Characterization of the Gene Transcriptional Response in Mouse Mesocorticolimbic Circuitry Following Adolescent Social Isolation

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Abstract

Adolescent social isolation is a developmental stressor that increases vulnerability to neuropsychiatric disorders including substance use disorder and eating disorders. The lasting consequences of isolation are thought to arise from disrupted experience-dependent maturation of mesocorticolimbic circuits during adolescent sensitive periods. My work found that adolescent isolation (P22–42) sex-specifically disrupts transcriptional coordination between the medial amygdala and ventral tegmental area without affecting physical connectivity or baseline dopamine neuron firing (Chapter 2) and fundamentally reorganizes prefrontal cortex maturation and reward-related behaviors in adulthood (Chapter 3).

At the molecular level, adolescent isolation induced opposing sex-specific effects on glutamatergic gene expression (elevated in females, reduced in males) and selectively increased dopamine receptor expression in males across the medial amygdala and ventral tegmental area. Using rank-rank hypergeometric overlap analysis, we determined that isolation dysregulates drug- and stress-responsive transcriptional profiles, with females showing enhanced PFC-NAc concordance but loss of PFC-VTA coordination following cocaine exposure (Chapter 3).

We examined the behavioral and hormonal correlates of these neural changes by assessing palatable food consumption and serum hormone profiles. Isolated males showed increased palatable food intake while females maintained typical consumption patterns. Critically, isolation altered thyroid hormone (T3/T4) ratios in males and reduced corticosterone in females, suggesting developmental reprogramming rather than stress-mediated effects (Chapter 3).

Together, this dissertation presents novel findings on how adolescent social isolation disrupts mesocorticolimbic maturation through deprivation of expected social inputs during hormone-primed sensitive periods (Chapters 2-3). Further research on how these sex-specific

transcriptional and molecular changes translate to vulnerability for substance use and eating disorders may inform interventions during critical developmental windows.

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Author Contributions & Acknowledgements

Chapter 1 was written by me, with Drs. Matt Lattal and Deena Walker editing and providing guidance. Chapters 2 and 3 were based on experiments designed by Dr. Deena Walker and me. Dr. Barbara Juarez was responsible for the electrophysiology work in Chapter 2. Data collected at OHSU were in large part from experiments performed by Dr. Natasha Fowler, Theresa Vu, Mandee Bell, Cari Bendersky, and Alison Milian; data analysis for these experiments was collaborative, with Dr. Deena Walker, Theresa Vu and me analyzing the datasets. I was responsible for the interpretation of the data collected at OHSU and the findings from the RNA sequencing data reanalysis from Dr. Deena Walker's postdoctoral work, which was guided by Drs. Matt Lattal and Deena Walker.

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This work is dedicated to Bill and Georgeanne Andrus.

Chapter 1: General Introduction

Adolescence is the transition from childhood to adulthood. The timecourse of this transition has been demarcated differently across human cultures according to the observations, expectations, and roles of those within it. But what underlies this transition is consistent: puberty, the developmental period when sexual maturation occurs. The process of sexual maturation spans vertebrate clades. As an organism develops, orientation toward the behaviors necessary for survival and reproduction must also develop. The conservation of fundamental mechanisms behind the processes of sexual maturation and behavioral re-orientation enables translational study of features of psychopathology that may develop during adolescence (Spear, 2013).

In mammals, adolescence is a period of development that affects brain systems responsible for navigating social contexts to facilitate survival and reproduction. These regions undergo dramatic biological and functional reorganization, principally in the form of changes in dopamine (DA) dynamics, excitatory and inhibitory signaling, and synaptic connectivity. Because these neural systems mediate a broad range of behaviors and neural processes, perturbations during this period can induce wide-ranging behavioral pathologies, including but not limited to substance use disorders (SUDs) and binge-eating disorders (BEDs) (Culbert et al., 2013; Paus et al., 2008; Wills, 1986).

This introduction establishes three main points. The first is that adolescence is a hormone-driven sensitive period of dramatic neural reorganization, particularly in mesocorticolimbic reward circuits. Subcortical regions that mediate reward-related learning and behavior mature rapidly while maturation of the prefrontal cortex (PFC), which exerts top-down control of these regions, lags; this creates a developmental window of imbalance in motivation, leading to an enhancement in risk-taking behaviors (Walker et al., 2017).

The second point is that the establishment of healthy neuropsychiatric phenotypes is experience-dependent: peer interactions during this window are essential for healthy maturation, and lacking or experiencing adverse peer interactions disrupts normative development (Andersen & Teicher, 2008; Loades et al., 2020; Paus et al., 2008; Thapar et al., 2012). This indicates that adolescent social experiences persistently impinge on how the brain circuits process stress and reward.

And third, this experience-dependence means that the biological foundations of neuropsychiatric disorders can be modeled in rodents by manipulating social environment during adolescence. Adolescent social isolation is a well-established preclinical model for neuropsychiatric risk (Butler et al., 2016; Mumtaz et al., 2018; Walker et al., 2019). Examining the brain changes that occur during, and because of, adolescent isolation can reveal how disrupted social development creates risk for the development of psychiatric disease.

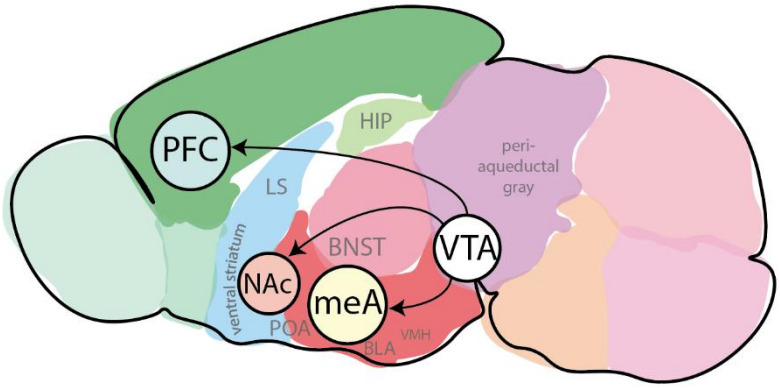
The neural substrate of social behavior and reward processing

To appropriately contextualize how social isolation leads to lasting behavioral and cognitive consequences, it is important to first discuss the shared evolutionary rationale and biological foundations of adolescence.

In social organisms, wide swaths of behavioral competency are necessary for survival and reproduction (Taborsky & Oliveira, 2012). Navigation of social milieu may necessitate conflict with conspecifics for access to resources and mates (among innumerable other reasons) or may involve more cooperative behaviors such as grooming, playing, and beyond. Alongside courtship and copulation with mates, these behaviors all require sophisticated cognitive processing to be successful.

The brain regions mediating social cognition and behavior are widespread and evolutionarily conserved. The neural basis of the processing of environmental and social stimuli has been called the social decision-making network (SDN). The SDN is an integrated network

composed of two canonically identified networks, the social behavior network (SBN) and mesolimbic reward system (O'Connell & Hofmann, 2011). Classically (though not without contention, see (Kelly, 2022), these networks are integrate input from the brain's primary sensory modules to 'label' the transduced environmental input from primary sensory regions with behavioral relevance. The SBN is composed of the bed nucleus of the stria terminalis (BNST), medial amygdala (meA), lateral septum (LS), periaqueductal gray/central gray (PAG/CG), preoptic area (POA), and anterior and ventromedial aspects of the hypothalamus (AH, VMH; (Newman, 1999). The mesolimbic reward system, in addition to sharing the LS, BNST, and amygdala, is comprised of the striatum (Str), nucleus accumbens (NAc), basolateral amygdala (BLA), hippocampus (Hip), and ventral tegmental area (VTA), all of which are orthologous across each major vertebrate species and serve similar general functions (O'Connell & Hofmann, 2011). And finally, in mammals, the prefrontal cortex (PFC) is a key hub for top-down control and integration of these functions in regulation of social- and reward-related behaviors within both the SBN and mesolimbic reward system. The PFC is listed separately because its homolog, if existent, across other vertebrates is contended (O'Connell & Hofmann, 2011). These interconnected regions undergo coordinated changes during adolescence, strongly influenced by the hormonal cascades of puberty.



Circuit diagram of the social decision-making network. Bed nucleus of the stria terminalis (BNST), medial amygdala (meA), lateral septum (LS), periaqueductal gray/central gray (PAG), preoptic area (POA), ventromedial the hypothalamus (VMH; anterior not shown). Striatum (Str), nucleus accumbens (NAc), basolateral amygdala (BLA), hippocampus (Hip), and ventral tegmental area (VTA). Arrows indicate dopaminergic projections.

The work contained within this dissertation will focus on four of these regions: meA and VTA (Chapter 2), the PFC (Chapter 3), and the NAc (Chapter 2 & 3).

Hormonal foundations of pubertal brain changes

To facilitate independence in adulthood, brain regions within the SDN undergo dramatic changes during adolescence; the relative timing of this is approximately evolutionarily conserved (Spear, 2013). This process is driven by gene transcriptional reorganization throughout the SDN thought to be initiated by hormonal actions under central nervous system control which serve to shape the SDN through engagement in and refinement of social behaviors such as play (Ahmed et al., 2008; Brinkmann, 1994; Li et al., 2024; Nugent et al., 2011; Schulz & Sisk, 2006).

Synaptic change throughout adolescence

Sensitive periods are developmental phases when neural circuits are primed to be shaped by experience, allowing experience to mould neural architecture in ways that have lasting – but not irreversible – effects on behavior and cognition. Fundamentally, these periods involve extensive synaptic remodeling through experience-dependent mechanisms including synapse formation, elimination, and consolidation, where repeated neural activity strengthens specific connections while unused synapses are pruned (Knudsen, 2004). This process is centered around long-term potentiation (LTP) of neuronal activity, where “cells that fire together wire together,” so that connections between neuron pairs that are active at the same time are strengthened, making them more likely to activate together in the future. Its converse is long-term depression (LTD), a process where the probability of a pair of cells firing together decreases. This is understood to be the fundamental mechanism behind the formation and augmentation of memory engrams, the physical instantiation of memory in neural tissue (Josselyn & Tonegawa, 2020).

Adolescence is a sensitive period, meaning it is a biological window within which the effects of experience on the brain and later behavior are uniquely persistent (Fuhrmann et al.,

2015). This is evidenced by substantially greater rates of adolescent neurite outgrowth and elimination than in mature adult brains (Gan et al., 2003; Zuo et al., 2005). A mix of both expansion and reduction, the adolescent brain prunes initially-overproduced synaptic connections and increases in myelination in response to social and other experiences (Spear, 2013).

While synaptic pruning shapes the structure of adolescent neural circuits, changes in neuromodulatory systems, particularly dopamine, fundamentally alter how these circuits process information and drive behavior.

Changes in dopamine dynamics during adolescence increase risk and are associated with peer affiliation

Coupled with these synaptic changes, motivational-emotional circuits drastically alter course in adolescence: dopamine, the primary motivation and learning neuromodulator, becomes heightened in its activity in the adolescent brain (Wahlstrom et al., 2010; Walker et al., 2017).

Briefly, dopamine functions as a neuromodulator through two families of G-protein coupled receptors (GPCRs): D1-like (D1 and D5) and D2-like (D2, D3, and D4). Unlike ionotropic receptors that directly gate ion channels for rapid neurotransmission, dopamine receptors operate through slower, second-messenger cascades. D1-like receptors couple to Gαs/olf proteins to stimulate adenylyl cyclase, while D2-like receptors couple to Gαi/o proteins to inhibit it (Tritsch & Sabatini, 2012). Adenylyl cyclase catalyzes ATP conversion to cyclic AMP (cAMP), which primarily activates protein kinase A (PKA); PKA then phosphorylates various targets that modulate ion channel conductances and neuronal excitability (Trantham-Davidson et al., 2004). However, dopamine's effects on target neurons are remarkably diverse and context-dependent, varying with receptor expression, cell type, concurrent neuromodulator

activity, and the target cell's electrical and biochemical state (Sippy & Tritsch, 2023; Tritsch & Sabatini, 2012). Dopaminergic modulation contributes to synaptic plasticity, with D1 signaling often facilitating LTP and D2 signaling associated with LTD (Sheynikhovich et al., 2013), though these effects depend on the specific brain region, timing, and cellular context.

Accordingly, during adolescence, there is an enhancement in the salience of rewarding and stressful stimuli, which is attributed at least in part to heightened mesolimbic activity (Ernst et al., 2005; Galvan et al., 2006). What this means in psychological terms, from data in humans, is that adolescence is a period of heightened emotionality and risk (Casey et al., 2008). Learning is strongly influenced by emotional strength, both in general and throughout adolescence (Emanuel & Eldar, 2023). The behavioral sum of these changes (and more that are beyond the scope of this work) is a shift in motivation toward interactions with peers and an enhancement in risky behaviors.

Adolescent changes in social reward

The adolescent shift toward peer affiliation is a robust, cross-species phenomenon among social mammals. An eight-year longitudinal study in humans shows that time spent with peers increases from mid-childhood through late adolescence, with time affiliating with same-sex peers peaking in mid-adolescence and cross-sex interactions increasing differentially by gender and age (Lam et al., 2014). Analyzing human social networks, one group found that age significantly predicted greater relative closeness to peers over parents, with the majority of participants having closer relationships with friends than caregivers by mid-adolescence (Morningstar et al., 2019).

This shift toward peers is also present in rodents. Adolescent mice and rats will, in conditioned place preference (CPP) paradigms, a Pavlovian conditioning task where reward motivation is inferred by examining time spent in a compartment previously paired with rewarding or aversive stimuli, form stronger preference for social CPP than adults; and in social-

reward operant tasks, adolescents lever press more than adults for social rewards (Douglas et al., 2004; J. B. Panksepp & Lahvis, 2007; Ramsey et al., 2022). The augmentation of social behavior in rats has been identified with sex-specific dopamine receptor dynamics (Kopec et al., 2018). In humans, peer-reward is mediated differently in adolescents versus adults. One study by Chein et al. (2011) demonstrates the relationship between peers, reward, and risk-taking behavior in a driving task with or without peer observation. In this study, adolescents, but not adults, exhibited greater activation in reward-related brain regions (in the VS and orbitofrontal cortex) when observed by peers during the task, and this heightened reward sensitivity predicted subsequent risk-taking behavior in the task (Chein et al., 2011). The motivational shift toward peer-reward and risky behaviors facilitates the acquisition of social status and competency as a non-dependent adult, and because increasing exploratory behavior and novelty-seeking can decrease the likelihood of inbreeding (Ellis et al., 2012).

Imbalance in adolescent mesocorticolimbic development drives risk-taking

The mesolimbic dopamine system, projecting from VTA to NAc, amygdala, and hippocampus, mediates incentive salience, which is the attribution of motivational value to stimuli (Berridge & Robinson, 1998; Björklund & Dunnett, 2007). During adolescence, this system's heightened reactivity appears to confer exaggerated motivational significance to social and novel cues independent of their rewarding outcomes, preceding the maturation of prefrontal regulatory control (Wahlstrom et al., 2010).

Riskier behavior during adolescence is mediated by enhanced motivational drive by DAergic innervation of limbic circuitry. Dopamine is essential for what Edward Thorndike (1911) termed “stamping in” stimulus-reward associations, a process whereby responses followed by rewarding outcomes increase in their probability of recurrence. Through this mechanism, dopamine contributes to the labeling of neutral environmental stimuli as motivationally relevant cues that elicit approach and seeking behaviors (Wise, 2004; Wise & Jordan, 2021). During

adolescence, evidence suggests that enhanced dopaminergic innervation of limbic circuitry facilitates the acquisition and consolidation of behavioral repertoires, particularly social behaviors; this heightened mesolimbic dopaminergic activity, occurring before the maturation of mesocortical regulatory regions, is associated with increased risk-taking and enhanced motivation for peer interactions that facilitate the stamping in of adult social competencies (Walker et al., 2017). According to evolutionary developmental theories, this evolutionarily conserved imbalance may promote adaptive risk-taking during the transition to adult independence (Ellis et al., 2012; Walker et al., 2017).

Substantial enhancements in mesolimbic dopamine system activity emerge during puberty. In the NAc, dopamine receptor density peaks at postnatal day (P) 40 in rodents, with this time-dependency being more pronounced for D1 than D2, while DA signaling undergoes dramatic reorganization, creating heightened sensitivity to rewarding stimuli, particularly social rewards (Andersen et al., 2000; Kopec et al., 2018; Teicher et al., 1995). The VTA shows enhanced phasic firing to both social and drug rewards during adolescence compared to adulthood (McCutcheon et al., 2012). Meanwhile, the meA undergoes hormone-driven reorganization during adolescence (De Lorme et al., 2012), with its dense populations of steroid-hormone-receptor-expressing neurons serving as a primary site for hormonal modulation of social and reproductive behaviors (Bergan et al., 2014). Through its connections with regions like the mPOA, sensory input via the meA triggers dopamine release in key projection targets, suggesting these pathways undergo experience-dependent maturation during the adolescent period when dopamine systems are being reorganized (Dominguez et al., 2001). This hormonal sensitivity positions the meA as a critical nexus between endocrine maturation and behavioral output, where testosterone and estradiol binding directly modulates neural responses to social stimuli, effectively 'gating' which social inputs trigger downstream dopaminergic reward signals. Though not definitively established, it is held that these mesolimbic changes create a

motivational drive toward novelty, risk, and – because of sex-hormone-induced neuropeptidergic changes – social rewards.

Maturation of the PFC during adolescence

The mPFC receives low dopaminergic innervation with fiber density continuing to increase linearly through adolescence into adulthood (Naneix et al., 2012; Willing et al., 2017). This is consequential because dopamine signaling in the PFC is crucial for the regulation of limbic regions through direct projections to the NAc, amygdala, and VTA in adulthood (Gabbott et al., 2005; Vertes, 2004, 2006). Without mature dopaminergic tone, the adolescent PFC exhibits reduced activation during tasks requiring inhibitory control and shows weaker functional connectivity with limbic regions compared to adults (Casey et al., 2008; Hwang et al., 2010). This delayed dopaminergic maturation is compounded by the PFC's protracted development of inhibitory neurotransmission, a sex-hormone-dependent phenomenon (Piekarski et al., 2017).

As mentioned previously, this creates a functional imbalance where hyperresponsive subcortical regions operate with insufficient cortical oversight, manifesting as heightened emotional reactivity, increased reward sensitivity, and diminished impulse control characteristic of adolescent behavior. The interplay between hormonal signaling, synaptic plasticity, and experience-dependent maturation during adolescence raises a question central to the work in this dissertation: what happens to these systems when the expected social experiences that these adolescent changes are taking place to prepare for are absent?

This question is increasingly pressing, as rates of loneliness among young adults have increased every year between 1976 and 2019 (Buecker & Horstmann, 2022), a phenomenon that appears to be only getting worse and is tightly associated with the spread of smartphone ownership and social media use (Matthews et al., 2023). Importantly, loneliness is associated with a suite of adverse mental health consequences, including SUD, depression, and anxiety (Loades et al., 2020; Paus et al., 2008; Thapar et al., 2012; Wills, 1986).

Adolescent social isolation as a model for neuropsychiatric disorders

Social isolation in rodents during adolescence serves as a paradigm for examining how the absence of species-typical social input disrupts brain development, informing both the mechanisms of normal development and the pathways to psychopathology (Butler et al., 2016; Mumtaz et al., 2018; Walker et al., 2019). Though isolation protocols differ between studies, across paradigms, the timing of isolation coincides with the beginning of adolescence (Walker et al., 2019). Some paradigms maintain social isolation into adulthood, preventing rehousing

Adolescent isolation induces social deficits

In rodents, adolescent social isolation (SI) induces sex-specific effects on a wide range of behaviors, though much of the literature has exclusively studied its influence on males. SI is associated with sociocognitive and behavioral deficits, ranging from impaired social recognition (Kercmar et al., 2011), enhanced aggression in males (Takahashi, 2025), and sexual deficits. Both sexes display incompetent copulatory behaviors following adolescent isolation (Cooke et al., 2000; De Lorme et al., 2019; Kercmar et al., 2014; Marquardt et al., 2022).

The behavioral consequences of preventing these formative social experiences are wide-ranging, affecting multiple domains of adult social competency. To understand the core of dysregulation, we examine the effects of SI on brain regions critical to species-typical social behaviors. The mPFC and meA are critical in both sexual and aggressive behaviors. The posterodorsal meA contains dense populations of steroid receptor-expressing neurons and serves as the primary site for hormonal modulation of reproductive behaviors (Bergan et al., 2014). The mPFC and meA are reciprocally connected, though (as in any behavior) this complex behavior is additionally mediated by secondary regional influence by way of the BNST and medial POA (mPOA; Knapska et al., 2007; Yamaguchi et al., 2020). Sensory input via the meA triggers mPOA dopamine release, suggesting this pathway may require sociosensory

experience to develop properly, experience that SI prevents during the sensitive period of adolescence, when dopamine systems are being reorganized (Dominguez et al., 2001).

Though steroid hormones are necessary for the development of adult-typical behaviors, they are not sufficient to initiate these behaviors, even if administered prior to puberty; this suggests that though hormones are necessary for the expression of age-appropriate behaviors once at the appropriate age, they are not sufficient on their own to affect behavioral maturation, which seems to require other processes (Schulz & Sisk, 2006). Beyond disrupting species-typical social behaviors, adolescent SI profoundly alters reward processing more broadly, as evidenced by enhanced sensitivity to drugs of abuse.

Adolescent isolation augments drug-associated behaviors

The work in this dissertation explores how adolescent isolation augments drug responding. Literature in rodents shows that adolescent isolation induces behavior associated with features of SUD. For example, SI increases drug self-administration for cocaine and methamphetamine in both sexes, and morphine in males (Fosnocht et al., 2019; Marks-Kaufman & Lewis, 1984; Yajie Ding et al., 2005). Rodents isolated through adolescence also show differences in associative drug learning, with SI in males enhancing preference for cocaine, methamphetamine, and ethanol and decreasing preference for morphine, and though data are limited, SI in females decreases preference for cocaine (Grotewold et al., 2014; Walker et al., 2020; Whitaker et al., 2013; Wongwitdecha & Marsden, 1996; Zakharova et al., 2009). These SI-induced changes in drug-associated behaviors make adolescent isolation a fertile preclinical model for characterizing the developmental dysregulations that lead to vulnerability to SUD.

Despite growing understanding of adolescent brain development and social isolation's effects, key questions remain about the mechanisms underlying these changes. The medial amygdala, while known to be sensitive to adolescent social experience, is understudied in the

context of reward processing compared to canonical mesocorticolimbic regions. Additionally, how adolescent social experiences persistently alter coordination between brain regions – rather than affecting individual regions in isolation – has received limited investigation. The biological mechanisms responsible for the observed sex differences following adolescent isolation studies remain unclear, as does whether these effects result from stress-mediated pathways or from altered developmental processes during adolescent sensitive periods.

This dissertation addresses these questions through complementary studies examining how adolescent social isolation affects mesocorticolimbic development and function. Chapter 2 demonstrates that isolation disrupts transcriptional coordination between the medial amygdala and ventral tegmental area without affecting interregional connectivity or baseline dopamine neuron firing rates. Using rank-rank hypergeometric overlap analysis of RNA-sequencing data, we show that isolation sex-specifically alters how these regions coordinate their transcriptional responses to cocaine and stress, accompanied by opposing effects on glutamatergic gene expression in males versus females and elevated dopamine receptor expression in isolated males. Chapter 3 explores how isolation reorganizes transcriptional regulation across prefrontal and subcortical networks, producing sex-specific changes in natural reward consummatory behavior and stress- and drug-responsive gene expression. Assessment of mid-isolation hormone profiles indicates that, rather than elevating corticosterone levels, corticosterone levels are reduced females and unaffected in males, suggesting that adolescent isolation is not inducing lasting behavioral and neurobiological change through stress-mediated pathways but through inadequate exposure to developmentally appropriate stimuli, supported by altered thyroid hormone ratios in males. Furthermore, findings in Chapter 2 using RT-qPCR indicate that adolescent isolation induced persistent disruptions in PFC neurotransmitter system maturation, with isolation preventing normal developmental transitions in dopaminergic, glutamatergic, and GABAergic gene expression that likely impinge on circuit function. This work indicates that adolescent social isolation disrupts the normal maturation of mesocorticolimbic

circuits, inducing circuit-wide dysregulation of gene transcription both across development and in adulthood. These disruptions in gene transcription may potentially manifest in the aberrant behavioral and cognitive phenotype associated with a history of adolescent isolation.

Chapter 2: Adolescent Isolation Disrupts Medial Amygdala and Ventral Tegmental Area Maturation and Sex-Specifically Dysregulates Transcriptional Responses to Cocaine and Stress

This chapter is adapted from the following publication:

Andrus, M. D., Vu, T., Juarez, B., & Walker, D. M. (2025). Adolescent isolation disrupts medial amygdala and ventral tegmental area maturation and sex-specifically dysregulates transcriptional responses to cocaine and stress. Neuropharmacology, 279, 110629. https://doi.org/10.1016/j.neuropharm.2025.110629

In rodents, post-weaning social isolation has long been used preclinically as a stressor that alters reward-, anxiety-, and depression-related behaviors (Bendersky et al., 2021; Burke et al., 2017; Walker et al., 2019). In this model, animals are isolated from weaning throughout the rest of their lives, making it difficult to distinguish between the adolescent-specific effects of social isolation/deprivation from chronic isolation effects. This is critical given the enormous developmental processes that occur during the adolescent period (Walker et al., 2017). To address this, the Walker lab and others have used an approach which restricts isolation specifically to the adolescent period, followed by social rehousing (Baarendse et al., 2014; Kinley et al., 2021; Walker et al., 2022b; Whitaker et al., 2013), to show that isolation specifically during adolescence impacts both drug reward and anxiety/depression-related behaviors (Einon & Morgan, 1977; Lukkes et al., 2009; Walker et al., 2019; Weintraub et al., 2010; Wright et al., 1991). With regards to reward, Walker et al. (2022a & b) found that adolescence-specific isolation, from P22–P42, persistently increases the preference for cocaine in adult males but not females and is associated with sex-specific alterations in cocaine-induced transcriptional profiles throughout the reward circuitry. Others have found that this same paradigm enhances acquisition of self-administration of and motivation for cocaine (Baarendse et al., 2014). Together, these data suggest that isolation, constrained to the adolescent period, results in sex-

and region-specific effects on the behavioral and transcriptional response to cocaine in adult animals.

One understudied region that is sensitive to the effects of adolescent isolation is the medial amygdala (meA). The meA, critical for social behaviors (Bergan et al., 2014; Li et al., 2017), undergoes sex-specific developmental changes during adolescence that are driven at least in part by pubertal hormones (Cooke, 2011; Cooke & Woolley, 2005; De Lorme et al., 2012), and it is dysregulated by post-weaning social isolation. The meA has recently emerged as an important sex-specific center for the effects of adolescent isolation on SUD vulnerability (Walker et al. 2022b). Although the meA is not considered a part of canonical mesocorticolimbic reward circuitry, it has a vast array of connections with reward circuitry, including the PFC, basolateral amygdala, and subiculum (Knapska et al., 2007), and sex-distinct reciprocal connections with the ventral tegmental area (VTA), the origin of mesocorticolimbic dopamine signaling (Cádiz-Moretti et al., 2016; Derdeyn et al., 2022; Pardo-Bellver et al., 2012). Females and males show differences in the meA transcriptional response to cocaine, including in the expression of c-Fos, an activity-dependent transcription factor, an effect that is sensitive to adolescent isolation (Walker et al., 2022b). Therefore, the meA may be a critical hub for the persistent effects of adolescent isolation on reward circuitry.

