06; P>0.15</td></tr><tr><td>Treatment</td><td>F (3,193) = 3.83; P<0.05</td></tr></table></td></tr>
<tr><td>Figure 5D</td><td>Pellet weight change 10 min post-NSF test</td><td>One-way ANOVA
F (3, 37) = 0.38; P>0.05</td></tr>
<tr><td>Figure 6A</td><td>Serotonin basal comparison</td><td>One-way ANOVA
F (3, 19) = 1.29; P>0.05</td></tr>
<tr><td>Figure 6C</td><td>Serotonin AUC comparison</td><td>One-way ANOVA
F (3, 19) = 0.82; P>0.05</td></tr>
<tr><td>Figure 6D</td><td>Dopamine basal comparison</td><td>One-way ANOVA
F (3, 18) = 0.19; P>0.05</td></tr>
<tr><td>Figure 6F</td><td>Dopamine AUC comparison</td><td>One-way ANOVA
F (3, 17) = 3.96; P<0.05</td></tr>
<tr><td>Figure 7B, C</td><td>Serotonin basal, CIT, and CIT+KOR comparison across treatments</td><td>Two-way ANOVA<table><tr><td>Drug x treatment</td><td>F (6, 14) = 4.21; P<0.05</td></tr><tr><td>Drug</td><td>F (2, 14) = 75.44; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 7) = 4.70; P<0.05</td></tr><tr><td>Subject</td><td>F (7, 14) = 3.79; P<0.05</td></tr></table></td></tr>
<tr><td>Figure 7E, F</td><td>Dopamine basal, CIT, and CIT+KOR comparison across treatments</td><td>Two-way ANOVA<table><tr><td>Drug x treatment</td><td>F (6, 14) = 0.19; P>0.05</td></tr><tr><td>Drug</td><td>F (2, 14) = 16.5; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 7) = 0.71; P>0.05</td></tr><tr><td>Subject</td><td>F (7, 14) = 10.0; P>0.05</td></tr></table></td></tr>
</table>

<table>
<tr><td>Figure S1A</td><td>Weight change over pregnancy</td><td>Two-way ANOVA<table><tr><td>Time x treatment</td><td>F (21, 497) = 3.42; P<0.001</td></tr><tr><td>Time</td><td>F (7, 497) = 578; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 71) = 2.64; P<0.06</td></tr><tr><td>Subject</td><td>F (71, 497) = 7.91; P<0.001</td></tr></table></td></tr>
<tr><td>Figure S1B</td><td>Water consumption over pregnancy</td><td>Two-way ANOVA<table><tr><td>Time x treatment</td><td>F (21, 280) = 5.60; P<0.001</td></tr><tr><td>Time</td><td>F (7, 280) = 31.6; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 40) = 8.71; P<0.001</td></tr><tr><td>Subject</td><td>F (40, 280) = 17.6; P<0.001</td></tr></table></td></tr>
<tr><td>Figure S1C</td><td>E15 dam weights</td><td>One-way ANOVA
F (3, 69) = 1.33; P>0.05</td></tr>
<tr><td>Figure S1D</td><td>Litter size</td><td>One-way ANOVA
F (3, 71) = 0.44; P>0.05</td></tr>
<tr><td>Figure S2A</td><td>P7 weights and maternal liter size</td><td>Control: R² = 0.77; P<0.001
CUS: R² = 0.68; P<0.05
CUS+CIT: R² = 0.67; P<0.05
CIT: R² = 0.67; P<0.05</td></tr>
<tr><td>Figure S2B</td><td>P14 weights and maternal liter size</td><td>Control: R² = 0.70; P>0.16
CUS: R² = 0.98; P<0.05
CUS+CIT: R² = 0.87; P<0.07
CIT: too few pairs</td></tr>
<tr><td>Figure S3A</td><td>Dam distance traveled across time</td><td>Two-way ANOVA<table><tr><td>Time x treatment</td><td>F (15, 280) = 2.24; P<0.01</td></tr><tr><td>Time</td><td>F (2.6, 146) = 204; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 56) = 4.97; P<0.01</td></tr><tr><td>Subject</td><td>F (56, 280) = 9.19.; P<0.001</td></tr></table></td></tr>
<tr><td>Figure S3B</td><td>Dam speed across time</td><td>Two-way ANOVA<table><tr><td>Time x treatment</td><td>F (15, 280) = 2.20; P<0.01</td></tr><tr><td>Time</td><td>F (2.6, 146) = 203; P<0.001</td></tr><tr><td>Treatment</td><td>F (3, 56) = 4.96; P<0.01</td></tr><tr><td>Subject</td><td>F (56, 280) = 9.18.; P<0.001</td></tr></table></td></tr>
<tr><td>Figure S3C</td><td>Dam total distance traveled in OFT</td><td>One-way ANOVA
F (3, 56) = 4.97; P<0.01</td></tr>
<tr><td>Figure S3D</td><td>Dam distance traveled in center zone of OFT</td><td>One-way ANOVA
F (3, 55) = 3.47; P<0.05</td></tr>
<tr><td>Figure S3E</td><td>Dam peripheral zone speed</td><td>One-way ANOVA
F (3, 56) = 5.02; P<0.01</td></tr>
</table>

<table>
<tr><td>Figure S3F</td><td>Dam center zone speed</td><td>One-way ANOVA
F (3, 55) = 2.60; P<0.07</td></tr>
<tr><td>Figure S3G</td><td>Dam time spent in center zone</td><td>Kruskal-Wallis test; P>0.05</td></tr>
<tr><td>Figure S3H</td><td>Dam latency to enter center zone</td><td>Kruskal-Wallis test; P>0.05</td></tr>
<tr><td>Figure S3I</td><td>Dam total entries to center zone</td><td>Kruskal-Wallis test; P>0.05</td></tr>
<tr><td>Figure S4A</td><td>Latency to feed in home cage between treatments</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 169) = 0.65; P>0.05</td></tr><tr><td>Pregnancy</td><td>F (1, 169) = 4.87; P<0.05</td></tr><tr><td>Treatment</td><td>F (3,169) = 0.99; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S4B</td><td>Latency to feed in novelty arena between treatments</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 172) = 0.73; P>0.05</td></tr><tr><td>Pregnancy</td><td>F (1, 172) = 13.0; P<0.001</td></tr><tr><td>Treatment</td><td>F (3,172) = 1.08; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S4C</td><td>Pregnancy effects in latency to feed in home cage</td><td>Mann-Whitney test; P<0.06</td></tr>
<tr><td>Figure S4D</td><td>Pregnancy effects in latency to feed in novelty arena</td><td>Mann-Whitney test; P<0.001</td></tr>
<tr><td>Figure S5B</td><td>Dam time spent in the open arm of the EPM</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 198) = 1.34; P>0.05</td></tr><tr><td>Sex</td><td>F (1, 198) = 0.75; P>0.05</td></tr><tr><td>Treatment</td><td>F (3,198) = 1.72; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S5C</td><td>Dam % distance traveled in open arms of the EPM</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 198) = 1.35; P>0.05</td></tr><tr><td>Sex</td><td>F (1, 198) = 0.26; P>0.05</td></tr><tr><td>Treatment</td><td>F (3,198) = 2.42; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S5D</td><td>Dam entries to the open arms of the EPM</td><td>Two-way ANOVA<table><tr><td>Interaction</td><td>F (3, 200) = 1.19; P>0.05</td></tr><tr><td>Sex</td><td>F (1, 200) = 0.22; P>0.05</td></tr><tr><td>Treatment</td><td>F (3,200) = 1.47; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S6A</td><td>Change in temperature after DPAT across treatment</td><td>Repeated measures one-way ANOVA<table><tr><td>Treatment</td><td>F (2.06, 10.3) = 0.96; P>0.05</td></tr><tr><td>Time</td><td>F (5, 15) = 100; P<0.001</td></tr></table></td></tr>
</table>

<table>
<tr><td>Figure S6B</td><td>Change in temperature after DPAT across sex</td><td>Two-way ANOVA<table><tr><td>Time x Sex</td><td>F (5, 225) = 2.69; P<0.05</td></tr><tr><td>Time</td><td>F (1, 225) = 141; P<0.001</td></tr><tr><td>Sex</td><td>F (5,225) = 0.43; P>0.05</td></tr><tr><td>Subject</td><td>F (45, 225) = 1.84; P<0.01</td></tr></table></td></tr>
<tr><td>Figure S8B, C</td><td>Serotonin basal vs. KOR comparison across treatments</td><td>Two-way ANOVA<table><tr><td>Drug x treatment</td><td>F (2, 1) = 2.46; P>0.05</td></tr><tr><td>Drug</td><td>F (1, 1) = 0.35; P>0.05</td></tr><tr><td>Treatment</td><td>F (2, 1) = 16.2; P>0.05</td></tr><tr><td>Subject</td><td>F (1, 1) = 0.84; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S8E, F</td><td>Dopamine basal vs. KOR comparison across treatments</td><td>Two-way ANOVA<table><tr><td>Drug x treatment</td><td>F (2, 1) = 844; P<0.05</td></tr><tr><td>Drug</td><td>F (1, 1) = 5477; P<0.001</td></tr><tr><td>Treatment</td><td>F (2, 1) = 0.11; P>0.05</td></tr><tr><td>Subject</td><td>F (7, 14) = 96977; P>0.05</td></tr></table></td></tr>
<tr><td>Figure S9</td><td>Analyte concentration changes over time</td><td>Simple linear regression
Serotonin: Y = 0.01385*X + 9.457; R^2 = 0.25
Dopamine: Y = -0.001606*X + 1.158; R^2 = 0.02</td></tr>
</table>

Table II.S2: Statistical analyses for all data.

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CHAPTER III

Optogenetic Stimulation of Midbrain Dopamine Neurons Produces Striatal Serotonin Release

Optogenetic Stimulation of Midbrain Dopamine Neurons Produces Striatal Serotonin Release. Merel Dagher, Katie A. Perrotta, Sara A. Erwin, Ayaka Hachisuka, Rahul Iyer, Sotiris C. Masmanidis, Hongyan Yang, and Anne M. Andrews. *ACS Chemical Neuroscience* 2022 *13* (7), 946-958.DOI: 10.1021/acschemneuro.1c00715.

Introduction

Optogenetics entails expressing light-driven ionotropic receptors in neurons or other excitable cells to enable spatially and temporally restricted activation or inhibition.[4-7] Gene constructs for microbial or engineered rhodopsins packaged in viruses are used to transduce brain-region-specific gene expression following local delivery. Gene expression can be further targeted using Cre recombinase under the control of cell-type-specific promotors, in combination with Cre-activated opsin constructs.[8] Opsins produce excitatory (*e.g.,* channelrhodopsin-2, Chrimson) or inhibitory (*e.g.,* halorhodopsin, archaerhodopsin) effects on neural activity.[9-11] The discovery and use of opsins have enabled the identification of neural pathways involved in the modulation of behavior.[12-15]

Opsin-targeted cell types, however, do not operate autonomously. Dopamine and serotonin are examples of functionally interconnected neurotransmitter systems. For instance, while dopamine signaling is often associated with reward prediction error, serotonin transmission also plays a role in processing reward-associated information.[16-18] Moreover, while widely used therapeutics for mood disorders target the serotonin system,[19] the dopamine system encodes information associated with anhedonia, a core symptom of major depressive disorder.[17,20-25] Interactions between the dopamine and serotonin systems are evident in drug mechanisms of action, *e.g.,* cocaine, methamphetamine, and 3,4-methylenedioxymethamphetamine.[26-29] Thus, these systems act in concert to modulate subjective states.[30,31]

Microdialysis is a tissue sampling technique. When combined with chemical separation and detection methods, microdialysis enables the identification and quantification of neurotransmitters, metabolites, and drugs in the extracellular space.[32]

Several groups, including ours, have optimized microdialysis to monitor brain extracellular dopamine or serotonin levels *via* online coupling with fast separations by high performance liquid chromatography (HPLC) and electrochemical detection in awake mice and rats.[33-39] Dopamine and serotonin can be resolved in the same dialysate samples enabling biologically relevant changes in basal and stimulated levels of these neurotransmitters to be simultaneously monitored.[39,40]

Here, we set out to determine the magnitude of extracellular dopamine release in the dorsal striatum (dSTR) upon optogenetic stimulation of midbrain dopaminergic neurons. The excitatory opsin Chrimson was expressed under the control of the dopamine transporter promoter in mice. Optical activation of dopamine neurons has been used to study dopaminergic encoding of reward and movement.[15,41] In addition to dopamine, we observed optically induced increases in the dopamine metabolite 3-methoxytyramine (3-MT) and in serotonin levels. These findings demonstrate a functional link between the dopamine and serotonin systems in the basal ganglia. They illustrate the importance of monitoring multiple neurotransmitters simultaneously. And they suggest that opsin-induced behavioral changes may not be attributable solely to the neurotransmitter system or cell type targeted by opsin expression. That is to say, while optogenetics imparts highly selective control of specific types of neurons, brain function and behavior arise from distributed and interconnected networks.

Materials and Methods

Animal procedures

Mice were generated at the University of California, Los Angeles (UCLA) from a DAT^{IREScre} line (The Jackson Laboratory, stock no. 006660) on a C57Bl/6J background *via* heterozygous matings. Mice were housed in groups of 2-5 same-sex siblings prior to surgery, same-sex-sibling pairs after the first surgery to deliver viral vectors and to implant optical fibers and head bars, and singly after the second surgery to implant a microdialysis guide cannula. Food and water were available *ad libitum* throughout, with the exception of microdialysis testing days where mice were hand-fed a 2:1 sweetened condensed milk:water solution *via* pipette every 2 h.

The light-dark cycle (12/12 h) in the animal colony room was set to lights on at 0730 h (ZT0). The same light schedule was maintained in the room where microdialysis was performed. The Association for Assessment and Accreditation of Laboratory Animal Care International has fully accredited UCLA. All animal care and use met the requirements of the NIH Guide for the Care and Use of Laboratory Animals, 2011. The UCLA Chancellor's Animal Research Committee (Institutional Animal Care and Use Committee) preapproved all animal procedures.

Surgeries were carried out under aseptic conditions with isoflurane anesthesia on a KOPF Model 1900 Stereotaxic Alignment System (KOPF, Tujunga, CA). A pair of rectangular stainless steel head-bars (9 mm × 7 mm × 0.76 mm, 0.6 g each, Fab2Order, Brownsburg, IN) were attached to the sides of the skull by C&B Metabond (Parkell, Edgewood, NY) for head fixation (**Fig. III.S1A,B**). Viral vectors, 600 nL of 7.8×10^{12}/mL AAV5/Syn-Flex-ChrimsonR-

tdTomato (for experimental groups) or 4.4 × 10^{12}/mL AAV5/EF1a-DIO-eYFP or 3.3 × 10^{12}/mL AAV5/EF1a-DIO-mcherry (for control subjects), were delivered unilaterally into the SN/VTA (AP-3.08 mm, ML ±1.20 mm, DV -4.00 mm from Bregma) using a Nanoject II (Drummond Scientific, Broomall, PA). A 200 μm diameter optical fiber (0.22 NA, Thorlabs, Newton, NJ) with a total length of 1 cm was lowered *via* the same track to reach the AAV injection site for optogenetic stimulation. Optical fibers were secured on the skull with C&B Metabond. The top of each optical fiber outside the skull was covered by a sleeve until coupling to a laser device for testing. All AAV Cre-dependent adeno-associated viral vectors were obtained from the University of North Carolina Vector Core (Chapel Hill, NC).

After the first surgery, animals recovered for 2-3 weeks (**Fig. III.1B**) to allow for viral vector expression prior to guide cannula implantation for microdialysis. During recovery, subjects were acclimated to being head-fixed over the course of 6-10 training sessions, each lasting 15-30 min. A second surgery was carried out on each mouse to implant a CMA/7 guide cannula for a microdialysis probe aimed at the dSTR (AP+1.00 mm, ML±1.75 mm, DV-3.10 mm from Bregma) in the same hemisphere as the viral delivery and fiber implant site. Each guide cannula was secured to the skull with C&B Metabond. Animals recovered from the second surgery for at least three days before microdialysis. Following each surgery, mice were given daily carprofen injections (5 mg/kg, 1 mg/mL, subcutaneously) for the first three days and a combination of an antibiotic (amoxicillin, 0.25 mg/mL) and a second analgesic (ibuprofen, 0.25 mg/mL) in their drinking water for 14 days postoperatively.

Microdialysis

Virgin female mice (*N*=23) underwent microdialysis at 3-6 months of age. Microdialysis was carried out over two consecutive days for Chrimson-transfected mice (*N*=14) and one day for control mice (*N*=9). On the night before the first testing day (ZT10-12), each mouse was transferred to the testing room in its home cage and briefly anesthetized with isoflurane (1-3 min) for insertion of a CMA/7 microdialysis probe (1 mm length, 6 kDa cutoff, CMA8010771) into the guide cannula. Subjects were returned to their home cages and aCSF was continuously perfused through the probe *via* a liquid swivel (375/D/22QM, Instech Laboratories Inc., Plymouth Meeting, PA) at 2-3 µL/min for 30-60 min followed by a 0.3 µL/min flow rate for an additional 12-14 h to allow the tissue surrounding the probe to recover from acute changes associated with probe insertion. Subjects were tethered to the liquid swivel but otherwise could move freely in their home cages.

Prior to microdialysis, the tubing connecting the microdialysis probe to the liquid swivel was disconnected. The mouse was transferred from its home cage and mounted to the head-fixed stage *via* its head-bars in the same testing room. The microdialysis probe was connected between the microdialysis syringe pump and the online autoinjector. The aCSF was perfused at 1.8 uL/min throughout each testing day, and samples were collected at 5-min intervals. Subjects were habituated for at least 10-min before the optical fiber was coupled for stimulation delivery.

An MGL-III-532 or MGL-III-589 laser (Opto Engine LLC, Ltd, Changchun, P. R. China) was used to deliver light pulses. The excitation spectrum of Chrimson has a λ_{max} at 590 nm. Due to the broad excitation spectrum, either 532 nm (green) or 589 nm (yellow) light were

used to excite this opsin.[10] The output of the optical fiber was calibrated to deliver 10 mW/mm^2 immediately before coupling on each testing day.

The stimulation pulse width (50 ms), frequency (10 Hz), and train duration (5 min) were selected to generate neurotransmitter release detectable by microdialysis using a 5-min dialysate sampling time. In preliminary experiments, we investigated stimulation pulse widths that varied from 5-2500 ms. We also investigated laser powers ranging from 5-20 mW/mm^2. Longer pulse widths were ultimately favored over higher laser power with shorter pulses to avoid tissue damage over longer stimulation times needed for microdialysis. There were no significant differences in stimulation output for frequencies over 10-30 Hz using 50% duty cycle and a 5-min train duration. A longer train duration was used previously by Correia *et al.* to investigate the role of serotonin transmission in locomotion.[42]

The first stimulation was delivered at ~ZT2 after 6-18 basal dialysate samples were collected and analyzed. Prior to reverse dialysis of drugs, three optical stimulations were delivered at 1-h intervals (**Fig. III.1B**). After 90-120 min of intrastriatal drug perfusion, an additional three optical stimulations were delivered at 1-h intervals while drug perfusion continued. On day 1, four Chrimson-transfected mice were perfused with the D1-like antagonist SCH-23390 (100 μM) through the dialysis probe. On day 2, the same four Chrimson-transfected mice were perfused with 100 μM eticlopride (D2-like antagonist).

Eleven mice not receiving D1- or D2-like antagonists underwent brief (5 min) perfusion with 120 mM K^+ (KCl substituted isotonically for NaCl in aCSF) to stimulate neurochemical overflow[38,39,43] for peak identification. In **Fig. III.S2,** data from a representative K^+-stimulated mouse are shown. Three Chrimson-transfected mice were

perfused with an SSRI (10 μM escitalopram) on day one to confirm serotonin peak identity (**Fig. III.3A, B**). Four mice (three control and one Chrimson-transfected) were administered the COMT inhibitor tolcopone (10 mg/kg, intraperitoneal) to identify the 3-MT peak (**Fig. III.2C**).

Dialysate analysis

High performance liquid chromatography was performed using an Amuza HTEC-500 integrated system (Amuza Corporation [formally known as Eicom], San Diego, CA). An Eicom Insight autosampler was used to inject standards and Eicom EAS-20s online autoinjectors were used to collect and inject dialysate samples online.[33] Chromatographic separation was achieved using an Eicom PP-ODS II column (4.6 mm ID x 30 mm length, 2 μm particle diameter) and a phosphate-buffered mobile phase (96 mM NaH_2PO_4 (Fluka #17844), 3.8 mM Na_2HPO_4 (Fluka #71633), pH 5.4, 2-2.8% MeOH (EMD #MX0475), 50 mg/L EDTA·Na_2 (Sigma #03682), and 500 mg/L sodium decanesulfonate (TCI #I0348) in water purified *via* a Milli-Q Synthesis A10 system (EMD Millipore Corporation, Billerica, MA). The column temperature was maintained at 21 °C. The volumetric flow rate was 450-500 μL/min. Electrochemical detection was performed using an Eicom WE-3G graphite working electrode with an applied potential of +450 mV *vs.* a Ag/AgCl reference electrode.

