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Long-Term Neurobehavioral Consequences of Adolescent Social Isolation

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Long-Term Neurobehavioral Consequences of Adolescent Social Isolation

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Abstract

Long-Term Neurobehavioral Consequences of Adolescent Social Isolation
By Elizabeth Hinton

Adolescence is a developmental period during which significant prefrontal cortical (PFC) maturation occurs in tandem with emerging, age-typical changes in social behavior. Unperturbed, these maturational shifts collectively aid juveniles in the transition to adulthood. Clinical research suggests that perturbations during adolescence, such as experiencing social adversity, constitute a risk factor for certain neuropsychiatric-related outcomes in adulthood. The findings presented in this dissertation are situated upon decades of preclinical social isolation research in rodents, which have attempted to investigate these lingering neurobehavioral consequences of prior social insults. However, this research largely excludes females and assesses outcomes while animals remain in a state of isolation, precluding an understanding of lasting consequences that are present despite correcting the social environment. We hypothesize that isolation during adolescence disrupts ongoing PFC maturation, such as dendritic spine pruning processes. Further, dysregulated PFC-dependent behaviors (*e.g.* a premature reliance on habitual responding at the expense of goal-directed decision making) may emerge. We show that previously-isolated mice more readily engage in habitual response strategies and exhibit adolescent-like densities of dendritic spines in the orbital PFC as adults. We then show that correcting dendritic architecture by inhibiting the cytoskeletal regulatory factor Rho-kinase during the adolescent isolation period blocks decision-making deficits in mice with a history of isolation. A history of isolation also shapes adult social interaction subtleties and medial PFC (mPFC) outcomes. We investigated dendritic spine densities and postsynaptic regulators of synaptic function, revealing elevated densities of thin-type dendritic spines and increased postsynaptic density-95 protein content. Finally, we use a chemogenetic approach to modulate the mPFC during a time in which animals should be consolidating information necessary to modify behavioral responses that no longer produce valued outcomes. This chemogenetic strategy blocks decision-making deficits and corrects anhedonic-like behavior in previously-isolated mice. Together, these findings implicate disrupted orbital PFC and mPFC function in the enduring neuropsychiatric-related behavioral deficits present following a history of isolation. Disruption of ongoing maturational processes during adolescence and associated impairments in related behaviors may constitute a mechanism by which social adversity experienced during adolescence becomes a risk factor for the occurrence of certain neuropsychiatric-related disorders in adulthood.

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CHAPTER 1: REVIEW OF THE LONG-TERM CONSEQUENCES OF SOCIAL ISOLATION DURING ADOLESCENCE

1.1 CONTEXT AND AUTHOR'S CONTRIBUTION

The following chapter reviews prefrontal cortical and behavioral consequences of a history of social isolation during adolescence. It focuses on findings from rodent models that combine isolation and resocialization protocols to investigate these long-term consequences that are either corrected by social enrichment or present despite social reintegration following the isolation period. This work was researched, organized, and written by the dissertation author, with editorial feedback from Dr. Shannon Gourley.

1.2 ABSTRACT

While social isolation during development is a commonly used rodent model to emulate early-life social adversity, the majority of the findings to date result from experiments conducted while animals remain in a current state of isolation and therefore do not address long-term neurobehavioral consequences of isolation. In order to better address this question, animals can be re-housed into social groups, or "resocialized" prior to testing. Here, findings from studies implementing resocialization have been reviewed. We focus on depressive-like and anxiety-like phenotypes, social behavior, and function and neurodevelopment of the prefrontal cortex, which is otherwise shaped by social experience during adolescence.

1.3 INTRODUCTION

Atypical social experiences can have profound neurobiological and behavioral effects. One well-documented example concerns the consequences of inadequate orphanage care in infants and children. Early-life neglect correlates with later abnormalities in corticolimbic connectivity. For instance, adolescents with early-life experience in orphanages have ventral medial prefrontal cortical (vmPFC)-amygdala connectivity patterns resembling those in significantly older individuals. This

"premature maturation" is accompanied by atypical anxiety levels, even despite adoption (Gee et al., 2013).

Lack of typical social interaction affects adult populations as well. Recently, a meta-analysis examining mortality risk factors in adults revealed that social isolation, in and of itself, carries a risk for death comparable to previously established factors such as insufficient healthcare access and obesity (Holt-Lunstad et al., 2015). As with social deprivation during childhood and adulthood, social adversity during adolescence poses long-term health risks. For example, Thapar et al. (2012) found that social stress exposure during adolescence constitutes a primary risk factor for depression in females, and that depression that emerges during adolescence is associated with greater risk for recurrent major depressive episodes throughout the lifespan.

Healthy, age-typical social engagement during adolescence fosters the maturation of behaviors necessary for independence in adulthood (Spear, 2000). In humans, the amount of time spent socializing within the family decreases in early adolescence as individuals spend more time engaging with peers (Larson et al., 1996). This reallocation of time from family to peers during adolescence is present in multiple mammalian species. Social play behavior emerges in rodents in adolescence, peaking between postnatal days (P) 30-40 (Thor and Holloway, 1984), then declining as rodents enter adulthood (Panksepp 1981). Social play behavior is described as non-aggressive forms of reciprocal physical behaviors traditionally thought to serve as "practice" for later aggressive bouts of fighting and/or mating behavior in adulthood. Others have argued that the maintenance of physical activity in a reciprocal manner that continues without escalating to the point of aggression recruits top-down cortical control of behavior, which in turn aids in the development of executive functioning skills and emotion regulation (Pellis and Pellis, 2017). In summary, socialization with peers (or cage mates in the case of laboratory rodents) increases during adolescence as individuals transition into adulthood, and is thought to play a role in an individual's burgeoning independence

from the family unit. Perturbations in the typical social environment during adolescence may thereby confer long-term maladaptive consequences that persist into, or manifest in, adulthood.

In the research laboratory, multiple procedures are used to study the neurobiological effects of social adversity in adolescence. Many studies in rodents capitalize on aggressive behavior and/or the disruption of social hierarchies in males. Female subjects have historically been understudied, given that they do not reliably engage in conspecific-directed aggression. Nevertheless, female rodents engage in communal nesting and play behavior during adolescence, suggesting that social experience during this time is developmentally impactful.

Another widely used model of social adversity in rodents is social isolation. Isolation procedures typically involve housing rodents individually over a period of time, during which control animals remain in typical group-housed settings. While an isolation protocol is simple to implement, many studies have drawn criticism, in terms of the conclusions that can and can't be drawn. One common critique concerns the timing of the isolation period. As discussed by Lukkes et al., (2009), many studies implement isolation shortly after animals are weaned, and the isolation persists for multiple weeks. This procedure makes it impossible to determine the specific developmental periods during which social isolation confers neurodevelopmental changes (for instance, failing to dissociate adolescent and peri-adolescent periods). Another critique is that many studies lack adult control groups, in which isolation occurs for the same duration of time, but following the developmental period of interest. Thus, one cannot conclude that any particular outcomes are attributable to isolation experienced during adolescence.

Finally, the majority of social isolation investigations test animals while they are experiencing isolation. Thus, one cannot dissociate neurobiological consequences of developmental *vs.* acute isolation. In our view, this weakness is considerable. ***The purpose of this review is to summarize investigations in which rodents were isolated during adolescence, then resocialized prior to***

testing, allowing the investigators to reveal the long-term consequences of social adversity experienced during adolescent sensitive periods. We initially focus on neurodevelopment of the prefrontal cortex, which is otherwise shaped by social experience during adolescence. Long-term behavioral outcomes related to prefrontal cortical function are discussed.

1.4 PREFRONTAL CORTICAL DEVELOPMENT DURING ADOLESCENCE

Adolescence constitutes a period of significant neurodevelopment, with the prefrontal cortex maturing throughout this period and well into early adulthood (Petanjek et al., 2011). A prominent component of adolescent cortical development is a gradual thinning of gray matter and increases in white matter. One component argued to underlie the thinning of gray matter is neuronal and synaptic remodeling that ultimately makes for a more energetically efficient adult brain (Chugani 1998). Specifically, synaptic and dendritic spine densities in both humans and rodents undergo a period of refinement and pruning, potentially to allow more efficient neurotransmission that requires fewer synaptic contacts (Bourgeois et al., 1994; Huttenlocher and Dabholkar, 1997; Gourley et al., 2012; Shapiro et al., 2017). Meanwhile, aforementioned increased prefrontal cortical white matter involves increased production of myelin across human and rodent species (Barnea-Goraly et al., 2005; Jacobson 1963). This increase in myelin results in insulation necessary for rapid neurotransmission and maintenance of signal transduction throughout axons. In summary, adolescent prefrontal cortical maturation consists of neuronal pruning and myelination trajectories that are conserved across humans and rodents. This plasticity, while presumably adaptive in many ways, may render the adolescent prefrontal cortex vulnerable to the effects of insults, such as adverse social experiences, encountered during this time.

The following section reviews what is known about the impact of isolation during adolescence on factors that may lead to suboptimal function of the medial prefrontal cortex.

Beginning with the influence of isolation at the synapse, we review alterations in dendritic spine densities and markers of excitatory and inhibitory synaptic transmission. These results are discussed in terms of the influence of isolation onset during the adolescent period, as well as how these results may contribute to suboptimal synaptic function in adulthood. Aside from changes at the synapse, dysregulated neurotransmission also arises from myelination deficiencies. The effects of adolescent isolation on myelin levels and factors contributing to myelination are then reviewed. Finally, electrophysiological results are discussed and provide evidence for long-lasting excitation/inhibition imbalance in the medial prefrontal cortex of adult animals with a history of isolation during adolescence.

1.5 LONG-TERM MPFC OUTCOMES FOLLOWING SOCIAL ISOLATION DURING ADOLESCENCE

1.5.1 Synaptic and cellular changes following isolation during adolescence

Adolescence is characterized by marked changes in synapse density. In humans, synaptic density in the prefrontal cortex decreases throughout adolescence until stabilizing by early adulthood (Rakic et al,. 1994). A similar pruning trajectory takes place in the rodent mPFC: For instance, by staining for synaptophysin, a presynaptic protein used as a marker of synapses, Drzewiecki et al., (2016) demonstrated synaptic pruning in the mPFC of both male and female adolescent rats. Social isolation during adolescence alters total synapse density in the mPFC, as ascertained immediately following an isolation period (but still during adolescence) and in adulthood, and despite correction of the social environment in mid-adolescence (Leussis et al., 2008, Leussis and Andersen, 2008). Specifically, Leussis et al., (2008) found reduced synaptophysin immunoreactivity in the infralimbic subregion of the mPFC in adults with a history of isolation. A similar loss in synaptophysin in previously-isolated rats was observed immediately following isolation (from P30-35) in adolescence

(Leussis and Andersen, 2008). Taken together, this pattern suggests that adolescent isolation (P30-35) leads to a rapid reduction of synaptophysin during early adolescence that fails to recover even when the social environment is corrected.

Like synaptic density during adolescence, dendritic spines, the primary sites of excitatory neurotransmission in the brain, are also pruned throughout adolescence in both humans and rodents. While synapse and dendritic spine refinement during this time is typical of the adolescent period under normal developmental circumstances, changes in the social environment during this time may redirect dendritic spine pruning in the prefrontal cortex, one of the final regions of the brain to finish maturing. Indeed, social isolation initiating at a timepoint when dendritic spine pruning should be beginning (P31), and lasting until dendritic spines densities should be largely stable (P60), results in both short-term and long-lasting changes in dendritic spine densities. Specifically, isolation during adolescence leads to an elevation in mPFC dendritic spine densities on layer V neurons, detectable both during the isolation period (at P49) and weeks following resocialization in adulthood (resocialization at P60, dendritic spines enumerated at P82) (Hinton et al., in prep., Chapter 2). The elevation in dendritic spine densities may be driven specifically by an atypical elevation in thin-type dendritic spines (Chapter 2). Thin spines are considered immature and highly motile, but they nevertheless can form and carry synapses. Together, these results suggest isolation during adolescence may predispose this excitatory cell population within the mPFC towards strengthen synaptic contact and/or a hyperexcitable phenotype in adulthood.

Multiple protein and cellular abnormalities resulting from adolescent isolation suggest possible mechanisms by which isolation during adolescence could result in long-term synaptic alterations in the mPFC (see Table 1-1). Importantly, functional consequences likely depend on the period during which animals experience isolation. For instance, we identified elevated postsynaptic density-95 (PSD95) protein content following social isolation from P31-60 (Hinton et al., in prep.;

Chapter 2). While these patterns are generally consistent with increased dendritic spine densities on layer V excitatory neurons discussed above (and see Hinton et al., in prep.; Chapter 2), they differ from the effects of short-term isolation from P30-35, which instead *reduces* synaptic marker levels in the mPFC, as also discussed above. Thus, it is possible that early-adolescent isolation reduces excitatory synapses, and if isolation then extends into later adolescent periods, synaptic contacts are not pruned, as would be typical, resulting in *elevated* synaptic marker expression in adulthood.

PSD95 acts as a postsynaptic protein at excitatory synapses where it regulates synaptic strength and activity-dependent plasticity of dendritic spines (Beique and Andrade, 2003). In adult mice with a history of prolonged social isolation in adolescence, we detected increased PSD95 protein content by western blot, but not immunofluorescent puncta counts, in the mPFC (Hinton et al., in prep.; Chapter 2). This pattern suggests enhanced strength of existing synapses, but not necessarily an increase in the total number of synapses. The precocious strengthening of synapses could render them resistant to elimination, even when their elimination would be adaptive. For instance, action-outcome learning and memory (discussed later in this chapter) requires dendritic spine elimination in certain prefrontal cortical regions, and blocking their elimination blocks response-outcome expectancy updating (Whyte et al., 2019), as also occurs in mice with a prolonged history of social isolation (Hinton et al., 2019; Chapter 3). Whether a history of social isolation blocks learning-related dendritic spine elimination should be explicitly tested in future investigations.

In addition to changes in factors involved in excitatory neurotransmission, adolescent isolation may also influence inhibitory synaptic function in the mPFC. Though current evidence suggests these changes may be transient, recovering with resocialization. Bator et al., (2018) discovered increased levels of a synaptic membrane protein, Glutamic Acid Decarboxylase-65 (GAD65), known to facilitate activity-dependent GABA release from vesicles. GAD65 protein levels in the mPFC of isolated animals were markedly increased immediately following isolation

during early adolescence (P30-40). However, this effect was transient and not present in adulthood. Similarly, in adults with a history of isolation we do not observe changes in mPFC gephyrin (Chapter 2; Table 1-1). A future experiment could assess levels of gephyrin puncta counts in the mPFC during the adolescent isolation period to determine whether the effects of isolation on levels of this inhibitory postsynaptic marker are immediate and transient in a manner similar to expression levels indicative of inhibitory function described above. Another reported mechanism by which adolescent isolation may impact mPFC neuronal inhibition is through its effects on parvalbumin interneurons. Parvalbumin cells are a subtype of GABAergic interneuron that preferentially inhibit subcortically-projecting neurons within the mPFC (Lee et al., 2014). In response to isolation during adolescence, parvalbumin protein levels transiently increase when examined a week following resocialization. However, parvalbumin protein levels are significantly reduced in adulthood following protracted social housing after the adolescent isolation period (Mackowiak et al., 2019).

Another potential mechanism by which adolescent isolation could translate into lasting changes in mPFC neurotransmission in adulthood is through its effects on myelin. Isolation during adolescence is known to correlate with aberrant myelination of the mPFC (Makinodan et al., 2012, 2017). Unperturbed, myelination of axons supports conduction velocity needed for optimal neurotransmission within networks. Social isolation during adolescence disrupts factors contributing to myelin development such as oligodendrocyte maturation (Makinodan et al., 2012, 2017, Hinton et al., 2019; Chapter 3; Table 1-1). For instance, multiple studies exposed both male and female mice to social isolation during periods that overlap with a known deposit of myelin in rats (P30-40; Jacobson, 1963), or a period of oligodendrocyte maturation in mice (P21-35; Makinodan et al., 2012, 2017), revealing long-lasting changes in mPFC myelination in adulthood. Intriguingly, myelin thickness in the mPFC is reduced in previously-isolated adult mice that were re-housed with previously-isolated cage mates, but not previously-isolated adult animals that were then re-housed

with previously-socialized conspecifics. In other words, hypomyelination in the mPFC of adults with a history of isolation could be recovered depending on the cage composition following isolation, suggesting that specific social contexts later in life could buffer against long-term alterations in myelination and myelin-related factors due to early-life social isolation.