To better understand the meA's role in mediating the persistent effects of isolation on sex-specific cocaine-induced transcription, we first analyzed a publicly available RNA-sequencing dataset from animals exposed to adolescent isolation and treated with different dosing paradigms of cocaine to determine if cocaine-responsive transcriptional profiles in the meA overlap with those in canonical regions of mesocorticolimbic reward circuitry. We next identified region- and stimulus-dependent differences in transcriptional profiles that may explain the sex-specific effects of isolation on transcriptional concordance and discordance between the meA and VTA. Finally, because the meA is composed of both GABA- and glutamatergic projection neurons, we used a candidate gene approach using qPCR to determine if maturation of cell-

type specific markers is disrupted by adolescent SI. We found that sex-specific similarities in stimulus dependent transcriptional profiles between the meA and VTA are disrupted by adolescent isolation and that maturational profiles of glutamatergic markers in the meA are associated with these adult-specific effects of adolescent isolation. Our results suggest that the meA undergoes isolation-induced developmental disruptions, influencing gene transcription programs in the adult VTA. Together our findings indicate an integral role of meA–VTA in mediating the lasting effects of adolescent social isolation, and position it as a key component of mesocorticolimbic reward circuitry.

Materials and Methods

All animal protocols were conducted in accordance with the ARRIVE guidelines and the *Guide for the Care and Use of Laboratory Animals* and approved by Oregon Health & Science University and Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee. All animals were housed in a humidity and temperature-controlled space (21–22°C) that was set on a 12-hour light/dark cycle. (Lights were set to turn on at 06:00 and off at 18:00.) Food and water were available *ad libitum*.

For animals used in sequencing, tracing, and electrophysiology experiments, C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and shipped to Icahn School of Medicine when they were exactly postnatal day (P)20, arriving in the animal facility on P21. The animals were isolated from P22–P42 then rehoused with their original cage mates until adulthood (Fig. 1a; detailed in Walker 2022a & b). Sequencing data from these animals, given ten injections of experimenter-administered vehicle or cocaine (7.5mg/kg), have been published and made publicly available on the Gene Expression Omnibus (GSE146472). For animals used in electrophysiology experiments, 1 adult animal per day was used for recordings. All females were in diestrus on the day of recording to match the sequencing results (Walker 2022a & b).

For qPCR experiments conducted at Oregon Health & Science University, C57BL/6J mice were purchased from Jackson Laboratory (Sacramento, CA) for in-house breeding. After acclimating to the facility, animals were bred and pregnancies monitored with the day of birth recorded at P0. To control for any differences in maternal behavior, animals were weaned into mixed litter groups between postnatal day P20 and P24 into a clean standard acrylic transparent cage with 4–5 animals per cage. The following day, mice were individually housed in a new cage. We ensured that one male and female sibling per litter was isolated and one male and female sibling was group housed at each age to further control for maternal behavior. This resulted in age ranges for the following timepoints during the treatment period: 10 days post-Isolation = P31–34 and adult = P72–75 which were rehoused between P41–P44. In the 10 days following weaning, animals were monitored daily and provided soft food to ensure nutrition intake and prevent dehydration. To control for any effects that may be driven by the timing of puberty, equal distributions of pre- and post-pubertal animals were included in each experimental group (group-housed control, “Ctrl” vs. socially isolated, “Iso”). In addition to these developmental variables, we counterbalanced for cohort, light cycle, body weight, time of euthanasia, and order of experimental groups euthanized. All mice were housed in the same colony room; thus, isolated mice could hear and smell and see other mice. Body weights were monitored every 10 days throughout the life cycle. After 3 weeks of isolation (~P42), isolated mice were group-housed (4–5 animals per cage) with their socially isolated peers and original cage mates from the day of weaning for the remainder of the experiment.

Experimenter administered intraperitoneal cocaine injections

Mice in Walker et al. (2022a & b) underwent either adolescent social isolation or group housing, then as adults received a series of 10 daily intraperitoneal (IP) injections. All animals received 9 injections of saline over 9 consecutive days. On day 10, animals received either: (1) a 10th

saline injection and were euthanized 1 hour later (“acute saline”), (2) their first cocaine injection and were euthanized 1 hour later (“acute cocaine”), or (3) a 10th saline injection and were euthanized 24 hours later (“habituation control”). The habituation control group serves as a baseline for quiescent gene expression, as transcriptional responses to the injection stress have subsided by 24 hours. This design allows us to distinguish between stress-responsive (acute saline vs. habituation) and cocaine-responsive (acute cocaine vs. acute saline) transcriptional changes in animals with different adolescent social experiences.

Stereotaxic surgeries and tissue collection for immunohistochemistry

Stereotaxic surgeries infusing retrograding AAV5-hsyn-EYFP (AV-5-PV1696; purchased from the University of Pennsylvania Viral Core) into the VTA (Coordinates from Bregma: A/P: -3.3mm; M/L 0.9 mm; D/V: -4.6mm; 7° angle) were conducted as previously described (Calipari et al., 2017). Adult (~P90) male and female mice who were isolated or group housed during adolescence were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg) then positioned on a stereotaxic frame (Kopf Instruments). Ophthalmic ointment was applied to the eyes to prevent drying; a midline incision was made down the midline of the scalp, and craniotomy was performed using a dental drill. Virus (0.5µL/hemisphere) was delivered at a rate of 0.1µL/min using a 33G needle and Hamilton syringe. Following injection, needles were left in the injection site for ~5 minutes before being withdrawn slowly to reduce backflow. Animals were then placed in a clean cage with prior cage mates and were left undisturbed for ~4 weeks to allow for robust viral retrograde and expression. Once peak expression was reached, animals were anesthetized with ketamine (100mg/kg) and xylazine (10mg/kg) then transcardially perfused with ice cold 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS; pH 7.5) and delivered at 20ml/min for ~7min with a peristaltic pump. Brains were post fixed for 24 hours in 4% PFA at 4°C and stored in PBS + Azide (0.1%) until processing. Prior to sectioning on a cryostat, brains were transferred to a 30% sucrose soluction for 24-48 hours and snap

frozen in isopentane in dry ice. Once frozen, brains were sectioned at 40mm on the frontal plane on cryostat (Leica Biosystems, Buffalo Grove, IL). Sections were stored in PBS + Azide (0.01%) until staining occurred.

Tissue collection and storage for molecular endpoints

For the animals used in the qPCR, a total of 4 cohorts were used. Animals from each cohort were included at each endpoint. Every effort was made to minimize differences in the timing, procedures, and methods of tissue collection protocols detailed in (Walker et al., 2022a & b). Animals were euthanized via rapid decapitation between 10:00–14:00 hours, before lights out, at two separate timepoints: (1) 10 days post-isolation (~P32; n=6–9/group), and (2) 30 days after rehousing (~P72; n=6–9/group). Brains were extracted, chilled in cold phosphate-buffered saline (PBS), and sectioned in a cold brain matrix (1 mm sections). Punches from the meA (2 15G) and VTA (2 16G) were snap frozen on dry ice and stored at -80°C until RNA isolation. Animal groups were euthanized in rotation to control for possible circadian effects.

RNA isolation and preparation for quantitative reverse-transcriptase PCR (RT-qPCR)

RNA was isolated from frozen meA and VTA punches using a modified protocol (Walker et al., 2018) for the PureLink RNA Mini Kit (ThermoFisher, Waltham, MA). Tissue was homogenized in QIAzol (Qiagen, Fredrick, MD) followed by chloroform extraction and on-column RNA clean-up (Walker et al. 2018, 2022a, & 2022b) Concentration was determined on a NanoDrop Spectrophotometer (ThermoFisher, Waltham, MA). RNA (300 ng) was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and diluted 1:15 with ultrapure water before qPCR reactions. Gene expression was assessed using RT-qPCR that was performed using PowerUp™ SYBR™ Taq polymerase (Fisher, Waltham, MA) on an ABI QuantStudio5 qPCR machine under the following parameters: 50°C for 2 min; 95°C for 10 min and 45 cycles (95°C

for 15 sec then 60°C for 15 sec); a melt curve was run at the end of every plate to confirm primer specificity and absence of genomic DNA. Primers were designed in-house using the NCBI website and generated by IDT (Coralville, IA) and efficiency was validated prior for each target (100+10%). For a complete list of genes and primer sequences, see Suppl. Table 1. All targets were run in triplicate and relative expression was determined using the comparative CT method (Livak & Schmittgen, 2001) using median CT values for each gene. Samples were normalized to the geometric means for two housekeeping genes (*Bactin* and *Hprt1*) after confirmation of no significant impacts of sex, age, or housing on housekeeping gene expression. We noted that many samples with low RNA quality (260:280 ratio between 1.6 and 1.8) had reduced expression of housekeeping genes, often without affecting the expression of our genes of interest. Therefore, prior to analysis, any sample with housekeeping gene expression greater than 2.5 standard deviations from the mean for all samples in the experiment was excluded along with samples with high coefficients of variance (>2.5 standard deviations) for housekeeping gene CTs across the 3 runs of qPCR. RT-qPCR experiments were run in three rounds. Exclusion predicated on housekeeping gene expression variance resulted in 4 samples excluded from the meA (round 1: 55/59; round 2: 55/59; round 3: 54/58) and 5 samples from the VTA (round 1: 51/56; round 2: 39/44; round 3: 51/56). Additionally, samples with CT values >35 cycles for a specific gene were removed as expression was outside of the dynamic range for assay efficiency. Samples that were deemed of exceptional quality were then calibrated to the mean δ - CT of the control group for each sex to determine fold change in expression for each animal as previously described (Walker et al., 2018).

Immunohistochemistry and Imaging

Free floating sections were rinsed three times for 10 min in PBS before permeabilization in PBS + 0.2% Triton X-100 (PBST) for 15 min. Sections were blocked in 3% donkey serum in

PBS (v/v) after 3 additional washes in PBS. To boost the GFP signal within the meA, sections were then incubated with anti-GFP primary antibody raised in chicken (GFP 1020; Aves, Davis CA) overnight in PBS + 3% donkey serum (v/v), washed 3x in PBST, and incubated in anti-chicken secondary antibody for 1 hour (Jackson, West Grove, PA). Rinsed sections were mounted on slides and imaged using a LSM 710 laser-scanning confocal microscope (ZEISS) at 10X objective using identical illumination parameters for all prepared sections. Z-stack images (2 µm planes) were obtained and stacked images were subsequently overlaid with the corresponding atlas sections. Images were analyzed in ImageJ (version 1.53, National Institutes of Health, USA) and GFP+ positive cell bodies were counted per section and averaged per animal. Anterior and posterior sections of the meA were analyzed separately.

Electrophysiology and analysis

For measurements of the spontaneous activity of VTA dopamine neurons, cell-attached recordings using patch-clamp electrophysiology were performed (Chaudhury et al., 2013). The experimenter was blinded to experimental groups. Acute brain slices containing the VTA were collected from C57 mice. Mice were anesthetized and perfused with ice-cold aCSF (artificial cerebrospinal fluid), which contained 128 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 24 mM $NaHCO_3$, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (oxygenated with 95% O_2 and 5% CO_2, pH 7.4, 295–305 mOsm). Acute brain slices containing VTA dopamine neurons were cut using a vibratome (Leica VT-1000) cold, slushy sucrose aCSF, with 254 mM sucrose fully replacing NaCl and saturated by 95% O_2 and 5% CO_2. Slices were then allowed to recover in holding chambers with aCSF for 1 h at 37 °C. Patch pipettes (3–5 mΩ) were filled with internal solution containing the following: 115 mM potassium gluconate, 20 mM KCl, 1.5 mM $MgCl_2$, 10 mM phosphocreatine, 10 mM HEPES, 2 mM magnesium ATP and 0.5 mM GTP (pH 7.2, 285 mOsm). Cell-attached recordings were carried out using aCSF at 34 °C (flow rate ~2.5 mL/min). Recordings were performed in current-clamp mode using the Multiclamp 700B

amplifier and data acquisition was done in pClamp 10 (Molecular Devices). Series resistance was monitored during the experiments. For cell-attached action potential recordings, signals were band-pass filtered at 300 Hz–1 kHz to identify dopamine neurons and were then Bessel filtered at 10 kHz (gain 50).

Putative dopamine cells in the VTA were identified according to standard anatomical and electrophysiological criteria: a stark, triphasic waveform with set filters. Action potential frequency was measured using Threshold Search in Clampfit 10.2 and data were further processed using R as previously described (Morel et al., 2014). (Number of cells: control male = 26; isolated male = 33; control female = 29; isolated female = 29; control male = 4; isolated male = 5; control female = 5; isolated female = 6; average cells per animal: control male = 6.5; isolated male = 6.6; control female = 5.8; isolated female = 4.8.) We analyzed the data in two different ways. One with the total number of cells as the N (Fig. 4d) and one with the average firing rate per animal (Figure A1). Both produced the same null finding.

Bioinformatic analyses

Differential expression analyses: Publicly available differential lists from the PFC, NAc, and VTA were generated using DESeq2 as previously described in Walker et al. (2022a & b).

Rank-rank hypergeometric overlap: Stratified rank-rank hypergeometric overlap (RRHO) analysis was performed using the RRHO2 script in R as described in (Cahill et al., 2018). RRHO is a method which allows for threshold-free comparisons of transcriptional profiles by ranking transcripts in two lists of genes by their p-value (lowest to highest) and direction of change (up- or downregulated) to determine if shared genes are regulated in the same or opposite directions across two comparisons (Cahill et al., 2018). Throughout this dissertation, “transcriptional concordance” refers to genes showing statistically significant regulation in the same direction (both upregulated or both downregulated) across compared conditions, while “discordance” indicates opposite regulation (upregulated in one condition/region while downregulated in the

other). The strength of regulation is indicated by -log10(p) values, where higher values indicate more genes showing coordinated regulation. Importantly, high overlap can be either concordant or discordant - the RRHO heatmaps show concordance in red (bottom-left and top-right quadrants) and discordance in blue (top-left and bottom-right quadrants).

Statistical Analysis of Electrophysiological, IHC and qPCR data

Statistical analyses were conducted using SPSS version 30.0.0.0 (SPSS, Chicago, IL, USA). For all endpoints, a generalized linear model (GLM) was used to examine differences in cell firing, number of cell bodies, and relative expression (qPCR) with sex (male vs. female), housing (Ctrl vs. Iso), and age (qPCR only; P32 vs. P72) included as factors. Main effects and interactions of each factor were assessed. When interactions were observed in our analysis, we split data by that factor and analyzed the effects of the other factors. When appropriate, *post-hoc* analyses were conducted using the Šídák test. For the qPCR data in particular, our original analysis revealed interactions of sex in many of the candidate genes of interest (See Stats Table 1b). Because our RRHO analysis showed sex-specific effects of isolation and the GLMs indicated sex-specific interactions of housing and age on candidate gene expression, we analyzed maturational changes in relative expression in males and females separately (See Stats Table 1a). In the VTA, age-related effects in our housekeeping genes (*Bactin* and *Hprt1*) were detected. To avoid bias in our data due to these age-related changes, we analyzed expression changes in mid- and post-isolation endpoints separately using paired t-tests, or, when data distributions were nonparametric, Welch's tests were used. Prior to analysis, outliers were removed using standardized residuals (z-score of +2.5 SD from the line of best fit), or Grubbs's test when nonparametric (critical z-score value 95% confidence).

Results

SI disrupts sex-specific transcriptional concordance/discordance between the meA and VTA

Here, we hypothesized that prior findings of isolation-induced differences in c-Fos may be associated with transcriptional dysregulation between the meA and the canonical reward circuitry that are persistently disrupted by adolescent isolation (Walker et al. 2022b). To test this, we used stratified rank-rank hypergeometric overlap (RRHO) to compare cocaine-induced

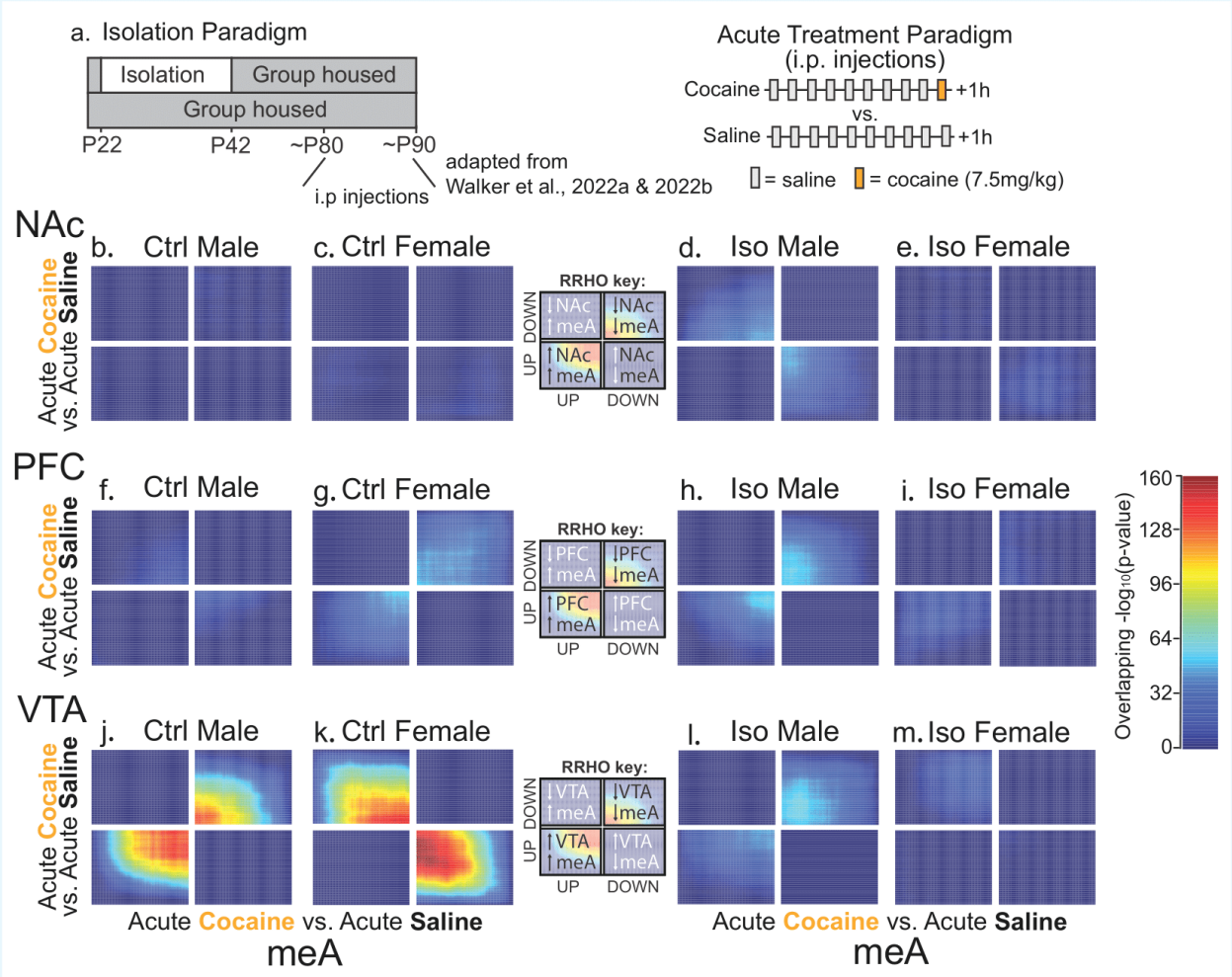


Figure 1. Adolescent social isolation induces loss of concordance and discordance in male and female meA–VTA gene expression. (a) Schematic of timeline for adolescent isolation, and cocaine and saline intraperitoneal injections. Key for reading rank-rank hypergeometric overlap plots. RRHO plots comparing transcriptional response contrasts made between acute cocaine (euthanized 1 hr post-cocaine injection) vs. acute saline (euthanized 1 hr post-saline injection) between the medial amygdala (meA) and the nucleus accumbens (NAc, b–e; b, maximum $-\log_{10}$ p-value=7; c, maximum $-\log_{10}$ p-value=9; d, maximum $-\log_{10}$ p-value=50; e, maximum $-\log_{10}$ p-value=15), prefrontal cortex (PFC, f–i; f, maximum $-\log_{10}$ p-value=21; g, maximum $-\log_{10}$ p-value=54; h, maximum $-\log_{10}$ p-value=58; i, maximum $-\log_{10}$ p-value=22), and ventral tegmental area (VTA, j–m; j, maximum $-\log_{10}$ p-value=138; k, maximum $-\log_{10}$ p-value=160; l, maximum $-\log_{10}$ p-value=62; m, maximum $-\log_{10}$ p-value=30).

transcriptional profiles in the meA and other canonical regions of the reward circuitry (Fig. 1). We used publicly available RNA-sequencing data to identify overlap in transcriptional profiles from mice given 9 prior habituating doses of saline over the previous 9 days then, on the 10th day, euthanized one hour ("acute") after being administered either their first dose of cocaine ("acute cocaine") or their 10th dose of saline ("acute saline"; For paradigm see: Fig. 1a).

RRHO revealed transcriptional concordance in the meA and VTA of control males (Fig. 1j, $-\log_{10}$ p=138) and discordance in control females (Fig. 1k, $-\log_{10}$ p=160), which is decreased in isolated male (Fig. 1l, $-\log_{10}$ p=62) and female (Fig. 1m, $-\log_{10}$ p=30) mice in comparison to control conditions. Importantly, when transcriptional profiles were compared in other regions of the reward circuitry, overlap was much weaker than those between the meA and VTA (Fig. 1b–i; max $-\log_{10}$ p=58), suggesting that a single dose of cocaine has similar effects on transcription in the meA and VTA of males but opposite effects in female mice and that this overlap in transcriptional regulation is disrupted by adolescent isolation.

While the transcriptional profiles in the meA and VTA are disrupted in isolated animals, it should be noted that the cocaine-induced transcriptional profiles in Figure 1 are compared to animals euthanized one hour after an injection of saline (Fig. 1a). While this comparison controls for injection and handling stress, it could conceal differences in stimulus-dependent transcription between isolated and group-housed control animals. This is important because published data indicates that an injection of saline coupled with handling results in robust transcriptional activity and enrichment of IEG expression throughout the reward circuitry in mice (Cates et al., 2018; Walker et al., 2018; Walker et al., 2022a & b), and recent data suggests that resilience to stress is a transcriptionally dynamic event (Bagot et al., 2017). Therefore, we sought to determine if the lack of cocaine-induced transcriptional concordance/discordance (Fig. 1j–m) in isolated animals was due to differences in stimulus-induced transcription. We hypothesized that differences in stimulus-induced transcription could explain the relative loss of transcriptional concordance and discordance in isolated animals. To test this, we compared

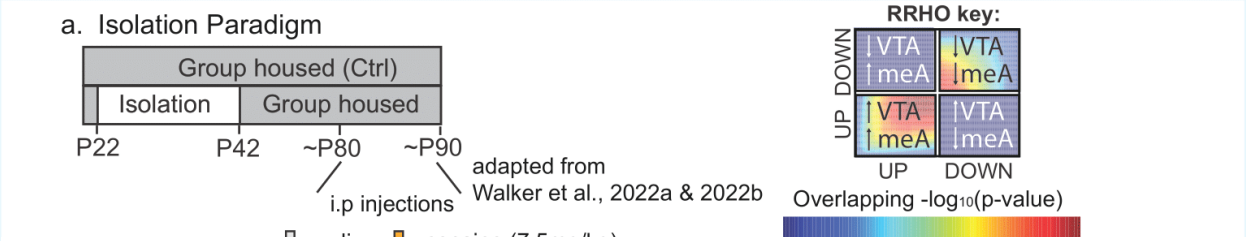


Figure 16. **Overlap between mesolimbic cocaine- and stimulus-responsive transcriptional profiles is enhanced by adolescent social isolation.**

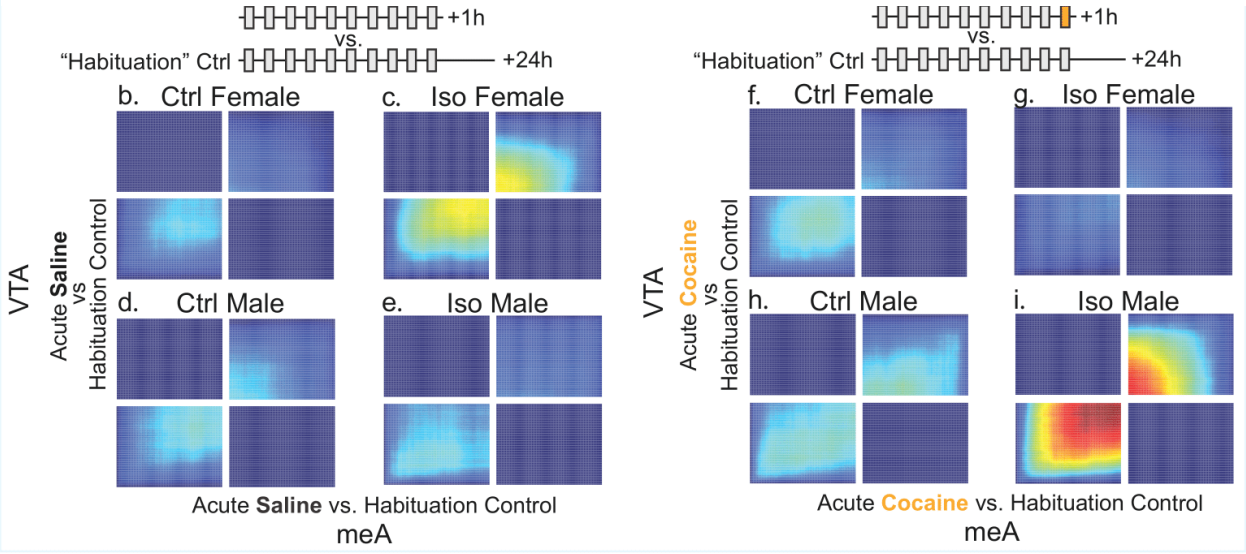


Figure 2. Overlap between mesolimbic cocaine- and stimulus-responsive transcriptional profiles is enhanced by adolescent social isolation. (a) Schematic of timeline for adolescent isolation, and cocaine and saline intraperitoneal injections. Key for reading rank-rank hypergeometric overlap plots. RRHO plots comparing transcriptional response to acute saline (1 hr post-saline injection, b–e; b, maximum −log10 p-value=168; c, maximum −log10 p-value=301; d, maximum −log10 p-value=194; e, maximum −log10 p-value=184) and cocaine (1 hr post-cocaine injection, f–i; f, maximum −log10 p-value=208; g, maximum −log10 p-value=120; h, maximum −log10 p-value=215; i, maximum −log10 p-value=463) vs. habituation control (24 hr post-saline injection) between the medial amygdala (meA) and ventral tegmental area (VTA).

saline-injection-induced (Fig. 2b–e) or cocaine-injection-induced (Fig. 2f–i) transcriptional profiles in comparison to a more quiescent transcriptome (habitation controls) of animals euthanized after 10 intraperitoneal (IP) saline injections then euthanized 24 hours after the final injection. We consider this transcriptomic baseline quiescent because gene transcription is temporally distant from the stimulus-induced transcriptional responses to experimenter injections, a consideration supported by a lack of differentially expressed genes (Walker et al., 2022a&b; adj. $p<0.05$) in the isolated vs. group-housed control animals (meA and VTA: Isolated males vs. control males = 0; Isolated females vs. control females = 0).

