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Charles Lester Ford IV Date

Oxytocin in Translation:
Manipulating the Social Brain with Pharmacology and Contextual Stimuli

By

Charles Lester Ford IV
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Neuroscience

_____
Larry J. Young, PhD
Advisor

_____
Joseph F. Cubells, MD, PhD
Committee Member

_____
Paul S. García, MD, PhD
Committee Member

_____
Shannon L. Gourley, PhD
Committee Member

_____
Robert C. Liu, PhD
Committee Member

Accepted:

_____
Kimberly Jacob Arriola, PhD, MPH
Dean of the James T. Laney School of Graduate Studies

_____
Date

Oxytocin in Translation:
Manipulating the Social Brain with Pharmacology and Contextual Stimuli

By

Charles Lester Ford IV
BS, Davidson College, 2012

Advisor: Larry J. Young, PhD

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Science
Neuroscience
2022

Abstract

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By Charles Lester Ford IV

Social behavioral neuroscience has recently achieved in animal models feats that just a decade ago were relegated to creative works of science fiction. By manipulating specific neural circuits with temporal and contextual precision, neuroscience has developed the ability to "incept" false memories, induce monogamous social bonds, and turn complex parenting behaviors on and off with the flip of a switch. These remarkable capabilities contrast starkly with the limited efficacy and decades-old paradigms of psychiatry, which still relies on purely behavioral diagnoses and chronic, brain-wide pharmacological interventions. In this dissertation, I draw from theoretical and experimental publications I authored in collaboration with Dr. Larry Young and members of his laboratory to examine the disjunction between neuroscience and psychiatry, and to illustrate a translational process by which this disjunction might be ameliorated, with a focus on oxytocin and social behavior. In Chapter 1, we describe how a circuit-level approach to studying, understanding, and manipulating the social brain might facilitate translation from neuroscience to psychiatry and enable a shift in therapeutic paradigms from behavioral pharmacology to neural rewiring. We examine a prominent failure of the behavioral pharmacology paradigm – a clinical trial that failed to improve social behavior in autism using chronic intranasal oxytocin administered independent of context – and propose an alternative paradigm utilizing pharmacologically enhanced behavioral therapy and context-dependent oxytocin administration to rewire the brain. However, this approach requires understanding how specific neural circuits are affected by particular pharmacological manipulations, so Chapter 2 describes in great detail the anatomical distribution of oxytocin receptors in the prairie vole brain and the functional implications thereof. Chapter 3 models pharmacologically enhanced behavioral therapy for social deficits and demonstrates that administering a novel pharmaceutical in a social context activates the endogenous oxytocin system and thereby selectively increases the activity of a brain area critical for social reward learning. As the therapeutic approach we advocate involves using psychological interventions to rewire the brain, in Chapter 4, I explore other psychosomatic phenomena, including the neurobiological basis of the placebo effect, translational possibilities for the salubrious properties of love, and the evolutionary origins of the increased mortality associated with widowhood.

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Acknowledgments

Larry Young was nothing short of a phenomenal mentor. He was patient when I was foolish and trusted me before I trusted myself. He was supportive of me as both a student and an individual, and he gave me incredible opportunities both professionally and personally. In addition to sharing his opportunities and successes, he shared his ideas with me and showed me how to communicate them both orally and in writing. Most valuable of all, he taught me how to think – how to think generally, skeptically, critically, scientifically, and pragmatically. All the work I do for the rest of my career will be deeply shaped by his influence, and I can think of no better influence to be shaped by. The members of my dissertation committee – Joe Cubells, Paul García, Shannon Gourley, and Robert Liu – generously donated their time and patience to support, encourage, and teach me for years. In the most constructive and friendly manner, they repeatedly reviewed and improved my work, answered my questions, shared their wisdom and expertise, troubleshot my problems, provided personal advice, given me opportunities to learn and grow, and on occasion even welcomed me into their homes. The other faculty of the Neuroscience Graduate Program were fantastic as well; every interaction – formal and casual – I had with these exceptional individuals expanded my mind in some way. Additionally, Anne Murphy at Georgia State University, and Larry's wife, went out of her way to encourage me and support me. My fellow Young Lab members were wonderful friends, teachers, role models, colleagues, and collaborators. Larry recruited the best and brightest from all over the world and it was an incredible opportunity to learn from each of them. In particular, Amélie Borie, Arjen Boender, and Megan Warren were daily sources of scientific support, guidance, and friendship, and my work directly profited from their skillsets (behavior, electrophysiology, viral vectors, data analytics, ultrasonic vocalizations). Kiyoshi Inoue elevates histology to an art form, and he spent a great deal of time teaching me subtle aspects of his craft. Moreover, he graciously allowed me to use his exhaustively detailed anatomical work in Chapter 2. I benefited greatly from the work of several undergraduates – Joon Baek assisted with behavior and histology; Aashna Sahni advanced and made an honors thesis out of the "jealousy" project; Anna McDonough generated Supplemental Figure 1 of Chapter 3 with only the slightest supervision; Drayson Campbell took data Amélie and I collected but did not know how to use, and he learned (from Megan Warren and Robert Liu) how to analyze it, ultimately leading to a publication. Hasse Walum taught me statistics; Zack Johnson and James Burkett repeatedly offered detailed advice and help troubleshooting; Jamie LaPrairie and Aurélie Menigoz provided regular logistical, administrative, and scientific support; Lorra Julian and Zakia Sathi provided daily support in the lab and with voles; Kengo Horie made and gave me transgenic voles; my fellow graduate students Desiree DeLeon, Tobias Pohl, Jim Kwon, Byron Gardner, Sena Agezo, Sarah Blumenthal, and others were constant sources of support; the veterinary and animal care staff at the primate center worked tirelessly to enable my research; and Vaunita Parihar at Emory's CTPSR Core provided most of the imaging for Chapter 3. Beyond the lab, Emory's Neuroscience Graduate Program and Medical Scientist Training Program (MSTP) supported me for years, particularly Erica Weaver, Cathy Quinones-Maeso, Bob Gross, and Joanna Bonsall from the MSTP. Last but not least, my parents, Beth and Charles, sister, Carol Anne, girlfriend, Courtney Wolpov, and my dog, Buckley, were indefatigably tolerant and supportive of me throughout graduate school, and all made personal sacrifices to facilitate my academic success. I am deeply grateful to all of these people for their support, contributions to my growth as both a person and a scientist, and their contributions, both direct and indirect, to this dissertation.

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Chapter 1

Advancing 21st-Century Psychiatry: Translational Opportunities for Circuit-Based Social Neuroscience

1.1 Acknowledgment of Reproduction and Authors' Contributions

This chapter is adapted from:

Ford CL, Young LJ (2021) Translational opportunities for circuit-based social neuroscience: advancing 21st century psychiatry. *Current Opinion in Neurobiology*. 68:1-8.

Ford CL, Young LJ (2022) Refining oxytocin therapy for autism: context is key. *Nature Reviews Neurology*. 18(2):67-68.

I wrote the manuscripts with input and revisions from, and under the guidance of, L. Young.

1.2 Abstract

The recent advancements of social behavioral neuroscience are unprecedented. Through manipulations targeting neural circuits, complex behaviors can be switched on and off, social bonds can be induced, and false memories can be "incepted." Psychiatry, however, remains tethered to concepts and techniques developed over half a century ago, including purely behavioral definitions of psychopathology and chronic, brain-wide pharmacological interventions. Drawing on recent animal and human research, we outline a circuit-level approach to the social brain and highlight studies demonstrating the translational potential of this approach. We then suggest ways both clinical practice and translational research can apply circuit-level neuroscientific knowledge to advance psychiatry, including adopting neuroscience-based nomenclature, stratifying patients into diagnostic subgroups based on neurobiological phenotypes, and pharmacologically enhancing psychotherapy. We conclude with a case study of a failed clinical trial that used intranasal oxytocin to treat the social deficits of autism spectrum disorders, before outlining our approach to modeling an alternative paradigm for the treatment of social deficits.

1.3 Introduction: The Gulf between Psychiatry and Neuroscience

Mental illness is a leading cause of global disease burden, yet progress in treating psychiatric disorders has largely stalled since the advent of modern psychopharmacology in the 1950s and 1960s. Currently, psychiatric disorders are treated by modulating neurotransmitter activity throughout the brain via a handful of cellular and molecular targets. The majority of "new" psychiatric pharmaceuticals are variations of old drugs with marginal improvements rather than drugs with novel mechanisms of action. This homogeneous repertoire of pharmaceuticals is ill-matched for treating heterogeneous psychiatric disorders. For example, depression is diagnosed behaviorally by the presence of at least five of nine possible symptoms and is typically treated with selective serotonin reuptake inhibitors (SSRIs). Two patients may therefore share the same diagnosis while having only a single symptom in common, yet divergent behavioral presentations often have different neurobiological etiologies and responses to treatment (Drysdale et al., 2017; Wu et al., 2020). Furthermore, as first-line treatment for at least nine different diagnoses, SSRIs serve as a sort of modern-day panacea for a myriad of conditions ranging from depression and panic disorders to bulimia and premature ejaculation. Although the focus on behavioral diagnoses and chronic, brain-wide neurotransmitter manipulation was a great advancement in the 1950s, today it is perhaps psychiatry's greatest limitation.

This clinical stagnation contrasts with the unprecedented progress of behavioral neuroscience. In rodents, it is possible to control even complex social behaviors – monogamous pair bond formation can be biased (Amadei et al., 2017), distinct parental behaviors can be switched on and off (Kohl et al., 2018), and false social memories can be "incepted" (Okuyama et al., 2016). These advances have been enabled by techniques like optogenetics and chemogenetics (Roth, 2016; Kim et al., 2017), viral vector-mediated transgenics (Nectow & Nestler, 2020), and

Cre-dependent expression systems (Tsien et al., 1996) that enable the activation or inhibition of specific neural circuits and cell types with temporal precision. Understanding the circuit components regulating social behavior is a key element of the National Institute of Mental Health Strategic Plan. A challenge for psychiatry is translating these circuit-level discoveries of today into the transformative interventions of tomorrow (**Figure 1**).

Many psychiatric disorders manifest with disruptions in social cognition and behavior, and there have been great advances in understanding the neural circuit mechanisms regulating social behavior in animal models. Here, we discuss select examples of how animal research has informed our conception of the social brain, examine human research offering insight into the translational potential of this conception, and outline steps for how clinical practice can be improved by acting on the current state of neuroscientific knowledge. We conclude by applying these considerations to the use of oxytocin to treat the social deficits of autism spectrum disorders (ASD), using a failed clinical trial as a case study, before outlining our approach to modeling an alternative paradigm for treating social deficits.

1.4 Conception of the Social Brain from Animal Research

The social brain evolved to facilitate adaptive processes from reproduction to cooperation and communication, ultimately enabling human civilization. Despite tremendous variation in social behavior across species, there is remarkable conservation in the neuromodulators regulating social behaviors. Oxytocin and vasopressin, for example, modulate social behaviors across vertebrate taxa, from flocking in birds to pair-bonding in voles. Interspecies and intraspecies variation in social behavior is related to variation in the distribution of oxytocin and vasopressin receptors in the brain (Freeman & Young, 2016; Walum & Young, 2018). This variation in brain receptor

distribution is determined by sequence variation in the receptor genes (Okhovat et al., 2015; King et al., 2016). In prairie voles, for instance, such sequence variation determines individual variation in striatal oxytocin receptor density (King et al., 2016), which predicts resiliency to early-life neglect with respect to adult social bonding (Barrett et al., 2015).

In mammals, social information is first processed by sensory areas including olfactory bulb for olfaction; superior colliculus, pulvinar, and primary visual cortex for vision; and primary auditory cortex for hearing. These regions express oxytocin receptors in various species, depending on the sensory pathways most relevant for a particular species' social interactions (Freeman & Young, 2016). In sensory pathways, oxytocin signaling increases the salience of social stimuli by modulating neuron excitability and facilitates the flow of social information across the brain (Johnson et al., 2017). This has been hypothesized to occur in pulvinar in primates (Freeman & Young 2016) and shown to occur in olfactory bulb in rodents, where oxytocin enhances social discrimination by increasing the signal-to-noise ratio of olfactory bulb output (Oettl et al., 2016). Likewise, oxytocin enhances the auditory cortex response to pup calls to promote maternal nurturing (Marlin et al., 2015).

Socio-sensory information is subsequently conveyed to a network of subcortical structures including the amygdala, which is involved in the integration of information from multiple sensory modalities (Gothard, 2020) (**Figure 2**). In rodents, olfactory information is transmitted to the medial amygdala, where oxytocin-dependent signaling is necessary for social recognition (Walum & Young, 2018; Gangopadhyay et al., 2020). Projections from the medial amygdala to basolateral amygdala may integrate valence with social cues (Beyeler et al., 2018). The medial and basolateral amygdala both project to the hippocampus, which expresses oxytocin receptors and is necessary for social recognition and memory formation (Freeman et al., 2016; Okuyama et al., 2016). The

hippocampus projects back to basolateral amygdala and nucleus accumbens (NAc), where oxytocinergic, dopaminergic, and serotonergic innervation from the hypothalamic paraventricular nucleus, ventral tegmental area, and dorsal raphe nucleus, respectively, are necessary for social reward learning (Dölen et al., 2013; Hung et al., 2017). The NAc integrates social memories and contextual information from hippocampus, goal-directed information from prefrontal cortex (PFC), and emotional valence from amygdala to influence an organism's behavioral output through its projections to the ventral pallidum (Love, 2014). For more details on the circuitry underlying social behavior, see (Chen & Hong, 2018; Walum & Young 2018).

The ability to manipulate social behaviors through circuit modulation is remarkable. By activating or inhibiting specific subsets of galanin-positive neurons in the medial preoptic area, Kohl et al. (2018) inhibited infanticidal behavior in males and increased pup grooming in females (projections to periaqueductal gray), increased parental motivation to interact with pups (projections to ventral tegmental area), and inhibited male-male aggression (projections to medial amygdala). Okuyama et al. (2016) identified a social engram, or collection of cells encoding a memory trace for an individual conspecific, in ventral CA1 of hippocampus; by activating this engram while administering a foot shock, they incepted a false memory that made the mice fearful of that conspecific. Inhibiting or stimulating the PFC-to-NAc projections of D1 dopamine receptor-expressing neurons impaired or restored, respectively, social recognition memories in mice (Xing et al., 2020), while stimulating PFC projections to NAc induced pair-bonding in prairie voles without mating (Amadei et al., 2017). Additionally, oxytocin signaling in insular cortex, central amygdala, and anterior cingulate cortex modulates emotion detection and responses to the social affect of conspecifics (Burkett et al., 2016; Ferretti et al., 2019; Rogers-Carter et al., 2019).

These are but a few studies from a growing body of literature that demonstrates the ability of distinct neural circuits to robustly control specific behaviors and illustrates the potential of treating psychiatric disorders from a circuit perspective. Indeed, accumulating evidence from mouse models of autism suggests that stimulating oxytocin neurons can rescue social deficits. *Cntnap2* knockout mice show deficits in social behavior and reduced numbers of oxytocin neurons, while chemogenetic stimulation of oxytocin neurons or pharmacologically-evoked release of endogenous oxytocin rescues the deficits (Peñagarikano et al., 2015). Additionally, *Nlgn3* knockout mice show impaired social novelty preference due to decreased oxytocin signaling in ventral tegmental area (Hörnberg et al., 2020). Both neural and behavioral deficits can be rescued with a MAP kinase-interacting kinase inhibitor, providing a novel translational target that affects oxytocin signaling (Hörnberg et al., 2020).

1.5 Insights from Human Research

The noninvasive nature of most human research limits its mechanistic insights. However, recent work using genetic analyses, fMRI, and intranasal oxytocin (IN-OT) administration supports the circuit-level conception of the social brain emerging from animal research and provides insight into the heterogeneity of human populations. Quintana et al. (2019) mapped the mRNA of oxytocin pathway genes throughout human brains and found the highest expression in olfactory bulbs and pallidum, high expression in hypothalamus, thalamus, caudate, and putamen, and more moderately elevated expression in amygdala, anterior cingulum, and hippocampus. Additionally, there is strong correlation between the expression of the oxytocin receptor gene (*OXTR*) and dopamine receptors (especially D2R) and muscarinic acetylcholine receptors. Comparing *OXTR* expression and fMRI activity associated with cognitive states revealed a robust correlation between *OXTR*

expression and activity maps for “sexual,” “motivation,” “incentive,” and “anxiety” cognitive states. These data suggest the oxytocin systems in humans and animals are similar in their anatomical distribution, interaction with other neurotransmitter systems, and role in regulating socioemotional processes.

Similar to findings in prairie voles, multiple studies suggest that genetic variation in human *OXTR* contributes to variation in behavior. Single nucleotide polymorphisms in *OXTR* are associated with impaired social memory (Skuse et al., 2014), behavior in romantic relationships (Walum et al., 2012), and autism diagnosis (LoParo & Waldman, 2015). Polymorphisms in *OXTR* also contribute to sexually dimorphic alterations in functional connectivity between the NAc and PFC (Hernandez et al., 2017; Hernandez et al., 2020). Hypermethylation at specific sites in *OXTR* is associated not only with ASD, but with distinct ASD behavioral phenotypes (Andari et al., 2020). Moreover, these distinct clinical profiles and epigenetic biomarkers are also associated with alterations in resting state connectivity between areas critical for social cognition and behavior, such as the NAc, PFC, amygdala, and cingulate cortex.

The effects of IN-OT have been researched extensively since studies showed it promotes prosocial behavior and decreases activity in the amygdala and neural circuits associated with fear (Grinevich & Neumann, 2020). A recent meta-analysis of 82 studies including 3950 subjects confirm that the amygdala is the brain region most likely to be modulated by IN-OT followed by the insula, cingulate cortex, inferior frontal and orbitofrontal cortices, midbrain and basal ganglia, temporal gyrus, precuneus, and occipital cortex (Grace et al., 2018). However, results across studies are highly heterogeneous and vary based on the population and context of the study. Divergent responses to IN-OT have been associated with *OXTR* polymorphisms, gender, personality traits, attachment style, and underlying psychopathology (Andari et al., 2018; Grace et

al., 2018). Notably, IN-OT enhances the coordination of corticostriatal networks involved in social emotive, motivational, and communicative processes (Bethlehem et al., 2017), which parallels findings in voles that show oxytocin enhances correlated activity across a network of social brain regions involved in pair-bonding (Johnson et al., 2016). Similarly, in pair-bonded men, IN-OT enhances the perceived attractiveness of their female partners' faces but not that of other equally attractive female faces (Scheele et al., 2013); in voles, pharmacologically-increased oxytocin signaling enhances partner preference (Modi et al., 2015). For reviews of lessons learned and controversies of IN-OT research, see (Walum et al., 2016; Quintana et al., 2020).

In psychiatric cohorts including patients with ASD, borderline personality disorder, social anxiety disorder, post-traumatic stress disorder (PTSD), and schizophrenia, IN-OT often exerts a "normalizing" effect on the amygdala, increasing or decreasing activity to align with that observed in healthy controls (Grace et al., 2018). The possibility of using oxytocin to modulate specific neural circuits based on context and individual characteristics is particularly intriguing. In one study using resting state fMRI, females with PTSD displayed increased connectivity between the right basolateral amygdala and right anterior cingulate cortex while males with PTSD displayed decreased activity between the right centromedial amygdala and right ventromedial PFC (Koch et al., 2016). In these subjects, IN-OT restored functional connectivity to levels observed in healthy controls and also decreased subjective experiences of anxiety and nervousness. Six weeks of IN-OT treatment in ASD patients rescued social reciprocity deficits and enhanced task-dependent functional connectivity between anterior cingulate cortex and PFC (Watanabe et al., 2015). In a separate study, acute IN-OT enhanced functional connectivity between PFC and NAc while viewing biological motion (Gordon et al., 2016). There are, however, failures of replication in well-powered IN-OT clinical trials for ASD that urge caution in supporting IN-OT alone as a

therapy (Yamasue et al., 2020). Taken together, human studies highlight the neural heterogeneity of the population and further demonstrate the potential of approaching the social brain, psychiatric disorders, and interventions from a circuit perspective.

1.6 Translational Implications

Psychiatry is mired in the psychopharmacological techniques of the 1950s and must move beyond nonspecific behavioral diagnoses and chronic, brain-wide pharmacological interventions to adopt more neuroscience-based diagnoses and targeted interventions. The first step is embracing neuroscience-based nomenclature, a system of classifying drugs based on pharmacological profile and neurobiological mechanism rather than diagnostic or behavioral indication (Zohar et al., 2015); clinical language that omits the neurobiological basis of diseases and treatments precludes the incorporation of neuroscientific precision into clinical practice. Similarly, the neurobiological basis of psychiatric disorders at the level of circuits should receive increased emphasis in the education of patients and medical trainees. The next generation of psychiatrists should be trained in both the behavioral pharmacological techniques employed today and the circuit neurophysiology that will drive clinical practice in the future. Regarding patients, it is worth considering that recasting psychiatric conditions in terms of "misfiring circuits" rather than disorders of behavior might reduce stigma, a key barrier to seeking treatment (Schnyder et al., 2017).

A circuit-based approach also suggests immediate solutions for addressing the glaring mismatch between the heterogeneity of psychiatric disorders and the homogeneity of both diagnoses and treatments. To improve the precision of psychiatric medicine, patients within diagnostic categories must be subdivided by finding correlations between behavioral phenotypes,

neural activity, genetic markers, and treatment responsiveness (Drysdale et al., 2017; Andari et al., 2018; Andari et al., 2020; Wu et al., 2020). Simultaneously, treatments' circuit-level neural effects must be studied in addition to their behavioral effects so that more precise diagnoses can be matched with more precise treatment options. Doing so should be a top priority for psychiatry as it has been demonstrated unambiguously to be possible (Chekroud et al., 2016; Drysdale et al., 2017; Wu et al., 2020) and the potential benefits are immense (Insel & Cuthbert, 2015; William, 2016). Additionally, more cross-species research, including reverse translation from humans to animal models, would help elucidate the mechanisms underlying clinical interventions and maximize the translational value of preclinical studies (Grimm et al., 2015; Keysers & Gazzola, 2017).

Psychotherapy is an alternative and effective means of treating mental illness that alters activity within neural circuits by harnessing the brain's inherent capacity for experience-dependent plasticity (Marwood et al., 2018). As pharmacology-based and psychotherapy-based interventions are independently capable of modulating circuit-level neural activity, pharmacologically-enhanced psychotherapy would seem an obvious opportunity to target specific circuits more effectively and potentially reduce the need for chronic pharmacological therapy (Sartori & Singewald, 2019). Promising results have been obtained by enhancing psychotherapy with methylenedioxymethamphetamine (MDMA) (Mitchel et al., 2021)[1], oxytocin (Flanagan et al., 2018), L-DOPA (Gerlicher et al., 2018), and other compounds (for review, see Sartori & Singewald, 2019). As therapeutic information in psychotherapy comes from a social source (the therapist), concurrently activating the oxytocin system is especially promising for disorders with

[1] At the time this section of Ford & Young, 2021a, was published online in 2020, MDMA-assisted psychotherapy for PTSD was being assessed in phase III clinical trial NCT03537014. The results have since been published in Mitchel et al., 2021, and will be discussed further in Chapters 3 and 4.

social deficits like ASD. In rodents, social stimuli, including social touch, robustly activate hypothalamic oxytocin neurons and enhance social motivation, effects that could be utilized in psychotherapy (Resendez et al., 2020; Tang et al., 2020). Oxytocin-enhanced therapy would have the combined benefits of increasing the salience of information coming from the therapist (Gordon et al., 2013; Shamay-Tsoory & Abu-Akel, 2016), promoting synaptic plasticity (Rajamani et al., 2018), and facilitating learning by modulating circuit-level neural activity (Eckstein et al., 2015; Andari et al., 2020). Despite the promising results of augmenting psychotherapy for PTSD with IN-OT (Flanagan et al., 2018), no studies have been reported for ASD; to date, most research on IN-OT interventions for ASD has involved administrations independent of context. Since oxytocin enhances the salience of social stimuli, we suggest that context-dependent IN-OT administration, preferably prior to therapy, may be a more beneficial intervention for ASD patients than the daily, context-independent administration schedules typical of other pharmacotherapies.

The circuit-based conception of the brain, psychopathologies, and treatments also suggests new directions for translational research. For instance, a circuit-targeted approach to manipulating oxytocin signaling increases the feasibility of resetting transcriptional or translational abnormalities (Hörnberg et al., 2020), altering the course of neurodevelopmental disorders through interventions during developmental sensitive periods (DeMayo et al., 2019), or reopening critical periods in adults to enable new social learning (Nardou et al., 2019). Moreover, single-cell sequencing has the potential to identify cell-type-specific molecular targets for the development of new pharmaceuticals that would affect specific neural circuits rather than the entire brain Romanov et al., 2017). However, bypassing the blood-brain barrier remains a perennial problem for pharmacological interventions, including IN-OT; the effect sizes in most IN-OT studies are small (Walum et al., 2016) and there are questions surrounding its ability to cross the blood-brain

barrier and diffuse to subcortical structures (Yamamoto et al., 2019; Higashida et al., 2019). We predict that the evolution of oxytocin interventions will move beyond administering exogenous oxytocin to second-generation strategies utilizing pharmaceuticals like melanocortin receptor agonists that potentiate endogenous oxytocin signaling much like how SSRIs and L-DOPA are used to potentiate serotonin and dopamine signaling (Young & Barrett, 2015). Beyond the oxytocin system, emerging technologies like transcranial magnetic stimulation, deep brain stimulation, and the application of focused ultrasound to circumvent the blood-brain barrier are enabling more targeted interventions. Such innovations are critical as while psychiatric precision will always lag behind neuroscientific precision due to ethical and technological constraints, the advancement of psychiatry requires that we develop ways to approximate in humans the circuit manipulations that have proven so efficacious in animal research (**Figure 3**).

1.7 Refining Oxytocin Therapy for Autism: A Case Study

A phase II clinical trial that tested the ability of IN-OT to improve social behavior in children with ASD recently made headlines for unambiguously demonstrating no clinical effect (Sikich et al., 2021). However, many of us in the oxytocin research community were unsurprised by these negative results. In fact, the results are consistent with what we have been advocating for years: oxytocin enhances social salience rather than social behavior, and for oxytocin administration to be therapeutic and influence behavior, it must be paired with an appropriate social context (Young, 2013; Young, 2015; Young & Barrett, 2015; Shamay-Tsoory & Abu-Akel, 2016; Ford & Young, 2021a). Published in *The New England Journal of Medicine*, the randomized, double-blind, parallel-group, placebo-controlled study by Sikich et al. (2021) was rigorous, robust, and thorough in both design and execution. Children and adolescents with ASD aged 3 – 17 years received

twice-daily target doses of 24 – 40 international units (IU) of IN-OT or placebo for a maximum of 24 weeks. Social behavior was assessed by the primary outcome – a clinical questionnaire completed by parents or guardians at 4-week intervals – and secondary outcomes that included additional social behavioral assessments and an IQ test. Subtler neurocognitive effects of the sort often reported in the preclinical literature were not assessed, but such effects would not be clinically meaningful without appreciable behavioral changes. Data were collected from 277 of the 290 participants enrolled across seven academic sites, a sample size more than adequate to detect an effect of clinically meaningful size. However, oxytocin administration had no effect on any of the outcome measures. As ASD is highly heterogeneous, it remains possible that chronic oxytocin could be beneficial for a small phenotypic subset of individuals despite this effect being undetectable in large, diverse populations. Nevertheless, these results align with those of a clinical trial from Japan, published in 2020, and provide strong evidence that children should not be prescribed chronic IN-OT independent of context (Yamasue et al., 2020).

Although chronic, context-independent pharmacological intervention is often effective in other organ systems – for example, the use of angiotensin-converting enzyme (ACE) inhibitors to lower blood pressure – this type of approach is likely to be less effective for addressing complex psychiatric issues such as social behavioral deficits (Young, 2013; Ford & Young, 2021a). Indeed, oxytocin does not directly cause prosocial behavior in the mechanistic sense in which ACE inhibitors directly lower blood pressure. Instead, oxytocin changes the way in which incoming social stimuli are perceived and processed, which can influence behavior and social learning in various ways (Froemke & Young, 2021). Oxytocin increases the salience of social stimuli and fine-tunes neural processes so that an organism can better attend and respond to those stimuli. The drug enhances the signal-to-noise ratio of social information in brain regions that process incoming

sensory signals and facilitates the flow of that information to areas involved in affective and cognitive processes, reward learning, and memory (Walum & Young, 2018; Froemke & Young, 2021). The behavioral effects of this increased social salience depend largely on context and the type of social information the organism receives. This effect of context is one reason the literature has shown ostensibly contradictory behavioral effects of oxytocin: the hormone can enhance trust or suspicion, affiliation or aggression, sexual arousal or learning and memory. Given the context-dependent nature of oxytocin's effects, the fact that Sikich et al. (2021) found no effect of context-independent oxytocin administration is unsurprising.

So how should we approach the use of oxytocin to treat the social deficits of ASD? Evidence that ASD is caused by decreased oxytocin signaling is lacking, and now we have compelling evidence that chronic administration of oxytocin is not beneficial. However, oxytocin is a powerful neuromodulator: it makes the brain more attentive to social stimuli, promotes synaptic plasticity, and facilitates social learning by modulating neural circuits (Walum & Young, 2018; Froemke & Young, 2021). Therefore, oxytocin administration might open a brief window of time for the brain to learn new social information and behaviors. We believe that oxytocin should be used as a tool to prime the brain for receiving, processing, and learning social information during cognitive and behavioral therapy interventions (Young & Barrett, 2015; Young & Ford, 2021) (**Figure 4**).

Cognitive and behavioral therapies can effectively address the social deficits of ASD, but up to 40 hr of therapy is required per week. Administration of oxytocin immediately before therapy might enhance the efficacy of therapy and reduce the number of hours required to achieve results. Oxytocin might also be beneficial when administered before positive social experiences, with the aim of promoting social engagement and reinforcing skills learned in therapy. In addition,

oxytocin-assisted therapy might be most effective during sensitive periods very early in life when the brain can more easily learn social skills through experience, similar to the way that language is learned (DeMayo et al., 2019). Future research must elucidate the interaction between oxytocin administration and social context so that interventions can be designed to activate the appropriate neural circuitry and influence clinically meaningful behaviors (Ford & Young, 2021a; Froemke & Young, 2021).

Even in a therapeutic context, IN-OT is a suboptimal method for enhancing oxytocin signaling. Oxytocin has a short half-life, its ability to penetrate the blood-brain barrier is hotly contested, and its ability to diffuse intracerebrally to reach relevant brain areas has been questioned (Young, 2013; Young & Barrett, 2015; Ford & Young 2021a). These limitations are likely to contribute to the relatively small effect sizes observed in previous studies of IN-OT – studies that use radiolabeled imaging probes to examine the dynamics of oxytocin in the human brain are greatly needed. Nevertheless, no other clinical intervention intended to increase the signaling of a neurochemical involves administering the neurochemical itself. Instead, the compounds that are administered increase the neurochemical's synthesis (for example, levodopa for dopamine), synaptic release (for example, amphetamines for dopamine and norepinephrine), time in synapses (for example, acetylcholinesterase inhibitors for acetylcholine or SSRIs for serotonin) or effect on receptors (for example, benzodiazepines and barbiturates for GABA). We also use compounds that bind directly to the target receptor (for example, opioids). Therefore, effective clinical oxytocin intervention might require a second generation of therapeutics – compounds that cross the blood-brain barrier and either enhance endogenous oxytocin signaling or bind specifically to oxytocin receptors (Young, 2015; Young & Barrett, 2015; Ford & Young, 2021a).

Although the data presented by Sikich et al. (2021) clearly demonstrate that chronic IN-OT administration independent of context is not beneficial for children with ASD, they do not provide a reason to abandon translational oxytocin research. On the contrary, these data are a clarion call for investigators to re-evaluate flawed assumptions, re-examine oxytocin neurophysiology, and redesign interventional approaches. Oxytocin is a powerful tool, but chronic supplementation is not the correct application. Enhancing oxytocin signaling remains a promising therapeutic possibility that must continue to be pursued with this newfound knowledge. Future clinical studies must explore the potential of pairing oxytocin with therapeutic contexts, and we must redouble translational efforts to design second-generation pharmacotherapies that enhance endogenous oxytocin signaling.

1.8 Concluding Remarks

As research on the social brain has demonstrated, the potential of incorporating circuit-level approaches into the diagnosis and treatment of psychiatric disorders is no longer theoretical – it is manifestly evident. Moreover, we presently possess the technological capabilities and scientific understanding required to begin implementing this approach. The sooner circuit-level approaches are embraced, the sooner the field of psychiatry can move beyond twentieth-century behavioral pharmacology and into twenty-first-century precision medicine.

Pharmacologically enhancing psychotherapy or behavioral therapy is one potential method for targeting and rewiring specific neural circuits that could be implemented immediately with current technologies. As discussed, combining behavioral therapy with pharmacological activation of the endogenous oxytocin system could succeed in treating social deficits in ASD where the current therapeutic paradigm of chronic oxytocin supplementation has failed. To assess how

increased oxytocin signaling might affect neural circuits, we first mapped oxytocin receptor mRNA expression throughout the prairie vole brain (Chapter 2). Then, to assess the plausibility that enhanced endogenous oxytocin signaling during behavioral therapy might increase activity in brain regions involved in social learning, we combined social stimulation with pharmacological activation of the endogenous oxytocin system in prairie voles using a melanocortin receptor agonist with translational potential (Chapter 3). We conclude with an examination of psychosomatic phenomena, including the neurobiological basis of the placebo effect, the possibility of harnessing the salubrious effects of love, and an evolutionary explanation for the increased mortality associated with widowhood (Chapter 4).

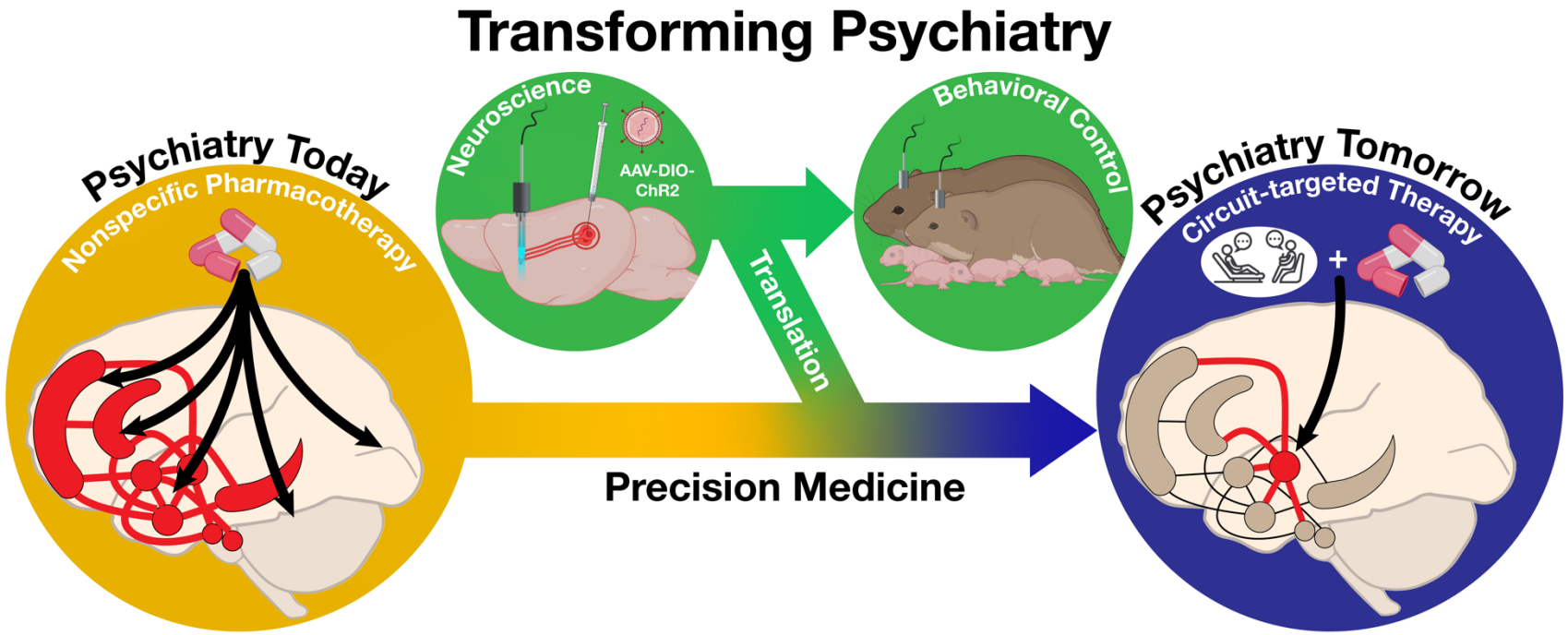


Figure 1. Visual representation of the transformation that must occur for psychiatry to become more precise.