1.5.2 Electrophysiological evidence for long-lasting changes in excitation/inhibition balance

In vitro recordings have hinted at impaired excitation/inhibition balance in the mPFC following social isolation. Baarendse et al., (2013) utilized whole cell patch clamp to record amplitudes of excitatory postsynaptic potentials (EPSPs) from layer V neurons. Tissue was derived from adult male rats (P84) with a history of either group-housing or isolation from P21-43. An application of dopamine (DA) receptor D1 and D2 agonists reduced EPSP amplitude in group-housed control mice, but this reduction was absent in previously-isolated rats. DA fiber innervation of the mPFC has been documented by quantifying tyrosine hydroxylase immunoreactive fibers in the mPFC. DA innervation of the mPFC abruptly increases during early adolescence and then proceeds more gradually into adulthood in both male and female rats (Willing et al., 2017). Dopaminergic receptor subtype mRNA expression peaks in the mPFC during late adolescence (P45) and subsequently declines and stabilizes in adulthood (Naneix et al., 2012). Given that the isolation timeline encompassed much of this development, this suggests that normative dopaminergic system function in the adult mPFC may be shaped by prior social isolation through an interaction between isolation and the maturation of DA fiber innervation of the mPFC.

A final piece of evidence that effects of prior social isolation could linger in terms of mPFC function in adulthood comes from electrophysiological recordings and analysis of immediate early gene expression levels in the mPFC evoked by social interaction. Here, Makinodan et al., (2017)

utilized electroencephalogram recordings to investigate oscillatory activity within layer V of the prelimbic and infralimbic cortices while mice socialized with novel conspecifics. Recordings were performed in adulthood at P65 in previously-isolated mice that were re-housed at P35 in social environments consisting of either previously-isolated cage mates or previously-socialized cage mates. Control animals were mice with a history of group housing. The social interaction began when an age-matched unfamiliar male was placed into the cage with the experimental mouse, and both subjects were allowed to freely roam throughout the recording. Oscillation activity was reported as a percentage of the baseline activity. Makinodan et al., (2017) observed a general decrease in γ-oscillation and cFos mRNA in mice with a history of isolation. These losses were only partially recovered if isolated mice were subsequently re-housed with group-housed cage mates following the adolescent isolation period. γ-oscillation has been described as being involved in stimulus-driven shifts in attention (Pinto et al., 2013) and is known to contribute to the balance of excitatory-inhibitory tone involved in mood and cognition (Yizhar et al., 2011, Canetta et al., 2016); thus its loss could be associated with related behavioral dysfunction following isolation discussed below.

1.6 LONG-TERM BEHAVIORAL CONSEQUENCES

1.6.1 Depressive-related behavior following adolescent social isolation

Experiencing depression that emerges during adolescence is associated with an increased risk for depressive episodes that persists across the lifespan (Kolaitis, 2012). A strong predictor of developing depression during adolescence is experiencing social stress, and following puberty, adolescent women are more likely to experience depression than men (Thapar, 2012). These patterns underscore the need to continue improving existing animal models of adolescent social adversity so as to recapitulate persistent depressive-like behaviors that endure into adulthood. Animal models

that induce these persistent depressive-like phenotypes can then be used to characterize and correct neurobiological mechanisms.

A range of depression-related symptoms can be modeled using rodents (*e.g.* social behavioral abnormalities, cognitive impairments, anxiety-like behavior, *etc.*), but these behaviors are not unique to depression and are not always investigated and discussed in terms of their applicability to depressive-like symptomology. Within the literature focused on identifying depressive-like behavioral consequences in animal models of adolescent social adversity, few report *persistent* depressive-like effects and even fewer incorporate female subjects. Limited findings regarding depression-like learned helplessness behavior and anhedonic-like effects of prior isolation are discussed below. However, it should be noted that long-term behavioral outcomes such as anxiety-like behavior, social behavioral deficits, and decision-making impairments discussed in subsequent sections are either often co-morbid with depression or known to be within the range of depression symptomology.

1.6.1.1 Passive reactivity to acute stressors

Two commonly used assays investigating depressive-related behavior in rodents are the forced swim and learned helplessness tests. Both assays have been previously considered to model behavioral despair that takes place when an animal is exposed to an inescapable stressor (*e.g.* forced swimming, inescapable foot shock) and subsequently stops struggling to escape the stressful stimuli or "gives up". In the forced swim test, this behavioral profile includes a decreased latency to the first bout of immobility in the water (*i.e.* floating), increased immobility, and decreased swimming. In the learned helplessness procedure, this behavioral profile includes increased latency to escape a foot shock, and increased number of failures to escape a foot shock. Importantly, the interpretation that these behavioral outcomes are depressive-like has been debated. An alternative perspective is that

the forced swim test assesses an animal's reactivity to an acute stressor, and that the learned helplessness procedure examines how controllability of a stressor impacts the resulting biological and/or behavioral toll of the stressor itself (Maier and Watkins, 2005). In this respect, while maladaptive behavioral responses observed using these protocols can resemble aspects of depressive-like behavior, these behaviors are not unique to depression symptomology (Maier and Watkins 2005).

As mentioned previously, depression is more common in adolescent women than in men (Thapar et al., 2012), and a susceptibility to depressive-related behaviors has been observed in adolescent female subjects exposed to the forced swim test and learned helplessness procedures during early adolescence (Leussis and Andersen 2008). Specifically, female adolescent group-housed rats exhibit decreased latency to immobility, increased immobility, and decreased time swimming in the forced swim test. They are also more susceptible to learned helplessness behavior relative to male group-housed subjects, as indicated by increased latency to escape and increased escape failures following inescapable stress. Social isolation during the early adolescent period (P30-35) triggered depressive-like responses in both tests in males; in females this was observed only in the learned helplessness procedure (Table 1-2). While this general susceptibility to depressive-like behaviors in group-housed female adolescents is interesting, social isolation during early adolescence does not appear to have as great of an effect in females as it does in previously-isolated males. While previously-isolated females exhibited the learned helplessness phenotype that emerged in response to inescapable footshock, how long-lasting the effect is remains unclear. Specifically, it is unclear whether this effect would persist into adulthood or recover with prolonged social housing, as animals were tested immediately following resocialization.

1.6.1.2 Anhedonic-like behavior

Anhedonia is a loss of interest in activities that one formerly enjoyed and is a core symptom of depression. A measure of anhedonic-like behavior in rodents is sucrose consumption, in which decreased preference for – or consumption of – sucrose-infused water is considered anhedonic-like (Der-Avakian and Markou, 2011). We have observed anhedonic-like behavior in female mice with a history of adolescent social isolation (Chapter 3). Here, isolation lasted nearly the entire adolescent period (P31-60) until mice were resocialized at P60. After a period of ~3 weeks, anhedonic-like behavior was assessed in previously-isolated and previously group-housed female mice. Mice with a history of social isolation during adolescence consumed less sucrose. Importantly, testing occurred well following resocialization in order to determine whether depressive-related behavior would be present despite correcting the social environment.

Taken together, these results begin to resemble patterns of depression prevalence in women. First, they reveal that adolescent females demonstrate a susceptibility to maladaptive behavioral responses to stress, associated with depression-like behavior. Additionally, social adversity in the form of isolation during adolescence evokes short-term depressive-like behavioral strategies during adolescence (Leussis and Andersen, 2008). Finally, long-lasting aspects of depressive-like behavior are present in adult females with a *history* of adolescent social isolation (Hinton et al., 2019; Chapter 3).

1.6.2 Long-term consequences of adolescent social isolation on decision-making behavior

Goal-directed decision making improves throughout adolescence, and this pattern holds true for both humans and rodents (Blakemore and Robbins, 2012; Naneix et al., 2012). There are numerous requirements for a decision to be considered "goal-directed" or "goal-oriented." First, goal-directed decisions require associating an action with its likely consequence, in other words, learning contingencies between actions and outcomes. Second, goal-directed decision making

requires an understanding of the outcome value. Third, a goal-directed decision is one that requires updating and consolidation of changes in previously-learned action-outcome contingencies and changes in outcome values at the time these changes are encountered. In the event that an organism fails to initially learn or consolidate this new information appropriately, behavioral strategies that were once goal-directed actions can then shift to habitual responses. Habits are insensitive to changes in action-outcome contingencies. As such, habits are behaviors that don't appropriately modify when an action's consequential outcome loses its value. Habits are also known to be stimulus-elicited and can develop as behaviors become more familiar. Goal-directed and habit-based behavior can be studied in rodents and humans using multiple tasks such as instrumental contingency degradation and outcome devaluation.

Little is known regarding the long-term effects of social isolation during adolescence on decision-making behavior in adulthood. We implemented an instrumental contingency degradation procedure to characterize decision-making strategies in previously-isolated mice. Here, mice are first trained to generate two distinct actions for food reinforcers. Following this step, one action-outcome contingency remains intact, while the alternate action-outcome contingency is degraded. Here, a food pellet outcome is delivered in a manner noncontingent upon the animal's action. Thus, information regarding familiar action-outcome relationships must be updated.

During an initial probe test, we found that both previously-socialized and previously-isolated (P31-60) mice show that they have updated action-outcome expectations and respond preferentially toward the intact instrumental contingency, exhibiting goal-directed decision making (Table 1-3). We further mapped the stability of goal-directed behavior and found that previously-isolated mice exhibit a premature shift towards relying on habitual response strategies as behaviors become familiar (Hinton et al., 2019). Importantly, this decision-making bias is detectable well after social reintegration (at P82), is complemented by outcome value insensitivity, assessed in a separate

outcome devaluation task, and is not present in mice exposed to isolation for an equivalent duration beginning in adulthood, suggesting that adolescence is a critical period for the development of neurocircuits supporting this complex cognitive function. An alternative possibility, which is not mutually exclusive, is that social isolation *strengthens* systems that support habitual behavior; future studies could attempt to parse apart these possibilities.

These findings contribute to evidence that adolescent social isolation exerts long-term effects on general executive function in adulthood. For example, in situations of uncertainty that are inherent during the rat gambling task, male rats exhibit decision-making abnormalities in adulthood stemming from prior acquisition failures (Baarendse et al., 2013). A second observation of acquisition impairment in male mice with a history of isolation has been reported in the context of deficient working memory in a water T-maze. While these acquisition impairments contribute to different behavioral phenotypes, it is worth noting that they result from a similar isolation timeline (P21-43, Baarendse et al., 2013; P21-35, Makinodan et al., 2012). This isolation timeline overlaps with various maturational processes (*e.g.* increased myelination, synaptic refinement) that are ongoing in the prefrontal cortex during this time. Collectively, these few findings confirm that a history of social isolation during adolescence contributes to various decision-making biases in adulthood that are present despite correcting the social environment and may be due to selective forms of learning and memory impairment.

1.6.3 Anxiety-like effects of social isolation during adolescence

Long-term effects of adolescent social isolation on anxiety-like behavior have been studied using a variety of isolation protocols and tasks (Table 1-4). The following section focuses on anxiety-like behavior assessed using the open field task and elevated plus maze.

1.6.3.1 Open field test

The open field test consists of placing a rodent into a novel, open chamber and recording locomotor activity over a period of time. The open field is meant to resemble a naturally aversive scenario for a rodent – one in which the immediate environment lacks an escape route and space to hide from a predator. Therefore, behaviors considered anxiety-like include: elevated locomotor activity, in and of itself, as well as elevated locomotor activity that does not decline over time, increased distance traveled along the perimeter, increased latency to enter the center of the open field arena, reduced time spent in the center of the open field, and freezing behavior (Lezak et al., 2017).

Long-term anxiogenic effects of adolescent social isolation (those detectable despite social reintegration) have largely not been explored in female rodents, so the following summary is focused on experiments using male subjects. Wright et al., (1991) reported elevated locomotor activity following isolation that spanned from the juvenile to early adult period (P21-51). Testing occurred prior to resocialization. Following resocialization, an anxiogenic phenotype has been observed in rats isolated from the juvenile period onward (P21-42; Lukkes et al., 2009a) (P18-32; Pascual et al., 2006). Notably, the anxiety-like phenotype reported by Lukkes et al., (2009a) – decreased entry into the center of the arena – was transient, in that previously-isolated males habituated to the open field within 10 minutes.

Other studies report no effects of prior isolation on anxiety-like behavior (Makinodan et al., 2017, Bator et al., 2018). It is worth noting Makinodan et al., (2017) derived results from distance traveled *during* a social interaction test, which suggests behavioral output of the experimental mice could have been influenced by the presence of a stimulus mouse. Additionally, various anxiety-like characteristics are quantified in these reports (*e.g.* distance traveled in arena vs. entries to the center of the chamber). While these behaviors describe features of an anxiogenic phenotype, the

inconsistent measures make it difficult to draw a conclusion regarding whether isolation in male rodents consistently produces an anxiety-like phenotype or not. A step forward would be for future studies to include more than one behavioral measure for this assay, even if this is a list of null findings briefly mentioned in manuscripts.

Here, we report no effects of prior isolation on anxiety-like behavior in adulthood. While this is in agreement with studies that have assessed anxiety-like behavior in female subjects exposed to ongoing isolation (see Walker et al., 2019), future work incorporating female subjects should focus on resocializing animals prior to testing in order to determine long-term effects, if any, on anxiety-like behaviors.

1.6.3.2 Elevated plus maze

The design of the elevated plus maze is intended to partially emulate another environmental scenario to which rodents are averse – that which is open, brightly lit, and elevated – similar to the motivation behind the design of the open field test. However, the elevated plus maze also includes two dark, enclosed arms, in addition to two open arms, all of which the animal can explore freely. In this assay, an animal that spends more time exploring the open arms and/or demonstrates an increased number of entries into the open arms is considered less anxious, because these arms of the maze are exposed and therefore pose a greater likelihood of encountering a predator. Similarly, more time spent in the closed arms and a greater number of entries into the closed arms represents anxiety-like behavior.

Leussis and Andersen (2008) investigated time spent in the open arms of the elevated plus maze in male and female Sprague-Dawley rats with a history of group-housing or isolation from P30-35. This experiment revealed multiple sex differences in anxiety-like behavior when rats were tested the day after being resocialized. First, female control subjects exhibited reduced anxiety-like

behavior relative to male control subjects, as indicated by a significant elevation in the time they spent in the open arms of the elevated plus maze. However, prior isolation induced an anxiety-like phenotype. Meanwhile, a history of isolation in males had no effect on anxiety-like behavior; *i.e.* time spent in the open arms did not differ from male group-housed control rats.

Sex differences in anxiety-like behavior that were present despite resocialization were also reported by Weintraub et al., (2010). Here, male and female rats were first isolated or group-housed beginning at P30, similar to the isolation procedure of Leussis and Andersen (2008), discussed above. However, the isolation period then spanned multiple phases of development – early, mid, and late adolescence – until re-housing at P50, and testing at P80. Regardless of prior housing condition, adult females demonstrated reduced anxiety-like behavior relative to males, in that they spent more time in the open arms of the maze, reminiscent of the decreased anxiety-like behavior observed in group-housed control female *vs.* male subjects discussed above. However, unlike in the prior study, a history of more prolonged isolation (and recovery time) had no obvious effect on anxiety-like behavior in female rats. Further, isolation appeared to *decrease* anxiety-like behavior in males, again in contrast to the above study, which deployed a shorter isolation procedure and minimal recovery time. One caveat, though, is that Weintraub et al., (2010) used rats that had been exposed to repeated restraint stress between P50-P80 as part of a separate experiment. This detail calls into question whether the reported anxiolytic-like effects of isolation were influenced by stressor exposure in adulthood.

Interestingly, Wright et al., (1991) followed a similar isolation timeline (P21-51) to investigate long-term anxiety-like behavior that persists despite resocialization (tested at ~P84). To do so, they utilized multiple groups, analyzing anxiety-like behavior prior to resocialization, following resocialization, in age-matched isolated animals that did not experience resocialization, in socially-housed control rats, and in an adult isolation comparator group in which isolation was delayed until

adulthood. In summary, anxiety-like behavior in males was present prior to resocialization, persisted despite resocialization, and was induced by isolation experienced during adolescence rather than in adulthood. While this profile opposes the anxiolytic-like phenotype observed by Weintraub et al., (2010), differences cannot necessarily be attributed to repeated restraint stress in Weintraub et al., (2010). The developmental age at which isolation was initiated also differs between the two studies (P21 *vs.* 30), so an interaction between isolation and the developmental period (juvenile *vs.* early adolescence) may also contribute to the opposing results.

In summary, the open field and elevated plus tests are predicated on typical approach and avoidant strategies exhibited by rodents in response to environments that evoke conflict. Both the open field test and elevated plus maze have been implemented in multiple resocialization studies to determine long-term effects of isolation on anxiety-like behavior, though it is worth reiterating that a paucity of findings pertain to females. One additional assay that has been used to examine approach and avoidance behaviors is the social interaction test (Lezak et al., 2017). Social behavior following a history of adolescent social isolation has been investigated using various adaptations of the social interaction test. For the purposes of this review, these investigations are distinguished from the aforementioned investigations and discussed in the next section, but we acknowledge that changes in anxiety-like and social behavior can be intimately related.