Indeed, when acute saline and cocaine were contrasted against habituation controls, we observed a sex-specific enhancement of concordance of meA–VTA transcriptional profiles in isolated mice (Fig. 2c, e, g, & i). Isolated females displayed comparatively enhanced overlap in meA–VTA expression 1 hour after an injection of saline (Fig. 2c, -$\log_{10}$ p=301) and diminished overlap following cocaine (Fig. 2e, -$\log_{10}$ p=120). In isolated males, the converse is true, where a comparative increase in concordance after a single injection of cocaine (Fig. 2i, -$\log_{10}$ p=463) and reduced concordance after an injection of saline (Fig. 2e, -$\log_{10}$ p=184) was observed. We observe comparatively lower concordance of meA and VTA transcriptional profiles in control mice (Fig. 2b, d, f &h; for max -$\log_{10}$ p-values, see figure legends). Together, these data not only support our hypothesis that isolation disrupts stimulus dependent transcription in the meA and

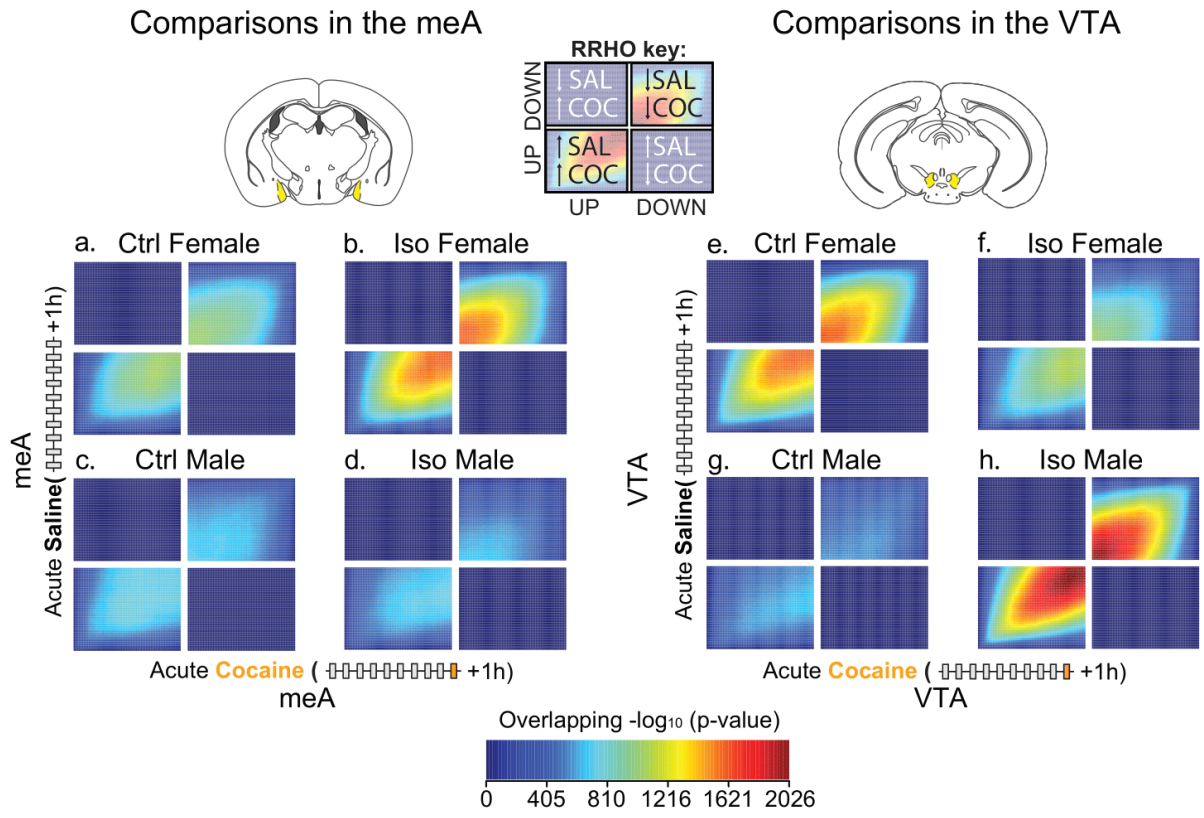


Figure 3. Adolescent social isolation induces a loss of transcriptional distinction between cocaine and saline. RRHO plots comparing intraregional transcriptional response to acute saline. (1 hr post-saline injection; a–d) or cocaine (1 hr post-cocaine injection; e–h) vs. habituation control (24 hr post-saline-injection) within the medial amygdala (meA; a, maximum $-\log_{10}$ p-value=1594; b, maximum $-\log_{10}$ p-value=1094; c, maximum $-\log_{10}$ p-value=777; d, maximum $-\log_{10}$ p-value=774) and ventral tegmental area (VTA; e, maximum $-\log_{10}$ p-value=1594; f, maximum $-\log_{10}$ p-value=1094; g, maximum $-\log_{10}$ p-value=683; h, maximum $-\log_{10}$ p-value=2026).

VTA but also suggest that isolation specifically impacts the cocaine-induced transcriptional concordance between the meA and VTA in males but not females.

The sex differences in stimulus-dependent transcription between the meA and VTA of isolated animals provide intriguing evidence that isolation results in region-specific dysregulation of stimulus dependent transcription. To determine whether gain of stimulus dependent transcriptional concordance in isolated animals (Fig. 2c & 2i) is driven by isolation-induced disruption in the meA, VTA, or both regions, we compared acute saline (y-axes, Fig. 3) and acute cocaine (x-axes, Fig. 3) transcriptional profiles within the meA (Fig. 3a–d) or VTA (Fig. 3e–h). In control mice (Fig. 3a, c, e & g), we observed transcriptional concordance in both the meA and VTA (males Fig. 3c & g $-\log_{10}$ p=777 & $-\log_{10}$ p=683; control females Fig. 3 a & e, $-\log_{10}$ p=1050 & VTA: $-\log_{10}$ p=1594) but noted that concordance was more pronounced in control females compared to males.

Interestingly, region-specific effects were present in isolated animals (Fig. 3b, d, f & h). Isolated females displayed the most transcriptional concordance in the meA when compared to all other groups (Fig. 3b, $-\log_{10}$ p=1598). Conversely, isolated males displayed the most transcriptional concordance in the VTA (Fig. 3h, $-\log_{10}$ p=2026) when compared to all other groups, but we found comparable overlap in control males and isolated males the meA (Fig. 3c, -log10 p=777 & Fig. 3d, $-\log_{10}$ p=744, respectively). We interpret the overlap of acute saline and acute cocaine-induced transcriptional profiles within a brain region as indicative of an inability to distinguish between injection stimuli and cocaine at the transcriptional level. Together, these data in isolated animals suggest that injection stress (saline injection + handling) and cocaine result in similar transcriptional profiles in the meA of females and VTA of males, which may provide insight into how adolescent isolation persistently disrupts reward-related behaviors.

Adolescent isolation does not affect meA–VTA connectivity

Our RRHO analysis suggests that adolescent isolation impacts the transcriptional response of the meA and VTA to stress (*i.e.*, handling stimuli associated with an injection of saline) and cocaine. Given that the meA projects to the VTA and develops during adolescence, we hypothesized that isolation might disrupt connectivity between the meA and VTA. To test this, we injected a retrograding AAV (AAV5-hSyn-eYFP) into the VTA of adult animals after adolescent isolation or group-housed control conditions (Fig. 4a) and counted then number of GFP+ cell bodies in the meA. Because the posterodorsal meA is sexually dimorphic and sensitive to post-weaning isolation (Cooke & Woolley, 2005; Raam & Hong, 2021), we counted GFP+ cell bodies in both the anterior and posterior meA then analyzed cell-count data via GLM with sex, subregion, and housing as factors. While no effects of isolation were observed on the number of GFP+ cells in the meA [Wald$\chi^2(1)=0.418$, $p=0.518$], GLM did reveal a subregional difference in connectivity between the meA and VTA with more projection in the anterior meA than posterior meA [Wald$\chi^2(1)=11.489$, $p<0.001$] and a nominal sex-difference in the number of meA to VTA projections [Wald$\chi^2(1)=3.836$, $p=0.05$], which are more numerous in males than females regardless of sub-region or housing conditions (Fig. 4b).

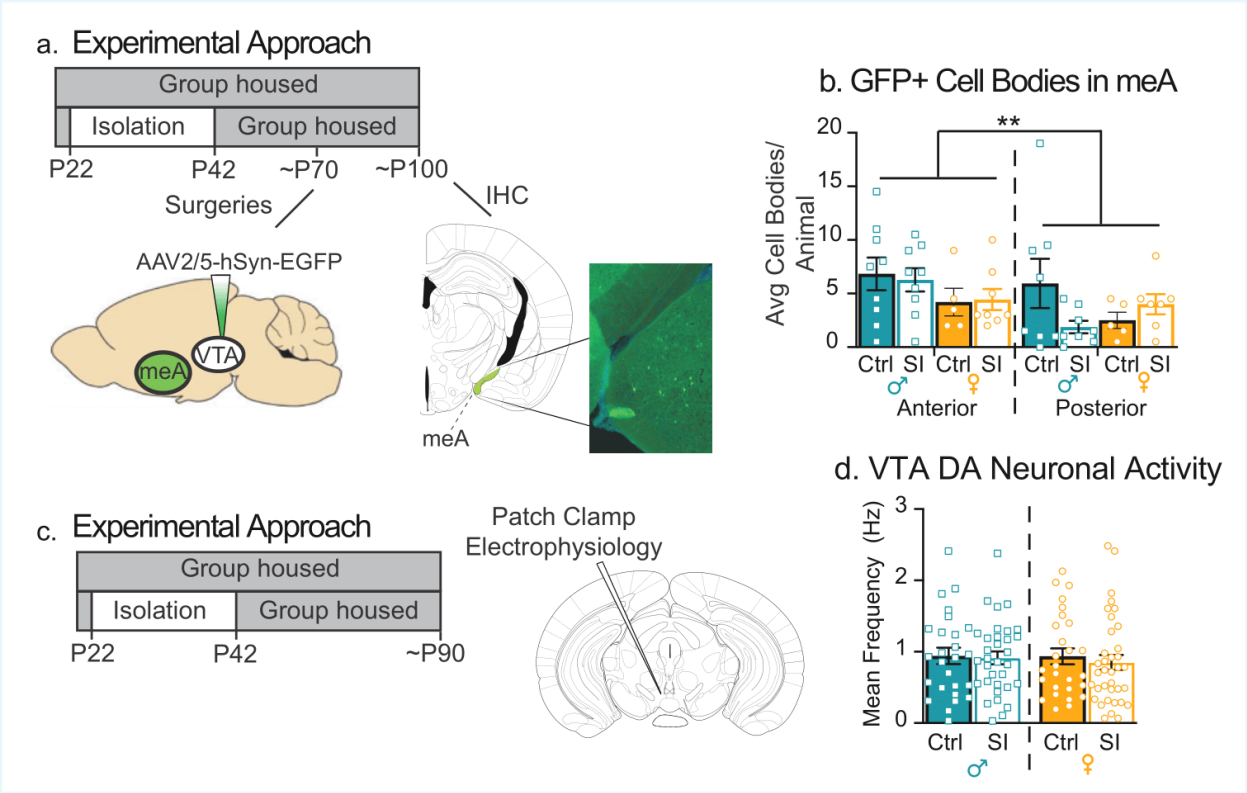


Figure 4. Retrograde tracing suggests meA to VTA projections are unaffected by adolescent isolation. (a) Schematic of timeline for adolescent isolation, AAV2/5-hSyn-EGFP surgery, and euthanasia with representative fluorescent image of meA→VTA AAV2/5-hSyn-EGFP expression in meA cell bodies. (b) Quantification of meA→VTA AAV2/5-hSyn-EGFP+ neurons in the meA. (c) Schematic of timeline for adolescent isolation and tissue collection for patch-clamp electrophysiology. (d) Quantification of mean cell firing of dopamine neurons from ventral tegmental area. Significance is indicated as *p<0.05, **p<0.01. Error bars indicate SEM. Ctrl, group housed; Iso, socially isolated; P, postnatal day.

Adolescent Isolation has no effect on dopamine cell firing rates

Given that isolation is associated with enhanced cocaine-induced transcriptional concordance between the meA and VTA, and that cocaine acts via the dopaminergic system, we hypothesized that isolation may impact dopaminergic neuron firing rate in the VTA. To test this, we characterized electrophysiological properties of putative dopaminergic neurons within the VTA using patch clamp electrophysiology. GLM revealed that isolation had no effect on firing of putative VTA DA neurons [Waldχ^2(1)=1.549,p=0.213] and no interactions of factors were observed (Fig. 4d). Additionally, as expected, GLM revealed no sex differences in DA

neuronal firing because all females were in diestrus when recordings occurred [(Calipari et al., 2017); Waldχ^2(1)=0.009, p=0.925].

Maturation of dopamine receptor gene expression in the meA is disrupted by adolescent isolation

Because we did not observe changes in baseline firing rates in DA neurons of the VTA of isolated animals, we hypothesized that changes in dopamine receptor expression within the meA may be related to the cocaine-induced changes in transcription observed in our RRHOs. The meA is rich in dopamine receptors which are necessary for reproductive behaviors in females and are sensitive to circulating gonadal hormones (Holder et al., 2015). Because adolescence is associated with the onset of puberty and expression of reproductive behaviors, we tested the hypothesis that isolation might impact maturational profiles of dopamine receptor gene expression (*Drd1*, Fig. 5b; *Drd2*, Fig. 5c) in the meA using qPCR in tissue collected in the middle of isolation, 10 days after start of isolation (mid), and ~30 days after rehousing (post; Fig. 5a). While no effects were observed in females (Suppl. Table 2), GLM revealed a main effect of housing on male expression of *Drd1* [Fig. 5b; Wald $\chi^2(1)=4.89$, p=0.027] and Drd2 [Fig. 5c; Wald $\chi^2(1)=12.16$, p<0.001], with isolated males expressing higher levels of *Drd1* & *Drd2* than control males. Together, these data suggest that adolescent isolation leads to a sex-specific enhancement of dopamine receptors in the meA and could point to a possible explanation for the disruption of cocaine-induced transcriptional profiles between the meA and VTA.

Maturation of cell-type specific markers in the meA is disrupted by adolescent isolation

While we did not observe differences in connectivity between the meA and VTA, it is important to note that the retrograding virus we used was under an hsyn promoter, meaning that transport occurred in neurons regardless of cell type. The meA is composed of both glutamatergic and GABAergic projection neurons (Raam & Hong, 2021) which control different

aspects of sex-specific social behaviors and are differentially distributed along the medial–lateral axis, particularly within the posterodorsal meA (Johnson et al., 2021; McIntyre et al., 2022). Neurons in the meA undergo important developmental changes during adolescence, including dendritic pruning (Zehr et al., 2006), and post-weaning isolation has been shown to alter neuronal activity and the number of neurons in meA (Adams & Rosenkranz, 2016; Cooke et al., 2000). Therefore, we hypothesized that isolation may disrupt maturation of specific subtypes of neurons that manifest in differences in the transcriptional response to cocaine and handling stimuli and stress associated with saline administration.

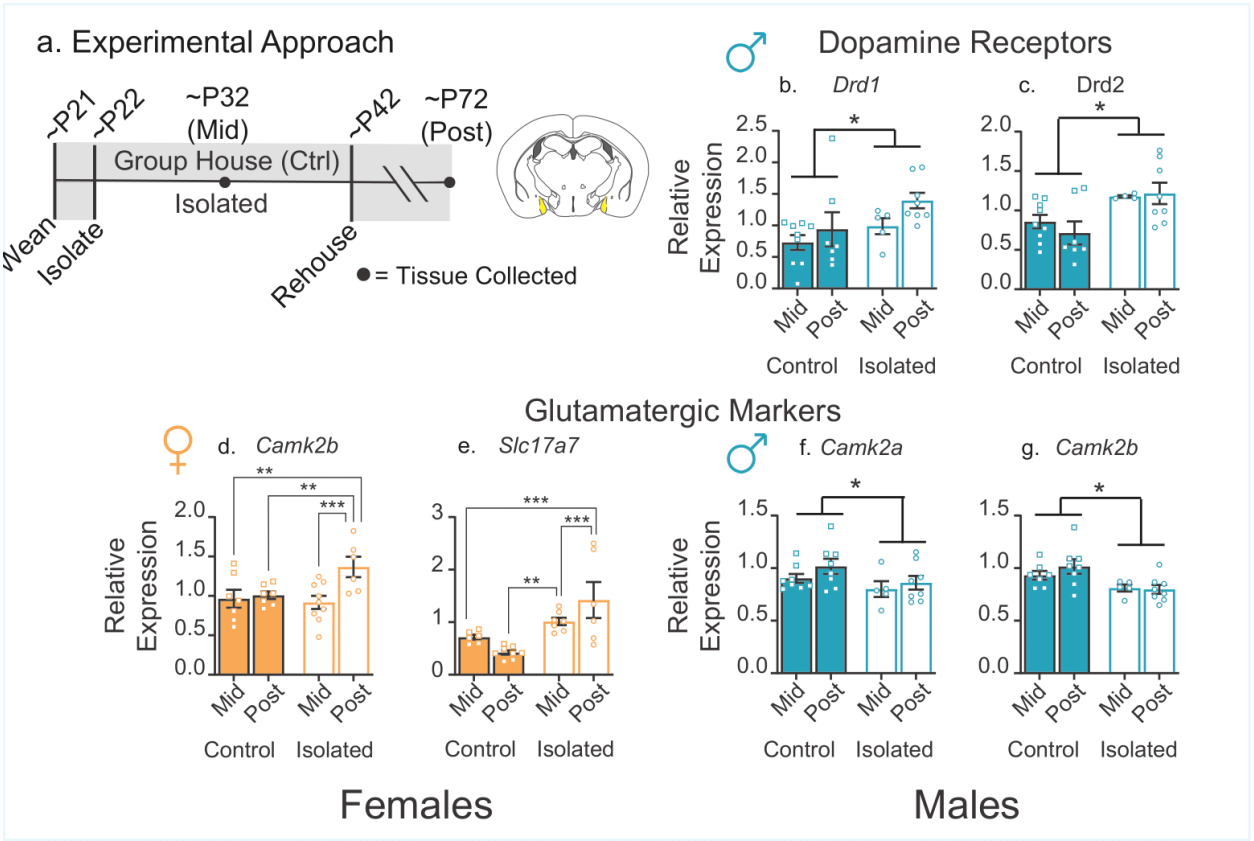


Figure 5. Medial amygdala maturation of cell-type specific and dopamine receptor gene transcription is disrupted sex-specifically by social isolation. (a) Schematic of adolescent social isolation experimental timeline. Tissue collected from medial amygdala at two timepoints: 10 days into social isolation (P32) or 50 days after social isolation (~P72). Using RT-qPCR, expression of cell-type specific gene targets (*Camk2a*, f; Camk2b, d/g; *Slc17a7*, e; *Drd1*, b; *Drd2*, c) were measured in the meA. Significance is indicated as *p<0.05, **p<0.01, ***p<0.001. Error bars indicate SEM. Control, group housed; Isolated, socially isolated; P, postnatal day.

To test this, we measured mRNA expression of candidate genes using qPCR (for full results see Suppl. Table 1) associated with specific cell types in the meA in the middle of isolation and in adulthood (Fig. 5a). We observed that glutamatergic but not GABAergic markers were disrupted in a sex-specific manner (Suppl. Table 2). Specifically, in female mice we observed a significant age × housing interaction for *Camk2b* [Fig. 5d; Waldχ^2(1)=5.197, p=0.023]. Post-hoc Šídák testing showed that in adulthood, post-isolation females had significantly higher *Camk2b* expression compared to mid-adolescence control females (1.40-fold higher, p=0.013) and in adulthood (1.36-fold higher, p=0.037), and mid-isolation adolescent females (1.45-fold higher, p=0.002). We observe a main effect of age on *Camk2a* expression [Waldχ2(1)=17.694; p<0.001], 1.23-fold higher in adult females than adolescents (p<0.001). Additionally, we observed a significant age × housing interaction in female *Slc17a7* expression (Fig. 5e; Waldχ^2(1)=5.663, p=0.017], which encodes for vesicular glutamate transporter 1. Post-hoc analysis revealed that adult, post-isolation females express more *Slc17a7* than mid-isolation females by 2-fold (p<0.001) and their adolescent control counterparts by 1.7-fold (p=0.008). Additionally, mid-isolation females express less *Slc17a7* than adult control females by 0.59-fold (p=0.015). On the other hand, in males, GLMs assessing age and housing as factors revealed a main effect of housing on two canonical markers of glutamatergic neurons, *Camk2a* [Fig. 5g; Wald χ^2(1)=4.564, p=0.033] and *Camk2b* [Waldt χ^2(1)=11.760, p<0.001; Fig. 5 g). On average, control male expression of *Camk2a* was 1.129-fold higher (p=0.033) than in isolated males, and control males had 1.169-fold higher expression of *Camk2b* (p=0.001) than isolated males. Together, these data suggest that glutamatergic, but not GABAergic (Suppl. Table 2), markers in the meA are affected by isolation in both males and females.

Post-isolation gene transcription in the VTA is affected by adolescent SI

We next asked if isolation impacted maturation of related GABA- and glutamatergic-related candidate genes in the VTA. However, because of a main effect of age on housekeeping genes, we were unable to compare expression values assessed mid-isolation to those in post-isolation (Suppl. Table 3).

Instead, the influence of housing was assessed in mice that were the same age, in mid-adolescence (P32), and in adulthood (P72). Interestingly, we observed no effects of isolation on GABA- or glutamatergic-related genes mid-isolation (Suppl Table 3). However, in adults, sex-specific effects were observed in glutamatergic-related genes (Fig. 6b–e). T-tests revealed that isolated females had higher expression of the obligate subunit of the NMDA receptor, *Grin1* [t(11)2.252, p=0.046], the GluN2B subunit of the NMDA receptor, *Grin2b* [t(11)2.336, p=0.04], and vesicular glutamate transporter 2, *Slc17a6* [t(7.538)2.628, p=0.032], compared to adult

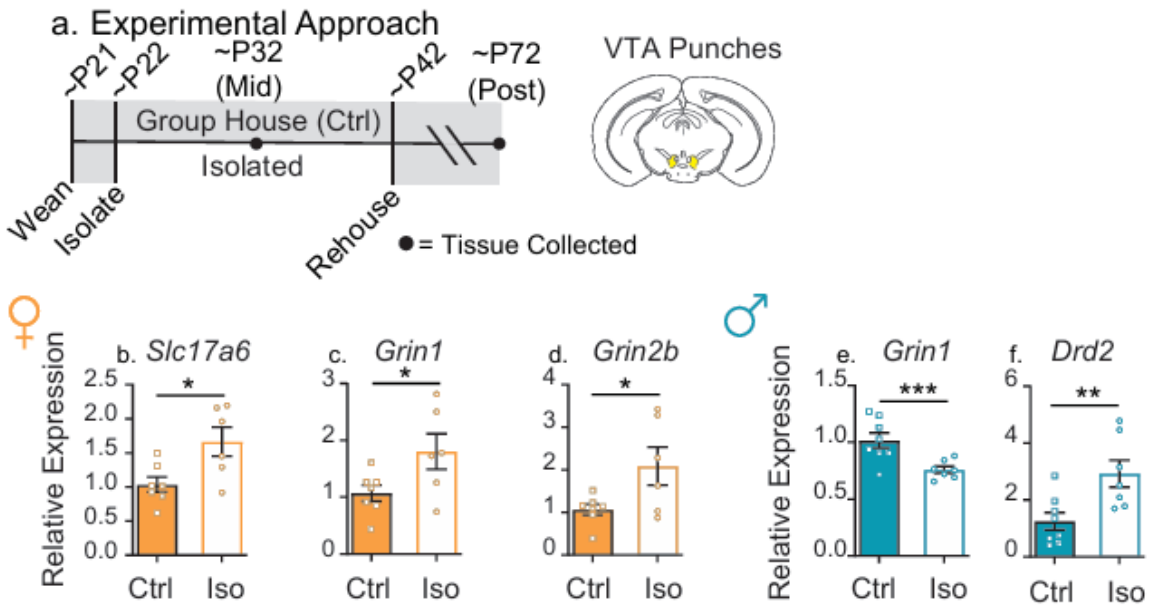


Figure 6. Adult ventral tegmental area gene expression of glutamate-related genes and dopamine receptors is sex-specifically disrupted by social isolation. Tissue collected from the ventral tegmental area (a). RT-qPCR plots showing relative gene expression of glutamatergic markers and receptors and dopamine receptors were measured in the VTA (*Slc17a6*, b; Grin1, c & e; *Grin2b*, d; *Drd2*, f). Significance is indicated as *p<0.05, **p<0.01, ***p<0.001. Error bars indicate SEM. Control, group housed; Isolated, socially isolated; P, postnatal day.

females reared in group housing (Fig. 6b–d). On the other hand, adult isolated males expressed significantly lower levels of *Grin1* [Fig. 6e; t(9.687)=3.513, p=0.006], and higher levels of *Drd2* [Fig. 6f; t(13)=3.003, p=0.01], which is primarily an autoreceptor expressed on VTA dopamine neurons, than adult control males in comparison to adult control males. Together these data suggest that isolation leads to developmental changes in meA glutamatergic neurons which may contribute to changes in sensitivity of VTA neurons to glutamate through changes in expression of NMDARs in adulthood.

Discussion

Using threshold-free rank-rank hypergeometric overlap (RRHO) analysis, cell-tracing techniques, and targeted gene expression assays, we show that adolescent social isolation disrupts transcriptional concordance between the medial amygdala (meA) and ventral tegmental area (VTA) in a sex-specific manner. These transcriptional disruptions were accompanied by sex-specific alterations in glutamatergic, but not GABAergic, neuronal markers in both regions, suggesting that disrupted excitatory signaling maturation underlies the loss of meA-VTA transcriptional coordination. Our findings underscore the importance of meA–VTA connectivity in adolescent isolation-induced disruptions and highlight a potential role for dopaminergic and glutamatergic signaling in mediating these effects. These data reinforce the need to consider cell-type-specific mechanisms underlying developmental social stress, essential for identifying therapeutic targets in adolescent-onset neuropsychiatric disorders.

While our findings provide important insights into the lasting effects of adolescent social isolation, we acknowledge several limitations that should be considered. Notably, the animals used in the sequencing, electrophysiology, and tracing studies were shipped from Jackson Laboratory on P20 with arrival on P21, whereas the animals for the qPCR experiments were bred on site. This difference in early rearing experiences could potentially influence the adolescent social isolation model or other aspects of development, including stress responsivity

and cocaine responses. Shipping stress during the late pre-weaning period might interact with our isolation paradigm in ways that differ from animals bred in-house, potentially affecting the generalizability of our findings across experiments. Future studies should standardize rearing conditions across all experimental endpoints to minimize this potential confound. Additionally, while we controlled for estrous cycle in our electrophysiology experiments by testing all females in diestrus, hormonal fluctuations across the estrous cycle could contribute to variability in other endpoints and warrant further investigation.

RRHO reveals adolescent isolation results in sex-specific alterations in stimulus-dependent transcription in the meA and VTA

Concordance in transcriptional profiles has been shown in preclinical models to be indicative of functional integration of brain regions in a functional network, where behavioral phenotypes of stress sensitivity are associated with a loss of transcription concordance within the reward circuitry (Bagot et al., 2016). Here we used this approach to determine if transcriptional disruptions in the meA, a region sensitive to adolescent experience, might be reflected within the canonical reward pathway.

In comparing the overlap of transcriptional profiles from the meA and VTA of animals euthanized one hour after being administered either their first dose of cocaine (acute cocaine) or their tenth dose of saline (acute saline), we found that the group-housed gene transcriptional programs are lost in adolescent isolation (Fig. 1). Despite documented connectivity of the meA with the prefrontal cortex and nucleus accumbens (Cádiz-Moretti et al., 2016; Knapska et al., 2007; Pardo-Bellver et al., 2012), we observed the strongest transcriptional concordance between the meA and VTA in males and discordance in control females. Importantly, this overlap is lost in isolated males and females indicating that, when controlling for handling-stress-associated stimuli (in the acute condition), cocaine-induced transcriptional responses in the meA and VTA are disrupted by adolescent isolation.

On the other hand, when we examine meA–VTA transcriptional profiles, comparing acute cocaine or saline against more “quiescent” transcriptomes (Fig. 2), we found isolation leads to sex-specific increases in comparative overlap in saline and cocaine gene programs. While control mice regulate these conditions comparably, we found that isolation induced a sex difference in transcriptional regulation in response to cocaine and saline between the meA and VTA. In comparisons assessing transcriptional response to acute saline between the meA and VTA (Fig. 2), isolation increased concordance of saline-responsive gene transcription in females, but not males, when compared to group-housed control counterparts. Conversely, cocaine-responsive transcriptional concordance between the meA and VTA was enhanced in isolated males but comparatively decreased in isolated females versus group-housed controls. This suggests that the loss of transcriptional concordance and discordance observed in isolated males and females, respectively, between the meA and VTA may be driven by sex-specific differences in stimulus-dependent transcription in isolated animals. These transcriptional disruptions provide a molecular basis for isolation-induced SUD vulnerability; the sex-specific loss of stimulus discrimination we observe may underlie the enhanced cocaine preference in males and altered stress responsivity in both sexes (Baarendse et al., 2014; Walker et al., 2019); Walker et al., 2022b).