Dopamine (Sigma #H8502), 3-MT (Sigma #65390), and serotonin (Sigma #H9523) standards were prepared in ice-cold 1:1 mobile phase/aCSF (147 mM NaCl (Fluka #73575), 3.5 mM KCl (Fluka #05257), 1.0 mM $CaCl_2$ (Aldrich #499609), 1.0 mM NaH_2PO_4, 2.5 mM $NaHCO_3$ (Fluka #88208), 1.2 mM $MgCl_2$ (Aldrich #449172), pH 7.3 ± 0.03. (See supplemental information in Liu et al., 2020 for detailed information on formulating aCSF).[44] Standard curves encompassed physiological concentration ranges (0-10 nM; **Fig. III.S5**). The limit of

detection was ≤300 amol (6 pM) for each analyte; the practical limit of quantification was ≤900 amol (18 pM). Dialysate samples were collected online at 5-min intervals using a dialysate flow rate of 1.8 µL/min and injected immediately onto the HPLC system for analysis.

In situ hybridization

We used RNAscope® technology (Advanced Cell Diagnostics Inc., Newark, CA) for *in situ* hybridization to colocalize mRNAs for D1 receptors in dorsal raphe neurons expressing SERT, VGLUT3, or both.[1,2,45,46] A DAT$^{IRES\textit{cre}}$ mouse not transfected with Chrimson was sacrificed by cervical dislocation without isoflurane and the brain was removed, cryoprotected, and frozen. Coronal sections were cut at 16-µm on a cryostat at -15-20 °C and mounted on polylysine-coated slides.

In situ hybridization was conducted using the RNAscope® fresh-frozen V2 protocol. Briefly, sections were incubated in freshly prepared 4% paraformaldehyde (Sigma-Aldrich Cat#441244) in phosphate buffered saline for 15 min followed by sequential dehydration in 50% EtOH, 70% EtOH, and 100% EtOH for 5 min each. Sections were then incubated with the necessary reagents from the Multiplex Fluorescent Reagent Kit V2 (ACD #323110) in a HybEZ® oven. Probes were as follows: *Sert* (Mm-Slc6a4 Cat#315851) channel 1, *Vglut3* (Mm-Slc32a1 Cat#319191-C2) channel 2, and *Drd1* (Mm-Drd1a-C3 Cat# 406491-C3) channel 3. Opal dyes 520, 570, and 690 were paired with each probe, respectively (Cat#FP1487A, FP1488A, FP1497A). ProLong™ Diamond Antifade Mountant with DAPI (Molecular Probes P36966) was added to stain cell bodies.

Visualization was carried out using a Leica DMI8 or Zeiss LSM800 microscope and images were processed with LAS X and Zen software. Cell nuclei in each field of view were

identified *via* DAPI staining. The DAPI labeled nuclei associated with puncta for one or more mRNA probes were then counted. Data are reported as percent positive cells calculated by dividing the number of cells labeled with *Sert*, *Drd1*, and/or *Vglut3* by the total number of *Sert* labeled cells.

Histology

At the end of each experiment, the microdialysis probe was removed and the brain of each mouse was prepared for histology to verify probe and optical fiber placements, and Chrimson, mCherry, or eYFP expression. Subjects were exsanguinated with an overdose of 100 mg/kg pentobarbital (2 mL/kg administered at 50 mg/mL, ip) followed immediately by transcardial perfusion with 4% paraformaldehyde in PBS. Sections from the midbrain and dSTR were cut using a vibratome and mounted on microscope slides. Images were acquired using a Zeiss Axio Examiner microscope as follows: tdTomato and mCherry (550 nm excitation/605 nm emission), or eYFP (470 nm excitation/525 nm emission). Microdialysis probe and optical fiber tracks were visualized *via* light microscopy. Three of the 23 microdialysis subjects failed histology verification for probe or fiber placement. Data for these subjects were excluded from analyses.

Data analysis and statistics

The microdialysis time-course data were analyzed in terms of absolute neurochemical concentrations (nM) and as percents of mean pre-stimulation basal neurochemical levels (%basal). Overflow peaks following optical stimulation were identified and analyzed individually using the following criteria and procedures. (1) For each control mouse, the concentrations of six dialysate samples for each neurochemical immediately preceding the onset of the first optical stimulation were averaged (nM) and converted to mean 100% basal

levels. (2) For Chrimson-expressing mice on experimental days 1 and 2, basal levels of individual neurochemicals were determined separately by day. The concentrations of the six dialysate samples immediately preceding the onset of the first pre-drug or post-drug optical stimulation were averaged (nM) and converted to mean 100% basal levels. (3) The AUC for each stimulation peak, defined by the four dialysate samples after the onset of stimulation, was calculated by trapezoidal integration and is reported in nM or as a percent of mean pre-stimulation basal levels.

Statistical analyses were carried out using Prism, v.9.0.2 (GraphPad Inc., La Jolla, CA). Data are expressed as group means ± SEMs. Two-tailed *t*-tests (either unpaired or ratio paired, as appropriate) were used for two-group comparisons. Throughout, $P<0.05$ was considered statistically significant. Detailed statistics are summarized in **Table III.S1**.

Results and discussion

Using microdialysis,[33,38,39] we quantified extracellular dopamine in a dopamine-rich projection region—the dSTR—during optical stimulation of midbrain dopamine cell bodies (**Fig. III.1, Fig. III.S1**). To induce dopamine release, we applied 50-ms square pulses at 10 Hz and 10 mW/mm^2 laser power. Stimulation pulse train durations were 5 minutes to match dialysate sampling times. These parameters were optimized to produce reproducible neurotransmitter release detectable *via* microdialysis. Activation of the excitatory opsin Chrimson[10] produced temporally specified increases in striatal extracellular dopamine levels (**Fig. III.2A**). Control mice expressing mCherry or yellow fluorescent protein (YFP) in dopamine cell bodies showed no detectable changes in dopamine upon optical stimulation (**Fig. III.2B**).

Basal (unstimulated) dialysate dopamine levels were not statistically different in Chrimson-expressing *vs.* control mice (**Fig. III.2C**; see **Table III.S1** for detailed statistics). Basal dopamine levels for control animals were normally distributed around the mean. In contrast, basal dopamine levels for Chrimson-expressing animals were not normally distributed. Individual dopamine concentrations fell mostly below the mean, apart from three animals, one of which was an outlier. Notably, this outlier is not the same animal that is an outlier for basal serotonin levels in Chrimson-transfected mice (**Fig. III.4A** *vide infra*). As such, we chose to not to exclude outliers from analysis, although exclusion would have led to a statistically significant reduction in basal dopamine levels in Chrimson-transfected *vs.* control mice. Stimulated dopamine overflow, quantified as area under the curve (AUC), was greater in Chrimson-expressing *vs.* control mice (**Fig. III.2D**; t_{18}=3.0, P<0.01). Dopamine levels were increased ~200 pM by optical stimulation.

In addition to dopamine, optical activation appeared to lead to increases in two other chromatographic peaks (**Fig. III.S2**). We initially hypothesized that the larger peak (peak 2) was serotonin. However, since retention times commonly shift between standards and brain dialysate samples, we could not definitively identify peak 2 using serotonin-containing standards. We perfused a selective serotonin reuptake inhibitor (SSRI) through the dialysis membrane during intracerebral dialysis to investigate peak identity. Increases in peak areas in response to serotonin transporter inhibition identified a small, later eluting peak (peak 3) as serotonin (**Fig. III.3A, B**; t_2=5.7, P<0.05).

Previous experience analyzing striatal tissue samples then led us to suspect that the remaining optically responsive peak was 3-methoxytyramine (3-MT). Dopamine is metabolized by catechol-*O*-methyltransferase (COMT) to produce 3-MT, which is hypothesized to function as a neuromodulator.[47,48] We administered the COMT inhibitor tolcapone systemically[49] and found that peak 2 was selectively decreased (**Fig. III.3C**; t_3=9.6, P<0.01). We also perfused 3-MT through the dialysis membrane into dSTR, *i.e.*, *in vivo* standard addition, and observed a retention time match confirming the identity of peak 2 as 3-MT and ruling out the possibility that this peak was serotonin (**Fig. III.3D**).

Having identified two optically (*i.e.*, biologically) responsive neurochemicals, in addition to dopamine, we quantified their basal dialysate levels. We found no differences in basal 3-MT or serotonin levels in Chrimson-expressing *vs.* control mice (**Fig. III.4A**). Optogenetic stimulation of midbrain dopaminergic neurons evoked reproducible increases in 3-MT and serotonin in Chrimson-expressing but not control mice (**Fig. III.4B,C**; t_{18}=3.1, P<0.01, t_{15}=4.4, P<0.001, respectively). Since basal neurochemical levels varied across individual mice (**Fig. III.2C, 4A**), we also analyzed optically stimulated neurochemical levels

normalized to mean pre-stimulation basal levels (**Fig. III.S3**). Concentration and %basal analyses similarly indicated that in addition to dopamine, 3-MT and serotonin overflow were increased in response to optogenetic stimulation of Chrimson-transfected dopamine neurons. Optical stimulation of control mice lacking opsin expression showed no nonspecific increases in neurochemicals associated with light-induced arousal.

We parsimoniously hypothesized that the increased overflow of serotonin associated with optical stimulation of dopamine neurons was mediated by activation of dopamine receptors on serotonin terminals in striatum. The mRNAs for DRD2 and DRD3 receptors (*i.e.*, D2-like) were previously identified in dorsal raphe.[50,51] Ren *et al.*[45] and Spaethling *et al.*[52] used single-cell transcriptomics to localize *Drd2* transcripts to serotonergic neurons. Using RNAseq, Dymecki and colleagues identified *Drd2* mRNA in dorsal raphe serotonin neurons specified by *Pet1* expression.[53]

A small number of DRN serotonin neurons has also been reported to contain *Drd1a* mRNA.[45,53] We carried out *in situ* hybridization to investigate colocalization of D1 receptor (*Drd1*) and serotonin transporter (*Sert*) mRNAs in the dorsal raphe nucleus (**Fig. III.5A,B**). We included a probe for the vesicular glutamate transporter type 3 (VGLUT3) because a subpopulation of serotonergic neurons co-expresses VGLUT3[54] and projects to the striatum.[54,55] We found that ~25% of total *Sert*-positive cells in the dorsal raphe were positive for *Sert* mRNA alone (**Fig. III.5C**). Approximately 10% of total *Sert*-positive cells showed colocalization of *Sert* and *Drd1* mRNA, while an additional 35% of *Sert*-positive cells showed *Drd1* and *Vglut3* mRNA colocalization. Positive and negative *in situ* hybridization controls are shown in Figure S4.

Since our data suggested that almost half of dorsal raphe serotonin neurons may express heterologous D1 receptors, we investigated whether blocking striatal D1-like receptors prevents optically stimulated serotonin overflow. We perfused SCH 23390, a D1-like receptor antagonist, into the dSTR. Basal dopamine (t_3=4.4, P<0.05) and serotonin (t_3=3.5, P<0.05) levels were increased by local D1-like receptor inhibition (**Fig. III.6A,B**). Stimulated dopamine (t_3=6.2, P<0.01), 3-MT (t_3=4.8, P<0.05), and serotonin (t_3=4.5, P<0.05) levels were also increased by local perfusion of SCH 23390 (**Fig. III.6B,C**). Elevations in striatal dopamine levels in response to SCH-23390 have been previously reported.[56] In addition to serotonin neurons, medium spiny neurons (MSNs) in striatum express D1 receptors. Blocking D1-receptors on MSNs disinhibits dopamine neurons causing an increase in dopamine levels.[57,58]

To focus on optically stimulated neurochemical levels, we normalized the SCH 23390 time-course data. Data prior to drug perfusion were normalized to pre-drug/pre-stimulation basal neurochemical levels determined in each mouse (**Fig. III.7A**). Data collected during drug perfusion were normalized to post-drug/pre-simulation basal neurochemical levels. When normalized to the respective basal levels, elevation of stimulated 3-MT (t_3=3.7; P<0.05) remained (**Fig. III.7A,B**). In contrast, potentiation of optically stimulated dopamine and serotonin levels were no longer evident during striatal SCH 23390 perfusion (**Fig. III.7A,B**). Thus, increases in the stimulated AUC for serotonin calculated using dialysate concentrations (**Fig. III.6C**) was largely the result of SCH 29930-induced increases in basal dialysate concentrations.

The D1-like receptor inhibitory increase in basal serotonin levels (**Fig. III.6A**) can be explained by a circuit connecting dSTR to the DRN.[1] Approximately, 95% of projections from

the dSTR to the DRN are D1-expressing MSNs,[1] which tonically inhibit DRN (and presumably serotoninergic) neurons. Blocking D1-like receptors on MSNs dendritic spines[59] could reduce tonic inhibition of DRN cell populations leading to increased serotonin levels in the dSTR. Regardless, local inhibition of D1 heteroreceptors on serotonin terminals and/or MSNs did not *prevent* optically evoked striatal serotonin.

Mice that received the D1-like inhibitor on day 1 of microdialysis were perfused with eticlopride (ETC), a D2-like receptor antagonist, on day 2 (**Fig. III.1A**). Inhibition of D2-like receptors, which are expressed as dopaminergic heteroreceptors and autoreceptors in striatum,[60] was not associated with changes in basal levels of dopamine, 3-MT, or serotonin (**Fig. III.8A**). Though not statistically significant due to small sample sizes, eticlopride perfusion into the dSTR potentiated optically evoked dopamine and 3-MT analyzed either as basal (nM) concentrations (**Fig. III.8B,C**) or %basal levels normalized to pre-stimulation basal (**Fig. III.9A,B**). Previous studies have shown that extracellular dopamine is increased upon inhibition of presynaptic D_2 receptors.[61,62] Importantly, in the context of our current hypothesis, and similar to striatal D1-like receptor inhibition, D2-like inhibition did *not* block serotonin overflow associated with optically evoked dopamine release.

Functional interactions between the dopamine and serotonin systems have been investigated for more than 50 years.[63-65] In prefrontal cortex, dopamine receptor activation by the nonselective agonist apomorphine, local dopamine perfusion, or D2 autoreceptor inhibition by haloperidol each produced increases in serotonin levels in rats.[66] Systemic administration of apomorphine was also shown to increase extracellular serotonin in striatum and hippocampus.[67] Our findings indicate that optogenetic activation of midbrain

dopamine neurons expressing the excitatory opsin Chrimson produces temporally specified increases in striatal serotonin, as well as an active dopamine metabolite, 3-MT.

We tested hypotheses linking striatal dopamine and serotonin based on the idea that these neurotransmitters are released from different terminals in striatum. We found that serotonin overflow was not prevented by inhibition of striatal D1-like or D2-like receptors (**Figs. III.6-9**). Our findings suggest that optically evoked dopamine does not produce serotonin release by stimulating dopamine receptors on striatal serotonin terminals (or direct/indirect pathway MSNs).

Our findings contrast with those of Jacobs and coworkers where apomorphine-induced or behaviorally evoked increases in striatal extracellular serotonin were inhibited by systemic and intrastriatal D2-like receptor inhibition.[67,68] Differences in species (rats *vs.* mice), drug (raclopride *vs.* eticlopride) and/or perfusion concentration (10 uM *vs.* 100 uM) might account for the discrepancies between studies. Jacobs and colleagues did not report on striatal dopamine levels in their studies, *i.e.*, apomorphine and the tail-pinch and light-dark-transition behaviors may have direct receptor/serotonin system effects that are different from those mediated by evoked dopamine.[69] Artigas and colleagues reported that reverse dialysis of D1-like or D2-like agonists into striatum in rats did not alter serotonin levels supporting the idea that dopamine-serotonin interactions are not mediated by striatal dopamine receptors.[40]

Another possibility is that serotonin is released from dopaminergic terminals *via* co-transmission or co-release. Co-transmission involves release of different neurotransmitters from different vesicle populations within the same neurons; co-release entails release of two or more neurotransmitters from the same vesicles.[70] Anatomical, genetic, and functional

evidence shows that neurons can have mixed neurochemical phenotypes (for review see[70-73] among others) and argues specifically for a serotonin/glutamate mixed phenotype.[54,55]

Regarding serotonin/dopamine interactions, under conditions where serotonin transporters are genetically or pharmacologically inactivated, serotonin appears to be taken up by dopamine transporters into dopamine neurons, indicated by double serotonin/tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta and ventral tegmental area.[74] Thus, SSRI treatment may result in serotonin being used as a 'false' transmitter by dopamine neurons. Studies on chronic SSRI administration using *in vivo* neurochemical monitoring are needed to test this hypothesis further. In any case, mice with wildtype serotonin transporter expression did not show serotonin colocalization in midbrain dopamine neurons suggesting that under typical circumstances, such as those investigated here, evidence is lacking for serotonin co-transmission or co-release by dopaminergic neurons.[74]

Beyond striatum, a dopaminergic pathway connects the substantia nigra to the dorsal raphe, which contains a majority of forebrain-projecting serotonin cell bodies (**Fig. III.10**). Mesostriatal serotonergic afferents project from the dorsal raphe to the striatum.[2] In addition to striatum, optical activation of dopamine neurons could increase extracellular dopamine in the vicinity of midbrain dopamine cell bodies. Substantia nigra dopamine neurons exhibit activity-dependent somatodendritic dopamine release and D2-mediated autoinhibition.[3,75-77] Activation of nigral D2 autoreceptors might increase extracellular serotonin in the dorsal raphe *via* disinhibition.[78] Furthermore, optical stimulation of dopamine cell bodies could activate dopamine projections to the dorsal raphe (**Fig. III.10**).

Both scenarios produce dopamine interactions with dorsal raphe serotonin neurons and ostensibly, could increase release of serotonin in striatum (and other brain regions).

Alternately, indirect mechanisms involving SNr-thalamus-cortex-dSTR and/or SNr-thalamus-cortex-DRN pathways cannot be ruled out.[79-82] Moreover, recent reports describe the presence of dopamine neurons in the rostral dorsal raphe nucleus.[83,84] Future experiments to parse out specific contributions from dopamine neurons in the SNr, SNc, VTA and DRN to dopamine-induced serotonin release will be informative. It is also possible that optical stimulation of dopamine neurons in Chrimson-transfected mice, in addition to releasing dopamine, is interoceptively detected by mice.[85] The perception, increased arousal, and/or reward associated with dopaminergic activity could lead to increases in extracellular serotonin by complex mechanisms not involving direct connections between the dopamine and serotonin systems.

Regardless of mechanism, the present findings indicate that optogenetic stimulation of midbrain dopamine neurons evokes striatal serotonin release. We recently reported similar findings elucidated by rapid-pulse voltammetry.[86] Dopamine-serotonin coupling is likely to be of importance to the facilitation of reward prediction, locomotor control, habit formation, and anhedonia.