1.6.4 Social behavioral consequences of adolescent isolation and resocialization

1.6.4.1 Resident-intruder test

One assay of aggressive behavior in rodents is the resident intruder test. Tulogdi et al., (2014) isolated male rodents for three days, then placed a smaller male "intruder" in the home cage. Attack episodes were categorized as vulnerable-area attacks (targeting head, throat, belly, paws of intruder). A nonvulnerable area-attack occurred if the resident targeted the back and hindlimb areas.

Isolated male rats delivered more attacks to vulnerable areas of the intruder's body relative to socially-housed control rats. Interestingly, this pattern persists despite resocialization over a subsequent three-week period. Attacks were also characterized as being either "signaled," such that they were delivered in response to the intruder exhibiting an offensive threatening behavior, or "non-signaled" in the case that a resident attacked the intruder despite a nonthreatening antecedent. When repeatedly tested following resocialization, previously-isolated animals delivered more non-signaled attacks relative to socially-housed control rats, indicating an abnormally aggressive phenotype.

1.6.4.2 Home cage social interaction observations

Home cage social interactions have been monitored in both male and female rats following resocialization. Data on female subjects are limited, but one study detected no differences in social behaviors (social contact, rearing, *etc.*) in female rats isolated from P21-42 that were rehoused with other previously-isolated rats as cage mates (Lukkes et al., 2013). By contrast, Tulogdi et al., (2014) recorded aggressive behavior in male rats during the hour immediately following resocialization and again five days later. Here, behaviors scored were offensive threatening behaviors, defensive behaviors classified as either those provoked by a cage mate or unprovoked, dominant postures, submissive postures, and attack counts. One behavior that emerged in previously-isolated rats was defensive behaviors that were unprovoked, specifically, defensive behaviors in reaction to events such as noises occurring outside of the home cage and/or a cage mate exploring an area within close proximity to the animal being observed. This defensive behavioral phenotype did not subside and was still present five days following resocialization.

A separate series of observations examined huddling behavior in males following resocialization. Here, cameras positioned at three different angles photographed sleeping animals

throughout a period of inactivity. Huddling was defined as direct physical contact with at least one cage mate while sleeping. Animals that were previously-isolated demonstrated less huddling behavior than previously-socialized control rats, and this effect was only present within the first week following resocializing all animals. A separate experiment was conducted to determine whether the prior social environment of cage mates influenced huddling behavior. Here, previously-isolated rats still demonstrated reduced huddling within the first days of resocialization, regardless of the cage composition – meaning, isolated rats re-housed with other isolated rats demonstrated a comparable decrement in huddling during the first week relative to isolated rats re-housed with previously-socialized rats.

Similarly, Bator et al., (2018) observed post-resocialization home cage interactions following isolation (P30-40) in male rats. Immediately following resocialization, previously-isolated rats engaged in a great deal of social contact, but by the end of the observation period (P42-64), this was no longer the case, and previously-isolated rats made significantly less social contact with cage mates. Play behavior was also initially elevated in previously-isolated rats following resocialization. Robust social activity following a prolonged isolation period has also been observed outside of the home cage environment in both male and female rats (Wall et al., 2012) and may reflect an isolated animal's first responses to social stimuli, rather than an enduring effect of isolation experienced during the adolescent period. One way to discern these effects is to assess social interaction prior to and well following resocialization, as in Bator et al., (2018) and some of our own experiments (Chapter 2 and summarized below).

1.6.4.3 Free roaming social interaction test

A free roaming social interaction test consists of placing an experimental rodent in an open chamber with a stimulus conspecific and recording behaviors initiated by the experimental mouse

such as sniffing, pushing, allogrooming, following, and huddling as both animals move freely throughout the chamber. Social behaviors can be scored as both frequencies of their occurrence and durations that they last. The unfamiliar stimulus animal is typically matched to the experimental mouse in terms of species, strain, age, weight, and sex. Using this procedure, we investigated social investigation of female mice that had been isolated in early adolescence (P31) and tested either just prior to resocialization (at P60) or three weeks after resocialization (Hinton et al., in prep.; Chapter 2). Social investigation was initially intense; however, three weeks following resocialization, social investigation had largely normalized or even diminished. For instance, while control, previously group-housed mice spent a significantly greater amount of time in contact with a stimulus mouse than a novel object, previously-isolated mice did not display the same preference. Long-term effects on social behavior in male subjects are less clear. While reduced social contact has also been reported in adult male mice with a history of isolation (Lukkes et al., 2009a), others reported no changes in social behavior of previously-isolated male rats (Bator et al., 2018).

The free roaming social interaction test can be adapted to analyze approach-avoidant behaviors within a social setting. In this design, the stimulus mouse is contained under a wire cup or box, while the experimental mouse is still able to freely roam. Researchers may choose to adapt this design further by including an empty identical container at the opposite end of the chamber as a nonsocial stimulus. As revealed using this design, isolation from P21-35 induces a social approach deficit present in early adulthood (~P50) in male mice that does not recover with resocialization (Makinodan et al., 2012). Specifically, mice spend less time exploring a container housing a stimulus mouse. Notably, this phenotype was also detected in a separate study in which mice were tested slightly later, at P65 (Makinodan et al., 2017). Interestingly, the same investigation revealed that resocialization with previously group-housed cage mates blocked the social approach deficit,

indicating that housing composition can have protective benefits, buffering against the effects of prior social isolation (Makinodan et al., 2017).

Another minor addition to this test design is to designate a certain proximity (*e.g.* 3 centimeters) surrounding the container housing as an "interaction zone." The number of entries and duration of time spent within the interaction zone can then be quantified. Using this approach, we found that female mice that had been isolated throughout the majority of adolescence (P31-60) and tested in adulthood exhibit aberrant social interactions that resemble darting maneuvers. Here, previously-isolated mice enter the interaction zone with greater frequency relative to controls, but the durations of their entries are significantly less than the average time spent by control subjects (Hinton et al., in prep; Chapter 2). This behavioral profile could be interpreted as a socially avoidant phenotype. It also resembles darting behavior seen in juveniles rodents attempting to initiate play through repeated contact with another mouse (Thor and Holloway, 1984), and thus could signal a failure in the maturation of "typical" social behavior. Lastly, the repeated initiation and evasion of social interactions resembles a phenotype that emerges in adult male rats with mPFC damage, discussed in Chapter 2.

In summary, social isolation triggers long-lasting changes in social behavior that are present despite resocialization in both male and female rodents. Upon initial resocialization, both males and females exhibit increased frequency and durations of social behaviors, and this intensity attenuates over time. This initial social exuberance is especially apparent when observing social behavior in a free roaming social interaction test. Within the home cage, previously-isolated males tend to exhibit aggressive behaviors and even diminished huddling, while home cage behavior in female rats is apparently typical.

These studies highlight considerations that should be made if choosing to use male subjects in combination with isolation procedures that then involve resocializing animals. Namely, isolation

evokes defensive and aggressive behavior in the home cage in male subjects of multiple strains and species. Therefore, investigating home cage behavior may not be a useful strategy for answering questions about the nuanced, complex, or atypical consequences of isolation since aggressive behavior predominates and is considered a "natural consequence" of isolation, reflecting territoriality. Aggressive encounters can also affect subsequent behaviors, even outside of the home cage, being a major stressor. Notably, Tulogdi et al., (2014) measured and attempted to match resident rats' aggression levels when resocializing previously-isolated males; this high standard of care may need to be systematically incorporated into future studies to circumvent cage composition effects. Finally, social behavior within the home cage following resocialization appears to be disrupted for at least up to a week in males, and so any behavioral testing before then could be confounded by the home cage environment, precluding one from clearly answering questions regarding the enduring effects of a history of isolation.

1.7 OVERVIEW AND RESEARCH STRATEGY OF THE DISSERTATION

This dissertation attempts to identify and correct long-term consequences of a history of social isolation during adolescence. Here, we socially isolate female mice during adolescence, from postnatal day (P) 31-60. Control mice are housed in social groups during this time. We then socially reintegrate all mice in early adulthood to ascertain the long-term consequences of adolescent social isolation that manifest or persist despite normalization of the social environment.

In Chapter 2 we report that social behaviors in adulthood are influenced by a history of adolescent isolation. When mice first encounter a conspecific following prolonged isolation, they exhibit intense social investigation, an unsurprising outcome given that this test serves as the initial instance of social interaction following isolation. However, when social interactions are observed in the weeks following social reintegration, previously-isolated mice engage with conspecifics in an

atypical manner that resembles "darting." Atypical social behavior, anhedonic-like behavior, and impaired goal-directed action have all been linked with impairments in mPFC function (Ferenczi et al., 2016), leading us to focus the rest of Chapter 2 on this brain region.

As mentioned previously, the mPFC undergoes dendritic spine pruning starting in early adolescence (Shapiro et al., 2017), a period encompassed by our social isolation procedure. We find evidence that social isolation during adolescence interferes with dendritic spine pruning in the mPFC. Specifically, isolated mice harbor higher dendritic spine densities both during adolescence and in adulthood, despite social reintegration in adulthood. We classified dendritic spines into subtypes to reveal that elevations in gross dendritic spine densities were driven by large populations of immature, thin-type dendritic spines.

Thin-type dendritic spines are considered immature, and they have also been referred to as "learning spines," because they have the ability to form stable synapses (Bourne and Harris, 2007). Higher densities suggest that prior isolation disrupts optimal signal:noise balance in the mPFC. The prelimbic subregion of the mPFC is necessary for rodents to form action-outcome associations necessary for goal-directed behavior, such that damage to the mPFC causes biases towards habit-based behaviors; meanwhile, the infralimbic region is necessary for habitual behavior (Gourley and Taylor, 2016). We attempted to normalize decision-making behaviors and also block anhedonic-like behavior in previously-isolated mice through chemogenetic inhibition of the mPFC using Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (Gi-DREADDs). Gi-DREADDs stimulation in the mPFC facilitated the consolidation of new information regarding the action-outcome relationships, resulting in the restoration of goal-directed decision making in previously-isolated mice otherwise prone to habit-based response strategies. Additionally, repeated Gi-DREADDs stimulation blocked anhedonic-like sucrose neglect in previously-isolated mice.

Chapter 3 initiates with data concurring with findings from others (*e.g.* Makinodan et al., 2012, 2017) that social isolation during adolescence impacts myelination in the mature prefrontal cortex. In mice with a history of adolescent isolation, Makiondan et al., (2012, 2017) discovered a simplification of oligodendrocyte morphology and reduced myelin thickness in the prefrontal cortex. We report that levels of CNPase, a marker of mature oligodendrocytes, which compose the myelin sheath, were lower in multiple cortico-striatal regions involved in goal-directed decision making.

Next, to quantify decision-making behavior in mice, we utilized instrumental contingency degradation procedures and outcome devaluation tasks. In the contingency degradation procedures, mice with a history of social isolation during adolescence were initially capable of forming action-outcome associations, inhibiting responding when one of two trained behaviors was un-reinforced. They, however, failed to update expectancies when nose poking became more familiar, deferring to habit-based behaviors. Responding was also insensitive to changes in outcome value – another marker of habits. Thus, social isolation in adolescence weakens goal-directed behavior later in life, causing mice to favor habit-based response strategies. Notably, mice isolated in adulthood did not demonstrate these precocious habits, suggesting adolescence may serve as a critical period during which socialization influences later decision-making behavior in adulthood.

At the same timepoint as previously-isolated mice exhibited a premature reliance on habit-based responding, we measured dendritic spine densities in the orbitofrontal cortex (OFC), given its role in allowing organisms to update goal-oriented response strategies when expectations change (Sul et al., 2010). Previously-isolated adult mice retained adolescent-like spine densities (*i.e.* higher densities than would be typical) in the OFC. PSD95, a marker of mature synapses, was also elevated here, suggesting that overabundant spines contained functional synapses (Berry and Nedivi, 2017). Interestingly, chemogenetic stimulation of excitatory CaMKII-expressing neurons in the OFC of typical mice recapitulated habit biases otherwise observed in previously-isolated mice.

We also investigated circulating levels of corticosterone (CORT; the rodent equivalent of cortisol) at multiple timepoints throughout adolescence as a potential mechanism by which social isolation during adolescence could have influenced the dendritic spine pruning process. During adolescence, circulating CORT increases (see several references in Chapter 3). Temporarily high CORT concentrations occupy low-affinity glucocorticoid receptors (GRs) and high-affinity mineralocorticoid receptors (MRs), coordinating high rates of cortical dendritic spine turnover and pruning during adolescence (Liston and Gan, 2011). Here, we found that social isolation caused CORT insufficiency in isolated mice early in the wake cycle, when levels should be high (cf. Sanchez et al., 1998).

We next determined that GR occupancy during adolescence affects decision making in adulthood. Specifically, we treated group-housed mice with a GR inhibitor at the same adolescent age when socially-isolated mice experienced low CORT (~sixth week of life). This GR inhibition resulted in failures to update action-outcome associations in adulthood. Thus, some minimal degree of GR occupancy in adolescence is necessary for optimal OFC function later in life, presumably acting by coordinating age-appropriate dendritic spine pruning (Liston and Gan, 2011).

In an effort to link isolation-induced decision-making biases with dendritic spine plasticity, another important experiment utilized a Rho-kinase (ROCK) inhibitor, administered systemically during the early isolation period (P39-47). Here we aimed to block the long-term neurobehavioral consequences of isolation. This strategy was motivated by previous findings that ROCK inhibitors can augment neuronal structural plasticity, enhancing dendritic spine elimination in response to certain appetitive conditioning procedures (Swanson et al., 2017), glutamate-mediated plasticity (Schubert et al., 2006), and adolescent development (Shapiro et al., 2019). Consistent with these patterns, the brain-penetrant ROCK inhibitor fasudil normalized dendritic spine excess in socially-isolated mice, decreasing the proportion of OFC dendrites with high spine densities. Fasudil also

corrected habit biases in adult, previously-isolated mice. Notably, a history of social isolation also reduced sucrose intake, an anhedonic-like behavior, which was blocked by fasudil administration.

Lastly, Chapter 4 concludes with discussion of limitations of certain isolation protocols, as well as the importance of the post-isolation social environments. Finally, I propose next directions and considerations in designing and interpreting future isolation and resocialization experiments.

CITATION	METHOD	SPECIES	SEX	ISOLATED	TEST	EFFECT*
BAARENDSE ET AL., 2013	Whole cell patch clamp in LV pyramidal cells	Long-Evans rats	M	P21-43	P84	Absent reduction in EPSP amplitude in response to D1 and D2 receptor agonist bath application
MAKINODAN ET AL., 2017	Intracranial EEG recordings from LV of PL and IL	C57BL/6J mice	M	P21-35 *Re-IS*	P65	↑ Power of β oscillation ↓ γ-oscillation **Relative to g-h controls and Re-SH*
MAKINODAN ET AL., 2017	Intracranial EEG recordings from LV of PL and IL	C57BL/6J mice	M	P21-35 *Re-SH*	P65	↓ γ-oscillation
MAKINODAN ET AL., 2017	Intracranial EEG recordings from LV of PL and IL	C57BL/6J mice	M	P21-35	P65	No change in δ, θ, α, σ-oscillations
MAKINODAN ET AL., 2012	qRT-PCR LV and VI	FVB/N x C57BL/6J mice	M	P21-35	P65	↓ Myelin basic protein, myelin associated glycoprotein
MAKINODAN ET AL., 2017	qRT-PCR from PL/IL LV and VI	C57BL/6J mice	M	P21-35 *Re-IS*	P65	↓ c-fos mRNA expression
MAKINODAN ET AL., 2017	qRT-PCR from PL/IL LV and VI	C57BL/6J mice	M	P21-35 *Re-SH*	P65	↓ c-fos mRNA expression
MAKINODAN ET AL., 2017	qRT-PCR from PL/IL LV and VI	C57BL/6J	M	P21-35 *Re-IS*	P65	↓ Arc and Npas4 expression
PASCUAL ET AL., 2006	ICC	Sprague-Dawley rats	M	P18-32	P62	↑ Density of VIP-expressing neurons
PASCUAL ET AL., 2006	Sholl analysis	Sprague-Dawley rats	M	P18-32	P62	↓ Dendritic complexity
MAKINODAN ET AL., 2017	Electron microscopy of LV axons	C57BL/6J mice	M	P21-35	P65	↓ Myelin thickness in previously-isolated mice and Re-IS. *vs. g-h mice and Re-SH mice*
HINTON ET AL., CHAPTER 2	Confocal microscopy of LV neurons	*thy*1-YFP mice (C57BL/6)	F	P31-60	P49 and P82	↑ Gross dendritic spine density ↑ Thin-type dendritic spine subtype density
HINTON ET AL., CHAPTER 2	IHC	C57BL/6J mice	F	P31-60	P82	No differences in PSD95 puncta count
HINTON ET AL., CHAPTER 2	Western blot	C57BL/6J mice	F	P31-60	P82	↑ PSD95
LEUSSIS AND ANDERSEN, 2008	Western blot IL subregion	Sprague-Dawley rats	M + F	P30-35	P36	↓ Spinophilin, synaptophysin
LEUSSIS ET AL., 2008	Western blot IL subregion	Sprague-Dawley rats	M	P30-35	P60	↓ Synaptophysin In vehicle-treated previously-isolated rats vs. vehicle-treated g-h rats
BATOR ET AL., 2018	Western blot	Wister Han rats	M	P30-40	P45	↑ Total GAD65, ↑ surface GAD65

MACKOWIAK ET AL., 2019	Western blot	Wister Han rats	M	P30-40	P47	↑ Parvalbumin
MACKOWIAK ET AL., 2019	Western blot	Wister Han rats	M	P30-40	P70	↓ Parvalbumin

Table 1-1. Summary of long-term mPFC outcomes following social isolation during adolescence. *Significant effect in previously-isolated rodents compared to group-housed control rats/mice, unless otherwise noted. P: postnatal day; F: female; M: male; G-h: group-housed control animals; LV: Layer 5; L VI: Layer 6; PL: Prelimbic; IL: Infralimbic; D1: Dopamine D1 Receptor; D2: Dopamine D2 receptor; DA: Dopamine; Re-IS: Previously-isolated rodents resocialized with previously-isolated cage mates; Re-SH: Previously-isolated rodents resocialized with previously group-housed cage mates; IHC: Immunohistochemistry; GAD65: glutamic acid decarboxylase 65; PSD95: Postsynaptic density-95; mPFC: medial prefrontal cortex; ICC: Immunocytochemistry; VIP: Vasoactive intestinal polypeptide; YFP: Yellow fluorescent protein. Horizontal lines separate sections using common techniques.