When we compared intraregional, *i.e.*, meA vs. meA and VTA vs. VTA, cocaine and saline transcription profiles, we observed a sex- and region-specific effect of adolescent isolation (Fig. 3). In the female meA, isolation induced substantial overlap in cocaine and saline transcriptional responses (Fig. 3b). Conversely, in the male VTA, isolation increased the overlap of cocaine-saline transcriptional regulation. Together, we interpret these data to indicate that isolation results in a sex- and region-specific loss in the transcriptional distinction between cocaine and saline injections. These findings suggest isolation may have regional specific effects on sex differences in stimulus-dependent transcription which account for the isolation-induced loss of transcriptional concordance and discordance between the meA and VTA, indicating that the

meA–VTA circuit may have a unique role in drug- and stress-responsive gene transcription programs. Taken together data indicate that adolescent isolation augments transcriptional regulation of both stress and drugs of abuse in a novel, understudied meA–VTA circuit. Further work using cell-type specific approaches such as single-cell RNA-seq alongside circuit-level investigations should be leveraged to understand how specific neuronal populations within this circuit are affected by adolescent isolation and contribute to sex-specific vulnerability to SUD. Understanding these mechanisms is particularly relevant given the development of SUD during adolescence and the pronounced sex differences in their trajectories (Becker et al., 2017).

Adolescent isolation induces sex-specific changes in expression of dopamine receptors in the meA and VTA without impacting baseline DA firing from the VTA

The VTA is a critical site of dopaminergic signaling integral to motivation and reward (Saunders & Richard, 2011). Cocaine acts through VTA dopaminergic signaling via second messenger cascades to influence transcription (Nestler, 2005). Therefore, one obvious candidate underlying these circuit effects is changes in dopamine (DA) signaling. Importantly, we found no influence of isolation on the number of meA→VTA neurons (Fig. 4b) or on average DA cell firing rates (Fig. 4d). However, we did observe higher expression of *Drd1* and *Drd2* mRNA in the meA and *Drd2* in the VTA of isolated males. Because we did not find differences in DA neuron firing, we interpret this to mean that isolation influences sensitivity to DA in males, particularly within the meA, through changes in DA receptors. While the meA is a dense site of DA receptors (Lein et al., 2007), less is known about their function. However, recent evidence suggests that D1-R/*Drd1* is highly expressed in the meA and mediates approach/avoidance conflict behaviors (Miller et al., 2019). Isolated males have been shown to be more aggressive (Biro et al., 2023), indicating a potential behavioral consequence of higher *Drd1* expression observed here. Compared to *Drd1*, there is less *Drd2* mRNA expression in the in the meA (Lein et al., 2007; Walker, Zhou, Cunningham, Ramakrishnan, et al., 2022) and our results indicate

that *Drd2* expression is higher in the meA and VTA of isolated males. While it's function in the meA is not well characterized, it is well established as an inhibitory G_o-coupled GPCR, that is principally found as an autoreceptor on DA neurons in the VTA (Beaulieu & Gainetdinov, 2011). While the literature is mixed in the influence of isolation on DR-2/*Drd2* throughout the reward circuitry (Del Arco et al., 2004; Fitzgerald et al., 2013; Han et al., 2012; Holder et al., 2015; Walker et al., 2019; Yorgason et al., 2016), our data indicate that the dopaminergic system is sensitive to adolescent-specific social experience in the canonical and noncanonical reward circuitry. Future work should examine pathway- and cell-type-specific influences of adolescent isolation on dopamine receptor expression within the meA and VTA to gain more understanding of how adolescent experiences influence sensitivity to dopamine throughout the mesocorticolimbic reward circuitry.

Adolescent isolation induces sex-specific changes in markers of excitatory neurons in the meA and glutamate receptors in the VTA

Our IHC data suggest that connectivity between the meA and VTA is not affected by adolescent isolation but maturation of shared stimulus dependent transcriptional profiles within the meA and VTA are disrupted by adolescent isolation. Our retrograding virus was not cell-type specific and it is well established that different population of GABA- and glutamatergic neurons in the meA influence different behavioral outcomes. Therefore, we hypothesized that isolation-induced transcriptional disruptions result from altered maturation of neuronal subtypes rather than changes in baseline connectivity. Using an RT-qPCR candidate gene approach, we found sex-specific disruptions in the developmental expression of markers of glutamatergic (Fig. 5 & 6) but not GABAergic (Suppl. Table 2 & 3) signaling in both the meA and VTA. Our finding of intact meA-VTA connectivity despite profound transcriptional dysregulation suggested that functional, rather than structural, changes underlie isolation effects. Remarkably, we found that adolescent isolation selectively disrupted glutamatergic, but not GABAergic, gene expression

trajectories in both regions, providing a mechanistic explanation for the sex-specific transcriptional dysregulation observed in our RRHO analyses. Broadly, isolated females exhibited increased expression of glutamatergic-related genes in adulthood (Fig. 5 & 6), whereas isolated males showed reductions in these markers in the meA during isolation which were maintained into adulthood (Fig. 5). In the VTA of isolated males, changes in glutamatergic receptor expression were not observed until adulthood. Together, these data suggest that isolation may disrupt maturation of the glutamatergic neurons in the meA which result in altered sensitivity to glutamate in the VTA of adults and indicate that the meA may be especially sensitive to social experience in adolescence.

With regards to specific genes that are impacted by adolescent isolation, we found isolated females express higher levels of two plasticity-related genes, *Camk2b* and *Slc17a7* in adulthood (Fig. 5). Females express higher levels of *Camk2a* in adulthood than adolescence, but no effect of housing was observed; we take this to be a characterization of typical *Camk2a* maturation (Suppl. Table 2). In isolated males, we found lower expression of two canonical markers of glutamatergic neurons, *Camk2a* and *Camk2b* across development. This is consistent with work finding reductions in meA size in isolated males (Cooke et al., 2000), and could be indicative of altered synaptic plasticity. Both *Camk2a*, alpha-CaMKII, and *Camk2b*, beta-CaMKII, are integral to synaptic plasticity and long-term potentiation (LTP). Alpha-CaMkII is especially enriched in the postsynaptic density and plays a critical role in synaptic plasticity (Yasuda et al., 2022). Beta-CaMKII is critical for synaptic plasticity through its interaction with F-actin, which influences dendritic spine morphology and synaptic strength, facilitating long-term potentiation (LTP) by targeting alpha-CaMkII to synapses (Borgesius et al., 2011) and contributes substantially to total CaMKII activity (Hinds et al., 1998; Silva et al., 1992). Taken together, these findings support and provide potential mechanisms underlying previous findings of gross anatomical and electrophysiological changes within the meA (Adams & Rosenkranz, 2016; Cooke et al., 2000).

In the VTA, adult isolated females showed increased *Grin1* expression, the obligatory unit of the NMDA receptor, and *Grin2b*, an NMDA receptor subunit associated with greater Ca^{2+} permeability (Paoletti et al., 2013), suggesting an increase in NMDA receptor function in the VTA over their group-housed counterparts. Comparatively enhanced expression of *Grin1* and *Grin2b* may indicate greater synaptic plasticity and differentially regulated dopamine release in isolated females. Adult males showed an opposing gene expression pattern to isolated females, where males with a history of isolation expressed lower *Grin1* in the VTA compared to control males. These findings dovetail with other work finding isolation-induced changes in the glutamate system in the VTA (Whitaker et al., 2013).

Our data indicate that the meA may be particularly sensitive to isolation-induced developmental disruptions, subsequently influencing VTA transcriptional responses in adulthood. This highlights the role of the meA in mediating long-term consequences of adolescent social experiences within mesocorticolimbic reward circuitry.

Conclusion

We observed that adolescent social isolation induces substantial, sex-specific transcriptional dysregulation in the meA and VTA across development and in adulthood, both at baseline and in response to drugs or handling- and injection-associated stimuli. These differences occur in the absence of disrupted connectivity between the meA–VTA and without differences in basal averages in dopamine neuron firing. Critically, these effects were specific to glutamatergic signaling pathways, highlighting mesolimbic excitatory neurotransmission as a key target of adolescent social experience. In the context of other work, our findings indicate that the aberrant behavioral and transcriptional phenotypes observed following social isolation may be attributable to dysregulated maturation of glutamatergic signaling in the meA. Overall, the molecular findings indicate that the persistent phenotypic differences that result from

adolescent social isolation are associated with sex-specific alteration in the maturational trajectories of glutamatergic and dopaminergic genes during adolescence.

Chapter 3: Sex-specific disruption of prefrontal maturation following adolescent isolation: behavioral, hormonal, and transcriptional consequences

In Chapter 2, I examined the influence of adolescent social isolation on neuronal connectivity, firing, and gene expression in the medial amygdala (meA) and ventral tegmental area (VTA). I found that social isolation during adolescence augments gene expression within and between the meA and VTA without affecting connectivity between the two regions or dopamine neuron firing. Assessing the bulk transcriptional response of cells in the meA and VTA in response to either cocaine or saline, I identified that adolescent social isolation was associated with sex-specific dysregulation of gene transcription profiles within and between these regions. This was identified by comparing the overlap in differentially expressed gene (DEG) lists in the meA and VTA from animals euthanized one hour after an injection of saline or cocaine.

When comparing these DEG lists between regions, I found that isolation is associated with altered overlap in meA–VTA drug- and saline-injection-responsive transcription profiles compared to controls that were group-housed throughout adolescence, with isolated animals showing comparatively enhanced transcriptional overlap between the meA and VTA; females showed enhanced overlap in meA–VTA saline-responsive transcriptional profiles and males cocaine-responsive profiles. Assessing how the transcriptional response to cocaine and saline overlaps in within-region comparisons of the meA and VTA, *i.e.*, meA vs. meA and VTA vs. VTA, I found that, in comparison adolescent isolation enhances overlap between cocaine and saline. Compared to group-housed controls, isolated females show greater overlap between cocaine and saline in the meA and lesser overlap in the VTA. Males are not substantially different in comparison to their group-housed counterparts in the overlap between cocaine and saline in the meA but show comparatively greater overlap in the VTA.

In a candidate gene approach using RT-qPCR, we find that within both the meA and VTA, adolescent isolation influenced the expression of glutamatergic markers in opposing directions in females and males. In comparison to their group-housed counterparts, social isolation led to elevations of glutamatergic markers in females but reductions in males. Isolation increased the expression of dopamine receptors in males but not in females, elevating expression of both D1 and D2 in the meA and D2 in the VTA in comparison to male group-housed mice. Though further work should validate the functional implications of these gene expression changes by assessing whether they manifest in different levels of their respective proteins between group-housed and isolated animals, these findings indicate that adolescent isolation disrupts targets critical to normative circuit maturation and motivated behavior by altering glutamatergic and dopaminergic signaling.

The medial amygdala undergoes reorganization during adolescence and is a region important for social cognition and social behaviors. Because of this, it is a prime region of interest in the development of disorders in neuropsychiatric diseases that where risk begins in adolescence, such as substance use disorder, anxiety disorders, and depression. However, the meA is understudied in the context of adolescent-onset neuropsychiatric risk in comparison to other limbic subregions that are part of canonical reward circuitry. The work presented in Chapter 2 identifies the medial amygdala as transcriptionally responsive to cocaine and stress along with the ventral tegmental area – a responsivity which is dysregulated by adolescent isolation; furthermore, Chapter 2 finds that adolescent isolation sex-specifically augments glutamatergic- and dopamine-related targets in these regions, which may illuminate, in part, a mechanism by which adolescent social adversity induces behavioral and cognitive pathology. This work indicates that the ventral tegmental area is influenced by adolescent social isolation and identifies the medial amygdala as a key brain region in the development of adolescent-onset psychopathologies such as substance use disorder, positioning it among canonical mesocorticolimbic reward in its importance.

While the meA and VTA represent critical subcortical nodes of adolescent neurodevelopment and reward processing, the prefrontal cortex (PFC) serves as the primary cortical hub orchestrating top-down regulation of these regions. Like the meA, the PFC undergoes extensive reorganization during adolescence, but uniquely, is the last brain region to reach adult-typical organization (Naneix et al., 2012; Willing et al., 2017). Through direct projections to the nucleus accumbens, amygdala, and ventral tegmental area, the PFC exerts top-down regulation of social- and reward-related behaviors by modulating glutamatergic, GABAergic, and dopaminergic neurotransmission (Alexandra Kredlow et al., 2022; Del Arco & Mora, 2008; Gabbott et al., 2005; Morales & Margolis, 2017; O'Connell & Hofmann, 2011; Russo & Nestler, 2013). Development of the PFC is characterized by increases in dopaminergic innervation, hormone-dependent refinement of inhibitory circuits, and synaptic pruning and increased myelination (Kritzer, 1998; Piekarski, 2017; Spear, 2013; Willing et al., 2017). Corresponding with overproduction of axons and synapses in early adolescence and marked pruning in late adolescence, human magnetic resonance imaging (MRI) indicates gray matter volume changes in an inverted U-shaped manner during adolescence, with frontal gray matter volume peaking in early adolescence and decreasing into adulthood (Andersen et al., 2000; Crews et al., 2007; Giedd, 2004; Giedd et al., 1999). These developmental changes are speculated to be the biological basis of developmental neuroplasticity within the PFC during adolescence, which promotes learning necessary for the successful acquisition of adult behaviors. However, this period of plasticity leaves the developing brain vulnerable to adverse experiences; accordingly, dysregulation of the PFC is a central neurobiological feature of psychiatric conditions like substance use disorders (SUDs), eating disorders including binge-eating disorder (BED), where adverse adolescent experiences are thought to induce maladaptive top-down regulation of behavioral and cognitive responding (Ahn et al., 2022; Goldstein & Volkow, 2011; Koob & Volkow, 2016).

As reviewed in the General Introduction, isolation or negative peer experiences during adolescence are associated with risk for the emergence of anxiety and depression (Begni et al., 2020; Blasio et al., 2014; Bonati et al., 2022; Guessoum, 2020; Levine, 2012; Loades, 2020; Mates & Allison, 1992).

To characterize the biological mechanism through which adolescent isolation disrupts the development of the PFC, we employed complementary behavioral and physiological assessments. Previous work has established the PFC as centrally involved in binge eating in both humans and rodent models (Blasio et al., 2014; Boeka & Lokken, 2011; Gluck et al., 2017). Because these sex differences in food intake develop during adolescence (Culbert et al., 2013; Marshall et al., 2017) in tandem with a peak in reward sensitivity for and intake of palatable food (Friemel et al., 2010), we examined the lasting sex-specific influence of adolescent isolation on feeding behavior. First, we examined palatable food intake, a naturalistic reward-seeking behavior with established sex differences (Buczek et al., 2020; Freeman et al., 2021; Klump et al., 2013; Marshall et al., 2017; Murray & McCormick, 2022). Next, given the importance of hormones across adolescence in inducing organizational and epigenetic changes throughout brain development, we examined levels of hormones that regulate both stress responses and neurodevelopment, corticosterone (CORT), thyroid hormones (triiodothyronine, T3; thyroxine, T4), and progesterone (P4), to determine whether adolescent isolation is accompanied by endocrine dysregulation. Then, to understand how a history of adolescent isolation influences stress- and drug-responsive gene transcription in the PFC, we analyzed publicly available RNA-sequencing dataset from experiments conducted in mice isolated through adolescence and then administered cocaine or saline via experimenter injection. We identified region-, sex- and stimulus-dependent differences in the regulation of cocaine and saline within and between the PFC, nucleus accumbens (NAc), and (VTA), three key hubs of dopaminergic activity. Given that lasting transcriptional changes can reflect altered epigenetic regulation of these genes, we then examined the expression of genes encoding epigenetic regulators, to identify potential

mechanisms through which isolation-induced changes become persistently encoded in the PFC transcriptome. And finally, because the PFC is comprised of excitatory and inhibitory projection neurons and receives dopaminergic input from the VTA, we employed a candidate gene approach using qPCR to determine whether isolation disrupts the normal developmental trajectory of key neurotransmitter systems, examining the expression of dopaminergic, GABAergic, and glutamatergic genes at two critical timepoints: mid-isolation (P32) when active remodeling occurs, and in adulthood (P72) to assess persistent changes.

Together, our findings indicate that adolescent isolation induces sex-specific dysregulations of serum hormones, where isolation altered thyroid hormone (T3/T4) ratio in males but not females and reduced corticosterone levels in females without augmenting levels in males. Additionally, we find sex-specific alterations of dopaminergic, excitatory, and inhibitory gene expression profiles across development in the prefrontal cortex. Further, we find that adolescent isolation is associated with substantial changes in drug- and handling-stress-responsive transcriptional profiles, where isolation enhances transcriptional concordance between the PFC and NAc following stress (saline injection) in both sexes but particularly in males, reorganizes cocaine-responsive coordination with females losing PFC-VTA synchrony while gaining PFC-NAc overlap, and alters within-region stimulus discrimination such that isolated males show reduced cocaine-saline discrimination in the NAc but enhanced overlap in the PFC.

Materials and Methods

Animals

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by Oregon Health & Science University Institutional Animal Care and Use Committee. Figure 7 illustrates the timeline of the housing manipulations, testing, and tissue collection. Black C57BL/6J mice were purchased from Jax Jackson Laboratory and bred in-house and housed under constant humidity and temperature (21–22°C) and on a 12 h light/12 h dark cycle (lights off at 6:00 pm). Food and water were available ad libitum. On postnatal day (P) 21 (P21), animals were weaned into mixed litter groups, 4–5 animals per cage. To control for litter effects, animals were combined across litters at weaning. The following day animals were either housed either alone in standard acrylic transparent cage or placed in a clean cage with their P21 cage. Therefore, animals were weaned between P20 and P23 to have enough animals per cage per group. All mice were housed in the same room as our colony; thus, isolated mice could hear, smell, and see other mice in the room. Body weights were monitored every 10 days throughout the life cycle. Mice were monitored daily for secondary sex characteristics indicative of puberty onset (preputial separation in males and vaginal opening in females). After the onset of puberty, estrous cycles were monitored via daily vaginal smears. After 3 weeks of isolation (~P42), isolated mice were group-housed (4–5 animals per cage) for the remainder of the experiment. Behavioral tests began four weeks later (~P72).

Palatable food intake test

To aid in the determination of each mouse's food intake, all mice (n=89) were singly housed the day prior to and the day of the feeding test. Additionally, to prevent neophobia to the palatable food (Supreme Mini Treats™, Chocolate Flavor, 1g Pellets; BioServ, Flemington, NJ), all mice

received 1-hour access to a pre-measured amount of palatable food in a paper feeding cup the day before the feeding test. On the test day, mice were single housed for a 1-hour acclimation period prior to the feeding test. Access to the palatable food occurred in the presence of *ad libitum* chow. Approximately 4 hours after lights on (10:00 am), ~5g of palatable food was given to each mouse for 1 hour in their home cage in addition to ad lib chow. All testing was conducted under dim red light. Body weight and chow were measured 1 hour prior to the start of the feeding test. At the end of the 1-hour palatable food access period, all remaining palatable food and chow were measured, and both palatable food and feeding cups were removed from each mouse's home cage. Palatable food testing was conducted in two cohorts of animals: Cohort 1 Ctrl: 15 females, 15 males (total = 30); Cohort 1 Iso: 14 females, 11 males (total = 25); Cohort 2 Ctrl: 7 females, 9 males (total = 16); Cohort 2 Iso: 8 females, 10 males (total = 18)

Serum hormone assays

Submandibular blood draws were used to collect serial blood draws in a separate cohort of animals. Using a longitudinal study design, repeated blood draws were conducted in the same animals on the day of isolation (P22; prior to isolation), 10 days after isolation (P32), on the day of rehousing (P42), and in adulthood (P72) to determine how SI might disrupt serum hormone concentrations. After collection, blood was allowed to clot at room temperature for approximately 1 hour and centrifuged at 4°C for 10 min at 5,000 X G to separate serum. Serum was collected and stored at -80°C until processing.

At each age, 8–10 animals per group were used. However, because a small amount of serum was collected in young animals, serum was pooled across two animals on P22 and P32 to ensure enough sample was available for processing. Samples were extracted using acetonitrile and pellets were resuspended in 50µL assay buffer for the Luminex steroid hormone multiplex kit (MilliporeSigma, St Louis, MO) to measure multiple hormones from the same sample (corticosterone, CORT; progesterone, P4; and thyroid hormones, triiodothyronine, T3;

thyroxine, T4). Samples were run in the laboratory of Dr. Andrew Wolfe at Johns Hopkins University according to manufacturer's protocol. All samples were collected and analyzed in a single run across three plates.

Experimenter administered intraperitoneal cocaine injections

Mice in Walker et al. (2022a & b) underwent either adolescent social isolation or group housing, then as adults received a series of 10 intraperitoneal (IP) injections, one per day. All animals received 9 injections of saline over 9 consecutive days. On day 10, animals received either: (1) a 10th saline injection and were euthanized 1 hour later (“acute saline”), (2) their first cocaine injection and were euthanized 1 hour later (“acute cocaine”), or (3) a 10th saline injection and were euthanized 24 hours later (“habituation control”). The habituation control group serves as a baseline for quiescent gene expression; though the handling associated with saline injections induces a stress and transcriptional response even after repeated habituating exposures, these responses subside by 24 hours. Accordingly, this design enables the within-group distinction between stress-responsive (*i.e.*, acute saline vs. habituation) and cocaine-responsive (*i.e.*, acute cocaine vs. habituation controls) gene transcription profiles against a transcriptional background that keeps both housing and handling experiences consistent.

Tissue collection and storage for molecular endpoints

For the animals used in the qPCR, a total of 58 animals were used. Methods of tissue collection were the same as those used in Walker et al. (2022a & b). Animals were euthanized via rapid decapitation between 10:00–14:00 hours, before lights out, at two separate timepoints: (1) 10 days post-isolation (~P32; n=4–8/group), and (2) 30 days after rehousing (~P72; n=6–8/group). Brains were extracted, chilled in cold phosphate-buffered saline (PBS), and sectioned

in a cold brain matrix (1 mm sections). One 12-gauge PFC punch was snap frozen on dry ice and stored at -80°C until RNA isolation.

RNA isolation and preparation for quantitative PCR (qPCR)

RNA was isolated from frozen PFC punches using a modified protocol (Walker, 2018) for the PureLink RNA Mini Kit (ThermoFisher, Waltham, MA). Tissue was homogenized in QIAzol (Qiagen, Fredrick, MD) followed by chloroform extraction and on-column RNA clean-up. Concentration was determined on a NanoDrop Spectrophotometer (ThermoFisher, Waltham, MA). RNA (300 ng) was converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol and diluted 1:15 with ultrapure water before qPCR reactions. Gene expression was assessed using RT-qPCR that was performed using PowerUp™ SYBR™ Taq polymerase (Fisher, Waltham, MA) on an ABI QuantStudio5 qPCR machine under the following parameters: 50°C for 2 min; 95°C for 10 min and 45 cycles (95°C for 15 sec then 60°C for 15 sec); a melt curve was run at the end of every plate to confirm primer specificity and absence of genomic DNA. Primers were designed in-house using NCBI Primer-BLAST to span exon–exon junctions, synthesized by Integrated DNA Technologies (Coralville, IA), and validated for efficiency (100 ± 10%) using standard curve analysis. For a complete list of genes and primer sequences, see (Appendix A1). All targets were run in triplicate and relative expression was determined using the comparative CT method (Livak & Schmittgen, 2001) using median CT values for each gene.

Statistical analysis of palatable food test, hormone assays, and qPCR data

Statistical analyses were conducted using SPSS version 30.0.0.0 (SPSS, Chicago, IL, USA). For all endpoints, a generalized linear model (GLM) was used to examine differences in palatable food consumption data (normalized to body weight), serum hormone concentration differences, and relative expression (qPCR) with sex (male vs. female), housing (group-housed,

"Ctrl" vs. socially isolated, "Iso"), age, and cohort (in PFT analyses) included as factors. Main effects and interactions of each factor were assessed. When interactions were observed in our analysis, we split data by that factor and analyzed the effects of the other factors. When appropriate, post-hoc analyses were conducted using the Šídák test. Because our RRHO analysis showed sex-specific effects of isolation, we analyzed maturational changes in PFC relative gene expression in males and females separately.

RT-qPCR experiments were run in three rounds. Samples were normalized to the geometric means for two housekeeping genes (*Bactin* and *Hprt1*) after confirmation of no significant impacts of sex, age, or housing on housekeeping gene expression. Exclusion based on housekeeping gene expression variance resulted in 2 samples excluded from the PFC. Prior to analysis, outliers were removed using standardized residuals (z-score of +2.5 SD from the line of best fit), or Grubbs's test when nonparametric (critical z-score value 95% confidence). Samples that met inclusion criteria were then calibrated to the mean δ-CT of the control group for each sex to determine fold-change in expression for each animal as described in (Walker et al., 2018).

Bioinformatic analyses

Differential expression analyses: Publicly available differential gene expression lists from the PFC, VTA, and NAc were generated using DESeq2 as previously described in (Walker et al., 2022a & b). We utilized publicly available RNA-sequencing data from mice (n=6–8 animals/group; total=116 samples) that underwent adolescent social isolation (P22–42) or group housing, then ~P80 received an injection of saline each day for 9 days, then, on the 10th day, received a final injection of either saline or cocaine (7.5 mg/kg) before being euthanized 1 hour or 24 hours later (Walker et al., 2022a & b).

Rank-rank hypergeometric overlap: Stratified rank-rank hypergeometric overlap (RRHO) analysis was performed using the RRHO2 script in R as described in (Cahill et al., 2018). RRHO

is a method which allows for threshold-free comparisons of transcriptional profiles by ordering lists of differentially expressed genes by their p-value (lowest to highest) and direction of change (up- or downregulated) to determine the direction of overlapping transcripts across two comparisons (Cahill et al., 2018).

Epigenetic factor gene classification: We analyzed 718 genes that express epigenetic factors from the EpiFactors database (Medvedeva et al., 2015). Our analyses were conducted on categories based upon enzymatic function (writers: n=246, erasers: n=115, readers: n=88) and chromatin regulatory effect (repressive: n=250, permissive: n=195, context-dependent: n=348). Gene lists were manually curated based on current literature to ensure accurate functional classification.

Statistical analysis of epigenetic factor expression: All analyses were performed in R using custom scripts. Statistical significance was set at $p<0.05$.

Count-based analysis: Fisher's exact test compared the proportion of epigenetic factor DEGs between Iso and Ctrl groups, testing whether isolation altered the expression of epigenetic-regulation-related genes (*e.g.*, number of significant epigenetic factor DEGs/total DEGs).

Expression magnitude analysis: We compared $\log_2$ fold-change values of all epigenetic factor genes between Iso and Ctrl groups using unpaired t-tests – a threshold-free approach to assess coordinated expression changes. Effect sizes were quantified using Cohen's d.

Results

Adolescent SI disrupts reward- and anxiety-related feeding behaviors in adulthood

To test the influence of adolescent isolation on natural reward, we used the palatable food test (PFT). While prior work has shown that SI has sex-specific effects on reward-associated behaviors (Bendersky et al., 2021; Li et al., 2021; Walker et al., 2019), no previous work has assessed how isolation influences consumption of palatable food. Mice were isolated

or weaned into mixed-litter groups from approximately P22–P42 then rehoused. Mice then remained group-housed until behavior testing around P72 (Fig. 7a). GLM revealed expected baseline differences: a main effect of sex on body weight [Fig. 7b; Wald $\chi^2(1)=212.59$, $p<0.001$] with no housing influence, and females consuming more palatable food than males overall [Fig. 7c; Wald $\chi^2(1)=15.31$, $p<0.001$], consistent with prior work (Klump et al., 2013; Sinclair et al., 2017). There was also a significant effect of cohort [Fig. 7c; Wald $\chi^2(1)=170.21$, $p<0.001$], and a cohort × sex interaction [Wald $\chi^2(1)=4.03$, $p=0.045$] indicated that the baseline sex difference in consumption was more pronounced in Cohort 1 (female=0.052 units, male=0.022 units; $p=0.041$) than Cohort 2 (female=0.024 units, male=0.022 units; $p=0.746$).