Figures

Figure III.1

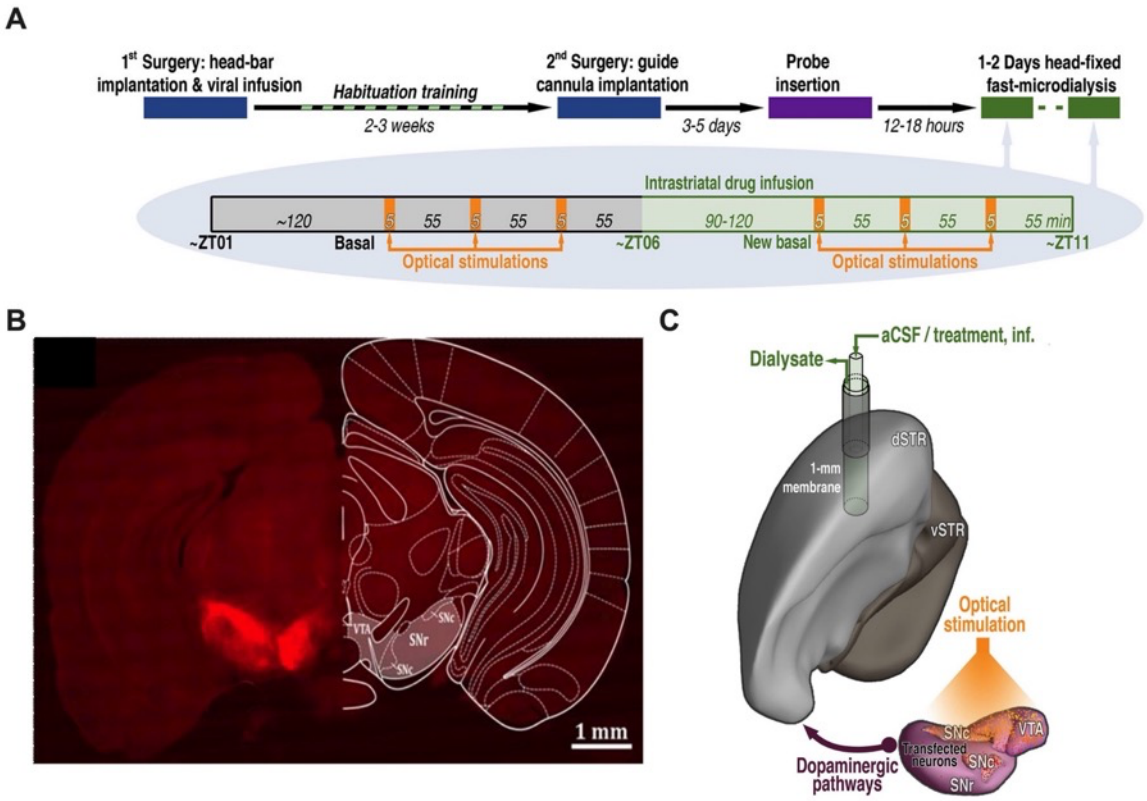

Figure III.1: Optogenetic stimulation of dopamine cell bodies. A. Experimental paradigm and timelines. Chrimson-expressing mice underwent microdialysis over two consecutive days. Control mice (transfected with mCherry or eYFP) were dialyzed only on Day 1. **B.** Representative optical microscopy image of unilateral Chrimson-positive neurons in the substantia nigra and ventral tegmental area. The coronal brain atlas plate 58, adapted from *The Mouse Brain in Stereotaxic Coordinates,* Paxinos and Franklin, *2nd edition (2001) Academic Press*, is overlaid on the hemisphere contralateral to transfection. Ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and substantia nigra pars reticulata (SNr) **C.** Model showing the location of the microdialysis probe in the dorsal striatum (dSTR) relative to Chrimson transfection and optical stimulation in the ipsilateral VTA, SNc, and SNr. Ventral striatum (vSTR), artificial cerebrospinal fluid (aCSF).

Figure III.2

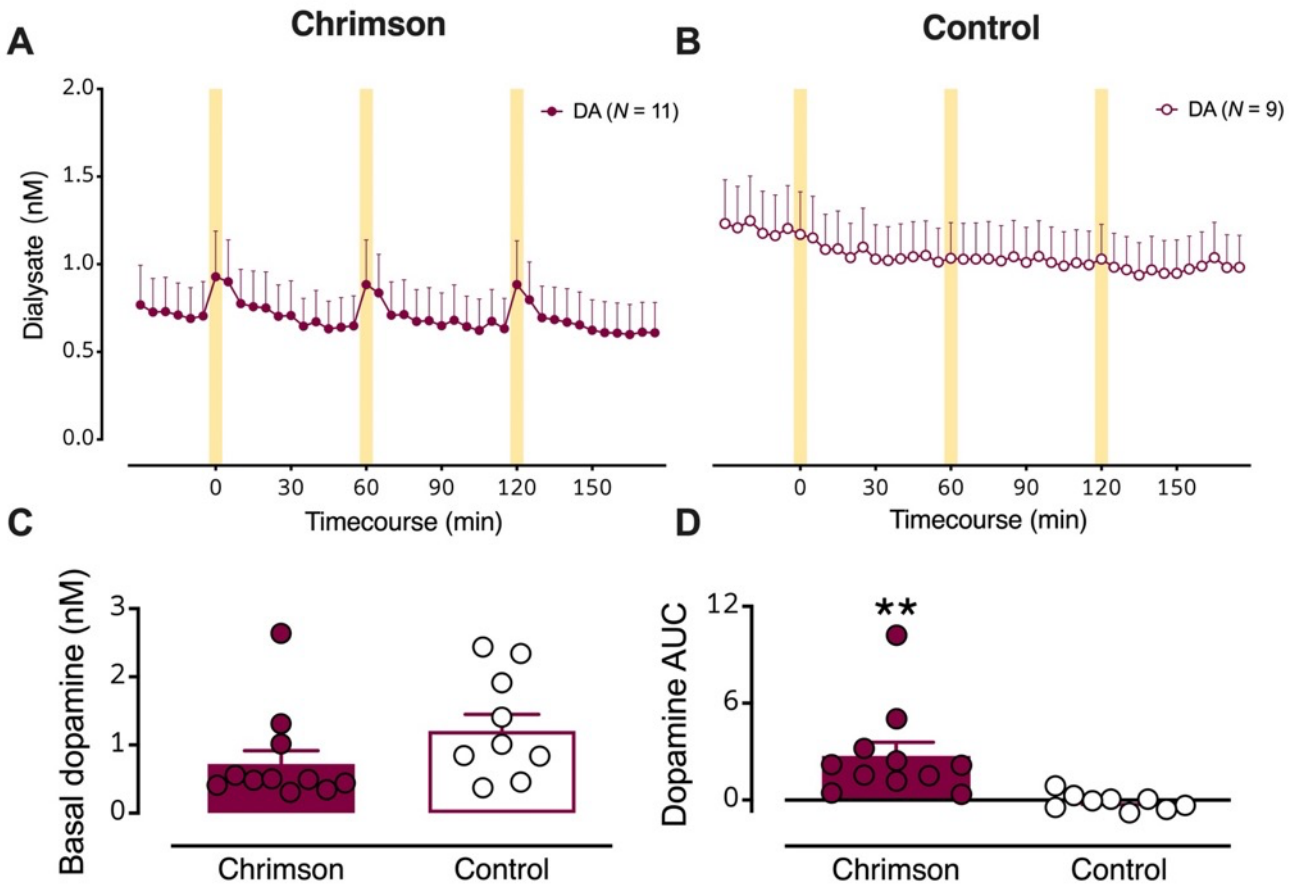

Figure III.2: Optical stimulation of dopaminergic cell bodies produces dopamine release in striatal terminal regions. **A.** Dialysate dopamine levels were increased in response to optical stimulation in mice expressing Chrimson (*N*=11) **B.** but not in control mice (*N*=9). The yellow bars indicate optical stimulations (10 mw/mm^2, 50 ms pulse width @ 10 Hz for 5 min). **C.** Basal dopamine levels in mice transfected with Chrimson relative to control mice. **D.** Dopamine overflow, quantified by area under the curve, was increased in Chrimson expressing but not control mice. Data are means ± SEMs. ***P*<0.01.

Figure III.3

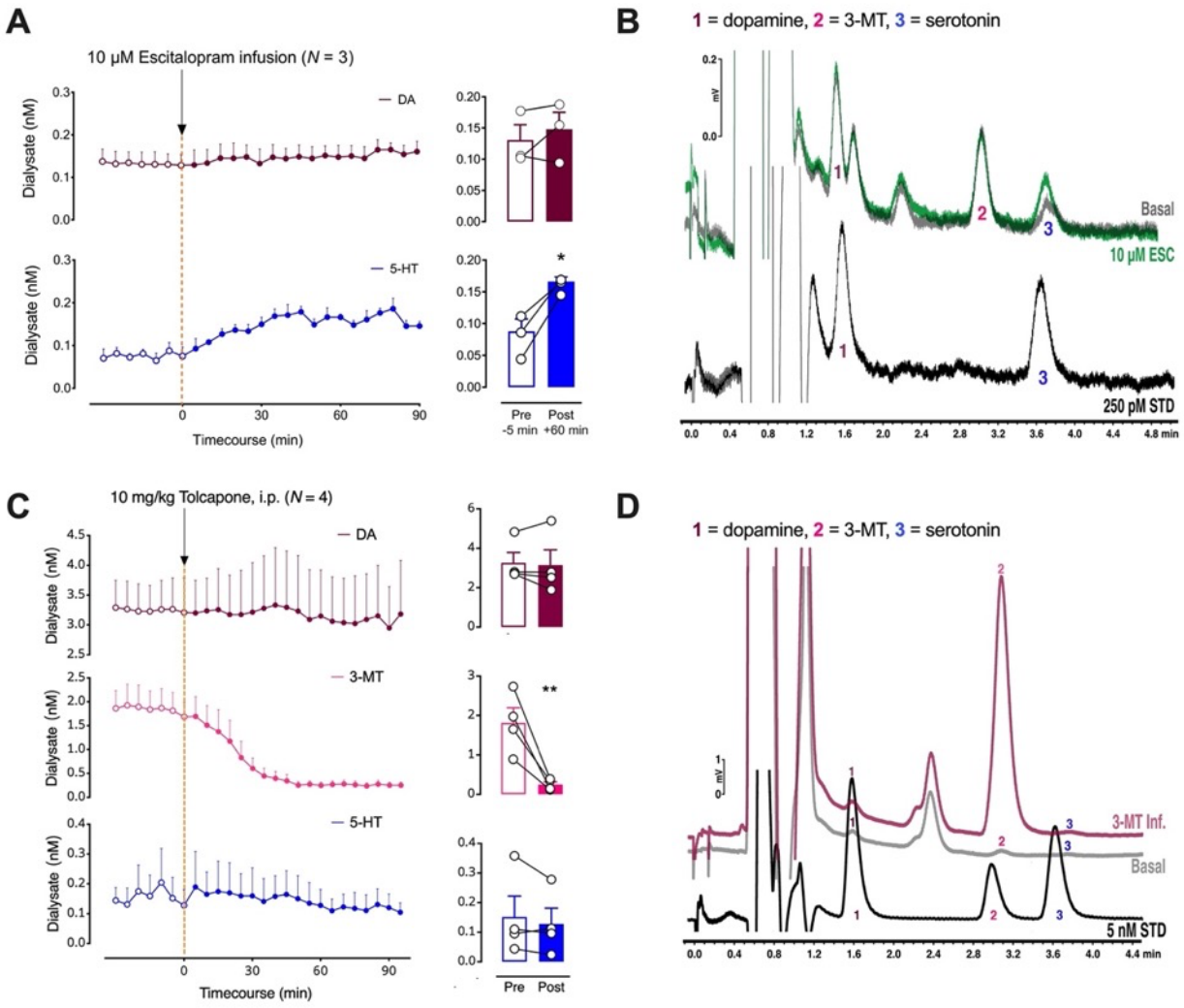
A
10 µM Escitalopram infusion (N = 3)
DA
5-HT
Dialysate (nM)
Timecourse (min)
Pre -5 min
Post +60 min
*
B
1 = dopamine, 2 = 3-MT, 3 = serotonin
Basal
10 µM ESC
250 pM STD
C
10 mg/kg Tolcapone, i.p. (N = 4)
DA
3-MT
5-HT
**
Pre
Post
D
1 = dopamine, 2 = 3-MT, 3 = serotonin
3-MT Inf.
Basal
5 nM STD

Figure III.3: A. The left panel shows the effects of intrastriatal perfusion of 10 μM escitalopram on dopamine (DA; top, red) and serotonin levels (5-HT; bottom, blue). Basal serotonin levels were significantly increased after escitalopram administration (right). **B.** Representative chromatograms showing dopamine (peak 1), 3-MT (peak 2), and serotonin (peak 3) from a control mouse under basal conditions (gray) and during perfusion of the selective serotonin reuptake inhibitor (SSRI) escitalopram (green). Peak 3 showed a large increase in response to local delivery of the SSRI suggesting that this peak was serotonin. A standard containing 500 pM dopamine (peak 1) and serotonin (peak 3) is overlaid in black. **C.** The left panel shows the effects of systemic administration of tolcapone, a catechol-*O*- methyltransferase (COMT) inhibitor on dopamine (DA; top, red), 3-methyltyramine (3-MT; middle, pink), and serotonin levels (5-HT; bottom, blue). The enzyme COMT converts dopamine to 3-MT. Only 3-MT (pink) was significantly reduced after tolcapone administration (right). **D.** Representative chromatograms showing dopamine (peak 1), 3-MT (peak 2), and serotonin (peak 3) after the intrastriatal perfusion of 50 nM 3-MT (red) *vs.* a basal dialysate sample from the same control mouse (gray). Reverse dialysis of 3-MT confirms peak 2 as 3-MT. A standard containing 5 nM dopamine (peak 1), 3-MT (peak 2), and serotonin (peak 3) is overlaid in black. Data in A and C are means ± SEMs. **P*<0.05, ***P*<0.01. A peak sometimes appearing between peaks 1 and 2 was not responsive to optical stimulation or high K^+ perfusion, therefore, we did not attempt to identify this peak.

Figure III.4

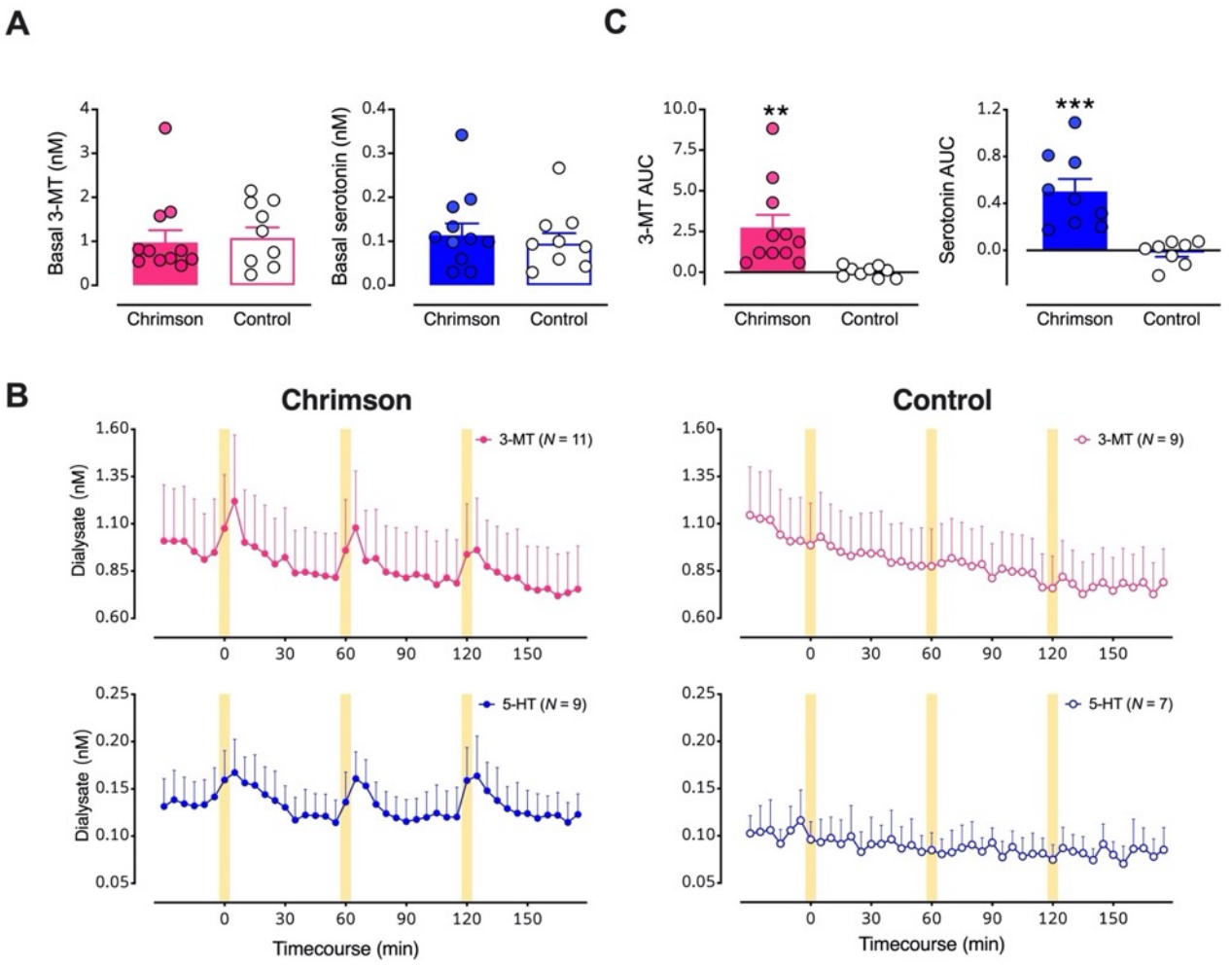

Figure III.4: Optical stimulation of midbrain dopamine neurons evokes overflow of 3-methyltyramine (3-MT) and serotonin in dorsal striatum (dSTR). A. Basal dialysate levels of 3-MT (left, pink) and serotonin (right, blue) in mice with *vs.* without Chrimson transfection. **B.** Time course of stimulated 3-MT (pink) and serotonin (blue) in mice transfected with Chrimson (left) compared to mice transfected with a control protein (right). Yellow bars indicate 5-min optical stimulations. **C.** Comparisons of areas under the curve (AUC) for the overflow of 3-MT or serotonin produced by optical stimulation of dopamine neurons expressing Chrimson with respect to control mice. Data are means ± SEMs. $^{**}P<0.01$, $^{***}P<0.001$. In two mice per group, data for serotonin were below the detectable limit.

Figure III.5

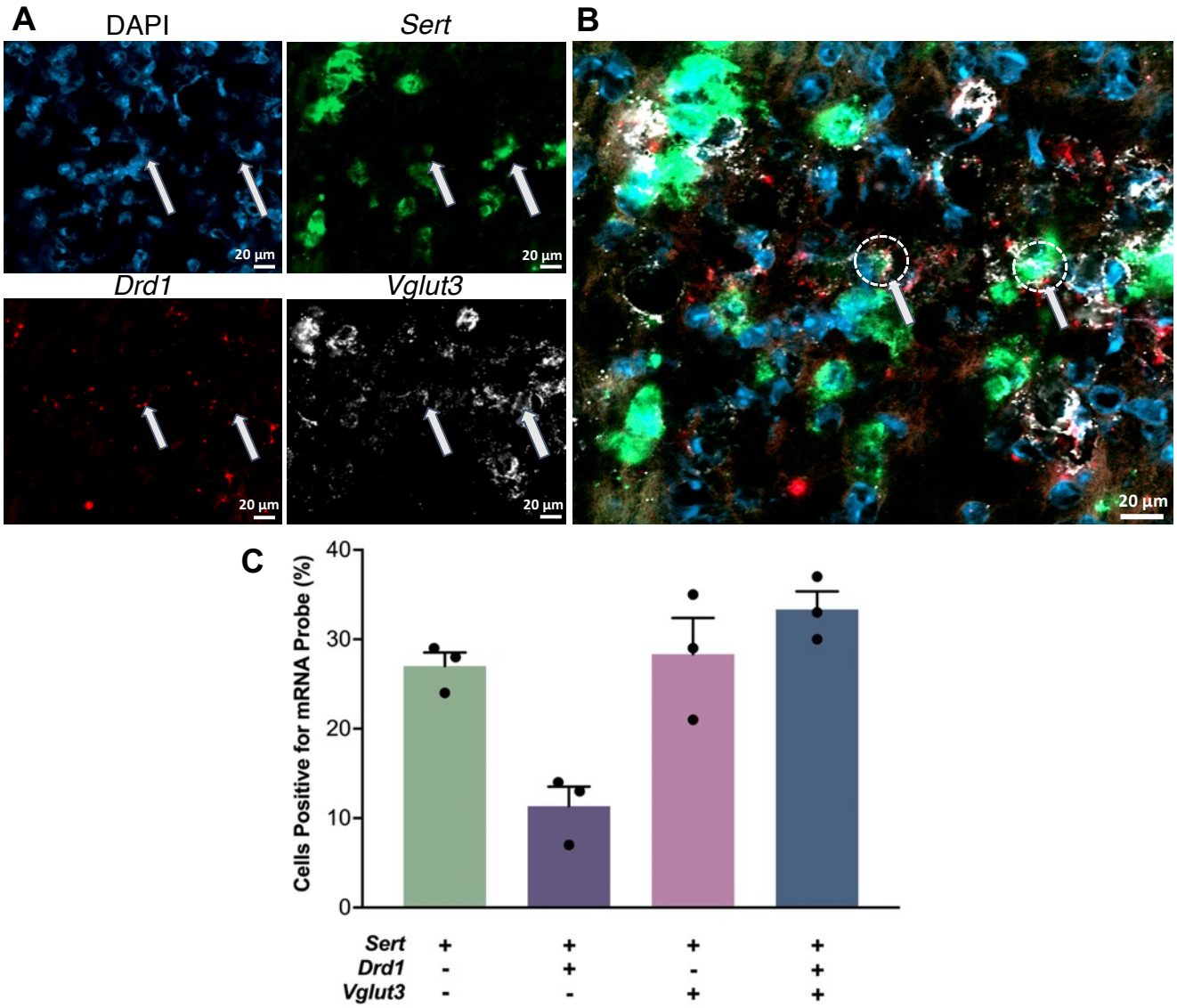

Figure III.5: Co-localization of serotonin transporter (*Sert*), D1 dopamine receptor (*Drd1*), and vesicular glutamate transporter 3 (*Vglut3*) mRNA in the dorsal raphe nucleus. A. Cell nuclei were stained with DAPI (top left, blue). Antisense probes to localize *Sert* (top right, green), *Drd1* (bottom left, red), and *Vglut3* (bottom right, white) mRNA were visualized. Puncta for each mRNA were colocalized in some nuclei but did not necessarily overlap. **B.** Overlay of images in A. Arrows indicate examples of the three mRNAs colocalized in the same nuclei. **C.** Relative quantification of cells containing *Sert*, *Drd1*, and *Vglut3* mRNA with respect to the total number of *Sert* expressing cell bodies. (SEMs are for *n*=3 z-stack planes in a single mouse. A total of 248 cells were counted).