CITATION	BEHAVIORAL ASSAY	SPECIES	SEX	ISOLATED	TEST	EFFECT*	PHENOTYPE
HINTON ET AL., CHAPTER 3	Sucrose consumption test	C57BL/6J mice	F	P31-60	P82	↓ Sucrose consumption	Anhedonic-like
LEUSSIS AND ANDERSEN 2008	Forced swim test	Sprague-Dawley rats	M	P30-35	P36-37	↑ Time immobile ↓ Time swimming ↓ Latency to immobile *vs. male g-h rats*	Passive response to acute stressor
LEUSSIS AND ANDERSEN 2008	Forced swim test	Sprague-Dawley rats	F	P30-35	P36-37	No differences in time immobile, swimming, latency to immobile *vs. female g-h rats*	No effect
LEUSSIS AND ANDERSEN 2008	Learned helplessness test	Sprague-Dawley rats	M	P30-35	P36-38	↑ Latency to escape ↑ Escape failures following *both* inescapable and escapable shock *vs. male g-h rats*	Passive response to acute stressor
LEUSSIS AND ANDERSEN 2008	Learned helplessness test	Sprague-Dawley rats	F	P30-35	P36-38	↑ Latency to escape ↑ Escape failures in previously-isolated exposed to inescapable shock *vs. following escapable shock or no shock*	Passive response to acute stressor

Table 1-2. Summary of depressive-related behavior following adolescent social isolation.

*Significant effect in previously-isolated rats/mice compared to previously group-housed controls, unless otherwise noted. P: postnatal day; F: female; M: male; G-h: group-housed. Horizontal lines separate sections using common behavioral assays.

CITATION	BEHAVIORAL ASSAY	SPECIES	SEX	ISOLATED	TEST	EFFECT*	PHENOTYPE
HINTON ET AL., 2019; CHAPTER 3	Instrumental contingency degradation	C57BL/6J mice	F	P31-60	P82	Premature shift to habitual response strategy	Impaired goal-directed decision making
HINTON ET AL., 2019; CHAPTER 3	Instrumental contingency degradation	C57BL/6J mice	F	P60-89	P110	No difference	None
HINTON ET AL., 2019; CHAPTER 3	Outcome devaluation	C57BL/6J mice	F	P31-60	P82	Habitual behavior, insensitive to outcome value	Impaired goal-directed decision making
MAKINODAN ET AL., 2012	Water T-maze	FVB/N x C57BL/6J mice	M	P21-35	P50+	Impaired acquisition in non-matching to place task	Working memory deficit
BAARENDSE ET AL., 2013	5CSRTT	Lister Hooded rats	M	P21-43	P84	↑ Premature responding	Impulsive action
BAARENDSE ET AL., 2013	Rat gambling task	Lister Hooded rats	M	P21-43	P84	Impaired acquisition of advantageous choice	Decision-making abnormality in scenarios of uncertainty
BATOR ET AL., 2018	Novel object recognition task	Wister Han rats	M	P30-40	P45 or P70	No differences	None

Table 1-3. Summary of long-term consequences of adolescent social isolation on decision-making behavior *Significant effect in previously-isolated rats/mice compared to previously group-housed controls, unless otherwise noted. P: postnatal day; F: female; M: male; 5CSRTT: 5-choice serial reaction time test.

CITATION	BEHAVIORAL ASSAY	SPECIES	SEX	ISOLATED	TEST	EFFECT*	PHENOTYPE
WRIGHT ET AL., 1991	Elevated plus maze	Lister Hooded rats	M	P21-51 *or* P21-84	P84	↓ Time in open arms ↓Time at end of open arms ↓ % Of time and entries into open arm relative to total time and all arm entries *Relative to group-housed controls and separate controls isolated in adulthood at ~P54*	Anxiogenic
LEUSSIS AND ANDERSEN 2008	Elevated plus maze	Sprague-Dawley rats	M + F	P30-35	P36	↓ Time spent in open arms *Females only; no effect in males*	Anxiogenic
WEINTRAUB ET AL., 2010	Elevated plus maze	Sprague-Dawley rats	M + F	P30-50	~P80	↑ % Time in open arms ↓ % Time in closed arms *Males only*	Anxiolytic
WEINTRAUB ET AL., 2010	Elevated plus maze	Sprague-Dawley rats	M + F	P30-50	~P80	↑ % Time in open arms ↓ % Time in closed arms *In females vs. males, regardless of prior housing.*	Anxiolytic
WEINTRAUB ET AL., 2010	Elevated plus maze	Sprague-Dawley rats	M + F	P30-50	~P80	No effect on # entries into open arms, % of time spent in open or closed arms *Females only*	No effect
HINTON ET AL., CHAPTER 2	Open field	C57BL/6J mice	F	P31-60	P82	No effect on time in center No effect on number of center entries	No effect
PASCUAL ET AL., 2006	Open field	Sprague-Dawley rats	M	P18-32	P32 or P62	↑ Locomotor activity at both time points	Anxiogenic
LUKKES ET AL., 2009	Open field	Sprague-Dawley rats	M	P21-42	P56	↓ Entries to center during first 10min No effect on time in center	Anxiogenic
LUKKES ET AL., 2009	Open field *following habituation*	Sprague-Dawley rats	M	P21-42	P56	↓ Distance traveled in arena	Anxiogenic
MAKINODAN ET AL., 2017	Open field *during social interaction test*	C57BL/6J mice	M	P21-35 or P21-65	~P65	No difference in distance traveled	None
BATOR ET AL., 2018	Open field	Wister Han rats	M	P30-40	~P45 or ~P70	No difference in distance traveled	None

Table 1-4. Summary of anxiety-like effects of social isolation during adolescence.

*Significant effect in previously-isolated rats/mice compared to previously group-housed controls, unless otherwise noted. P: postnatal day; F: female; M: male. Horizontal lines separate sections using common behavioral assays.

CITATION	BEHAVIORAL ASSAY	SPECIES	SEX	ISOLATED	TEST	EFFECT*	PHENOTYPE
HINTON ET AL., CHAPTER 2	Social interaction test *with novel conspecific contained in box*	C57BL/6J mice	F	P31-60	~P82	↑ Entries into interaction zone ↓ Entries with durations lasting longer than avg. duration of g-h mice	Darting behavior
MAKINODAN ET AL., 2012	Social interaction test *with novel conspecific contained in box*	C57BL/6J mice	M	P21-35	P50+	↓ Ratio of time spent exploring box containing stimulus mouse to total time approaching "social" and "nonsocial" boxes	↓ Social exploration
LIU ET AL., 2012	Social interaction test *with novel conspecific contained in box*	C57BL/6J mice	M	P56	P84	No difference in time spent within social interaction zone	No effect
MAKINODAN ET AL., 2017	Social interaction test *with novel conspecific contained in box*	C57BL/6J mice	M	P21-35 *Resocialized with previously-isolated mice*	P65	↓ Ratio of time taken to approach box containing mouse vs. empty box **vs. g-h and isolated mice re-housed with previously-isolated cage mates*	↓ Social approach
MAKINODAN ET AL., 2017	Social interaction test *with novel conspecific contained in box*	C57BL/6J mice	M	P21-35 *Resocialized with previously g-h mice*	P65	No difference	No effect
HINTON ET AL., CHAPTER 2	Social interaction test *Free roaming*	C57BL/6J mice	F	P31-60	P82	No difference in time spent in contact with conspecific vs. novel object	No preference for social stimulus over nonsocial
LUKKES ET AL., 2009	Social interaction test *Free roaming*	Sprague-Dawley rats	M	P21-42	P56	↑ Latency to first approach novel conspecific ↑ Duration of freezing during test ↓ Social contact counts ↓ Duration of social contact	Social avoidance and anxiety-like behavior
LUKKES ET AL., 2013	Social interaction test *Free roaming*	Sprague-Dawley rats	F	P21-42	P59	No differences	No effect
BATOR ET AL., 2018	Social interaction test *Free roaming*	Wister Han rats	M	P30-40	~P45 or P70	No difference in time spent interacting with conspecific	No effect
LUKKES ET AL., 2013	Home cage social behavior	Sprague-Dawley rats	F	P21-42	P60	No differences in social contact, rearing, self-grooming, burying	No effect

BATOR ET AL., 2018	Home cage social behavior	Wister Han rats	M	P30-40	P42-64	↓ Amount of social investigations ↓ Total amount of social contacts made over time, despite initial increase following resocialization No difference in amount of play behavior over time, despite initial increase following resocialization	Play behavior intact, but rats otherwise avoid social encounters

Table 1-5. Summary of social behavioral consequences of adolescent isolation and resocialization. *Significant effect in previously-isolated rats/mice compared to previously group-housed controls, unless otherwise noted. P: postnatal day; F: female; M: male; G-h: group-housed control animals. Horizontal lines separate sections using common behavioral assays.

CHAPTER 2: CORRECTING THE DURABLE CONSEQUENCES OF ADOLESCENT SOCIAL ISOLATION

2.1 CONTEXT, AUTHOR'S CONTRIBUTION, AND ACKNOWLEDGEMENT OF REPRODUCTION

The following chapter focuses on the mPFC and characterizes long-term neurobehavioral consequences of a history of social isolation in adolescence. I then use a chemogenetic approach to correct certain behavioral consequences of isolation that otherwise remain present in adulthood. The dissertation author contributed by designing and conducting the experiments, analyzing the data, and writing the manuscript under the guidance of Dr. Shannon Gourley. The PSD95 western blot included here was performed and analyzed by Dan Li.

2.2 ABSTRACT

Social adversity during development is a risk factor for multiple psychopathologies with neurodevelopmental origins. Animal models may help to elucidate both the durable consequences of adolescent social adversity – those that persist despite improvement or normalization of the social environment – and corrective strategies. Here, we socially isolated female mice from postnatal days 31-60, the majority of adolescence, then re-housed them in social groups. Adolescent-onset isolation caused long-term changes in the social behavior, inducing intensive social investigation upon re-socialization that then becomes fragmented with social experience. A history of social isolation also weakened the ability of adult mice to select actions based on expected outcomes, causing them to defer to habit-based behaviors, and it induced anhedonic-like behavior in a sucrose consumption task, collectively pointing to mPFC dysregulation following isolation during adolescence. Indeed, in the mPFC, the densities of thin-type dendritic spines on layer V neurons were elevated. Postsynaptic density-95 (PSD95) protein content was also elevated. Meanwhile, the stimulation of Gi-coupled Designer Receptors Exclusively Activated by Designer Drugs (Gi-DREADDs) in the mPFC improved the ability of previously-isolated mice to consolidate new information about action-

outcome relationships, correcting decision-making biases. Additionally, repeated stimulation of Gi-DREADDs in the mPFC reversed anhedonic-like behavior in adult mice with a history of social isolation. These findings indicate that social isolation during adolescence disrupts typical mPFC neuron development, such that mitigating excitatory plasticity in adulthood corrects behavioral abnormalities.

2.3 INTRODUCTION

During adolescence, prefrontal cortical dendrites, dendritic spines, and synapses mature and refine (Bourgeois et al., 1994; Huttenlocher and Dabholkar, 1997; Shapiro et al., 2017). Social experience contributes to the process, such that depriving rodents of typical (Himmler et al., 2013) or any (Hinton et al., 2019) social interaction disrupts the maturation of principal pyramidal neurons. Atypical social experiences during adolescence can have long-lasting behavioral consequences that manifest in adulthood (Burke et al., 2017). However, whether long-term neurobehavioral consequences remain present in adulthood, even despite normalization of the social environment, and/or can be corrected remains unclear.

Throughout, this chapter focuses on neuronal and behavioral consequences pertaining to the mPFC. The interaction between social experiences during adolescence and regulation of mPFC dendritic spine subtype populations (if any) is unknown. Here, we report increased densities of thin-type dendritic spine subtype populations both during the adolescent isolation period and well into adulthood following resocialization. Additionally, levels of PSD95 protein content suggest the potential for increased synaptic contact in this subregion. Much of mPFC function adheres to an inverted U-shaped curved, such that "too much" or "too little" plasticity is suboptimal. Thus, these alterations in mPFC neuronal anatomy suggested to us that performance in mPFC-dependent tasks would be impaired in previously-isolated mice.

The mPFC is necessary for rodents to form (consolidate) action-outcome associations that facilitate goal-directed behavior, such that damage to the prelimbic mPFC causes biases towards habit-based behaviors (reviewed Gourley and Taylor, 2016). To quantify decision-making behavior, we used an instrumental contingency degradation procedure described in the previous chapter. Briefly, mice are first trained to generate two food-reinforced actions. Then, the predictive relationship between one action and its outcome is violated by providing the associated food pellet in a manner that is not dependent on the animal's action, thus "degrading" the action-outcome relationship. Following this session, mice should be consolidating information regarding the newly degraded relationship between their recent nose-poke action and its now noncontingent outcome. In a subsequent test on the following day, inhibiting this nose-poke, while continuing to engage in the nose-poking action associated with an intact action-outcome relationship, is interpreted as evidence of the consolidation of new action-outcome associations. This consolidation is necessary for goal-directed responding. By contrast, habit-based responding, which is insensitive to action-outcome associations, is resistant to updating.

The mPFC is also involved in the expression of anhedonic-like behavior, such that optogenetic stimulation, elevating mPFC activity, causes anhedonic-like sucrose neglect in rodents (Ferenczi et al., 2016). Here, we replicated prior findings that adolescent isolation causes anhedonic-like behavior, and also habit-based response biases, in adulthood (Hinton et al., 2019; Chapter 3). These findings motivated us to utilize a chemogenetic approach to attempt to correct these long-lasting behavioral consequences. We also took the opportunity to investigate social behavior following long-term isolation, given evidence that the social environment during adolescence impacts social behavior in adulthood, even despite normalization of housing conditions (reviewed Chapter 1).

2.4 MATERIALS AND METHODS

2.4.1 Subjects.

C57BL/6 mice bred in-house from Jackson Labs stock (Bar Harbor, ME) were used. For dendritic spine imaging, C57BL/6-back-crossed mice expressed *thy1*-derived YFP (Feng et al., 2000). Mice were provided food and water *ad libitum* except during instrumental conditioning when mice were food-restricted to ~90-93% of their original weight to motivate food-reinforced responding. Procedures were in accordance with the Emory University IACUC, and females were used unless otherwise noted.

2.4.2 Social isolation.

Mice were weaned at postnatal day (P) 21-22 and housed in single-sex groups of 6-8. At P31, mice were either rehoused in groups of 6-8 with novel conspecifics, or housed in isolation, living in individual "shoebox"-style cages on ventilated racks. At P60, mice were socially reintegrated, housed in new cages with novel conspecifics, with each cage containing 3-4 previously-isolated mice and 3-4 previously-socialized mice. All mice subject to social isolation were female.