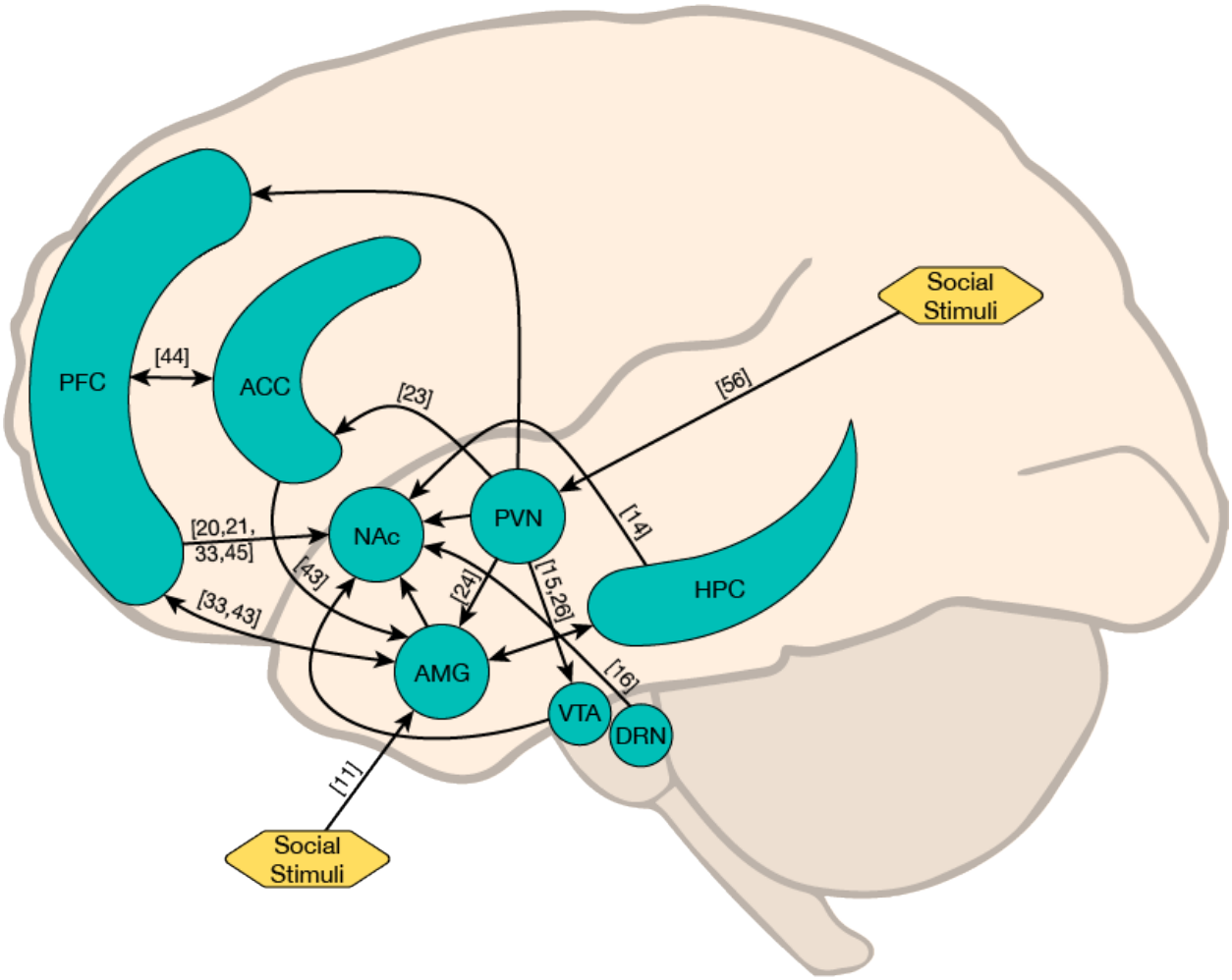


Figure 2. Select pathways involved in social cognition and associated references suggesting interventional potential. Recent studies in both animals and humans have identified pathways in the social brain that can be modified with behavioral consequences and thus merit further investigation for therapeutic targeting. These pathways include sensory input to the amygdala (AMG) ([11] refers to Gothard, 2020) and paraventricular nucleus of the hypothalamus (PVN) ([56] refers to Tang et al., 2020); PVN projections to AMG ([24] refers to Ferretti et al., 2019), ventral tegmental area (VTA) ([15] refers to Hung et al., 2017; [26] refers to Hörnberg et al., 2020), and anterior cingulate cortex (ACC) ([23] refers to Burkett et al., 2016); reciprocal connections between prefrontal cortex (PFC) and both AMG ([33] refers to Andari et al., 2020; [43] refers to Koch et al., 2016) and ACC ([44] refers to Watanabe et al., 2015); ACC projections to AMG ([43]

refers to Koch et al., 2016); projections to nucleus accumbens (NAc) from PFC ([20] refers to Xing et al., 2020; [21] refers to Amadei et al., 2017; [33] refers to Andari et al., 2020; [45] refers to Gordon et al., 2016), hippocampus (HPC) ([14] refers to Okuyama et al., 2016), and the dorsal raphe nuclei (DRN) ([16] refers to Dölen et al., 2013).

It should be noted that in the version of this figure published in Ford & Young, 2021a, the bracketed numbers in the figure do not correspond to the correct publications listed in the references section. This mistake occurred because I failed to update the bracketed numbers in the figure after adding additional references prior to publication. The references as listed in the figure caption of this dissertation are correct.

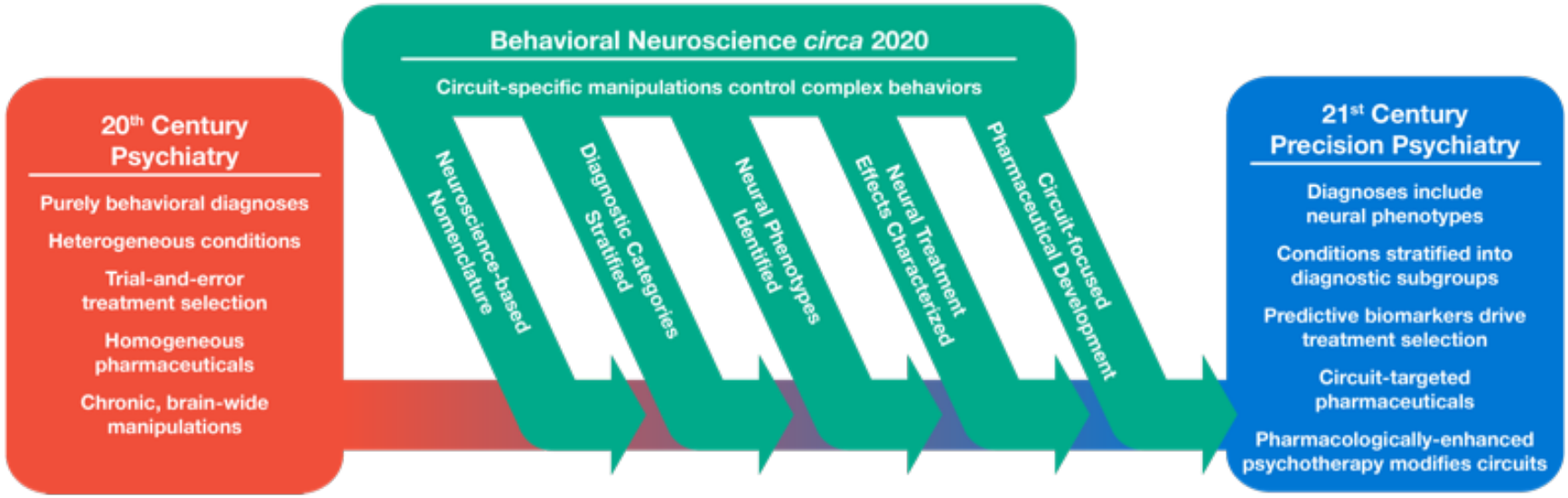


Figure 3. Suggested steps for advancing psychiatric practices based on recent progress in neuroscience.

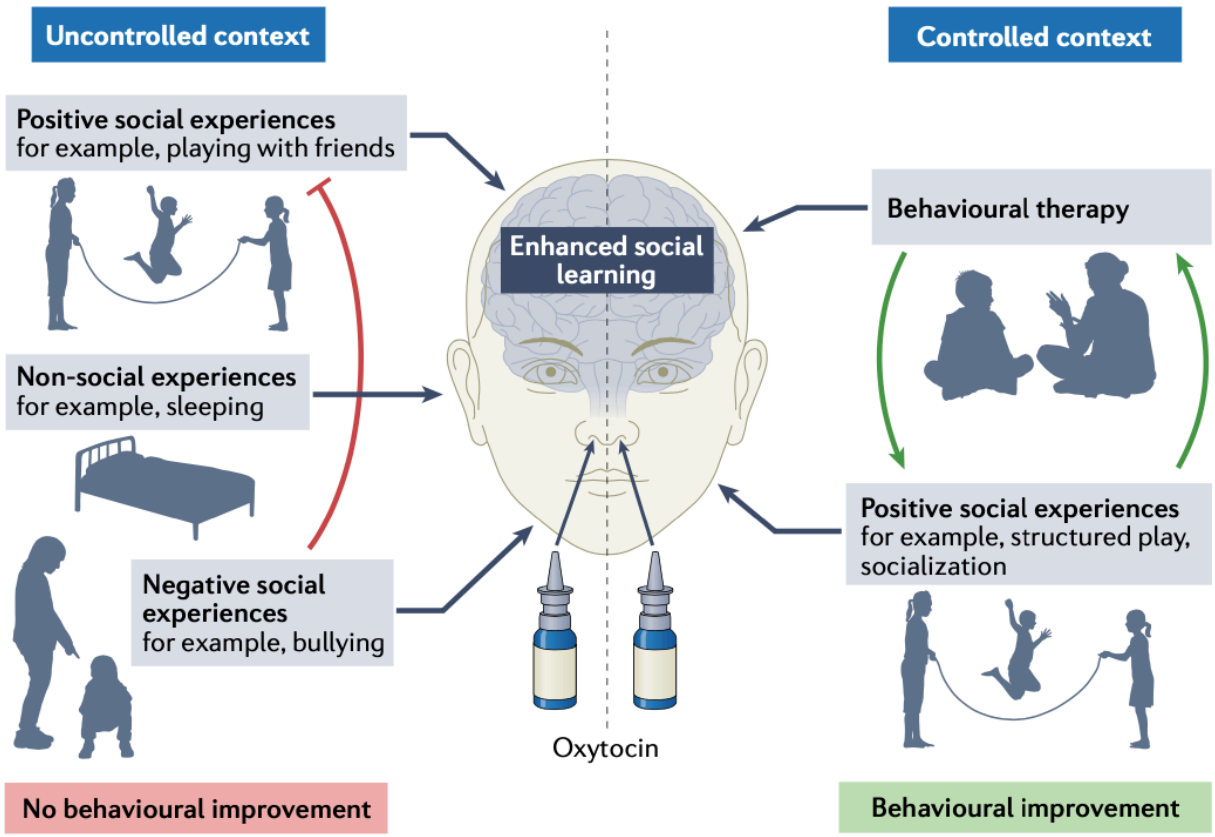


Figure 4. The effects of oxytocin depend on social context. Oxytocin enhances the salience of social information and primes the brain for social learning. Administering oxytocin without controlling social context is unlikely to yield clinically beneficial results, and learning from negative experiences might counteract learning from positive experiences (red line). To improve social behavior, oxytocin administration should be paired with opportunities for constructive social learning, such as behavioral therapy sessions and positive social experiences, which can positively reinforce learned social behaviors (green arrows).

Chapter 2

Oxytocin Receptors are Widely Distributed in the Prairie Vole Brain: Relation to Social Behavior, Genetic Polymorphisms, and the Dopamine System

2.1 Acknowledgment of Reproduction and Authors' Contributions

This chapter is adapted from:

Inoue K, Ford CL, Horie K, Young LJ (2022) Oxytocin receptors are widely distributed in the prairie vole (*Microtus ochrogaster*) brain: Relation to social behavior, genetic polymorphisms, and the dopamine system. *Journal of Comparative Neurology*. Online ahead of print.

K. Inoue performed the extensive histological work presented, with his permission, in this chapter, including sectioning, in situ hybridization, autoradiography, imaging, image analysis, and figure construction, in addition to editing the manuscript. I assisted with the optimization of the in situ hybridization protocol, performed the statistical analyses, and wrote the manuscript. K. Horie assisted with tissue processing and figure construction, and edited the manuscript. All work was performed under the guidance of L. Young, who conceptualized the project and also edited the manuscript.

2.2 Abstract

Oxytocin regulates social behavior via direct modulation of neurons, regulation of neural network activity, and interaction with other neurotransmitter systems. The behavioral effects of oxytocin signaling are determined by the species-specific distribution of brain oxytocin receptors. The socially monogamous prairie vole has been a useful model organism for elucidating the role of oxytocin in social behaviors, including pair bonding, response to social loss, and consoling. However, there has been no comprehensive mapping of oxytocin receptor-expressing cells throughout the prairie vole brain. Here, we employed a highly sensitive in situ hybridization, RNAscope, to construct an exhaustive, brain-wide map of oxytocin receptor mRNA-expressing

cells. We found that oxytocin receptor mRNA expression was widespread and diffuse throughout the brain, with specific areas displaying particularly robust expression. Comparing receptor binding with mRNA revealed that regions of the hippocampus and substantia nigra contained oxytocin receptor protein but lacked mRNA, indicating that oxytocin receptors can be transported to distal neuronal processes, consistent with presynaptic oxytocin receptor functions. In the nucleus accumbens, a region involved in oxytocin-dependent social bonding, oxytocin receptor mRNA expression was detected in both the D1 and D2 dopamine receptor-expressing subtypes of cells. Furthermore, natural genetic polymorphisms robustly influenced oxytocin receptor expression in both D1 and D2 receptor cell types in the nucleus accumbens. Collectively, our findings further elucidate the extent to which oxytocin signaling is capable of influencing brain-wide neural activity, responses to social stimuli, and social behavior.

2.3 Introduction

Oxytocin is a neuromodulatory nonapeptide that regulates social behavior and reproductive physiology in a wide variety of taxa through its signaling via oxytocin receptors (OXTRs) in the brain (Jurek & Neumann, 2018; Froemke & Young, 2021; Grinevich & Ludwig, 2021). Largely conserved through 700 million years of evolution (Donaldson & Young, 2008), oxytocin and its analogs control egg laying in annelids (Oumi et al., 1996), social preference in fish (Landin et al., 2020), and flocking in birds (Goodson et al., 2012). In mammals, oxytocin plays a critical role not only in reproductive and parenting behaviors (Rilling & Young, 2014; Numan, 2020) but also in complex social dynamics such as consolation of conspecifics (Burkett et al., 2016), intergroup conflicts (Samuni et al., 2017), and interspecies bonds (Nagasawa et al., 2015). Oxytocin may also modulate social behaviors believed to be uniquely human, such as love (Young & Alexander,

2012; Walum & Young, 2018) and altruism (De Dreu et al., 2010). Perhaps most importantly, the oxytocin system is a promising therapeutic target for neuropsychiatric conditions with social dysfunction, including autism spectrum disorders, schizophrenia, and posttraumatic stress disorder (Andari et al., 2018, 2021; DeMayo et al., 2019; Ford & Young, 2021a, 2022).

Despite its evolutionary conservation, oxytocin mediates remarkably diverse behaviors across species via species-specific patterns of OXTR distribution throughout the brain (Johnson & Young, 2017; Young & Zhang, 2021). For instance, OXTR signaling in the nucleus accumbens (NAc) and prelimbic cortex (PLC) is critical for pair bonding in monogamous prairie voles, and monogamous and polygamous vole species differ in OXTR expression in these regions (Young et al., 1996, 2001; Young & Wang, 2004). Subsequent studies in rodents and primates have supported the idea that interspecies variation in social behavior results from variation in OXTR distribution (Francis et al., 2000; Smeltzer et al., 2006; Campbell et al., 2009; Freeman & Young, 2016; Freeman et al., 2020; Rogers Flattery et al., 2021). A recent quantitative analysis of data from 13 rodent species compared in seven papers demonstrates that OXTR density in the NAc and lateral septum predicts social group size, behavior toward conspecifics, and reproductive strategies (Olazábal & Sandberg, 2020). However, a recent study comparing OXTR distributions in monogamous and polygamous lemur species indicated that, in some primates, OXTR binding does not predict mating strategies, suggesting that other mechanisms also contribute to social behavioral diversity (Grebe et al., 2021). Nevertheless, differences among primates, including humans, in the brain regions innervated by oxytocinergic fibers or containing OXTR may contribute to certain behavioral differences across species (Rogers Flattery et al., 2018, 2022).

Differences in OXTR distribution mediate intraspecies variations in behavior as well (Ross et al., 2009; Ophir et al., 2012; Keebaugh et al., 2015). In prairie voles, single nucleotide

polymorphisms (SNPs) in the OXTR gene, *Oxtr*, predict individual differences in OXTR density in the NAc but not in other areas, which, in turn, predicts social attachment behaviors and resilience to early-life neglect (Barrett et al., 2015; King et al., 2016; Ahern et al., 2021). Additionally, decreased OXTR density in the lateral septum of male prairie voles is associated with higher rates of social investigation of females (Ophir et al., 2009). In rat dams, higher densities of OXTR in the central nucleus of the amygdala and medial preoptic area (MPOA) are associated with increased maternal behaviors (Francis et al., 2000; Champagne et al., 2001). Many changes in an individual's social behavior are also mediated by changes in *Oxtr* expression. Rats undergo an estrogen-dependent increase in *Oxtr* mRNA expression in the ventromedial hypothalamus (VMH) during pregnancy and the MPOA and the bed nucleus stria terminalis (BNST) at parturition (Young et al., 1997; Meddle et al., 2007). OXTR density in the VMH is highly sensitive to sex steroids, with gonadal steroids having opposite effects on OXTR density in the VMH in mice and rats (Insel et al., 1993). Paternal behavior is also regulated, at least in part, by changes in *Oxtr* expression. Male mandarin voles' behavior toward pups changes from infanticidal to parental after they sire offspring, a change that requires upregulation of OXTRs in the MPOA (Yuan et al., 2019).

OXTRs localized to specific regions have specific behavioral functions. In rodents, OXTRs are heavily expressed throughout olfactory pathways, including the anterior olfactory area (also known as the anterior olfactory nucleus) (Freund-Mercier et al., 1987; Freeman & Young, 2016). Oxytocin signaling in the anterior olfactory area facilitates social recognition by enhancing the signal-to-noise ratio of olfactory information processing via top-down projections to inhibitory granule cells in the main olfactory bulb (Oettl et al., 2016). In primates, OXTRs are more densely expressed in areas responsible for visual information processing and attention, including the

nucleus basalis of Meynert and superior colliculus (Freeman et al., 2014; Freeman & Young, 2016; Grebe et al., 2021). In the auditory cortex of maternal mice, OXTRs enhance the salience of pup calls and facilitate pup retrieval by regulating cortical inhibition (Marlin et al., 2015). OXTRs in the insular cortex mediate the recognition of emotion in rats (Rogers-Carter et al., 2018), in the anterior cingulate cortex they mediate partner-directed consolation behavior in voles (Burkett et al., 2016), and in the PLC and NAc they regulate pair bonding in voles (Walum & Young, 2018).

Beyond its brain region-specific effects, oxytocin is a modulator of modulators; it coordinates activity across a network of social brain regions (Johnson et al., 2016; Johnson & Young, 2017), and it influences the activity of other neurotransmitters, including serotonin and dopamine (Froemke & Young, 2021). OXTRs synthesized in the dorsal raphe nucleus, a primary source of serotonergic projections, and in the ventral tegmental area, a primary source of dopaminergic projections, are both critical for social reward (Dölen et al., 2013; Hung et al., 2017). In particular, the interplay of OXTRs, D1 dopamine receptors (D1Rs), and D2 dopamine receptors (D2Rs) on medium spiny neurons (MSNs) in the striatum mediates many complex social behaviors, including social reward learning, pair bonding, and selective aggression. Specifically, D1R activity appears to inhibit pair bonding, D2R activity appears to facilitate pair bonding, and an increase in the ratio of D1R:D2R expression appears to underlie selective aggression in pair-bonded male voles (Aragona et al., 2006).

Although OXTRs have been mapped in mice using immunohistochemistry (Mitre et al., 2016), the translational relevance of social neuroscience research in mice and rats is limited by these models' social behavioral repertoire, particularly in relation to pair bonding, partner loss, biparental care, and empathy-based consoling behavior. In contrast, the prairie vole has emerged as the premier model organism for studying neurochemistry and neural circuit mechanisms of these

social behaviors (McGraw & Young, 2010). The prairie vole has already proven useful for elucidating the role of oxytocin in social behavior and cognition, and its utility as a model organism will continue increasing now that transgenic and gene-editing tools once available only in mice have become available in voles (Horie et al., 2019, 2020; Boender & Young, 2020).

Despite the value of the prairie vole model for understanding the effects of oxytocin on behavior, there has been no comprehensive mapping of *Oxtr* mRNA expression throughout the vole brain. Previous OXTR localization studies in voles have relied on autoradiographic detection, which does not provide cellular resolution. Here, we used RNAscope in situ hybridization to label *Oxtr* mRNA throughout the prairie vole brain. We compared the subcellular localization of *Oxtr* mRNA to the localization of OXTR protein using autoradiography, which revealed localized mismatches, suggesting a presynaptic function for OXTRs. We also examined the colocalization of *Oxtr* mRNA with mRNA for D1R (*Drd1*) and D2R (*Drd2*) on cells in the NAc. Additionally, we leveraged the naturally occurring genetic variation in our outbred prairie vole colony to examine how SNPs known to affect social behavior affect *Oxtr* mRNA expression in *Drd1*-expressing cells compared to *Drd2*-expressing cells in the NAc.

2.4 Materials and Methods

2.4.1 Subjects

A total of 16 male and 16 female adult (150–220 days old) prairie voles (*Microtus ochrogaster*) were selected from our outbred laboratory colony at Emory that was originally derived from field-caught voles in Champaign, Illinois. Eight males and eight females were homozygous for the high *Oxtr* expression allele (High-*Oxtr*; C/C at NT213739), and eight males and eight females were homozygous for the low *Oxtr* expression alleles (Low-*Oxtr*; T/T at NT213739), as described in

King et al. (2016). Voles were group-housed at 22˚C under a 14:10 h light/dark cycle in ventilated 26 × 18 × 19 cm Plexiglass cages containing Bedo'cobbs Laboratory Animal Bedding (The Andersons; Maumee, Ohio) with ad libitum access to water and food (Lab Rabbit Diet HF #5326, LabDiet). All experiments were done in accordance with the Institutional Animal Care and Use Committee at Emory University.

2.4.2 Brain sectioning

Voles were euthanized by isoflurane overdose followed by decapitation, and the brains were removed and immediately frozen on powdered dry ice. Brains were stored at −80°C until they were sectioned coronally into 14 μm sections using a cryostat, mounted onto Superfrost Plus slides (Fisherbrand; Pittsburgh, Pennsylvania), and stored at −80˚C.

2.4.3 Chromogenic RNAscope in situ hybridization for mapping Oxtr *mRNA*

To map *Oxtr* mRNA for Figures 1–7 and Supplementary Table 1, an RNAscope 2.5 High Definition Red Assay (Advanced Cell Diagnostics; Newark, California) was used to perform chromogenic in situ hybridization of *Oxtr* mRNA according to the manufacturer's instructions on every 10 sections from four brains (one high- and one low-*Oxtr* male and one high- and one Low-*Oxtr* female). For regions included in sections used for quantitative analysis of *Oxtr* genotype effects (OB, NAc, CPu, Tu, VP, and SNR), at least six brains per genotype per sex were used. Briefly, slide-mounted sections were thawed and fixed in 10% neutral buffered formalin for 15 min prior to 30 min of treatment with RNAscope Protease IV. Sections were then incubated in a hybridization probe specific for prairie vole *Oxtr* (Cat. No. 500721-C3) for 2 h at 40°C. Following six amplification steps, hybridized probes were reacted with chromogenic RNAscope RED

substrate for 10 min at room temperature and then counterstained with a 50% hematoxylin staining solution for 2 min at room temperature. Slides were then dried for 15 min at 60°C, dipped in xylene, and coverslipped with EcoMount mounting medium (Biocare Medical; Pacheco, California). Two negative controls, one performed without a probe and one performed with a bacterial DapB probe (Cat. No. 310043), appeared similar and showed no specific labeling. All sections shown in figures were taken from High-*Oxtr* voles, except where indicated otherwise.

2.4.4 Oxytocin receptor autoradiography for mRNA–protein comparison

To compare the location of *Oxtr* mRNA and OXTR protein, adjacent sections from the brains used for chromogenic in situ hybridization described above underwent OXTR autoradiography as previously described (Ross et al., 2009). Briefly, sections were thawed and then fixed for 2 min with 0.1% paraformaldehyde in PBS at room temperature. They were then incubated in 50 pM ^{125}I-OVTA (2200 Ci/mmol; PerkinElmer; Boston, MA), a selective, radiolabeled OXTR ligand, for 1 h. Unbound ^{125}I-OVTA was then removed with Tris-$MgCl_2$ buffer, and sections were allowed to dry. Sections were exposed to BioMax MR film (Kodak; Rochester, New York) for 5 days, and a QCAM CCD digital camera (Qimaging; Surrey, Canada) was used to capture 1200 dpi images in 8-bit grayscale. Adobe Photoshop CS6 (San Jose, California) was used to adjust the contrast and brightness of the images. Acetylcholine esterase staining was performed after OXTR binding as described previously to enable the accurate identification of brain regions (Lim et al., 2004a). All sections shown in the figures were taken from High-*Oxtr* males, except where noted otherwise.

2.4.5 Fluorescent RNAscope for colocalizing Oxtr *mRNA within D1R- and D2R-expressing cells*

An RNAscope Fluorescent Multiplex Reagent Kit (Advanced Cell Diagnostics; Newark, California) was used to perform fluorescent in situ hybridization triple labeling of *Oxtr*, *Drd1*, and *Drd2* mRNA according to the manufacturer's instructions on sections from 24 brains (six High- and six Low-*Oxtr* males, and six High- and six Low-*Oxtr* females). Briefly, slide-mounted sections were thawed and fixed in 10% neutral buffered formalin for 15 min prior to 30 min of treatment with RNAscope Protease IV. Sections were then incubated in hybridization probes specific for prairie vole *Oxtr* (Cat. No. 500721-C3), *Drd1* (Cat. No. 422581), and *Drd2* (Cat. No. 534471-C2) mRNA for 2 h at 40˚ C. Sequences of prairie vole *Drd1* and *Drd2* and several other behaviorally relevant genes are available from BAC clones described previously (McGraw et al., 2012). Following three amplification steps, hybridized probes were labeled with fluorophores Alexa 488 (*Drd1*), Atto 550 (*Drd2*), and Atto 647 (*Oxtr*). Sections were coverslipped with ProLong Gold Antifade Mountant with DAPI (ThermoFisher Scientific; Waltham, Massachusetts) for nuclear labeling. Two negative controls, one performed without a probe and one performed with a bacterial dapB probe (Cat. No. 310043), appeared similar and showed no specific labeling. Autoradiography was performed on a small subset of sections from each brain to confirm that the genotype predicted NAc OXTR density as expected. All sections shown in figures were taken from male voles.

2.4.6 Microscopy

A Keyence BZ-X710 microscope was used to take all in situ hybridization images. For chromogenic in situ hybridization, whole slides were imaged at 10× and stitched together using Keyence BZ X Analyzer Software. Fluorescent in situ hybridization images were taken with a 40× lens in four channels (blue, green, red, and infrared) using the Z-stack function. These images were

processed using the Keyence BZ X Analyzer Software to merge the four channels and convert the Z-stack into a single two-dimensional image using the Full Focus function.

2.4.7 Image analysis and statistics

For the semiquantitative brain-wide assessment of chromogenic RNAscope in situ hybridization, brain regions were defined according to the seventh edition of *The Rat Brain in Stereotaxic Coordinates* (Paxinos & Watson, 2013). For each brain region, 80 cells, detected by hematoxylin staining, were randomly selected and assessed for positive labeling of *Oxtr* mRNA. The number of cells expressing *Oxtr* was counted and binned into four groups: 1–20 cells (green in Figure 1, + in Supplementary Table 1), 21–40 cells (yellow in Figure 1, ++ in Supplementary Table 1), 41–60 cells (orange in Figure 1, +++ in Supplementary Table 1), and 61 or more cells (red in Figure 1, ++++ in Supplementary Table 1). Brain regions in which *Oxtr* was unevenly distributed were subdivided, and *Oxtr* was quantified separately for each subregion. Only the male High-*Oxtr* brain was used to generate Supplementary Table 1, although this expression pattern was consistent across the other three brains except for the lower expression in genotype-dependent regions such as the NAc and CPu of Low-*Oxtr* voles.

ImageJ with Fiji (version 2.1.0/1.53c; Schindelin et al., 2012) was used to quantify fluorescent RNAscope in situ hybridization of *Oxtr* mRNA. Sections were selected from the anterior portion of the NAc immediately posterior to the formation of the genu of the corpus callosum. The area selected for analysis was approximately midway between the anterior commissure and the ventral tip of the lateral ventricle. In each of 24 voles, 20 *Drd1*-expressing and 20 *Drd2*-expressing cells (D1R and D2R cells, respectively) were randomly selected for analysis in each hemisphere, yielding a total of 40 D1R and 40 D2R cells from each animal. Each

of the selected cells was outlined, and the mean gray value for the infrared (*Oxtr*) channel was measured to quantify the level of *Oxtr* mRNA. These values were averaged for D1R cells and D2R cells to yield one value per cell type in each animal. The statistical software Prism (version 9.2.0; GraphPad; San Diego, California) was used to conduct a three-way mixed model ANOVA with cell type (D1R vs. D2R) as a repeated-measures, within-subjects factor and with sex (male vs. female) and genotype (High- vs. Low-*Oxtr*) as between-subjects factors. A post hoc Šidák's multiple comparisons test was used to make pairwise comparisons.

2.5 Results

2.5.1 Brain-wide mapping of Oxtr *mRNA*

To investigate the expression of *Oxtr* mRNA throughout the prairie vole brain, we performed chromogenic RNAscope in situ hybridization on sections extending from the olfactory bulb to the brainstem. Labeling reflected specific binding of the *Oxtr* mRNA probe as a negative control using an mRNA probe for the bacterial gene *dapB* produced no signal. In total, we identified *Oxtr* mRNA in over 250 distinct brain regions, which were assessed in a semiquantitative manner and cataloged in Supplementary Table 1. Semiquantitative schematic illustrations of *Oxtr* expression at 14 points along the rostral-caudal axis are provided in **Figure 1**, where colors reflect the percentage of cells expressing *Oxtr*. These illustrations were made by overlaying representative images of mRNA labeling onto schematics from a rat brain atlas (Paxinos & Watson, 2013). Diffuse expression of *Oxtr* can be seen in numerous regions throughout the brain, with robust expression concentrated in specific regions.

2.5.2 Regions involved in social salience and memory

Oxtr mRNA expression was particularly pronounced in areas involved in social recognition, reward, valence, and memory, processes thought to be important for pair bonding (**Figure 2**) (Walum & Young, 2018). Olfaction is a highly social sensory modality for rodents, and both the olfactory bulb and anterior olfactory area express *Oxtr* (**Figure 2a,b**). *Oxtr* mRNA was detected throughout the amygdala, which processes olfactory information and modulates valence, and expression was particularly dense in the central and basolateral regions (**Figure 2e**) and the posteromedial cortical amygdala (**Figure 1**). Both the core and shell of the NAc, a critical reward center, contained high levels of *Oxtr* mRNA (**Figure 2d**), as did all regions of the PFC, including the prelimbic and infralimbic cortices, although strong expression was more prominent in deeper cortical layers, particularly 5 and 6, in the PLC (**Figure 1,2c**). Certain regions of the hippocampal formation, including CA2, ventral CA1, and the dentate gyrus, contained high levels of *Oxtr* mRNA, although it was primarily restricted to the stratum pyramidale of CA fields and the granule cell and polymorph layers of the dentate gyrus (**Figure 6c,d, 4d, 2f**).

2.5.3 Hypothalamic, thalamic, and septal regions

Oxtr mRNA-producing cells were identified in certain hypothalamic areas and related structures involved in social and reproductive behavior, including the VMH (**Figure 3f**), lateral septum (**Figure 3a**), medial preoptic area (**Figure 3c**), paraventricular thalamus (**Figure 3d**), paraventricular nucleus of the hypothalamus (**Figure 3e**), and BNST, with a more intense signal in the lateral division (**Figure 3b**). The BNST, VMH, and paraventricular thalamic nucleus displayed exceptionally strong expression, while the medial preoptic area and lateral septum contained more moderate levels of *Oxtr* mRNA. The paraventricular nucleus of the hypothalamus

and supraoptic nucleus, two primary sites of oxytocin synthesis, contained only sparse labeling (**Supplementary Table 1**).

2.5.4 Cortical regions

Oxtr mRNA expression in the cerebral cortex was relatively diffuse, with certain regions containing moderate-to-high levels of expression (**Figure 1**). These signals were primarily located in cortical layers 2, 5, and 6 (**Figure 4**). In the piriform cortex, expression was mostly restricted to the posterior piriform cortex, with the anterior portion displaying only sparse labeling (**Figure 1, 4c**). In contrast, *Oxtr* mRNA coursed through both the anterior and posterior insular cortex (**Figure 4b**). Both the anterior cingulate cortex (**Figure 4a**) and auditory cortex (**Figure 4d**) showed relatively modest levels of *Oxtr* mRNA labeling overall, although expression was mostly restricted to and relatively dense in layers 5 and 6. The interstitial nucleus of the posterior limb of the anterior commissure, a part of the extended amygdala receiving dense innervation from tyrosine hydroxylase-containing fibers, can be seen in these images and contained very high levels of *Oxtr* mRNA.

2.5.5 Neuromodulatory areas

Certain areas in the hindbrain displayed robust *Oxtr* expression, including the hypothalamic retromamillary nucleus (**Figure 5a**), posterior hypothalamic nucleus (**Figure 5b**), and the alpha part of the central gray (**Figure 5d**). The ventral tegmental area (**Figure 5a**), periaqueductal gray area (**Figure 5c**), dorsal raphe nucleus (**Figure 5c**), the laterodorsal tegmental nucleus (**Figure 5d**), and the nucleus basalis of Meynert (not shown) contained relatively sparse *Oxtr* expression. Barrington's nucleus and the locus coeruleus displayed strong *Oxtr* expression, although no signal

was observed in the caudal portion of the locus coeruleus (**Figure 5d**). The vagus nerve nucleus (**Figure 1**) also displayed strong expression.