Our key finding was a significant sex × housing interaction for palatable food intake normalized by body weight [Fig. 7c; Wald $\chi^2(1)=4.62$, $p=0.032$], revealing that isolation selectively increased palatable food intake in males [Wald $\chi^2(1)=13.45$, $p<0.001$] but had no effect on female consumption.

Together, these findings demonstrate that adolescent isolation sex-specifically increases hedonic feeding in males while leaving female reward-related consumption intact. These findings suggest that adolescent isolation disrupts normal sex differences in hedonic feeding, enhancing male, but not influencing female, consumption.

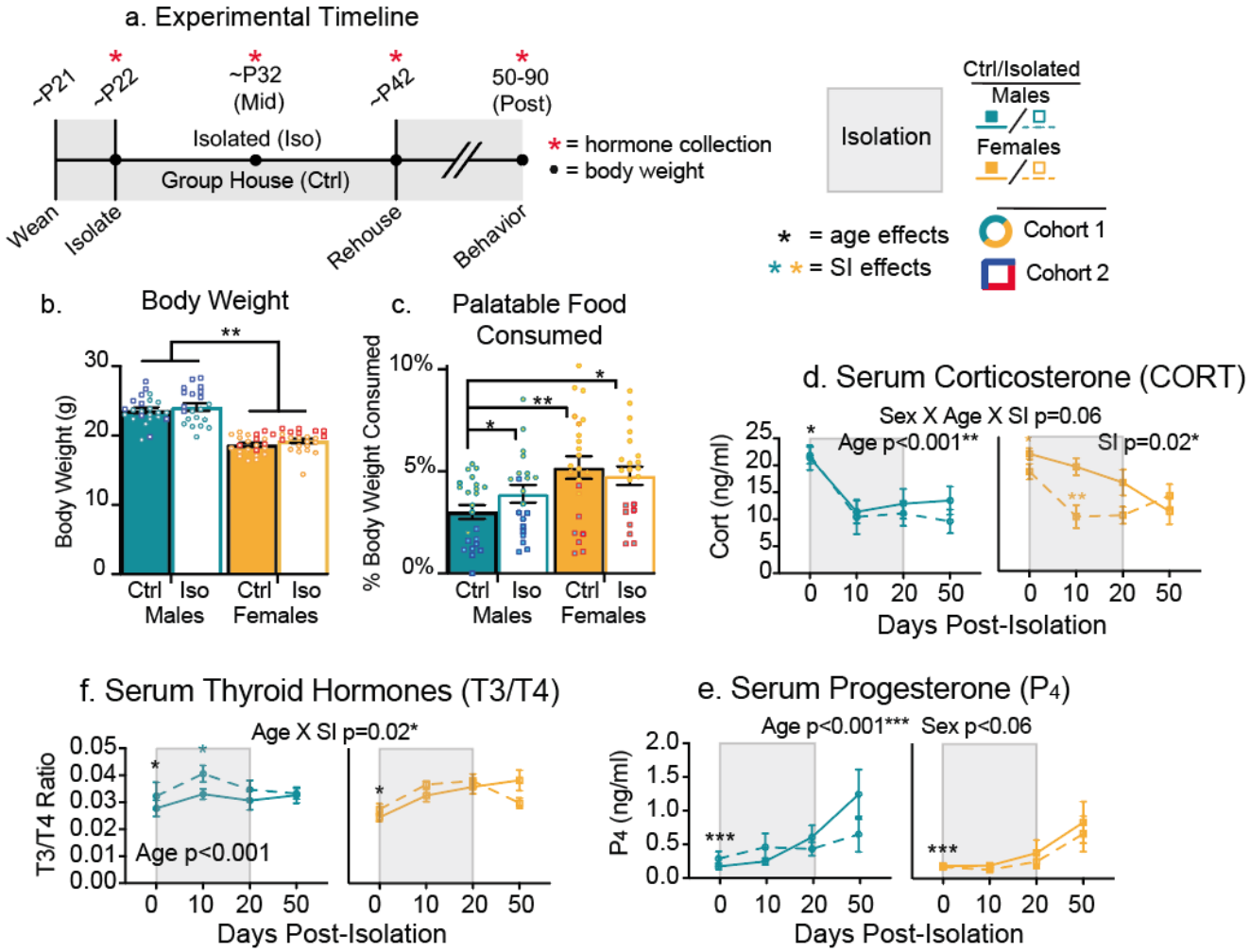


Figure 7. Adolescent isolation sex-specifically influences palatable food intake and hormone levels. (a) Schematic of timeline for adolescent isolation, weight measurement, and tissue/hormone collection. (b) Quantification and comparison of mouse body weight. (c) Palatable food intake weight as a percentage of animal body weight; BEP, binge-eating prone; BER, binge-eating resistant. (d) Serum levels of corticosterone. (f) Serum levels of the ratio of active vs. inactive thyroid hormone (T3 and T4, respectively). (e) Serum levels of progesterone hormone. Significance is indicated as *p<0.05, **p<0.01, ***p<0.001. Error bars indicate SEM. Solid lines, group housed; dashed lines, socially isolated; P, postnatal day.

Adolescent SI disrupts developmental levels of serum hormones in males and females

Developmental changes in hormone concentrations result in long-term brain and behavioral consequences; prior work has found that thyroid hormone is implicated in pubertal onset and more generally in brain developmental periods of heightened plasticity (Batista &

Hensch, 2019; Taylor et al., 2017; Wang et al., 2021) and a thyroid-signaling-related protein was found to be causally related to phenotypic differences observed as a result of adolescent isolation (Walker, 2022a). We hypothesized that isolation-induced alterations in sex-specific behaviors may be due to disruption of developmental hormones. We tested the hypothesis that adolescent isolation alters developmental levels of corticosterone (CORT), progesterone (P4), and thyroid hormones (triiodothyronine, T3; thyroxine, T4) across the lifespan of mice.

Longitudinal GLM analysis revealed significant age-dependent reductions in serum corticosterone [Fig. 7d; Wald $\chi^2(1)=5.857$, $p=0.016$] and age-dependent increases in P4 (Fig. 7e) across development in both sexes (mean difference=0.727 ng/mL, $p<0.001$). However, only corticosterone levels were significantly affected by isolation, with isolated females showing significantly lower corticosterone 10 days post-isolation compared to control females (mean difference=9.73 µg/dL, $p=0.049$); corticosterone levels return to levels comparable to that of controls following the end of isolation and into adulthood. The T3/T4 ratio serves as a more complete picture of functional thyroid status at the tissue level, beyond what TSH and individual hormone levels can reveal (Hadi et al., 2024; Holtorf, 2014). Accordingly, we assessed the T3/T4 ratio across isolation. Longitudinal GLM analysis revealed that isolation resulted in altered T3/T4 ratio in a significant age × housing effect [Fig. 7f; Wald $\chi^2(1)=8.268$, $p=0.004$], where isolated males showed a higher T3/T4 ratio in mid-isolation adolescence compared to in adulthood (mean difference=0.007 units, $p=0.011$), which recovered to control-comparable levels following rehousing. These longitudinal analyses suggest that serum hormone dysregulation by adolescent isolation may contribute to brain and behavioral differences observed in isolated animals, though their levels are not persistently altered following rehousing.

Drug- and stress-responsive transcriptional regulation of mesocorticolimbic circuitry is reorganized by adolescent isolation

Because of our palatable food findings and because palatable food intake is, in part, a PFC-mediated behavior (Ahn et al., 2022; Blasio et al., 2014; Sinclair et al., 2017), we hypothesized that adolescent isolation induces lasting transcriptional dysregulation between the PFC and the NAc and VTA. To test this, we used stratified rank-rank hypergeometric overlap (RRHO) to compare publicly available RNA-sequencing PFC gene transcription profiles of mice that were isolated during adolescence then given injections of cocaine or vehicle (Walker et al., 2022a & b). Previous work has demonstrated that overlap between regions in reward circuitry

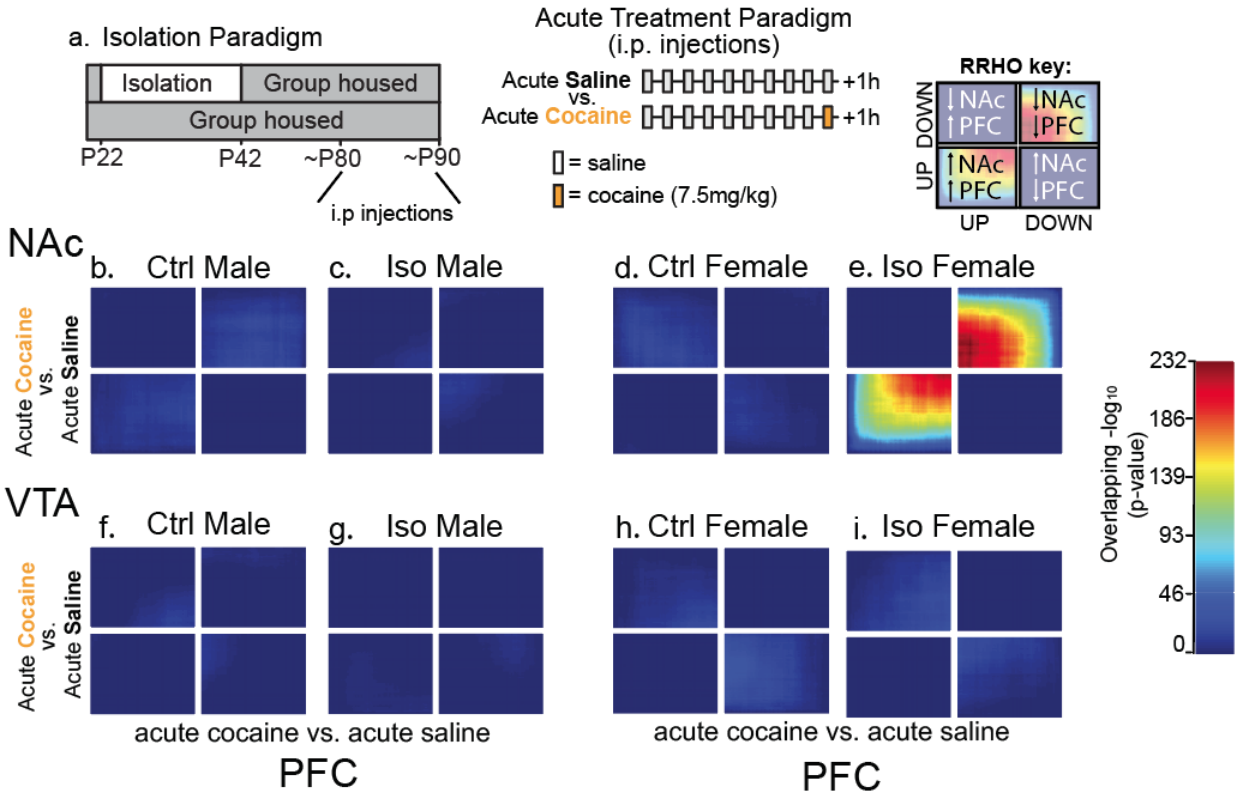


Figure 8. Isolation enhances overlapping regulation of acute cocaine and saline transcriptional profiles between the prefrontal cortex and nucleus accumbens in females. (a) Schematic of timeline for adolescent isolation, and cocaine and saline intraperitoneal injections. Key for reading rank-rank hypergeometric overlap plots. RRHO plots comparing transcriptional response to acute cocaine (1 hr post-cocaine injection) vs. acute saline (1 hr post-saline injection) between the prefrontal cortex (PFC) and the nucleus accumbens (NAc, b–e; b, maximum −log10 p-value=23; c, maximum −log10 p-value=13; d, maximum −log10 p-value=22; e, maximum −log10 p-value=232) and ventral tegmental area (VTA, f–i; f, maximum −log10 p-value=16; g, maximum −log10 p-value=8; h, maximum −log10 p-value=37; i, maximum −log10 p-value=27).

has been associated with different behavioral outcomes (Bagot et al., 2016). To identify overlap in injection-stress- and cocaine-responsive transcriptional profiles, these mice were all treated with 9 prior habituating daily doses of saline over 9 days. On the 10th day, these mice were either injected with saline then euthanized 1 hour later (“acute saline”) or 24 hours later (“habituation controls”), or were injected with a 7.5mg/kg dose of cocaine (“acute cocaine”; for paradigm see: Fig. 8a).

RRHO indicates that females that were isolated during adolescence show greater concordant overlap between the PFC and NAc (-log10 p=232), in comparison to control females (-log10 p=22), control males (-log10 p=23), and isolated males (-log10 p=13). Little overlap in gene transcriptional response was observed between the PFC and VTA in all groups (Fig. 8f–I,

max -log10 p=37 in isolated males). This indicates that there is a comparatively greater level of concordant overlap in gene transcription profiles in response to acute cocaine and saline administration in the PFC of isolated female mice versus their group-housed counterparts and versus males. While these transcriptional profiles are augmented in isolated females, these acute-cocaine-induced differential gene expression lists are from comparisons where both animals were euthanized one hour after an injection of saline (Fig. 8a). While this comparison controls for injection and handling stress by comparing conditions where mice were euthanized one hour after injections, controlling for handling stress may conceal key differences in stimulus-dependent transcription between isolated and group-housed-control animals because both acute timepoints include the same injection-associated stimuli. Prior work (including that described in Chapter 2) indicates that the handling and injection stimuli associated with the administration of saline stimuli can induce robust transcriptional responses, leading to the enrichment of IEG expression throughout the reward circuitry in mice (Cates et al., 2018; Walker et al., 2022a & b). Analyzing overlap of differential gene expression contrasts of acute saline or cocaine are made against the same quiescent transcriptional background may inform whether isolation influences the transcriptional response to cocaine and handling-stress or handling-stress alone.

Due to these considerations of stimulus-responsive transcription, we sought to uncover whether cocaine-induced transcriptional profile overlap and injection-stress-induced transcriptional profiles differ in control and isolated animals. To test this, we compared cocaine-injection-induced (Fig. 9b–i) or saline-induced (Fig. 10b–i) transcriptional profiles against a more quiescent transcriptome (habitation controls) of animals euthanized after 10 intraperitoneal (IP) saline injections then euthanized 24 hours after the final injection. We consider this to be a quiescent transcriptome because gene transcription is temporally distant from the stimulus of experimenter injections, a consideration supported by a lack of differentially expressed genes

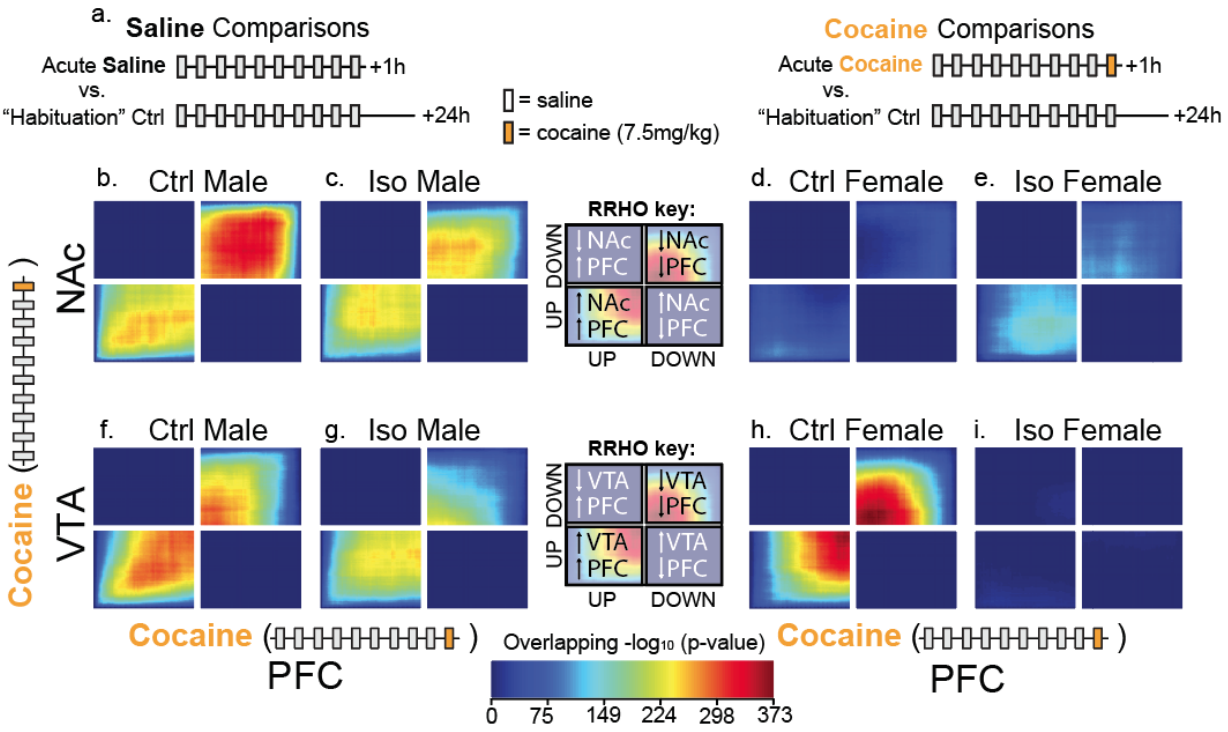


Figure 9. Adolescent isolation is associated with circuit-wide dysregulation of mesocorticolimbic transcriptional response to cocaine in adulthood. (a) Schematic of timeline for adolescent isolation, and cocaine and saline intraperitoneal injections. Key for reading rank-rank hypergeometric overlap plots. RRHO plots comparing transcriptional response to cocaine (1 hr post-cocaine injection) vs. habituation control (24 hrs post-saline injection) between the prefrontal cortex (PFC) and the nucleus accumbens (NAc, b–e; b, maximum −log10 p-value=340; c, maximum −log10 p-value=267; d, maximum −log10 p-value=104; e, maximum −log10 p-value=158) and ventral tegmental area (VTA, f–i; f, maximum −log10 p-value=299; g, maximum −log10 p-value=254; h, maximum −log10 p-value=373; i, maximum −log10 p-value=20).

(Walker et al., 2022a & b; adj. $p<0.05$) in the isolated vs. group-housed control animals (PFC, NAc, and VTA: Isolated males vs. control males = 0; Isolated females vs. control females = 0) and the enrichment of immediate early and activity-dependent genes in both the acute saline

and acute cocaine genes lists when compared to the habituation control group (Walker et al., 2022a & b). We posit this to be the case because the stimulus-responsive transcriptional response following handling and injection has subsided. As a result, the habituation control provides a within-housing-condition quiescent transcriptomic control where all features of experimental manipulation are matched save for the timing following the final injection of saline. Therefore, as in Chapter 2, differential gene expression contrasts of acute saline or cocaine vs. habituation controls allows for the separation of injection-responsive and cocaine-responsive gene transcriptional profiles to inform, using RRHO analyses, which component differs in isolation vs. control animals' transcriptional regulation within the PFC, NAc, and VTA.

When examining overlap in transcriptional profiles between the PFC and canonical reward circuitry, specifically the NAc and VTA, we find sex-specific influences of adolescent social isolation. We observe slight differences in concordant transcriptional regulation of cocaine

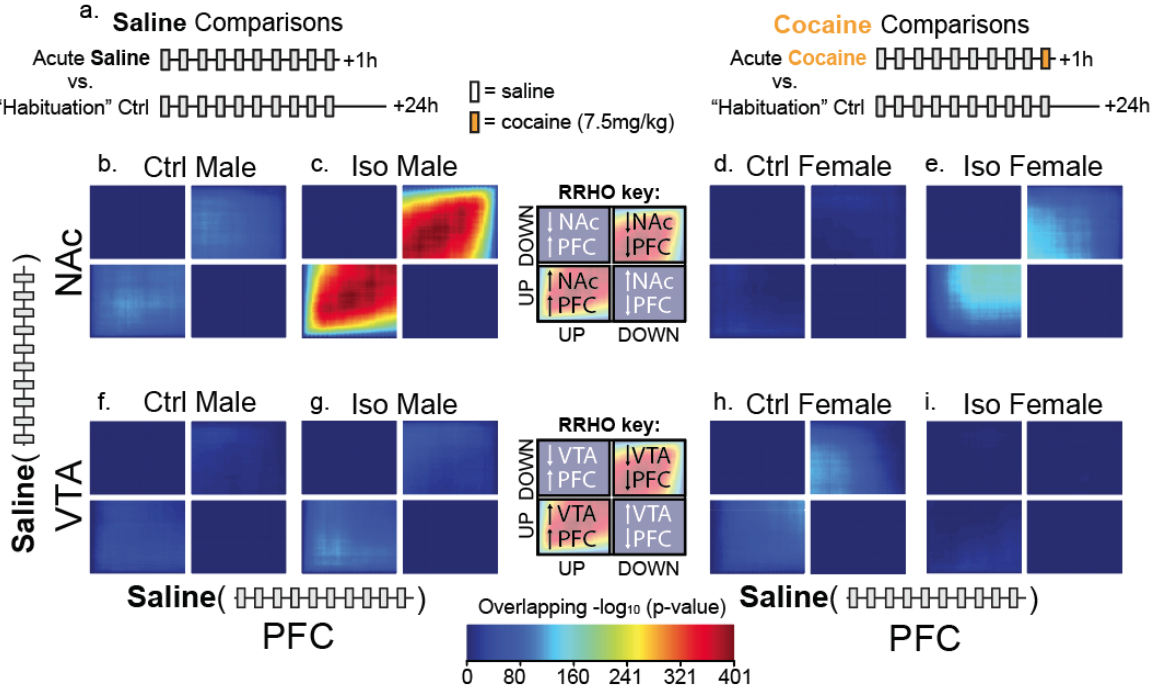


Figure 10. Adolescent isolation induces persistent enhancement in stress-responsive transcriptional overlap between prefrontal cortex and nucleus accumbens in males. (a) Schematic of timeline for adolescent isolation, and cocaine and saline intraperitoneal injections. Key for reading rank-rank hypergeometric overlap plots. RRHO plots comparing transcriptional response to saline (1 hr post-saline injection) vs. habituation control (24 hrs post-saline injection) between the prefrontal cortex (PFC) and the nucleus accumbens (NAc, b–e; b, maximum −log10 p-value=340; c, maximum −log10 p-value=267; d, maximum −log10 p-value=104; e, maximum −log10 p-value=158) and ventral tegmental area (VTA, f–i; f, maximum −log10 p-value=299; g, maximum −log10 p-value=254; h, maximum −log10 p-value=373; i, maximum −log10 p-value=20).

between the PFC, NAc, and VTA in both control (Fig. 9d, max -log10 p=340) and isolated males (Fig. 9b, max -log10 p=267), indicating that adolescent isolation does not substantially perturb the regulation of these cocaine-responsive transcriptional profiles in males. However, it appears that there is a substantial decrease in comparative concordant overlap between the PFC and VTA associated with a history of adolescent isolation in females; isolated females exhibit low levels of overlap in transcriptional response to cocaine (Fig. 9b, -log10 p=20) versus control females (Fig. 9b, -log10 p=373). Conversely, regarding regulation of cocaine response between the NAc and PFC, there is a comparative enhancement of concordant regulation in isolated females (Fig. 9b, -log10 p=340) versus control females. Taken together, these findings indicate that the circuit-wide regulation of cocaine responsivity is more affected by isolation in females than in males.

To understand how adolescent isolation might persistently alter stress- and handling-stimulus-responsive gene transcription profiles, we next assessed the overlap between the PFC, NAc, and VTA in response to injections of saline (Fig. 10). Injections and their associated handling stimuli are stressful to rodents and even following repeated habituating exposures, they persistently elicit a stress response, though after a 24-hour period the transcriptional response does return to baseline (Ryabinin et al., 1999; Sharp et al., 2003; Walker, 2022b). When examining the transcriptional response to injection-handling stress, we find that adolescent isolation is associated with comparatively greater overlap of the saline-injection-responsive gene transcription between the PFC and NAc in isolated males (Fig. 10b, -log10 p=401) and females (Fig. 10d, -log10 p=169) versus their group-housed control counterparts (control males Fig. 10a, -log10 p=116; control females Fig. 10c, -log10 p=25). With regards to transcriptional regulation of saline between PFC–VTA, isolated males (Fig. 10f, -log10 p=116) and control females (Fig. 10h, -log10 p=132) exhibit comparable levels of concordant overlap in gene transcription profiles over the relatively low levels of transcriptional overlap in both control males (Fig. 10e, -log10 p=50) and isolated females (Fig. 10h, -log10 p=25). These data suggest

that there are sex-specific differences in stimulus-dependent transcription between the PFC, NAc, and VTA associated with a history of adolescent isolation.

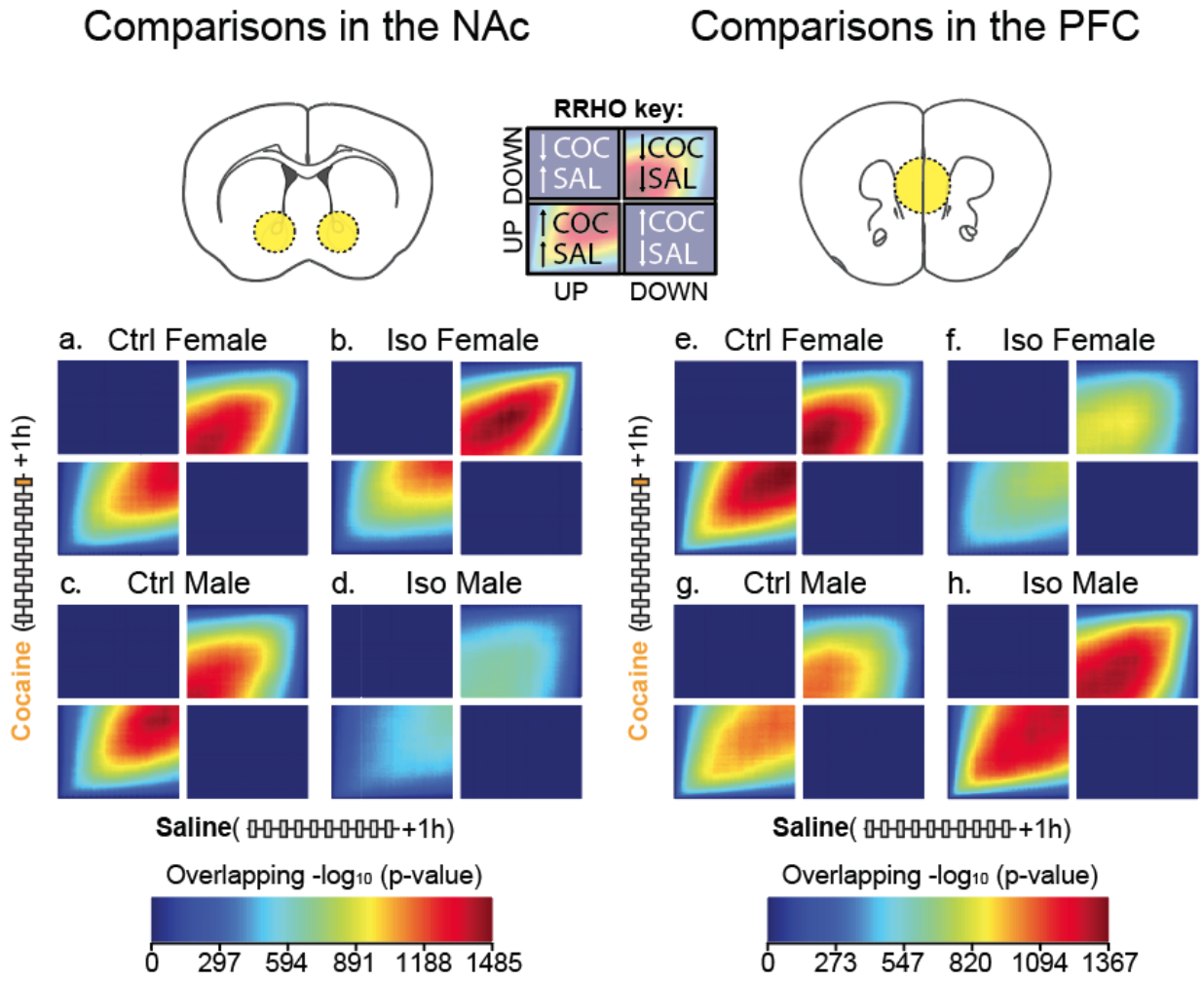


Figure 11. Adult transcriptional regulation of cocaine and saline within the nucleus accumbens and prefrontal cortex is sex-specifically altered by adolescent isolation. Key for reading rank-rank hypergeometric overlap plots. RRHO plots comparing transcriptional response to cocaine (1 hr post-cocaine injection) vs. habituation control (24 hr post-saline injection) against saline (1 hr post-saline injection) vs. habituation control (24 hr post-saline injection) within the nucleus accumbens (NAc, a–d; a, maximum −log10 p-value=1409; b, maximum −log10 p-value=1485; c, maximum −log10 p-value=1435; d, maximum −log10 p-value=683) and prefrontal cortex (PFC, e–h; e, maximum −log10 p-value=1367; f, maximum −log10 p-value=867; g, maximum −log10 p-value=1069; h, maximum −log10 p-value=1306).