2.4.3 Social interaction test.

Social interactions were observed in mice either immediately before resocialization at P60 or, in a separate cohort, 2-3 weeks following resocialization, as in prior reports (*e.g.* Makinodan et al., 2012). Here, an unfamiliar adult female mouse of similar size as the experimenter mouse was used as the stimulus mouse. Experimental and stimulus mice were placed in a large chamber (41x20x20 cm) and allowed to freely roam for 5min while being video-recorded. Recordings were scored by a blinded rater. Behaviors scored were: nose-to-nose sniffing (sniffing the nose of the stimulus mouse), nose-to-anogenital sniffing (sniffing the anogenital region of the stimulus mouse), body

sniffing, front approach, push-crawl (push = pushing the head underneath the stimulus mouse's body and/or squeezing between the wall/floor and the stimulus mouse; crawl = crawling over or under the stimulus mouse's body), and following (walking straight behind the stimulus mouse) (Yang et al., 2012). Total social contact consisted of all behavioral counts and durations.

To rule out the possibility that behaviors observed during a social interaction test may simply be attributable to novelty, frequency and duration of interaction with an unfamiliar, non-social object (Falcon tube) were scored. Here, mice were placed in a large chamber (41x20x20 cm) containing a clean Falcon tube in the center of the chamber. Duration and frequency of contact with the Falcon tube were videotaped for 5min and scored by a blind rater. Chambers were cleaned with 70% EtOH between sessions throughout both experiments.

We next addressed the hypothesis that behavioral outcomes of this test may be influenced by the sex of the stimulus mouse. We generated a separate cohort of female mice that were group-housed during adolescence. We then ran a series of social interaction tests occurring in adulthood at the time matched to social interaction tests occurring in prior cohorts. Stimulus mice were age-matched adult female mice as above or adult male mice. Social interaction tests were performed as above.

2.4.4 Social approach test.

One limitation of the social interaction test described above is the ability of the stimulus mice to freely roam and therefore influence the duration and frequency of social interactions. To assess social interaction in a more controlled setting, stimulus mice were placed in wire containment cups that were positioned in the center of a large chamber. Wire containment cups were large enough to allow for airflow while preventing direct physical contact between the stimulus mouse on the inside and the experimental mouse on the outside. Stimulus mice were allowed to habituate for

15min prior to testing. Experimental mice were then placed in the chamber, and their behavior was video-recorded for 5min. Recordings were scored by a blinded rater. Behaviors scored were: the number of entries and duration of time within an interaction zone (3 cm surrounding the containment cup), duration of direct contact with the containment cup, and the proportion of entries into the interaction zone that lasted longer than the average duration that control mice spent (6.5 seconds). Chambers and containment cups were cleaned with 70% ethanol between sessions.

2.4.5 Open field test.

Previously-isolated and previously group-housed female mice were tested in adulthood. Mice were habituated to the behavioral testing room 30min prior to testing. Mice were then placed in the center of a rectangular field (41x20x20 cm). Exploratory activity was videotaped under dim light for 10min, and the number of entries and time spent in a 6x6 cm center square was recorded.

2.4.6 Dendritic spine imaging and quantification.

Methods were in concordance with prior reports (Hinton et al., 2019; Chapter 3). YFP-expressing mice were euthanized by rapid decapitation at P49 (during isolation) or P82 (following social reintegration). Fresh brains were submerged in chilled 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 40 μm sections on a microtome held at -15°C. Unobstructed dendritic arbors running parallel to the surface of the section were imaged on a Leica SP8 laser scanning confocal microscope.

Z-stacks were collected with a 40X 1.3NA oil-immersion objective at a resolution of 1,024 x 1,024 pixels using a 0.1 μm step size, sampling above and below the dendrite. Dendrites were collected from the mPFC. They consisted of secondary branches within 50-150 μm of the soma and were 20-25 μm in length. Collapsed z-stacks were then analyzed by a single blinded rater using

ImageJ. Each protrusion ≤4 μm was considered a spine, and bifurcated spines were considered singular units. To generate density values, spine number for each segment was normalized to the length of the segment. 6-8 dendrites/mouse were imaged and scored, with each mouse considered an independent sample.

Dendritic spines were subsequently reconstructed in 3D using Imaris software (Bitplane AG) and classified into specific spine subtype populations to allow for further spine density analyses. According to methods previously described (Swanger et al., 2011; Gourley et al., 2013; Swanson et al., 2017), the Filament Tracer module of Imaris was used here. Briefly, dendrites 20-25 μm in length were drawn using the autodepth function, dendritic spine head locations were indicated manually, and Filament Tracer processing algorithms calculated morphological parameters. Dendritic spines were then classified into stubby, mushroom or thin subtypes populations. Stubby spines were defined as those with a length ≤0.6 μm, and a head to neck ratio of ≤1.5 μm. Mushroom spines were classified as having a head to neck ratio of ≥1.5 μm. Thin spines were defined as spines with a length >0.6 μm, and a head to neck ratio of ≤1.5 μm. Dendritic spines with a head to neck ratio ≥1.5 μm and a head size of ≤0.3 μm, were also classified as thin spines. Blinded individuals quantified all dendritic spines within a single experiment, with inter-rater reliability established between two experimenters.

2.4.7 Immunoblotting for PSD95.

Mice were rapidly decapitated at P82; brains were frozen at -80°C and then sectioned into 1 mm sections. The mPFC was dissected by a single experimenter using a 1 mm tissue core. Tissue was homogenized by sonication in lysis buffer [100 μl: 137 mM NaCl, 20 mM tris-Hcl (pH=8), 1% igepal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma), 1:1000 Protease Inhibitory Cocktail (Sigma)]. Protein concentrations were determined by Bradford colorimetric assay

(Pierce, Waltham, MA), and 15 μg/sample was separated by SDS-PAGE on a 12% gradient tris-glycine gel (Bio-rad, Hercules, CA). Following transfer to PVDF membrane, membranes were blocked with 5% nonfat milk.

Primary antibodies were anti-PSD95 (Cell Signaling; 1:1000), with anti-HSP-70 (Santa Cruz; 1:5000, Dallas, TX) serving as a loading control. Immunoreactivity was assessed using a chemiluminescence substrate (Pierce) and measured using a ChemiDoc MP Imaging System (Bio-rad).

2.4.8 Immunostaining for PSD95.

Mice were euthanized by rapid decapitation, and brains were stored for 48 hours in chilled 4% paraformaldehyde, then transferred to 30% w/v sucrose, and sectioned into 40 μm sections on a microtome held at -15ºC. All immunohistochemical work used free floating sections. Before each step, the sections were washed 3X in 1X PBS. Sections were blocked with PBS and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO), 2% NGS, and 1% bovine serum albumin (BSA) for 1.5 hours at room temperature. Sections were then incubated 48 hours at 4ºC in 1 X PBS and 0.3% Triton X-100, 0.3% NGS, 1% BSA, and a primary antibody against PSD95 (Cell Signaling Technology; 1:500, Danvers, MA). Tissue was then incubated for 1 hr at room temperature in 1X PBS and 0.3% Triton X-100, 1% NGS, and a secondary antibody Alexa Fluor 594 (Jackson ImmunoResearch Laboratories; 1:400, West Grove, PA).

The mPFC was located using *The mouse brain in stereotaxic coordinates* (Franklin and Paxinos, 2007). Images were collected in a single session using a Leica SP8 confocal microscope. Images were captured using a 63X oil-immersion 1.4NA objective at a resolution of 1,024 x 1,024 pixels with a pinhole of 0.40 Airy units, scanning speed of 100, and the line averaging of 2. The prelimbic and infralimbic subregions were each captured in distinct images with the image size held constant.

Particle analysis (puncta count) was quantified using ImageJ software. Brightness and contrast settings were first adjusted consistently for all grayscale 8-bit images while leaving pixel values unchanged. Next, all images were adjusted using a threshold for signal intensity in order to maximize puncta signal and minimize background noise. These images were then converted into binary versions. The total number of PSD95-stained puncta with sizes ranging 0.01 – 4.00 μm^2 pixel units was then quantified from these binary images. Sample area size was held constant. Data from the prelimbic and infralimbic subsections were averaged (2 images/mouse), as there were no differences in PSD95 puncta counts between mPFC subsections. Each mouse was considered an independent sample.

2.4.9 Immunostaining for gephyrin.

YFP-expressing mice were euthanized at P82 by rapid decapitation, and brains were stored for 48 hours in chilled 4% paraformaldehyde, then transferred to 30% w/v sucrose, and sectioned into 40 μm sections on a microtome held at -15ºC. All immunohistochemical work used free floating sections. Before each step, the sections were washed 3X in 1X PBS. Sections were blocked with 3% bovine serum albumin (BSA), 5% normal goat serum (NGS), PBS and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. Sections were then incubated at 4ºC overnight in 3% BSA, 5% NGS, PBS with 0.3% Triton X-100, and a primary mouse-monoclonal antibody against gephyrin (Synaptic Systems; 1:750, Goettingen, Germany). The following day, sections were incubated for 1 hour at room temperature in 0.5% NGS, PBS with 0.3% Triton X-100, and goat anti-mouse Alexa Fluor 594 (Life Technologies; 1:1000, Waltham, MA) serving as the secondary antibody.

Z-stacks were collected with a 40X oil-immersion objective (1.3 NA) at a resolution of 1,024 x 1,024 pixels using a 0.3 μm step size on a Leica SP8 laser scanning confocal microscope with

settings and the number of stacks (9 total) held constant. Gephyrin-immunoreactive puncta were imaged using a sequential scan on the 594 channel. Layer V neurons were then imaged on the 488 channel. All tissue was imaged during a single uninterrupted session with microscope settings held constant. ImageJ was used to calculate mean intensity values from 3 samples per animal. Averages of these 3 values were then normalized to controls within each of 2 independent cohorts. As in dendritic spine imaging experiments, puncta were initially quantified 50-150 μm from the soma in the mPFC. We also imaged gephyrin adjacent to the soma, given that inhibitory synapses are primarily localized to the perisomatic region. Again, imaging settings and sampling parameters were held constant and each animal contributed 2 samples and was considered an independent sample.

2.4.10 Surgery.

Experiments utilized intracranial placement of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Mice were P60-63. Mice were anesthetized with Ketamine/Dexdomitor (75mg/kg + 1mg/kg, *i.p.,* MedVet, Cincinnati, OH). AAV5-CaMKII-HA-rM$_4$D(Gi)-IRES-mCitrine or AAV5-CaMKII-GFP (UNC Viral Vector Core, Chapel Hill, NC) were infused bilaterally (0.5μl/side) at AP+2.0, ML$\pm$0.1, DV-2.8. Infusions were delivered 0.05μl/minute, with the needle left in place for 5 additional min. The scalp was sutured and mice were revived with Antisedan (1mg/kg, *i.p.*, MedVet, Cincinnati, OH). 2-3 weeks were allotted for viral vector expression and recovery time. Following behavioral testing, mice were euthanized by rapid decapitation, brains collected and stored in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose. Brains were sectioned at 40 μm, and fluorescence within the mPFC was imaged and transposed onto images from the Mouse Brain Library (Rosen et al., 2000).

2.4.11 Instrumental conditioning.

2.4.11.1 Instrumental response training.

Mice were food-restricted to ~90-93% of their original weights and trained to nose poke for grain-based food reinforcers (20 mg, Bio-Serv, Flemington, NJ) in Med-Associates operant conditioning chambers (St. Albans, VT) equipped with two nose poke recesses and a separate food magazine. Responding was reinforced using a fixed ratio 1 (FR1) schedule wherein 30 pellets were available for responding on the 2 distinct nose poke recesses, resulting in 60 pellets/session. Mice acquired the responses within 7 70min sessions (1/day). We additionally applied random interval training, as in Chapter 3, to ensure robust response rates (not shown). Importantly, there were no side preferences that could influence our findings.

2.4.11.2 Instrumental contingency degradation.

A modified version of classical action-outcome contingency degradation was used, as previously described (*e.g.*, Gourley et al., 2012; Swanson et al., 2013). In a 25min "non-degraded" session, one nose poke aperture was occluded, and responding on the other aperture was reinforced using a variable ratio 2 schedule of reinforcement. In the 25min "degraded" session, the opposite aperture was occluded, and reinforcers were delivered into the magazine at a rate matched to each animal's reinforcement rate from the previous session. Responses produced no programmed consequences. In this case, only ~7% of reinforcers are delivered (by chance) within 2sec following a response (Butkovich et al., 2015). Thus, this response becomes significantly less predictive of reinforcement than the other. The following day, both apertures were available during a 10min probe test. As is standard practice, the probe test was conducted in extinction, and response rates during this period are shown.

2.4.12 Sucrose consumption.

A sucrose consumption protocol that allows for standard laboratory group housing was implemented (Gourley et al., 2008, 2013b). First, mice were water-restricted overnight. Then, each mouse was allowed access to a 1% w/v sucrose solution in its home cage while cagemates were housed in a clean cage in the same room. Each mouse had 1 hour access such that the *average* water deprivation period across the cage of mice was 19 hours. We also assessed estrous phase by visual examination at the time of testing (Byers et al., 2012), which did not appear to have any effect on sucrose intake.

2.4.13 Clozapine-N-Oxide (dosing and timing relative to behavioral testing).

Clozapine N-Oxide (CNO; Sigma-Aldrich, 1mg/kg, *i.p.,* 1ml/100g) was dissolved in 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich) solution in 0.9% sterile saline and prepared on the day of injection. Injection timing is graphically indicated. In one experiment, injection occurred immediately following the contingency degradation training session, and then response preferences were tested the next day, when mice were drug-free. In another experiment, injections occurred 1 hour prior to a sucrose consumption test. The same mice received a vehicle injection 1 hour prior to another sucrose consumption test. The order of these tests was counter-balanced and separated by 1 day. Then, the same mice received once daily CNO injections over the next five days. These mice then received a final CNO injection 1 hour before another sucrose consumption test. Also, they received a final vehicle injection 1 hour prior to another sucrose consumption test. The order of these final 2 sucrose consumption tests was counter-balanced and separated by 1 day.

Importantly, in both experiments, all mice, regardless of viral vector, received CNO, equally exposing all mice to any unintended consequences of the drug (see Gomez et al., 2017). Also, note that our dosing is commonly utilized within the field (see tables in Urban and Roth, 2015).

2.4.14 Statistics.

Social interaction zone counts, interaction durations, proportion data, and average duration of time spent in open field center, as well as puncta count, mean intensity, and protein content levels for PSD95 and gephyrin were compared by independent t-tests. Contact duration with a same-sex conspecific, a different-sex conspecific, and a non-social stimulus were compared by 1-way ANOVA. Contact with social *vs.* nonsocial stimuli, dendritic spine densities, nose poke response rates, and sucrose consumption values were compared by 2- or 3-factor ANOVA with repeated measures when appropriate. Values greater than two standard deviations above the mean were considered outliers and excluded. Post-hoc comparisons were generated using Student's *t*-tests and were applied following interactions or main effects between >2 groups. $p<0.05$ was considered significant, and comparisons are indicated graphically. SPSS and Sigmastat were used.

2.5 RESULTS

2.5.1 Social behavior in adulthood is influenced by social experience in adolescence.

Little is known regarding the long-term consequences of social isolation during adolescence. To address this issue, we implemented a social isolation procedure in which mice are socially isolated throughout most of the adolescent period in rodents [P31-60 (Spear 2000; Green and McCormick, 2013)]. During this time, control mice (*i.e.*, socialized mice), are housed in groups of 6-8. At P60, all animals are re-housed in social groups in order to elucidate the durable consequences of social isolation in adolescence (*i.e.*, those that manifest despite normalizing the social environment).

One of our goals was to assess social behavior following prolonged isolation. We first confirmed that typically-housed adult female mice interact with a novel conspecific to a measurable degree in an open field in which both mice can freely roam. Importantly, the sex of the conspecific was critical, with exploration counts of females exceeding those of males and a non-social stimulus

(a Falcon tube) (main effect $F_{(2, 12)}$=4.39,p=0.03)(fig.2-1a). Significant post-hoc comparisons are indicated in the figure captions throughout.

Next, we quantified social interaction in socially-isolated mice either at P60 (just prior to re-socialization) or 2-3 weeks following re-socialization. Regardless of testing time, socially-housed control mice spent a greater duration of time in contact with the conspecific mouse than a non-social object. By contrast, isolated mice spent a considerable amount of time when encountering a conspecific for the first time in one month; however, following resocialization, these mice no longer spent a discernably greater amount of time in contact with a stimulus mouse *vs.* a non-social object (housing condition x test time x stimulus type, $F_{(1, 48)}$=13.35,p<0.05)(fig.2-1b). Thus, mice with a history of social isolation fail to prefer a social conspecific over a non-social stimulus.