2.5.6 Mismatch of Oxtr *mRNA and OXTR protein*

Next, we performed autoradiography on adjacent sections to compare the locations of OXTR protein and *Oxtr* mRNA. Protein and mRNA expression were closely aligned in most brain regions but not all. Both OXTR protein (**Figure 6a, b**) and *Oxtr* mRNA (**Figure 6c, d**) were strongly expressed in the stratum pyramidale of hippocampal CA2, but even stronger protein labeling in the absence of any mRNA signal was evident immediately superficial and deep to the stratum pyramidale (**Figure 6e, f**). The resolution of receptor autoradiography is too low to identify with certainty which layers contained this protein labeling, but the two layers immediately superficial to the stratum pyramidale, the stratum radiatum, and stratum lacunosum-moleculare, as well as the stratum oriens immediately deep to the stratum pyramidale, are plausible options. This disparity between the locations of OXTR protein and *Oxtr* mRNA suggests that OXTRs may be synthesized in the cell body in one location and transported to projections in another, enabling synaptic transmission.

We also examined differences in mRNA and protein expression between voles with genetically determined differences in *Oxtr* expression (**Figure 7**). High-*Oxtr* voles expressed far more OXTR protein (**Figure 7a**) and *Oxtr* mRNA (**Figure 7e**) in the NAc than Low-*Oxtr* voles (**Figure 7b, f**), as expected. Interestingly, a similar genotype effect on OXTR binding was observed in the substantia nigra pars reticulata (SNR) (**Figure 7c,d**), which is heavily innervated by the striatum. However, no *Oxtr* mRNA-positive cells were detected in the SNR in voles of either genotype (**Figure 7g,h**), suggesting that OXTR binding in the SNR is on projections from

another brain area, most likely the striatum, where *Oxtr* expression is genotype dependent (King et al., 2016) (**Figure 7**).

2.5.7 Colocalization of Oxtr *with* Drd1 *and* Drd2 *mRNA in the NAc*

Finally, we investigated the colocalization of *Oxtr* with *Drd1* and *Drd2* mRNA in the NAc of male and female High- and Low-*Oxtr* voles (**Figure 8a–d** and **Figure 8e–h**, respectively). As in other rodents, *Drd1* (**Figure 8a, e**) and *Drd2* (**Figure 8b, f**) were largely expressed in separate cells. *Oxtr* mRNA labeling (**Figure 8c, g**) was detected in approximately 80% of cells. To determine how *Oxtr* expression varied between cell types (D1R vs. D2R), genotypes (High- vs. Low-*Oxtr*), and sexes, we measured the intensity of *Oxtr* labeling in a subset of D1R and D2R cells and analyzed the data using a three-way mixed model ANOVA with cell type as a repeated-measure, within-subjects factor and with sex and genotype as between-subjects factors. There was a main effect of genotype (F (1, 20) = 74.20, $p < .0001$), accounting for 70.81% of the variation in *Oxtr* expression (**Figure 9**), which aligns closely with previous studies (King et al., 2016). There was no main effect of sex, but there was a main effect of cell type (F (1, 20) = 20.90, $p = .0002$); although slightly more *Oxtr* signal was detected in D2R cells than in D1R cells, cell type accounted for just 2.18% of the variation. Weak genotype-by-sex (F (1, 20) = 4.41, $p = .0485$) and genotype-by-cell type (F (1, 30) = 4.80, $p = .0405$) interactions were also detected, accounting for 4.21% and 0.50% of variation, respectively, in which genotype had slightly larger effects on females than on males and on D2R cells than on D1R cells. A post hoc Šidák's multiple comparisons test revealed significant differences in *Oxtr* expression between High- and Low-*Oxtr* genotypes in each sex-by-cell-type comparison ($p < .01$). There was also a significant difference between D1R and D2R cells in High-*Oxtr* males ($p = .0053$), with D2R cells expressing slightly more *Oxtr*, but there

was no significant difference between D1R and D2R cells in Low-*Oxtr* males or in females of either genotype.

2.6 Discussion

Here, we provide the first detailed description of the distribution of *Oxtr*-producing cells in the prairie vole brain and the first information on the colocalization of *Oxtr* mRNA with *Drd1* and *Drd2*. Our comprehensive analysis of *Oxtr* mRNA revealed widespread expression of *Oxtr* across the brain, with enrichment in particular brain regions, indicating that OXTR signaling may exert more profound modulation of brain function than expected from earlier receptor-binding studies. Oxytocin is released in response to a myriad of social stimuli, including social touch (Tang et al., 2020), visual observation of maternal behavior (Carcea et al., 2021), infant vocalizations (Valtcheva et al., 2021), and even interspecies eye contact between humans and dogs (Nagasawa et al., 2015). Once released, oxytocin is capable of modulating the activity of neural networks at three levels: (i) by directly depolarizing projection neurons, (ii) by affecting the activity of interneurons that regulate local circuit activity, and (iii) by modulating the activity of other neuromodulator systems like dopamine, acetylcholine, and serotonin (Putnam et al., 2018; Froemke & Young, 2021). Our findings further expand the potential influence of oxytocin signaling as the widespread *Oxtr* expression we observed, coupled with the diverse social triggers of oxytocin release, and the many neural processes oxytocin has been shown to affect, suggesting that social encounters can profoundly impact brain function through oxytocin-dependent modulation of vast neural networks.

Our observations are consistent with previous studies labeling *Oxtr* mRNA in prairie voles, although these earlier efforts analyzed fewer anatomical regions and utilized in situ hybridization

techniques that were far less sensitive and had lower resolution than those we employed in the present study (Young et al., 1996; Lim et al., 2004b). Although species differences exist, our findings are generally consistent with protein and mRNA analyses of *Oxtr* expression patterns in other species. Mitre et al. (2016) mapped OXTR protein in mouse brain using immunohistochemistry and showed labeling in many of the same regions we found *Oxtr* mRNA in prairie voles, including hippocampus, amygdala, BNST, certain hypothalamic nuclei, and certain cortical regions. There were also some differences from our findings. Intermediate to high levels of OXTR immunolabeling were detected in the mouse PVN, SON, and SCN, whereas only a small population of cells expressed *Oxtr* mRNA in these nuclei of the prairie vole. Although Mitre et al.'s labeling in mice is convincing, antibodies targeting OXTRs are notorious for producing nonspecific labeling (Yoshida et al., 2009). In humans, *Oxtr* mRNA expression appears similarly widespread and diffuse with isolated regions of high expression, including the striatum, thalamus, hypothalamus, hippocampus, amygdala, and olfactory bulbs (Bethlehem et al., 2017; Quintana et al., 2019). Notably, the high level of *OTXR* mRNA expression in the human CPu and NAc is consistent with prairie vole but not in the mouse or rat brain. There is also a notable overlap of *OXTR* mRNA with dopamine receptor mRNA expression (Quintana et al., 2019).

It should be noted that species- and brain region-specific developmental changes in OXTR density have been reported in rodents, which our study cannot address since only adult animals were used. For instance, in the NAc, CPu, BLA, and PVA, OXTR-binding increases from juvenile to adulthood in prairie voles, whereas OXTR binding is significantly decreased in several of the same regions as rats mature into adults (Smith et al., 2016; Prounis et al., 2018). Future studies should examine developmental changes in *Oxtr* mRNA in the prairie vole brain, particularly since

developmental oxytocin signaling is thought to influence later life social behaviors in adults (Barrett et al., 2015).

2.6.1 Oxytocin receptor mRNA expression in select areas influencing behavior

Although widespread, it is evident that OXTRs are concentrated in certain areas that subserve specific functions. We observed *Oxtr* mRNA in many subregions of the hippocampus, but the expression was particularly strong in dorsal CA2 and ventral CA1, regions studied extensively for their role in social memory. Multisensory social information is believed to be processed in the dorsal CA2 and then transmitted to the ventral CA1 for social memory storage (Watarai et al., 2021). Dorsal CA2 and its projections to ventral CA1 are critical for social memory encoding, consolidation, and recall (Meira et al., 2018; Oliva et al., 2020), and OXTRs in CA2 are necessary for the discrimination of social stimuli and the formation of social recognition memory (Raam et al., 2017; Lin et al., 2018). Indeed, dorsal CA2 provides strong excitatory input to the ventral CA1 (Meira et al., 2018), and in mice OXTR-binding depolarizes CA2 pyramidal cells, which regulates the short-term plasticity of CA2-to-CA1 synaptic transmission (Tirko et al., 2018). Ventral CA1 is believed to house social engrams or memory traces for specific individuals (Okuyama et al., 2016; Okuyama, 2018), and oxytocin signaling in CA1 fast-spiking interneurons enhances the signal-to-noise ratio in CA1 pyramidal cells (Owen et al., 2013). OXTR binding in hippocampal subfields, particularly dorsal CA2 and ventral CA1, therefore appears to modulate hippocampal network activity and facilitate the flow of social information through the hippocampus, thus enabling social discrimination and memory.

Oxytocin signaling in the medial amygdala is also essential for social recognition in mice (Ferguson et al., 2001), and in the central amygdala oxytocin signaling modulates emotion

discrimination (Ferretti et al., 2019). In prairie voles, we observed extensive labeling in the amygdala, with particularly strong expression in the basolateral and central nuclei. Interestingly, while prairie voles have high densities of OXTR binding in the basolateral amygdala, mice do not (Young & Zhang, 2021). Strong *Oxtr* labeling was also detected in the anterior olfactory area and granule cell layer of the olfactory bulb. OXTR signaling in the anterior olfactory area enhances social recognition in mice by increasing excitatory output to inhibitory granule cell interneurons in the main olfactory bulb (Oettl et al., 2016). This increased inhibition enhances the signal-to-noise ratio of social olfactory information conveyed via mitral and tufted cell projection neurons from the olfactory bulb to the olfactory cortex (Oettl et al., 2016). OXTR binding in the prairie vole anterior olfactory area is highly sensitive to estrogen, suggesting that oxytocin-dependent salience of olfactory social cues is enhanced during mating, which facilitates pair bonding (Witt et al., 1991). Very strong *Oxtr* expression was also observed in the posteromedial cortical amygdaloid nucleus, which is the only cortical target of the direct projection from the accessory olfactory bulb. The accessory olfactory bulb relays vomeronasal information. Thus, oxytocin signaling might be involved in the information processing of the accessory olfactory system and the influence of this system on sociosexual behavior.

We observed very strong *Oxtr* mRNA expression in the NAc, where oxytocin signaling is necessary for the formation of monogamous pair bonds (Young et al., 2001), and decreases in oxytocin signaling appear to mediate some of the effects of partner loss, such as passive stress coping (Bosch et al., 2016; Pohl et al., 2019). There is emerging evidence that the neurophysiological effects of OXTR signaling in the NAc dynamically change with social experience. OXTR signaling in the NAc of virgins decreases the amplitude of spontaneous excitatory postsynaptic currents (EPSCs), while it increases the amplitude of electrically evoked

EPSCs in an endocannabinoid-dependent manner in the NAc of pair-bonded voles (Borie et al., 2022b). BNST also showed very strong labeling. Oxytocin signaling in the BNST and NAc mediates social vigilance and the regulation of social approach behaviors (Duque-Wilckens et al., 2020; Williams et al., 2020). We only detected sparse labeling of *Oxtr* mRNA in the VTA and PAG. Although there are some reports of OT behavioral effects on these regions (Song et al., 2016; Hung et al., 2017), to our knowledge, there is no report of robust expression of *Oxtr* in these regions. Consistent with our sparse *Oxtr* labeling in the adult prairie vole VTA, *Oxtr* expression in the rat VTA was decreased during development, and only weak expression was detected in the adult (Yoshimura et al., 1996). *Oxtr* mRNA in the mouse PAG also showed a decline from juvenile to adulthood (Olazábal & Alsina-Llanes, 2016). We speculate that either few *Oxtr*-expressing neurons in the VTA are physiologically sufficient to exert behavioral effects in rodents or that mice have greater numbers of Oxtr neurons than prairie voles and OXTR signaling plays a lesser role in prairie vole behavior than mice. Notably, the vagus nerve nucleus showed strong labeling as well, as peripheral oxytocin signaling in the vagus nerve can influence feeding and drug self-administration behaviors (Iwasaki et al., 2015; Everett et al., 2021) and therefore could potentially mediate some of the behavioral effects of intranasally administered oxytocin in humans.

We detected *Oxtr* mRNA expression in numerous hypothalamic nuclei. Oxytocin is synthesized and released primarily by the magnocellular and parvocellular neurons of the paraventricular and supraoptic nuclei as well as accessory nuclei (Althammer & Grinevich, 2018; Grinevich & Ludwig, 2021). Via their projections into the posterior pituitary, magnocellular neurons are the primary source of oxytocin released as a hormone into the peripheral circulation. The anatomical origins and physiological regulation of oxytocin release in the central nervous system are more complicated and not fully understood but certainly rely on parvocellular

projections, magnocellular axon collaterals, and extrasynaptic oxytocin release (Althammer & Grinevich, 2018; Grinevich & Ludwig, 2021). Intrahypothalamic oxytocin signaling appears particularly important. For example, the somatodendritic release of oxytocin in the paraventricular and supraoptic nuclei is believed to have local autocrine and paracrine effects with important behavioral consequences (Ludwig & Leng, 2006; Modi et al., 2015; Ludwig et al., 2016). Additionally, a recent study found that a small population of parvocellular neurons in the paraventricular nucleus is selectively activated by social touch and that these cells project to and regulate the activity of magnocellular neurons (Tang et al., 2020). High levels of OXTR immunolabeling were reported in the mouse paraventricular and supraoptic nuclei, whereas *Oxtr* mRNA expression in these nuclei in prairie voles was notably less robust than in many other hypothalamic nuclei. Despite the small proportion of neurons expressing *Oxtr*, they may be sufficient to trigger functional effects through collateral activation of interconnected magnocellular neurons. The VMH, which is critical for satiety as well as defensive behavior (Wang et al., 2019a) and aggressive behavior in both males (Karigo et al., 2021) and females (Hashikawa et al., 2017), contained particularly high levels of *Oxtr* mRNA. Notably, expression in the prairie vole VMH was least dense in the ventrolateral portion. In contrast, rats have robust *Oxtr* expression in the ventrolateral portion of the VMH, and this expression influences sexual behavior and is regulated by estrogen and progesterone (Schumacher et al., 1990; Bale et al., 1995). The medial preoptic area, which is critical for sexual behavior and both maternal and paternal parenting behaviors, also contains robust levels of *Oxtr* mRNA (Pedersen et al., 1994; Kohl et al., 2018; Yuan et al., 2019; Numan, 2020).

Many cortical areas displayed sparse labeling of *Oxtr* mRNA, but some, including the anterior cingulate and insular cortices, showed strong labeling in restricted layers, primarily layers

two, five, and six. Oxytocin signaling in the anterior cingulate cortex is critical for consoling and helping behaviors in prairie voles (Burkett et al., 2016; Kitano et al., 2020), mandarin voles (Li et al., 2020), and rats (Yamagishi et al., 2020). In the insular cortex, OXTR activity is necessary for rats to demonstrate appropriate social affective preference, that is, to approach stressed juveniles and avoid stressed adults (Rogers-Carter et al., 2018). The anterior cingulate and insular cortices share reciprocal connectivity, suggesting a mechanism by which OXTRs in these regions could coordinate activity between two distinct cortical areas and thus regulate emotion detection and behavioral responses. Notably, in humans, the dorsal anterior cingulate cortex and anterior insular cortex constitute the key nodes of a salience network, which is critical for the detection of behaviorally relevant stimuli and commonly disrupted in neuropsychiatric conditions (Seeley et al., 2007; Uddin, 2015). Moreover, the insular cortex has been implicated as a central node linking social affective cues to the social decision-making network (SDMN), which consists of brain regions such as the MeA, BNST, MPOA, LS, HIP, NAc, and BLA (Rogers-Carter & Christianson, 2019). *Oxtr* was consistently expressed in the insular cortex and all the nodes of the SDMN.

2.6.2 Mismatches in the localization of Oxtr *mRNA and OXTR protein*

We compared *Oxtr* mRNA expression to OXTR protein distribution by applying in situ hybridization and receptor autoradiography to adjacent sections. To our knowledge, this is the first brain-wide comparison of OXTR protein (i.e., binding) and *Oxtr* mRNA distribution with cellular resolution in any species. Throughout most of the brain, protein and mRNA expression overlapped. There were, however, two notable instances in which we observed OXTR binding (i.e., protein) in the absence of mRNA, which provides compelling evidence that *Oxtr* mRNA can be located in cell somas in one region, while the cells' OXTR proteins reside in projections elsewhere in the

brain. This has important implications for the application of targeted OXTR knockdown techniques, such as viral vector-mediated CRISPR, as genome editing in one area could eliminate OXTR signaling on distal projections (Boender & Young, 2020).

The first instance of misaligned protein and mRNA localization was in the hippocampus. The CA2 region displayed strong expression of both *Oxtr* mRNA and OXTR protein. The mRNA was restricted to the stratum pyramidale, while protein labeling was evident both superficial and basal to the stratum pyramidale. It is important to note that OXTR-binding patterns have been shown to vary widely between rodent species (Raggenbass et al., 1989; Young & Zhang, 2021). Nevertheless, in mice, CA2 receives afferent projections from the paraventricular nucleus of the hypothalamus, and CA2 pyramidal cells express robust levels of OXTRs, which maintain the long-term potentiation of synapses with entorhinal cortex projections (Yoshida et al., 2009; Cui et al., 2013; Lin et al., 2018). It is therefore plausible that the OXTR protein labeling we observed superficial and basal to the stratum pyramidale is located on the dendritic arbors of pyramidal neurons. However, other neurons could also be responsible for this protein signal; numerous types of interneurons throughout the hippocampus express OXTRs, some interneurons with somas in the stratum pyramidale send projections into the strata radiatum and lacunosum moleculare, and the retromamillary nucleus, which had a very high expression, has reciprocal connections with CA2 (Botcher et al., 2014; Young & Song, 2020). Although the origin of the OXTR protein signal we observed cannot be determined definitively from our images, it is clear that the proteins reside and act in receptive fields a considerable distance from the somas in which the mRNA resides. Interestingly, hippocampal OXTR binding has been reported to vary with sex and reproductive physiology and is speculated to be involved in sex differences in spatial memory and fidelity in voles (Zheng et al., 2013; Ophir, 2017); therefore, future studies using Cre-dependent retrograde

viral tracers in *OXTR-Cre* prairie voles may provide behaviorally relevant insight into neural circuitry contributing to mating tactics by identifying the sources of OXTR protein in the hippocampus (Horie et al., 2020).

The second instance of misaligned protein and mRNA localization was in the SNR, but this misalignment was contingent on the animal's *Oxtr* genotype. We previously identified a set of SNPs in linkage disequilibrium in the prairie vole *Oxtr* gene that predicts both the animal's ability to form a pair bond and the density of OXTR protein expressed in the NAc, CPu, OB, and anterior olfactory area (King et al., 2016). Levels of *Oxtr* mRNA in the NAc also varied in accordance with the protein (King et al., 2016). Here, we again observed that OXTR protein and *Oxtr* mRNA levels in the NAc varied with genotype. We also observed that High-*Oxtr* voles had considerable OXTR binding in the SNR, while Low-*Oxtr* voles showed no detectable binding there. More interestingly, no *Oxtr* mRNA signals were detected either in the SNR of Low-*Oxtr* or High-*Oxtr* voles. As NAc MSNs send significant projections to the SNR (Matamales et al., 2009), it is likely that the OXTR protein labeling we observed in the SNR is from OXTRs located on the presynaptic terminals of NAc MSNs. This observation supports the idea that OXTRs can serve presynaptic functions far from the anatomical location of the cells expressing the *OXTR* gene (Dölen et al., 2013; Mairesse et al., 2015; Mitre et al., 2016; Putnam et al., 2018). It also expands our understanding of how SNPs in the *Oxtr* gene are capable of influencing oxytocin signaling throughout the brain. A similar genotype-dependent pattern of OXTR binding was also observed in the ventral pallidum, which had a few sparse *Oxtr* mRNA-positive cells. Similar to the SNR, the ventral pallidum receives strong direct projections from the NAc, and we suspect that the genotype-dependent OXTR binding we observed in the ventral pallidum of High-*Oxtr* voles is from OXTR proteins on the terminals of axons from the NAc. However, as the ventral pallidum

receives projections from both D1R and D2R MSNs, while the SNR receives projections from only D1R MSNs (Pardo-Garcia et al., 2019), our observation of genotype-dependent OXTR expression in these brain regions raises the question of whether the High- and Low-*Oxtr* alleles influence *Oxtr* expression in both D1R and D2R MSNs or only D1R MSNs. Given that D1R in the NAc inhibits, while D2R facilitates, pair bond formation (see below) and both MSN types express *Oxtr*, it would be important to determine whether presynaptic OXTR signaling in the VP and SNR modulates pair bonding and parental care as well, particularly in relation to proposed circuit mechanisms of pair bonding involving the release of VP from NAc inhibition to promote maternal care and pair bonding (Numan & Young, 2016).

2.6.3 Comparing the localization of Oxtr *mRNA with* Drd1 *and* Drd2 *mRNA in the NAc*

The primary projection neurons of the NAc, GABAergic MSNs, constitute approximately 95% of all neurons in the NAc and can be subdivided into those that express *Drd1* and those that express *Drd2*. Both D1R and D2R MSNs receive afferent input from a wide range of brain regions (Li et al., 2018). Although oversimplified, the canonical notion of D1R and D2R MSNs is that they project to the SNR either directly via the monosynaptic "direct pathway" or indirectly via the multisynaptic "indirect pathway," respectively (Calabresi et al., 2014). In the direct pathway, D1R MSNs project to and inhibit the SNR, which sends inhibitory projections to the thalamus, resulting in disinhibition of thalamocortical projections. More recent experimental evidence has shown that D1R MSNs can also project to the ventral tegmental area and ventral pallidum (Yang et al., 2018). In the indirect pathway, D2R MSNs project indirectly to the SNR through a multisynaptic pathway, including the globus pallidus externa and subthalamic nucleus. This pathway excites the

inhibitory SNR neurons that project to the thalamus, resulting in the inhibition of thalamocortical projections.

Activation of D1R versus D2R MSNs is associated with antagonistic behavioral effects, including reward (D1R) and punishment (D2R) (Soares-Cunha et al., 2016) and pair bond formation (D2R) and maintenance (D1R) (Aragona et al., 2006). In prairie voles, D2R activation facilitates pair bond formation, and either blocking D2Rs or activating D1Rs inhibits pair bond formation (Aragona et al., 2006). However, 2 weeks after pair bonds are formed, male voles display upregulation of D1R in the NAc (Aragona et al., 2006). This upregulation of D1R promotes selective aggression toward unfamiliar conspecifics, which serves to help maintain the pair bond (Aragona et al., 2006). Most importantly, pair bond formation requires activation of both D2R and OXTR in the NAc (Liu & Wang, 2003).

Given the influence of *Oxtr* gene SNPs on pair bond formation and *Oxtr* expression in the NAc and the critical interaction of oxytocin and D1R versus D2R signaling in this region, we quantified and compared *Oxtr* mRNA expression in *Drd1*- and *Drd2*-expressing cells in the NAc of males and females homozygous for either the High-*Oxtr* or Low-*Oxtr* alleles. We found that 71% of the variation in *Oxtr* expression in the NAc was determined by genotype, which is remarkably close to our previous estimate of 74% (King et al., 2016). Although D2R cells expressed slightly more *Oxtr* than D1R cells, the vast majority of variation in *Oxtr* expression is determined by SNPs in the *Oxtr* gene. Of critical importance, these SNPs affect *Oxtr* expression more or less equally in both D1R and D2R cells.

2.7 Conclusion and Future Directions

Our comprehensive, brain-wide analysis of *Oxtr* mRNA expression revealed diffuse, widespread distribution throughout the brain as well as robust expression restricted to specific brain regions. These regions of concentrated expression suggest a mechanism by which oxytocin signaling can modulate the activity of neural networks throughout the brain in response to social stimuli. Furthermore, our comparison of *Oxtr* mRNA and OXTR protein localization in adjacent slices suggests that OXTRs may act in receptive fields after being transported a considerable distance from somas containing *Oxtr* mRNA, consistent with both pre- and postsynaptic signaling mechanisms. Lastly, we determined that SNPs in the *Oxtr* gene, which are predictive of an animal's ability to pair bonds, influence *Oxtr* expression in both D1R and D2R cells in the NAc. Comparative studies are needed to determine how these expression patterns differ from those of other organisms, especially other rodents and organisms with both similar and divergent behavioral repertoires. However, these data should inform future work in voles, particularly experiments attempting region-specific manipulations of *Oxtr* expression. Indeed, tracing experiments could help identify which of these oxytocin-sensitive brain regions communicate with each other, and site-specific knockdown of *Oxtr* expression could determine how oxytocin signaling in these regions and networks influences behavior. Additionally, we have identified many brain regions that express *Oxtr* mRNA in which the function of oxytocin signaling has not been studied. Exploring oxytocin signaling in these understudied regions could yield new insights into how social stimuli and oxytocin signaling affect the brain and behavior.

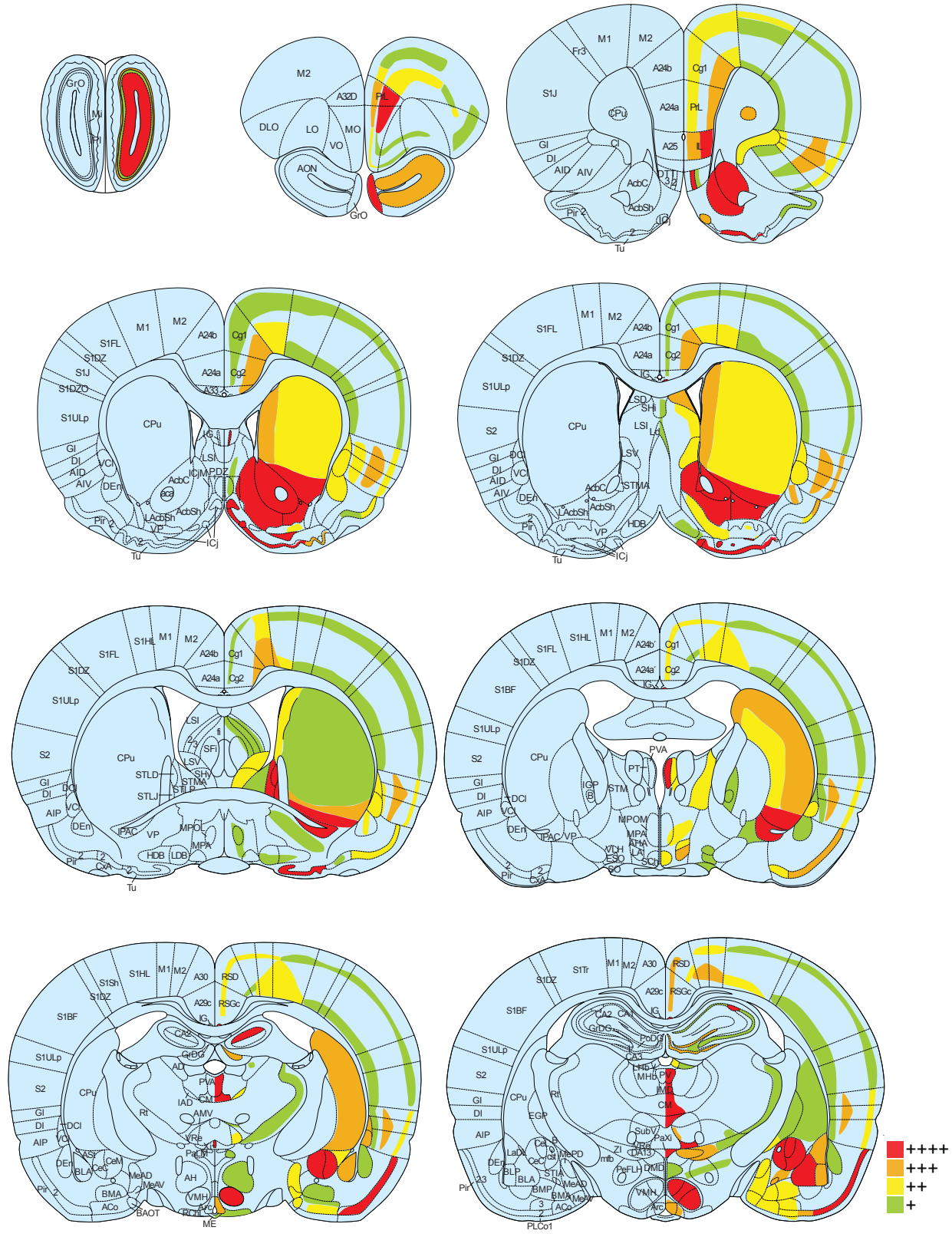


Figure 1. (continued on next page)

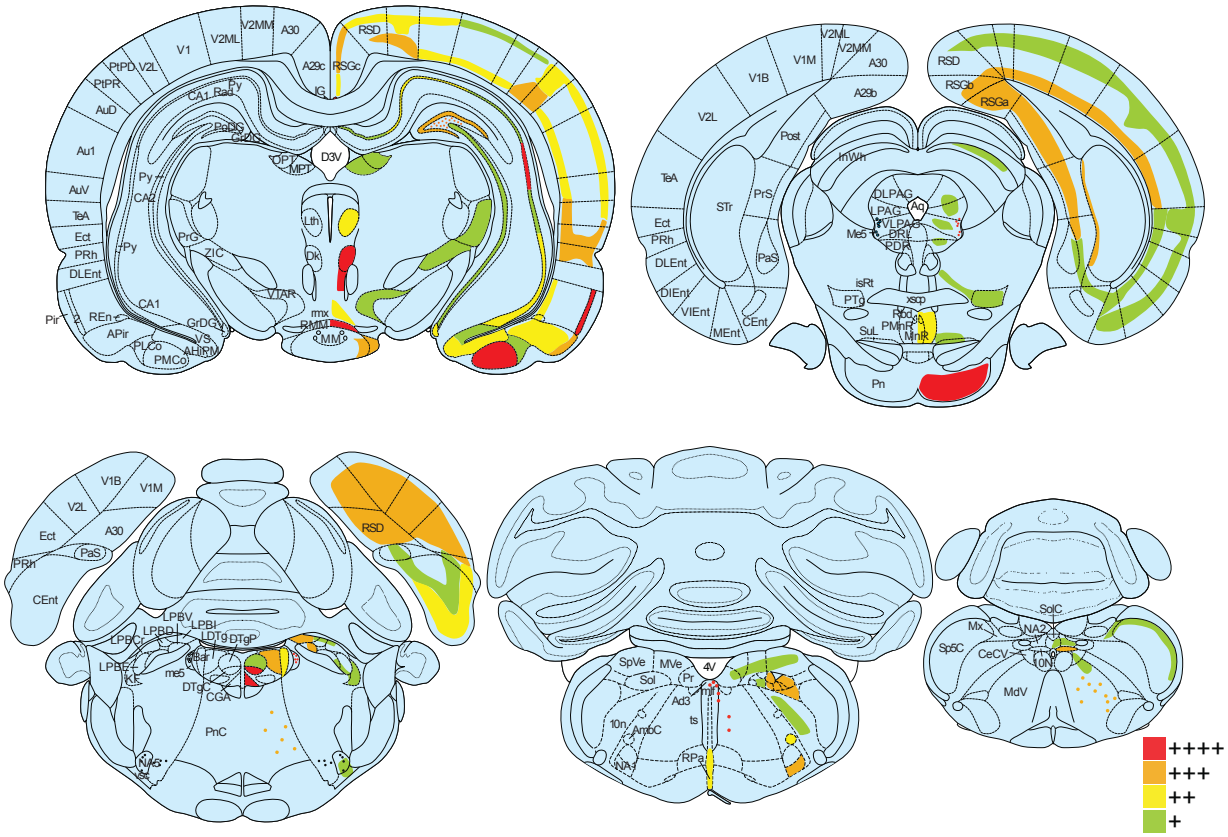


Figure 1. *Oxtr* mRNA expression throughout prairie vole brain. *Oxtr* mRNA was labeled with in situ hybridization, and the percentage of cells positive for *Oxtr* was calculated for each brain region. Brain regions are color coded according to *Oxtr* expression density (green for 1-25% of cells expressing *Oxtr*, yellow for 26-50%, orange for 51-75%, and red for 76-100%) in coronal sections from a rat brain atlas at 14 points along the rostral-caudal axis (Paxinos & Watson, 2013). For some regions (e.g., reticular formation, molecular layer of the hippocampus) with sparse cells containing exceptionally strong expression, red dots were used. Abbreviations on the left half of the images correspond to new nomenclature used in the seventh edition of *The Rat Brain in Stereotaxic Coordinates* (Paxinos & Watson, 2013), while the abbreviations on the right half of the images correspond to traditional nomenclature used in previous editions. (See Supplementary Table 1 for definitions of abbreviations.)

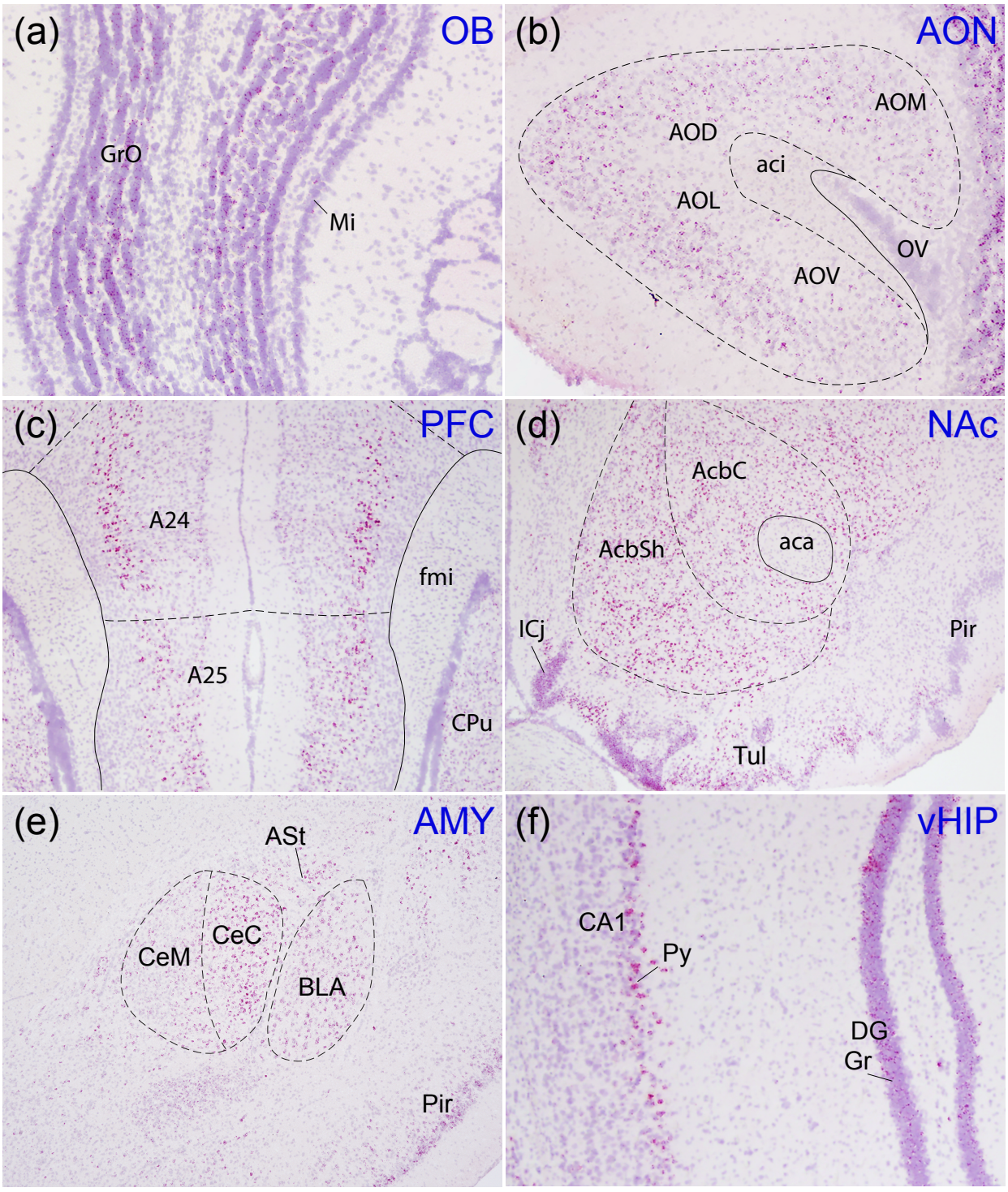


Figure 2. *Oxtr* expression in regions involved in social salience and memory. *Oxtr* mRNA (red) was labeled with in situ hybridization against a hematoxylin counterstain (purple). The olfactory bulb (a), anterior olfactory area (AO; AOV, ventral; AOL, lateral; AOD, dorsal; AOM,

medial; (b), granule cell layer of the olfactory bulb; Mi, mitral cell layer of the olfactory bulb; aci, anterior commissure, intrabulbar; OV, olfactory ventricle; A24, cingulate cortex previously known as prelimbic cortex; A25, cingulate cortex previously known as infralimbic cortex; fmi, forceps minor of the corpus callosum; CPu, caudate putamen; ICj, the island of Calleja; Tul, olfactory tubercle; aca, anterior commissure, anterior part; CeC, central amygdaloid nucleus, capsular; CeM, central amygdaloid nucleus, medial; BLA, basolateral amygdaloid nucleus, anterior; ASt, amygdalostriatal transition area; Pir, piriform cortex; Py, stratum pyramidale; DG, dentate gyrus; Gr, granule cell layer of the dentate gyrus.