The sex differences in stimulus-dependent transcription between the PFC and NAc of isolated animals provide evidence that isolation results in region-specific dysregulation of drug- and stimulus-dependent transcription. Next, we sought to examine how overlap in regulation of gene transcription *within* the PFC and NAc differ (*i.e.*, comparisons of PFC vs. PFC and NAc vs. NAc) as a function of adolescent isolation to determine whether gain of injection-stimulus-

responsive transcriptional concordance in isolated males (Fig. 10c & 10i) and acute drug- and injection-responsive transcription in females (Fig. 8e) are associated with isolation-induced disruption of injection- or cocaine-responsive transcription in the PFC, NAc, or both regions. (Similarly to as shown for the meA and VTA in Chapter 2 in Figure 3e–h, where isolation was associated with enhanced overlap in female meA and male VTA and decreased overlap in female VTA.) We compared acute saline (y-axes, Fig. 11) and acute cocaine (x-axes, Fig. 11) transcriptional profiles within the NAc (Fig. 11a–d) or PFC (Fig. 11e–h). When examining overlap in gene transcription profiles within the NAc (*i.e.*, DEG contrasts of acute cocaine vs. habituation control against acute saline vs. habituation control), we observe comparatively lower overlap in isolated males (Fig. 11d, −log10 p-value=683) versus control males (Fig. 11c, −log10 p-value=1435) in their transcriptional response to cocaine and saline injections within the NAc. We find that the converse is the case in the PFC, where isolated males show greater concordant overlap (Fig. 11h −log10 p-value=1306) than control males (Fig. 11g, −log10 p-value=1069) in transcriptional response between cocaine and saline. The overlap in transcriptional response of the NAc to cocaine and saline does not appear to be influenced by social isolation in females, where they show similarly high levels of concordance in transcriptional overlap (control females, Fig. 11a, −log10 p-value=1409; isolated females, Fig. 11b, −log10 p-value=1485). However, in the PFC, we observe comparatively lesser overlap in isolated females than control females (control females, Fig. 11e, −log10 p-value=1367; isolated females, Fig. 11f, −log10 p-value=867). These findings suggest that adolescent isolation persistently augments the bulk gene transcriptional response between both cocaine and stress (*i.e.*, the handling stimuli associated with an injection of saline) in the PFC and NAc, where isolated animals show region- and sex-dependent shifts in transcriptional overlap, such that cocaine- and saline-responsive transcription become more distinct in some contexts (male NAc, female PFC) but more convergent in others (male PFC), while remaining largely unchanged in female NAc.

Adolescent isolation persistently dysregulates the epigenetic-related gene transcription response of PFC and NAc to saline and cocaine administration

Given that differences in gene transcription can reflect altered epigenetic regulation, we examined the expression of genes encoding epigenetic regulators – the molecular machinery that writes, erases, and reads chromatin modifications; functionally, these regulators establish differences in the accessibility of chromatin to govern gene expression by altering the steric properties and charge distribution of chromatin (Hamilton & Nestler, 2019; Hyun et al., 2017). This determines which transcriptional programs are accessible, inducing permissive or repressive gene transcription that impinges on neural responsivity. Using a curated database with translationally relevant epigenetic factors (Medvedeva et al., 2015), we categorized these epigenetic factor genes based on their functional roles (writers, erasers, readers) and their effects on chromatin state (whether they are transcriptionally repressive, permissive, or if their effect on transcription is context-dependent). While we did not directly measure chromatin modifications or protein levels of epigenetic factors, differential expression of these regulatory genes indicates a potential for altered capacity of epigenetic remodeling and may reveal which stimulus- and drug-responsive chromatin-modifying pathways are disrupted by adolescent isolation.

Isolation alters expression of epigenetic erasers in a sex- and region-specific manner

To determine whether adolescent isolation alters the expression of proteins related to epigenetic regulation, we analyzed the expression the epigenetic factors (from Medvedeva et al., 2015) following saline or cocaine administration. We employed two complementary approaches: (1) count-based analysis using Fisher's exact tests (FET) examining the proportion

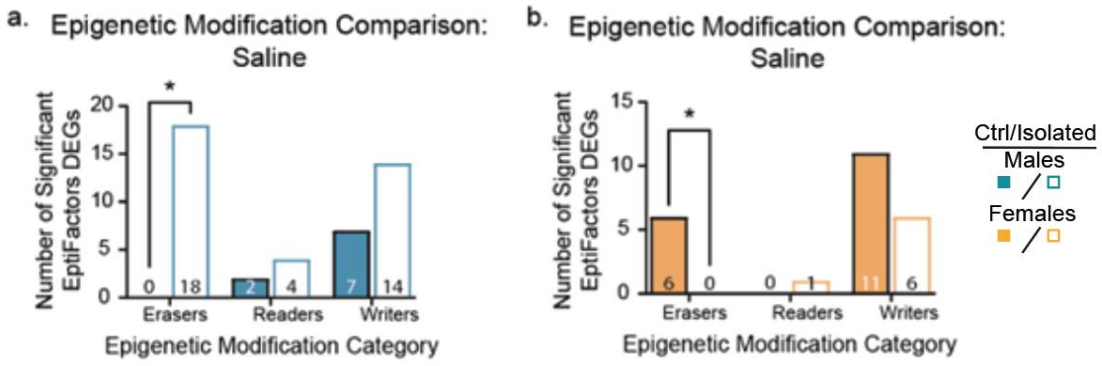


Figure 12. Stress-responsive gene expression for epigenetic erasers is differentially influenced by adolescent housing condition history. Quantitative comparison of significantly (adjusted p<0.05) differentially expressed genes by category (erasers, readers, and writers). Expression levels of erasers following saline injection are higher in isolated males (a) and control females (b). Significance is indicated as *p<0.05. Ctrl, group housed; Isolated, socially isolated.

of epigenetic factor DEGs (adj.p<0.05), and (2) magnitude-based analysis comparing aggregate expression changes across all genes within each category (regardless of p-value).

After first assessing for significant (adjusted p<0.05) enrichment over background gene expression within conditions then comparing differential representation between groups, we found that only epigenetic modification erasers showed significant differential expression between isolated and control animals, and this occurred exclusively in the PFC following saline injection. Specifically, isolated males had 18 erasers that significantly responded to saline-injection-stress (enrichment p<0.001) compared to zero in controls (0% of total DEGs in control males vs. 1.8% of total DEGs in isolated males, p=0.019). This indicates that, in males, isolation confers stress-responsiveness to the transcription of epigenetic erasers (see Appendix B1 for list of erasers) that is absent in male mice reared in a group-housed environment throughout adolescence. Females showed the opposite pattern with control females expressing 6 erasers (see Appendix B1 for list of erasers; enrichment p=0.023) versus zero in isolated females (1.9% of total DEGs in control females vs. 0% of total DEGs in isolated females, p=0.042). No other epigenetic factor categories showed significant count differences in differentially expressed

genes between control and isolated animals. These findings suggest adolescent isolation induces sex-specific effects on stress-responsive epigenetic eraser gene expression in the PFC.

To capture coordinated expression patterns, we also examined the magnitude of expression changes across all genes within categories regardless of individual significance, which indicated further differences in epigenetic-regulatory-related gene expression as a result of adolescent isolation. In the PFC of saline-treated males, isolation was associated with increased expression of epigenetic factors across multiple categories (all values shown as mean $\log_2$ fold change): writers (Iso 0.04 vs. Ctrl -0.04, p=0.043), repressive factors (Iso 0.064 vs. Ctrl 0.006, p=0.004), and permissive factors (Iso 0.066 vs. Ctrl 0.03, p=0.003). In response to cocaine administration, isolated males also showed substantially higher magnitude of expression of writer factors (Iso 0.08 vs. Ctrl -0.01, p=0.012). This coordinated upregulation suggests a potential for heightened capacity of chromatin remodeling in the PFC of male mice that underwent adolescence in isolation. Conversely, in females, there was an isolation-associated decrease in the expression of writer (Iso 0.025 vs. Ctrl 0.05, p=0.019) and reader (Iso 0.016 vs. Ctrl 0.068, p<0.001) factors in the PFC following cocaine administration.

In the NAc, only one statistically significant difference was observed in the magnitude of expression of epigenetic-related factors. Control males showed markedly higher expression of permissive factors compared to isolated males (Iso -0.015 vs. Ctrl 0.046, p<0.0001; Cohen's d=-0.415) following cocaine administration compared to isolated males. No differences in expression magnitude existed in response to saline injections in the NAc.

These findings indicate that adolescent isolation alters epigenetic factor expression in a sex- and region-specific manner. In the PFC, isolated males showed significant expression of histone erasers following saline exposure and broad upregulation of writer, repressive, and permissive factor expression across both saline and cocaine conditions, whereas isolated females displayed reduced expression of erasers and downregulation of writer and reader categories following cocaine. By contrast, the NAc showed minimal differences, with only

permissive factors significantly reduced in expression levels in isolated males relative to controls following cocaine. Together, these results demonstrate that the impact of adolescent isolation on epigenetic factor expression is more pronounced in the PFC than the NAc and differs in directionality between males and females.

Adolescent SI disrupts developmental GABAergic and dopaminergic gene expression profiles in the PFC

Our findings suggest isolation persistently alters stress- (i.e., the handling stimuli associated with an injection of saline) and drug-responsive gene expression. To elucidate how these changes in regulation of cocaine and stress develop, we hypothesized that adolescent isolation dysregulates maturation of candidate gene expression of receptors and enzymes related to dopaminergic, GABAergic, and glutamatergic neurotransmission in the PFC. Because

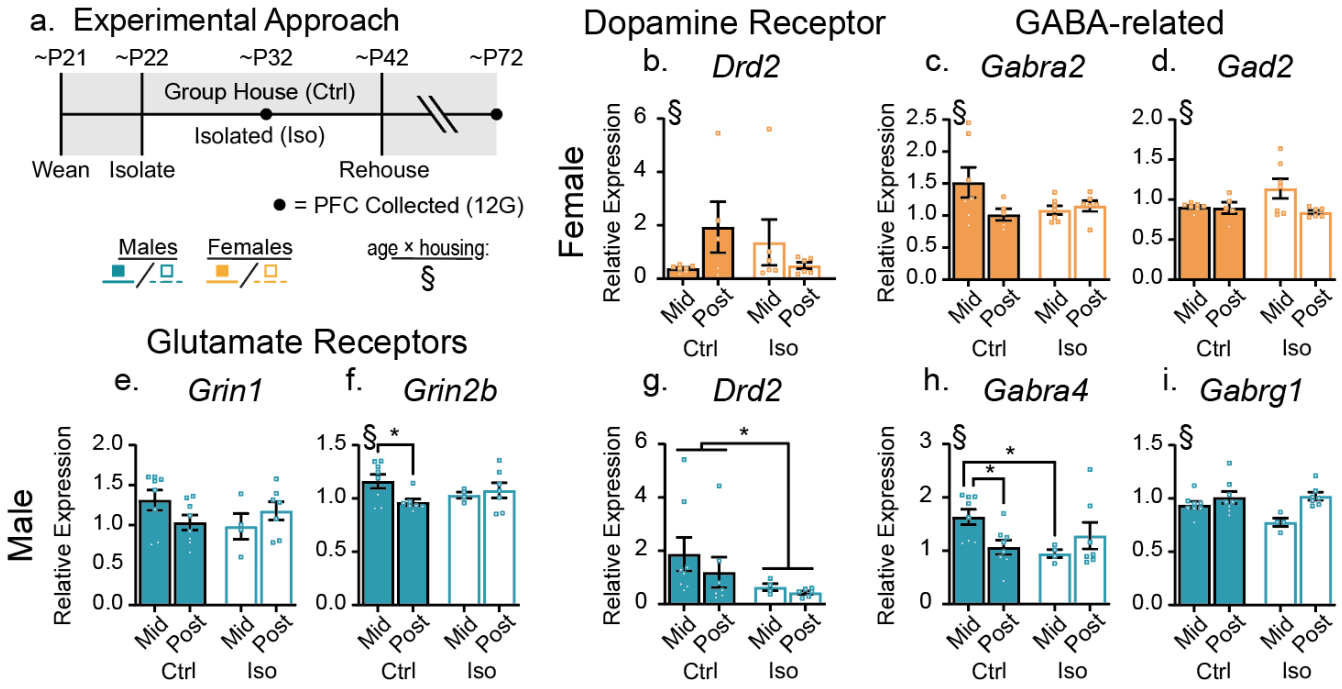


Figure 13. Isolation disrupts prefrontal cortex maturation of gene transcription profiles of dopamine receptors and markers of excitatory and inhibitory neurons. (a) Schematic of adolescent social isolation experimental timeline. Tissue collected from prefrontal cortex at two timepoints: 10 days into social isolation (P32) or 50 days after social isolation (~P72). Using RT-qPCR, gene expression of dopamine receptors (*Drd2*, b & g), glutamate- (*Grin1*, e; *Grin2b*, f) and GABA-related (*Gabra2*, c; *Gad2*, d; *Gabra4*, h; *Gabrg1*, i) targets were measured in the PFC. Significance is indicated as *p<0.05, **p<0.01, ***p<0.001. Error bars indicate SEM. Ctrl, group housed; Iso, socially isolated; Mid, mid-adolescence/isolation; Post, post-isolation. P, postnatal day.

the normative adolescent development of the PFC involves dopaminergic and excitatory/inhibitory signaling (Caballero et al., 2016; Peters & Naneix, 2022), we hypothesized that developmental profiles of GABAergic, glutamatergic, and dopaminergic gene expression would be sex-specifically disrupted by SI. To test this, we measured PFC mRNA expression of candidate genes related to dopamine signaling and excitatory and inhibitory neurotransmission using qPCR (while experiments for each candidate gene were run for both males and females, only genes with significant statistical findings are shown; for full results see Appendix B1).

Maturation of dopamine receptor gene expression in the PFC is disrupted by adolescent isolation

In both sexes, we find that adolescent isolation is associated with aberrant maturation of dopamine receptor expression. While no statistical differences were observed for expression of *Drd1* (Appendix B1), we observed statistically significant influences of adolescent isolation on *Drd2* expression. In males, we observe a main effect of housing [Fig. 13g; Wald $\chi^2(1)=4.035$, $p=0.045$], with Šidák post-hoc analyses indicating that isolated males express 0.997-fold less *Drd2* ($p=0.045$). In females, we find a significant interaction of age × housing [Fig. 13b; Wald $\chi^2(1)=5.097$, $p=0.024$] on the maturation of *Drd2* expression. However, Šidák post-hoc analyses did not reveal significant pairwise comparisons. These findings indicate that adolescent isolation is associated with sex-specific differences in the maturation of *Drd2*. Males express persistently lower levels in adolescence and adulthood. Control females showed a modest increase in expression from adolescence to adulthood, but this maturational change was not apparent in isolated animals.

Adolescent isolation sex-specifically dysregulated GABAergic gene expression in the prefrontal cortex

We also observe sex-specific differences in the influence of adolescent housing history on GABA-related candidate genes. In males, we observe a significant age × housing effect on *Gabra4* [Fig. 13h; Wald $\chi^2(1)=7.074$, $p=0.008$], with Šidák post-hoc analyses indicating that

adolescent control males expressed significantly higher *Gabra4* (0.69-fold) than adolescent isolated males (p=0.047) and adult control males (0.567-fold higher, p=0.043). We also find significant effect of age [Fig. 12i; Wald $\chi^2(1)=13.538$, p<0.001] on *Gabrg1* in addition to an Age × Housing interaction [Wald $\chi^2(1)=3.984$, p=0.046] where Šidák post-hoc analyses reveal that adult males express 0.16-fold higher levels than adolescents (p<0.001) and control males express higher levels during adolescence than isolated males (fold-difference=0.16, p=0.015).

In females, we observe a significant age × housing interaction on female *Gabra2* expression [Fig. 13c; Wald $\chi^2(1)=4.279$, p=0.039] but Šidák post-hoc analyses were not significant. In *Gad2*, the enzyme that catalyzes the GABA from L-glutamic acid, we find in females a significant age [Fig. 13d; Wald $\chi^2(1)=4.742$, p=0.029] and age × housing interaction [Fig. 12d; Wald $\chi^2(1)=4.096$, p=0.043] effect. Šidák post-hoc analyses indicate that expression levels are 0.15-fold higher in adolescence than adulthood (p=0.029).

Together, these findings suggest that adolescent isolation influences the developmental trajectories of genes important to GABAergic neurotransmission in a sex-specific fashion.

Maturation of NMDA receptor subunit expression in the prefrontal cortex is dysregulated by adolescent isolation

Adolescent social isolation changes N-methyl-D-aspartate (NMDA) glutamate receptor expression in males, blunting heightened adolescent expression observed in control males. We observed a significant age × housing interaction [Fig. 12e; Wald $\chi^2(1)=4.161$, p=0.041] on *Grin1*, the obligate subunit of the NMDA receptor. We find a significant age × housing interaction [Fig. 12f; Wald $\chi^2(1)=4.633$, p=0.031] on *Grin2b*, encoding GluN2B. GluN2B-containing receptors maintain longer channel open times, enabling greater calcium influx, and their C-terminal domain preferentially binds CaMKII – interactions critical for synaptic plasticity during cortical maturation and dopamine modulation in the PFC (Caballero et al., 2016; Paoletti et al., 2013).

Šidák post-hoc analyses reveal that adolescent control males expressed 0.2-fold higher *Grin2b* than adult control males ($p=0.035$), a difference absent in isolated males.

Discussion

Our findings suggest that adolescent isolation induces sex-specific alterations in natural reward-seeking behavior and transcriptional responses to stress and drugs of abuse that are associated with dysregulated maturation of adolescent gene transcription and differences in serum hormone levels. Males isolated during adolescence showed increased palatable food intake, enhanced PFC-NAc transcriptional concordance following stress, reduced cocaine-saline discrimination in the PFC, and selective expression of stress-responsive epigenetic erasers. Developmentally, isolated males exhibited disrupted dopaminergic, glutamatergic, and GABAergic gene expression maturation in the PFC, alongside altered thyroid hormone (T3/T4) ratios during isolation. Isolated females displayed loss of PFC-VTA cocaine-responsive transcriptional synchrony while gaining PFC-NAc concordance, reduced corticosterone during isolation, and transient elevation of Gad2 expression. Together, these findings suggest that adolescent isolation disrupts experience-dependent neural maturation through developmental reprogramming rather than classical stress-mediated mechanisms.

Palatable food intake peaks during adolescence (Murray & McCormick, 2022) and is a PFC-mediated behavior disrupted in both human and rodent models of binge eating (Blasio et al., 2014; Boeka & Lokken, 2011). Isolated males consumed more palatable food than group-housed controls, while females showed no isolation effect, maintaining their typically higher baseline consumption, aligning with literature indicating social isolation influences behavior more in males (Guo et al., 2004; Marquardt et al., 2023). To our knowledge, this is the first demonstration that adolescent isolation alters palatable food consumption. While one other study found no enhancement in food-reward-seeking in male mice following adolescent isolation, these mice remained in isolation for the duration of operant responding, had a history of surgery, and the data were from an operant food self-administration task (Fosnocht et al., 2019). The discrepancy between our findings and this previous work likely reflects fundamental differences between more naturalistic consumption, as in the palatable food task, and learned

instrumental behaviors, in addition to factors pertaining to persistent isolation and surgical history.

Adolescent isolation sex-specifically alters hormone levels

Because PFC maturation is influenced by hormones (Kritzer, 1998; Piekarski, 2017), the sex-specific behavioral alterations we observed raised questions about hormones that may be contributing to changes in adolescent brain development and later adult behavior. Examining serum levels of hormones that both regulate stress responses and neurodevelopment, corticosterone (CORT), thyroid hormones (T3/T4), and progesterone (P4), we find sex-specific influences of isolation on CORT and T3/T4.

In males, isolation does not influence levels of CORT. However, in females, we observed lower circulating CORT during isolation compared to controls. These findings align with reports of adolescent isolation producing variable effects on glucocorticoid levels in rodents and contribute to sex-specific hormonal influences of isolation (Hawkley et al., 2012).

Thyroid hormones are regulators of developmental plasticity (Batista & Hensch, 2019) that change at the onset of and across puberty (Marwaha et al., 2012; Wang et al., 2021). Additionally, previous work has shown a role of crystallin mu, a thyroid-hormone sequestration protein, in the induction of adolescent isolation behavioral phenotypes (Walker et al., 2022a). In males, we observe altered T3/T4 ratio during isolation, with elevated mid-isolation T3/T4 ratios during that normalized by adulthood. This transient thyroid hormone dysregulation is particularly significant given recent work demonstrating that thyroid hormone mediates the influence of early-life stress on behavior (Bennett et al., 2024), and aberrant thyroid hormone signaling at critical developmental periods, other tissues via epigenetic reprogramming of thyroid hormone sensitive genes (Fonseca et al., 2021).

RRHO Reveals Sex-Specific Reorganization of Stimulus-Responsive Transcription Across Mesocorticolimbic Circuits

Using threshold-free rank-rank hypergeometric overlap (RRHO) analysis of RNA-sequencing data, we found that adolescent isolation fundamentally reorganizes transcriptional coordination between the PFC, NAc, and VTA in a sex- and stimulus-specific manner. Comparing transcriptional profiles in these regions from mice euthanized one hour after being intraperitoneally (IP) administered cocaine (acute cocaine) or saline (acute saline), a comparison that controls for the handling-stress-stimuli associated with IP injections, we find that females show a substantial increase in concordant overlap between the PFC and NAc that is associated with a history of adolescent isolation.

Because controlling for handling stress may conceal differences in gene transcription profiles, we examined cocaine- and handling-stress-responsive gene profiles against the quiescent transcriptome of mice euthanized 24 hours after their 10^{th} and final injection of saline (habituation control). In males, we observe reductions in the overlap in regulation of PFC cocaine-responsive transcription profiles with the NAc and VTA (Fig. 9b & c, f & g). In females, we find a modest increase in PFC–NAc (Fig. 9d & e) and a marked loss of PFC–VTA overlap (Fig. 9h & i) in transcriptional overlap in response to cocaine. When examining injection-stress-responsive transcriptional profiles (acute saline vs. habituation control), we find that in males, a history of adolescent isolation is associated enhanced overlap of injection-stress-responsive transcriptional response between the PFC and NAc (Fig. 10b). Isolated females also show elevated overlap of stress-responsive PFC–NAc transcriptional responsive compared to their group-housed counterparts, though not as substantial (Fig. 10d). Transcriptional synchrony between PFC and NAc has been associated with stress resilience (Bagot et al., 2016). Prior work indicates that male mice subjected to adolescent social isolation exhibit reduced anxiety-like behavior in adulthood (Walker et al., 2019). In consideration of these other findings, our

observation of elevated transcriptional overlap between PFC and NAc in isolated males following saline injection may represent a transcriptional signature underlying the anxiolytic phenotype observed in adult males with a history of adolescent isolation. Little change in stress-responsive PFC–VTA overlap was observed. These patterns suggest sex-specific reorganization of how stress-responsive transcription is coordinated across reward circuits following isolation.

To determine whether enhanced interregional concordance reflected changes within specific brain regions, we examined intraregional overlap between cocaine and saline responses (Fig. 12). Within the NAc, isolated males showed reduced concordance between cocaine and saline transcriptional profiles compared to controls (Fig. 12a & b), indicating enhanced discrimination between drug and stress stimuli. The opposite pattern emerged in the PFC, where isolated males showed increased cocaine-saline overlap compared to controls (Fig. 12c & d). Conversely, in females, isolation is associated with lesser comparative overlap in transcription profile regulation in the PFC (Fig. 12e & f). This suggests a shift in transcriptional regulation stimulus responsivity in response to social isolation that is particularly pronounced in isolated males.

Gene expression of epigenetic-regulating factors, particularly erasers, is influenced by adolescent isolation

Our analysis of epigenetic factor genes from a curated, translationally relevant database (Medvedeva et al., 2015) revealed sex- and stimulus-specific alterations following adolescent isolation. While we measured gene expression rather than chromatin modifications or epigenetic factor protein levels, differential expression of genes coding for epigenetic regulators suggests altered capacity for chromatin remodeling.

A history of adolescent isolation was associated with – one hour following an injection of saline – a significant difference in the regulation of epigenetic eraser differentially expressed genes in the PFC. This effect was sex-specific, where isolated males showed significant enrichment of erasers genes expressed compared to group-housed counterparts that expressed no genes meeting significance thresholds. The converse was true for females, where group-housed controls expressed high levels in response to a saline injection and isolated females expressed none. This difference was only found in the PFC in response to saline injections; no other count-based comparisons of epigenetic factors were significant.

However, when comparing the differences in category-wide expression levels of epigenetic factors for all genes, regardless of whether they met significance thresholds, evidence of broader dysregulation of epigenetics-related gene expression associated with adolescent isolation emerged. Because writers, erasers, and readers act together in coordinated modules, category-wide magnitude analyses are especially informative for epigenetic regulators (Hyun et al., 2017; Marmorstein & Zhou, 2014). Gene-set methods show that small, coordinated shifts across a set can be biologically meaningful even when few single genes are significant (e.g., GSEA, CAMERA, QuSAGE; Subramanian et al., 2005; Wu & Smyth, 2012; Yaari et al., 2013). In the male PFC after saline injection, isolation was associated with coordinated increases in writers, repressive factors, and permissive factors. Following cocaine injection, writers were elevated in isolated males compared to their control counterparts. In the female PFC following cocaine, isolation was associated with lower expression of writers and readers. By contrast, the NAc showed fewer differences; apart from a reduction in permissive factor expression in isolated males after cocaine, magnitude differences were not prominent, and no saline-responsive magnitude effects were detected in this region.

Together, these data indicate that adolescent isolation reprograms epigenetic regulatory potential in a sex- and region-specific manner. Because epigenetic regulators act in concert, such coordinated shifts can be biologically meaningful even when individual genes do not cross

significance thresholds. These sex-specific patterns align with evidence that males and females employ distinct epigenetic strategies during development (McCarthy et al., 2009). These differences in epigenetic factor gene expression may relate to how isolation establishes distinct cocaine- and stress-responsive transcriptional profiles across and within regions of mesocorticolimbic circuitry. However, confirming functional chromatin alterations would require direct assessment of histone modifications and DNA methylation, as transcript levels do not necessarily correlate with enzymatic activity.