One limitation of the social interaction test described above is the ability of the stimulus mice to freely roam and therefore influence the duration and frequency of social interactions, irrespective of an experimental mouse's volitional engagement. To address this issue, we adapted a social approach assay in which a stimulus mouse is constrained within a wire cup while an experimental mouse is free to roam. Here, the duration of time spent inside an interaction zone, a 3 cm area surrounding the wire containment cup (Kaidanovich-Beilin et al., 2011), and duration of time spent in contact with the wire cup did not differ between groups [(t_{12}=1.06,p=0.30;fig.2-1c) and (t_{12}=-1.52,p=0.15;fig.2-1d), respectively]. Nevertheless, previously-isolated mice entered the interaction zone a greater number of times (t_{12}=-2.77,p=0.01;fig.2-1e), and fewer of these entries lasted longer than the average duration of time spent by control mice (t_{12}=2.55,p=0.02;fig.2-1f). This behavioral pattern results in a "darting" phenotype in which previously-isolated mice initiate social interactions more frequently, but then quickly evade these interactions.

Importantly, this "darting" phenotype is not obviously due to an anxiogenic effect of prior isolation. The number of entries and the average time spent in the center of an open field is commonly

used as an indicator of an anxiety-related behaviors and did not differ between previously group-housed and previously-isolated mice (entries: t_{24}=-0.24,p=0.60, not shown; time: t_{24}=-0.24,p=0.80;fig.2-1g). In summary, social isolation during adolescence shapes subtleties of social interactions in adulthood, which might contribute to an overall decrease in social investigation in a naturalistic social interaction test (again, fig.2-1b).

Prior investigations revealed a similar social interaction pattern (repeated initiation followed by quick evasions) following damage to the mPFC in rodents (Bell et al., 2009). Motivated by these findings, we next investigated how social isolation during adolescence influences mPFC maturation in female mice.

2.5.2 Elevations in specific dendritic spine sub-types and synaptic marker levels in the mPFC of socially-isolated mice.

To determine whether social experience during adolescence impacts neuronal maturation during the same period, we enumerated dendritic spines on layer V neurons in the prelimbic mPFC at P49 (during isolation) and P82 (following re-socialization). Manual counting revealed elevated densities in socially-isolated mice, regardless of time point (main effect of housing $F_{(1,20)}$=13.47,p=0.002, no interactions)(fig.2-2a), suggesting that one immediate neuronal response to a lack of social engagement during adolescence is an elevation of mPFC dendritic spines (or alternatively, a failure in the elimination of dendritic spines). Correcting the social environment does not appear to ameliorate this morphological phenotype.

Dendritic spines were next reconstructed in 3D (fig.2-2b) in order classify their subtype, which is closely linked with spine function. Social isolation increased thin-type dendritic spine densities (main effect of housing $F_{(1,20)}$=4.87,p=0.03, no interactions)(fig.2-2c). Meanwhile, mushroom-type dendritic spine densities trended lower with age, as would be expected, given

dendritic spine elimination in adolescence (reviewed Chapter 1) (trend for main effect of age $F_{(1,20)}$=4.05,p=0.058), but we identified no effect of housing condition or interactions (no effect of housing $F_{(1,20)}$=0.125,p=0.727)(fig.2-2d). Finally, no differences between groups were observed in stubby-type spine densities (no effect of housing $F_{(1,20)}$=0.193, p=0.665; not shown). Thus, social engagement during adolescence appears necessary for maintaining normative levels of labile, thin-type dendritic spines during adolescence. In its absence, dendritic spine excess appears persistent, detected even following social reintegration.

Next, we used western blotting to measure PSD95, a marker of synaptic strength (Beique and Andrade, 2003). Adult mice with a history of social isolation had elevated PSD95 protein content (t_{16}=-2.36,p=0.03;fig.2-2e-f). PSD95 is also often used as a marker of synaptic density, which we did not necessarily expect to differ between groups, given similar densities of mature mushroom-shaped spines above. To confirm this speculation, we quantified PSD95 puncta, revealing no group differences (t_{16}=0.72,p=0.47;fig.2-2g). Thus, the elevation in PSD95 protein level likely reflects synapse strengthening, rather than an elevation in excitatory synapse counts, *per se*.

Additionally, we quantified gephyrin, a postsynaptic marker of inhibitory synapses. Gephyrin acts as a scaffolding protein at postsynaptic sites of inhibitory synapses where it facilitates the clustering and organization of glycinergic and $GABA_A$ receptors (Choii and Ko, 2015). Immunofluorescent staining revealed no differences between groups when sampled in proximity to dendrites on layer V neurons (t_{16}=-1.26,p=0.22;fig.2-2h-i). We also imaged gephyrin adjacent to the soma, given that inhibitory synapse location can vary between cell types with densities primarily localized to the perisomatic region in some cases (Hikoi et al., 2013). Again, no differences between groups emerged (t_{15}=-1.12,p=0.28;fig.2-2j).

2.5.3 Gi-DREADDs stimulation in the mPFC corrects decision-making biases and anhedonic-like behavior in previously-isolated mice.

Considering our current findings – elevated PSD95 and thin-type dendritic spines, transient extensions with the potential for synapse formation (Bourne and Harris, 2007) – we next questioned whether braking mPFC activity would normalize behavioral abnormalities in mice with a history of social isolation.

We first expressed Gi-DREADDs or a control virus in the mPFC of mice with and without a history of isolation using a 2 x 2 experimental design (fig.2-3a). Mice were first trained to generate two distinct nose poke actions for food reinforcers. Neither a history of social isolation (no effect of prior housing $F_{(1,43)}$=1.41,p=0.24), nor virus ($F_{(1,43)}$=1.54, p=0.22) impacted response rates during this instrumental response training (no session x housing x virus interaction $F_{(6, 258)}$=1.30, p=0.25)(fig.2-3b). Then, the relationship between one nose poke action and its outcome was degraded by delivering pellets non-contingently. Thus, one nose poke became significantly less predictive of reinforcement than the other, and mice must consolidate new information about the action-outcome contingency. During this consolidation period, all mice received the Gi-DREADDs ligand CNO. The following day, mice underwent a brief probe test to assess response preference. Goal-directed decision making consists of responding preferentially toward the intact instrumental contingency, while nondiscriminatory responding on both nose poke apertures represents a failure in action-outcome conditioning, causing mice to favor familiar, habit-like response strategies.

A three-way interaction between housing x virus x aperture ($F_{(1,43)}$=5.306, p=0.02)(fig.2-3c) with Student's t-test comparisons indicated that control mice expressing the control virus (GFP) favored the intact action-outcome contingency (p=0.007), while control, previously-socialized mice expressing Gi-DREADDs mice did not (p=0.075), likely because the prelimbic mPFC region is necessary for action-outcome memory consolidation (see for review, Gourley and Taylor, 2016).

While previously-isolated mice expressing the control virus exhibited habit biases (p=0.985) as expected, previously-isolated mice expressing Gi-DREADDs responded in a goal-directed manner (p=0.015).

We next investigated long-term effects of adolescent social isolation on anhedonic-like behavior using a sucrose consumption test (fig.2-3d). Despite an acute administration of CNO, previously-isolated mice nevertheless demonstrated anhedonic-like sucrose neglect compared to previously-socialized controls (main effect of housing $F_{(1,13)}$=5.507,p=0.035, no effect of CNO ($F_{(1,13)}$=0.171, p=0.686)(fig.2-3e). However, with repeated CNO treatment, previously-isolated mice consumed comparable sucrose amounts to those of controls (no effect of housing $F_{(1,13)}$=0.0772, p=0.786)(fig.2-3e).

2.6 DISCUSSION

2.6.1 Isolation during adolescence shapes social behavioral subtleties in adulthood

The majority of reports investigating long-term consequences of isolation in adolescence on subsequent social behavior in adulthood have focused on findings in male subjects or have reported no lasting effects in females (see Chapter 1 for review). Here we report long-term changes in the timing and frequency of social interactions of previously-isolated female mice. Importantly, this change in the nature of social interactions of previously-isolated females is present long after mice have been resocialized.

Two behavioral assays were used to probe social interactions following resocialization. During the initial social interaction test, in which both the experimental and stimulus, conspecific mice were free to roam, a difference in the duration of social contact emerged. While previously-socialized mice spent a greater duration in contact with a conspecific mouse vs. a novel object, previously-isolated mice did not show more engagement with a novel mouse over a novel object.

One limitation of the design of this free roaming social interaction test is that the social behavior of the experimental mice can be influenced by the behavioral output of the stimulus mouse, making it difficult to draw conclusions about the influence of prior social experiences on subsequent social interactions in adulthood.

A common adaptation to the free roaming social interaction test is to contain the stimulus mouse within a chamber while allowing the experimental mouse to socially engage on its own volition. Implementing this behavioral assay revealed a behavioral subtlety in the way in which previously-isolated mice engage in social interactions as adults. Overall, the amounts of time spent engaging in social interactions were comparable between previously-isolated mice and previously-socialized control mice. However, the nature of these interactions differs based on prior social experience in adolescence. Surprisingly, previously-isolated mice initiated more social interactions than control mice. However, the previously-isolated mice then subsequently evaded these interactions more rapidly. This aberrant sequence of social behaviors resembles a "darting" phenotype in the mice with a history of adolescent isolation.

This repeated initiation and evasion of social interactions could represent a variety of long-lasting consequences of social isolation during adolescence. First, it could represent a socially anxious or avoidant phenotype in the previously-isolated mice, given that the proportion of interactions that lasted as long or greater than the average interaction bout was significantly reduced compared to previously-socialized mice. Alternatively, it could reflect an age-atypical social phenotype, as this type of darting behavior is a common characteristic in juvenile rodents, more so than adults, and is commonly observed when attempting to initiate bouts of social play (Thor and Holloway, 1984). Finally, the repeated initiation and evasion of interactions parallels a social behavioral profile seen in adult male rats with mPFC damage. Bell et al., 2009 found that mPFC lesions led to an increase in the initiation of play, a disruption in the sequencing of complex

maneuvers otherwise typically exerted to maintain bouts of social play, and an increase in the number of play evasions. Bell et al., 2009 suggest that the failure to coordinate complex actions needed to maintain normative bouts of social play may result in less rewarding social interactions and therefore more frequent evasions. Then, subsequent social interactions are posited to continue being initiated as a means to compensate for this decreased reward value of interactions. These initiation increases are thought of as repeated attempts for the animal to become "socially sated." Motivated by these findings, we next investigated how social isolation during adolescence impacted aspects of mPFC development.

2.6.2 mPFC dendritic spine density subtype changes following adolescent isolation

The primary sites of excitatory transmission in the brain, dendritic spines, undergo a protracted period of pruning during adolescence in both humans and rodents (Spear 2000). We hypothesized that the social environment interacts with dendritic spine dynamics in the mPFC during adolescence to set the stage for neurobehavioral function later in life. This idea is supported by research showing that a lack of social play experience during the juvenile period leads to decreased mPFC dendritic plasticity in response to later experience (Himmler et al., 2013) and imbalanced excitation/inhibition of the mPFC (Makinodan et al., 2017).

Despite this prior research, the interaction between social experience during adolescence and mPFC dendritic spine subtype population (if any) is unknown. Here, we report increased densities of thin-type dendritic spine subtype populations both during the adolescent isolation period and well into adulthood following resocialization. Additionally, expression levels of PSD95 protein content but not puncta counts suggested the potential for increased synaptic strength of excitatory synapses in this subregion.

2.6.3 Improvement of action-outcome decision making following isolation during adolescence

Alterations in mPFC neuronal anatomy suggested to us that behaviors relying on intact mPFC function would be atypical in previously-isolated mice. Further, behavioral impairments should be rescued by modulating mPFC plasticity. The prelimbic compartment of the mPFC is necessary for the formation (consolidation) of action-outcome associations. In conjunction with other structures like the ventrolateral orbitofrontal cortex, which is involved in updating (retaining) new associations, the prelimbic mPFC is essential to goal-directed behavior in rodents, such that damage to the prelimbic mPFC causes biases towards habit-based behaviors (reviewed Gourley and Taylor, 2016). Here, we attempted to normalize decision-making behaviors that were otherwise biased towards habit-based strategies in previously-isolated mice (Hinton et al., 2019; Chapter 3).

To quantify decision-making behavior, we used an instrumental contingency degradation procedure in which the predictive relationship between one action and its outcome is violated by providing the associated food pellet outcome in a manner that is not dependent on the animal's action. Following this degraded session, mice should be consolidating information regarding the newly degraded relationship between their recent nose-poke action and its now noncontingent outcome. Gi-coupled DREADDs were placed in the ventral prelimbic mPFC. CNO is the presumed ligand for DREADDs, and it was delivered to all animals immediately following the degradation session in order to target the action-outcome memory consolidation period. Previously-socialized animals expressing the control virus engaged in goal-directed behavior, as expected. By contrast, Gi-DREADDs in previously-socialized mice induced habitual responding, as also occurs with lesions and inducible inactivation of the prelimbic cortex (see several references reviewed in Gourley and Taylor, 2016). Previously-isolated mice expressing the control virus exhibited habit-based behavior, replicating prior findings (Hinton et al., 2019; Chapter 3). Meanwhile, Gi-

DREADDs stimulation in the mPFC rescued goal-directed responding in mice with a history of isolation during adolescence.

I suggest that our results provide evidence that habit biases rooted in disrupted action-outcome memory consolidation are both a symptom *and* outcome of mPFC dysfunction. Specifically, both perturbations – isolation-induced mPFC dendritic spine density elevation and DREADDs-mediated disruption of the mPFC during action-outcome memory consolidation in otherwise healthy control subjects – were associated with the same habit bias. Notably, habit biases can be corrected if previously-isolated mice receive Gi-DREADDs stimulation during the consolidation period, potentially by moderating neuronal plasticity on dendrites with excessive spine densities.

2.6.4 Correcting isolation-induced anhedonic-like behavior

The mPFC includes Brodmann's Areas (BA) 32 and 25 in humans, corresponding with the prelimbic and infralimbic subregions in rodents, respectively. Elevated metabolic activity involving a compartment of this region (BA 25) has been correlated with depression symptomology in patients (Mayberg et al., 2005), and recent work using optogenetic stimulation in rodents has shown that elevated mPFC activity causes anhedonic-like sucrose neglect (Ferenczi et al., 2016). Specifically, Ferenczi et al., 2016 reported that optogenetic stimulation of glutamatergic neurons in the mPFC causes anhedonic-like behavior in rats via top-down control of the striatal response to dopamine. Given that dendritic spines are the primary sites of excitatory plasticity in the brain, dendritic spine excess could result in an aberrant excitation/inhibition balance in the mPFC, ultimately leading to anhedonic-like behavior in adult mice with a history of social isolation. Indeed, previously-isolated mice here exhibited anhedonic-like sucrose neglect in adulthood, detectable weeks following normalization of the social environment.

We again placed CaMKII-driven, Gi-coupled DREADDs in the mPFC, reversing anhedonic-like sucrose neglect in mice with a history of social isolation. Interestingly, reversal of anhedonic-like behavior occurred only after repeated Gi-DREADDs stimulation, once per day over the course of a week, and one stimulation was not sufficient. This repeated stimulation parallels the approach used by Ferenczi et al., 2016, discussed above. In this case, the emergence of anhedonic-like behaviors required repeated optogenetic stimulation once daily over the course of three days. Together, these combined experiments suggest that shifts in hedonic and anhedonic-like behaviors may require broad changes in functional connectivity that do not necessarily arise rapidly, but instead, evolve with time and repeated modulation.

2.6.5 Conclusions

We initiated our investigation by probing long-term changes in the social behaviors of previously-isolated mice. One question that arises is whether differences in social interactions observed are due to resocialization and/or the subsequent home cage social environment, or compounding effects of prior isolation and resocialization, rather than a history of isolation itself. Importantly, all mice throughout these experiments – including previously group-housed females – were re-housed in adulthood. Previously group-housed mice nevertheless show greater social engagement with conspecifics relative to previously-isolated mice; this group difference indicates that the re-housing component alone is not enough to skew behavioral characteristics of control mice to the point that measurable differences in social behavior of previously-isolated mice could not be observed.

As mentioned previously, home cage social interactions in the hours-to-days following resocialization have been observed in both sexes, with no evidence for social behavioral changes (including defensive and anxiety-like behaviors) following resocialization of females (Lukkes et al.,

2013). Considered in light of the present data, one conclusion is that long-lasting changes in social interactions observed here are unlikely to be caused by social experiences immediately following resocialization of female mice. By contrast, resocialized male rodents display numerous defensive and aberrant social behaviors in the home cage environment following resocialization. These findings are reviewed in more detail in Chapter 1.