Figure III.6

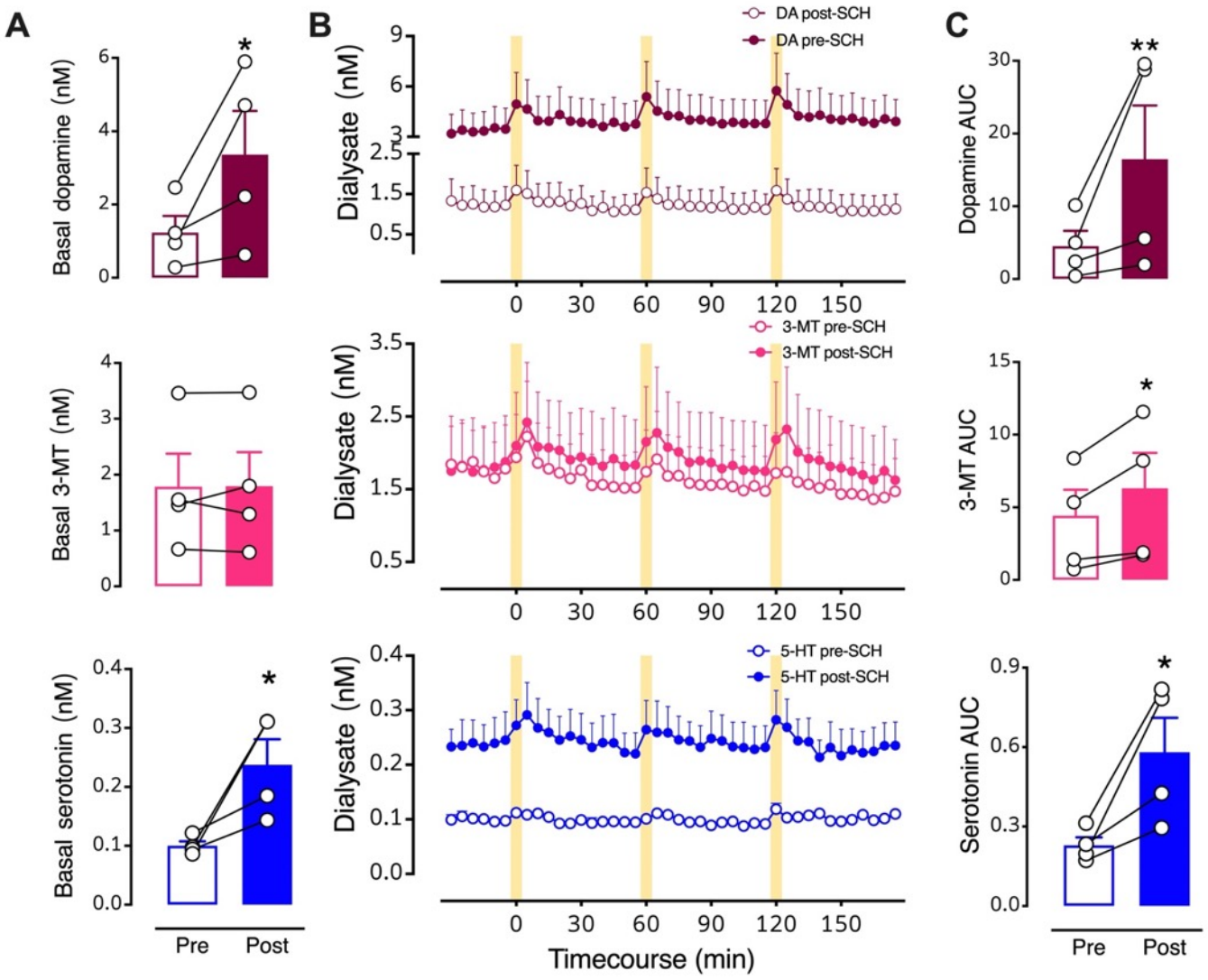

Figure III.6: Intrastriatal perfusion of a D1-like receptor inhibitor. A. Basal levels for the three neurochemicals pre- *vs.* post-SCH 23390. **B.** Time courses before and during intrastriatal perfusion of 100 μM SCH 23390 showing optically stimulated increases in dopamine (red, top), 3-methyltyramine (3-MT; pink, middle), and serotonin (blue, bottom). **C.** Area under the curve (AUC) comparisons of overflow induced by optical stimulation prior to (Pre) and during (Post) SCH 23390 striatal perfusion. Following three pre-drug stimuli, SCH 23390 was perfused for 90-120 min in each mouse prior to the first post-drug stimulation (see Fig. III.1A for timeline). The data 30 min prior to the first post-drug stimulus were used to calculate post-drug basal levels. The drug was continuously perfused throughout the post-drug stimulation period. Data are means ± SEMs. Some error bars in B cannot be seen due to scale. **P*<0.05, ***P*<0.01 pre- *vs.* post- initiation of drug perfusion. *N*=4 mice. Each basal data point in A represents the mean of six measurements taken just prior to the first pre- or post-drug stimulation (errors not shown). The AUC data points in C are means of each of the three stimuli (errors not shown).

Figure III.7

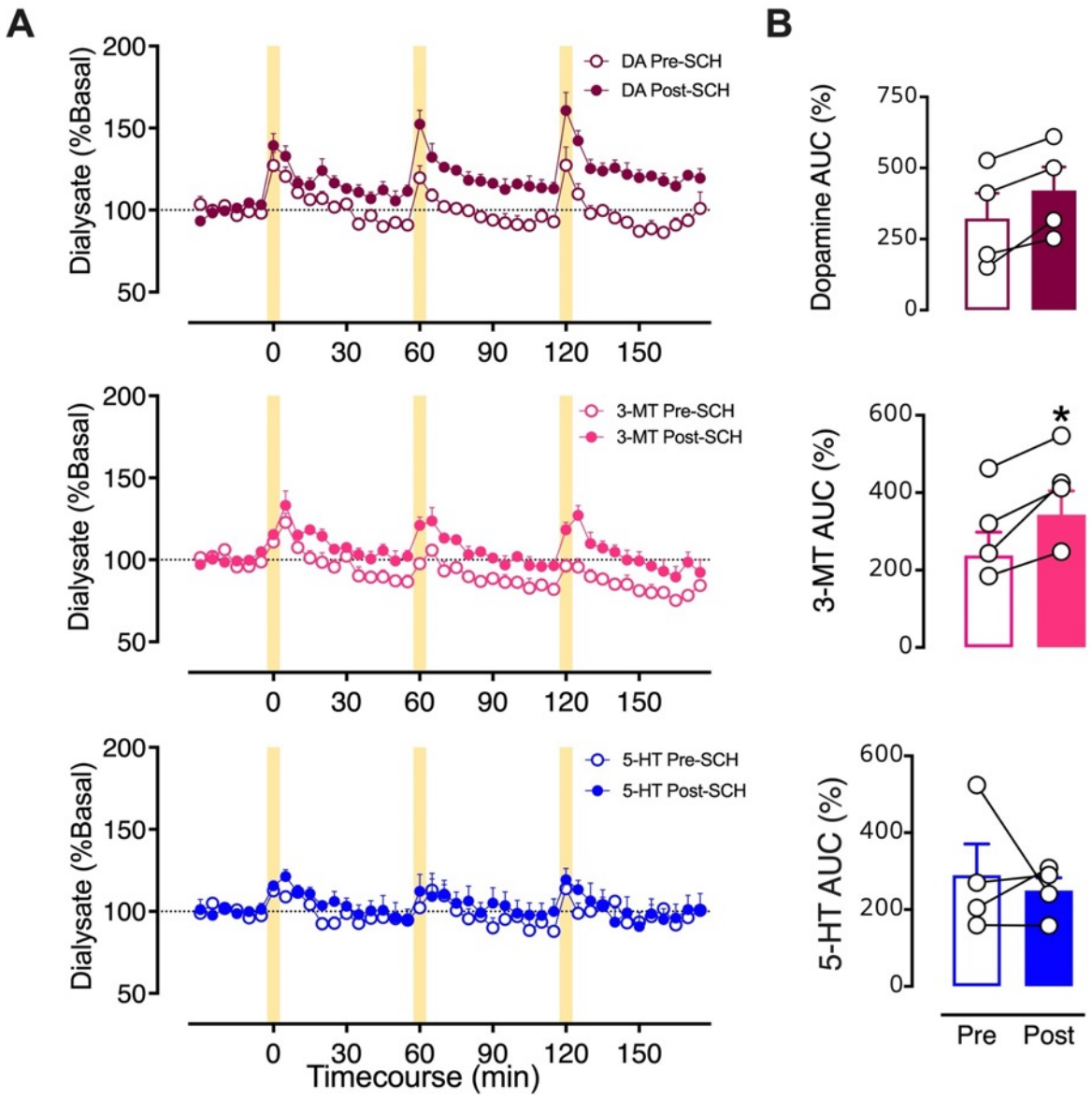

Figure III.7: Effects of intrastriatal perfusion of a D1-like receptor inhibitor analyzed with respect to pre-stimulation basal levels. A. Time courses of optically stimulated neurochemical levels before and during intrastriatal perfusion of SCH 23390 (100 ⍰M). **B.** Optically evoked overflow expressed as area under the curve for data normalized to pre-stimulation basal levels (AUC (%)). Data are means ± SEMs. *N*=4 mice. **P*<0.05. The AUC datapoints in C are means of the three stimuli for each mouse (errors not shown).

Figure III.8

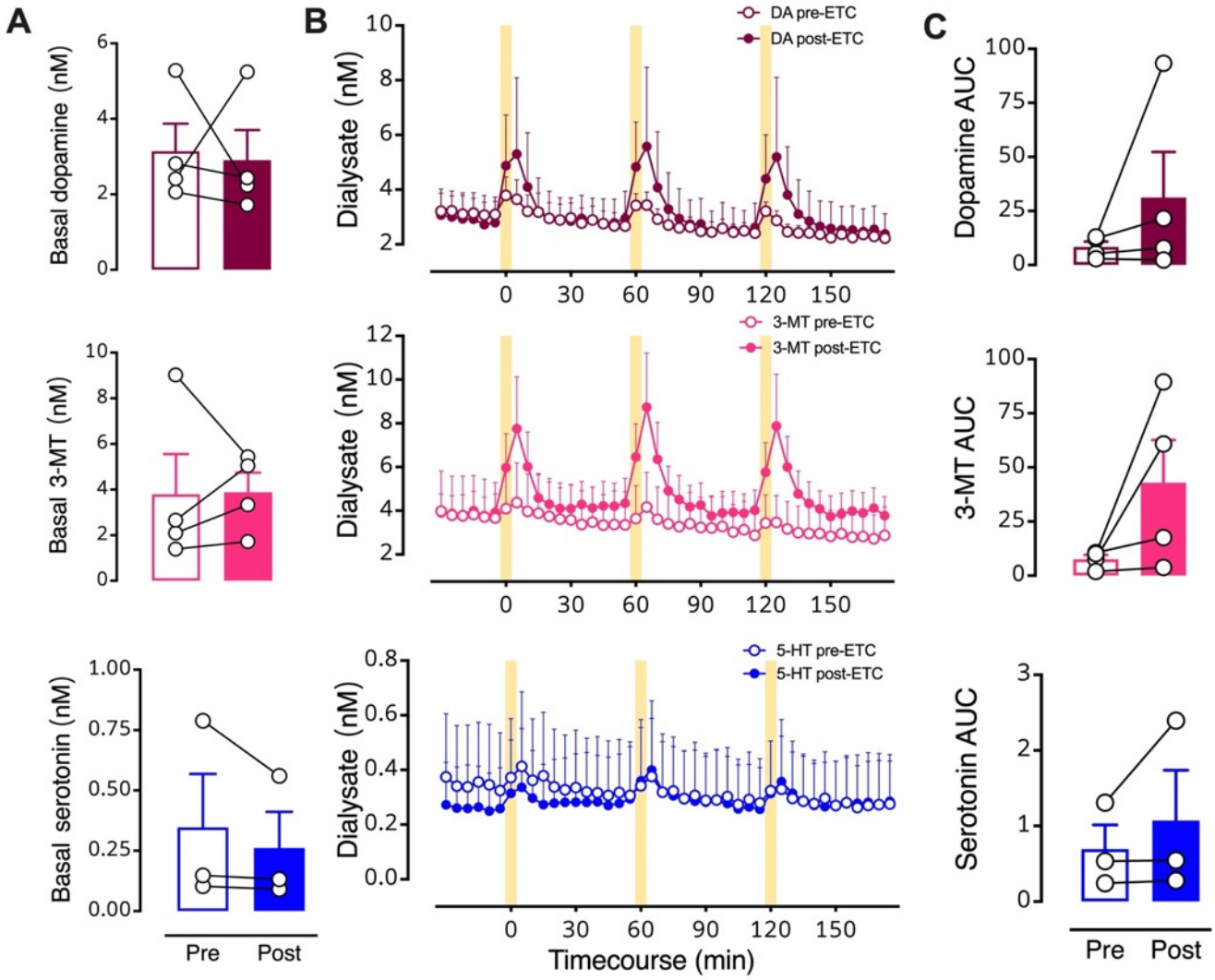

Figure III.8: Intrastriatal perfusion of a D_2 antagonist does not prevent optically evoked serotonin. **A.** Pre- *vs.* post-eticlopride basal levels of all three neurochemicals. **B.** Time course before and during intrastriatal infusion of the D2-like receptor inhibitor eticlopride (100 ?M). **C.** Areas under the curve (AUC) for optically stimulated neurochemical release prior to (Pre) and during (Post) eticlopride perfusion into striatum. Data are means ± SEMs for *N*=4 mice. Serotonin levels for one mouse were not detectable. Basal data points in A represent the means of six measurements just prior to the first stimulation (errors not shown). The AUC datapoints in C represent the means of three stimuli (errors not shown).

Figure III.9

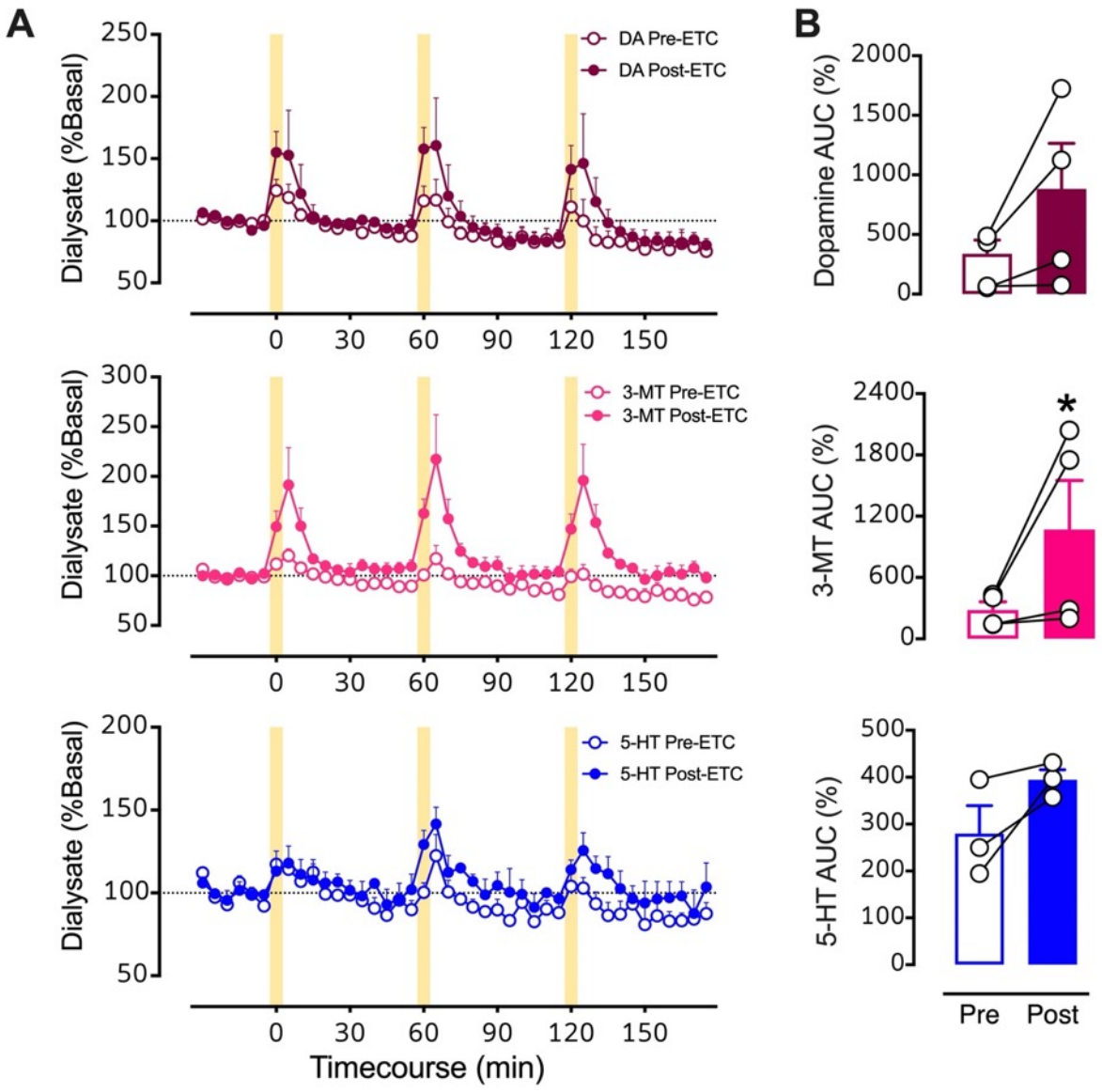

Figure III.9: Effects of intrastriatal perfusion of a D_2 antagonist analyzed with respect to pre-stimulation basal levels. A. Time courses before and during intrastriatal perfusion of eticlopride (100 □M) showing basal and stimulated neurochemical levels expressed as percents of respective pre-stimulation basal levels. **B.** Optically evoked overflow expressed as area under the curve for data normalized to pre-stimulation basal levels (AUC (%)). Data are means ± SEMs for *N*=4 mice. Serotonin levels for one mouse were not detectable. **P*<0.05 *vs.* pre-drug. %Basal data points in A represent the means of six measurements just prior to the first stimulation (errors not shown). The AUC datapoints in C are the means of the three stimuli (errors not shown).