Sex-specific effects of social isolation on dopaminergic, GABAergic, and glutamatergic gene expression

To illuminate the mechanism by which stress- and reward-related transcriptional and behavioral phenotypes are induced by isolation, we characterized PFC gene expression throughout isolation using qPCR. We found that adolescent isolation disrupts *Drd2* in both sexes, inverting or diminishing sex-typical gene expression levels in females and males in adolescence and adulthood, respectively. Our finding of reduced Drd2 expression in isolated males aligns with electrophysiological evidence showing abnormal PFC responses to VTA stimulation in isolated animals (Peters & O'Donnell, 2005). The loss of D2-mediated inhibitory control could underlie the aberrant firing patterns and inappropriate hyperpolarization observed following VTA stimulation, suggesting that disrupted dopaminergic feedback mechanisms contribute to PFC dysfunction following isolation. Relatedly, we observe a significant effect of adolescent isolation on *Grin2b* expression. The *Grin2b* subunit, which confers higher calcium permeability and slower kinetics to NMDA receptors, normally decreases during adolescent development as part of the maturation of dopamine-glutamate interactions in the PFC (Caballero et al., 2016). We observed this expected decrease in control males, but isolated males failed to show this developmental transition, *i.e.*, they did not exhibit a decrease in *Grin2b*

expression levels in adulthood. Aberrant developmental NMDA receptor composition may interact with reduced *Drd2* expression to change PFC functional output. These changes may be relevant to findings of altered PFC responses to VTA stimulation following isolation previously observed by (Peters & O'Donnell, 2005) and, perhaps through an insufficiency of inhibitory control as proposed above, may contribute to enhanced binge-eating-like behavior and enhancement in transcriptional overlap in the PFC between cocaine and saline injection conditions. However, more work is needed to investigate how these differences manifest in altered regulatory control over mesolimbic reward circuitry.

Further, we observe dysregulation of GABAergic gene expression that showed distinct sex-specific patterns. In males, *Gabra4* failed to show the same expression levels in adolescence that were observed in controls. *Gabra4* encodes the α4 subunit of the $GABA_A$ receptor, which is an extrasynaptic receptor that mediates tonic inhibition, and normally increases during adolescence to refine cortical circuits (Smith, 2013). In the PFC, the developmental upregulation of α4-containing $GABA_A$ receptors during adolescence has been observed on pyramidal neurons layers 3 and 5, where they mediate tonic inhibition and regulate synaptic pruning and spine density (Evrard et al., 2021; Smith et al., 2024). *Gabrg1* expression, which encodes the γ-1 subunit of the $GABA_A$ receptor, is also influenced by adolescent isolation. Further, we observed that *Gabrg1* expression was lower in adolescence than adulthood, but importantly, expression was also lower in isolated males than group-housed males. Trucco et al2020) found a sequential risk gradient in genotype for the human variant GABRG1, where heterozygous and homozygous minor alleles expressed lower functioning γ-1 subunit expression, which was associated with heightened sensitivity to peer influence on externalizing behaviors. These findings suggest that there may be persistent reduction of GABAergic function adolescence as a result of adolescent isolation. In females, we found *Gad2* expression during adolescent isolation that returned to control-typical levels in adulthood. *Gad2* encodes the enzyme responsible for catalyzing the synthesis GABA from L-glutamic acid, suggesting an

enhanced capacity for GABA synthesis that is indicative of the potential for altered excitatory-inhibitory balance during adolescence, depending upon what cell types these changes are occurring within. Together with the dopaminergic and glutamatergic alterations, these findings may suggest that isolation prevents the coordinated maturation of excitatory-inhibitory balance required for appropriate stimulus discrimination and behavioral control.

It is important to note here that all molecular measures in this chapter were limited to mRNA-level expression, so the association of these measures with protein levels and whether they have functional consequences is untested. In addition, the use of bulk tissue makes it difficult to identify what cell types these changes are taking place in for many of these targets, meaning that the functional consequences of these observations could differ substantially depending on whether they occur in, ***e.g.***, pyramidal neurons versus interneurons. For ionotropic receptor subunits, changes in transcript levels may not necessarily alter receptor composition at the membrane: excess subunits could be retained intracellularly without assembling into functional complexes. Given the contrasting roles these cell types play in excitatory–inhibitory balance and circuit maturation, the direction and impact of transcriptional changes may diverge sharply across them, limiting interpretation of region-level results.

Conclusion

Adolescent social isolation persistently and sex-specifically disrupts prefrontal cortical maturation, producing lasting behavioral and transcriptional alterations. Isolated males exhibited increased palatable food consumption, which is first demonstration of isolation-induced alterations in palatable food intake. Females showed reduced serum CORT mid-isolation while males displayed altered thyroid hormone (T3/T4) ratios, with our evidence suggesting that the experience of adolescent isolation may be interpreted as disrupted developmental programming more than stress-mediated, though disruptions in developmental programming are not incompatible with stress-mediated effects.

RRHO analyses revealed substantial reorganization of mesocorticolimbic transcriptional regulation. Critically, isolated males showed enhanced PFC–NAc transcriptional concordance following saline injection, a pattern that may underlie the low-anxiety phenotype observed in isolated males. Combined with reduced cocaine-saline transcriptional discrimination within the PFC but enhanced discrimination in the NAc, these data indicate circuit-wide reorganization of transcriptional responsivity to stress and drugs. The stress-responsive expression of epigenetic erasers in male PFC and broader epigenetic factor dysregulation suggests there may be altered chromatin remodeling that capacity maintains these persistent transcriptional differences between isolated and group-housed mice, though further work must be done to confirm differences in chromatin state and regulatory landscape beyond merely measuring gene expression.

Developmental candidate gene expression qPCR analyses reveal a potential molecular basis for these phenotypes through dysregulation of normative maturation of gene expression profiles. Isolated males showed reduced expression of *Drd2* and lacked the normative downregulation of juvenile NMDA receptor expression of *Grin2b* expression from adolescence to adulthood, potentially disrupting normal dopamine-glutamate interactions. GABAergic maturation was altered: males lacked the normal adolescent *Gabra4* peak and expressed lower levels of *Gabrg1*, while females displayed elevated *Gad2* during isolation. These disruptions in dopaminergic modulation, glutamatergic signaling, and GABAergic development suggest impaired maturation of excitatory-inhibitory balance in the PFC, providing potential molecular correlates for the altered reward-seeking behavior and transcriptional dysregulation observed in isolated animals.

Chapter 4: General Discussion

Summary of Major Findings

The primary goal of this dissertation was to characterize persistent brain and behavioral differences induced by adolescent isolation. To understand these mechanisms, this work compared the behavioral, hormonal, and neural profiles of mice that were isolated throughout adolescence to those that were group housed. The key findings of this dissertation are that adolescent isolation induces changes in serum hormone concentrations as well as GABA-, glutamatergic, and dopaminergic gene expression across mesocorticolimbic circuitry that are associated with sex-specific dysregulation of adult reward-related behavior and drug- and stress-responsive gene transcription. In the context of broader literature on adolescent isolation, these findings suggest that isolation disrupts brain development not primarily through corticosterone but rather through deprivation of expected social inputs during a hormone-primed sensitive period, leading to persistent changes in how environmental stimuli are processed within mesocorticolimbic circuitry.

Chapter 2

Chapter 2 assessed the influence of social isolation on the medial amygdala and ventral tegmental area, examining connectivity, cell activity, and gene expression between and within these regions. Despite not altering connectivity between the meA and VTA or inducing persistent differences in firing rates of dopamine neurons in the VTA, adolescent isolation sex-specifically disrupted the transcriptional responses to cocaine and injection stress between and within these regions. In both the meA and VTA, isolation induced opposite effects on glutamatergic markers in males and females, where isolated female mice expressed elevated levels of glutamatergic markers and males expressed reduced glutamatergic markers versus their group-housed counterparts. Dopamine receptor expression was also sex-specifically

affected, with isolation inducing persistently elevated male dopamine receptor expression in the meA (D-1 and D2) and VTA (D-2 only) but not influencing female expression. Analyses of RNA sequencing data using RRHO reveal that isolation dysregulates drug- and stress-responsive meA–VTA transcriptional profiles sex specifically, where isolation is associated with enhanced transcriptional overlap between cocaine and stress in the female meA and the male VTA, indicating similar gene transcriptional responses to drug and stress. This work identifies the medial amygdala as a key brain region in the development of adolescent-onset psychopathologies such as substance use disorder, positioning it among canonical mesocorticolimbic reward in its importance.

Chapter 3

Chapter 3 examined how adolescent social isolation disrupts prefrontal cortex development, assessing reward behavior, hormonal profiles, and transcriptional concordance with VTA and NAc. Isolation sex-specifically altered natural reward-seeking, with males showing increased palatable food consumption while females maintained typical intake patterns. Analyses of serum hormones revealed sex-specific endocrine disruptions during isolation. Isolation altered thyroid hormone (T3/T4) ratio in males but not females and reduced CORT levels in females without augmenting levels in males, suggesting the lasting behavioral and brain influences of adolescent isolation are not stress mediated. RRHO revealed that isolation fundamentally reorganized stimulus-responsive gene transcription profile overlap between the PFC, nucleus accumbens, and ventral tegmental area. Both sexes showed enhanced PFC–NAc transcriptional concordance following stress exposure compared to controls. For cocaine responses, isolated females showed enhanced PFC–NAc overlap but markedly reduced PFC–VTA overlap compared to controls, while males maintained relatively stable interregional coordination. Within-region analyses revealed that isolated males showed reduced discrimination between cocaine and stress responses in the PFC but enhanced discrimination in

the NAc. Gene expression analyses of epigenetic regulatory factors revealed sex-specific alterations, with isolated males and females showing opposing patterns of chromatin modifier gene expression, particularly for histone erasers, suggesting altered capacity for epigenetic regulation. Developmental gene expression profiling identified persistent disruptions in neurotransmitter system maturation, with isolation preventing normal developmental transitions in dopaminergic, glutamatergic, and GABAergic signaling. These data indicate that isolation induces alterations to dopaminergic, excitatory, and inhibitory gene targets that may manifest in altered inhibitory regulation that are associated with lasting changes in gene regulatory programs underlying reward processing and stress responsivity into adulthood.

Implications

Taken together, these findings support the view that social isolation may lead to persistent dysregulation behavior more through a failure of adequate developmentally appropriate experiences rather than through stress-dependent mechanisms during adolescence. However, further experimentation is needed to assess the relative contribution of stress, and interpreting the mechanism of social isolation's lasting influence on the brain and behavior in this way is not without contention.

Adolescent isolation as stress

Some researchers frame the isolated-phenotype as attributable to stress (*e.g.*, Butler et al., 2016; Hueston et al., 2017; McElroy et al., 2025). The contention is that adolescent isolation induces phenotypic change is because of the chronic engagement of stress systems throughout adolescent development. However, the work of this dissertation contributes to a body of evidence suggesting that the behavioral phenotypes induced by adolescent isolation are due not to the stress of isolation per se but rather to failures of neural circuitry to be properly tuned by adequate stimulus exposure during the period of elevated adolescent neuroplasticity. This

may sound tautological: of course isolation implies deprivation, but there is an important distinction in stress- versus deprivation-formed phenotypic aberrances.

Behavioral adaptation requires changes in neural activity (Cajal, 1894; Kandel, 2001). This necessitates that the brain augment gene expression to adapt behavioral outputs in response to environmental experience (Harris & Hofmann, 2014). An important medium through which an organism's behavior responds to its environment is brain-level gene expression (Sinha et al., 2020). Therefore, it is reasonable to posit that steroid hormones, given their role in transcriptional regulation (Brinkmann, 1994), are centrally implicated in lasting phenotypic change induced by experiences like adolescent isolation. But appealing to stress-induced change implies action through effector proteins implicated in the body's stress response mediated by activity in the hypothalamic-pituitary-adrenocortical (HPA) axis involving the secretion of glucocorticoids (GCs). Commonly, the main biological mover that is meant in the context of "stress-induced changes" is the GC cortisol or corticosterone, cortisol's rodent homolog. However, claiming that the phenotype that results from adolescent social isolation is stress-induced in rats and mice is not as well supported if this is the criterion.

Though it appears to be the case that in primates and pigs, social isolation does establish higher basal GC levels during the period during which the organism is isolated, the findings are mixed in rodents; some investigators (ourselves included: see Ch. 3, Fig. 7d) find either lower or comparable levels of GC in isolated versus group-housed, and others report higher levels (as reviewed in Hawkley et al., 2012). Despite mixed findings, it is well supported that social isolation can create a lasting neurobiological imprint that makes the HPA axis more reactive to future stressors, even if baseline glucocorticoid levels appear normal. Akana et al. (1992) describe this as leaving a "facilitatory trace." As one example, in rats, Djordjevic et al. (2012) find that, despite lower comparative level of serum CORT versus controls, isolated rats had enlarged adrenals, indicative of greater ability to mount surges of CORT release in response to stressors. However, evidence supporting a facilitatory trace is not indicative that the

lasting neurobiological changes that are induced by isolation throughout the adolescent sensitive period are attributable to chronic stress. Much of the literature referencing adolescent-social-isolation-as-stress does not measure circulating CORT levels, making it difficult to directly attribute biological and behavioral to the function of isolation-induced HPA dynamics. Moreover, focusing solely on GCs as the hormonal mediators of SI phenotypes overlooks other neurosteroids that influence gene transcription and neural excitability. Besides GCs, alternative neurosteroidal baseline differences have been documented, implicating lower brain allopregnanolone levels in behavioral abnormalities and hyperexcitability of neuronal populations in rodents that underwent adolescence in isolation (and importantly, these experiments did not include females), though exhaustive analyses of this literature are beyond the scope of this dissertation (Pibiri et al., 2008; Talani et al., 2016). However, accounts of elevated levels of allopregnanolone do not explain why the social-isolation phenotype occurs. This is because, similarly to the observation that isolation induces a facilitatory trace, enhanced levels of allopregnanolone are an effect of isolation, but allopregnanolone has not been satisfactorily characterized as casual in the establishment of the SI phenotype. The findings of this dissertation suggest that both thyroid hormone and social deprivation are better positioned as a mediator of the persistent phenotypic changes induced by adolescent isolation stress.

Adolescent isolation as failure of adequate social experience during the adolescent sensitive period

A brief overview of adolescent brain and behavioral changes will help situate these findings.

Puberty initiates brain changes

As the transition from childhood into adulthood, adolescence is when puberty, the development of sexual maturity, takes place. In adolescence, the brain enters a sensitive period

within which the effects of experience on the brain and later behavior are uniquely persistent. The adolescent brain is in a state of heightened plasticity to facilitate the acquisition of sociobehavioral competencies necessary for independence (Ellis et al., 2012; Fuhrmann et al., 2015; Spear, 2013). This period of heightened plasticity occurs alongside surges in hormonal activity that begin when the hypothalamic-pituitary-gonadal (HPG) axis re-activates during puberty (after its initial activation in infancy). Pubertal onset begins when kisspeptin, a neuropeptide, stimulates gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus initiate pulsatile release of GnRH. GnRH causes the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the pituitary, leading to the initiation of sexual maturity by activation of the gonads (Herbison, 2016).

As a result of kisspeptin activating GnRH's signal to the gonads via LH and FSH, the sex hormones estrogen and testosterone circulate throughout the body and brain, establishing the biological and neurobiological foundations of sexual maturity. Testosterone, estradiol, and dehydroepiandrosterone (DHEA; both acts on its own receptors and is a precursor to testosterone and estradiol, is released earlier in adrenarche) act as powerful reorganizers of neural circuits by binding receptors that are highly concentrated in limbic regions within the social decision-making network (SDN), initiating gene transcriptional changes that fundamentally alter neurotransmitter synthesis, receptor expression, and synaptic connectivity (Spaziani et al., 2021; Vijayakumar et al., 2021). Subcortical limbic regions undergo comparatively rapid hormone-driven reorganization, which creates a developmental mismatch with the prefrontal cortex, which must integrate and regulate these newly reorganized limbic circuits while still undergoing its own maturation.

The timing of PFC plasticity, which extends well beyond the maturation of subcortical structures, represents an adaptive feature of brain development that spans species (Selemon, 2013). The PFC exerts top-down regulation of social- and reward-related behaviors (Gabbott et al., 2005; O'Connell & Hofmann, 2011; Russo & Nestler, 2013) through projections to the

nucleus accumbens, amygdala, and ventral tegmental area, modulating dopaminergic, glutamatergic, and GABAergic neurotransmission (Alexandra Kredlow et al., 2022; Del Arco & Mora, 2008; Morales & Margolis, 2017). Because subcortical circuits reorganize earlier, the delayed maturation of the PFC allows it to be shaped by social and environmental experience. This prolonged development is adaptive: it permits the regulation of limbic reward and social circuits to be tuned to the individual's environment, establishing enduring patterns of cognition and behavior (Friedman & Robbins, 2022; Reynolds & Flores, 2021).

The evolutionary logic and neuropeptidergic basis of pubertal changes in motivation

Beyond influencing neurotransmitter synthesis, receptor expression, and synaptic excitability, estrogen upregulates oxytocin and oxytocin receptor synthesis while testosterone promotes vasopressin production (Auger et al., 2011; Lim & Young, 2006). Oxytocin remains important across the lifespan, but vasopressin assumes a greater role in adolescence, particularly in shaping peer relationships and social recognition. This developmental rebalancing is crucial because oxytocin primarily facilitates parent-child bonding in childhood, while vasopressin becomes the dominant neuropeptide for peer relationships and social recognition during adolescence (Shorey et al., 2023; Veenema et al., 2012; Weinstein et al., 2014). The surge in vasopressin signaling, particularly in the lateral septum and extended amygdala, drives the reorientation of social motivation from parental to peer-directed affiliative behaviors. These changes in social neuropeptides are integral to adolescent sociobehavioral development and occur within the context of the developing mesocorticolimbic dopamine system, creating heightened sensitivity to social rewards. Without enhancements in vasopressin, the adolescent does not re-orient affiliative behavior toward peers, as evidenced by experiments showing that vasopressin receptor knockout or pharmacological blockade induces a failure to develop normal peer recognition and preference, maintaining juvenile-like social patterns (Bielsky et al., 2004; Paul et al., 2016). Occurring alongside enhancements in risk-seeking behaviors, this change in

social reward is robust, occurring in many vertebrates, facilitating social competency and status, and increasing exploratory behavior and novelty-seeking can decrease the likelihood of inbreeding (Ellis et al., 2012).

In rodents, adolescence is when play behavior begins in earnest, peaking in rats between P32–40, gradually declining thereafter and ending when play behavior decreases in adulthood (Baenninger, 1967; J. Panksepp, 1981; Pellis & Pellis, 2017). Though not quite the same rough-and-tumble play seen in rats and higher clades of mammals, adolescent mice engage in rudimentary play behaviors including non-contact chasing, social sniffing, and brief physical interactions, which peaks around P23–32 (J. B. Panksepp & Lahvis, 2007; Terranova et al., 1993). Across many mammalian species, males typically engage in play behavior more frequently and intensely than females (DiPietro, 1981; Pellis & Pellis, 2017), though this pattern shows variation across species and contexts (Marley et al., 2022).

Social isolation and hormonal manipulations both induce social deficits

Adolescent social isolation (SI) induces sex-specific effects on a wide range of behaviors, though much of the literature has exclusively studied its influence on males. SI is associated with sociocognitive and behavioral deficits, ranging from impaired social recognition (Kercmar et al., 2011), enhanced aggression in males (Takahashi, 2025), and sexual deficits, where much of the literature finds that both sexes display incompetent copulatory behaviors following adolescent isolation (Cooke et al., 2000; De Lorme et al., 2019; Kercmar et al., 2014; Marquardt et al., 2022). These deficits can be arguably attributed to restrictions on play behavior, though literature examining this is limited and mixed, with some work suggesting that minimal physical contact alongside olfactory, auditory, and visual cues may be sufficient for appropriate sexual competency (Holloway & Suter, 2004). This experience-dependent priming of neural circuits during the juvenile period suggests that adequate behavioral maturation

requires not only hormonal signaling but also appropriate social experiences during critical developmental windows.

In a fascinating set of experiments, adult-level steroid hormones administered prior to puberty are not sufficient to induce adult sexual behavior (Schulz & Sisk, 2006). Even when given the same hormonal signals that would activate sexual behavior in adults, prepubertal hamsters cannot respond because their brains have not yet developed the capacity for hormonal responsiveness. This may be attributable to immature neuronal populations in the meA among other regions (Page et al., 2022) that do not yet express receptors necessary to induce processes of differentiation of neuronal functioning needed to adequately mediate mature sexual behavior, though the particulars of the biological foundation of this has not, to my knowledge, been uncovered. This suggests that though hormones are necessary for the expression of age-appropriate behaviors once at the appropriate age, they are not sufficient on their own to effect behavioral maturation, which seems to require other central coordination.

The relationship between hormones and experience in shaping adult behavior becomes evident when examining the limitations of hormonal manipulations alone. When the brain reaches the appropriate developmental stage and hormones are present, experience remains essential for proper behavioral maturation. De Lorme et al. (2019) demonstrated that pubertal testosterone programs sexual proficiency through experience-dependent ΔFosB induction in the infralimbic cortex (IL). ΔFosB is a splice variant of the *Fosb* gene that accumulates in neurons following chronic or repeated stimulation that, unlike regular Fos proteins that degrade within hours, ΔFosB is stable and accumulates over time, lasting weeks to months and promotes structural changes including dendritic spine formation and synaptic remodeling (Ej et al., 1999; Robison & Nestler, 2022). Males deprived of testosterone during puberty failed to show behavioral improvements or ΔFosB expression despite repeated sexual experience, but viral overexpression of ΔFosB in the IL rescued sexual competency. Viral overexpression of ΔFosB led to similar mounting to normally developed males, even without prior sexual experience. This

suggests that hormonal organization of ΔFosB-mediated plasticity during adolescence is critical for experience-dependent behavioral adaptation. This shaping is thought to occur through experience-induced ΔFosB accumulation, which acts as a molecular switch driving neuronal and circuit specialization. ΔFosB regulates downstream genes that promote synaptic plasticity and dendritic remodeling, essentially solidifying experience-dependent neural adaptations that guide populations toward their mature behavioral functions. Relatedly, though not directly assessing the role of ΔFosB, other behavioral aberrances beyond mating correspond in SI mice with impaired plasticity and immature dendritic spine morphology in the PFC (Medendorp et al., 2018).

These findings demonstrate the importance of not only social experience but also adequate hormonal environment in the development of mature a brain and behavioral phenotype. The normative differentiation of neuron populations that underlies behavioral competency requires coordinated hormonal, behavioral, and transcriptional changes across the adolescent period, and without any one of these components, aberrant phenotypes occur.

Adolescent isolation induces a failure of sensitive period transcriptional differentiation of neuronal population functioning

Not only do gonadal hormones augment circuit-wide brain maturation, but thyroid hormone also plays an integral role, particularly within periods of heightened plasticity (Batista & Hensch, 2019), with aberrant signaling during critical periods capable of affecting other tissues through epigenetic reprogramming of thyroid hormone-sensitive genes (Fonseca et al., 2021). Prior work has shown that early-life stress is associated with diminished thyroid hormone signaling and causally implicated in the aberrant development of VTA gene targets, and that administration of thyroid hormone rescues these changes (Bennett et al., 2024). Further, and more central to the findings of this dissertation, crystallin mu, a thyroid-sequestration protein,

has been identified as causally implicated in the induction of adolescent isolation behavioral phenotypes (Walker, 2022a).

Because of these findings, I hypothesize that social isolation prevents the recruitment of transcriptionally differentiated neuronal subpopulations, “neuronal phenotypes,” that are normally shaped through developmentally appropriate stimulus exposure during the sensitive period induced by pubertal surges.

Short-term (7 day) social isolation in adult rats induces lasting bulk RNA-seq transcriptional (and protein) changes in the rat medial amygdala, with differentially expressed genes found to be enriched in BDNF signaling and axon guidance pathways that seem to normalize after rehousing (Lavenda-Grosberg et al., 2022). This suggests that even outside of sensitive periods, signals pertaining to proliferation and projections of neurons are influenced by a lack of social experience. Supporting this concept of experience-dependent neural differentiation of neuronal population functioning, single-nucleus RNA-seq analysis of mouse visual cortex reveals that sensory deprivation during *critical periods* disrupts normal cell type specification, with UMAP clustering showing that dark-reared mice fail to develop the three distinct L2/3 glutamatergic subtypes seen in normally-reared animals, instead displaying poorly separated transcriptomic identities that partially recover with brief light exposure (Cheng et al., 2022). It stands to reason that, (1) because adolescence is a sensitive period for the development of neural structures and (2) data regarding the role of transcription-regulatory-related proteins in sociobehavioral competency (*e.g.*, De Lorme et al., 2019), similar clustering in cell identity and function likely follows adolescent socialization. This is a process which SI likely truncates. Thus, the SI phenotype emerges not from stress-induced changes but from the absence of experience-dependent sculpting that normally refines neuronal phenotypes and neural circuits into their mature, functionally specialized forms. Further research employing both behavioral and hormonal manipulations in tandem with single-cell RNA sequencing to adequately test this hypothesis.

Limitations

There are several important considerations in the interpretation of the findings in this dissertation. Centrally, the correlational nature of our data precludes establishing causal relationships between transcriptional changes and behavioral phenotypes. I interpret the data to mean that social isolation prevents experience-dependent differentiation of neuronal function rather than inducing stress-mediated changes. However, confirming this mechanism would require combined behavioral and hormonal manipulations.

Further, while informative for regional transcriptional changes the use of bulk RNA sequencing, it cannot distinguish cell-type-specific responses within heterogeneous brain regions. The PFC, NAc, meA, and VTA are comprised of extraordinarily distinct cell types (Bergan et al., 2014; Bhattacherjee, 2019; Chen et al., 2021; Tiklová et al., 2019). Testing our hypothesis that isolation prevents the recruitment of differentiated neuronal subpopulations would require single-cell RNA sequencing to identify whether specific neuronal subtypes fail to develop their mature transcriptomic identities. The gene transcription profiles in this study represent brief snapshots of bulk cellular responses to experience. Group-level (*e.g.*, housing and sex) transcriptional responses are subject to regulatory dynamics that influence the timecourse of gene expression following drug or stimulus exposure. For example, in Walker et al. (2022a & b), mice were euthanized 1 hr post-injection. The group-housed and isolated mice from these experiments may undergo similar transcriptional programs at different rates but their profiles would appear different because a fixed sampling timepoint does not account for varying regulatory dynamics.

Methodological differences across experiments may influence our findings. The animals used in sequencing, electrophysiology, and tracing studies were shipped from Jackson Laboratory on P20, whereas animals for qPCR experiments were bred on-site. This difference in early rearing experiences, including potential shipping stress during the late pre-weaning

period, could interact with our isolation paradigm in ways that differ from animals bred in-house, potentially affecting the generalizability of our findings across experiments. Additionally, control for hormonal fluctuations was limited to electrophysiology experiments, where all females were tested in diestrus. Hormonal variations across the estrous cycle in other endpoints could contribute to variability, particularly given our findings of sex-specific effects.

Our candidate gene approach, while hypothesis-driven, inherently limited our investigation to pre-selected targets and may have overlooked other critical genes undergoing developmental disruption. Though constrained by practical limitations, RNA-sequencing would be an ideal means of characterizing adolescent social isolation's influence on gene expression profiles. Additionally, some qPCR analyses had relatively small sample sizes (n=4-8/group) after quality control exclusions. This may have limited our statistical power to detect certain group differences, as evidenced by significant main effects without significant post-hoc comparisons in several analyses. Importantly, we did not validate gene expression changes at the protein level or assess functional consequences of altered transcript abundance. Changes in mRNA levels may not translate to proportional changes in protein expression or enzymatic activity, particularly for genes encoding receptors and neurotransmitter synthesis enzymes where post-translational modifications critically regulate function. Post-translational modifications not only regulate receptor/enzyme function, but also govern intracellular trafficking, determining whether proteins remain in precursor pools or are delivered to functional sites such as the membrane or synapse. Without electrophysiological or pharmacological validation, we cannot confirm that observed changes in dopamine receptor or GABA subunit expression translate to altered neurotransmission or behavioral outcomes. These limitations underscore the need for complementary approaches to validate the functional significance of transcriptional changes identified in our study.

Future Directions

The findings presented here establish a foundation for mechanistic studies of how social experience shapes adolescent brain development. Ideally, single-cell RNA sequencing would directly test the hypothesis that isolation prevents the establishment of transcriptionally differentiated neuronal phenotypes. By comparing transcriptomic identities of individual neurons from isolated versus group-housed animals, one could determine whether specific cell populations fail to mature, similar to the poorly differentiated glutamatergic subtypes observed in sensory-deprived visual cortex.

Validation of gene expression targets represents another important component in future work in this area. Combining our gene expression findings with electrophysiology would establish whether altered dopamine receptor and NMDA subunit expression translates to disrupted synaptic transmission. Chemogenetic or optogenetic manipulation of specific meA→VTA or PFC→NAc projections during adolescence could determine whether activating these circuits during isolation prevents later behavioral and transcriptional abnormalities – essentially providing artificial "social" stimulation to drive circuit maturation.