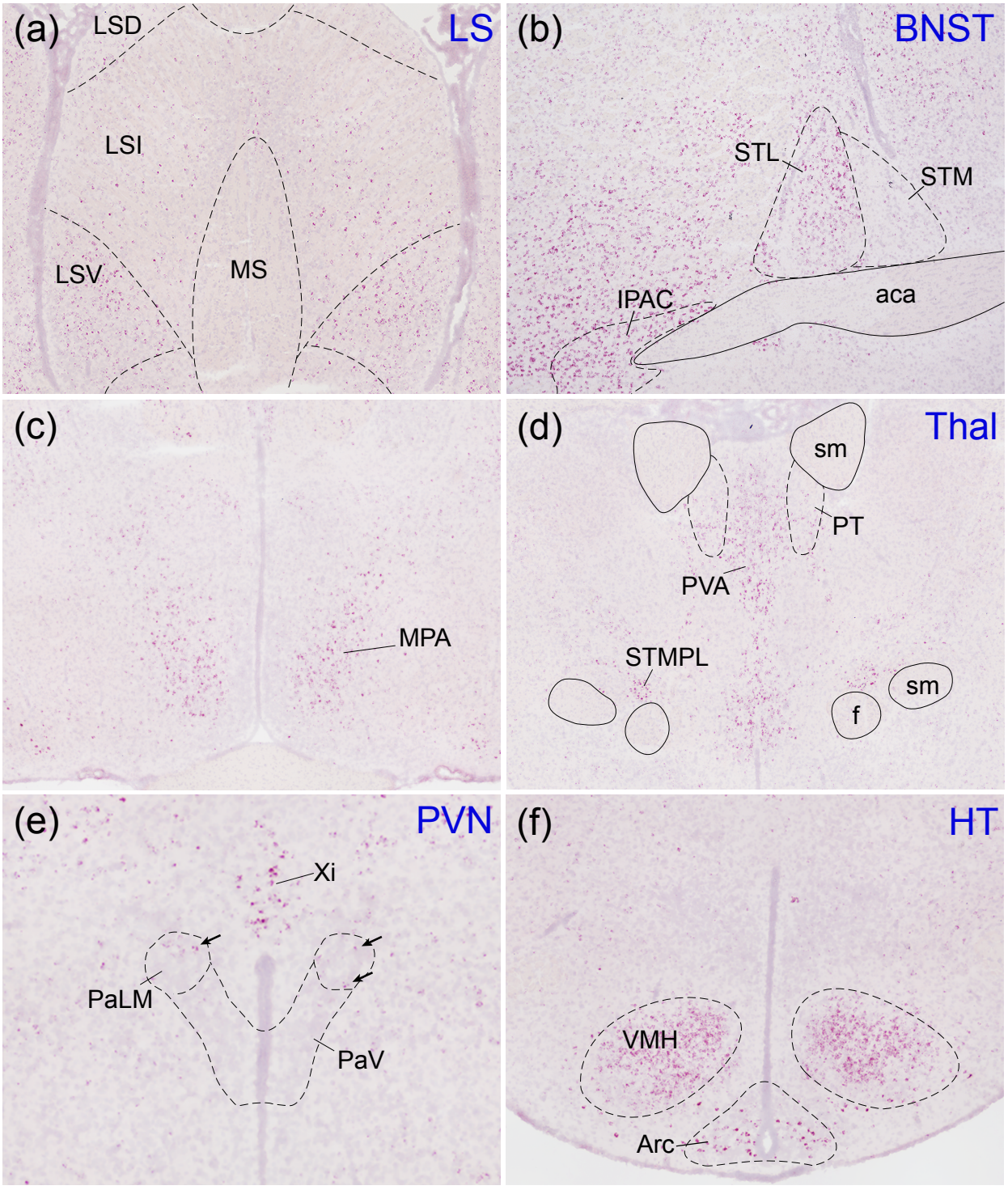


Figure 3. *Oxtr* expression in hypothalamic regions. *Oxtr* mRNA (red) was labeled with in situ hybridization against a hematoxylin counterstain (purple). The lateral septum (a) contained moderate expression concentrated mostly in the ventral (LSV) portion. *Oxtr* was robustly

expressed in the lateral division of the bed nucleus of the stria terminalis (STL) and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC; b) as well as the ventromedial nucleus of the hypothalamus (VMH; f). The medial preoptic area (MPA; c), thalamus (d), and arcuate nucleus of the hypothalamus (Arc; f) displayed moderate expression, while the paraventricular nucleus of the hypothalamus (PaLM, lateral magnocellular; PaV, ventral part; e; arrows indicate *Oxtr*-positive cells) displayed sparse expression. LSI, lateral septal nucleus, intermediate; LSD, lateral septal nucleus, dorsal; MS, medial septal nucleus; STM, bed nucleus of the stria terminalis, medial division; aca, anterior commissure, anterior part; PT, paratenial thalamic nucleus; PVA, paraventricular thalamic nucleus, anterior; sm, stria medullaris; f, fornix; STMPL, bed nucleus of the stria terminalis, medial, posterolateral; Xi, xiphoid thalamic nucleus.

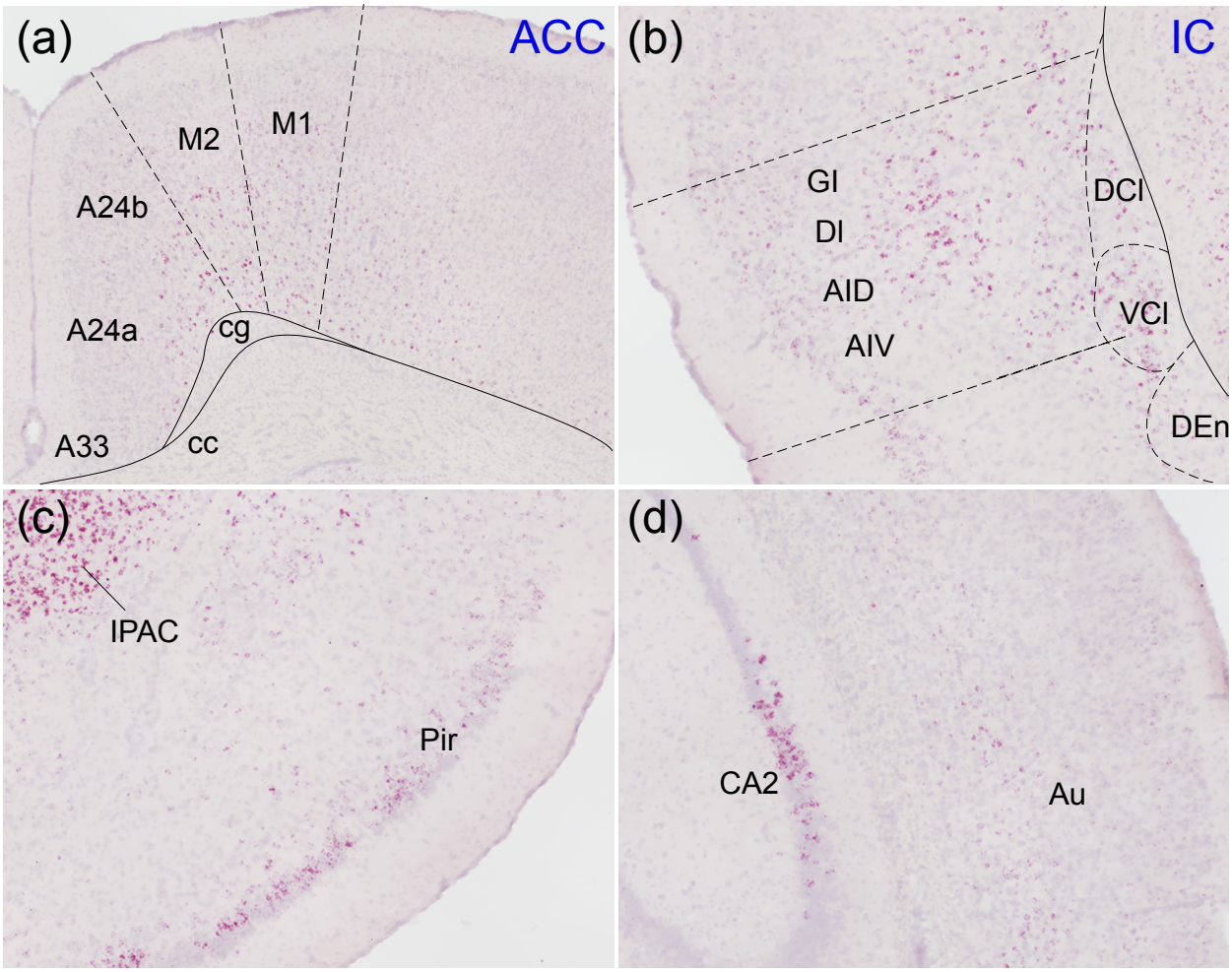


Figure 4. *Oxtr* expression in neuromodulatory areas. *Oxtr* mRNA (red) was labeled with in situ hybridization against a hematoxylin counterstain (purple). The anterior cingulate cortex (A24a, A24b, A33; a), insular cortex (b), piriform cortex (Pir; c), and secondary auditory cortex (Au; d) all contained *Oxtr* primarily restricted to cortical layers 2, 5, and 6. M1, primary motor cortex; M2, secondary motor cortex; cg, cingulum; cc, corpus callosum; GI, granular insular cortex; DI, dysgranular insular cortex; AID, agranular insular cortex, dorsal; AIV, agranular insular cortex, ventral; DCl, dorsal claustrum; VCl, ventral claustrum; DEn, dorsal endopiriform nucleus; IPAC, interstitial nucleus of the posterior limb of the anterior commissure.

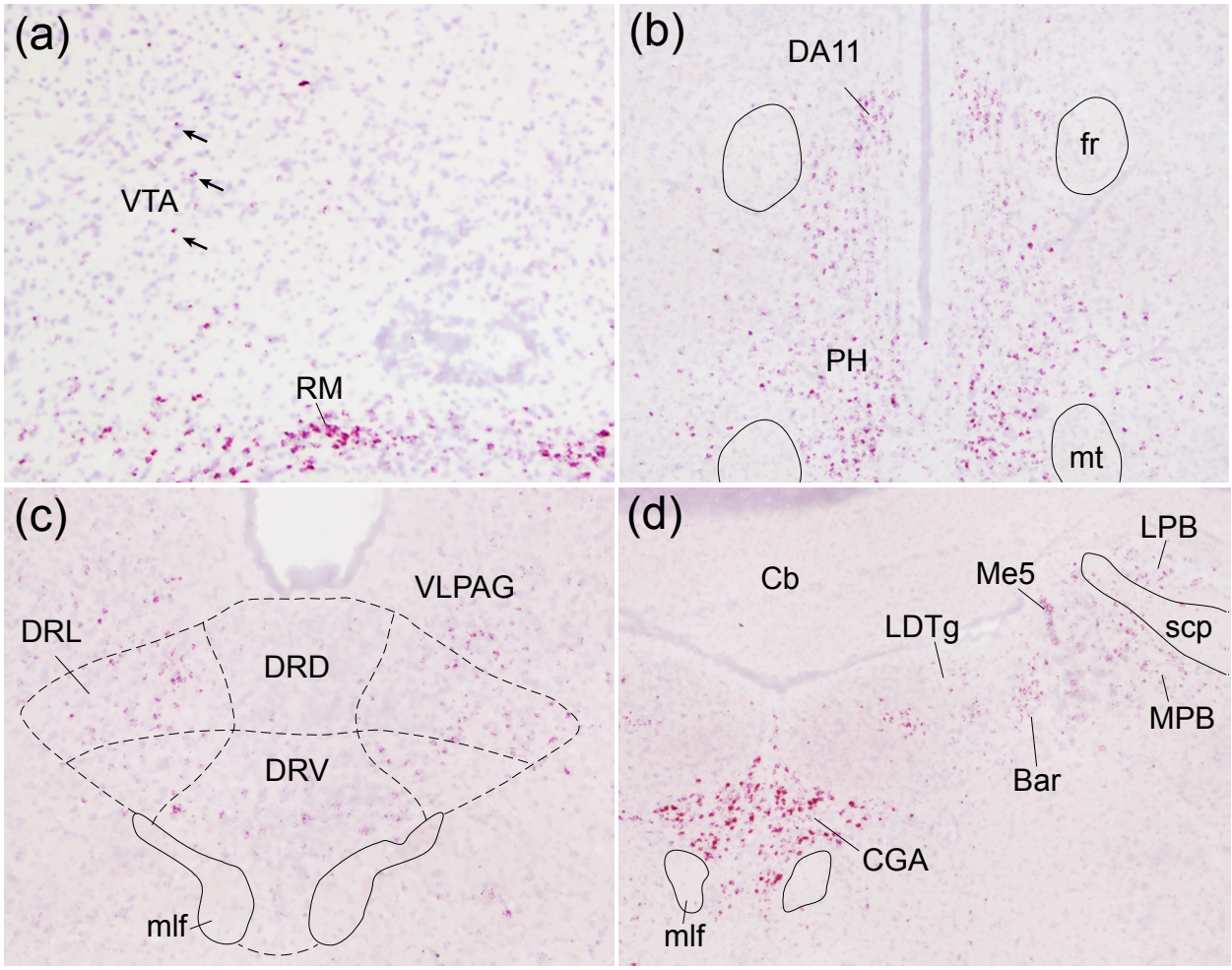


Figure 5. *Oxtr* expression in other regions of interest. *Oxtr* mRNA (red) was labeled with in situ hybridization against a hematoxylin counterstain (purple). The ventral tegmental area (VTA; a) and dorsal raphe nucleus (DR; c) displayed spare *Oxtr* labeling, while the retromammillary nucleus (RM; a), posterior hypothalamic nucleus (PH; b), and central gray, alpha part (CGA; d) displayed robust labeling. Arrows indicate *Oxtr*-positive cells within the VTA; DA11, DA11 dopamine cells; fr, fasciculus retroflexus; mt, mammillothalamic tract; DRD, dorsal raphe nucleus, dorsal part; DRL, dorsal raphe nucleus, lateral part; DRV, dorsal raphe nucleus, ventral part; VLPAG, ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus; CG, central gray, LDTg, laterodorsal tegmental nucleus; LPB, lateral parabrachial nucleus; MPB, medial

parabrachial nucleus; Me5, mesencephalic trigeminal nucleus; Bar, Barrington's nucleus; Cb, cerebellum; scp, superior cerebellar peduncle.

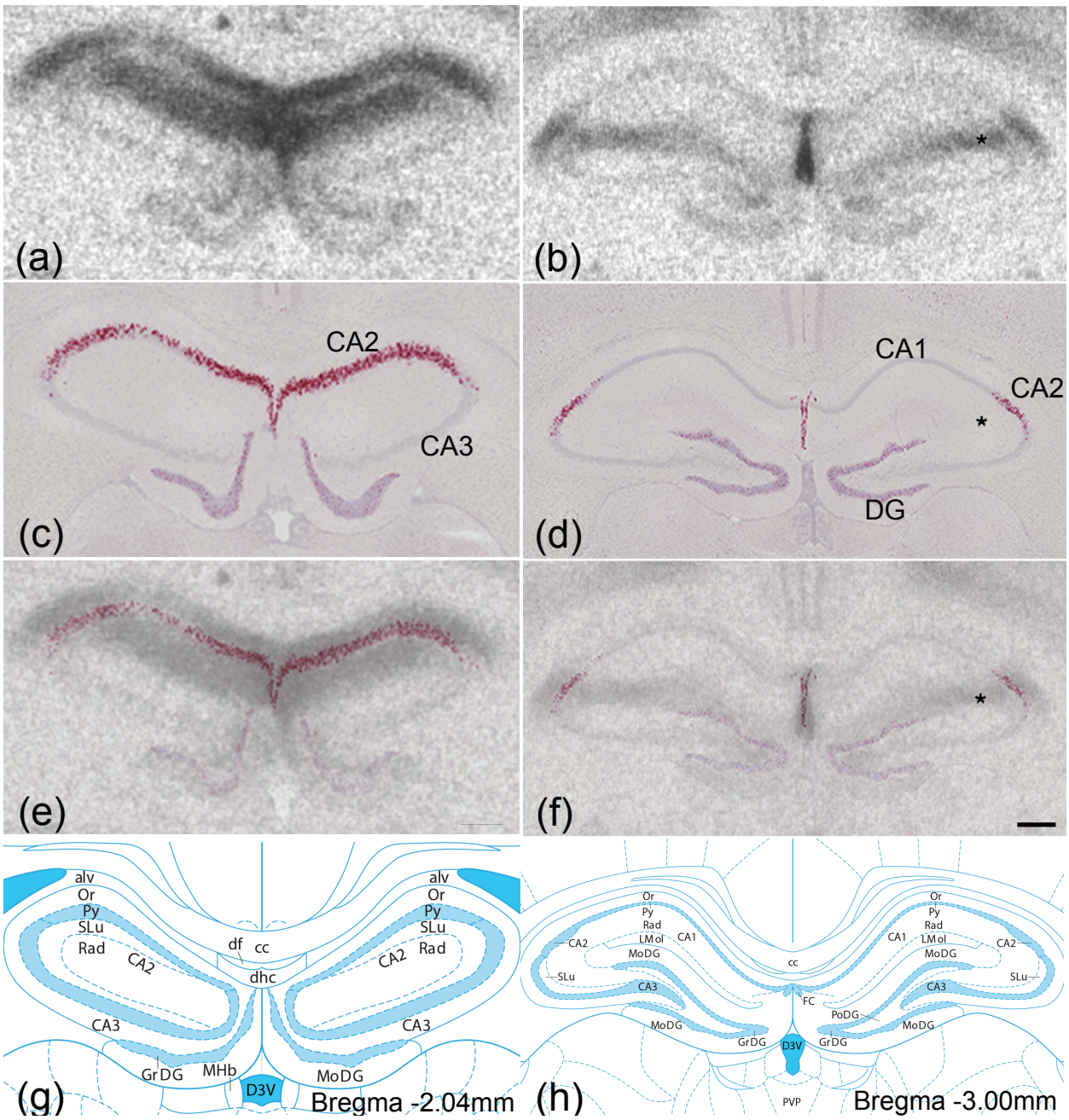


Figure 6. *Oxtr* mRNA and OXTR protein mismatch in the hippocampus. OXTR protein (black; a,b) was labeled with autoradiography, and in adjacent sections, *Oxtr* mRNA (red; c,d) was labeled with in situ hybridization against a hematoxylin counterstain (purple). Images from adjacent autoradiography and in situ hybridization sections were merged (e,f) to compare protein and mRNA localization. *Oxtr* mRNA was restricted to the stratum pyramidale, the principal cell

layer containing the somas of pyramidal cells, while robust OXTR protein binding was present superficial and basal to the stratum pyramidale, most likely in the stratum radiatum, stratum lacunosum moleculare, and stratum oriens. Illustrations from the *Rat Brain Atlas* indicate that *Oxtr* mRNA was enriched in CA2 in the Ammon's horn (g,h). DG, dentate gyrus; * denotes OXTR protein in the absence of *Oxtr* mRNA. Scale bar = 300 μm shown in (f).

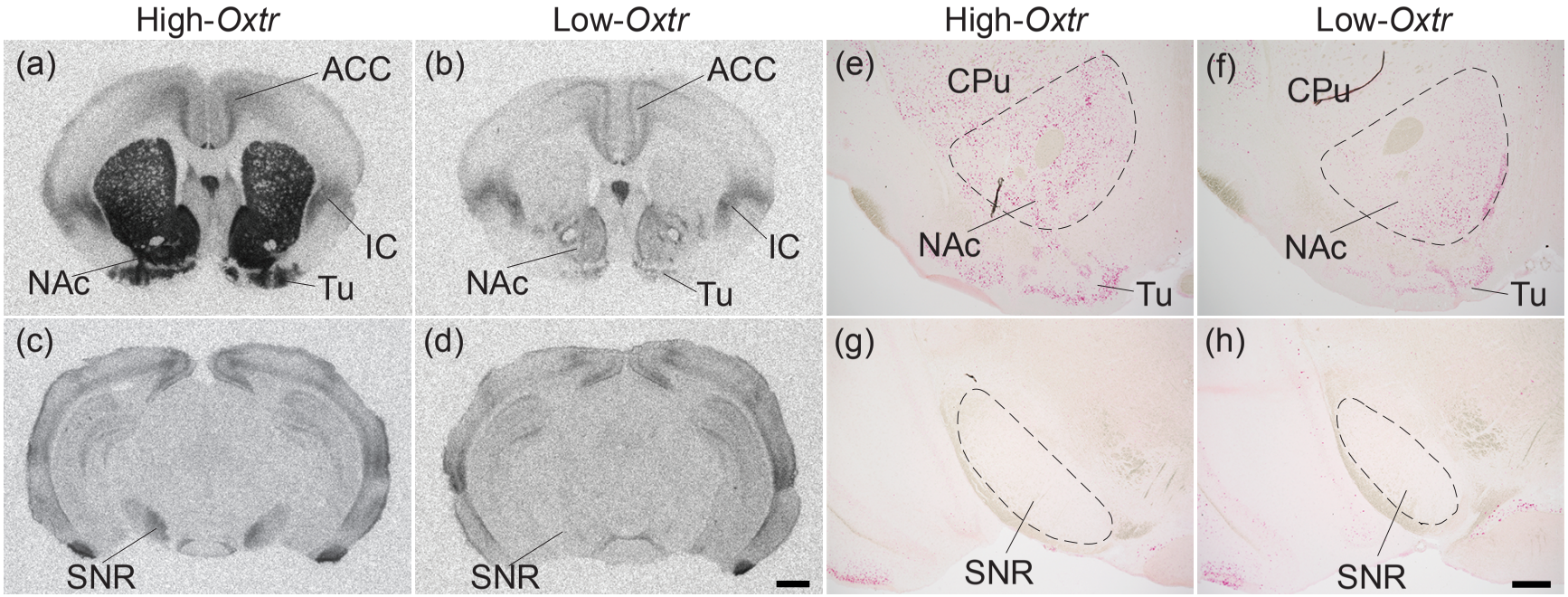


Figure 7. Genotype-dependent *Oxtr* mRNA and OXTR protein mismatch in substantia nigra pars reticulata. OXTR protein (black; a–d) was labeled with autoradiography, and in adjacent sections, *Oxtr* mRNA (red; e–h) was labeled with in situ hybridization against a hematoxylin counterstain (purple) in High-*Oxtr* voles (a,c,e,g) and Low-*Oxtr* voles (b,d,f,h). High-*Oxtr* and Low-*Oxtr* genotypes predicted protein binding in the nucleus accumbens (NAc) and substantia nigra pars reticulata (SNR) and mRNA expression in the NAc. However, no *Oxtr* mRNA was detected in the SNR of either genotype. CPu, caudate putamen; ACC, anterior cingulate cortex; Tu, olfactory tubercle; IS, insular cortex. Scale bars for (a–d) = 1 mm shown in (d), for (e-h) = 400 μm shown in (h).

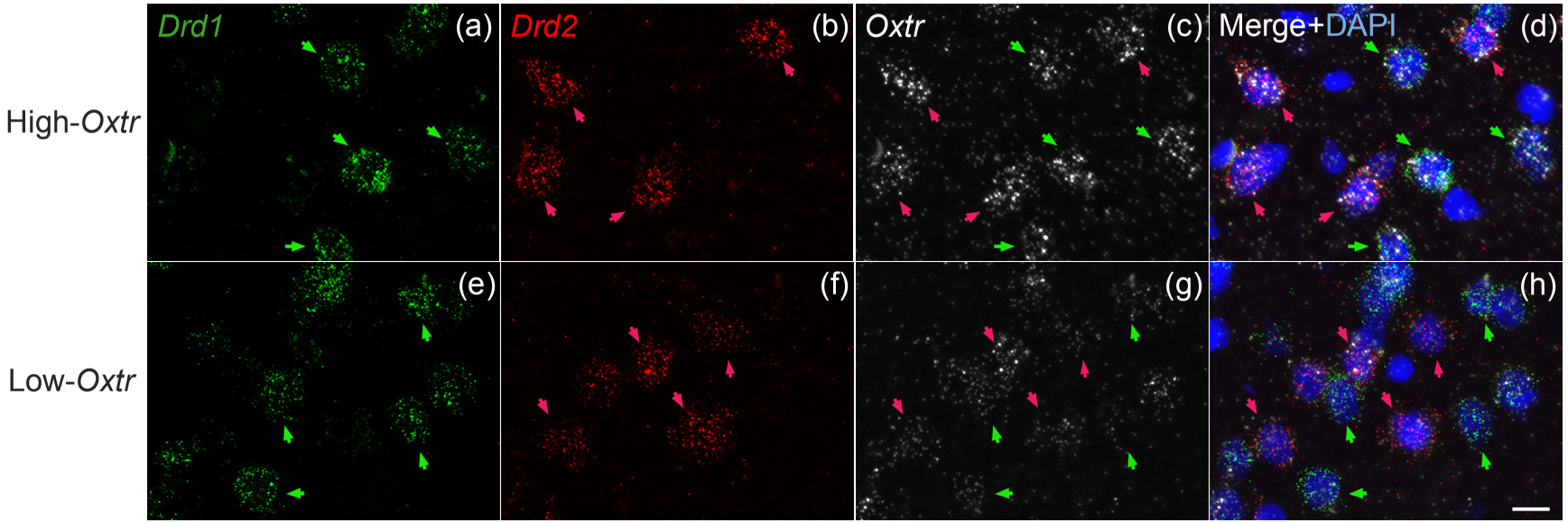


Figure 8. *Oxtr* mRNA colocalized with both *Drd1* and *Drd2* mRNA in the NAc. *Drd1* (green; a,e), *Drd2* (red; b,f), and *Oxtr* (white; c,g) mRNA were labeled with in situ hybridization in both High- and Low-*Oxtr* genotypes (a–d, e–h, respectively). *Oxtr* was expressed in both *Drd1*- and *Drd2*-expressing cells (d,h) in both genotypes. Scale bar = 10 μm shown in (h).

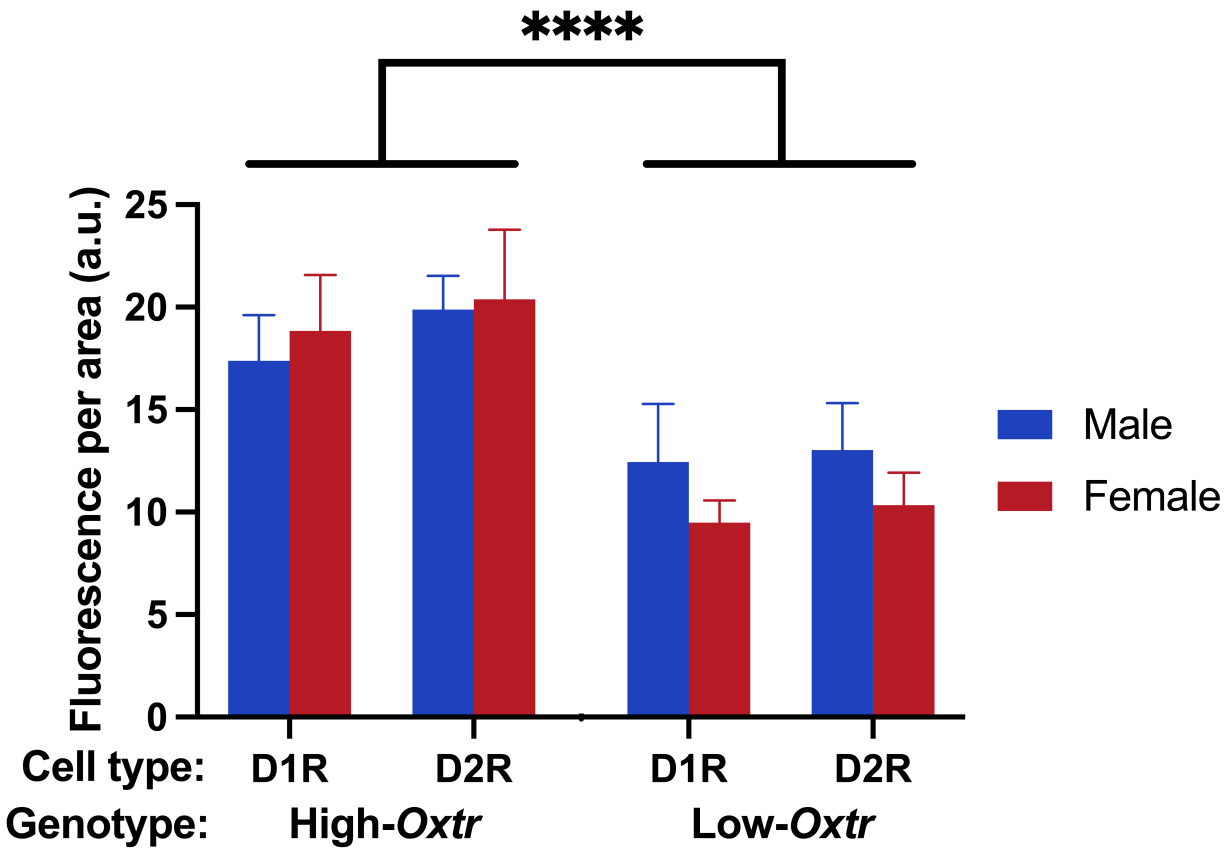


Figure 9. Genotype predicts *Oxtr* mRNA expression in both D1R and D2R cells in the NAc. The intensity of *Oxtr* labeling was quantified in D1R and D2R cells of both genotypes (High-*Oxtr* and Low-*Oxtr*) and sexes. A three-way mixed model ANOVA revealed a significant main effect of genotype, accounting for 70.81% of the variation in *Oxtr* expression. Post hoc tests revealed significant differences in *Oxtr* expression between High- and Low-*Oxtr* genotypes in each sex-by-cell-type comparison. ****, $p < .0001$.