Left unperturbed, the mPFC undergoes dendritic spine pruning starting in early adolescence (Shapiro et al., 2017), a period encompassed by our social isolation procedure. We find evidence that social isolation during adolescence interferes with dendritic spine populations in the mPFC. Specifically, isolated mice harbor higher dendritic spine densities both during adolescence and in adulthood, despite social reintegration in adulthood. We classified dendritic spines into subtypes to reveal that elevations in gross dendritic spine densities were driven by large populations of immature, thin-type dendritic spines.

These findings suggest that a lack of typical social engagement during adolescence results in an overabundance of labile, thin-type dendritic spines. This overabundance may reflect an initial redirection (due to isolation) of the pruning trajectory of this particular subtype. However, it is unclear whether individual dendritic spine pruning trajectories during adolescence exist for each unique dendritic spine subtype population.

Finally, we investigated the mPFC as a site of modulation through which long-term consequences of isolation could be blocked. We attempted to normalize decision-making behaviors and also block anhedonic-like behavior in adult, previously-isolated mice through chemogenetic inhibition of the mPFC using Gi-DREADDs. Gi-DREADDs in the mPFC facilitated the consolidation of new information regarding action-outcome relationships, resulting in the restoration of goal-directed decision making in previously-isolated mice otherwise prone to habit-

based response strategies. Additionally, repeated Gi-DREADDs stimulation blocked anhedonic-like sucrose neglect in previously-isolated mice.

Here, it's important to address three areas for future studies. First, in our experiments, all mice were temporarily housed individually during the sucrose consumption test. While this practice adheres to a commonly used sucrose consumption protocol that otherwise allows for communal group housing, future experimenters may consider how to adapt this test in a way that allows mice to remain in a social environment throughout testing. This improvement would circumvent potential confounding effects of acute isolation during testing. Second, it's unclear whether the reversal of anhedonic-like behavior reported is long-lasting following cessation of mPFC Gi-DREADDs stimulation. Future studies could incorporate additional testing timepoints following the final administration of CNO to determine the time frame within which the expression of anhedonic-like behavior may (or may not) reemerge in previously-isolated mice. Finally, future experiments could incorporate a sucrose habituation period in order to control for potential effects of novelty on sucrose consumption during the initial testing time point.

2.7 ACKNOWLEDGEMENTS

Aylet Allen created the operant conditioning chamber schematic. We would also like to acknowledge Kelly Vogel and Meghan Wynne for their valuable contributions towards scoring parts of the dendritic spine populations and social behavioral datasets, respectively.

2.8 FUNDING

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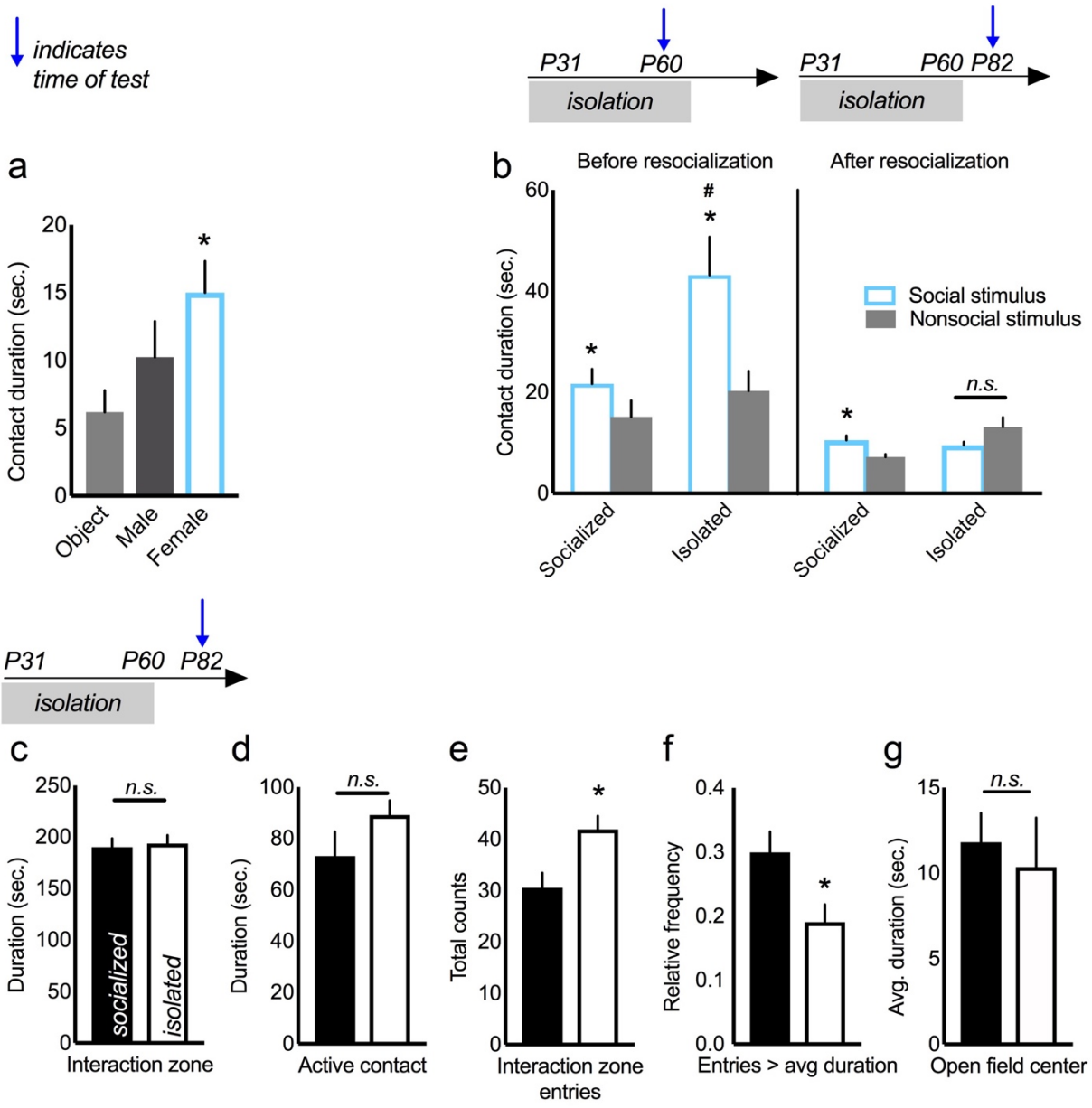

Figure 2-1: Social behavior in adulthood is influenced by the social environment during adolescence. Timelines correspond to the data below. (**A**) We first confirmed that typically-housed adult female mice spend a greater duration of time in contact with a social stimulus mouse of the same sex relative to stimuli either of the opposite sex or a nonsocial stimulus (n=7/group). (**B**) An interaction effect indicates that control, group-housed animals demonstrate greater contact duration with a stimulus mouse in comparison to time spent in contact with a novel object. Those exposed to social isolation

exhibit an aberrantly high duration of social contact with a stimulus mouse when tested while isolated (n=5-9/group). Two weeks following resocialization when mice are no longer housed in isolation, mice with a history of social isolation no longer spend a discernably greater amount of time in contact with a stimulus mouse vs. object (n=18-20/group). (**C**) Duration of time spent inside an interaction zone surrounding a wire containment cup containing a stimulus mouse and (**D**) the duration of time spent in contact with the wire cup containing the stimulus mouse did not differ between groups (n=6-8/group). (**E**) While previously-isolated mice entered the interaction zone a significantly greater number of times, (**F**) significantly fewer of these entries lasted longer than the average duration of time spent by control mice upon each entry, suggesting long-term changes in social approach (n=6-8/group). (**G**) Importantly, this phenotype is not obviously attributable to a general anxiogenic effect of prior isolation, as average time spent in the center of an open field did not differ between groups (n=7-13/group). Bars represent means + SEMs. *p<0.05 as indicated, #p=0.01 isolated vs. control.

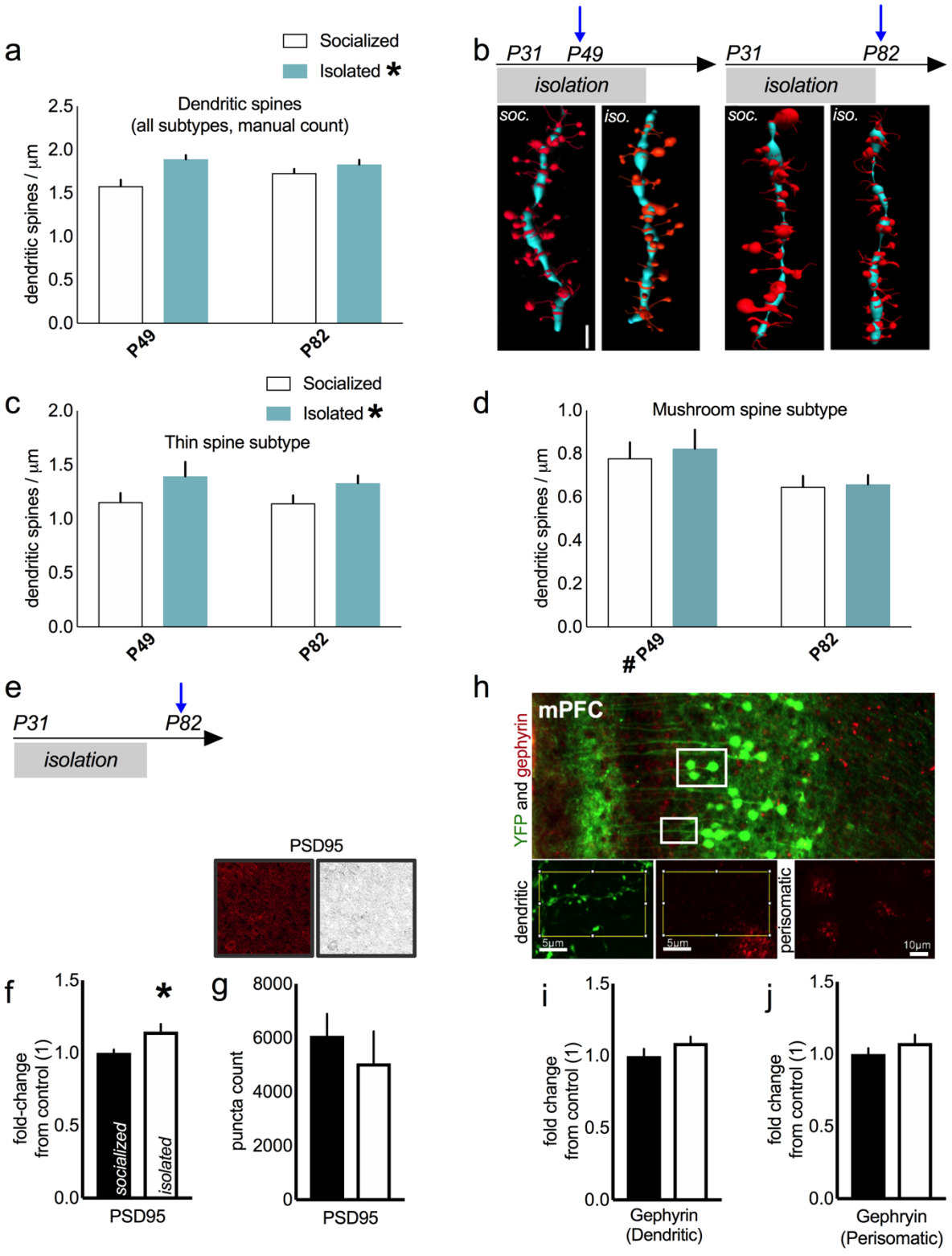

Figure 2-2: Elevations in specific dendritic spine sub-types and synaptic marker levels in the mPFC of socially-isolated mice. (A) Manual counts of dendrites revealed that dendritic spine

densities on excitatory neurons in the mPFC were elevated in mice exposed to isolation both during adolescence (P49) and in adulthood (P82) (*n*=6/group with each mouse considered an independent sample). (**B**) Timeline of experimental events for fig.2-2a-d. Representative dendrite reconstructions corresponding to the timeline shown below. Scale bar = 2 μm. (**C**) 3D reconstruction reveals elevated thin-type dendritic spine density present in isolated mice at both timepoints. (**D**) Mushroom subtype densities trend towards being elevated at P49 in both isolated and socialized mice. (**E**) Timeline for fig.2-2ef-j. (**F**) A history of social isolation elevated mPFC PSD95, as determined by western blotting (*n*= 8-10/group); (**G**) however, PSD95 puncta count did not change (*n*=8-10/group). **Above:** PSD95 immunofluorescence in the mPFC next to gray-scale inverted image used to quantify puncta. (**H**) Representative staining of gephyrin immunofluorescence in the mPFC of YFP-expressing mice. Representative staining proximal to layer V dendrites vs. soma inset below. (**I-J**) Gephyrin immunofluorescence does not differ between groups (*n*=7-11/group). Bars represent means + SEMs, **p*<0.05, #*p*=0.058.

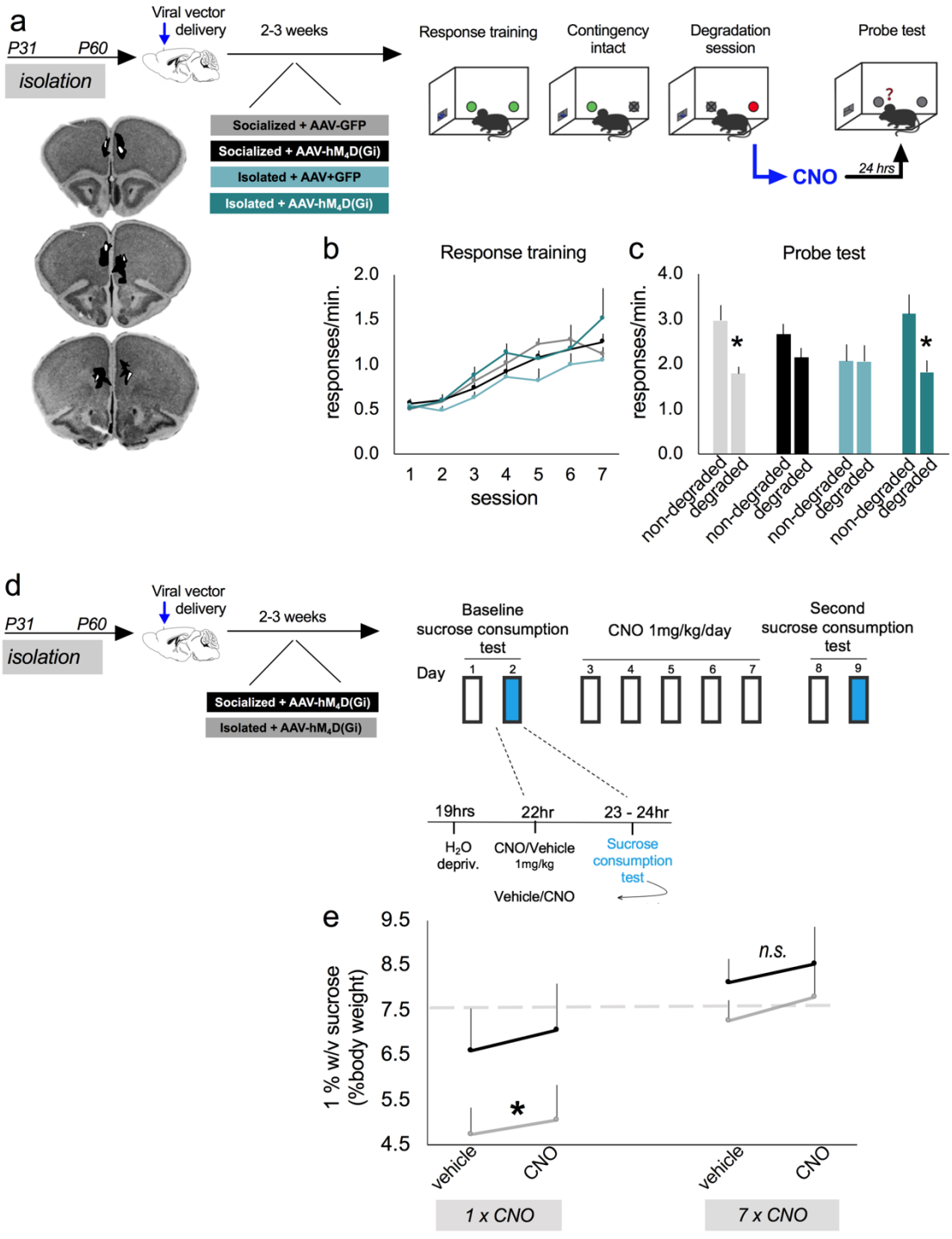

Figure 2-3: Stimulating CaMKII-driven Gi-DREADDs in the mPFC blocks habit formation and anhedonic-like behavior in previously-isolated mice. (**A**) Isolation and surgery timeline. Representative histology indicates largest viral spread (black) and smallest (white). Timeline and

histology, followed by schematic of instrumental response training and contingency degradation procedure. Mice were resocialized in early adulthood (P60) before undergoing surgery. Mice received either a Gi-DREADDs or GFP control viral vector in the mPFC. (**B**) All mice underwent instrumental response training sessions in which a nose-poke action on each aperture resulted in a food pellet outcome, and no group differences were found (n=8-16/group). Next, mice underwent a session in which one nose-poke action remained associated with a pellet reinforcer. In a subsequent "degraded session," this relationship between a nose-poke action and food pellet outcome was degraded via noncontingent pellet delivery. The DREADDs ligand CNO was administered immediately following the conclusion of this degraded session when mice should be consolidating new information about the action-outcome relationship. (**C**) A probe test was conducted the following day in which preferential responding on the "non-degraded" aperture was indicative of goal-directed decision making in previously-socialized mice expressing the control virus. Previously-socialized mice expressing the Gi-DREADDs virus exhibited habit-like responding, as expected. Previously-isolated mice with the control virus were also habitual. However, Gi-DREADDs stimulation corrected this decision-making deficit. (**D**) Experimental timeline and schematic of repeated sucrose consumption testing relative to drug administration. (**E**) Acute CNO did not ameliorate the anhedonic-like sucrose neglect in previously-isolated mice. However, repeated CNO administration reversed this anhedonic-like phenotype in previously-isolated mice, such that their consumption levels were comparable to those of control mice. Gray line indicates overall mean of control mice across all tests (n=8-9/group). Bars and symbols represent means + SEMs, *p<0.05.