Figure III.10

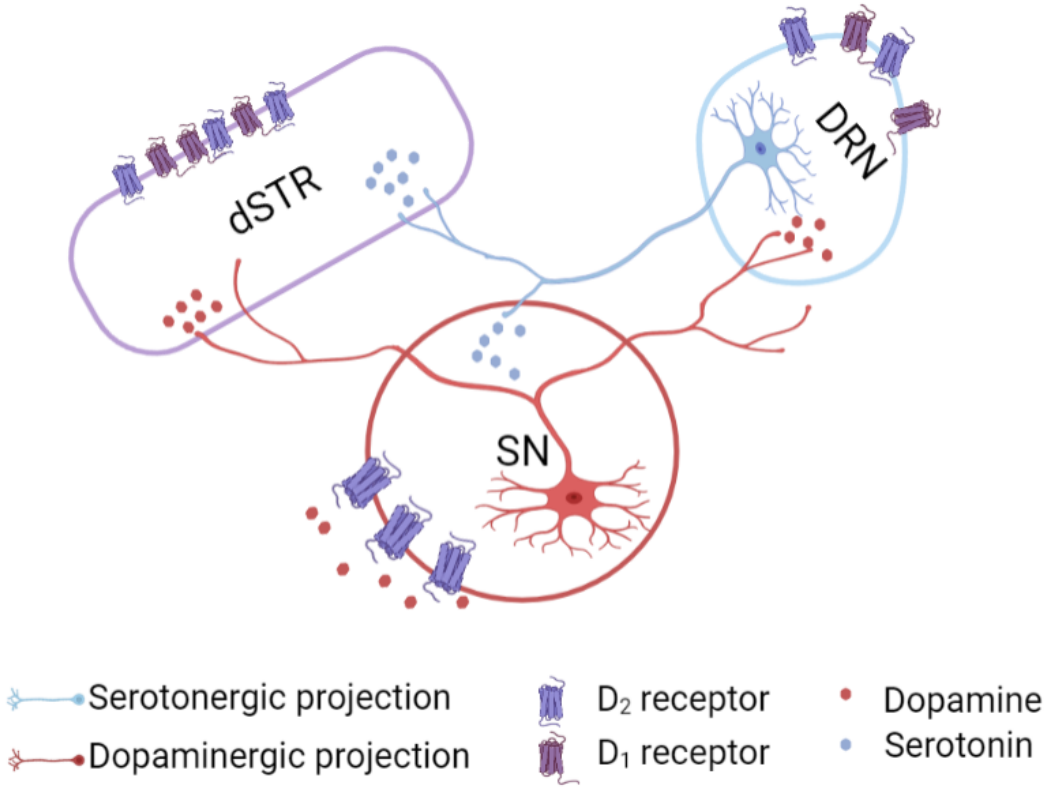

Figure III.10: Proposed mechanisms of dopamine-mediated serotonin release. The substantia nigra (SN) sends dense dopaminergic projections to the striatum (nigrostriatal pathway) and to the dorsal raphe nucleus (DRN).[1] The DRN sends serotonergic projections to dopaminergic cell bodies in the SN and to the striatum.[2] We found that optical activation of midbrain dopamine neurons produces striatal serotonin release that was not blocked by striatal D1- or D2-like receptor inhibition. Another possible mechanism for dopamine-mediated serotonin release is that an optogenetically induced increase in dopamine in the SN, which promotes D2 somatodendritic autoreceptor activation[3] and subsequent disinhibition of serotonin cell bodies in the DRN, produces serotonin release in the striatum. Alternately, optically induced dopamine release in DRN could act *via* local D1 or D2-like receptors to increase the probability of firing of DRN serotonin neurons projecting to dSTR.

Figure III.S1

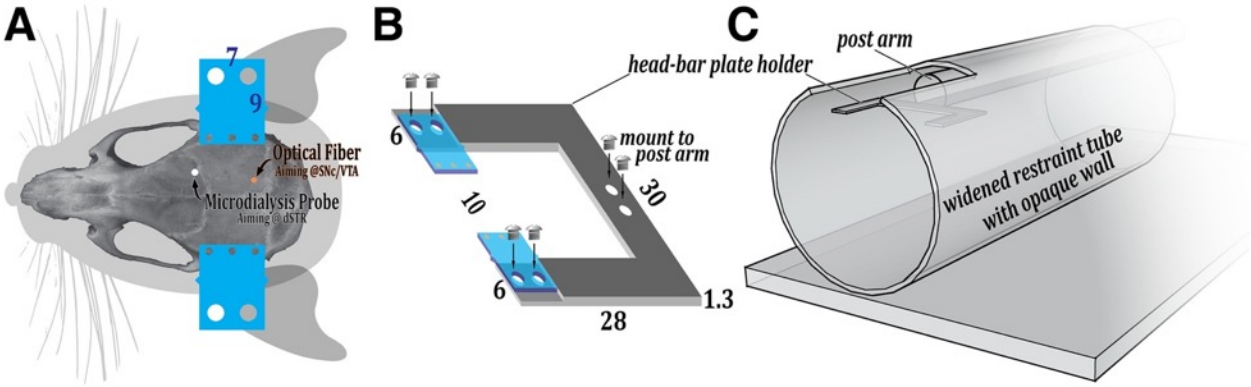

Figure III.S1: Head-fixed recording set-up. A. Schematic showing the locations of the head-bar implants (in blue and to scale) and the stimulation (stim) and recording (dSTR) site craniotomies relative to a mouse skull. **B.** Schematic of the head-bar plate holder (in gray and to scale). The head-bar plate holder was 30 mm long, 28 mm wide, and 1.3 mm thick. The mini-plates, which attach the holder to the head bars, were 9 mm long, 7 mm wide, and 0.65 mm thick, with a 10 mm gap between them. **C.** Schematic of the custom head-fixed tube used for fast microdialysis recordings with optical stimulation. The restraint tube (2" diameter), constructed of opaque (black) plexiglass, provided loose restraint to reduce spontaneous and stimulated physical movement, which can evoke movement-induced dopamine release artifacts in dorsal striatum.

Figure III.S2

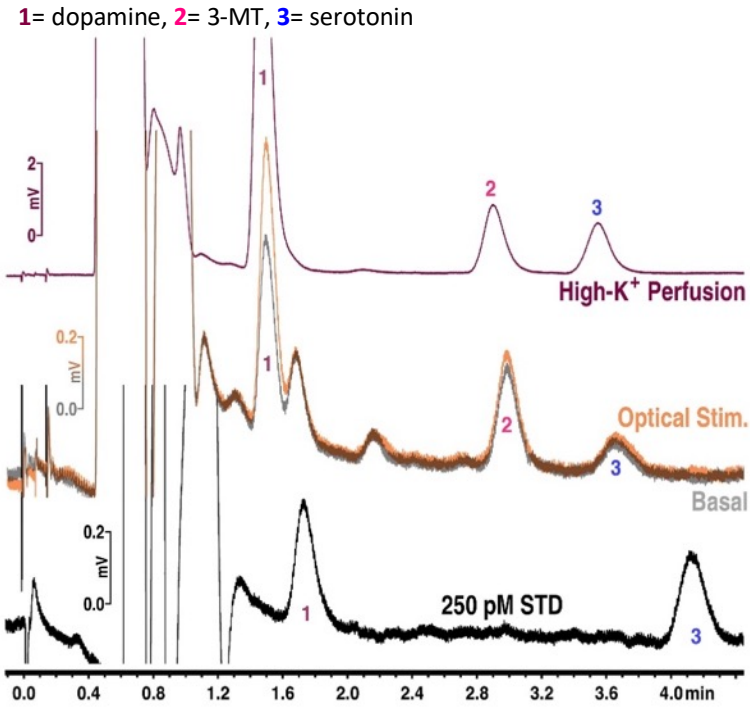

Figure III.S2: Optical stimulation of midbrain dopamine neurons increases in striatal serotonin and 3-methoxytyramine. Representative chromatograms from a Chrimson-transfected mouse under basal conditions (gray), and in response to optical stimulation (orange) or high-K^+ perfusion (red). Both optical stimulation and high-K^+ perfusion induced increases in neurochemicals (peaks 2 and 3), in addition to dopamine (peak 1). Chromatogram of a standard containing 250 pM dopamine (peak 1) and serotonin (peak 3) is shown in black. Peaks 2 and 3 in the dialysate samples could not be definitively identified based on comparison with retention times in the standard chromatogram.

Figure III.S3

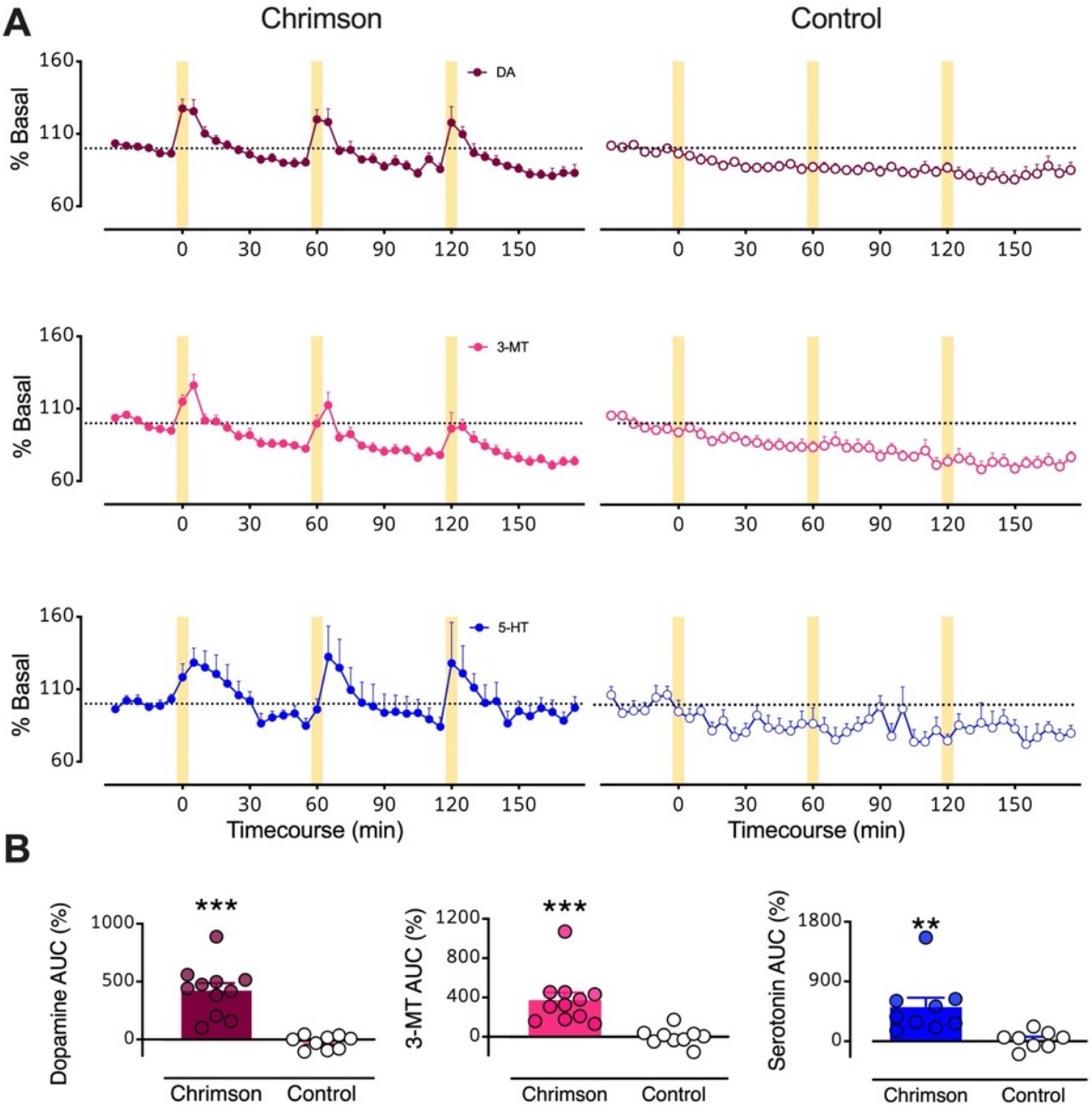

Figure III.S3: Normalized responses to optical stimulation. A. Time courses of %basal dialysate levels for DA (top, red), 3-MT (middle, pink), and serotonin (5-HT; bottom, blue) in mice expressing Chrimson (left) *vs.* mice transfected with a control protein (right). Optically induced overflow of dopamine, 3-MT, and serotonin were only detected in the Chrimson animals. **B.** The magnitudes of overflow are represented as areas under the curve percent (AUC (%)). Dialysate serotonin concentrations were below the detectable threshold in 2/11 Chrimson mice and 2/9 control mice. The yellow bars indicate optical stimulations (5 min). $^{**}P<0.01$ and $^{***}P<0.001$.

Figure III.S4

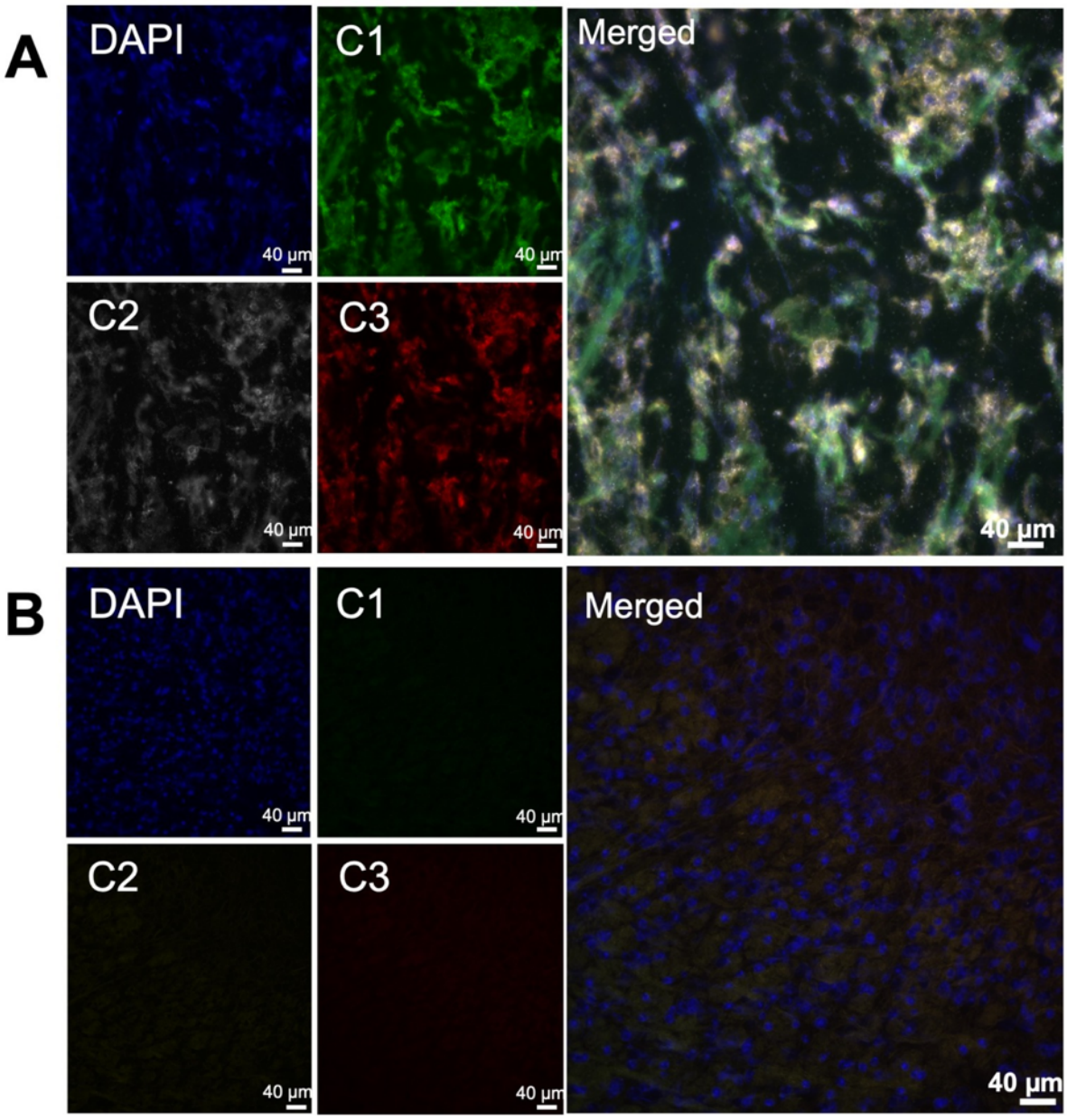

Figure III.S4: RNAscope *in situ* hybridization controls in dorsal raphe. A.The RNAscope® Multiplex Fluorescent Assay as a 3-plex positive control. The RNA polymerase II subunit RPB1 (Polr2a, C1 channel), cyclophilin B (PPIB, C2 channel), and ubiquitin C (UBC, C3 channel) are mRNAs found in all mouse cells. Cell nuclei stained by DAPI are shown in blue. The overlay is shown on the right **B.** The RNAscope® Multiplex Fluorescent Assay as a 3-plex negative control. A probe for DapB, an mRNA that codes for a reductase enzyme from *Bacillus subtilis*, was used in all three channels with each of the opal dyes to evaluate background staining.

Figure III.S5

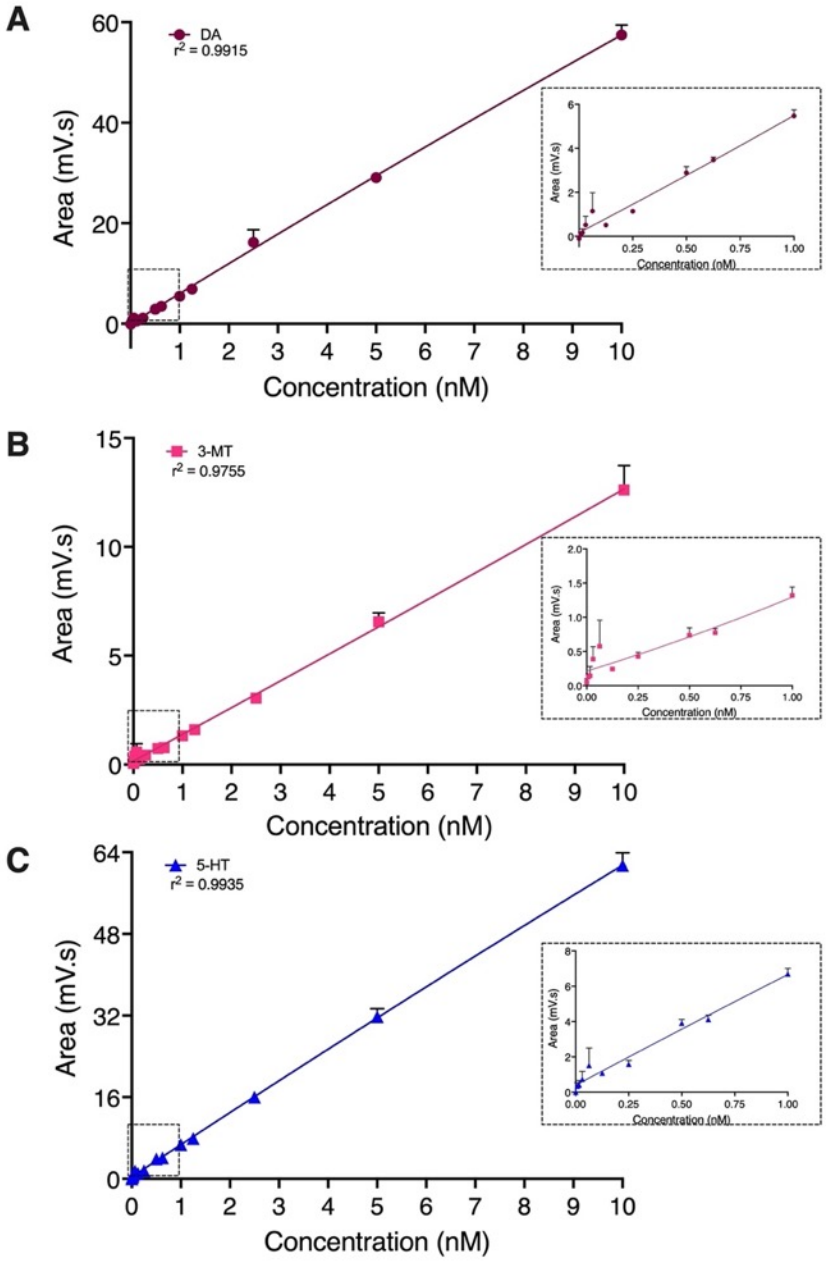

Figure III.S5: Standard curves for dopamine, 3-methyltyramine 3-MT), and serotonin. Fourteen standards (0 nM, 0.008 nM, 0.016 nM, 0.032 nM, 0.063 nM, 0.125 nM, 0.250 nM, 0.500 nM, 0.625 nM, 1 nM, 1.25 nM, 2.5 nM, 5 nM, and 10 nM) were injected into the HPLC (20 µL volumes) to create standard curves. Insets are zoomed in on the lower concentrations ranging from 0-1 nM. Quadratic curve-fits were applied to **A.** dopamine, **B.** 3-MT, and **C.** serotonin standards. Each point represents *N*=3 replicates measured on different days. Error bars (standard errors of the means) are too small to be visualized in some cases.