Supplementary Table 1

Region	Abbreviation	*Oxtr* mRNA
Prosencephalon		
Telencephalon		
Olfactory bulb		
Granule cell layer	GrO	++++
Mitral cell layer	Mi	+++
Internal plexiform layer	IPl	+
Anterior olfactory area	AO	+++
Cerebral cortex		
Frontal association cortex	FrA	+++
Medial orbital cortex	MO	++
Cingulate cortex A32D	A32D	++++
Cingulate cortex A32V	A32V	++++
Cingulate cortex A33	A33	++
Cingulate cortex A25	A25	++++
Cingulate cortex A24a	A24a	+++
Cingulate cortex A24b	A24b	+++
Cingulate cortex A30	A30	+++
Cingulate cortex A29	A29	+++
Dorsolateral orbital cortex	DLO	+
Lateral orbital cortex	LO	+
Ventral orbital cortex	VO	+
Secondary motor cortex	M2	+++
Primary motor cortex	M1	+++
Frontal cortex area 3	Fr3	+
Primary somatosensory cx	S1	++
Secondary somatosensory cx	S2	++
Insular cortex	I	+++
Piriform cortex (rostral)	Pir	+
Piriform cortex (caudal)	Pir	++++
Medial parietal association cx	MPtA	+++
Lateral parietal association cx	LPtA	++
Secondary auditory cx	Au	++
Ectorhinal cortex	Ect	+++
Perirhinal cx	PRh	+++
Dorsolateral entorhinal cx	DLEnt	++
Dorsal intermed entorhinal cx	DIEnt	++

Ventral intermed entorhinal cx	VIEnt	+
Medial entorhinal cx	MEnt	+
Caudomedial entorhinal cx	CEnt	+
Secondary visual cx, mediom	V2MM	+++
Primary visual cx	V1	++
Parietal cx, posterior area	PtP	+++
Temporal association cx	TeA	+++
Hippocampus		
Granule cell layer of dentate gyrus	GrDG	+++
Polymorph layer of dentate gyrus	PoDG	+
Field CA1 of the hippocampus (rostral)	CA1	+
Field CA1 of the hippocampus (caudal)	CA1	++
Field CA2 of the hippocampus	CA2	++++
Field CA3 of the hippocampus	CA3	+
Ventral subiculum	VS	++
Postsubiculum	Post	+++
Presubiculum	PrS	+++
Parasubiculum	PaS	+++
Dorsal tenia tecta	DTT	++++
Accumbens nu, shell	AcbSh	++++
Accumbens nu, core	AcbC	++++
Island of Calleja	ICj	++++
Olfactory tubercle	Tu	++++
Indusium griseum	IG	++++
Ventral claustrum	VCl	++
Dorsal claustrum	DCl	++
Dorsal endopiriform nu	DEn	++
Caudate putamen	CPu	+++
Caudate putamen (Ventral)	CPu	++++
Lateral septal nu, intermediate	LSI	++
Lateral septal nu, ventral	LSV	++
Lateral septal nu, dorsal	LSD	+++
Septohippocampal nu	SHi	+
Lambdoid septal zone	Ld	+
Ventral pallidum	VP	++
Nu of horiz limb of the diagonal band	HDB	+
Lateral nu of the diagonal band	LDB	+
Septohipothalamic nu	SHy	++
Interstitial nu of post limb on ac	IPAC	++++

Bed nu stria terminalis, lateral division	STL	++++
Bed nu stria terminalis, medial division	STM	+/++/++++
Medial preoptic area	MPA	+++
Internal part globus pallidus	IGP	+
External part globus pallidus	EGP	++
Basal nucleus (Meynert)	B	++
Bed nu of the accessory olfactory tract	BAOT	+
Amygdala		
Basolateral amygdaloid nu, ant	BLA	++++
Central amygdaloid nu	Ce	++++
Basomedial amygdaloid nu, ant	BMA	+
Anterior cortical amygdaloid nu	ACo	++
Medial amygdaloid nu	MeA	++
Amygdalostriatal transition area	ASt	++
Lateral amygdaloid nu, dorsolat	LaDL	+++
Lateral amygdaloid nu, ventrolat	LaVL	++
Lateral amygdaloid nu, ventromed	LaVM	+
Basolateral amygdaloid nu, post	BLP	+++
Bed nu st, intraamygdaloid div	STIA	+++
Intercalated nu of the amygdala	I	+++
Basomedial amygdaloid nu, post	BMP	++
Posterolat cortical amygdaloid area	PLCo	++/++++
Posteromed cortical amygdaloid a	PMCo	++++
Amygdalohippocamp, anterola	AHiAL	+++
Amygdalohippocamp, posterol	AHiPL	+
Amygdalohippocamp, posterom	AHiPM	+
Amygdalopiriform transition area	APir	++/+++
Hypothalamus		
Anterior hypothalamic, anterior	AHA	++
Lateroanterior hypothalamic nu	LA	+++
Suprachiasmatic nu	SCh	++
Ventrolateral hypothalamic nu	VLH	+
Episupraoptic nu	ESO	+
Supraoptic nu	SO	+
Paraventricular nu, med parvic	PaMP	+
Paraventricular nu, lat magnoc	PaLM	+
Paraventricular nu, posterior	PaPo	+
Anterior hypothalamic a, poster	AHP	+
Anterior hypothalamic a, central	AHC	+

Ventromedial hypothalamic nu	VMH	++++
Arcuate hypothalamic nu	Arc	+++
Retrochiasmatic area, lateral	RChL	+
Median eminence	ME	+
Dorsomedial hy, dorsal part	DMD	+
Dorsomedial hy, compact part	DMC	++
Dorsomedial hy, ventral part	DMV	+
Perifornical par of lateral hypothalamus	PeFLH	+
Peduncular lateral hypothalamus	PLH	++
Magnocellular nu of lat hypothalamus	MCLH	+++
Dorsal hypothalamic area	DA	+++
DA11 dopamine cells	DA11	+++/++
Posterior hypothalamic nu	PH	+++
Premamillary nu, ventral part	PMV	++++
Premamillary nu, dorsal part	PMD	+
Retromammillary nu	RM	++++
Submamillothalamic nu	SMT	+++
Lateral mamillary nu	LM	+++
Medial mamillary nu, lateral part	ML	+++
Posterior hypothalamic area	PHA	+++

Diencephalon

Thalamus

Paraventricular thalamic nu, ant	PVA	++++
Patenial thalamic nu	PT	++
Reticular nu (prethalamus)	Rt	+
Central medial thalamic nu	CM	++++
Interanterodosal thalamic nu	IAD	++
Ventral reuniens thalamic nu	VRe	+++/++++
Xiphoid thalamic nu	Xi	++++
Anteromedial thalamic nu, ventr	AMV	+
Anterodorsal thalamic nu	AD	+
Paracentral thalamic nu	PC	+++
Paraxiphoid nu of thalamus	PaXi	++++
Zona incerta, rostral part	ZIR	++
Zona incerta, caudal part	ZIC	+
Ventromedial thalamic nu	VM	+++
DA13 dopamine cells	DA13	+++
Interanteromedial thalamic nu	IAM	++
Rhomboid thalamic nu	Rh	++++

Submedius thalamic nu, ventral	SubV	+++
Medial habenular nu	MHb	++
Lateral habenular nu	LHb	+
Subthalamic nu	STh	+++
Paraventricular th nu, posterior	PVP	++++
Lateral post th nu, mediorostr	LPMR	+
Medial pretectal area	MPT	+
Olivary pretectal nu	OPT	+
Pregeniculate nu of the prethalamus	PrG	+

Mesencephalon (Midbrain)

Lithoid nu	Lth	++
Nucleus of Darkschewitsch	Dk	++++
Ventral tegmental area, rostral part	VTAR	+
Substantia nigra compact, dorsal tier	SNCD	+
Subbrachial nu	SubB	+++
Pararubral nu	PaR	+
Supraoculomotor periaqueduct	Su3	+++
Supraoculomotor cap	Su3C	+
Dorsomedial periaqueductal gray	DMPAG	+
Dorsolateral periaqueductal gray	DLPAG	+
Lateral periaqueductal gray	LPAG	++
Ventrolateral periaqueductal gray	VLPAG	+
Mesencephalic reticular formation	mRt	++
Interfascicular nu	IF	+
Interpeduncular nu	IP	+
Mesencephalic dosal raphe	mDR	+
Dorsal raphe nu	DR	++
Pedunculotegmental nu	PTg	+
Median raphe nu	MnR	++
Paramedian raphe nu	PMnR	++
Rhabdoid nu	Rbd	++
Retroisthmic nu	RIs	+
isthmic reticular formation	isRt	+
Microcellular tegmental nu	MiTg	+
Intermediate nu of lat lemniscus	ILL	+
Ventral nu of lateral lemniscus	VLL	+

Rhombencephalon (Hindbrain)

Pontine nuclei	Pn	++++

Pontine reticular nu, oral part	PnO	+
Laterodorsal tegmental nu	LDTg	+++
Reticulotegmental nu	RtTg	+
Lateral parabrachial nu	LPB	+++
Dorsal tegmental nu, pericentral	DTgP	+
Dorsal tegmental nu, central	DTgC	++++
Kolliker-Fuse nu	KF	+
Medial parabrachial nu	MPB	+/+++
NA7 noradrenalin cells	NA7	+
Mesencephalic trigeminal nu	Me5	++++
Lateral parabrachial nu, ventral	LPBV	++++
Pontine reticular nu, caudal part	PnC	+
NA5 noradrenalin cells	NA5	+
Central gray, alpha part	CGA	++++
Barrington's nu	Bar	++++
Subcoeruleus nu, ventral part	SubCV	+
Locus coeruleus (rostral)	LC	++++/?
Locus coeruleus (caudal)	LC	-
Posterodorsal tegmental nu	PDTg	+
Central gray	CG	+
Medial vestibular nu	MVe	+
Central gray, beta part	CGB	++
Central gray, gamma part	CGG	++
Lateral superior olive	LSO	+
Dorsal periolivery region	DPO	+
Supragenual nucleus raphe	SGe	++
Intermediate reticular nu, alpha	IRtA	+
Parvicellular retic nu, alpha	PCRtA	+
Medioventral periolivary nu	MVPO	+++
Lateral paragiganocel nu, external	LPGiE	+
Dorsal paragigantocellular nu	DPGi	+
Gigantocellular reticular nu	Gi	+
Gigantocellular reticular nu, alpha	GiA	+
Raphe magnus nucleus	RMg	+
Solitary nu	Sol	+++
Trigeminal-solitary transition	5Sol	+++
Prepositus nu	Pr	+
Spinal vestibular nu	SpVe	+
Rostroventrolateral reticular nu	RVL	+++
NA1 noradrenalin cells	NA1	+++

Ambiguus nucleus, compact	AmbC	++
Ad3 adrenalin cells	Ad3	+
Vagus nerve nu	10N	+++
Intermediate reticular nu	IRt	++
Parvicellular reticular nu	PCRt	++
Gracile nu	Gr	+++
Lateral reticular nu	LRt	++
NA2 noradrenalin cells	NA2	+++
Spinal trigeminal nu, interpolar	Sp5I	+
Inferior olive, cap of Kooy	IOK	+
Medullary reticular nu, ventral	MdV	++
Medullary reticular nu, dorsal	MdD	++
Central cervical nu	CeCv	+
Interstitial basal nu of medulla	IB	+
Cuneate nu	Cu	+
Gelatinous layer of caudal Sp5	Ge5	++
Ad1 adrenalin cells	Ad1	+++
Accessory nerve nu	11N	+

Chapter 3

Melanocortin Agonism in a Social Context Selectively Activates Nucleus Accumbens in an Oxytocin-Dependent Manner

3.1 Acknowledgment of Reproduction and Authors' Contributions

This chapter is adapted from:

Ford CL, McDonough AA, Horie K, Young LJ (under review) Melanocortin agonism in a social context selectively activates nucleus accumbens in an oxytocin-dependent manner. *Neuropharmacology*.

I designed and conducted the experiments, analyzed the data, and wrote the manuscript, with input from and under the guidance of L. Young. A. McDonough assisted with surgeries, behavioral experiments, and video analysis under my guidance. K. Horie provided the wildtype and oxytocin receptor-knockout voles.

3.2 Abstract

Social deficits are debilitating features of many psychiatric disorders, including autism. While time-intensive behavioral therapy is moderately effective, there are no pharmacological interventions for social deficits in autism. Many studies have attempted to treat social deficits using the neuropeptide oxytocin for its powerful neuromodulatory abilities and influence on social behaviors and cognition. However, clinical trials utilizing supplementation paradigms in which exogenous oxytocin is chronically administered independent of context have failed. An alternative treatment paradigm suggests pharmacologically activating the endogenous oxytocin system during behavioral therapy to enhance the efficacy of therapy by facilitating social learning. To this end, melanocortin receptor agonists like Melanotan II (MTII), which induces central oxytocin release and accelerates formation of partner preference, a form of social learning, in prairie voles, are promising pharmacological tools. To model pharmacological activation of the endogenous oxytocin system during behavioral therapy, we administered MTII prior to social interactions

between male and female voles and assessed its effect on oxytocin-dependent activity in brain regions subserving social learning using Fos expression as a proxy for neuronal activation. In non-social contexts, MTII only activated hypothalamic paraventricular nucleus, a primary site of oxytocin synthesis. However, during social interactions, MTII selectively increased oxytocin-dependent activation of nucleus accumbens, a site critical for social learning. These results suggest a mechanism for the MTII-induced acceleration of partner preference formation observed in previous studies. Moreover, they are consistent with the hypothesis that pharmacologically activating the endogenous oxytocin system with a melanocortin agonist during behavioral therapy has potential to facilitate social learning.

3.3 Introduction

Deficits in social behavior and cognition are debilitating features of many psychiatric disorders including autism spectrum disorder (ASD), schizophrenia, post-traumatic stress disorder (PTSD), and social anxiety disorder. One potential therapeutic target for treating these social deficits is the oxytocin system, which plays myriad roles in social behavior. Numerous studies have demonstrated prosocial effects of exogenous oxytocin administration, including increasing trust (Kosfeld et al., 2005), inclusion (Riem et al., 2013), communicational reciprocity (Spengler et al., 2017), empathy (Hurlemann et al., 2010), eye gaze (Guastella et al., 2008), social approach (Preckel et al., 2014), and altruism (Marsh et al., 2015). Although many oxytocin studies are underpowered (Quintana et al., 2021), show small effect sizes (Walum et al., 2016), fail to replicate (Lane et al., 2015), or show context-dependent effects (Shamay-Tsoory & Abu-Akel, 2016), collectively, they are consistent with animal research demonstrating that oxytocin is a powerful modulator of the social brain (Froemke & Young, 2021; Marsh et al., 2021).

The promising preclinical oxytocin literature has prompted many clinical trials, most commonly using intranasal oxytocin in patients with ASD. While small, early trials were inconclusive (Anagnostou et al., 2012, Parker et al., 2017, Yamasue & Domes, 2018, Wang et al., 2019, Yamasue et al., 2020), a recent Phase II clinical trial definitively demonstrated that daily oxytocin administration has no positive effect on social behavior in children and adolescents with ASD (Sikich et al., 2021). One plausible reason this oxytocin supplementation paradigm failed is that while oxytocin enhances the salience of social information (Shamay-Tsoory & Abu-Akel, 2016; Xue et al., 2020) and facilitates social learning (Hurlemann et al., 2010; Eckstein et al., 2015), it does not necessarily cause prosocial behavior (Shamay-Tsoory et al., 2009; De Dreu et al., 2010 & 2011; Bartz et al., 2011; Ne'eman et al., 2016). That is, the potential therapeutic effects of oxytocin are context-dependent (Ford & Young, 2022). Second, oxytocin has a short half-life and it is unclear how long intranasal oxytocin remains active, how much enters the brain, and how far it diffuses (Quintana et al., 2021). Regardless, oxytocin receptors (OXTR) are widespread in brain (Quintana et al., 2019; Inoue et al., 2022), so exogenous administration likely has less-targeted effects than endogenous release from oxytocinergic neurons.

A potentially more effective clinical application would be to pair oxytocin administration with a positive social learning experience like Applied Behavior Analysis (ABA) therapy (Meyer-Lindenberg et al., 2011; Ford & Young, 2022). ABA is the gold standard for treating social deficits in ASD, but it can require upwards of 40 hours per week to be effective (Eldevik et al., 2010; Linstead et al., 2017). Administering oxytocin immediately prior to ABA might improve the efficacy and efficiency of therapy by enhancing the salience of the therapeutic social information (Gordon et al., 2013; Gordon et al., 2016; Shamay-Tsoory & Abu-Akel, 2016; Xue et al., 2020), increasing the signal-to-noise ratio of that information as it is processed in the brain (Oettl et al.,

2016; Froemke & Young, 2021), and accelerating learning by promoting synaptic plasticity at a molecular level (Dölen et al., 2013; Rajamani et al., 2018; Nardou et al., 2019; Froemke & Young, 2021). Notably, the goal of oxytocin-enhanced therapy would not be to improve social behavior transiently through direct and immediate pharmacological activity, but rather to improve behavior more permanently by using oxytocin to enhance the learning and neural rewiring that occurs during behavioral therapy (Ford & Young, 2021 & 2022). One recent clinical trial demonstrated the potential of this therapeutic paradigm by showing a positive and enduring effect on social behavior in children with ASD when oxytocin was administered immediately prior to positive social interactions with their caregivers (Le et al., 2022).

A second potential improvement to oxytocin-based therapies would be to use blood-brain-barrier-penetrant pharmaceuticals that activate the endogenous oxytocin system rather than administering exogenous oxytocin (Modi & Young, 2012; Young & Barrett, 2015). This approach could allow for more targeted activation and therapeutic rewiring of specific circuits and brain regions, rather than activating OXTR everywhere exogenous oxytocin diffuses. One candidate pharmacological mechanism for activating the endogenous oxytocin system is agonism of melanocortin receptors, specifically melanocortin 4 receptors (MC4R) (Modi et al., 2015; Peñgarikano et al., 2015; Mastinu et al., 2018). MC4R are expressed on oxytocinergic cells in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON) (Siljee et al., 2013), and stimulating MC4R activates oxytocin neurons (Kublaoui et al., 2008), increases their firing rate (Paiva et al., 2017), and induces central oxytocin release (Sabatier et al., 2003; Sabatier, 2006). Critically, MC4R agonism appears safe and well tolerated in humans, with melanocortin agonist Bremelanotide approved to treat hypoactive sexual desire disorder (Dhillon & Keam, 2019).

The nonspecific melanocortin receptor agonist and alpha-melanocyte stimulating hormone (α-MSH) analog Melanotan II (MTII), the commercially-available metabolic precursor of Bremelanotide, improves social behavior in a mouse model of ASD (Minakova et al., 2019), protects against social deficits caused by neonatal isolation (Barrett et al., 2015), and accelerates the formation of partner preference, a type of social learning indicative of monogamous pair bond formation, in prairie voles (Barrett et al., 2014; Modi et al., 2015). MTII can also increase the amount of oxytocin released in the nucleus accumbens (NAc), an area critical for social learning, but only when oxytocin release is triggered by a physiological stimulus (Modi et al., 2015). It is unknown, however, if MTII can increase neuronal activity in social brain regions. Here, in order to model oxytocin-enhanced therapy via melanocortin receptor agonism, we administered MTII to prairie voles in social and non-social contexts using *Oxtr*-knockout (*Oxtr*-KO) voles (Horie et al., 2019) and OXTR antagonist to identify oxytocin-dependent effects. We then assessed the ability of melanocortin receptor agonism in these contexts to enhance activity in brain regions that subserve social learning by quantifying the immediate early gene product Fos, a marker of neuronal activation, in PVN, NAc, basolateral amygdala (BLA), prelimbic cortex (PLC), and dorsal CA2 of hippocampus (CA2).

3.4 Materials and Methods

3.4.1 Subjects

All experiments used adult (75 – 186 days of age), sexually-naïve male and female prairie voles (*Microtus ochrogaster*) from our outbred colony at Emory University, which was originally derived from field-caught voles in Champaign, Illinois. Voles were group-housed in ventilated 26 x 18 x 19 cm Plexiglass cages with Bedo'cobbs Laboratory Animal Bedding (The Andersons;

Maumee, Ohio) and *ad libitum* access to food (Lab Rabbit Diet HF #5326, LabDiet) and water, and were maintained at 22 ºC with a 14:10 h light/dark cycle. All procedures were performed in accordance with the Institutional Animal Care and Use Committee at Emory University.

3.4.2 Peripheral MTII administration in a non-social context

Three days prior to experimentation, subjects were removed from group housing, weighed, placed in cages by themselves, and randomly assigned to either the MTII (n = 12 (6M, 6F)) or saline group (n = 12 (6M, 6F)). On the day of the experiment, voles received intraperitoneal (i.p.) injections of either 0.3 mL 0.9% saline (Pfizer, New York, NY) or 10 mg/kg MTII (Toronto Research Chemicals, Toronto, ON) dissolved in 0.9% saline and were then returned, alone, to their home cages. The 10 mg/kg dose was chosen for the effects it demonstrated in prior studies (Barrett et al., 2014; Modi 2015). Two h and 15 min after injection, voles were anesthetized with an overdose of isoflurane and transcardially perfused.

3.4.3 Peripheral MTII administration in a social context

Three days prior to experimentation, wildtype (WT) and *Oxtr*-KO littermates were removed from group housing, weighed, placed in cages by themselves, and randomly assigned to receive either saline (WT n = 14 (6M, 8F); *Oxtr*-KO n = 12 (6M, 6F)) or MTII (WT n = 13 (6M, 7F); *Oxtr*-KO n = 15 (7M, 8F)). Vole genotypes were confirmed as previously described (Horie et al., 2019). On the day of the experiment, voles received i.p. injections of either 0.3 mL 0.9% saline or 10 mg/kg MTII and were then placed in clean test cages. Forty-five minutes after injection, a novel, opposite-sex stimulus animal (intact males, ovariectomized females) was placed in the test cage. The social interaction was videotaped (HF R800, Canon, Melville, NY) and later reviewed to ensure no

fighting or mating occurred. Thirty minutes after the stimulus animal was introduced, both animals were removed and the experimental animal was returned to its home cage. The experimental vole remained undisturbed for 1 h, at which time it was anesthetized with an overdose of isoflurane and transcardially perfused.

3.4.4 Central MTII administration dose-response

To determine the optimal MTII dose for central administration, we examined the dose-response relationship for MTII-induced behavioral side effects. WT voles were anesthetized with isoflurane and 26-gauge guide cannulas (Plastics One, Roanoke, VA) were implanted targeting the right lateral ventricle (from Bregma: -0.4 mm A/P, +1.0 mm M/L, -2.5 mm D/V with 1 mm internal cannula protrusion) and secured with glass ionomer cement (Harvard Apparatus, Holliston, MA). After surgery, voles were singly housed. One week after cannula implantation, voles were lightly anesthetized with isoflurane and received an intracerebroventricular (i.c.v.) injection of 2 µL aCSF (vehicle; Tocris Bio-Techne, Minneapolis, MN), 0.001 nmol MTII, 0.01 nmol MTII, 0.1 nmol MTII, or 1 nmol MTII dissolved in 2 µL aCSF (n = 6 (3M, 3F) per group). Following i.c.v. injection, voles were placed in clean test cages by themselves. From 45 – 60 minutes post-injection, their behavior was videotaped. Videos were scored for the time each animal spent ambulating, grooming, or stretching using Noldus Observer XT software (v. 14, Noldus, Wageningen, Netherlands) on a Dell Precision 3630 computer running Windows 10 (Dell, Round Rock, TX). Increased grooming and stretching (often in conjunction with yawning) are behavioral side effects of melanocortin receptor agonism in many animals (Ferrari, 1958; Adan et al., 1999; Bertolini et al., 2009), which may be mediated in part by oxytocin or vasopressin signaling at vasopressin-1A receptors (Schorscher-Petcu et al., 2010). The time animals spent engaged in each behavior was

graphed as a percent of the total time scored (**Supplementary Figure 1A**). Although grooming and stretching induced by melanocortin receptor agonism do not disrupt an organism's ability to respond to social or environmental stimuli (Bertolini et al., 2009), spontaneous ambulation was almost entirely eliminated at the 1.0 nmol dose (**Supplementary Figure 1B**). Based on these data, 0.1 nmol MTII was selected for subsequent experiments with central administration as the highest dose at which the subject's ability to engage socially with a stimulus animal was unlikely to be impaired.

3.4.5 Central MTII administration in a social context

WT voles were surgically implanted with guide cannula targeting the lateral ventricle as previously described and were singly housed thereafter. One week after surgery, subjects were lightly anesthetized with isoflurane and received i.c.v. injections of 2 µL aCSF (n = 20 (10M, 10F)), 0.1 nmol MTII dissolved in 2 µL aCSF (n = 19 (10M, 9F)), or 0.1 nmol MTII and 5 ng of the highly selective oxytocin antagonist (d(CH_2)$_5^1$,Tyr(Me)^2,Thr^4,Orn^8,des-Gly-NH_2^9)-Vasotocin (OTA; Bachem, Bubendorf, Switzerland) dissolved in 2 µL aCSF (n = 20 (10M, 10F)). Five ng of this OTA inhibits consoling behavior and mating-induced partner preference formation in prairie voles (Burkett et al., 2016 and Johnson et al., 2016). Subjects were then placed in a clean test cage and, 45 minutes later, a novel, opposite-sex stimulus animal (ovariectomized females, intact males) was placed in the test cage. The social interaction was videotaped and later reviewed to ensure no fighting or mating occurred. Thirty minutes after the stimulus animal was introduced, the stimulus animal was removed and the experimental animal was left in the test cage, where it remained undisturbed for 1 h. It was then anesthetized with an overdose of isoflurane and transcardially perfused.

3.4.6 Central MTII administration in a novel context

WT voles were surgically implanted with guide cannula targeting the lateral ventricle as previously described and were singly housed thereafter. One week after surgery, subjects were lightly anesthetized with isoflurane and received i.c.v. injections of either 2 μL aCSF (n = 10 (5M, 5F)) or 0.1 nmol MTII dissolved in 2μL aCSF (n = 15 (7M, 8F)). Subjects were then placed in a clean test cage and, after 45 minutes, a novel rodent figurine was placed in the cage. After 30 minutes, the figurine was removed, and the vole was left undisturbed for 1 hr, when it was anesthetized with an overdose of isoflurane and transcardially perfused.

3.4.7 Perfusion and sectioning

Immediately following isoflurane overdose, all subjects were transcardially perfused with 30-40 mL phosphate-buffered saline (PBS, pH 7.4; Teknova, Hollister, CA) followed by 30-40 mL 4% paraformaldehyde (Polysciences, Warrington, PA) in PBS at a rate of 4-8 mL/min, depending on vole size, using a peristaltic pump (Easy-Load II Masterflex, Cole-Palmer, Vernon Hills, IL). Brains were extracted and post-fixed in 4% paraformaldehyde in PBS for 24 h before being placed in 30% sucrose and 0.01% azide in PBS for at least one week prior to sectioning. Brains were then sliced into 40 μm coronal sections using a freezing microtome (HM 440 E, Microm, Walldorf, Germany) and stored in PBS with 0.01% azide until staining.

3.4.8 Fos immunohistochemistry

Every third section underwent immunohistochemical labeling of Fos at RT in custom 144- or 64-well staining trays on a rotator set to 100 revolutions per minute. Sections were washed three times

in PBS, then incubated in a peroxidase blocking solution for 10 min (Bloxall, Vector Laboratories, Newark, CA), washed twice in PBS, and incubated for 20 min in 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 2.5% horse serum (ThermoFischer Scientific, Waltham, MA) in PBS (PBSTN). Sections were then incubated in an avidin-blocking solution (Vector Laboratories) for 15 min, washed twice in PBS, incubated in a biotin-blocking solution (Vector Laboratories) for 15 min, washed twice in PBS, and incubated for 30 min in primary rabbit monoclonal anti-c-Fos antibody (EPR21930-238, Abcam, Cambridge, UK) diluted to 1:6000 in PBSTN. After incubation in primary antibody, sections were washed seven times in PBS before being incubated for 30 min in biotinylated goat anti-rabbit IgG antibody (Vectastain Elite ABC-HRP Kit, Vector Laboratories) in PBS with 0.05% Triton X-100 and 1.5% horse serum according to the manufacturer's instructions. Following secondary antibody incubation, sections were washed seven times in PBS and incubated in an avidin-biotin peroxidase system (Vectastain Elite ABC-HRP Kit, Vector Laboratories) for 30 min. Then, sections were washed seven times in PBS, stained with a nickel-DAB peroxidase substrate kit (SK-4100, Vector Laboratories), again washed seven times in PBS, and mounted onto Superfrost Plus slides (Fisher Scientific, Waltham, MA) in dH_2O. After drying, slides were coverslipped with VectaMount permanent mounting medium (Vector Laboratories). Due to slight variations in staining with each batch of immunohistochemistry, Fos scores from different experiments should not be compared to one another.

3.4.9 Nissl staining

Every third section underwent Nissl staining to facilitate identification of brain regions in Fos-labeled sections and to confirm that cannulas successfully targeted the lateral ventricle. Sections were mounted onto Superfrost Plus slides and allowed to dry before being submerged in xylene

for 5 min, 95% ethanol for 3 min, 70% ethanol for 3 min, and dH_2O for 3 min. Sections were then stained for 12 min in dH_2O containing 0.01% cresyl-violet, 0.5% acetic acid, and 0.07% sodium acetate. After staining, sections were submerged in 70% ethanol for 3 min, 95% ethanol for 2 min, and dipped once in 100% ethanol before being submerged in xylene for 5 min. While wet with xylene, slides were coverslipped with Eukitt mounting medium (Sigma-Aldrich) and then allowed to dry.

3.4.10 Imaging

Slides were scanned with a NanoZoomer 2.0-HT (Hamamatsu Photonics, Shizuoka, Japan) using NDP.scan software (version 3.3, Hamamatsu Photonics), and images were captured with NDP.view2 software (Hamamatsu Photonics). Using Nissl-stained sections and a rat brain atlas (Paxinos & Watson, 2013) as references to determine anatomical boundaries, a blinded experimenter outlined PVN, NAc, BLA, PLC, and CA2 bilaterally in three consecutive Fos-labeled sections of each subject with ImageJ (version 1.52) (Schindelin et al., 2012) on a MacBook Pro running OS version 12.3.1 (Apple, Cupertino, CA). These regions were chosen for their high levels of *Oxtr* expression (Inoue et al., 2022) and role in social reward, learning, and memory (Walum & Young, 2018; Watarai et al., 2021). PVN, BLA, PLC, and anterodorsal CA2 were outlined in their entirety; for NAc, a medial sample consisting primarily of NAc shell was analyzed due to its dynamic role in social learning and pair bonding (Aragona et al., 2006; Resendez et al., 2012; Dreyer et al., 2016). Fos-positive nuclei were quantified using the ImageJ "Analyze Particles" function with the threshold held constant across subjects for each brain region in each experiment. For each section, the Fos-positive nuclei count was divided by the area of the region analyzed in mm^2, and the resulting values were averaged for each subject to yield a single value

representing Fos-positive cell density in a given brain region. The Fos-labeled sections displayed in figures have Fos-positive cell densities that approximate the means for their respective groups. Damaged sections were excluded, as were subjects with less than two intact sections for a given region.

3.4.11 Statistics

All statistical analyses were performed in GraphPad Prism (version 9.4.1, GraphPad Software, San Diego, CA) using raw values for Fos-positive cell density (Fos-positive cell count per mm^2). However, cell density varies widely between brain regions, so to facilitate visualization of the data, graphs with data from multiple brain regions present the fold difference between the control group (saline- or aCSF-injected animals) and experimental groups for each brain region. Similarly, due to inherent differences in cell density between brain regions, there was a significant effect of brain region, which is not reported, in all experiments. The ability of MTII to activate oxytocinergic PVN cells via melanocortin receptor agonism is well established in the literature (Kublaoui et al., 2006 & 2008; Barrett et al., 2014; Modi et al., 2015), so the PVN served as a positive control and was analyzed separately from the four oxytocin-sensitive target areas (Inoue et al., 2022). ANOVAs were performed for all experiments unless a value was missing from the dataset due to tissue damage, in which case a mixed-effects analysis was performed instead. Initial experiments in both social and non-social conditions found no sex effect or interaction, so data were collapsed across sex for those initial experiments and sexes were pooled for subsequent experiments. Oxytocin-sensitive target brain regions (NAc, BLA, PLC, CA2) were treated as repeated measures because Fos expression is highly correlated between brain regions within subjects

(**Supplementary Figure 2**). Correlation matrices were generated from z-scores with means and standard deviations pooled within each brain region.

3.5 Results

3.5.1 Melanocortin receptor agonism in a non-social context

To determine how a peripherally administered melanocortin receptor agonist affects the activation of social brain areas in a non-social context, we injected voles with 10 mg/kg MTII or saline i.p. and returned them, alone, to their home cages. A one-tailed Welch's t test comparing saline-injected and MTII-injected voles found a significant increase in Fos-positive cell density in the PVN of MTII-treated animals (M = 1889, SEM = 297.6) compared to saline-treated animals (M = 525.7, SEM = 83.79; t(df) = 4.409(12.73), p = 0.0004) (**Figure 1A**), as expected (Kublaoui et al., 2006 & 2008; Barrett et al., 2014; Modi et al., 2015). In comparing Fos-positive cell density in NAc, BLA, PLC, and CA2 between MTII- and saline-injected groups, a three-way ANOVA with Geisser-Greenhouse correction and with brain region as a within-subjects repeated measure and sex and treatment as between-subjects factors found no effect of sex (F (1, 20) = 0.08448, p = 0.7743) and no sex-brain region interaction (F (3, 60) = 0.1750, p = 0.9129), sex-treatment interaction (F (1, 20) = 0.6280, p = 0.4374), or sex-treatment-brain region interaction (F (3, 60) = 0.01537, p = 0.9974), so treatment was collapsed across sex and sexes were pooled in subsequent experiments with non-social contexts. The resulting two-way, repeated-measures ANOVA found neither a treatment effect (F (1, 22) = 1.171, p = 0.2910) nor a treatment-brain region interaction (F (3, 66) = 0.6341, p = 0.5957), indicating that while peripheral MTII administration activates PVN cells, it does not significantly alter Fos immunoreactivity in the NAc, BLA, PLC, or CA2

(**Figure 1B**). Representative images of sections with Fos-positive cell densities that approximate their groups means are shown in **Figure 2**.

3.5.2 Melanocortin receptor agonism in a social context

Next, we injected 10 mg/kg MTII or saline i.p. and allowed voles to interact with a novel, opposite-sex vole for 30 minutes. A one-tailed Welch's t test comparing saline- and MTII-injected voles again found a significant increase in Fos-positive cell density in the PVN of MTII-treated animals (M = 2917, SEM = 231.3) compared to saline-treated animals (M = 1713, SEM = 224.5; t(df) = 3.735(24.88), p = 0.0005) (**Figure 3A**). In comparing Fos-positive cell density in NAc, BLA, PLC, and CA2 between MTII- and saline-injected groups, a three-way ANOVA with Geisser-Greenhouse correction and with brain region as a within-subjects repeated measure and sex and treatment as between-subjects factors found no effect of sex (F (1, 23) = 0.1324, p = 0.7193) and no sex-brain region interaction (F (3, 69) = 0.3423, p = 0.7948), sex-treatment interaction (F (1, 23) = 2.076, p = 0.1631), or sex-treatment-brain region interaction (F (3, 69) = 2.047, p = 0.1154), so treatment was collapsed across sex and sexes were pooled in subsequent experiments with social contexts. The resulting two-way, repeated-measures ANOVA found no main effect of treatment (F (1, 25) = 0.3749, p = 0.5458), but in contrast to the experiment with a non-social context, this ANOVA found a treatment-brain region interaction (F (3, 75) = 10.59, p < 0.0001) and the Holm-Šídák post hoc test was used to compare Fos cell counts within brain regions (**Figure 3B**). This post hoc test revealed that in a social context, MTII significantly increases Fos immunoreactivity in NAc (p = 0.0034) and decreases Fos immunoreactivity in BLA (p = 0.0214) compared to saline.

Previous research with MTII demonstrated behavioral effects that were dependent on oxytocin signaling (Modi et al., 2015), so to explore if these social context-dependent neural

effects were mediated by oxytocin, we repeated this experiment with *Oxtr*-KO voles from the same litters. A one-tailed Welch's t test indicated PVN Fos immunoreactivity was significantly increased in MTII-injected voles (M = 2825, SEM = 305.2) compared to saline-injected voles (M = 1038, SEM = 121.5; t(df) = 5.436(18.20), $p < 0.0001$) (**Figure 3C**). Fos immunoreactivity in NAc, BLA, PLC, and CA2 were compared using a mixed-effects model with Geisser-Greenhouse correction and with brain region as a within-subjects repeated measure and treatment as a between-subjects factor, which found neither a treatment effect (F (1, 25) = 0.8711, p = 0.3596) nor a treatment-brain region interaction (F (3, 74) = 0.6045, p = 0.6141) (**Figure 3D**).

To determine if the differences in NAc and BLA Fos immunoreactivity between wildtype (WT) and *Oxtr*-KO voles were mediated by oxytocin-dependent effects of MTII, we performed separate two-way ANOVAs for NAc and BLA with genotype and treatment as between-subjects factors. In NAc, this ANOVA revealed a significant treatment-genotype interaction (F (1, 50) = 9.338, p = 0.0036) in addition to significant main effects of treatment (F (1, 50) = 8.324, p = 0.0058) and genotype (F (1, 50) = 4.049, p = 0.0496), indicating that the MTII-induced increase in NAc Fos immunoreactivity observed in WT animals depends on OXTR. A Holm-Šídák post hoc test found significantly increased Fos-positive cell density in MTII-injected WT animals compared to saline-injected WT animals (p = 0.0006), saline-injected *Oxtr*-KO animals (p = 0.0063), and MTII-injected *Oxtr*-KO animals (p = 0.0031) (**Figure 3E**). In BLA, the ANOVA found a main effect of treatment (F (1, 50) = 12.56, p = 0.0009) but no effect of genotype (F (1, 50) = 0.01037, p = 0.9193) or treatment-genotype interaction (F (1, 50) = 0.1051, p = 0.7471), indicating that the effects of MTII on BLA Fos immunoreactivity do not dependent on OXTR. A Holm-Šídák post hoc test found a significant decrease in Fos-positive cell density in MTII-injected WT voles compared to saline-injected WT voles (p = 0.0496), and although MTII-injected *Oxtr*-

KO voles also showed a decrease in Fos-positive cell density relative to saline-injected *Oxtr*-KO voles, this difference was not statistically significant after adjusting for multiple comparisons ($p = 0.0804$) (**Figure 3F**). Representative images of sections with Fos-positive cell densities that approximate their groups means are shown in **Figure 4**.

3.5.3 Melanocortin receptor agonism in a social context with central administration

Next, we sought to replicate the social context-dependent effects of melanocortin receptor agonism with i.c.v. administration of aCSF, 0.1 nmol MTII, or 0.1 nmol MTII combined with OTA (MTII+OTA) dissolved in aCSF. There were two purposes of this experiment. The first was to determine if the changes in Fos expression observed with i.p. administration were due to indirect effects of MTII or oxytocin acting in the periphery rather than in the brain. For example, there is some evidence that MTII activation of peripheral receptors increases Fos expression in the nucleus of the solitary tract, which projects to and may regulate the activity of hypothalamic oxytocin neurons (Paiva et al., 2017). Second, we wished to confirm that the absence of a NAc Fos increase in *Oxtr*-KO voles reflected the oxytocin-dependence of this effect rather than abnormal physiology arising from congenital *Oxtr* knockout. Brown-Forsythe and Welch ANOVA tests found a significant effect of treatment on Fos-positive cell density in PVN ($W (2.0, 29.75) = 11.46$, $p = 0.0032$), and Dunnett's T3 multiple comparisons test found significant increases in both the MTII group ($p = 0.0056$) and MTII+OTA group ($p = 0.0040$) compared to the aCSF group (**Figure 5A**). We compared Fos-positive cell density in NAc, BLA, PLC, and CA2 using a mixed-effects model with Geisser-Greenhouse correction and with brain region as a within-subjects repeated measure and treatment as a between-subjects factor, and found a significant treatment-brain region interaction ($F (6, 163) = 3.583$, $p = 0.0023$) as well as a significant treatment effect ($F (2, 56) =$

5.025, p = 0.0098). A Holm-Šídák post hoc test revealed that Fos immunoreactivity in NAc was significantly increased in the MTII group compared to both the aCSF (p = 0.0029) and MTII+OTA (p = 0.0111) groups, but no significant differences were found in other brain regions (**Figure 5B**). Although BLA Fos immunoreactivity was lower in both the MTII and MTII+OTA groups compared to the aCSF group, the differences were not statistically significant (aCSF vs. MTII p = 0.1834, aCSF vs. MTII+OTA p = 0.3600). These results are consistent with those of the peripheral-injection experiments and confirm that the MTII-induced increase in NAc Fos immunoreactivity that occurs in a social context requires oxytocin signaling. Representative images of sections with Fos-positive cell densities that approximate their groups means are shown in **Figure 6**.