Direct manipulation of thyroid signaling during isolation, either through hormone supplementation or crystallin mu modulation, could establish causal relationships between endocrine disruption and neural phenotypes. Conversely, maintaining euthyroid states during isolation might prevent transcriptional and behavioral consequences, definitively separating deprivation from metabolic effects, potentially preserving or sensitive period plasticity upon rehousing. Testing interventions at different developmental stages would help to define particular windows of vulnerability.

Conclusion

This dissertation demonstrates that adolescent social isolation fundamentally reorganizes mesocorticolimbic circuits through developmental deprivation rather than chronic stress. Across the medial amygdala, ventral tegmental area, and prefrontal cortex, isolation induced sex-specific disruptions in dopaminergic, glutamatergic, and GABAergic maturation that persist into adulthood. These changes manifested as dysregulated interregional concordant and discordant transcriptional responses to both drugs and stress, altered natural reward-seeking behavior, and disrupted stimulus discrimination.

The distinction between stress and deprivation mechanisms has critical implications for understanding adolescent vulnerability to neuropsychiatric disorders. Our findings suggest that the behavioral and cognitive sequelae of adolescent isolation emerge not from chronic HPA axis activation, but from the absence of experience-dependent neural sculpting during a hormone-primed sensitive period. The sex-specific nature of these effects may help explain sex differences in the prevalence and presentation of adolescent-onset psychiatric conditions.

These results underscore that adolescent social experience is not merely beneficial but essential for the circuit-specific differentiation that determines lifelong patterns of reward processing and stress responsivity. As rates of adolescent loneliness continue to rise, understanding these mechanisms becomes increasingly urgent for developing interventions that can preserve or restore the neuroplasticity necessary for healthy brain development.

Appendix A: Supplemental Data for Chapter 2

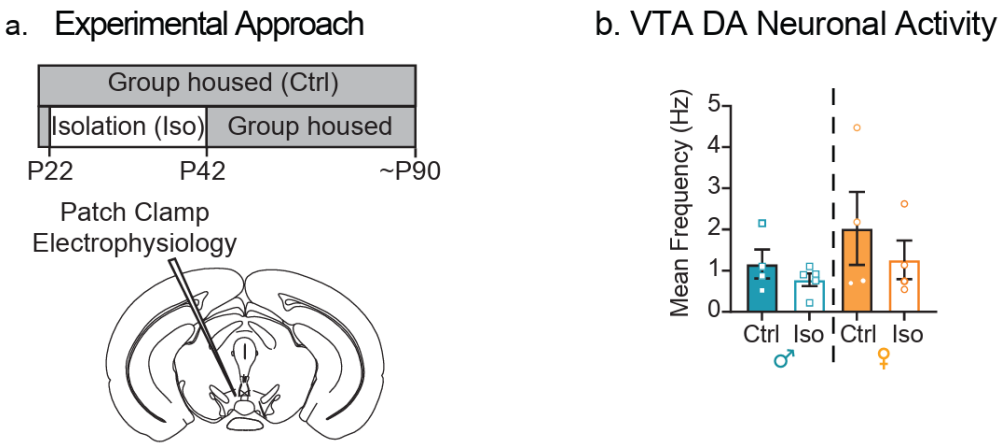


Figure. A1. **Social isolation does not influence mean firing frequency of ventral tegmental dopamine neurons.** (a) Schematic of timeline for adolescent isolation and tissue collection for patch-clamp electrophysiology. (b) Quantification of mean cell firing of dopamine neurons from ventral tegmental area. Error bars indicate SEM. Ctrl, group housed; Iso, socially isolated; P, postnatal day.

A2. Chapter 2/3: Primer Sequences	Gene ID	Gene Name	Forward Sequence	Reverse Sequence
Calmodulin kinase	*Camk2a*	Calmodulin-dependent protein kinase II alpha	AGCATCCCAGCCCTAGTTC	AGAAGGCTCCCTTTCCCA
	Camk2b	Calmodulin-dependent protein kinase II beta	GAAGCATTCCAACATTGTACGC	CTCTTGCCACGATGTCTTCA
GABA	*Gabra1*	GABA-A receptor subunit α1	CCAAGTCTCCTTCTGGCTCAAC	AAGCCACCTTCGGGAGGGAATT
	Gabra2	GABA-A receptor subunit α2	TACTAGCTGTTCAGCTTTGGC	GATGTTAGCCAGCACCAACC
	Gabra4	GABA-A receptor subunit α4	GTGCTCTGTCTGTCCGTCTC	TTTCACTTCTGTAACAGGACCTAAT
	Gabrb2	GABA-A receptor subunit β2	GATGGACCTAAGGCGGTATCCAC	GGAAGCTCAATCTTTGTCACTCCTG
	Gabrd	GABA-A receptor subunit Δ	GGCGCCAGGGCAATGAA	CATTCACAGGAGCACCTCCA
	Gabrg1	GABA-A receptor subunit δ1	AAAACAAGACTTCGGCTTCCC	TTGCCCTCCAAACACTGGTAG
	Gabrg2	GABA-A receptor subunit δ2	GGAGCATTGGAAGCTCAGTC	TTTTGGCTTGTGAAGCCTGG
	Gad1	Glutamate decarboxylase 1	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC
	Gad2	Glutamate decarboxylase 2	TCCTTGAAGTCAAACAGAAAGGATT	TGTCAGCTACAGCCAAGAGA
	Slc32a1	Vesicular inhibitory amino acid transporter	ACCCTCTGGCTCCAAGGAC	GCACGAACATGCCCTGAATG
Glutamate	*Gria1*	AMPA receptor subunit 1	CTAGGCTGCCTGAACCTTTG	GGGAAGATTGAATGGAAGCA
	Gria2	AMPA receptor subunit 2	AGTGCATTTCGGGTAGGGATG	CCTCCAAATTGTCGATATGGGGT
	Grin1	NMDA receptor subunit 1	GGCGTTGAGCTGTATCTTCC	TGCTTTTCTCCTGCTCCTTC
	Grin2a	NMDA receptor subunit 2a	ACGTGACAGAACGCGAACTT	TCAGTGCGGTTCATCAATAACG
	Grin2b	NMDA receptor subunit 2b	TGCTGTAGCTGTCTTTGTCTTTG	CTTTGCCGATGGTGAAAGAT
	Grin2c	NMDA receptor subunit 2c	ATCGGGGTCAACAATACCAAC	TTGTCCTCAAAGACGATGCCG
	Slc17a6	Vesicular glutamate tranporter 2	TGGAAAATCCCTCGGACAGAT	CAGGGGCTTACCGTCCTCT
	Slc17a7	Vesicular glutamate transporter 1	GGGGTCACATACCCTGCTTG	CCCAGCATAGGAACCGCAA
Dopamine	*Drd1*	Dopamine receptor D1	GTCTCCCAGATCGGGCATTTG	GTCACTTTTCGGGGATGCTG
	Drd2	Dopamine receptor D2	ACCTGTCCTGGTACGATGATG	GCATGGCATAGTAGTTGTAGTGG

A3. Supplemental Table 2a

		GLM p-values				P32		P72	
Category	Gene	Age	Housing	Age*Housing	Groups	Rel. Exp.	± SEM	Rel. Exp.	± SEM
Calmodulin	*Camk2a*	**<.001**	0.677	0.077	GHF	0.82	(+/- 0.11)	1.01	(+/- 0.05)
					SIF	0.64	(+/- 0.03)	1.12	(+/- 0.15)
	Camk2b	**0.006**	0.077	**0.023**	GHF	0.96	(+/- 0.11)	1.01	(+/- 0.05)
					SIF	0.92	(+/- 0.08)	1.37	(+/- 0.13)
GABA	*Gad1*	0.106	0.110	0.106	GHF	0.90	(+/- 0.05)	0.90	(+/- 0.10)
					SIF	1.40	(+/- 0.24)	0.90	(+/- 0.05)
	Gad2	0.899	0.125	0.678	GHF	1.04	(+/- 0.14)	1.01	(+/- 0.07)
					SIF	1.16	(+/- 0.12)	1.22	(+/- 0.08)
	Slc32a1	0.798	0.094	0.565	GHF	0.95	(+/- 0.19)	0.82	(+/- 0.08)
					SIF	1.12	(+/- 0.18)	1.17	(+/- 0.15)
Glutamate	*Slc17a6*	0.855	0.066	0.762	GHF	1.21	(+/- 0.24)	1.07	(+/- 0.15)
					SIF	1.65	(+/- 0.33)	1.68	(+/- 0.42)
	Slc17a7	**<.001**	0.699	**0.017**	GHF	0.71	(+/- 0.05)	1.01	(+/- 0.07)
					SIF	0.42	(+/- 0.04)	1.42	(+/- 0.34)
Dopamine	*Drd1*	0.053	0.277	0.452	GHF	1.36	(+/- 0.13)	1.06	(+/- 0.16)
					SIF	1.84	(+/- 0.42)	1.15	(+/- 0.22)
	Drd2	0.080	0.182	0.208	GHF	2.04	(+/- 0.34)	1.07	(+/- 0.15)
					SIF	7.15	(+/- 3.32)	1.21	(+/- 0.32)

GHF = Group-housed female

SIF = Socially isolated female

A4. Supplemental Table

		GLM p-values				P32		P72	
Category	Gene	Age	Housing	Age*Housing	Groups	Rel. Exp.	± SEM	Rel. Exp.	± SEM
Calmodulin	*Camk2a*	0.15	**0.033**	0.654	GHM	0.90	(+/- 0.04)	1.02	(+/- 0.07)
					SIM	0.80	(+/- 0.07)	0.86	(+/- 0.06)
	Camk2b	0.471	**<.001**	0.323	GHM	0.93	(+/- 0.04)	1.02	(+/- 0.07)
					SIM	0.81	(+/- 0.03)	0.80	(+/- 0.04)
GABA	*Gad1*	0.823	0.798	0.602	GHM	0.96	(+/- 0.08)	0.94	(+/- 0.05)
					SIM	0.94	(+/- 0.06)	0.99	(+/- 0.10)
	Gad2	0.481	0.21	0.443	GHM	0.88	(+/- 0.09)	0.88	(+/- 0.05)
					SIM	0.73	(+/- 0.07)	0.84	(+/- 0.09)
	Slc32a1	0.943	0.056	0.972	GHM	0.93	(+/- 0.15)	0.93	(+/- 0.09)
					SIM	0.73	(+/- 0.09)	0.72	(+/- 0.07)
Glutamate	*Slc17a6*	0.316	0.502	0.351	GHM	1.70	(+/- 0.31)	1.22	(+/- 0.26)
					SIM	1.31	(+/- 0.24)	1.29	(+/- 0.19)
	Slc17a7	0.82	0.99	0.915	GHM	1.50	(+/- 0.29)	1.59	(+/- 0.29)
					SIM	1.52	(+/- 0.43)	1.56	(+/- 0.23)
Dopamine	*Drd1*	0.056	**0.027**	0.546	GHM	0.73	(+/- 0.12)	0.94	(+/- 0.27)
					SIM	0.99	(+/- 0.13)	1.39	(+/- 0.12)
	Drd2	0.654	**<.001**	0.437	GHM	0.86	(+/- 0.09)	0.71	(+/- 0.15)
					SIM	1.18	(+/- 0.02)	1.22	(+/- 0.14)

GHM = Group-housed male

SIM = Socially isolated male

A5. Supplemental Table		t-test p-values		P32	
Category	Gene	Two-Sided p	Groups	Rel. Exp.	± SEM
GABA	*Gabra1*	0.821	GHF	0.84	(+/- 0.13)
			SIF	0.83	(+/- 0.17)
	Gabra2	0.829	GHF	0.65	(+/- 0.33)
			SIF	0.74	(+/- 0.25)
	Gabra4	0.985	GHF	0.82	(+/- 0.15)
			SIF	0.83	(+/- 0.17)
	Gabrb2	0.962	GHF	0.75	(+/- 0.25)
			SIF	0.77	(+/- 0.23)
	Gabrd	0.971	GHF	0.76	(+/- 0.21)
			SIF	0.44	(+/- 0.08)
	Gabrg1	0.175	GHF	0.68	(+/- 0.31)
			SIF	0.74	(+/- 0.25)
	Gabrg2	0.833	GHF	0.80	(+/- 0.19)
			SIF	0.73	(+/- 0.26)
	Gad1	0.886	GHF	0.83	(+/- 0.14)
			SIF	0.81	(+/- 0.19)
	Gad2	0.948	GHF	0.75	(+/- 0.25)
			SIF	0.72	(+/- 0.27)
	Slc32a1	0.775	GHF	0.79	(+/- 0.21)
			SIF	0.73	(+/- 0.26)
Glutamate	*Gria1*	0.936	GHF	0.83	(+/- 0.14)
			SIF	0.86	(+/- 0.13)
	Gria2	0.91	GHF	0.78	(+/- 0.22)
			SIF	0.79	(+/- 0.21)
	Grin1	0.964	GHF	0.74	(+/- 0.26)
			SIF	0.73	(+/- 0.26)
	Grin2a	0.974	GHF	0.50	(+/- 0.06)
			SIF	0.76	(+/- 0.24)
	Grin2b	0.448	GHF	0.50	(+/- 0.09)
			SIF	0.82	(+/- 0.18)
	Grin2c	0.229	GHF	0.73	(+/- 0.27)
			SIF	0.77	(+/- 0.23)
	Slc17a6	0.912	GHF	0.85	(+/- 0.10)
			SIF	0.77	(+/- 0.23)
	Slc17a7	0.372	GHF	0.73	(+/- 0.27)
			SIF	0.44	(+/- 0.13)
Dopamine	*Drd1*	0.98	GHF	0.72	(+/- 0.28)
			SIF	0.71	(+/- 0.27)
	Drd2	0.999	GHF	0.79	(+/- 0.21)
			SIF	0.79	(+/- 0.21)

A6. Supplemental Table		t-test p-values		P32	
Category	Gene	Two-Sided p	Groups	Rel. Exp.	± SEM
GABA	*Gabra1*	0.263	GHM	1.00	(+/- 0.03)
			SIM	0.85	(+/- 0.12)
	Gabra2	0.552	GHM	1.01	(+/- 0.08)
			SIM	0.93	(+/- 0.10)
	Gabra4	0.27	GHM	1.01	(+/- 0.10)
			SIM	1.44	(+/- 0.38)
	Gabrb2	0.356	GHM	1.00	(+/- 0.03)
			SIM	0.93	(+/- 0.06)
	Gabrd	0.295	GHM	1.59	(+/- 0.87)
			SIM	0.60	(+/- 0.07)
	Gabrg1	0.086	GHM	1.00	(+/- 0.03)
			SIM	1.19	(+/- 0.09)
	Gabrg2	0.527	GHM	1.00	(+/- 0.02)
			SIM	0.94	(+/- 0.08)
	Gad1	0.159	GHM	1.02	(+/- 0.11)
			SIM	0.74	(+/- 0.13)
	Gad2	0.167	GHM	1.01	(+/- 0.07)
			SIM	0.81	(+/- 0.10)
	Slc32a1	0.393	GHM	1.01	(+/- 0.08)
			SIM	0.87	(+/- 0.13)
Glutamate	*Gria1*	0.163	GHM	1.01	(+/- 0.07)
			SIM	1.38	(+/- 0.22)
	Gria2	0.237	GHM	1.00	(+/- 0.02)
			SIM	1.27	(+/- 0.20)
	Grin1	0.639	GHM	1.02	(+/- 0.14)
			SIM	0.95	(+/- 0.05)
	Grin2a	0.578	GHM	1.02	(+/- 0.10)
			SIM	0.93	(+/- 0.10)
	Grin2b	0.8	GHM	1.02	(+/- 0.10)
			SIM	1.07	(+/- 0.20)
	Grin2c	0.701	GHM	1.01	(+/- 0.07)
			SIM	0.95	(+/- 0.12)
	Slc17a6	0.793	GHM	1.00	(+/- 0.02)
			SIM	0.95	(+/- 0.18)
	Slc17a7	0.562	GHM	1.09	(+/- 0.26)
			SIM	0.85	(+/- 0.29)
Dopamine	*Drd1*	0.419	GHM	1.07	(+/- 0.22)
			SIM	1.30	(+/- 0.03)
	Drd2	0.072	GHM	1.09	(+/- 0.24)
			SIM	2.30	(+/- 0.50)

A7. Supplemental Table		t-test p-values		P72	
Category	Gene	Two-Sided p	Groups	Rel. Exp.	± SEM
GABA	*Gabra1*	0.088	GHF	1.05	(+/- 0.12)
			SIF	1.35	(+/- 0.10)
	Gabra2	0.707	GHF	1.04	(+/- 0.12)
			SIF	1.11	(+/- 0.14)
	Gabra4	0.314	GHF	1.03	(+/- 0.11)
			SIF	0.86	(+/- 0.12)
	Gabrb2	0.042	GHF	1.03	(+/- 0.10)
			SIF	1.29	(+/- 0.04)
	Gabrd	0.125	GHF	1.32	(+/- 0.40)
			SIF	0.50	(+/- 0.07)
	Gabrg1	0.201	GHF	1.15	(+/- 0.06)
			SIF	1.01	(+/- 0.08)
	Gabrg2	0.717	GHF	0.98	(+/- 0.01)
			SIF	0.99	(+/- 0.03)
	Gad1	0.124	GHF	1.06	(+/- 0.14)
			SIF	1.44	(+/- 0.18)
	Gad2	0.167	GHF	1.06	(+/- 0.13)
			SIF	1.37	(+/- 0.17)
	Slc32a1	0.296	GHF	1.03	(+/- 0.11)
			SIF	1.22	(+/- 0.13)
Glutamate	*Gria1*	0.403	GHF	1.06	(+/- 0.14)
			SIF	1.22	(+/- 0.10)
	Gria2	0.052	GHF	1.06	(+/- 0.13)
			SIF	1.53	(+/- 0.18)
	Grin1	0.046	GHF	1.07	(+/- 0.14)
			SIF	1.80	(+/- 0.31)
	Grin2a	0.058	GHF	1.09	(+/- 0.18)
			SIF	1.99	(+/- 0.41)
	Grin2b	0.039	GHF	1.07	(+/- 0.13)
			SIF	2.09	(+/- 0.45)
	Grin2c	0.231	GHF	1.07	(+/- 0.14)
			SIF	1.29	(+/- 0.11)
	Slc17a6	0.019	GHF	1.03	(+/- 0.11)
			SIF	1.66	(+/- 0.21)
	Slc17a7	0.874	GHF	1.33	(+/- 0.36)
			SIF	1.40	(+/- 0.27)
Dopamine	*Drd1*	0.153	GHF	1.11	(+/- 0.20)
			SIF	0.74	(+/- 0.04)
	Drd2	0.104	GHF	1.04	(+/- 0.11)
			SIF	0.72	(+/- 0.14)

A8. Supplemental Table		t-test p-values		P72	
Category	Gene	Two-Sided p	Groups	Rel. Exp.	± SEM
GABA	*Gabra1*	0.732	GHM	1.10	(+/- 0.16)
			SIM	1.17	(+/- 0.10)
	Gabra2	0.102	GHM	1.04	(+/- 0.11)
			SIM	0.81	(+/- 0.07)
	Gabra4	0.037	GHM	1.03	(+/- 0.10)
			SIM	1.39	(+/- 0.12)
	Gabrb2	0.527	GHM	1.03	(+/- 0.08)
			SIM	1.09	(+/- 0.05)
	Gabrd	0.246	GHM	2.43	(+/- 1.65)
			SIM	0.56	(+/- 0.13)
	Gabrg1	0.338	GHM	1.01	(+/- 0.06)
			SIM	1.09	(+/- 0.05)
	Gabrg2	0.55	GHM	1.03	(+/- 0.09)
			SIM	1.10	(+/- 0.05)
	Gad1	0.983	GHM	1.11	(+/- 0.17)
			SIM	1.11	(+/- 0.06)
	Gad2	0.681	GHM	1.06	(+/- 0.11)
			SIM	1.12	(+/- 0.07)
	Slc32a1	0.713	GHM	1.03	(+/- 0.09)
			SIM	1.07	(+/- 0.04)
Glutamate	*Gria1*	0.98	GHM	1.01	(+/- 0.07)
			SIM	1.02	(+/- 0.11)
	Gria2	0.131	GHM	1.02	(+/- 0.07)
			SIM	0.86	(+/- 0.07)
	Grin1	0.005	GHM	1.02	(+/- 0.07)
			SIM	0.76	(+/- 0.03)
	Grin2a	0.185	GHM	1.04	(+/- 0.12)
			SIM	0.85	(+/- 0.07)
	Grin2b	0.123	GHM	1.13	(+/- 0.22)
			SIM	0.74	(+/- 0.07)
	Grin2c	0.199	GHM	1.01	(+/- 0.04)
			SIM	0.91	(+/- 0.06)
	Slc17a6	0.265	GHM	1.09	(+/- 0.17)
			SIM	0.84	(+/- 0.13)
	Slc17a7	0.234	GHM	2.06	(+/- 1.42)
			SIM	0.28	(+/- 0.04)
Dopamine	*Drd1*	0.114	GHM	1.02	(+/- 0.09)
			SIM	1.42	(+/- 0.22)
	Drd2	0.01	GHM	1.25	(+/- 0.31)
			SIM	2.93	(+/- 0.48)

Appendix B: Supplemental Data for Chapter 3

B1. Supplemental Table Chapter 3
Significant Differentially Expressed Gene Epigenetic Factor List in Erasers Category

Ctrl Female	Iso Female	Ctrl Male	Iso Male
Kdm2a	N/A	N/A	*Bcorl1*
Kdm3a			*Eny2*
Kdm6b			*Gse1*
Kdm7a			*Hdac4*
Rcor1			*Hdac5*
Zmynd8			*Hif1an*
			Kdm2a
			Kdm2b
			Kdm5b
			Kdm5c
			Kdm6b
			Kdm7a
			Ncor2
			Phf8
			Rcor1
			Sin3b
			Usp16
			Zmynd8

B2. Supplemental Table Chapter 3
Female RT-qPCR Prefrontal Cortex Relative Expression Statistics

		GLM p-values				P32		P72	
Category	Gene	Age	Housing	Age*Housing	Groups	Rel. Exp.	± SEM	Rel. Exp.	± SEM
GABA	*Gabrd*	**<.001**	0.121	0.161	GHF	0.72	(+/- 0.028)	1.01	(+/- 0.065)
					SIF	0.71	(+/- 0.047)	0.86	(+/- 0.080)
	Gabrg1	**0.006**	0.218	0.799	GHF	0.82	(+/- 0.039)	0.96	(+/- 0.036)
					SIF	0.87	(+/- 0.041)	1.05	(+/- 0.109)
	Gad2	**0.029**	0.213	**0.043**	GHF	0.91	(+/- 0.019)	0.9	(+/- 0.070)
					SIF	1.14	(+/- 0.123)	0.84	(+/- 0.023)
	Gabra2	0.112	0.282	**0.039**	GHF	1.52	(+/- 0.235)	1.02	(+/- 0.091)
					SIF	1.24	(+/- 0.094)	1.54	(+/- 0.145)
	Gabra4	0.318	0.822	0.885	GHF	0.95	(+/- 0.069)	1.08	(+/- 0.093)
					SIF	0.99	(+/- 0.052)	1.07	(+/- 0.082)
Glutamate	*Gria1*	0.396	0.291	0.346	GHF	1.04	(+/- 0.058)	1.13	(+/- 0.094)
					SIF	1.18	(+/- 0.065)	1.13	(+/- 0.071)
	Gria2	**0.016**	0.221	0.663	GHF	0.89	(+/- 0.023)	1.05	(+/- 0.074)
					SIF	0.93	(+/- 0.033)	1.05	(+/- 0.046)
	Grin1	0.853	0.609	0.109	GHF	0.95	(+/- 0.074)	1.06	(+/- 0.087)
					SIF	1.1	(+/- 0.062)	0.97	(+/- 0.046)
	Grin2b	0.467	0.704	0.986	GHF	1.12	(+/- 0.123)	1.13	(+/- 0.137)
					SIF	1.08	(+/- 0.043)	1.07	(+/- 0.053)
	Grin2c	**0.043**	0.678	0.379	GHF	1.01	(+/- 0.084)	1.29	(+/- 0.146)
					SIF	1.08	(+/- 0.059)	1.17	(+/- 0.079)
	Slc17a7	**0.019**	0.55	0.385	GHF	0.85	(+/- 0.031)	0.96	(+/- 0.038)
					SIF	0.91	(+/- 0.026)	0.93	(+/- 0.032)
Dopamine	*Drd1*	0.543	0.465	0.65	GHF	0.91	(+/- 0.022)	0.87	(+/- 0.044)
					SIF	0.85	(+/- 0.027)	0.86	(+/- 0.033)
	Drd2	0.526	0.667	**0.024**	GHF	1.16	(+/- 0.057)	0.98	(+/- 0.057)
					SIF	0.93	(+/- 0.043)	1.02	(+/- 0.042)

B3. Supplemental Table Chapter 3
Male RT-qPCR Prefrontal Cortex Relative Expression Statistics

		GLM p-values				P32		P72	
Category	Gene	Age	Housing	Age*Housing	Groups	Rel. Exp.	± SEM	Rel. Exp.	± SEM
GABA	*Gabrd*	0.235	**0.035**	0.215	GHM	0.93	(+/- 0.048)	0.93	(+/- 0.070)
					SIM	0.76	(+/- 0.028)	0.88	(+/- 0.047)
	Gabrg1	**<.001**	0.084	**0.046**	GHM	0.84	(+/- 0.031)	1.07	(+/- 0.059)
					SIM	0.78	(+/- 0.025)	1.15	(+/- 0.044)
	Gad2	0.782	0.538	0.657	GHM	0.99	(+/- 0.040)	0.98	(+/- 0.025)
					SIM	0.92	(+/- 0.033)	0.95	(+/- 0.028)
	Gabra2	0.18	0.363	0.885	GHM	1.19	(+/- 0.068)	1.35	(+/- 0.134)
					SIM	1.2	(+/- 0.147)	1.31	(+/- 0.107)
	Gabra4	0.494	0.164	**0.008**	GHM	1.17	(+/- 0.068)	0.97	(+/- 0.072)
					SIM	0.89	(+/- 0.025)	1.1	(+/- 0.048)
Glutamate	*Gria1*	**0.042**	0.354	0.365	GHM	1.05	(+/- 0.051)	1.17	(+/- 0.057)
					SIM	0.99	(+/- 0.080)	1.03	(+/- 0.033)
	Gria2	0.9	0.977	**0.034**	GHM	1	(+/- 0.039)	0.96	(+/- 0.051)
					SIM	0.89	(+/- 0.023)	0.97	(+/- 0.023)
	Grin1	0.705	0.43	**0.041**	GHM	1.1	(+/- 0.038)	1.03	(+/- 0.052)
					SIM	0.93	(+/- 0.043)	1.03	(+/- 0.033)
	Grin2b	0.175	0.888	**0.031**	GHM	1.17	(+/- 0.073)	1.08	(+/- 0.064)
					SIM	0.96	(+/- 0.037)	1.16	(+/- 0.040)
	Grin2c	0.373	0.176	0.335	GHM	1.25	(+/- 0.085)	1.29	(+/- 0.082)
					SIM	1.06	(+/- 0.036)	1.24	(+/- 0.071)
	Slc17a7	0.777	0.892	0.343	GHM	0.94	(+/- 0.032)	0.91	(+/- 0.028)
					SIM	0.88	(+/- 0.025)	0.89	(+/- 0.012)
Dopamine	*Drd1*	0.37	0.106	0.812	GHM	0.92	(+/- 0.042)	0.98	(+/- 0.041)
					SIM	0.82	(+/- 0.019)	0.84	(+/- 0.025)
	Drd2	0.374	**0.045**	0.631	GHM	1.1	(+/- 0.045)	1.15	(+/- 0.052)
					SIM	0.96	(+/- 0.024)	1.08	(+/- 0.046)

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