Table III.S1

Table S1: Statistical summary

FIGURE	COMPARISON	TEST	RESULTS	SIGNIFICANT?
2C	Basal DA: control *vs.* Chrimson	Unpaired two-tailed t-test	t (18)=1.6; *P*>0.1	No
2D	**AUC DA: control *vs.* Chrimson**	**Unpaired two-tailed t-test**	**t (18)=3.0; *P*<0.01**	**
3A	DA: 5 mins pre- *vs.* 60 mins post-ESC	Paired two-tailed t-test	t (2)=0.92; *P*>0.4	No
3A	**5HT: 5 mins pre- *vs.* 60 mins post-ESC**	**Paired two-tailed t-test**	**t (2)=5.7; *P*<0.05**	*
3C	DA: 5 mins pre vs. 60 mins post TOL	Ratio paired two-tailed t-test	t (3) = 0.83; *P*>0.46	No
3C	**3MT: 5 mins pre vs. 60 mins post TOL**	**Ratio paired two-tailed t-test**	**t (3) = 9.6; *P*<0.01**	**
3C	5HT: 5 mins pre vs. 60 mins post TOL	Ratio paired two-tailed t-test	t (3) = 1.3; *P*>0.29	No
4A	Basal 3MT: control *vs.* Chrimson	Unpaired two-tailed t-test	t (18)=0.27; *P*>0.7	No
4A	Basal 5HT: control *vs.* Chrimson	Unpaired two-tailed t-test	t (18)=0.52; *P*>0.6	No
4C	**AUC 3MT: control vs. Chrimson**	**Unpaired two-tailed t-test**	**t (18)=3.1; *P*<0.01**	**
4C	**AUC 5HT: control vs. Chrimson**	**Unpaired two-tailed t-test**	**t (15)=4.4; *P*<0.001**	***
6A	**Basal DA: pre- *vs.* post-SCH**	**Ratio paired two-tailed t-test**	**t (3)=4.4; *P*<0.05**	*
6A	Basal 3MT: pre- *vs.* post-SCH	Ratio paired two-tailed t-test	t (3) = 0.17; *P*>0.87	No
6A	**Basal 5HT: pre- *vs.* post-SCH**	**Ratio paired two-tailed t-test**	**t (3) = 3.5; *P*<0.05**	*
6C	**AUC DA: pre- *vs.* post-SCH**	**Ratio paired two-tailed t-test**	**t (3) = 6.2; *P*<0.05**	**
6C	**AUC 3MT: pre- *vs.* post-SCH**	**Ratio paired two-tailed t-test**	**t (3) = 4.8; *P*<0.05**	*
6C	**AUC 5HT: pre- *vs.* post-SCH**	**Ratio paired two-tailed t-test**	**t (3) = 4.5; *P*<0.05**	*
7B	AUC (%) DA: pre vs. post SCH	Ratio paired two-tailed t-test	t (3) = 2.4; *P*<0.1	Trend
7B	**AUC (%) 3MT: pre vs. post SCH**	**Ratio paired two-tailed t-test**	**t (3) = 3.7; *P*<0.05**	*
7B	AUC (%) 5HT: pre vs. post SCH	Ratio paired two-tailed t-test	t (3) = 0.41; *P*>0.71	No
8A	Basal DA: pre vs. post ETC	Ratio paired two-tailed t-test	t (3) = 0.31; *P*>0.78	No
8A	Basal 3MT: pre vs. post ETC	Ratio paired two-tailed t-test	t (3) = 0.81; *P*>0.47	No
8A	Basal 5HT: pre vs. post ETC	Ratio paired two-tailed t-test	t (2) = 2.7; *P*>0.11	No
8C	AUC DA: pre vs. post ETC	Ratio paired two-tailed t-test	t (3) = 1.5; *P*<0.23	No

8C	AUC 3MT: pre vs. post ETC	Ratio paired two-tailed t-test	t (3) = 3.1; *P*<0.06	Trend
8C	AUC 5HT: pre vs. post ETC	Ratio paired two-tailed t-test	t (2) = 1.4; *P*>0.28	No
9B	AUC (%) DA: pre vs. post ETC	Ratio paired two-tailed t-test	t (3) = 2.6; *P*<0.08	Trend
9B	**AUC (%) 3MT: pre vs. post ETC**	**Ratio paired two-tailed t-test**	**t (3) = 4.4; *P*<0.05**	*
9B	AUC (%) 5HT: pre vs. post ETC	Ratio paired two-tailed t-test	t (2) = 1.8; *P*>0.21	No
S3B	**AUC (%) DA: control vs. chrimson**	**Unpaired two-tailed t-test**	**t (18) = 5.9; *P*<0.001**	***
S3B	**AUC (%) 3MT: control vs. chrimson**	**Unpaired two-tailed t-test**	**t (18) = 4.1; *P*<0.001**	***
S3B	**AUC (%) 5HT: control vs. chrimson**	**Unpaired two-tailed t-test**	**t (15) = 3.1; *P*<0.01**	**

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Chapter IV

Serotonin Transmission in Responses to Ambiguous Cues

Introduction and Background

The Diagnostic and Statistical Manual of Mental Disorders (DSM) characterizes anxiety disorders by "excessive anxiety and worry (apprehensive expectation), occurring more days than not for at least 6 months."[1] A hallmark of increased anxiety in humans is a greater tendency to interpret uncertainty or ambiguity as negative.[2-6] Increased negativity around uncertainty is a key facet of the personality trait neuroticism, which is one of the core personality traits. Neuroticism refers to experiencing high negative emotion.[7] Individuals who score higher on measures of neuroticism experience feelings of anxiety, depression, anger, and vulnerability to stress more often and/or to a greater degree than low-scoring counterparts.

High scores on neuroticism personality trait measurement subscales—typically in combination with low scores on extraversion—are associated with greater risk for developing an anxiety disorder.[6] Higher neuroticism may decrease confrontation of irrational fears, thus contributing to the development and maintenance of anxiety disorders.[8] Moreover, epidemiological studies show that individuals with anxiety disorders score higher on measures of neuroticism compared to healthy individuals.[9-11] Lastly, many studies have found a significant association between higher neuroticism and serotonin transporter (SERT) promoter polymorphisms.[7] Taken together, these findings suggest a link between negative emotions associated with anxiety, negative bias linked to ambiguity, and the serotonin system.

Mood and anxiety disorders are associated with additional variations in the serotonin system.[12] For example, genome-wide association studies (GWAS) and candidate gene studies have shown that the serotonin system plays an important role in mood and anxiety

disorders.[13,14] While studies show that polymorphisms in SERT predispose some individuals,[15] stronger evidence comes from research on selective serotonin reuptake inhibitor (SSRI) antidepressants. The SSRIs are the most commonly prescribed medications for the treatment of major depressive and anxiety disorders. The SSRIs prevent reuptake of serotonin into presynaptic neurons *via* SERT blockade.[16] Though not effective for all individuals, administration of SSRIs reproducibly improves positive mood outcomes compared to placebo drugs.[17]

Rodent models have corroborated the role of serotonin in depression and anxiety disorders. Many studies have found that disrupting the serotonin system leads to anxiogenic phenotypes. For example, using a constitutive SERT knockout model, Holmes *et al.* and Altieri *et al*. found that mice lacking SERT expression (SERT-/-) show elevated anxiety-related phenotypes in two behavior tests, the elevated plus maze (EPM) and the open field test (OFT).[18-20] The SERT-/- mice also have a reduced ability to extinguish a learned association between unconditioned-conditioned stimuli pairings in fear conditioning.[21] Conversely, mice overexpressing SERT show reductions in anxiety-related behavior in the EPM[22] and reductions in freezing behavior to an ambiguous-cue pairing in a fear conditioning assay.[23]

Using a $5HT_{1A}$ receptor knockout line of mice, which have been extensively shown to exhibit increased anxiety-like behavior,[24] Klemenhagen found that $5HT_{1A}$-/- mice exhibited increased freezing behavior following fear conditioning in a similar, but not identical context, as wildtype mice.[25] This similar environment had the same spatial cues as the original fear conditioning context but differed in olfactory and tactile cues. As such, the similar environment was ambiguous. These findings and others support the idea that the serotonin

system, particularly SERT and $5HT_{1A}$ receptors, plays an important role in anxiety-related behavior, and possibly, in assigning emotional valence to ambiguous environmental or contextual cues.

While differences in neuroticism and SERT expression may be important predictors of risk for developing anxiety or mood disorders, they neither cause depression nor anxiety disorders *per se*. Moreover, neither can be used to predict individual risk. Many factors have been identified as having associations with the onset and development of affective and anxiety disorders. However, all of the currently identified risk factors are factors of low (small) effect that work in complex association with other factors, many of which have yet to be identified.

Three meta analyses have found significant associations between carriers of the *5-HTTLPR* short '*S*' allele and neuroticism scores compared to individuals homozygous for the long '*L*' allele.[7] Moreover, functional magnetic resonance imaging has shown that variations in alleles of *5-HTTLPR* relate to variations in limbic structures *i.e.*, the amygdala and hippocampus, in response to threatening stimuli.[26-28] Impaired connectivity of these limbic structures with areas important for inhibitory control, *i.e.*, prefrontal cortex, are also linked with increased neuroticism scores.[29] Thus, while no direct study has examined brain region variation in individuals with variations in *5-HTTLPR* alleles and their respective neuroticism scores, overlap can be inferred.[7]

The *5-HTTLPR* '*S*' allele has been associated with 30-50% reductions in SERT expression and function.[30-34] By comparison, heterozygous SERT-deficient mice (SERT+/-) express 50% lower transporter levels, while SERT-/- mice show an absence of functional SERT expression compared to wildtype littermates.[35,36] Serotonin uptake rates are likewise

reduced.[37,38] Behaviorally, SERT-/- mice display increased avoidance of anxiogenic environments,[39] a robust phenotype that has been consistently observed.[40] The SERT+/- mice are generally characterized by an intermediate phenotype compared to wildtype littermates.[41]

In this chapter, I describe my initial work to investigate how mice with heightened anxiety-like behavior respond to ambiguous cues. I used an associative learning paradigm called fear conditioning,[42] which is based on classical conditioning first reported by Pavlov in the early 20th century. The same terms used to describe classical conditioning have been adapted for fear conditioning.[43]

Fear conditioning involves learning an association between a neutral stimulus and an unconditioned stimulus (US).[12] A neutral stimulus, *e.g.*, tone or light, evokes no inherent behavioral response. By contrast, an unconditioned stimulus, *e.g.*, foot shock, evokes a naturally occurring (unlearned, automatic) behavioral response. In the case of fear conditioning, the unconditioned stimulus specifically evokes a fear-related response, *e.g.*, freezing or darting[44,45] in rodents. Once an association is learned, the neutral stimulus is "conditioned". The conditioned stimulus (CS) now produces the same behavior as the unconditioned stimulus.

In fear conditioning, experimenters can change the predictive probability of pairing. A tone (CS) can always predict a shock (US), sometimes predict a shock, or never predict a shock. In cases where the CS always predicts the US, the CS is referred to as a "perfect" cue; it will always be followed by an aversive stimulus. When a CS never predicts a footshock (US), the CS is not expected to produce freezing behavior. Finally, in the condition where a CS is sometimes predictive of a shock, the CS is referred to as a "partial" cue.

My goal was to establish the translational validity of aversion to ambiguity to study intolerance to uncertainty in rodent models of stress-related disorders. I hypothesized that a genetic model of increased anxiety-like behavior will be associated with increased freezing or darting behavior to ambiguous cues. I studied mice with constitutive loss of expression of the serotonin transporter (SERT-/- mice), which show increased anxiety-related behavior.[20] Due to the COVID-19 pandemic, the number of cohorts of mice I could test was limited. In the future directions portion of this chapter, I outline a comprehensive study strategy for future experiments.

Materials and Methods

Animals

The SERT-deficient mice were generated as described previously[35] on a mixed 129S6/SvEv × CD-1 background. In the present study, mice produced in-house were weaned at 21 days of age and housed in groups of 2-4 same-sex siblings per cage in a temperature- and humidity-controlled room with food and water available *ad libitum* (12 h light/dark cycle, lights on at 0600 h). The SERT-deficient mice were generated by mating SERT+/- (heterozygote) females with SERT+/- males. The SERT+/- parent pairs were selected based on open-arm behavior in a pre-screening elevated plus maze (EPM) test. Female and male mice displaying behavior in the middle quantiles of historical data were selected for mating.

A total of 24 mice were studied herein. Individual group sizes are detailed in the figures. The Association for Assessment and Accreditation of Laboratory Animal Care International has fully accredited UCLA. All animal care and use met the requirements of the NIH Guide for the Care and Use of Laboratory Animals, 2011. The UCLA Chancellor's Animal Research Committee (Institutional Animal Care and Use Committee) preapproved all animal procedures.

Elevated Plus Maze

The elevated plus maze was used to assess avoidance of anxiogenic environments.[24,46] A single maze was used throughout the study having two opposing open arms (30 cm length x 5 cm width) with a 0.5-cm lip around the edges of the open arms to prevent animals from falling. The maze also had two opposing closed arms (30 cm length x 5 cm width x 15 cm height) and a center platform (5 cm x 5 cm) and was raised 38.5 cm from the floor. The walls of the closed arms were constructed of clear Plexiglas to ensure even light levels across all

arms. The floor of the maze was constructed of a continuous piece of red opaque Plexiglas. Mice were placed on the center platform facing a closed arm and allowed to explore the maze freely for 5 min. I cleaned the maze with Accel Solution and dried between same-sex mice. When switching between male and female mice, I cleaned the apparatus with 70% ethanol solution followed by Accel Solution.

Behavior was videotaped and visually scored using the ANYmaze behavioral tracking system (Stoelting Co., Wood Dale, IL). Parameters quantified included latency to first open arm entry, open arm distance, %open-arm time, and %open arm distance. Time in the open arms was analyzed as a percentage of total arm time excluding time spent in the center zone of the maze. Likewise, distance traveled in the open arms was analyzed as a percentage of total arm distance excluding the distance traveled in the center zone of the maze.

Fear Conditioning

Fear conditioning chambers in the UCLA Behavioral Core were used for these experiments. Mice were transferred from the Gonda vivarium to the Pritzker Hall vivarium at least one week in advance of behavior testing. On the day of an experiment, animals were transferred from the Pritzker Hall colony room to a holding room, where they were acclimated for at least 30 min. Immediately before experiments, animals were transferred to the behavior testing room. Fear conditioning chambers were cleaned with Strikeback solution before and between animals. One scent, *e.g.*, Simple Green, was used in association with the conditioning chambers on conditioning days and a different scent, *e.g.*, Windex, was used on the fear memory recall (FMR) day.

Tones were adapted from McHugh *et al.*, where the authors used three different tones to predict different CS-US pairings and to investigate freezing behavior in mice constitutively

overexpressing SERT.[23] In this study, the three types of tones were white noise, 2900 Hz, and 7000 Hz, all at 72 dB. Shock intensity was set to 0.3 mA in preliminary experiments and subsequently raised to 0.5 mA. The 0.3 mA shock was used in the first cohort of mice. Because animals did not appear to be displaying freezing behavior based on tracking software output, the shock intensity was increased to 0.5 mA for the two subsequent cohorts.

Tones were randomly assigned to ambiguous, aversive, and null pairings, *i.e.*, CS20%, CS+, and CS-, respectively. Each tone was presented a total of five times over a 30-min testing session. The first 5 min and the last 3 min of the testing session did not have any tones or shocks. The total paradigm consisted of one day of habituation to the fear conditioning apparatus (day 1, pre-exposure or PE), three training days (days 2-4, T1-T4), and one testing day (day 5, FMR). On FMR day, no shocks were administered.

Freezing behavior was analyzed using Med Associates VideoFreeze software (Med Associates, St. Albans, VT) . Variables including baseline freezing behavior, freezing during the 30-s period just prior to and the 30-s period just after each tone was presented, and freezing behavior while tones were presented were analyzed. Batch reports were exported, and data analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). A detailed protocol can be found in the Andrews' Lab Dropbox.

Results

A statistically significant increase in open arm latency was observed in SERT+/- *vs.* SERT+/+ groups (**Fig. IV.1A**). Increased sample sizes would have been needed to make comparisons involving the SERT-/- group, which only had two mice. Other parameters for avoidance behavior measured were open arm distance, % open arm time, and % open arm distance. No differences were seen between groups (**Fig. IV.1B-D**). The SERT+/- and SERT-/- groups require increased sample sizes to draw statistical conclusions.

The EPM apparatus and conditions of these experiments were not optimized. Most often, we use an EPM with a black floor, which has a historical average of ~20% open arm time. The red-floored EPM used here showed an average % open-arm time for the wildtype SERT+/+ group above 50% (**Fig. IV.1C**). While my starting point was replicating the open arm time % from previous studies, it is possibly that 50% open arm time was the baseline for the SERT+/+ mice. As noted by Andrews *et al*., determining proper experimental conditions for different strains of mice is more important than replicating previous conditions in other studies.[47] The first step for future studies is to increase the sample size in SERT+/- and SERT-/- groups and see if the observed trends remain the same. Future studies to may also seek to increase avoidance of the open arms in the red-bottomed EPM by increasing the light intensity, *e.g.*, using three lamps in the Gonda behavior room, or increasing the contrast between the bottom and the floor, *e.g.*, putting white construction paper below the apparatus.

Due to low sample sizes for the SERT+/- and SERT-/- groups, I did not attempt to correlate EPM and fear conditioning data. However, if these EPM experiments are repeated in the future and similar baselines for the SERT+/+ group are observed in % open arm time,

then data can be combined for increased sample sizes across groups. Replication of preliminary findings, *i.e.*, decreased time and distance in the open arms in SERT-/- mice, and increased sample size are two important steps for future studies.[48] Moreover, given the historical ~20% averages were observed in (1) adult offspring from commercially purchased CD-1 dams, and (2) SERT-deficient animals at Penn State University, the SERT-deficient animals at UCLA may display differences in baseline open arm activity.[47,48]

For the fear conditioning experiments, all genotypes froze to the CS+ (**Figure IV.2**). For SERT+/+ mice, the main effect of day was significant. Post-hoc analysis indicated that on the pre-exposure (habituation) day, freezing responses to the CS+ and CS- tones significantly differed. No day differences were observed for the CS- tone, suggesting that the animal freezing response did not change over time and that the unpaired tone did not result in freezing (**Fig. IV.2A**). The SERT+/+ mice froze significantly more to the CS+ tone on training day 3 than habituation day, suggesting that they learned the association between CS+ tone and footshock. No differences were seen for the CS20% tone in the SERT+/+ animals across day.

In the case of the SERT+/- animals, a significant effect of day but not tone was observed. The SERT+/- animals froze at equal levels to the CS+ and CS- tones (**Fig. IV.2B**), suggesting the animals did not differentiate between tones and did not learn the association between tone and footshock. In the second cohort, which was the only cohort that included SERT+/- mice, a higher shock intensity was focused on (0.5 mA *vs.* 0.3 mA) and the CS20% was omitted for simplicity.

For the SERT-/- mice, significant effects of day and tone were observed. On training day 3, there were significant differences in freezing response to the CS+ *vs.* CS- and CS20%

vs. CS- tones. Moreover, the SERT-/- mice froze significantly more to the CS20% tone on training day 3 than pre-exposure day, suggesting that the animals had learned the association between ambiguous tone and footshock. Increased sample sizes for SERT+/- and SERT-/- groups are necessary before drawing conclusions. Moreover, the VideoFreeze software frequently mistook grooming behavior for freezing behavior, thereby potentially confounding the data.

Animals were also tested for contextual conditioning. Here, freezing in response to the footshock context (conditioning apparatus) was quantified during the first 5 min (prior to the delivery of CS-US pairing) on the habitation, training, and memory recall days (**Fig. IV.3**). On the FMR day, the environmental cues were different than the training days. Thus, the first 5 min of FMR freezing behavior was quantified and compared to that of freezing behavior in the footshock environment. Significant effects of genotype and day were observed for habituation *vs.* training days, and for habituation *vs.* testing day. This suggests that animals overgeneralized fear, such that they froze even in the absence of environmental cues where they learned footshock-tone pairings.

Future studies may benefit from hand-scoring these videos to assess the accuracy of VideoFreeze at delineating freezing from grooming. Future experiments could switch to using mice on the B6 background instead of the CD1 background. Anecdotally, we observed that the CD1 background mice display less freezing behavior compared to B6 mice. While these preliminary data suggest differences with respect to genotype, repetition of cohorts using 0.5 mA shock intensity should be done to increase sample size in groups, particularly SERT+/- and SERT-/- groups. While I did run female mice (N=4, data not shown), I did not visually observe darting behavior.

Future Directions

Aim 1

This project on investigating the relationship between ambiguity aversion and anxiety-related behavior was conceptually developed for my doctoral oral examination. The data above were collected in support of proposed aim 1.1, in which investigating freezing or darting behavior in response to ambiguous cues was proposed with respect to SERT-deficient genotypes. For aim 1.2, studies were proposed to investigate ambiguity aversion associated with stress-induced increases in anxiety using chronic immobilization stress with or without SSRI treatment.