3.5.4 Melanocortin receptor agonism in a novel context

To address the possibility that the context-dependent effects of MTII we observed were due to the novelty of the social stimulus rather than its sociality, we allowed voles to interact for 30 minutes with a novel rodent figurine rather than a novel conspecific after administering i.c.v. either 0.1 nmol MTII or aCSF. A one-tailed Welch's t test again indicated PVN Fos immunoreactivity was significantly increased in MTII-injected voles (M = 829.3, SEM = 168.1) compared to aCSF-injected voles (M = 435.7, SEM = 124.8; t(df) = 1.879(22.88), p < 0.0365) (**Figure 7A**). We compared Fos-positive cell density in NAc, BLA, PLC, and CA2 using a mixed-effects model with Geisser-Greenhouse correction and with brain region as a within-subjects repeated measure and treatment as a between-subjects factor and found neither a treatment effect (F (1, 23) = 0.8086, p = 0.3778) nor a treatment-brain region interaction (F (3, 65) = 0.1308, p = 0.9414) (**Figure 7B**). These data are consistent with the hypothesis that the context-dependent effects of MTII observed in previous experiments require a social stimulus rather than simply a novel stimulus.

Representative images of sections with Fos-positive cell densities that approximate their groups means are shown in **Figure 8**.

3.6 Discussion

Our data demonstrate that combining melanocortin agonism with a social context induces a synergistic, selective, oxytocin-mediated increase in NAc activity. Consistent with previous research on MTII (Barrett et al., 2014; Modi et al., 2015) and other MC4R agonists (Kublaoui et al., 2008), MTII administration increased Fos expression in PVN, which sends dense oxytocinergic projections to NAc (Ross et al., 2009; Ross & Young, 2009). However, MTII only increased NAc Fos in a social context, and this increase required oxytocin signaling. MTII may also have decreased BLA Fos in a social context independent of oxytocin signaling, though the effect size was small and not statistically significant in most experiments.

Previously, Modi et al. (2015) showed that MTII can accelerate partner preference formation, a form of social learning indicative of pair bond formation, in monogamous prairie voles. They further showed that while MTII administration alone does not cause oxytocin release in NAc, MTII increases the amount of oxytocin released in the NAc when release is triggered by a physiologic stimulus. A wide range of social stimuli are believed to induce oxytocin release as well (Uvnäs-Moberg, 1998; Dobloyi et al., 2018), including social touch (Tang et al., 2020) and mutual eye gaze (Nagasawa et al., 2015). In mice, a five-minute exposure to an anesthetized juvenile is sufficient to activate oxytocin neurons in PVN (Resendez et al., 2020), and in voles, oxytocin release in NAc increases during both sexual and nonsexual social interactions (Ross et al., 2009). Therefore, it is plausible that the 30-min social interactions in our experiments triggered oxytocin release in NAc and that MTII increased the amount of oxytocin released by this social

stimulus. This mechanism likely mediates the oxytocin- and social-context-dependent increase in NAc activation we observed in the present experiment. Furthermore, this increase in NAc activation may represent a neural correlate of the MTII-induced acceleration of social learning reported by Modi et al. (2015). Indeed, Modi et al. found that a microinfusion of OTA into the NAc prevented partner preference formation following peripheral MTII injection.

The enhancement of oxytocin-dependent NAc activity has important translational implications due to the critical role the NAc and oxytocin play in social and reward learning in both humans (Cohen et al., 2009; Gordon et al., 2013) and voles (Keebaugh et al., 2015). Indeed, many forms of social learning appear to be mediated by dynamic NAc physiology involving oxytocin and its interaction with other neurotransmitter systems. Oxytocin and serotonin signaling interact in NAc to induce synaptic plasticity and confer rewarding properties to social interactions (Dölen et al., 2013). Furthermore, synaptic plasticity in NAc mediated by oxytocin-serotonin interactions can re-open a critical period for social reward learning in adult mice (Nardou et al., 2019). In prairie voles, concurrent activation of NAc OXTR and D2-type dopamine receptors is necessary for pair bond formation (Liu & Wang, 2003). This pair bonding then causes an upregulation of NAc D1-type dopamine receptors, which mediate selective aggression toward non-partner voles to help maintain the pair bond (Aragona et al., 2006). Additionally, pair bonding reverses the electrophysiological effect of OXTR agonism on NAc medium spiny neurons; OXTR agonism decreases the amplitude of excitatory postsynaptic currents in virgins but increases their amplitude in pair-bonded voles (Borie et al., 2022b). This experience-induced change in NAc physiology is mediated by the coupling of OXTR and endocannabinoid receptor signaling, and it appears to facilitate the subsequent expression of behaviors that maintain the pair bond (Borie et al., 2022a). Collectively, these findings indicate that the enhancement of oxytocin-dependent NAc

activity is an ideal target for translational interventions seeking to facilitate social learning, rewire neural circuitry, and effect lasting behavioral changes. This is particularly true of social learning intended to meliorate the social deficits of ASD, in which decreased social reward may be a core etiological component (Dölen, 2015; Clements et al., 2018; DeMayo et al., 2019).

The selectivity with which MTII enhanced NAc activation without affecting Fos expression in PLC or CA2 also carries important translational implications. First, a therapeutic intervention that causes targeted release of endogenous oxytocin would minimize the risk of receptor desensitization from chronic administration of exogenous oxytocin (Rajagopal & Shenoy, 2018; Freeman et al., 2018; Le et al., 2022). Second, interventions with more targeted neural effects may be less likely to have undesired off-target effects (Ford & Young, 2021). OXTR are widespread throughout the brain; in prairie voles, *Oxtr* mRNA is expressed in over 250 distinct brain regions (Inoue et al., 2022). In humans, the spatial distribution of *OXTR* mRNA is highly correlated with the fMRI-based localization of diverse cognitive processes ranging from aversive states like anxiety, stress, and fear, to appetitive states involving sexual and food stimuli, and anticipatory states involving motivation, incentive, and reward (Quintana et al., 2019). The widespread distribution of OXTR and the disparate behavioral and cognitive functions they subserve may contribute to the divergent and at times contradictory effects of intranasal oxytocin. Although most studies have focused on its prosocial effects, intranasal oxytocin can also have anti-social effects like increasing feelings of envy and schadenfreude (taking pleasure in the misfortune of others) in a game of chance (Shamay-Tsoory et al., 2009), and increasing aggressive behavior in which participants in a monetary game choose to hurt their opponent rather than help themselves (Ne'eman et al., 2016). Intranasal oxytocin can also promote ethnocentric bias by increasing favoritism for one's ethnic in-group and derogation of an ethnic out-group (De Dreu et al., 2011),

and it can decrease trust and cooperation in people with borderline personality disorder (Bartz et al., 2011). Although the importance of controlling context in oxytocin-based therapeutic interventions cannot be overstated (Hurlemann, 2017; Ford & Young, 2022), an intervention that selectively activates a specific brain area via the endogenous oxytocin system may have more reliably therapeutic effects than intranasal administration of exogenous oxytocin.

There are, however, some important limitations to our study. First, we did not test the ability of MTII to enhance social learning. As such, we cannot conclude that the neural effects we observed would correlate with social learning or any other behavioral outcome, though the similarities between our study and that of Modi et al. (2015) implicate our neural findings as a plausible mechanism underlying the accelerated partner preference formation Modi et al. observed. Furthermore, while partner preference and pair bond formation in voles are instances of social learning with potential translational utility for ASD (Modi & Young, 2011), they might not be valid models for certain forms of social learning in humans. Nevertheless, the social learning that occurs in pair bonding requires many oxytocin-dependent processes, including attending to social cues, determining the valence of social stimuli, and forming social memories (Keebaugh et al., 2015; Walum & Young, 2018; Rigney et al., 2022). As such, partner preference formation is a useful behavioral readout for assessing the ability of pharmacological manipulations to activate the endogenous oxytocin system, independent of its translational utility as a model for any particular form of social learning (Modi & Young, 2012).

Second, our quantification of Fos was agnostic to cell type. While we can make inferences based on the general functions of anatomical brain regions, we cannot make inferences about the specific functions of activated cells or how they might affect the activity of the brain regions in which they were located. For example, it is possible that the increase in Fos-positive NAc cells

was driven disproportionately by activation of D1-type dopamine receptor-expressing cells rather than those expressing D2-type dopamine receptors, or vice versa. Such a finding would have important behavioral implications. Similarly, it is also possible that the increase in NAc Fos was driven by the activation of many GABAergic interneurons, which could have resulted in net inhibition of NAc activity despite an increase in the number of Fos-positive cells. However, this particular scenario is unlikely given that GABAergic interneurons comprise less than five percent of NAc neurons (Tepper et al., 2018). Conversely, if MTII caused an important physiological change by activating a small number of highly influential cells, our Fos quantification would not have detected such an effect.

There are also important limitations to the translational potential of MTII stemming primarily from its promiscuity as a ligand and the widespread distribution and diverse functions of melanocortin receptors. MTII binds to four of the five melanocortin receptors: MC1R, MC4R, MC3R, and MC5R in order of decreasing affinity. MC1R are primarily expressed in peripheral melanocytes (Mountjoy, 2010). MC5R are mostly located in the periphery with some expression in the brain, including PVN, and they contribute to immune system modulation, exocrine function, and metabolism (Shukla et al., 2012; Xu et al., 2020). MC3R, which play a role in feeding and metabolism, are found in many brain areas but are particularly concentrated in the thalamus, the ventral tegmental area, and several nuclei of both the amygdala and hypothalamus (Bedenbaugh et al., 2022). They are expressed at relatively low levels in the BLA and PVN, although the PVN receives abundant projections from MC3R-expressing neurons (Bedenbaugh et al., 2022). MC4R are involved in feeding, metabolism, and reproduction and are even more widely expressed in the brain than MC3R, though they are most highly concentrated in the brainstem, numerous

hypothalamic nuclei including PVN, and several amygdala nuclei including BLA (Gelez et al., 2010; Mountjoy, 2010; Modi et al., 2015).

As a consequence of this expansive distribution and functionality, melanocortin receptor agonism has the potential for numerous off-target effects. Melanocortin receptor agonism has been reported to: increase skin pigmentation, sexual arousal, stretching, and yawning (Bertolini et al., 2009); reduce appetite (Bertolini et al., 2009); elevate blood pressure and heart rate, which can lead to nausea and flushing (Li et al., 2013; Dhillon & Keam, 2019); induce histamine release and hypothermia, though this has only been reported in mice (Jain et al., 2018); possibly increase the risk of more serious conditions including cutaneous (Habbema et al., 2017) and renal (Peters et al., 2020) complications, though the current evidence is anecdotal and inconclusive. Despite these concerns, off-target effects may be greatly reduced or even eliminated with careful dose calibration and intermittent (i.e. before behavioral therapy sessions) rather than chronic use. For example, Dhillon & Keam (2019) explain that in developing Bremelanotide, an active metabolite of MTII, "some patients reported an increase in blood pressure, which was attributed to variability in drug uptake with intranasal administration." Switching to subcutaneous administration to achieve more consistent dosing resolved this problem. Indeed, the recent FDA approval of Bremelanotide to treat hypoactive sexual desire disorder indicates that melanocortin agonists like MTII can be safe and well-tolerated in humans with careful dose calibration (Dhillon & Keam, 2019). Of course, whether MTII would be safe and well-tolerated at efficacious doses for the enhancement of behavioral therapy remains to be seen.

Future translational studies utilizing melanocortin agonists should, if possible, consider using Bremelanotide given the relative ease of repurposing drugs that have already been approved by the FDA. Additionally, to further minimize off-target effects, the possibility of using or

developing more selective MC4R agonists should be explored. It is also important to note that the mechanism by which melanocortin agonism increases oxytocin release in NAc only when release is triggered by another stimulus (Modi et al., 2015) remains to be elucidated. It has been suggested that MC4R agonism causes somatodendritic release of oxytocin (Sabatier et al., 2003), which acts in an autocrine and paracrine manner to synchronize oxytocin neurons and prime oxytocin vesicles for increased activity-dependent release when subsequent physiological stimulation occurs (Ludwig & Leng, 2006; Ludwig & Stern, 2015; Modi et al., 2015; Ludwig et al., 2016). However, the ability of melanocortin agonism to trigger somatodendritic release of oxytocin *in vivo* has been inconsistent; in one study, neither systemic (intravenous) nor i.c.v. administration of MTII caused somatodendritic oxytocin release in SON, but retrodialysis of α-MSH into SON did induce somatodendritic oxytocin release (Paiva et al., 2017). Notably, no studies have examined melanocortin-induced somatodendritic release of oxytocin in PVN, and the sparse expression of *Oxtr* mRNA in vole PVN further decreases the likelihood of this autocrine priming mechanism (Inoue et al., 2022). Focal knock-down of OXTR in PVN and SON would help elucidate the role of autocrine and paracrine oxytocin signaling in the effects of melanocortin receptor agonism (Boender & Young, 2020).

Despite lingering questions regarding the underlying mechanism, our results provide a proof of concept that combining a pharmaceutical manipulation with a particular environmental stimulus can influence neuronal activity in ways that neither the pharmaceutical nor the environmental stimulus can alone. That is, pharmacology and environmental stimuli can interact synergistically. Thus, this study provides additional support for the argument that oxytocin therapies should be paired with a therapeutic social context (Young & Barrett, 2015; Shamay-Tsoory & Abu-Akel, 2016; Hurlemann, 2017; Ford & Young, 2022; Le et al., 2022). Critically,

we demonstrated that such multimodal interventions can have highly targeted effects restricted to specific brain areas. Developing more targeted therapeutics is a critical goal for translational neuroscience (Ford & Young, 2022), and as such, other possible synergies between pharmacology and environmental stimuli should be explored. Arguably the most successful combination of pharmacology and environmental stimuli to date is 3,4-methylenedioxymethamphetamine (MDMA)-assisted psychotherapy for PTSD, which received Breakthrough Therapy Designation from the FDA in 2019 (Feduccia et al., 2019) and has since passed a Phase III clinical trial with very promising results recently published in *Nature Medicine* (Mitchell et al., 2021). It should be noted that the authors of this trial suggest the remarkable efficacy of MDMA-assisted therapy may be mediated by MDMA "reopening an oxytocin-dependent critical period of neuroplasticity" (Mitchell et al., 2021), which has been shown to occur in NAc for critical periods of social learning (Nardou et al., 2019).

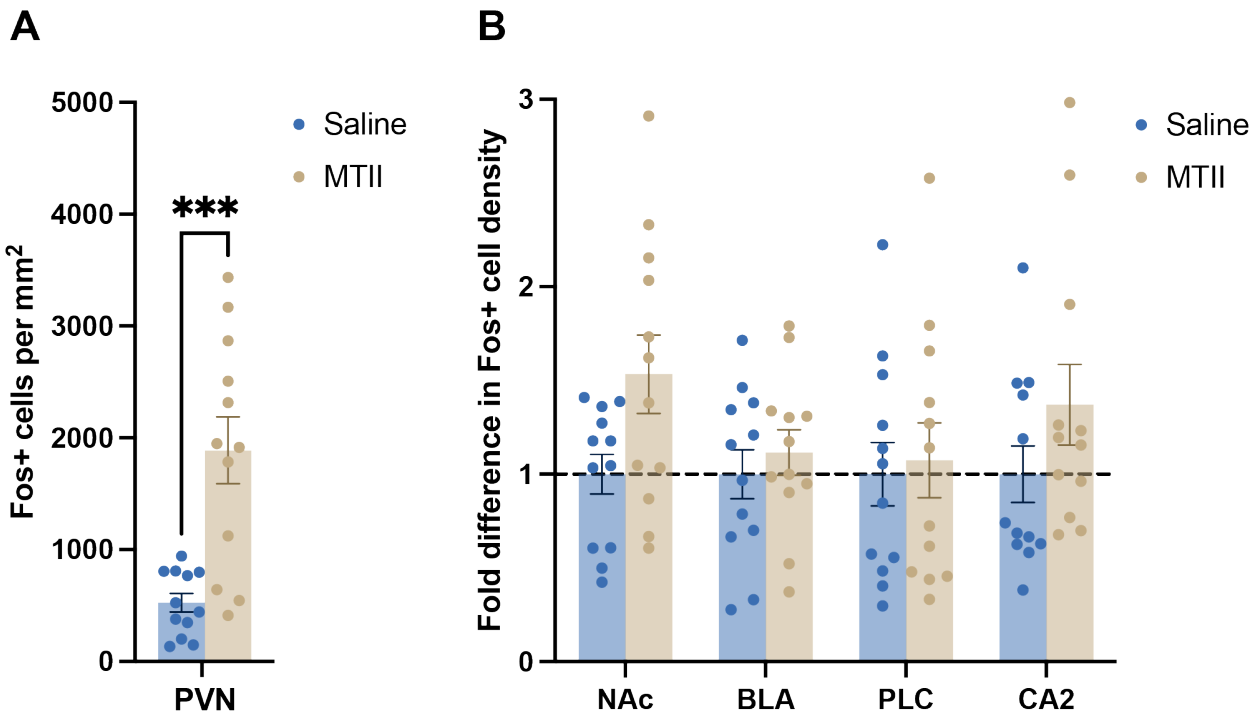


Figure 1. Peripheral MTII in a non-social context increases Fos in PVN but not other regions. Voles were injected i.p. with either saline (n = 12 (6M, 6F)) or MTII (n = 12 (6M, 6F)) and returned to their home cages alone. A one-tailed Welch's t test found a significant increase in Fos-positive cell density in MTII-injected voles (M = 1889, SEM = 297.6) compared to saline-injected voles (M = 525.7, SEM = 83.79; t(df) = 4.409(12.73), p = 0.0004) (**A**). In NAc, BLA, PLC, and CA2, a two-way, repeated-measures ANOVA failed to detect a treatment effect (F (1, 22) = 1.171, p = 0.2910) or a treatment-brain region interaction (F (3, 66) = 0.6341, p = 0.5957). Error bars show standard error of the mean.

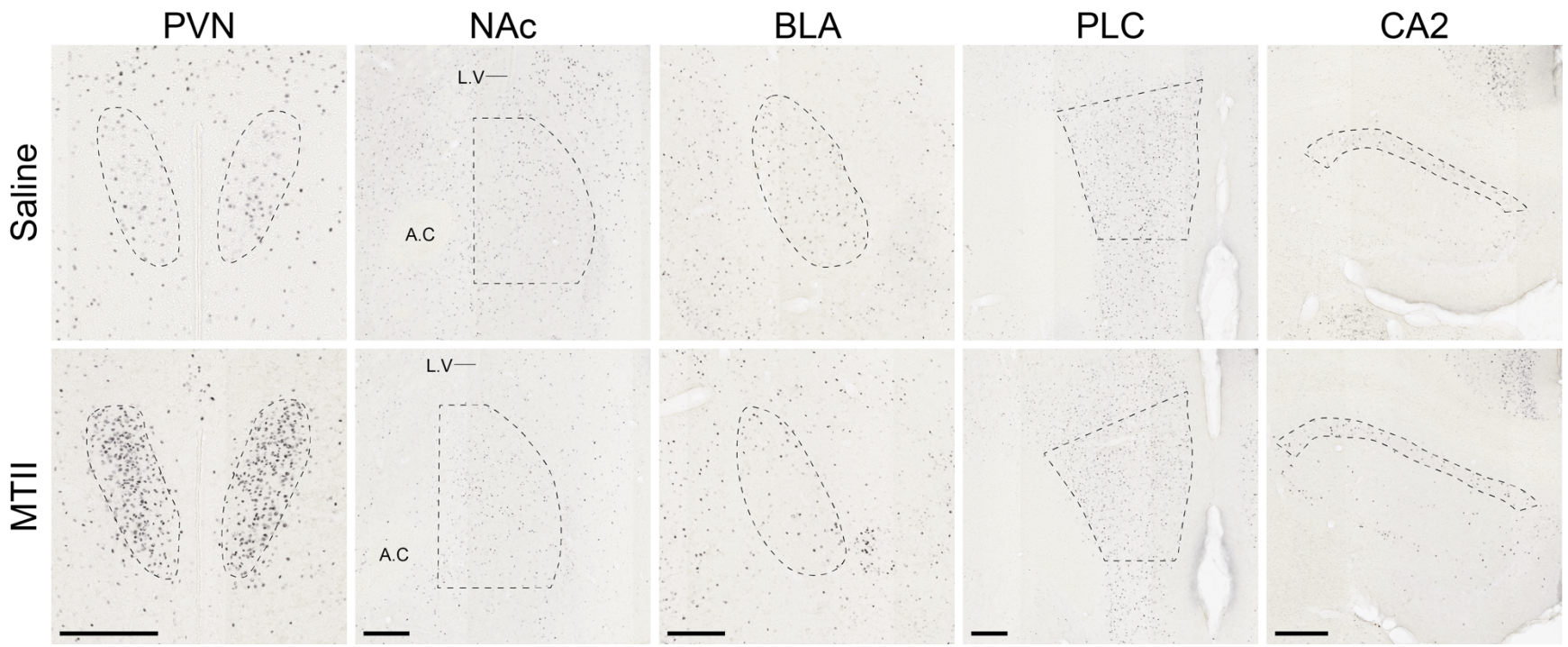


Figure 2. Representative images of Fos-labeled brain regions after a non-social context. Voles were injected i.p. with either saline or MTII and returned to their home cages alone. Representative sections of PVN, NAc, BLA, PLC, and CA2 with Fos-positive cell densities that approximate their group means are shown here. L.V. = lateral ventricle; A.C. = anterior commissure; all scale bars = 250 µm.

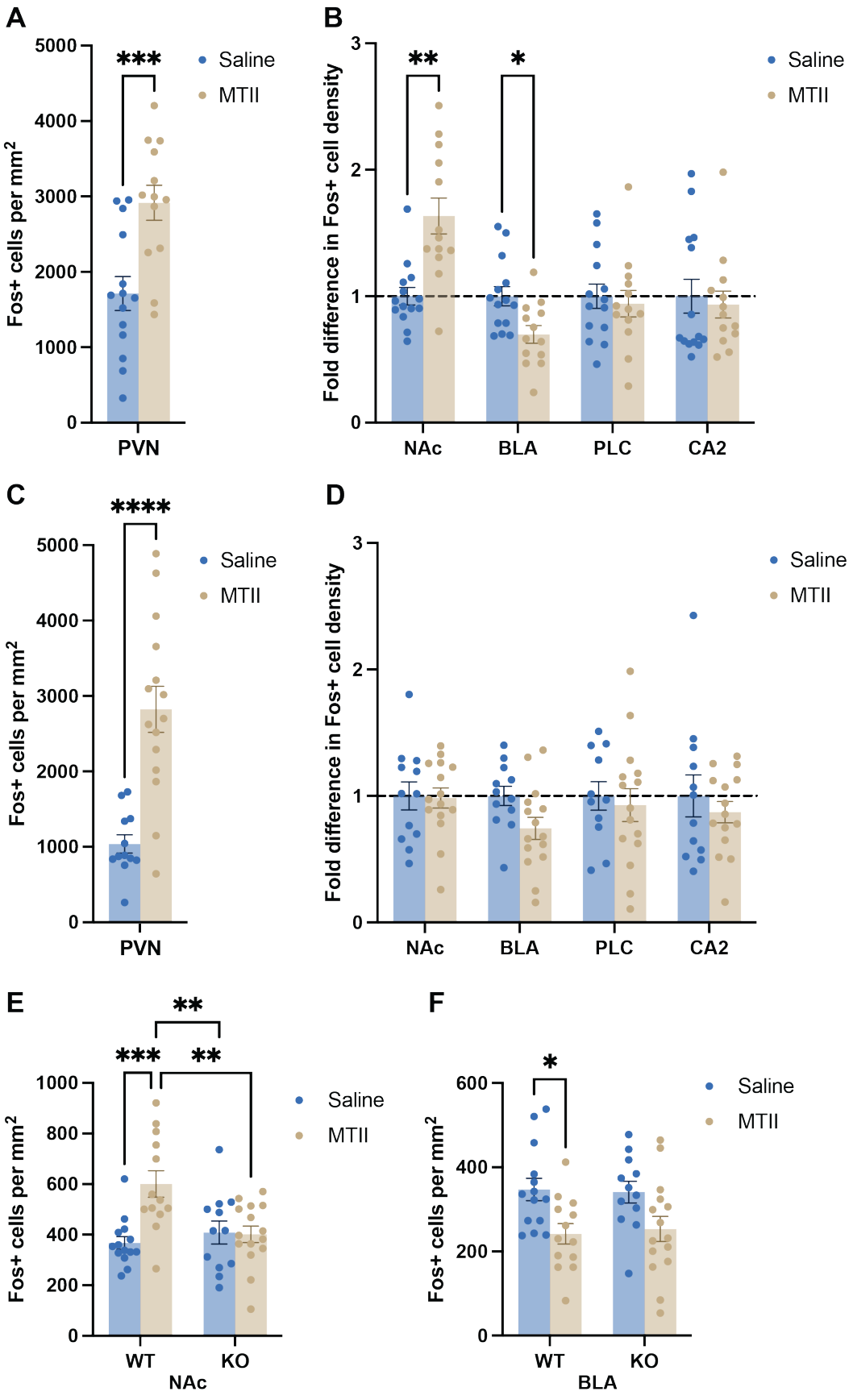

A
B
C
D
E
F
Saline
MTII
Fos+ cells per mm²
Fold difference in Fos+ cell density
PVN
NAc
BLA
PLC
CA2
WT
KO
***
**
*
****

Figure 3. Peripheral MTII in a social context increases Fos in NAc in WT, but not *Oxtr*-KO, subjects. WT and *Oxtr*-KO voles were injected i.p. with either saline (WT n = 14 (6M, 8F); *Oxtr*-KO n = 12 (6M, 6F)) or MTII (WT n = 13 (6, 7F); *Oxtr*-KO n = 15 (7M, 8F)) and allowed to interact socially with a novel, opposite-sex vole for 30 min. A one-tailed Welch's t tests found significant increases in Fos-positive cell density in the PVN of MTII-injected animals of both WT (M = 2917, SEM = 231.3; t(df) = 3.735(24.88), p = 0.0005) and *Oxtr*-KO (M = 2825, SEM = 305.2; t(df) = 5.436(18.20), p < 0.0001) genotypes compared to saline-injected animals (WT M = 1713, SEM = 224.5; *Oxtr*-KO M = 1038, SEM = 121.5) (**A**,**C**). A two-way, repeated-measures ANOVA analyzing NAc, BLA, PLC, and CA2 revealed a significant treatment-brain region interaction (F (3, 75) = 10.59, p < 0.0001) in WT voles, and a Holm-Šídák post hoc test found a significant increase in Fos immunoreactivity in NAc (p = 0.0034) and a significant decrease in Fos immunoreactivity in BLA (p = 0.0214) in the MTII group compared to the saline group (**B**). In *Oxtr*-KO voles, a mixed-effects model found neither a treatment effect (F (1, 25) = 0.8711, p = 0.3596) nor a treatment-brain region interaction (F (3, 74) = 0.6045, p = 0.6141) (**D**). A two-way ANOVA of NAc data from both genotypes found a treatment-genotype interaction (F (1, 50) = 9.338, p = 0.0036), and a Holm-Šídák post hoc test found Fos immunoreactivity significantly increased in the MTII-injected WT group compared to the saline-injected WT (p = 0.0006), saline-injected *Oxtr*-KO (p = 0.0063), and MTII-injected *Oxtr*-KO (p = 0.0031) groups (**E**). A two-way ANOVA of BLA data from both genotypes found only a main effect of treatment (F (1, 50) = 12.56, p = 0.0009), and a Holm-Šídák post hoc test found Fos immunoreactivity significantly decreased in the MTII-injected WT group compared to the saline-injected WT group (p = 0.0496) (**F**). Error bars show standard error of the mean.

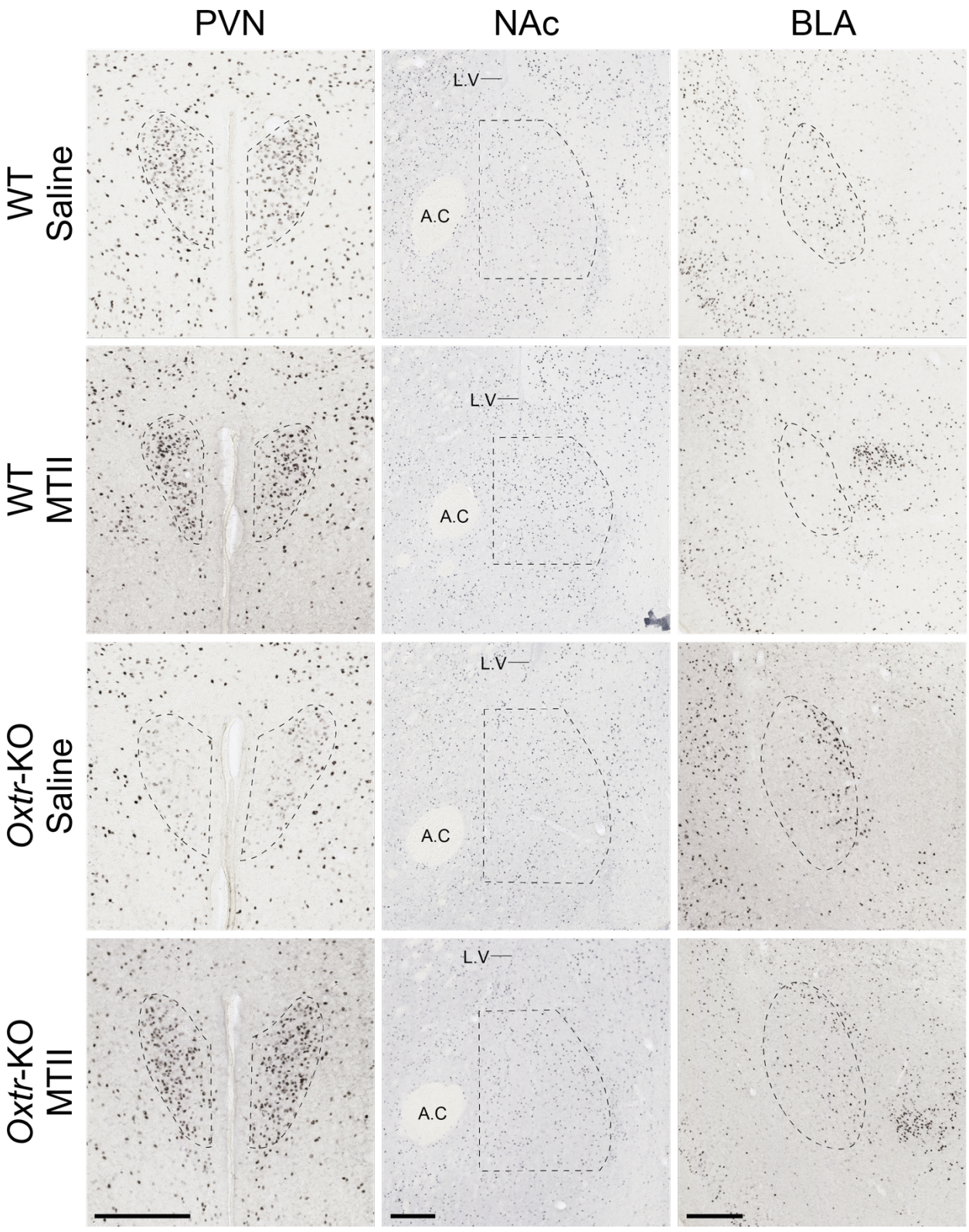

PVN
NAc
BLA
WT Saline
WT MTII
Oxtr-KO Saline
Oxtr-KO MTII
L.V
A.C

Figure 4. Representative images of Fos-labeled brain regions after a social context. Voles were injected i.p. with either saline or MTII and allowed to interact with a novel, opposite-sex vole for 30 minutes. Representative sections of PVN, NAc, and BLA with Fos-positive cell densities that approximate their group means are shown here. L.V. = lateral ventricle; A.C. = anterior commissure; all scale bars = 250 μm.

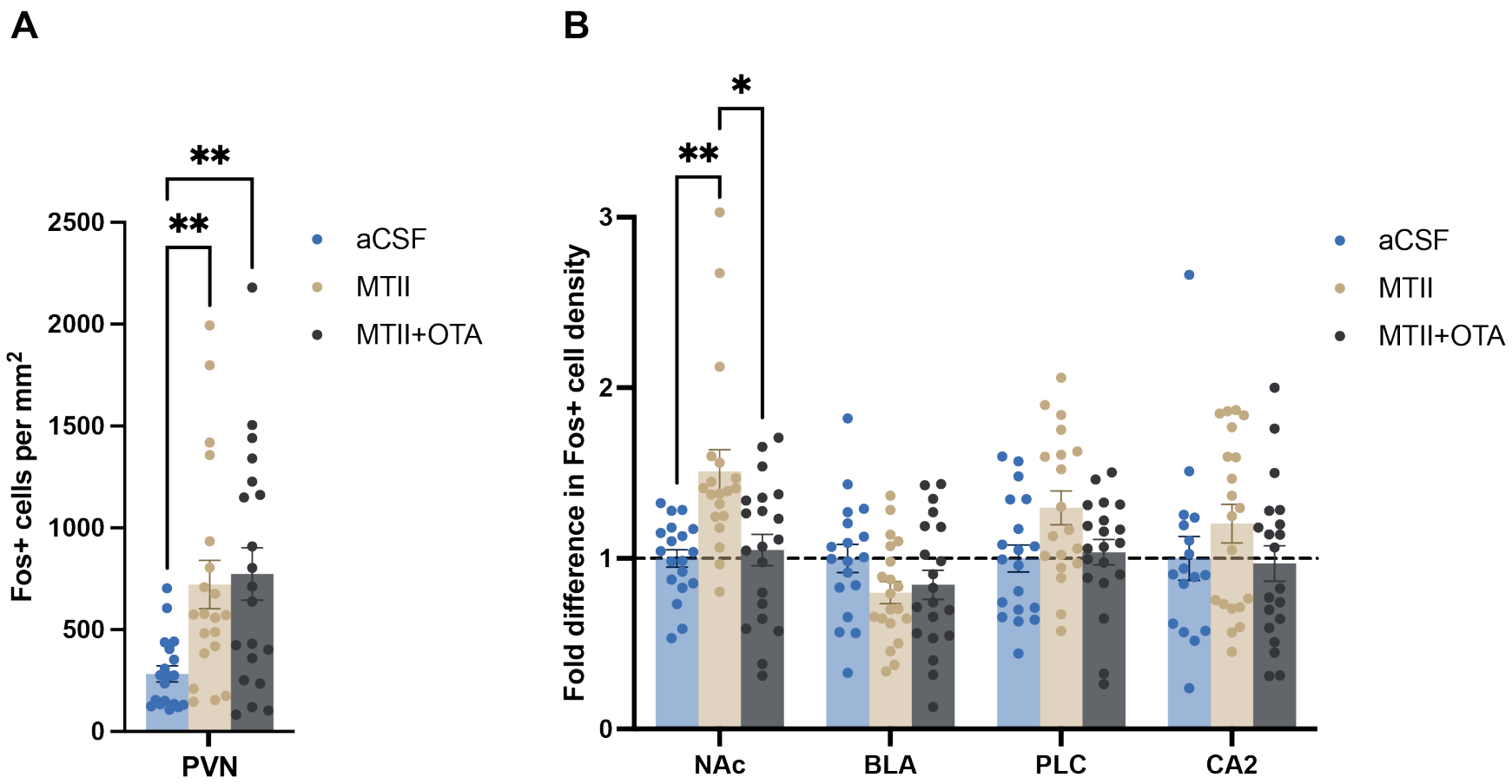


Figure 5. Central MTII in a social context induces an oxytocin-dependent increase in NAc Fos. Voles were injected i.c.v. with either aCSF (n = 20 (10M, 10F)), MTII (n = 19 (10M, 9F)), or MTII combined with OTA (MTII+OTA; n = 20 (10M, 10F)) and allowed to interact socially with a novel, opposite-sex vole for 30 min. Brown-Forsythe and Welch ANOVA tests found a significant treatment effect in PVN (W (2.0, 29.75) = 11.46, p = 0.0032), and Dunnett's T3 multiple comparisons test revealed significant increases in Fos immunoreactivity in both the MTII (p = 0.0056) and MTII+OTA (p = 0.0040) groups… (continued on next page)

(Figure 5 Caption continued) …compared to the aCSF group (**A**). A repeated-measures mixed-effects model analyzing NAc, BLA, PLC, and CA2 found a significant treatment-brain region interaction ($F (6, 163) = 3.583$, $p = 0.0023$), and a Holm-Šídák post hoc test indicated Fos immunoreactivity in NAc was significantly increased in the MTII group compared to both aCSF ($p = 0.0029$) and MTII+OTA ($p = 0.0111$) groups (**B**). Error bars show standard error of the mean.