The interplay between stress and stress-related disorders is complex.[49] Stress is an important risk factor for mood and anxiety disorders and numerous studies show that acute and chronic stress can have adverse outcomes on anxiety- and depressive-like behavior.[50] Chronic stress, in particular, causes changes to brain morphology and circuitry by inducing neuroinflammation, overactivating the hypothalamic-pituitary-adrenal (HPA) axis, and increasing cortisol levels.[50] Stress also leads to social isolation, drug abuse, and poverty, which pose additional risk factors for developing depression or anxiety disorders. Thus, stress is an important environmental factor to study when examining the susceptibility increased anxiety and depressive-like phenotypes.

While some molecular mechanisms associated with the adverse effects of stress have been characterized, others are yet to be uncovered. Despite not having a full picture of how stress contributes to mood and anxiety disorders, stress has repeatedly been documented to be a trigger for their onset.[52,53] Because of this, chronic stress paradigms are used in rodent

studies to model one of the significant risk factors associated with depression- and anxiety-related disorders.[54]

Preliminary findings from our group and others[19] suggest that SERT+/- mice may have increased sensitivity to stress indicated by increased anxiety-related behavior. Ultimately examining freezing or darting behavior of SERT-deficient mice will not only add to the existing literature regarding understanding the relationship between SERT function and anxiety-related behavior, but it will highlight important sex differences in behavioral responses to ambiguous cues. Moreover, if SERT+/- mice have increased negative bias to ambiguity, the spectrum of SERT expression may be used to model a component of neuroticism, complementary to human findings.[7]

Aim 2

The second aim of the proposed project was to investigate serotonin encoding of information about ambiguous cues by examining serotonin transmission in the median raphe nucleus to the ventral hippocampus pathway. The ventral hippocampus (vHPC) has been shown to play a role in anxiety-related behavior and freezing responses to ambiguity. Kjeistrup *et al.* found that lesioning the vHPC, but not the dHPC, impacts anxiety-related behavior in the EPM, *i.e.*, increases open arm behavior.[55] Jimenez *et al*. showed that optogenetic inhibition of glutamatergic pyramidal neurons in the vHPC increased open arm behavior in the EPM indicative of decreased anxiety-related behavior.[56] Lastly, Altieri *et al.* showed that increased *vs.* decreased EPM-anxiety behavior correlated with increased *vs.* decreased vHPC extracellular serotonin.[20] Findings from these three studies implicate the vHPC in the modulation of anxiety-related behavior.

Beyond neuroanatomical studies, other studies have shown that the serotonin system modulates vHPC function related to anxiety and ambiguity. Ohmura *et al.* found that optogenetically activating serotonergic neurons that project from the MRN increased anxiety-related behavior on the EPM.[57] These authors also observed increases in extracellular serotonin, measured by microdialysis, in the vHPC, when MRN was optically activated. In a recently published follow-up study, Ohmura *et al.* found that selectively exciting serotonin terminals in the vHPC increased anxiety-related behavior.[58]

Two recent studies examined serotonin release in the hippocampus during different behavior tasks. Abela *et al.* reported that optogenetic activation of serotonin neurons in the MRN increased anxiety-related behavior measured in three different behavior tests, including the EPM.[59] The authors also observed increases in serotonin in the dHPC, measured by microdialysis; these authors did not examine serotonin release in the vHPC. Mice were anaesthetized during microdialysis measurements, which may reduce extrapolation to behavior in awake mice. In the 2019 study by Ohmura *et al.*, the authors investigated the effects of optically stimulating either DRN- or MRN-projecting serotonin neurons on anxiety and impulsivity-like behaviors.[58] Using freely moving and awake mice, these authors also determined serotonin release in the vHPC. They observed increases in extracellular serotonin upon stimulation of serotonergic terminals in the vHPC. Taken together, the preponderance of evidence points to an important serotonergic pathway from the MRN to the vHPC that will require additional investigation to elucidate behavioral modulation regarding ambiguity.

Anticipated results and interpretations

In my first proposed aim, three core questions will be answered. First, are constitutive reductions in SERT associated with increased freezing or darting behavior to an ambiguous cue? Preliminary data indicate yes, particularly in the SERT-/- group. Second, does chronic stress potentiate freezing or darting behavior to ambiguous cues? Third, does SSRI administration during chronic stress attenuate elevated anxiety and freezing or darting behavior in the EPM and fear conditioning, respectively? I hypothesize the answer is yes to all three questions. I hypothesize that elevated anxiety-related behavior in the EPM, will be correlated with elevations in freezing or darting behavior to ambiguous cues in the fear conditioning paradigm in genetic and environmental models. Furthermore, I hypothesize that SSRI treatment in combination with stress-induced anxiety will attenuate ambiguity aversion.

In the second aim, three additional core questions were posed. First, does inhibition of MRN serotonergic input to the vHPC reduce freezing or darting behavior to ambiguous cue pairings in fear conditioning? Second, is there a correlation between increased anxiety-related behavior and freezing or darting behavior to ambiguous cues during serotonergic inhibition? Third, does inhibition of serotonergic input into the vHPC attenuate elevated anxiety-related behavior and freezing or darting to ambiguous cues induced by chronic stress? Again, I hypothesize the answer to the aforementioned questions is yes.

While many studies have directly examined serotonin signaling in the vHPC as a modulator of anxiety-related behavior, *no study has looked at this signaling in relation to ambiguous stimuli.* Examining signaling in the vHPC would provide a direct pathway by which negative bias to ambiguity is processed in the brain.

Power analysis

To reduce overall animals for use in the proposed study, I recommend a within-subjects design. Below is a power analysis for each aim to indicate how many animals are suggested initially. Group sizes would be adjusted as the study progresses and information on actual standard deviations takes shape upon intermediate data analyses.

Aim 1.1: Genotype

A statistical power analysis was performed for sample size estimation based on Altieri et al. (*N*=24 mice/group) data.[20] The effect size (ES) in this study was 0.67, considered to be medium using Cohen's criteria. With an alpha=0.05 and power=0.80, the projected sample size needed with this effect size is approximately *N*=24 for the simplest group comparison. With *N*=24 and 6 groups [3 genotypes (SERT+/+, +/-, -/-) x 2 sexes], the total anticipated number of animals is 144 mice.

Aim 1.2: Stress

With an alpha=0.05 and power=0.80, the projected sample size needed with this effect size is approximately *N*=17 for the simplest group comparison. With *N*=17 and 8 groups [4 treatments (Control, CIS, ESC, ESC+CIS) x 2 sexes], the total anticipated number of animals is 136 mice.

Aim 2.1: Excitatory opsin transfection

With an alpha=0.05 and power=0.80, the projected sample size needed with this effect size is approximately *N*=16 for the simplest group comparison. With *N*=16 and 8 groups [2 treatments (Control, CIS) x 2 transfections (Chrimson-, Chrimson+) x 2 sexes], the total anticipated number of animals is 128 mice.

Aim 2.2: Inhibitory opsin transfection

With an alpha=0.05 and power=0.80, the projected sample size needed with this effect size is approximately *N*=16 for the simplest group comparison. With *N*=16 and 8 groups [2 treatments (Control, CIS) x 2 transfections (NpHR-, NpHR+) x 2 sexes], the total anticipated number of animals is 128 mice.

The total number of animals needed is 536 mice.

Figures

Figure IV.1

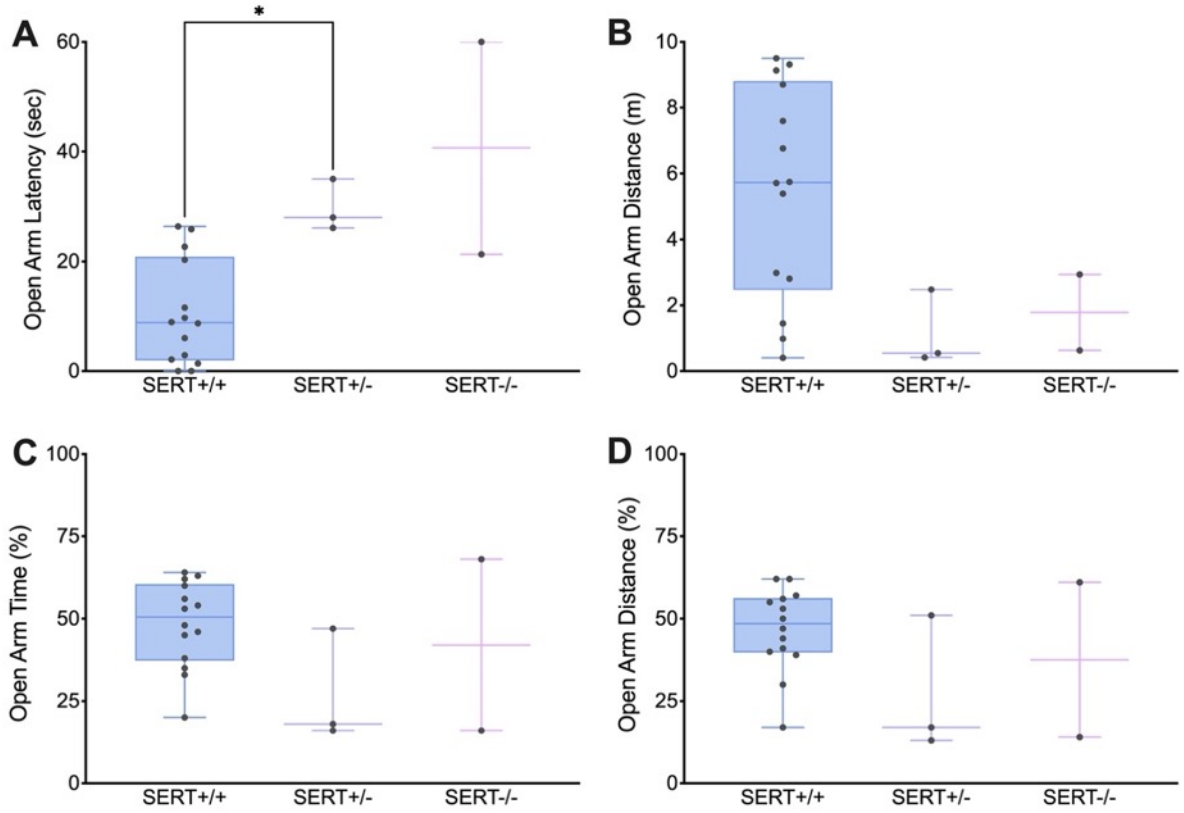

Figure IV.1. Elevated plus maze (EPM). Mouse behavior on the EPM with respect to serotonin transporter (SERT) genotype was evaluated by **A.** open arm latency, **B.** open arm distance, **C.** % open arm time (open arm time divided by total time spent in open and closed arms), and **D.** % open arm distance (open arm distance divided by open arm and closed arm distance). *N* = 15, 3, and 2 for SERT+/+ , SERT+/- , and SERT-/- male mice, respectively.

Figure IV.2

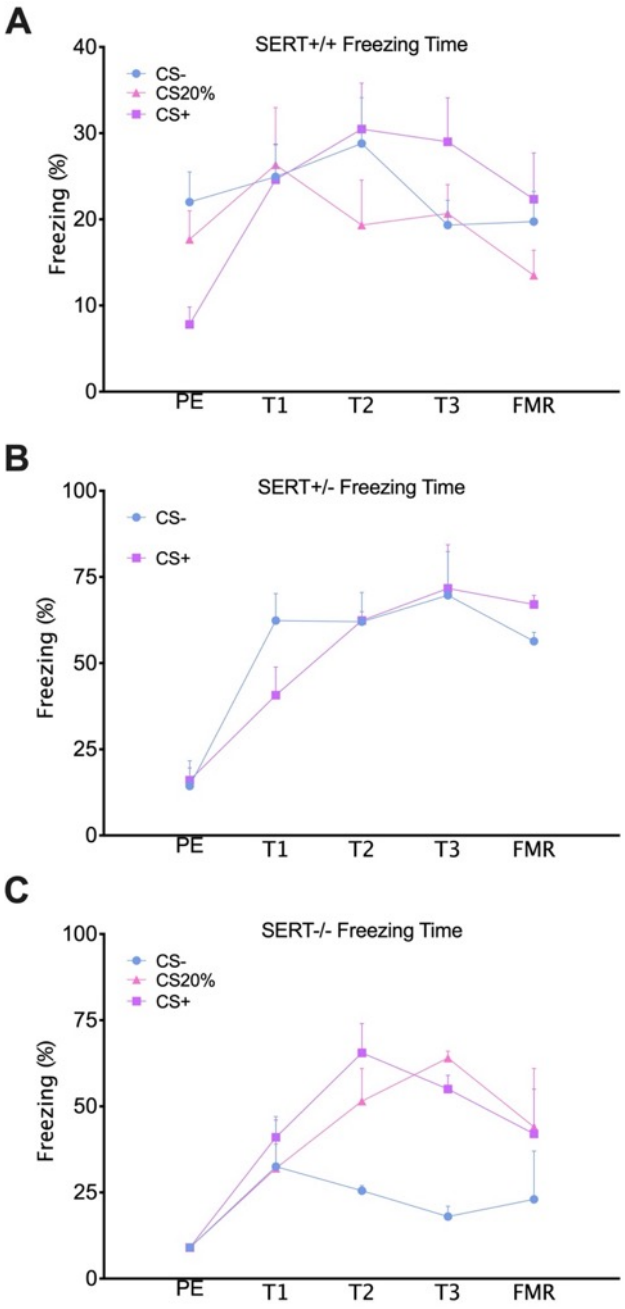

Figure IV.2. Percent freezing time to conditioned cues. Animal freezing time in response to CS+, CS-, and CS20% tones in **A.** SERT+/+, **B.** SERT+/-, **C.** and SERT-/- mice. N = 15, 3, and 2 for SERT+/+, SERT+/-, and SERT-/- male mice, respectively. No SERT+/- mice were conditioned to the CS20%. PE = pre-exposure; T1-3 = training days 1-3; FMR = fear memory recall

Figure IV.3

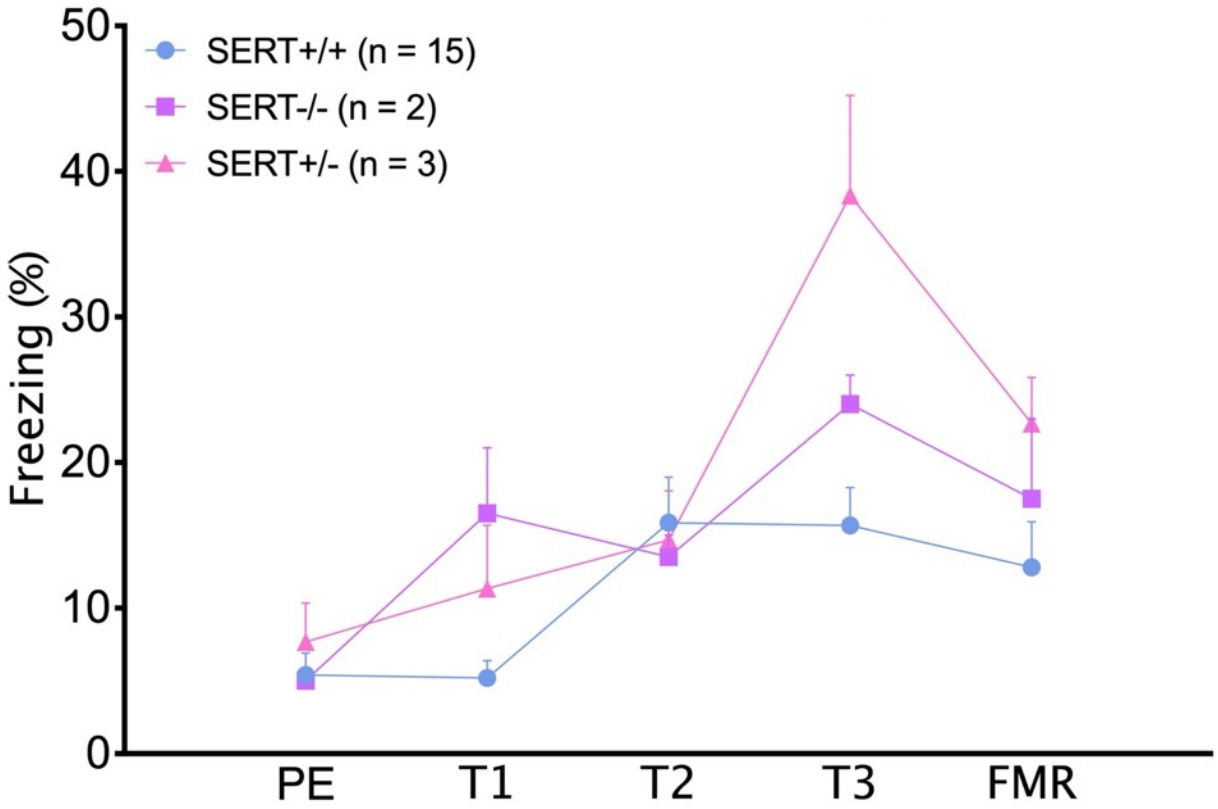

Figure IV.3. Percent freezing time to contextual cue. Animal freezing time during the first 5 minutes of each day, before any tones or shocks are presented. N = 15, 3, and 2 for SERT+/+, SERT+/-, and SERT-/- male mice, respectively.

Table IV.1

Figure	Comparison	Results
Figure 1A	***Genotype comparisons in open arm latency***	***Kruskal-Wallis test, P<0.01***
Figure 2A	Genotype comparisons in open arm distance	One-way ANOVA F (2,16) = 3.45; P<0.06
Figure 3A	Genotype comparisons in %open arm time	One-way ANOVA F (2,16) = 2.18; P>0.05
Figure 4A	Genotype comparisons in %open arm distance	One-way ANOVA, F (2,16) = 1.99; P>0.05
Figure 2A	***Freezing differences to tones in SERT+/+ mice***	***Mixed-effects ANOVA; significant main effect of day (P<0.05)*** *Day: F (3.26, 91.3) = 3.20; P<0.05* Tone: F (2, 33) = 0.18; P>0.05 Day x Tone: F (8, 112) = 1.34; P>0.05
Figure 2B	***Freezing differences to tones in SERT+/- mice***	***Two-way ANOVA; significant effect of day (P<0.001)*** Day x Tone: F (4, 16) = 1.73; P>0.05 *Day: F (1.94, 7.78) = 22.6; P<0.001* Tone: F (1, 4) = 0.035; P>0.05 *Subject: F (4, 16) = 3.27; P<0.05*
Figure 2C	***Freezing differences to tones in SERT-/- mice***	***Two-way ANOVA; significant effect of day (P<0.05) and tone (P<0.05)*** Day x Tone: F (8, 12) = 1.44; P>0.05 *Day: F (1.69, 5.07) = 7.99; P<0.05* *Tone: F (2, 3) = 29.95; P<0.05* Subject: F (3, 12) = 0.25; P>0.05
Figure 3	***Genotype comparisons in freezing response to context***	***Mixed effects ANOVA; significant effect of day (P<0.01) and genotype (P<0.05)*** *Day: F (2.46, 35.7) = 6.60; P<0.01* *Genotype: F (2, 17) = 3.67; P<0.05* Day x Genotype: F (8, 58) = 1.51; P>0.05

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Chapter V

Silicone neuroprobes are associated with reduced brain tissue injury compared to microdialysis probes

Introduction

Implantable devices enable direct and indirect neurochemical monitoring in vivo.[1-4] Common monitoring methods include microdialysis and voltammetry, which are used by our research group.[5-10] We have also developed silicon (Si) neuroprobes for multiplexed neurotransmitter detection *in vivo*.[11] Microfabricated neuroprobes have biosensors on their tips composed of field-effect transistors (FETs for signal transduction) coupled with aptamers (oligonucleotide receptors for target recognition).[12,13] When neurotransmitters (or other targets) bind to aptamers, their negatively charged oligonucleotide backbones undergo conformation changes that gate FET transconductance in a target-concentration-dependent manner.