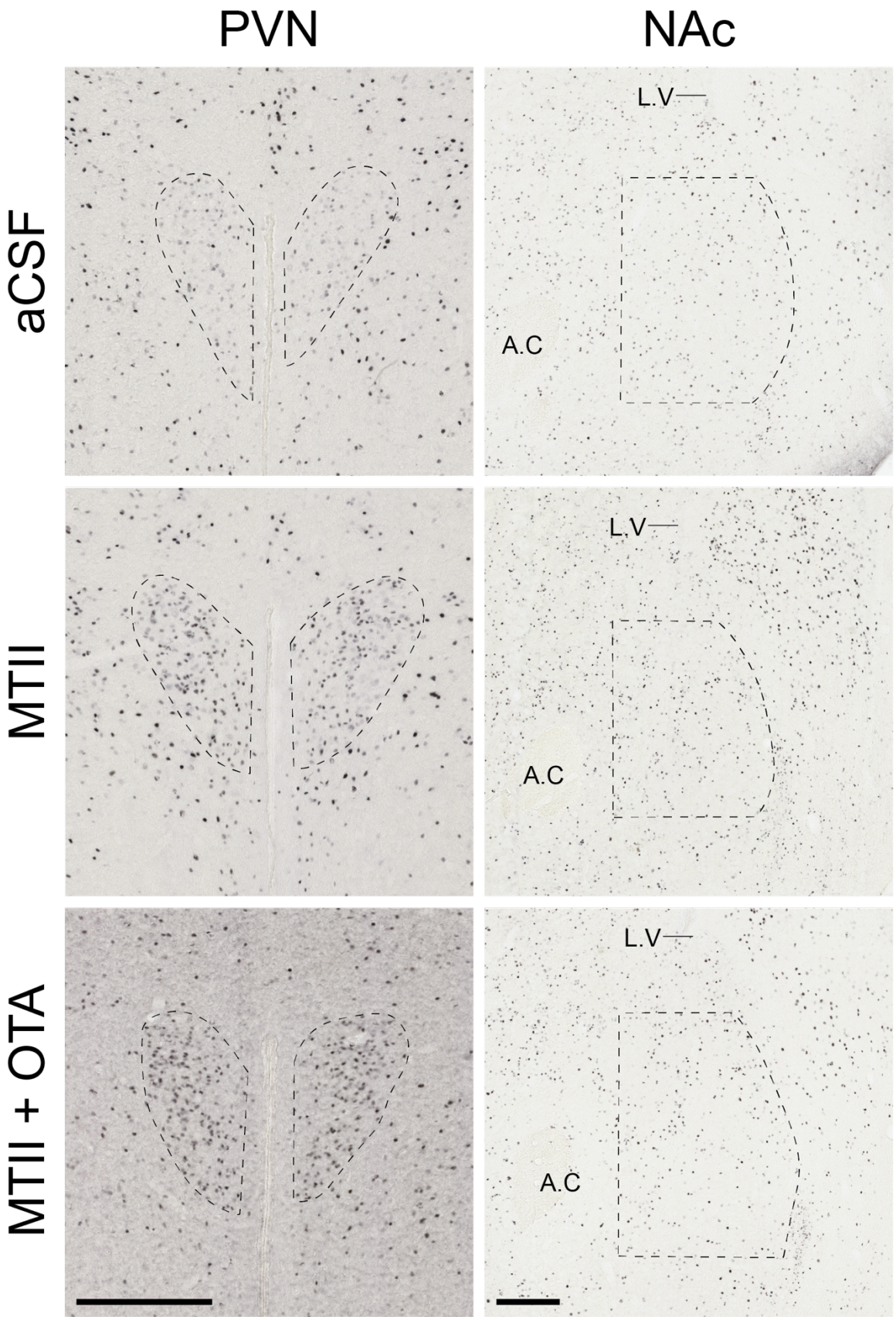

PVN
NAc
aCSF
MTII
MTII + OTA
L.V
A.C
L.V
A.C
L.V
A.C

Figure 6. Representative images of Fos-labeled brain regions after a social context and i.c.v. injection. Voles were injected i.c.v. with either aCSF, MTII, or MTII+OTA and allowed to interact with a novel, opposite-sex vole for 30 minutes. Representative sections of PVN and NAc with Fos-positive cell densities that approximate their group means are shown here. L.V. = lateral ventricle; A.C. = anterior commissure; all scale bars = 250 μm.

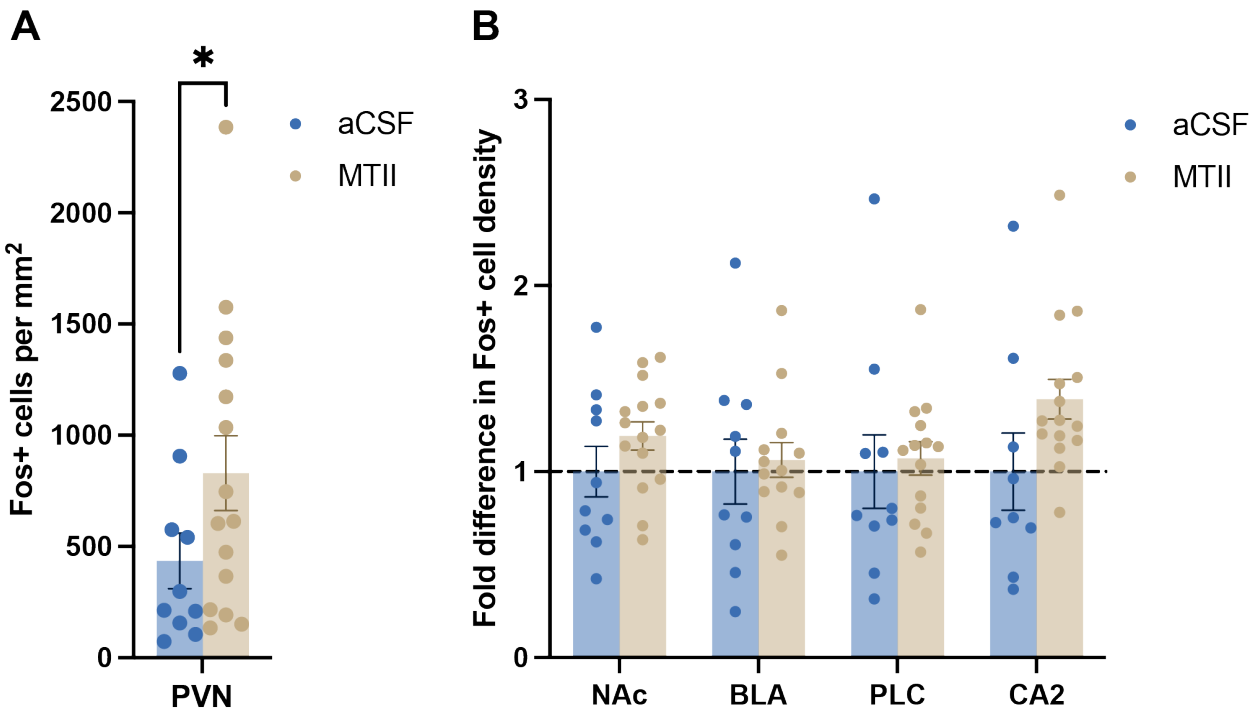


Figure 7. Central MTII in a novel context. Voles were injected i.c.v. with either aCSF (n = 10 (5M, 5F)) or MTII (n = 15 (7M, 8F)) and exposed to a novel rodent figurine for 30 min. A one-tailed Welch's t test indicated PVN Fos immunoreactivity was significantly increased in the MTII group (M = 829.3, SEM = 168.1) compared to the aCSF group (M = 435.7, SEM = 124.8; t(df) = 1.879(22.88), $p < 0.0365$) (**A**). In NAc, BLA, PLC, and CA2, a mixed-effects model failed to detect a treatment effect ($F(1, 23) = 0.8086$, $p = 0.3778$) or a treatment-brain region interaction ($F(3, 65) = 0.1308$, $p = 0.9414$) (**B**) Error bars show standard error of the mean.

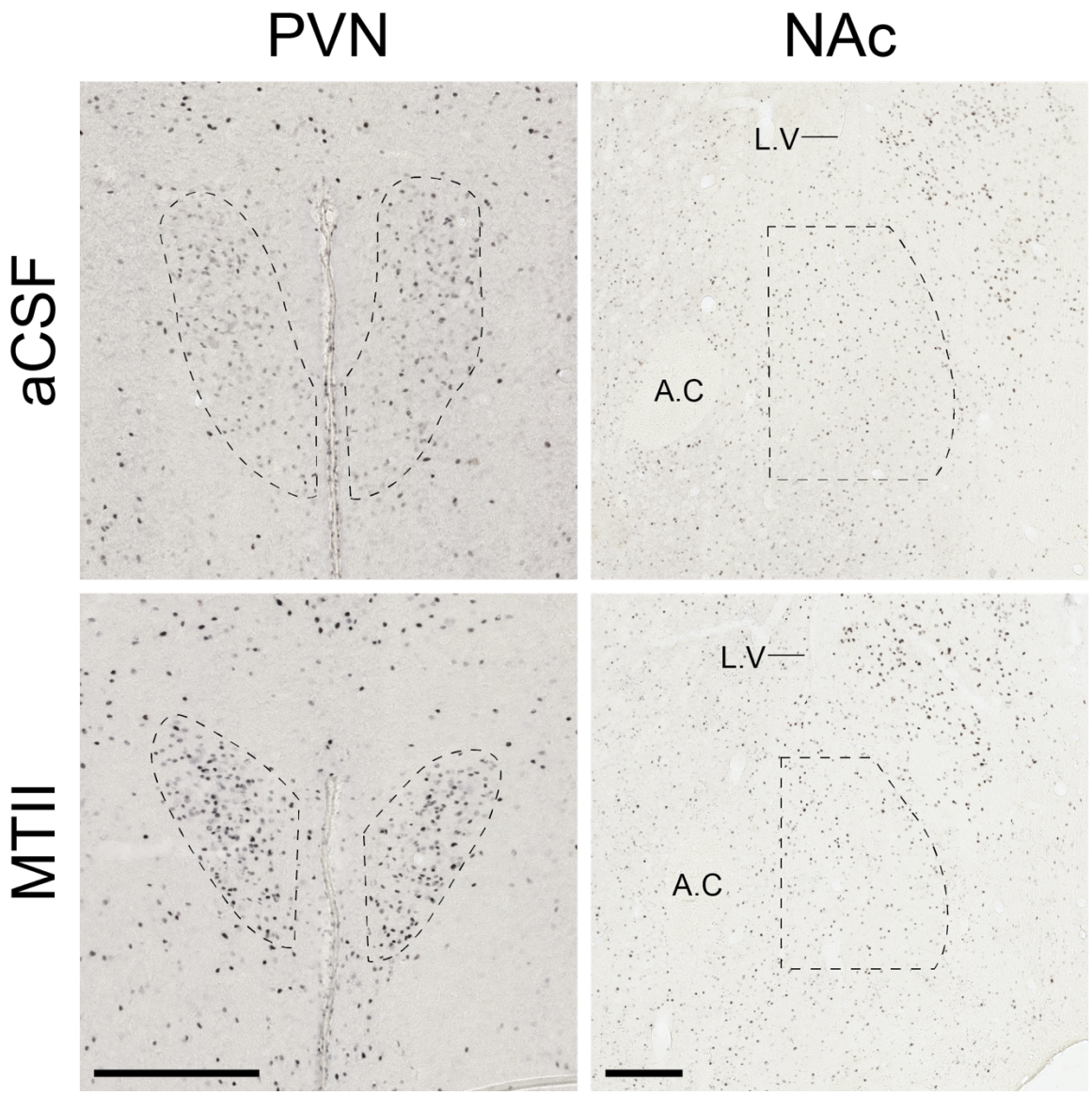
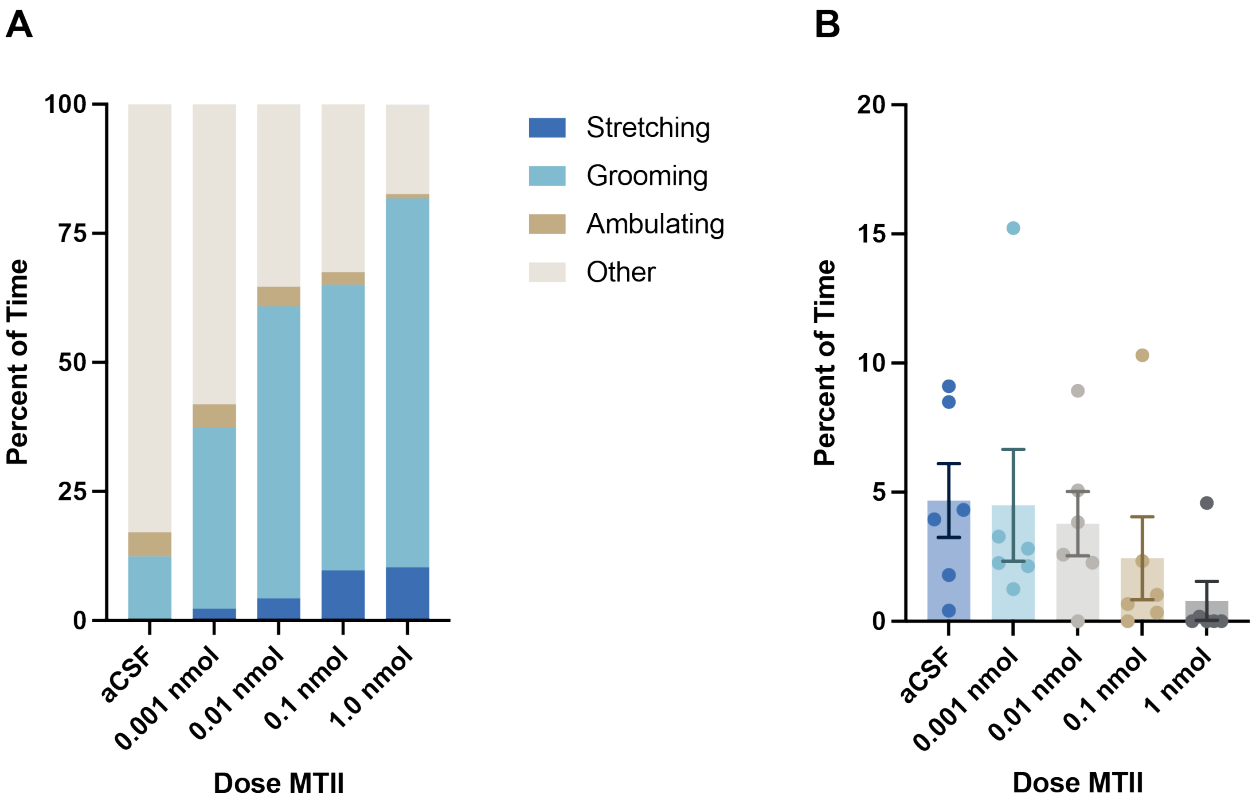
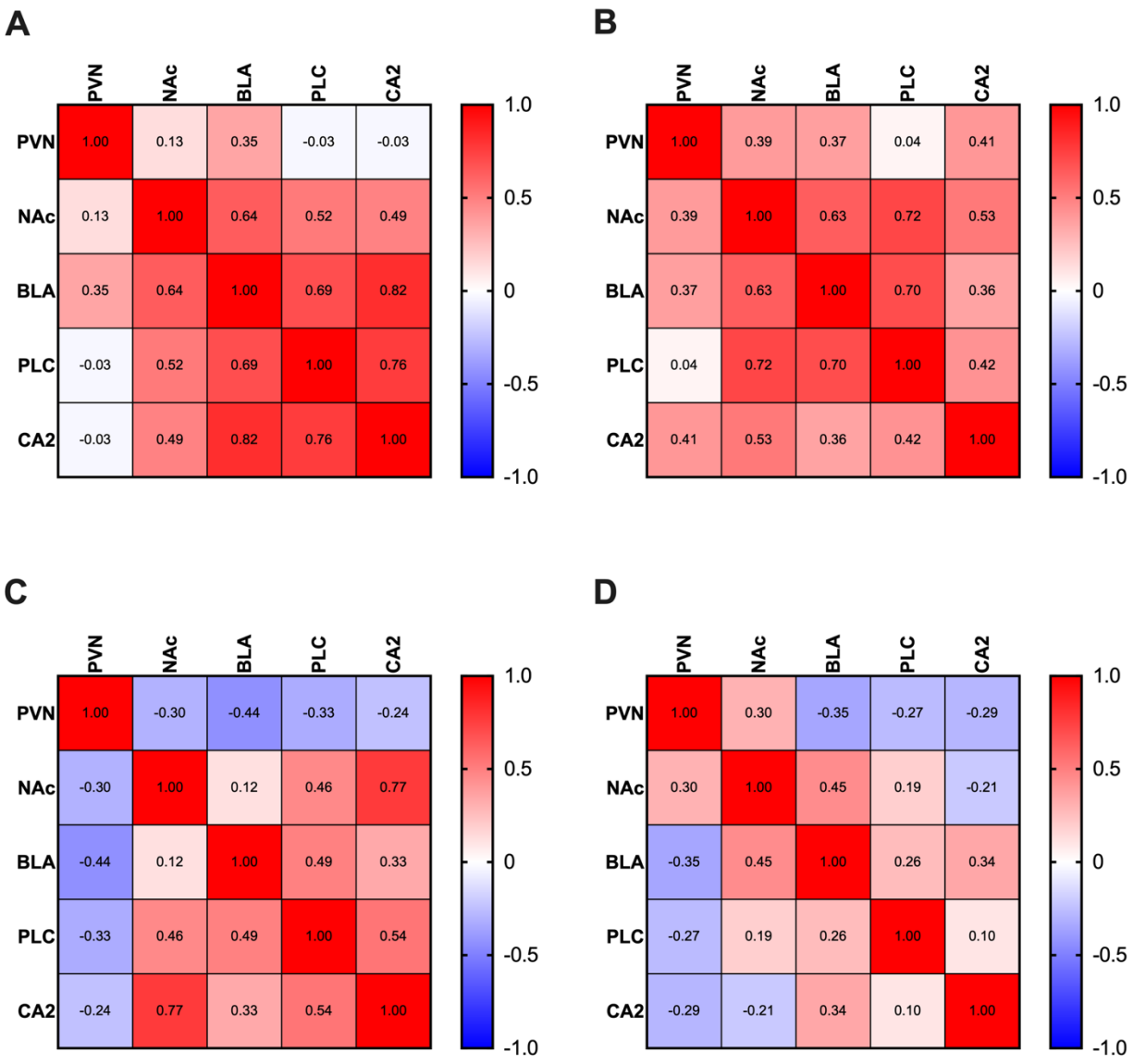


Figure 8. Representative images of Fos-labeled brain regions after a novel context and i.c.v. injection. Voles were injected i.c.v. with either aCSF, MTII, or MTII+OTA and allowed to interact with a novel rodent figurine for 30 minutes. Representative sections of PVN and NAc with Fos-positive cell densities that approximate their group means are shown here. L.V. = lateral ventricle; A.C. = anterior commissure; all scale bars = 250 μm.



Supplemental Figure 1. Dose-response of MTII and behavioral side effects. Voles were injected i.c.v. with 2 µL of aCSF, 0.001 nmol MTII, 0.01 nmol MTII, 0.1 nmol MTII, or 1 nmol MTII dissolved in aCSF (n = 6 (3M, 3F) per group). Their behavior was filmed for 15 min and scored for the time they spent ambulating, grooming, and stretching. The mean percent of time each group spent engaged in these behaviors is shown (**A**), as is the percent of time each individual subject spent ambulating (**B**). Notably, ambulation was greatly reduced at the 1.0 nmol MTII dose. Error bars show standard error of the mean.



Supplemental Figure 2. Correlations of Fos expression between brain regions within subjects. Fos expression levels were highly correlated (red) between target brain regions (NAc, BLA, PLC, CA2) within each subject regardless of context (non-social **A**,**B**; social **C**,**D**) or drug treatment (saline **A**,**C**; MTII **B**,**D**). While PVN Fos demonstrated less correlation with other regions, Fos expression levels in target brain areas were not independent of one another. Consequently, data from target areas were analyzed as a repeated measure in ANOVAs.

Chapter 4

Love, Death, and Placebos: A Foray into Psychosomatics

4.1 Acknowledgment of Reproduction and Authors' Contributions

This chapter is comprised of original writing, with the exception of section 4.5, entitled "Harnessing the Healing Power of Love," which is adapted from:

Ford CL, Young LJ (2021) Harnessing the healing power of love. *Trends in Molecular Medicine*. 27(9):833-834

I wrote the manuscript with input and revisions from, and under the guidance of, L. Young.

4.2 Abstract

Psychosomatic phenomena have been neglected and even ridiculed as a subject of study for ostensibly conflicting with a philosophically materialistic worldview. I argue that to dismiss psychosomatics as a study of immaterial phenomena is, ironically, to fail to recognize the material neurological basis of all psychological phenomena. I review the limited literature on the neurobiological basis of the most well-studied psychosomatic phenomenon – the placebo effect – and its translational implications. I then discuss a recent paper describing psychosomatic effects of pair bonding and partner loss on tumorigenicity in a monogamous rodent, and propose a model for how social stimuli might be transmuted into somatic effects on cancer cells via neural processing. Finally, I propose a novel hypothesis positing an evolutionary origin of the so-called "widowhood effect," or the increased mortality risk of widowed persons. In doing so, I discuss the neurobiological basis of this psychosomatic phenomenon, inclusive fitness and the "grandmother hypothesis" for the evolution of a long post-reproductive lifespan, Hamilton's Rule for the evolution of altruism and how it can be modified to predict altruistic mortality, genetically programmed organism death, selection pressures following partner loss, my hypothesis's ability to explain real-world observations, and the arresting possibility that some of our genes may be

trying to kill us. I conclude with a brief discussion of future directions for, and my personal interests in, psychosomatics.

4.3 Introduction: The Case for Psychosomatics

"The mysterious aspects of consciousness might disappear, just as the mysterious aspects of embryology have largely disappeared now that we know about the capabilities of DNA, RNA, and protein." – Dr. Francis Crick, *The Astonishing Hypothesis* (1994)

Psychosomatics, which is defined as "a branch of medical science dealing with interrelationships between the mind or emotions and the body and especially with the relation of psychic conflict to somatic symptomatology" (Merriam-Webster, n.d.), has not received from most clinicians or researchers the thoughtful consideration and study that attends other neuroscientific subjects. Although few would outright deny their existence, psychosomatic phenomena are most commonly dismissed as either false perceptions of psychiatric origin or physiological consequences of severe stress (Burton et al., 2015; Henningsen et al., 2018; Mostafaei et al., 2019). These explanations are undoubtedly correct in some cases, such as the psychosomatic blindness in Cambodian refugees who survived the Khmer Rouge (Rozée & Van Boemel, 1990). However, given the enormous complexity of the nervous system and its influence over the rest of the body, these explanations are, in many instances, likely to be oversimplifications of more complex and less understood neurobiological processes.

One perennial challenge of psychosomatics is that it raises the mind-body problem. While Cartesian dualism is the most common belief among nonscientists, most scientists accept some form of monism (Kalat, 2009). Consistent with the monistic philosophical positions of materialism and materialistic functionalism, I posit that every psychological event and state could, with

sufficient knowledge, be described fully in terms of neural events and states. That is, all psychological phenomena are neurological phenomena, and thus all psychosomatic phenomena are neuro-somatic phenomena. In this view, psychosomatics is the study of purely physiological processes like any other biological subject, except in that the underlying mechanisms are not well understood. As such, psychosomatic phenomena should be the subject of robust and rigorous scientific investigation to discover the as yet unknown neurophysiological processes behind them. Through empirical interrogation, the apparent necessity of psychological phenomena in explaining psychosomatic events might dissipate over the coming decades, just as the illusory necessity of a "vital force" in explaining embryological events dissipated in the last century. As Dr. Crick wrote in 1966, "Exact knowledge is the enemy of vitalism."

4.4 The Neurobiological Mechanisms of the Placebo Effect

"The wonderful influence of imagination in the cure of diseases is well known. A motion of the hand, or a glance of the eye, will throw a weak and credulous patient into a fit; and a pill made of bread, if taken with sufficient faith, will operate a cure better than all the drugs in the pharmacopœia." – Charles Mackay, *Extraordinary Popular Delusions and the Madness of Crowds* (1841)

The placebo effect is undoubtedly the most studied of all psychosomatic phenomena, and significant placebo effects have been demonstrated for a wide range of conditions, often with large effect sizes. For instance, in reducing migraine severity, a placebo was 60% as effective as Rizatriptan (a serotonin receptor agonist and common migraine medication) and equally as effective as Rizatriptan mislabeled as placebo (Kam-Hansen et al., 2014). Remarkably, even non-deceptive studies have shown significant effects of open-label placebos for migraines (Kam-

Hansen et al., 2014), irritable bowel syndrome (Kaptchuk et al., 2010), fatigue in cancer survivors (Hoenemeyer et al., 2018), allergic rhinitis (Schaefer et al., 2016), and chronic lower back pain (Carvalho et al., 2016). Although these examples all depend on subjective perceptions of unpleasant symptoms, placebos can impact objective physiological measures as well. After classical conditioning, a placebo flavored beverage (conditioned stimulus) that had been paired with immunosuppressive drugs (calcineurin inhibitors; unconditioned stimulus) was able to inhibit T cell proliferation in renal transplant recipients (Kirchhof et al., 2018). Additionally, a meta-analysis of beta-blocker efficacy including over 11,000 subjects with hypertension across 23 studies estimated that the placebo effect accounted for 34% of systolic and 47% of diastolic blood pressure reduction (Wilhelm et al., 2016). Unfortunately, very few studies have investigated the neural correlates of placebo effects; however, there are two conditions in which they have been partially elucidated – major depressive disorder (MDD) and Parkinson's disease.

Published in *JAMA Psychiatry*, one study on MDD showed that an acute intravenous placebo administration increased μ-opioid receptor binding in nucleus accumbens (NAc), and one week of oral placebo administration increased μ-opioid receptor binding in NAc, subgenual anterior cingulate cortex, amygdala, and midline thalamus (Peciña et al., 2015). Both placebos induced significant reductions in depression symptoms, and μ-opioid receptor binding in NAc was positively correlated with the magnitude of symptomatic improvement ($R^2 = 0.32$). After the placebo phases, these previously unmedicated patients underwent 10 weeks of standard, open-label antidepressant treatment primarily with selective serotonin reuptake inhibitors. Following antidepressant treatment, subjects who had previously experienced a placebo-induced improvement in depression symptoms (n = 14) had half the symptom severity of subjects who had not experienced placebo-induced symptom improvements (n = 21), and these placebo

"responders" were three times more likely to be in remission than placebo "non-responders." Additionally, placebo-induced μ-opioid receptor binding predicted 43% of the variance in antidepressant efficacy, and combined with subjective placebo responsiveness, it predicted 57% of the variance. Lastly, μ-opioid receptor binding in NAc after intravenous placebo administration was positively correlated with symptom reduction after 10 weeks of antidepressant treatment (R^2 = 0.41). It is unclear if the placebo-induced μ-opioid receptor activation enhanced the efficacy of antidepressants, or if inherent differences between placebo responders and non-responders were predictive of responsiveness to antidepressants. However, as the authors note, the partial μ-opioid agonist buprenorphine has antidepressant properties, and it is possible that the opioidergic effects of placebo and the monoaminergic effects of antidepressants interact synergistically to reduce depression symptoms (Peciña et al., 2015).

In Parkinson's disease, which is characterized by progressive motor dysfunction arising from the degeneration of dopaminergic neurons in the substantia nigra, placebo injections administered after verbally inducing an expectation of symptom improvement elicited dopamine release in both the dorsal (de la Fuente-Fernández et al., 2001) and ventral (de la Fuente-Fernández et al., 2002) striatum. The magnitude of this dopamine response was comparable to that elicited by therapeutic doses of levodopa or apomorphine (a dopamine receptor agonist). Interestingly, the dopamine response in dorsal striatum was greater in patients that perceived an improvement in motor symptoms, but this was not the case in ventral striatum, indicating that the dopamine response in ventral striatum is associated with the expectation of reward rather than the reward itself (de la Fuente-Fernández et al., 2002). Other studies have implicated dopaminergic activity in the substantia nigra pars reticulata, subthalamic nucleus, ventral anterior thalamus, and ventral lateral thalamus in the parkinsonian placebo response (Benedetti et al., 2009). A relatively recent

literature review concluded that placebo-induced motor improvement in Parkinson's disease requires dorsal striatal dopamine release to activate the entire nigrostriatal pathway, the magnitude of the improvement depends on the expectation of improvement mediated by ventral striatal dopamine release, and that prior exposure to dopaminergic therapeutics enhances the placebo effect, indicating that conditioning may play a role (Quattrone et al., 2018).

Although the neurobiological effects of placebos may be specific to the purpose of the treatment and the condition being treated (Colloca & Barsky, 2020), some neural networks and mechanisms, along with associated psychological phenomena, have been implicated in the placebo effect more generally (Jubb & Bensing, 2013). Verbally inducing the expectation of analgesia activates the endogenous opioid system, which mediates placebo-induced analgesia via opioid signaling in the NAc, amygdala, rostral anterior cingulate cortex, anterior insula, orbitofrontal cortex, and periaqueductal gray (Scott et al., 2008). Only one study (Scott et al., 2008) used positron emission tomography with a radioactive ligand to detect endogenous opioid signaling specifically, but other studies using fMRI have also implicated the thalamus and dorsolateral prefrontal cortex in this process (Petrovic et al., 2002; Bingel et al., 2011). Dopamine signaling in NAc, which canonically mediates motivation, reward, and expectation of reward, also contributes to placebo-induced analgesia (Scott et al., 2008). As relief from unpleasant symptoms is rewarding, it is plausible that NAc dopamine release might contribute to the placebo effect in other conditions as well, but research on this subject is scarce. It is also intriguing to note that nigrostriatal dopamine signaling is central to both the "false" perceptions and beliefs of the placebo effect and the positive symptoms (e.g. delusions and hallucinations) of schizophrenia (McCutcheon et al., 2019). In addition to opioids and dopamine, one study demonstrated that some forms of placebo-induced analgesia are mediated by endocannabinoid signaling at the CB1 receptor (Benedetti et al., 2011).

Social factors have powerful influences on the placebo effect, although the underlying neurobiological mechanisms are not well understood (Colloca & Barsky, 2020). In one study of the placebo effect on the common cold, "enhanced" patient-provider interactions in which the provider conveyed more positivity and empathy were compared to standard patient-provider interactions (Rakel et al., 2011). Patients who perceived their provider as more empathetic experienced illnesses with subjectively lower severity and shorter durations, and objectively, these patients had increased neutrophil counts and Interleukin-8 levels, which are both indicators of a more robust immune response (Rakel et al., 2011). A separate study on the placebo effect in irritable bowel syndrome found that the social quality of the patient-practitioner interaction accounted for significant differences in the magnitude of the placebo effect between groups of patients (Kelley et al., 2009). Furthermore, a systematic literature review of 34 studies examining the effects of practitioner characteristics on patient outcomes concluded that warm, positive demeanor and nonverbal behaviors in practitioners contributed to stronger placebo effects and reduced experiences of pain and anxiety in patients (Daniali & Flaten, 2019).

The influence of social factors on the placebo effect implicates oxytocin as a possible mediator. A recent review speculated on the role of oxytocin in placebo effects (Itskovich et al., 2022), although empirical research is limited. One study found that intranasal oxytocin enhances placebo analgesia arising from verbally induced expectation (Kessner et al., 2013). Another showed that intranasal oxytocin combined with a placebo and either negative or positive expectations could impair or enhance, respectively, working memory; however, neither oxytocin nor placebos alone had any effect (Zhao et al., 2019). A third study showed that intranasal vasopressin, but not oxytocin, enhanced placebo analgesia in women but not men (Colloca et al., 2016). However, three other studies failed to find any effects of oxytocin on placebo-induced

analgesia or nocebo-induced hyperalgesia (Skvortsova et al., 2018; Liu et al., 2020; Skvortsova et al., 2020). The inconsistency of these results is reminiscent of the context-dependent inconsistencies seen in the cognitive and behavioral intranasal oxytocin studies discussed in Chapters 1 and 3. As Itskovich et al. (2022) point out, experimental contexts involving pain induction may, depending on the design, impair trust in the subject-experimenter relationship and thus blunt oxytocin-dependent aspects of the placebo effect. These studies also varied in the doses administered, the sex of the subjects, and the sex of the experimenters, all of which could have influenced the effects of oxytocin (Colloca et al., 2016).

Research into the neurobiological mechanisms of the placebo effect is still nascent, but once the mechanisms are better understood, they should be exploited in creative ways to provide and enhance therapeutic benefit. As studies on MDD and Parkinson's disease have demonstrated, placebos can induce beneficial, targeted, endogenous opioid and dopamine release (de la Fuente-Fernández et al., 2001, 2002; Peciña et al., 2015). The possibility of using placebos to induce therapeutic neurotransmission in other conditions with opioid or dopamine signaling deficiencies, such as multiple sclerosis (Dworsky-Fried et al., 2021) or attention-deficit/hyperactivity disorder (Spencer et al., 2007), respectively, should be explored. Placebos can also affect, often through classical conditioning, somatic physiology in various ways, including immune system suppression (Kirchof et al., 2018), immune response enhancement (Rakel et al., 2011), blood pressure reduction (Wilhelmet al., 2016), insulin secretion (Stockhorst et al., 2000), and growth hormone secretion (Benedetti et al., 2003). This raises the possibility of using conditioning to sustain the effects of pharmaceuticals while tapering the dose, which could be helpful for toxic or addictive pharmacotherapy regimens (Colloca & Barsky, 2020). Additionally, researchers should note that prior positive experiences with a therapy create expectations that enhance the placebo effect,

potentially confounding clinical trials with crossover designs by enhancing the placebo effect in groups receiving active drug first and placebo second (Colloca & Barsky, 2020). Interestingly, the size of the placebo effect appears to have increased between the years 1990 and 2013, but only in the United States (Tuttle et al., 2015); this country-specific effect could be the result of direct-to-consumer pharmaceutical advertisements, which are not allowed in Europe, increasing expectations of efficacy (Sanders et al., 2020).

Lastly, with (i) the interactions of dopamine, opioid, endocannabinoid, and possibly oxytocin and vasopressin signaling, (ii) the central role of the nucleus accumbens, (iii) the involvement of the prefrontal cortex, amygdala, anterior cingulate cortex, and insula, and (iv) the importance of empathy and reward learning, there is remarkable similarity between the neurobiological underpinnings of the placebo effect and those of pair bonding (Burkett et al., 2016; Numan & Young, 2016; Walum & Young, 2018; Borie et al., 2022b). Given these similarities, it is worth considering if pair bonding might also be capable of inducing therapeutic psychosomatic effects. It is also interesting, if not scientifically useful, to reflect on phenomenological similarities between the placebo effect and love, such as how one's experience of something so viscerally real can rapidly and radically change when new information is revealed.

4.5 Harnessing the Healing Power of Love

"Love cures people – both the ones who give it and the ones who receive it." – Dr. Karl Menninger, *Sparks* (1973)

The salubrious effects of marriage and the deleterious effects of widowhood are well documented. Married men and women over 65 have life expectancies approximately two years longer than their unmarried counterparts, and the risk of mortality nearly doubles during the first three months after

the loss of a spouse (Jia & Lubetkin, 2020). Married cancer patients are less likely to die from the disease than unmarried cancer patients, although this disparity may be due to differences in treatment regimens and diagnosis delays (Chen et al., 2021). However, the mortality risk of bereaved partners is increased for almost all causes of death, including cancer, infections, and cardiovascular disease (Elwert & Christakis, 2008). The improved health outcomes of married people are often attributed to extrinsic factors, such as emotional and financial support, as well as spousal motivation of healthy behaviors, but the causes of the detrimental "widowhood effect" are largely unknown owing to the difficulty in discriminating between the behavioral and biological consequences of bereavement in humans.

Using monogamous Peromyscus mice as a model, a recent paper by Naderi et al. (2021) probes the biological basis of these phenomena and provides evidence that pair bonding is protective against tumor growth. They show in vitro that human lung cancer grows larger when exposed to sera from mice with disrupted pair bonds compared to sera from mice with intact pair bonds. Transcriptomic analyses reveal that tumor cells grown with sera from bonded animals, compared to those grown with sera from either virgin or bond-disrupted animals, exhibit differential expression of several cancer-related genes involved in cell migration and tissue morphogenesis. In addition, tumors transplanted in vivo from bonded mice into virgin immunocompromised mice show decreased tumorigenicity compared to those transplanted from bond-disrupted mice, indicating that the effects of the pair bond status of the initial animal persist even in the absence of any continuing influence from the pair bond itself.

Collectively, these findings indicate that the social relationships of an individual may influence cancer progression through intrinsic biophysiological mechanisms rather than through behavior and extrinsic lifestyle factors alone. Although the influence of the immune system on the

brain and psychiatric conditions is clear (Miller et al., 2017), the possibility of reciprocal, psychosomatic influences is often regarded as dubious. Nevertheless, the idea that melancholy can promote the pathogenesis of cancer, which dates back to Galen in the second century AD, has persisted through the centuries (Karamanou et al., 2016). As Naderi et al. (2021) note, studies in mice show that stimulation of a dopaminergic brain reward area (ventral tegmental area) can suppress tumor growth (Ben-Shaanan et al., 2016), and stress can induce tumor metastasis via sympathetic nervous system activity (Sloan et al., 2010). The data of Naderi et al. (2021) lend additional support to the possible significance of psychosomatic influences on cancer. Specifically, they indicate a biochemical difference between the sera of bonded and bond-disrupted animals which is capable of altering gene expression in, and thus the growth of, tumors.

The crucial question is – how are extrinsic social stimuli transduced into intrinsic biological effects that influence health outcomes? Elucidating this process may reveal opportunities for pharmacological interventions to recapitulate the health benefits of social bonds. Given the results of Naderi et al. (2021), the process most likely involves three steps: (i) social information is encoded in a neural signal, (ii) the neural signal directly or indirectly induces the release of some humoral factor, (iii) the humoral factor binds to a receptor on cancer cells that induces changes in gene expression (**Figure 1**). Fortunately, the way in which the brain detects and processes social information is already well understood: it relies on specific neural networks and signaling molecules, most notably oxytocin, vasopressin (also known as antidiuretic hormone), dopamine, and corticotropin-releasing factor, which directly regulate pituitary gland activity (Walum & Young, 2018). In addition to mediating pair bond formation and disruption in the brain, oxytocin, vasopressin, and stress hormones are also secreted by the pituitary into the peripheral circulation where they influence physiological processes throughout the body (Pohl et al., 2019). Triggered

by social information processed in the brain, pituitary endocrine secretions may affect cancer cells directly, or they may work indirectly as secretagogues that affect an intermediary endocrine or immune tissue. Alternatively, social signals in the brain could influence peripheral endocrine or immune tissues via neural innervation. Irrespective of the origin, the data of Naderi et al. (2021) strongly suggest that some humoral factor is released into the bloodstream and subsequently alters transcription in cancer cells, most likely through receptor binding. We propose that subsequent studies should investigate the biological pathway leading from the perception of social stimuli to the regulation of transcription in cancer cells because each underlying mechanism is a potential entry point for identifying molecular targets for novel drug interventions.

The demonstration by Naderi et al. (2021) of biologically mediated effects of bond status on cancer progression has implications beyond its physiological mechanisms. As the authors mention, it illustrates the value of using animal models that better reflect human socioemotional dynamics than conventional mice and rats. It also highlights the importance of considering the social support and relationship health of patients as consequential lifestyle factors similar to diet, exercise, or substance use. More provocatively, the authors suggest "that cancers at widowhood represent a distinct pathological entity that may deserve targeted therapeutic strategies." This idea is supported by evidence of bond-dependent alterations in tumor cell gene expression and the fact that cancers are often defined by, and their treatments tailored to, their gene expression profiles. Transcriptomic analyses of tumors from matched cohorts of married and recently widowed cancer patients could help to determine whether a similar phenomenon occurs in humans. The authors further suggest that "targeted therapeutic strategies ... should take into consideration social interactions." Certainly, the therapeutic benefits of social interactions and bonds should be maximized. However, although powerful social bonds such as love may be easily prescribed, they

are difficult to obtain. Therefore, the healing power of love may be more easily harnessed pharmacologically by targeting the biochemical processes that underlie the results of Naderi et al. (2021).

4.6 On the Origin of the Widowhood Effect

"Death is very likely the single best invention of life. It is life's change agent. It clears out the old to make way for the new." – Steve Jobs, Stanford University Commencement Address (2005)

4.6.1 A Novel Hypothesis

The increased mortality risk following the loss of a spouse, dubbed the "widowhood effect," is well established – meta-analyses drawing on data from millions or hundreds of millions of people have confirmed its existence (Moon et al., 2011; Shor et al., 2012). Although estimates regarding its magnitude vary widely depending on study methodology, one large meta-analysis that carefully controlled for covariates found that widows and widowers had a 41% increased relative risk of death compared to married peers (Moon et al., 2011). The increase in mortality risk appears to peak at 50 – 90% in the first three months following the loss of a spouse, decreasing over the next two years and remaining slightly elevated thereafter (Schaefer & Quesenberry, 1995; Boyle et al., 2011; Moon et al., 2011; Moon et al., 2014; Ding et al., 2021). Despite much research, the causes of the widowhood effect remain unclear. One possibility is selection bias, with characteristics of a couple's shared environment imparting increased mortality risk to the same individuals who are more likely to be widowed, though the contribution of selection appears to be minimal (Boyle et al., 2011; Ding et al., 2021). Another possible cause is changes to the surviving partner's living environment, which include a loss of social and emotional support as well as living alone after potentially decades of partnership (Bowling, 1987; Sullivan & Fenelon, 2014). However, given

that a widow(er)'s mortality risk increases for virtually all causes of death and peaks soon after the partner passes (Elwert & Christakis, 2008; Brenn & Ytterstad, 2016), the primary causes of the widowhood effect are most likely biological and neurobehavioral changes arising directly from the spouse's death.

As with other psychosomatic phenomena, research on the biological effects of widowhood is limited. Knowles et al. (2019) performed the only systematic review examining the association between bereavement and immune function. Collectively, the 33 papers published on the subject strongly support a causal association between bereavement and immune dysregulation (Knowles et al., 2019). Bereavement suppresses T cell proliferation (Buckley et al., 2012b) and the antibody response to vaccination (Phillips et al., 2006) while increasing markers of systemic inflammation including neutrophils, monocytes, eosinophils, and cytokines (Buckley et al., 2012a; Schultze-Florey et al., 2012; Cohen et al., 2015). In accordance with these findings, a study examining gene expression in the lymphocytes of bereaved persons found an upregulation of genes involved in general immune system activation and a selective downregulation of genes involved in the activation and proliferation of B cells, which produce antibodies (O'Connor et al., 2014). Notably, the time course of this dysregulation parallels the time course of increased mortality risk, peaking acutely after partner loss and gradually normalizing over the subsequent months (Buckley et al., 2012a). Additionally, the duration and severity of immune system dysfunction depends on the psychological response of the bereaved, with severe and prolonged grief associated with severe and prolonged immune dysfunction (Knowles et al., 2019). Widowhood is also associated with an increased risk of cancer (Elwert & Christakis, 2008; Breen & Ytterstad, 2016; Lu et al., 2016), cardiovascular events (Elwert & Christakis, 2008; Buckley et al., 2012a; Martikainen & Valkonen, 1996), and infection (Elwert & Christakis, 2008), and higher incidences of these conditions are

associated with higher levels of grief (Chen et al., 1999; Knowles et al., 2019). Intriguingly, the physiological changes induced by bereavement appear particularly well suited to increasing mortality in prehistoric humans, given that their leading cause of death was likely infections by a wide margin, followed by cancer and cardiovascular disease (Finch, 2009).

Regarding the psychosomatic or neuro-somatic mechanisms mediating these biological consequences of bereavement, researchers universally blame stress (Powell et al., 2013; Sullivan & Fenelon, 2014; Knowles et al., 2019). While stress and cortisol are likely core mechanistic components of the widowhood effect (Irwin et al., 1988; Khanfer et al., 2011; Richardson et al., 2015), this explanation may be an oversimplification of the process that obscures its evolutionary origin. Indeed, evidence that partner loss in monogamous rodents causes analogous increases in depression-like behavior, corticotropin-releasing factor, and tumorigenicity suggests that the widowhood effect is not just a sociopsychological or cultural phenomenon unique to humans (Bosch et al., 2008; Naderi et al., 2021). Rather, these observations indicate that the widowhood effect is the human instantiation of a common biological response to a particular social stimulus (partner loss) in monogamous species. It therefore requires an evolutionary explanation, of which there are two possibilities: it is either a deleterious but unavoidable side effect of evolving the capacity for monogamous pair bonding, or it is an advantageous biological response to partner loss that often evolves in parallel with monogamous pair bonding. I propose the novel hypothesis that the increased mortality risk resulting from bereavement-induced physiological changes following partner loss evolved through natural selection by increasing inclusive fitness (e.g. kin selection, group selection).

4.6.2 Natural Selection for Altruistic Mortality

In eusocial organisms like ants, bees, and naked mole rats, natural selection acts on an individual's genes, even when the individual never reproduces, through the ways in which the individual's behavior contributes to the success of the group composed of closely related individuals (Wilson & Hölldobler, 2005). In social species like humans, natural selection continues to act on an individual's genes, even after the individual has reared offspring and can no longer reproduce, through the ways in which the individual's behavior contributes to the success of kin or the group in which its kin reside (Hamilton, 1964; Bourke, 2014). This is best illustrated by the "grandmother hypothesis," which states that human females evolved long post-reproductive lifespans due to the inclusive fitness achieved by caring for grandchildren. Chapman et al. (2019) provide compelling evidence for the grandmother hypothesis using data from 5815 children born between AD 1731 – 1890 in pre-industrial Finland. Having a living grandmother between 50 – 75 years of age increased the likelihood of a child surviving from two to five years of age by 29.5% compared to children with no grandmother. However, the most striking finding from this work is that having a grandmother older than 75 years of age was detrimental to child survival, reducing the probability of surviving from birth to two years of age by 37.1%. The authors note that in pre-industrial societies, women often switch from being net producers of resources (e.g. calories, caregiving) to net consumers as their physical and mental abilities gradually decline between 70 – 80 years of age; thus the survival detriment incurred by having an older grandmother was likely due to the grandmother competing with the child for resources like food and attention from caregivers (i.e. the child's parents)[2]. Additionally, the authors found that women's mortality risk began increasing

[2] The strength of human social bonds and associated emotions have powerful evolutionary benefits; however, in so far as they are irrationally unselfish, they can be powerfully maladaptive in certain contexts. Consequently, selective pressures must favor the dissolution of bonds in specific contexts, such as when

rapidly at age 60; by age 70 it was three times greater than at age 50, and by age 80 it was six times greater. Collectively, these data suggest that a post-reproductive human's inclusive fitness can, in certain contexts, turn negative, at which point selection pressures favor an increase in mortality. Indeed, it is not difficult to imagine how a group in which adults survived long past the point of becoming net consumers would be at a disadvantage, all else being equal, compared to a group in which adults died at the approximate age they ceased to be net producers.

Altruism in evolutionary biology was originally conceived as a behavioral phenomenon (Hamilton, 1964). However, Longo et al. (2005) make a compelling argument that, through kin or group selection, organisms can evolve to have genetically programmed lifespan restrictions, patterns of aging, and even event-triggered accelerated aging and death, which they collectively describe as "altruistic aging." The rapid increase in mortality of aging grandmothers described in Chapman et al. (2019) may be an example of altruistic aging. However, I suggest that the term "altruistic mortality" is more inclusive, better reflecting the probabilistic and potentially reversible nature of increased mortality risk from phenomena like bereavement-induced immune system dysregulation, as opposed to specifying an acceleration of the unidirectional aging process. Regardless, whether it manifests as a behavioral act or as a genetically programmed physiological process, for altruism to evolve, it must satisfy Hamilton's Rule:

$$R \times B > C$$

where C is the reproductive cost incurred by the altruistic individual, B is the reproductive benefit received by the beneficiaries, and R is the relatedness of the beneficiaries to the altruistic individual

infidelity creates a risk of raising another's offspring (resolved by bond dissolution), or feeble grandmothers begin competing with grandchildren for resources (resolved by death).

(Hamilton, 1964). Thus, assuming all individuals have the same reproductive potential[3], selection pressures favor sacrificing one's life (C = 1) if in so doing one would save more than the lives of two siblings (R = 0.5, B = 2), four half-siblings (R = 0.25, B = 4), eight cousins (R = 0.125, B = 8), and so forth (Hamilton, 1964). When rearing offspring, R x B can become large, justifying self-sacrificial parenting behaviors. Under nearly all other conditions, R x B is very small, explaining the rarity of non-parental altruism. However, if the altruist does not have the potential to reproduce, then C = 0 as there is no reproductive cost to self-sacrifice, making it relatively easy to satisfy Hamilton's Rule. This explains the altruistic behavior of nonreproductive members of eusocial bee and ant species, and may explain the short post-reproductive lifespans of most animals, as the death of an organism can increase resource availability and thereby impart an advantage (B), no matter how small, to relatives (R), no matter how distantly related.

In social species, however, a post-reproductive organism's behavior can contribute to inclusive fitness and thus drive selection for longevity, as demonstrated by the "grandmother effect" described above. Therefore, to determine if selection pressures favor a social organism's altruistic mortality, Hamilton's Rule must be modified:

$$R \times B > C + N$$

where B is the reproductive benefit conferred to others by the altruist's death, R is the relatedness of the beneficiaries to the altruist, C is the reproductive cost incurred by the altruist in the event of the altruist's death, and N is the net behavioral contribution to inclusive fitness the altruist would make if it remained alive. Grandmothers, for example, may have C = 0, but as long as N remains positive (i.e. they are net producers rather than net consumers) and R x B remains small, selection

[3] To be more accurate, both B and C should be multiplied by coefficients representing each individual's reproductive potential

pressures will favor longevity over mortality[4]. However, if N turns negative, such as when a grandmother ages to the point that she consumes more resources (e.g. food, caregiver attention) than she produces, then selection pressures will favor her mortality even if R x B = 0[5].

4.6.3 The Case of Partner Loss

From an evolutionary standpoint, the challenge of selecting for increased mortality is developing a mechanism by which the mortality increase reliably occurs only when it confers a reproductive advantage to other copies of the organism's genes; that is, when R x B > C + N. Nature is rife with examples of genetically programmed organism death, known as phenoptosis. *E. coli* initiate a death sequence when infected by a phage to prevent the phage from spreading (Skulachev, 1999). Similarly, it has been hypothesized that septic shock in animals, including humans, evolved as a response to severe infection in order to prevent the pathogen from spreading (Skulachev, 1999). Although hypotheses for the evolutionary advantages conferred by specific instances of phenoptosis are typically speculative, many center on the act increasing resource availability for offspring, kin, or groups (Longo et al., 2005). It is critical to note that events in the reproductive cycle are common triggers – elevated glucocorticoids in some species of salmon facilitate navigation to spawning grounds and cause death after spawning (Gems et al., 2021); increased steroid production causes female octopus to stop eating and begin degenerating after laying eggs (Wang et al., 2022); increases in corticosteroids cause males of the marsupial genus Antechinus to

[4] An exception is "heroic" acts where R x B is large. It has been suggested that older adults are more likely to perform altruistic self-sacrifices due to the selection pressure dynamics described in Hamilton's Rule (Humphrey, 2018). Risk of suicide, which may be a derangement of the eusocial capacity for altruistic self-sacrifice (Joiner et al., 2016), is greater in older adults (Conwell & Thompson, 2008).

[5] Isaac et al. (2021) show that robust activation of skeletal muscles causes them to act like endocrine tissue, releasing myokines that promote neurogenesis and synaptic plasticity. This activity-dependent "brain preservation" signal could be a way of promoting healthy brain function while the organism is behaviorally productive, and encouraging the organism's senescence when productivity ceases.

age rapidly through the breeding season and die shortly after mating (Tian et al., 2022); decapitation of the male praying mantis during mating facilitates ejaculation and fertilization (Brown & Barry, 2016); mothers in some species of spiders enhance their reproductive success by allowing themselves to be eaten by their newly hatched spiderlings (Kim & Roland, 2000). The loss of a partner in a monogamous pair bond is a unique social stimulus impacting the reproductive cycle (Bosch et al., 2008; Knowles et al., 2019; Pohl et al., 2019). As such, it could serve as a highly specific signal for triggering neurological, endocrinological, physiological, and behavioral changes that increase mortality. However, such a mechanism would evolve only if partner loss reliably shifted the balance of selection pressures to favor increased mortality; that is, if partner loss was likely to make $R \times B > C + N$.

When a person of reproductive age loses their monogamous partner, their reproductive potential, reflected in C, drops dramatically in the short term. In addition to losing their mating partner, the social isolation and antisocial behaviors (e.g. increased rates of accidents, violence, and substance abuse) associated with bereavement (Bowling, 1987; Martikainen & Valkonen, 1996) reduce the likelihood of reproducing with someone else. Even if the bereaved did reproduce with someone outside of a pair bond, the offspring would have lower survival probability (in a prehistoric context) due to the benefits of biparental care. While grieving, the surviving partner's behavioral productivity is likely to be reduced by an amount and for a duration roughly proportional to the severity and duration of their grief – to this day, humans still commonly take time away from work to recover from the loss of a loved one. This reduction in productive behaviors reduces their net behavioral contribution to the fitness of kin and the larger group, resulting in a large drop in N. Consequently, C + N becomes very small, perhaps even negative if the individual is so consumed with grief that they become a net consumer of resources rather than

a net producer. Thus, in the short term, partner loss is likely to make R x B > C + N, indicating that selection pressures would favor a rapid increase in mortality. Additionally, the magnitude by which R x B is greater than C + N is likely to be proportional to the severity of the grief, and so selection pressures should favor a larger increase in mortality in individuals with more severe grief. Nevertheless, the reductions in both reproductive potential and behavioral contributions to inclusive fitness are likely to be temporary; a majority of bereaved individuals move on in time, resuming productive activities and pursuing new mating opportunities, though some remain consumed by grief for prolonged periods (Knowles et al., 2019). Thus, as time passes and grief lessens, the value of C + N will grow and selection pressures will gradually shift to favor reduced mortality in most individuals. However, a subset of persons who remain bereaved will continue to meet the conditions of R x B > C + N, and so selection pressures will continue to favor their increased mortality risk[6].

4.6.4 Evaluating the "Altruistic Mortality Hypothesis"

The altruistic mortality hypothesis I propose explains the origin of the widowhood effect by stating that humans evolved for partner loss to trigger bereavement-induced physiological changes that increase mortality, thus increasing inclusive fitness. In today's parlance – the widowhood effect is a feature, not a bug. Above, I explained why it is plausible that the stimulus of partner loss could act as a trigger for increased mortality, and how the selection pressures for mortality described by R x B > C + N are likely to change in the aftermath of partner loss: (i) selection pressures begin favoring mortality immediately after the partner's death, (ii) as grief abates over time, so does

[6] This may suggest an evolutionary explanation for consoling behaviors – members of the group support and attempt to reduce the grief of the bereaved so that the bereaved is more likely to resume making contributions to inclusive fitness. Resources shared with the bereaved are an investment with a probability of profitable returns.

selection pressure for mortality, (iii) individuals who experience prolonged grief experience prolonged selection pressure for mortality, (iv) the magnitude of the selection pressure for mortality is proportional to the severity of grief. If the altruistic mortality hypothesis is correct, these theoretical changes in selection pressure for mortality should parallel the changes in mortality risk, and underlying physiological dysfunctions, that occur following partner loss in the real world. Indeed, this is precisely what the literature shows: (i) grief, mortality, as well as the antecedent immune system dysregulation and heightened risk of infection, cancer, and cardiovascular events, increase rapidly after the partner's death, (ii) mortality risk, its antecedent conditions, and grief decrease over the following two years in most individuals, (iii) a subset of individuals experience prolonged grief and prolonged increases in mortality and its antecedent conditions, (iv) more severe grief is associated with greater increases in mortality risk and its antecedent conditions (Chen et al., 1999; Elwert & Christakis, 2008; Buckley et al., 2012a; Knowles et al., 2019; Ding et al., 2021). In summary, changes in the theoretical selection pressure for mortality, the real-world mortality risk, the physiological dysfunctions, and the severity of grief parallel one another perfectly over time both in individuals who exhibit a typical recovery from bereavement, and in those who experience prolonged bereavement. Although there are few, if any, empirical data to support a causal relationship between these four phenomena, there are theoretical reasons (described above) to suspect a causal relationship, and the synchrony of changes in these phenomena suggests coincidence is a highly unlikely explanation[7].

[7] It should be noted that increased mortality risk also occurs for behavioral causes of death, including motor vehicle accidents, suicide, violence, and substance abuse (Martikainen & Valkonen, 1996). However, consistent with the philosophical assumptions described in the introduction of this chapter, these bereavement-induced neurobehavioral changes need not be distinguished from the bereavement-induced neuro-somatic changes described here.

As an evolutionary explanation, the altruistic mortality hypothesis further predicts that in common circumstances in which bereavement-induced mortality would be selected against (i.e. R x B < C + N), mortality risk will either not increase or the increase will be significantly mitigated. One such scenario is remarriage after widowhood, which increases reproductive potential (C > 0) for men and, if young enough, women. Thus, selection pressures should favor increased longevity rather than mortality in bereaved men and young women, but not post-reproductive women, who remarry. Although research on the subject is scarce, two studies show that remarriage after widowhood decreases mortality for men but not for women (Helsing et al., 1981; Berntsen & Kravdal, 2012). One study did not have an adequate sample size to examine the age-dependence of this effect in women, and the other did not stratify the data by age. However, the surprising sex difference in the effect of remarriage on mortality demonstrated in two separate studies using cohorts from different countries (the United States and Norway) supports the altruistic mortality hypothesis.

A second scenario that could test the altruistic mortality hypothesis occurs when the widowed person has young children. In this case, the offspring's survival depends on the widowed parent's continued survival and productive behavior (large N), and so selection pressures would favor longevity over mortality despite bereavement. The current prevailing hypothesis suggests that bereavement-induced increases in physiological dysfunction and mortality risk are generic consequences of severe stress rather than adaptive responses to a specific social stimulus. Thus, the prevailing hypothesis would predict that, due to the challenges of raising a child alone, recently widowed single parents might be at an increased risk of biologically mediated mortality compared to widow(er)s without children. Conversely, if widowed single parents of young children were to show reduced mortality from biological causes, that would provide exceptionally strong support

for the altruistic mortality hypothesis. Although there are many quantitative studies on the effects of widowhood on children, none have examined the effect of children on the mortality of widowed parents. To my knowledge, only two studies have approached this question. In the first, positive support from adult children of widowed older adults decreased the surviving parent's symptoms of depression, anxiety, and anger (Ha, 2010). The second was a qualitative psychological analysis of interviews with recently widowed single parents with dependent children (Anderson et al., 2022). The authors concluded that "despite an overwhelming sense of sole responsibility, dependent children provided participants a purpose to survive and move forward after loss" and provided the surviving parent a continued sense of connection with their deceased partner (Anderson et al., 2022). Although these studies suggest that dependent children could potentially buffer against the bereavement-induced mortality increases of widowhood, quantitative studies are needed to test the altruistic mortality hypothesis directly. Notably, parent-child bonds utilize much of the same neural circuitry as monogamous pair bonds (Numan & Young, 2016), which raises the possibility that a widow(er)'s children, as a social stimulus, might continue activating pair bond circuitry after partner loss, thereby mitigating the detrimental effects of widowhood.

4.6.5 A Remarkable Implication

The available data on the widowhood effect are better explained by the altruistic mortality hypothesis than any available alternative. If validated, this hypothesis would have many interesting implications, but one in particular is remarkable. There is a common assumption that, if an evolved physiological response is harmful to us today, it must have been beneficial to us in our evolutionary past. If the altruistic mortality hypothesis is correct, it would mean this assumption of evolutionary benevolence is incorrect. It would mean, contrary to intuition, that some of our physiological

processes evolved specifically to harm us and hasten our demise. It would mean our selfish genes, interpreting environmental signals with probabilistic algorithms built on 3.7 billion years of experience optimizing their own replicative success, actively adjust our life expectancies to ensure we do not outlive our ability to contribute to a prehistoric model of genetic fitness. Our genes dictate the destruction of organelles that have outlived their usefulness to cells, the destruction of cells that have outlived their usefulness to tissues, and the destruction of tissues that have outlived their usefulness to organisms – why not the destruction of organisms that have outlived their usefulness to groups (Skulachev, 1999)?

As unsettling as this implication is, it includes a compelling silver lining. If our genes can actively adjust our mortality risk and aging rate, then our health and longevity are not inherently limited by the apparent imperfections of our biology as we perceive them today. If our health is actively diminished by physiological processes rather than passively diminished by physiological flaws, biomedical science can target and inhibit those processes. Indeed, for decades we have known that deletions of specific genes can significantly extended lifespan in mice (Liang et al., 2003). Unfortunately, the incredible promise of research on ways to inhibit our genetically programmed degeneration has itself been inhibited by the government edict that "aging is not a disease" and the consequent dearth of funding for these efforts (Bulterijs et al., 2015). Perhaps as evidence mounts that certain forms of pathophysiology are in fact intentionally deleterious features of normal physiology, support for anti-aging work will return. Then, through research, we can wrest the lifespan controls from our genes' proteinaceous grip and adjust the settings to "maximum longevity."

4.7 Conclusions and Future Directions

"The only way to discover the limits of the possible is to go beyond them into the impossible."

– Arthur C. Clarke, *Report on Planet Three and Other Speculations*, 1972

Psychosomatics needs a rebranding. Moving forward, the field should aggressively assert that it is the neuroscientific study of the brain's interaction with both the environment and the body, and especially of the neurobiological mechanisms through which external stimuli influence somatic physiology; and it should dispense with evocations of immaterial psychic entities like the mind. The vast majority of the literature on the topics covered in this chapter – the placebo effect, love and marriage, and the widowhood effect – come from the social sciences. There are incredible discoveries waiting to be made by natural scientists willing to explore this neglected subject; no discipline is better disposed to do so than behavioral neuroscience, and the subdiscipline of social behavioral neuroscience best of all.

Similarly, most research on "social determinants of health" is more social science than natural science, heavily influenced by political, ideological, and moral sensibilities. It asserts itself as "hard" science but lacks mechanistic teeth and legitimate, rather than speculative, explanatory power because it ignores the neurobiological processes that transmute social and environmental stimuli into physiological consequences. I would argue that there are no social determinants of health; there are only social causes of neurobiological processes that affect physiology and thereby influence health. Research on the latter subject is limited but fascinating, and from a translational perspective, carries the prescriptive authority of natural science (see Morr et al., 2022 for a recent example). Indeed, the effect of social influences on biological physiology and health are just beginning to be appreciated, and the underlying mechanisms have barely been explored; just yesterday (September 7th), a study in *JAMA Psychiatry* found that social loneliness prior to

infection with COVID-19 increased the relative risk of developing "long COVID" by 32 percent (Wang et al., 2022). I agree emphatically with the authors: "Future work should examine the biobehavioral mechanism linking psychological distress with persistent postinfection symptoms" (Wang et al., 2022).

Of all the subjects in translational neuroscience, I am most interested in pharmacologically and technologically enhanced psychotherapy and behavioral therapy. The various forms of psychotherapy are optimized psychosomatic techniques intended to rewire the brain, and the various forms of behavioral therapy are optimized "somato-neural" techniques for rewiring the brain. Although still nascent, I suspect that utilizing technologies like virtual reality, transcranial magnetic stimulation, and transcranial direct current stimulation to enhance the intensity and circuit-specificity of psychotherapy and behavioral therapy will gain traction as emerging treatment paradigms in the future (for review, see Sathappan et al., 2019; Wilkinson, 2019; Spagnolo et al., 2020). Excluding the shamanistic rituals that have been performed for thousands of years, the first, to my knowledge, published report of pharmacologically enhanced therapy came from Emory's own Drs. Kerry Ressler, Barbara Rothbaum, Michael Davis, and colleagues (Ressler et al., 2004). Today, MDMA-assisted therapy for PTSD and psilocybin-assisted therapy for depression are being developed collaboratively between psychiatry and clinical psychology, and they are two of the most promising new therapies in mental health. The efficacy of the former appears to be mediated by activation-dependent rewiring of amygdala-hippocampal connectivity caused by the synergistic effects of MDMA and psychotherapy, both of which independently increase functional connectivity between the amygdala and hippocampus (Singleton et al., 2022). Two recent clinical trials of psilocybin implicate global increases in brain network integration driven by 5-HT2A receptor activation as the mechanism underlying its efficacy in treatment-

resistant depression (Daws et al., 2022). This knowledge creates an opportunity for psychotherapies to be tailored to enhancing this effect. Ketamine has proven a powerful new treatment for depression as well, although few studies have attempted to combine it with psychotherapy (for review, see Joneborg et al., 2022). However, one recent study found preliminary evidence that cognitive behavioral therapy (CBT) might prolong the antidepressant effects of ketamine (Wilkinson et al., 2021). The authors also cite evidence that both CBT and ketamine independently have similar effects on cognitive control processes, and they hypothesize "that ketamine may provide an opportune window during which cognitive and behavioral interventions may be used to harness a state of enhanced neuroplasticity" (Wilkinson et al., 2021).

These studies on MDMA-, psilocybin-, and ketamine-assisted psychotherapies and behavioral therapies are precisely the sort of multi-modal, circuit-targeted interventions Dr. Larry Young and I advocate in Ford & Young, 2021a. The remarkable success of these interventions in recent years makes it likely that more expansive research on pharmacologically enhanced therapies will occur in the near future. Given the initial success of Le et al. (2022) in pairing intranasal oxytocin with positive social interactions, and given the ability of Melanotan II to accelerate social learning (Modi et al., 2015) and increase oxytocin-dependent NAc activity in a social context (Chapter 3), perhaps Bremelanotide-assisted Applied Behavior Analysis therapy for social deficits in autism will be next.

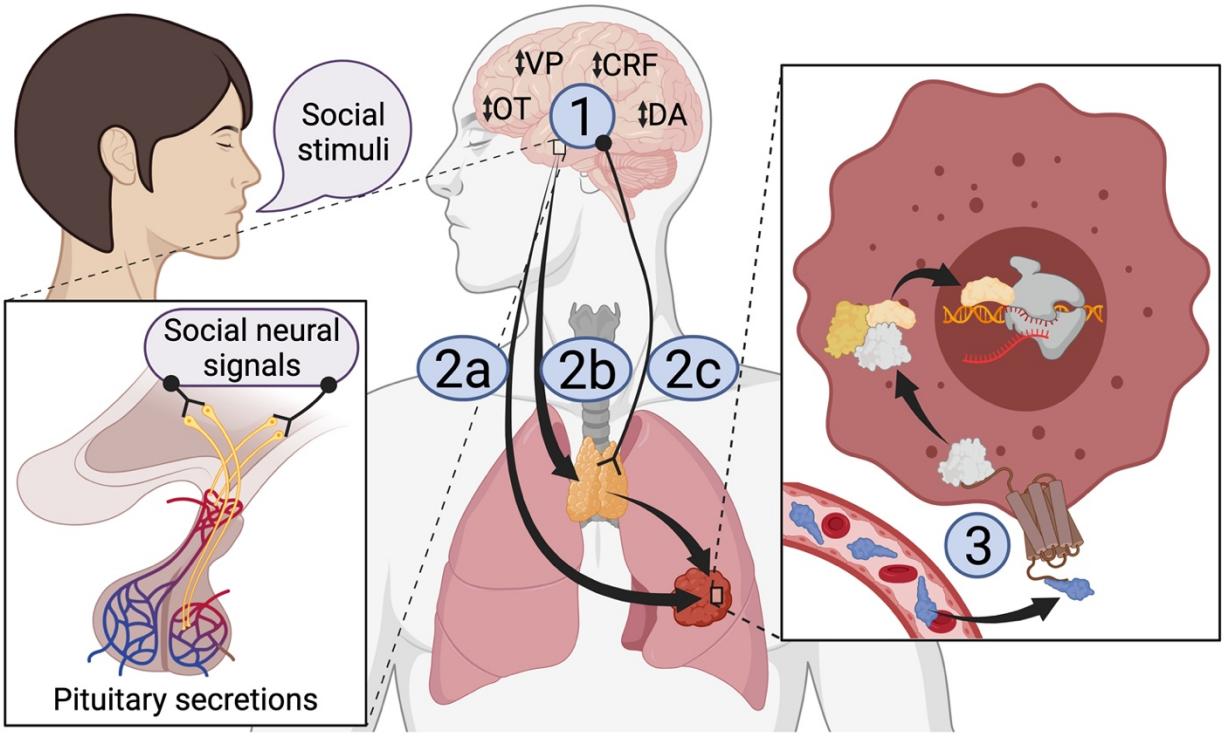


Figure 1. Proposed model for the transduction of social stimuli into a physiological signal that influences cancer progression. Social stimuli are processed by neural networks utilizing oxytocin (OT), vasopressin (VP), corticotropin-releasing factor (CRF), and dopamine (DA), which also regulate pituitary secretions into the circulation (1). These secretions may act as humoral factors that directly impact the tumor (2a) or as secretagogues that trigger the release of a humoral factor from other tissues (2b). Alternatively, neural innervation of intermediary tissues could trigger the release of a humoral factor (2c). The humoral factor binds to receptors on tumor cells, thereby altering tumor physiology and progression (3).

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