Implanted devices, however, induce injury and inflammation at implantation sites that interfere with neurotransmitter measurements, particularly when recording over longer time periods (days to weeks to months).[14,15] Glial cells, which are key modulators of brain injury, include astrocytes, oligodendrocytes, and microglia. Glia outnumber neurons by a 3:1 ratio in mammalian brains.[16] Glial have complex morphologies and functions that contribute to the survival of neurons, speed of signal transmission, glutamate signalling, and the immune response of the central nervous system.[16]

Astrocytes and microglia have primarily been implicated in inflammatory responses to implantable devices.[15,17,18] While I focused on astrocytic and microglial responses, other cells are also important in physiological responses to implantable devices.[19] For example, in response to acute and chronic implantation of microelectrode arrays, Wellman *et al.* found that neuronal loss and reductions in oligodendrocyte densities occurred most markedly within 0-50 μm from probe implant sites.[19]

Astrocytes become hypertrophic and are upregulated at the site of implantation injury and are visualized *via* their cytoskeletal component—glial fibrillary acidic protein (GFAP).[20] Astrocytes form scars around brain injury sites, which prevent integration with adjacent brain areas, inhibit axonal regeneration, and reduce or prevent recording signals from surrounding neurons.[14,21] Microglia are also implicated in inflammatory responses to implantable devices and are the "first responders" to implants.[22] Microglia play diverse roles in many brain processes, *e.g.*, neurogenesis, dendritic pruning.[23] Microglia have been dubbed the "immune cells" of the central nervous system and in pathological conditions, they expand and become reactive.[23]. A number of different markers have been used to study quiescent and reactive microglia.[17] One pro-inflammatory microglial marker is CD11b, a protein involved in the integrin complement receptor on activated microglia.[17]

I carried out the preliminary experiments described in this chapter to begin to investigate astrocytic and microglial responses to our newly developed Si neuroprobes and to compare these responses to microdialysis probes used widely in neuroscience research. The commercially available cylindrical microdialysis probes we use have 240-μm diameter active membrane tips and are slightly larger than our first-generation Si neuroprobes (150 μm × 150 μm). Microdialysis membranes are constructed from biocompatible polyurethane (*vs.* Si).

I hypothesized that neuroprobes would induce lower inflammatory responses, *i.e.*, less astrocytic and microglial activation and aggregation around implantation sites than microdialysis probes, mostly due to the smaller cross section of the former. I investigated acute, *i.e.*, 4 h or 1-d, and chronic, *i.e.*, 1 week or 2 weeks, implantation durations to evaluate the temporal evolution of inflammatory responses to these different devices. My goal was to

begin to construct a roadmap for future studies by our group aimed at characterizing and minimizing implantation site injury to improve *in vivo* neurochemical recordings.

Materials and Methods

Animals Procedures

The SERT-deficient mice were generated as described previously[24] on a mixed 129S6/SvEv × CD-1 background. These mice were selected as we maintain an in-house colony and were not used for other studies. Male SERT+/+ mice (*N*=13) at 3-10 months of age were used for this study. Initially, nine mice were used for surgery and immunocytochemistry optimization. Images were taken for all mice, but only images from *N*=4 are shown in the figures (one mouse/figure). Mice were housed in groups of 2-5 same-sex siblings prior to surgery. Food and water were available *ad libitum* throughout. The light-dark cycle (12/12 h) in the animal colony room was set to lights on at 0730 h (ZT0). The Association for Assessment and Accreditation of Laboratory Animal Care International has fully accredited UCLA. All animal care and use met the requirements of the NIH Guide for the Care and Use of Laboratory Animals, 2011. The UCLA Chancellor's Animal Research Committee (Institutional Animal Care and Use Committee) preapproved all animal procedures.

Surgeries were carried out under aseptic conditions with isoflurane anesthesia on a KOPF Model 1900 Stereotaxic Alignment System (KOPF, Tujunga, CA). Surgery was carried out on each mouse to implant a CMA/7 microdialysis probe or a Si neuroprobe aimed at the hippocampus (AP-3.4 mm, ML+/-3 mm, DV-3.10 mm from Bregma). For the last two mice (shown in figures 3 and 4), a microdialysis probe and a neuroprobe were implanted during the same surgery in contralateral hemispheres for within-subjects comparisons. Each implant was secured to the skull with C&B Metabond. Following surgery, mice were given

twice daily carprofen injections (5 mg/kg, 1 mg/mL, subcutaneously) for the first three days for chronic time points of 1 week and 2 weeks. For acute time points, animals were treated with carprofen until the time of perfusion, which occurred 4 h, or 1 d post-surgery.

Immunocytochemistry and microscopy

Mice were exsanguinated with an overdose of 100 mg/kg pentobarbital (2 mL/kg administered at 50 mg/mL, i.p.) followed immediately by transcardial perfusion with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). In two mice (figures 2 and 5), FluoSpheres™ Carboxylate-Modified Microspheres (Thermo Fisher Sci, Cat #F8808) were added to the 4% PFA solution. The microspheres allow for blood vessel visualization. Probes remained in the brain at the time of perfusion and were removed when the brains were removed from the skulls. Brains were post-fixed for 2 h in 4% PFA. They were then transferred to 30% sucrose where they remained until they sunk to the bottom of their tubes. After sinking, brains were frozen at -80 °C until the day of sectioning.

Initially, coronal and sagittal sections were collected at 40-µm thickness using a cryostat. However, due to the difficulty in locating and visualizing probe tracks in coronal and sagittal sections, as well as sections folding onto themselves, horizontal sections were ultimately used. Horizontal sections spanning the dorsoventral axis were collected at 40 µm using a cryostat. Sections were mounted on poly-L-lysine coated slides (VWR, Cat # 100500-998) and stored at -20 °C until immunocytochemistry was performed.

Antibodies against GFAP and CD11b were used to image astrocytes and activated microglia, respectively. The GFAP protocol was adapted and modified from Jaquins-Gerstl and Michael.[25] Conditions for including the CD11b antibody in the GFAP protocol were optimized empirically. Slides were removed from the freezer and washed three times with

1× Tris-buffered saline (TBS) (Sigma-Aldrich, Cat # T5912). After the last wash, slides were incubated for 30 min in a blocking solution consisting of 10% donkey serum (Sigma-Aldrich Cat # D9663), 0.5% Triton-X (Millipore Sigma, Cat # 11332481001), and 1× TBS on a shaker at room temperature.

The blocking solution was removed and sections were incubated in a monoclonal rabbit anti-GFAP antibody solution diluted 1:1000 (Thermo Fischer, Cat # 130300) in 1× TBS overnight at 4 °C. The next day, slides were washed three times with 1× TBS and incubated for 2 h at room temperature in a solution containing 1:1000 diluted secondary donkey anti-rabbit Alexa Fluor® 488 antibody (Abcam, Cat # ab150073), 1:500 diluted anti-CD11b antibody tagged with an eFluor 660 fluorophore (Thermo Scientific, Cat # 50-0113-82), and 10% donkey serum in 1× TBS. Slides were then dried, covered with a DAPI antifade mountant (Thermo Fischer, Cat # P36962), and stored at -20 °C for at least 24 h.

Sections were initially imaged using a Leica DMI8 epifluorescent microscope provided by the UCLA IDDRC. The DAPI, GFP, Cy3, and Cy5 channels were used for DAPI, GFAP, microspheres, and CD11b, respectively. Images in Figures **V.2** and **V.5** were taken with the epifluorescent microscope. In subsequent experiments, a Zeiss LSM 800 confocal microscope provided by the UCLA IDDRC core was used and sections were visualized with a 20× objective. The confocal microscope provided better-resolution images, particularly for microglia. For **Figures V.3** and **V.4**, DAPI and microsphere channels were omitted to reduce overall scan time for the tiled, z-stacked images.

Results

A general experimental overview is shown in **Figure V.1**. Neuroprobes and/or microdialysis probes (**Fig. V.1A**) were implanted into the hippocampus in contralateral hemispheres when both were implanted into the same brains (**Fig. V.1B**). Neither activated astrocytes nor microglia were observed at the 1 h and 4 h post-implantation time points (**Fig. V.2**). At the 1-d time point, the neuroprobes produced qualitatively less pronounced astrocytic and microglia responses in the tissue immediately surrounding the site of implantation compared to microdialysis probes (**Fig. V.3**). Similar qualitative findings were observed 1-week post-implantation (**Fig. V.4**). At 2 weeks, the neuroprobes produced an inflammatory response, however, no comparisons with microdialysis probes were made (**Fig. V.5**).

Discussion

My preliminary findings suggest that the Si neuroprobes developed by our group produce reduced inflammatory responses when compared to larger microdialysis probes. Previous studies have shown that lower inflammatory responses are associated with better signal resolution and less tissue damage.[22] Moreover, a 2020 study found that adding bioactive coatings to neural implants, such as a synthetic zwitterionic polymer, suppresses microglial encapsulation of the implants.[26] Zwitterionic polymers have many applications due to their hydrophilicity and low fouling properties. The authors found that this zwitterionic coating reduced protein adsorption and cell attachment *in vitro* and significantly reduced microglial encapsulation compared to non-coated probes. Thus, future iterations of Si neuroprobes could use zwitterionic polymers or other bioactive coatings to reduce inflammatory responses further, particularly for chronic measurements.[27]

In future studies, additional time points should be studied, *e.g.*, 4 days and 4 weeks. These time points will further elucidate the timeline of astrocytic and microglial encapsulation and glial scar formation after implanting various devices of different sizes and surface chemistries. Future studies should also integrate measurements using neuroprobes with immediate assessment of neuroinflammatory responses and determine differences in neuroinflammation between 150-μm Si neuroprobes and a significantly smaller version (50-μm) of these devices, which we also recently reported.[11] Understanding how size, duration of implantation time, and surface chemistries impact inflammatory responses will guide experimental design for future acute or chronic recordings. Lastly, studying additional markers, *i.e.*, oligodendrocyte, neuronal, and protein markers, will provide a holistic analysis of overall biological responses to Si neuroprobes (and other devices, e.g., carbon-fiber

microelectrodes used for voltammetry) across size and time.[19,22] Astrocytic and microglial radial diameters should be quantified and statistically analyzed using appropriately powered group sizes to draw firm conclusions on the influence of device size, shape, duration of implantation, surface properties, and electrical currents and fields generated during use on implantation injury and the viability of devices for longer recording times.

Figures

Figure V.1

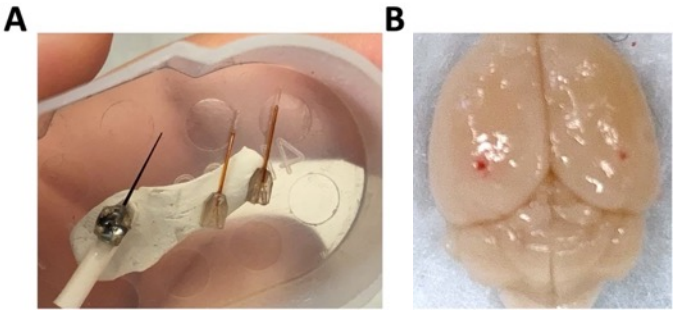

Figure V.1. Experimental overview. A. Comparison of a neuroprobe (left) and microdialysis probes (right). **B.** Example of a bilateral surgery where the red dots show the sites of probe implantation. A neuroprobe was implanted in the animal's right hemisphere and a microdialysis probe was implanted in the left hemisphere. The brain was photographed after fixation and removal of the probes

Figure V.2

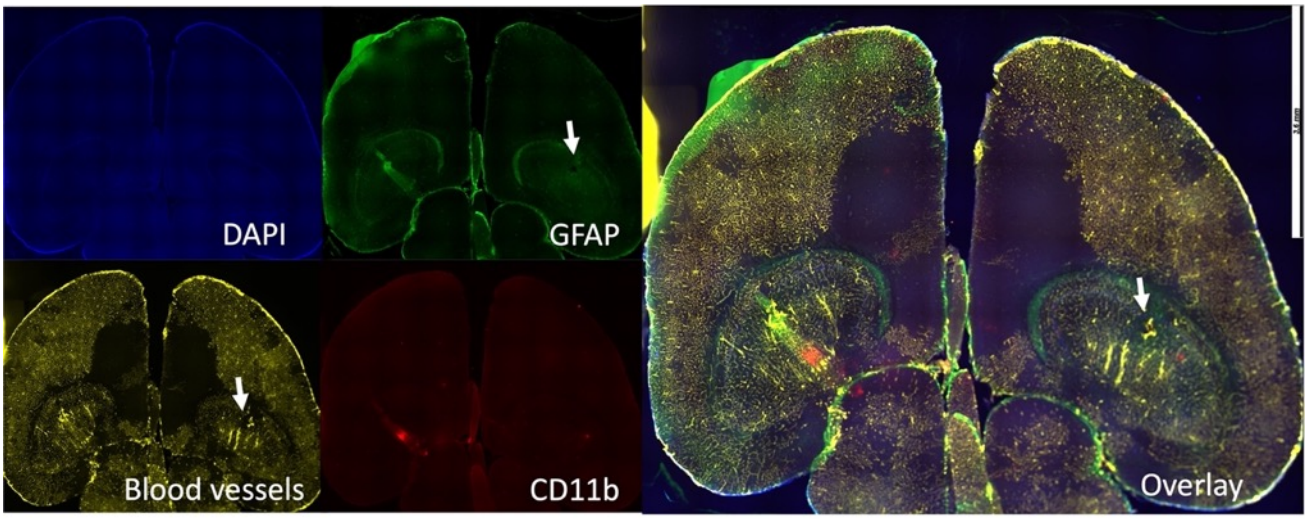

Figure V.2. Representative inflammatory responses 4 h after neuroprobe implantation. Images are taken with a 10x objective and are DAPI (blue), GFAP (green), microspheres (yellow), and CD11b (red), with all overlaid (right). Arrows point to the site of implantation, which is most clearly seen in the GFAP and microspheres (blood vessels) images. Astrocyte and microglial activation were mostly absent at this time point.

Figure V.3

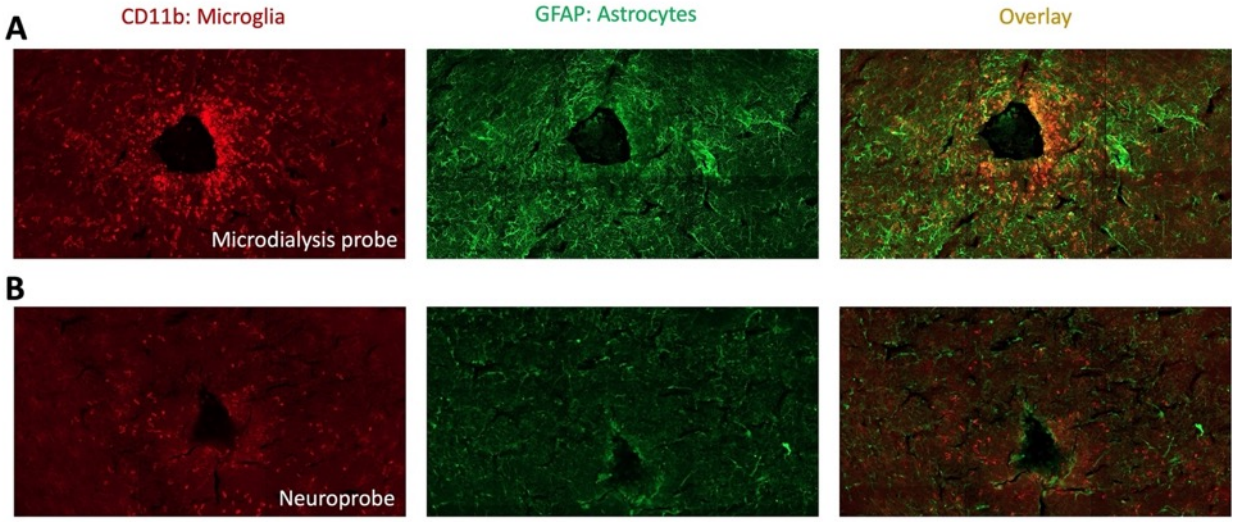

Figure V.3. Representative inflammatory responses 1 d after probe implantation. Left images show the microglial marker CD11b. The center images show the astrocyte marker GFAP. The right images are overlays showing colocalization of CD11b and GFAP. **A.** Neuroinflammation due to a microdialysis probe (240 μm) **B.** Neuroinflammation due to a neuroprobe (150 μm) implanted in contralateral hemisphere of the same mouse. Images are taken from the same mouse with a 20x objective in z-stacks .

Figure V.4

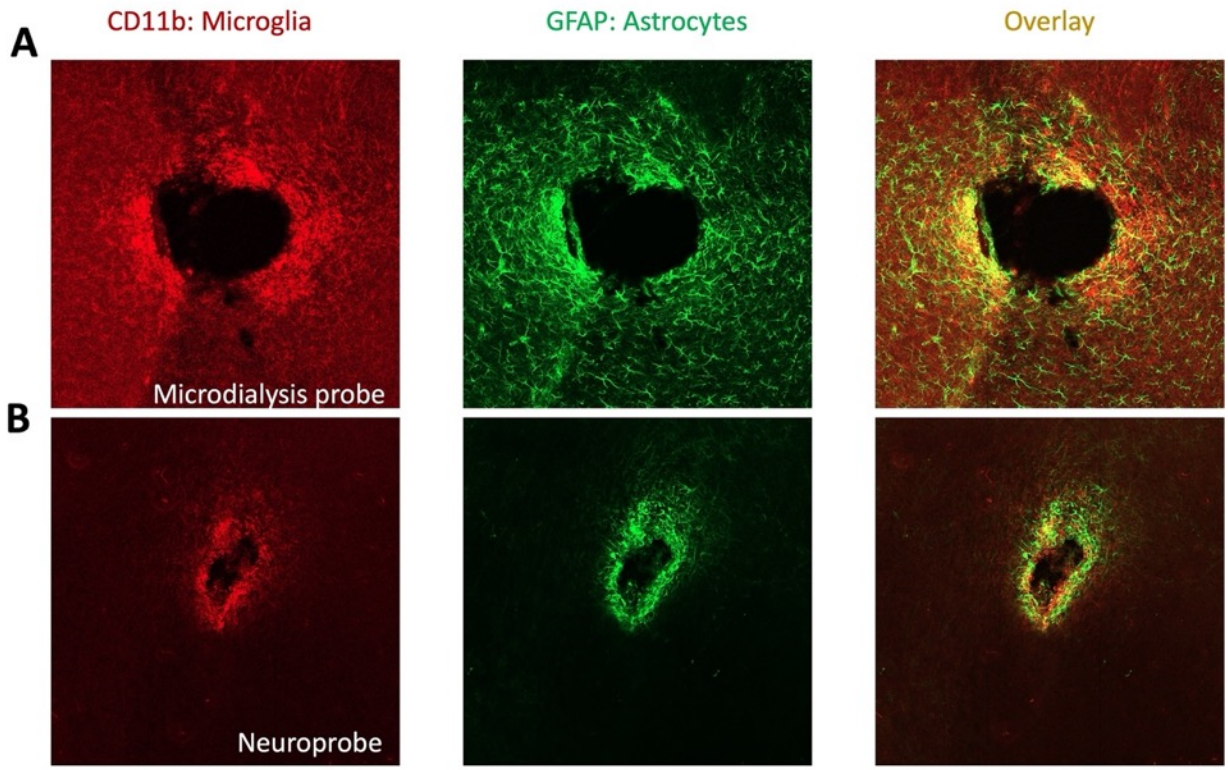

Figure V.4. Representative inflammatory responses one week after probe implantation. Images were acquired with a 20x objective in z-stacks and are CD11b (left), GFAP (center), and CD11b and GFAP overlaid (right). **A.** Neuroinflammation due to a microdialysis probe (240 μm). **B.** Neuroinflammation due to a neuroprobe (150 μm) implanted in contralateral hemisphere of the same mouse. Images are from the same mouse.

Figure V.5

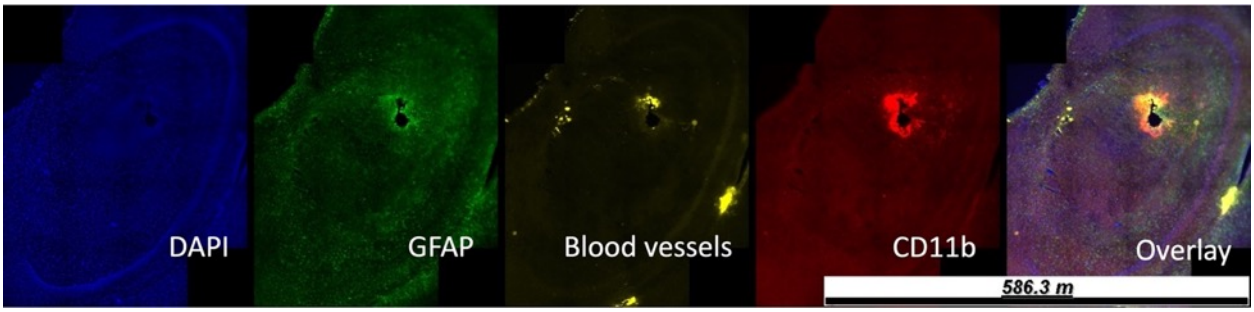

Figure V.5. Representative inflammatory responses two weeks after neuroprobe implantation. Images taken with a 20x objective and are DAPI (blue), GFAP (green), microspheres (yellow), CD11b (red), with all overlaid (right).

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