CHAPTER 3: SOCIAL ISOLATION IN ADOLESCENCE DISRUPTS CORTICAL DEVELOPMENT AND GOAL-DEPENDENT DECISION MAKING IN ADULTHOOD, DESPITE SOCIAL REINTEGRATION

3.1 CONTEXT, AUTHOR'S CONTRIBUTION, AND ACKNOWLEDGEMENT OF REPRODUCTION

The following chapter identifies and reverses long-term consequences of adolescent social isolation on neurobehavioral outcomes in adulthood. This chapter is reproduced with minor edits from Hinton EA, Li DC, Allen AG, & Goulrey SL (2019). Social isolation in adolescence disrupts cortical development and goal-dependent decision making in adulthood, despite social reintegration. eNeuro. doi: 10.1523/ENEURO.0318-19.2019. The dissertation author contributed by designing and running experiments, analyzing data, and was a main contributor to the writing of the manuscript, under the guidance of Dr. Shannon Gourley. Dan Li and Aylet Allen contributed to designing and running experiments and analyzing experimental results.

3.2 ABSTRACT

The social environment influences neurodevelopment. Investigations using rodents to study this phenomenon commonly isolate subjects, then assess neurobehavioral consequences while animals are still isolated. This approach precludes one from dissociating the effects of on-going *vs.* prior isolation, hindering our complete understanding of the consequences of social experience during particular developmental periods. Here we socially isolated adolescent mice from postnatal days 31-60, then re-housed them into social groups. We tested their ability to select actions based on expected outcomes using multiple reinforcer devaluation and instrumental contingency degradation techniques. Social isolation in adolescence (but not adulthood) weakened action-outcome response updating, causing mice to defer to habit-based behaviors. Decision-making biases were associated with glucocorticoid insufficiency in adolescence, oligodendrocyte marker loss throughout cortico-striatal regions, and dendritic spine and synaptic marker excess in the adult orbitofrontal cortex (OFC). Chemogenetic stimulation of the OFC in typical, healthy mice recapitulated decision-making

biases following isolation, causing habit-like behaviors. Meanwhile, correcting dendritic architecture by inhibiting the cytoskeletal regulatory protein ROCK remedied decision-making defects in socially-isolated mice. Our findings suggest that adolescence is a critical period during which social experience optimizes one's ability to seek and attain goals later in life. Age-typical dendritic spine elimination appears to be an essential factor, and in its absence, organisms may defer to habit-based behaviors.

3.3 INTRODUCTION

The early-life social environment influences neurodevelopment and behavior. For example, social adversity during adolescence is associated with lifetime risk of depression in women (Thapar et al., 2012). In rodents, considerable efforts have revealed that social isolation during the post-weaning period simplifies oligodendrocyte morphology in the prefrontal cortex (PFC), induces anxiety-like behavior, and impairs working memory (Makinodan et al., 2012; Palanza 2001), but investigations most commonly isolate subjects, then assess neurobehavioral consequences while animals are still isolated. This approach precludes one from dissociating the effects of on-going *vs.* prior isolation. Critical periods during which social experience impacts long-term neurobehavioral functions are still being identified.

During typical adolescence, prospective goal-oriented decision making improves as individuals become more sensitive to the real or perceived consequences of their behaviors (Blakemore and Robbins, 2012). In the absence of goal awareness or updating, organisms may instead defer to habits – familiar behaviors that are stimulus-elicited and insensitive to goals. Also during adolescence, PFC neurons undergo dramatic structural reorganization and synaptic remodeling. Some dendritic spines and synapses are stabilized and refined, whereas others – up to 50% in certain regions – are pruned (Bourgeois et al., 1994; Huttenlocher and Dabholkar, 1997;

Shapiro et al., 2017). Individuals who experience early-life adversities have an increased incidence of behaviors that can lead to addiction and obesity as adults, and Patterson et al. (2013) provided evidence that these behaviors may result from an overreliance on outcome-insensitive habits. Complementary investigations in rodents linked stress-related failures in goal-oriented action selection and resulting biases towards habit-based behaviors to changes in prefrontal cortical dendrite and dendritic spine structure (Dias-Ferreira et al., 2009; Barfield et al., 2017). Nevertheless, the long-term effects of social experience during adolescence have not been thoroughly investigated, and mechanistic factors in adversity-induced habit biases, occurring at the expense of goal-directed action, are only beginning to be understood.

Goal-directed action requires: ***1)*** associating behaviors with their likely consequences (*i.e.*, learning action-outcome contingencies), and ***2)*** updating expectations when action-outcome associations change. Using multiple tasks and several independent cohorts of mice, we discovered that adult mice with a history of social isolation during adolescence could learn action-outcome contingencies, but were less able to update action-outcome response strategies, even despite normalization of the social milieu in adulthood. Their deferral to habit-based modes of response was reminiscent of orbitofrontal cortical (OFC) inactivation across rodent and primate species (Gremel and Costa, 2013; Gourley et al., 2013a; Jackson et al., 2016; Zimmermann et al., 2018; Parkes et al., 2018; Whyte et al., 2019), as well as *hyper*-activation (artificial stimulation), which can cause compulsive-like behavior (Ahmari et al., 2013; Gillan et al., 2016; Pascoli et al., 2018). We found that a history of social isolation impoverished oligodendrocyte marker expression in this region and induced dendritic spine excess on layer V excitatory neurons, suggestive of failures in age-appropriate dendritic spine pruning. Based on these patterns, we then identified a sensitive period during adolescence when manipulating key regulators of the actin cytoskeleton – the structural

lattice that forms the shape and plasticity of dendritic spines – can correct structural and behavioral defects caused by social poverty.

3.4 MATERIALS AND METHODS

3.4.1 Subjects

C57BL/6 mice bred in-house from Jackson Labs stock (Bar Harbor, ME) were used. For dendritic spine imaging, C57BL/6-back-crossed mice expressed *thy1*-derived YFP (H line; Feng et al., 2000). Mice were provided food and water *ad libitum* except during instrumental conditioning when mice were food-restricted to ~90-93% of their original weight to motivate food-reinforced responding. Mice were maintained on a 12:12-hour light cycle (0700 on). Females were used unless otherwise noted, a decision discussed in the next section. Mice used for post-mortem measures were not subject to behavioral testing. Procedures were in accordance with the Emory University IACUC.

3.4.2 Social isolation.

Mice were weaned at postnatal day (P) 21-22 and housed in single-sex groups of 6-8. At P31, mice were either rehoused in groups of 6-8 with novel conspecifics, or housed in isolation, living in individual "shoebox"-style cages. All cages were positioned on racks that provided each cage, individually, with ventilation, minimizing the ability of mice to smell each other. Isolated mice lived on one rack and group-housed mice lived on another, minimizing visual contact. At P60, mice were assigned numeric ID's and tail-marked accordingly before being socially reintegrated. Social reintegration involved housing all mice in new cages with novel conspecifics, with each cage containing 3-4 previously-isolated mice and 3-4 previously-socialized mice.

In the one experiment using male mice, we did not socially reintegrate mice because humane concerns regarding high levels of aggression in C57BL/6 males preclude housing of unfamiliar adult males together (Simon 1979).

3.4.3 Corticosterone (CORT) ELISA.

Trunk blood was collected following decapitation at P31, 39, 49, 60, and 82 in chilled Eppendorph tubes between 17:15-20:30 hours. Tubes were centrifuged for 30 min at 4ºC, and blood serum was extracted. CORT levels were determined by ELISA in accordance with manufacturer's instructions (Enzo, Farmingdale, NY). Because this experiment utilized a between-subjects design, data points are connected by curve fits, rather than connected lines.

3.4.4 Adrenal and thymus gland extraction.

Immediately following euthanasia at P82, adrenal and thymus glands were exposed via mid-line dissection and extracted from surrounding adipose tissue. Weights represent both glands as a percent of body weight.

3.4.5 Immunostaining for 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase).

Mice were euthanized by rapid decapitation at P39 or P82, and brains were stored for 48 hours in chilled 4% paraformaldehyde, then transferred to 30% w/v sucrose, and sectioned into 40 μm sections on a microtome held at -15ºC. All immunohistochemical work used free floating sections. Before each step, the sections were washed 3X in PBS. Sections were blocked with PBS, 4% normal goat serum (NGS), and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 1.5 hours at room temperature. Sections were then incubated at 4ºC overnight in 2% normal goat serum, 0.3% Triton X-100, and primary antibody against CNPase (Millipore; 1:200, Billerica, MA). The following

day, sections were incubated for 1 hour at room temperature in 1% NGS and 0.3% Triton X-100, with Alexa Fluor 633 (Life Technologies; 1:500, Waltham, MA) serving as the secondary antibody.

Sections were imaged in a single session using a Nikon 4550s SMZ18 microscope with settings held constant. Fluorescence density was determined using ImageJ. A sampling area was drawn using *The mouse brain in stereotaxic coordinates* (Franklin and Paxinos, 2007) as reference to confirm that samples were collected from equivalent rostral-caudal, medial-lateral, and dorso-ventral positioning throughout. The sampling shape and area were held constant for each section. Imaging and scoring were completed by a single rater blinded to group. One brain from an isolated mouse generated values >2 standard deviations outside of the mean in 4 of the 13 brain regions tested. We excluded this mouse.

3.4.6 Immunostaining for PSD95.

Mice were euthanized by rapid decapitation at P82, and brains were stored for 48 hours in chilled 4% paraformaldehyde, then transferred to 30% w/v sucrose, and sectioned into 40 μm sections on a microtome held at -15ºC. All immunohistochemical work used free floating sections. Before each step, the sections were washed 3X in PBS. Sections were blocked with PBS and 0.3% Triton X-100 (Sigma-Aldrich), 2% NGS, and 1% bovine serum albumin (BSA) for 1.5 hours at room temperature. Sections were then incubated 48 hours at 4ºC in 1 X PBS and 0.3% Triton X-100, 0.3% NGS, 1% BSA, and a primary antibody against PSD95 (Cell Signaling Technology; 1:500, Danvers, MA). Tissue was then incubated for 1 hour at room temperature in PBS and 0.3% Triton X-100, 1% NGS, and a secondary antibody Alexa Fluor 594 (Jackson ImmunoResearch Laboratories; 1:400, West Grove, PA).

The ventrolateral orbitofrontal cortex was located using *The mouse brain in stereotaxic coordinates* (Franklin and Paxinos, 2007). Images were collected in a single session using a Leica SP8 confocal

microscope (Wetzlar, Germany) with a 63X oil-immersion 1.4NA objective at a resolution of 1,024 x 1,024 pixels with a pinhole of 0.40 Airy units, scanning speed of 100, and line averaging of 2.

Puncta count was quantified by a blinded rater using ImageJ software. Brightness and contrast settings were first adjusted consistently for all grayscale 8-bit images while leaving pixel values unchanged. Next, all images were adjusted using a threshold for signal intensity in order to maximize puncta signal and minimize background noise. These images were then converted into binary versions. The total number of PSD95+ puncta with sizes ranging 0.01-4.00 μm^2 pixel units were then quantified from these binary images.

3.4.7 Immunoblotting.

Mice were rapidly decapitated at P82; brains were frozen at -80°C and then sectioned into 1mm sections. The ventrolateral OFC was dissected by a single experimenter using a 1 mm tissue core. Tissue was homogenized by sonication in lysis buffer [100 μl: 137 mM NaCl, 20 mM tris-Hcl (pH=8), 1% igepal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma), 1:1000 Protease Inhibitory Cocktail (Sigma)]. Protein concentrations were determined by Bradford colorimetric assay (Pierce, Waltham, MA), and 15 μg/sample was separated by SDS-PAGE on a 12% gradient tris-glycine gel (Bio-rad, Hercules, CA). Following transfer to PVDF membrane, membranes were blocked with 5% nonfat milk.

Primary antibodies were anti-PSD95 (Cell Signaling; 1:1000), with anti-HSP-70 (Santa Cruz; 1:5000, Dallas, TX) serving as a loading control. Immunoreactivity was assessed using a chemiluminescence substrate (Pierce) and measured using a ChemiDoc MP Imaging System (Bio-rad).

3.4.8 Dendritic spine imaging and quantification.

YFP-expressing mice were euthanized by rapid decapitation at P39 or P82. Fresh brains

were submerged in chilled 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 40 μm sections on a microtome held at -15°C. Unobstructed dendritic arbors running parallel to the surface of the section were imaged on a spinning disk confocal (VisiTech International, United Kingdom) on a Leica microscope.

Z-stacks were collected with a 100X 1.4NA objective using a 0.1 μm step size, sampling above and below the dendrite. Dendrites were collected from secondary branches within 50-150 μm of the soma. They were 11-85 μm in length, but primarily 20-25 μm. After imaging, we confirmed at 10X that the image was collected from the ventrolateral OFC. Collapsed z-stacks were then analyzed by a single blinded rater using ImageJ. Each protrusion $\leq$4 μm was considered a spine, and bifurcated spines were considered singular units.

To generate density values, spine number for each dendritic segment was normalized to the length of the segment. 6-8 dendrites/mouse were imaged and scored, with each mouse contributing a single density (the mean of its dendrites) to initial comparisons between groups by ANOVA. Additional subsequent analyses are described in the Statistics section.

3.4.9 Surgery.

Some experiments utilized intracranial placement of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). Group-housed mice were P31 at the time of surgery. Mice were anesthetized with Ketamine/Dexdomitor (75 mg/kg + 1 mg/kg, *i.p.,* MedVet, Cincinnati, OH). AAV8-CaMKII-HA-rM_3D(Gs)-IRES-mCitrine or AAV8-CaMKII-GFP (UNC Viral Vector Core, Chapel Hill, NC) was infused bilaterally (0.5 μl/side) at AP+2.6, ML$\pm$1.2, DV-2.8. Infusions were delivered 0.05μl/minute, with the needle left in place for 5 additional minutes. The scalp was sutured, and mice were revived with Antisedan (1 mg/kg, *i.p.*, MedVet, Cincinnati, OH). Following behavioral testing, mice were euthanized, brains collected and stored in 4% paraformaldehyde for 48

hours, then transferred to 30% w/v sucrose. Brains were sectioned at 40 μm, and fluorescence within the OFC was imaged and transposed onto images from the Mouse Brain Library (Rosen et al., 2000).

3.4.10 Pharmacological treatments.

Experiments using Gs-coupled DREADDs capitalized on previously validated procedures (Gourley et al., 2016). Clozapine N-Oxide (CNO; 1 mg/kg in a volume of 1 ml/100 g, *i.p.,* Sigma-Aldrich) was dissolved in a 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich) solution in 0.9% sterile saline and prepared on the day of injection. Injections were delivered immediately following the contingency degradation training session, described below, then mice were tested the next day, drug-free. Importantly, all mice, regardless of viral vector, received CNO, equally exposing all mice to any unintended consequences of the drug, such as conversion to clozapine (Gomez et al., 2017). Notably, we have also confirmed that the same dose of CNO does not itself have any effects in the same task, nor does it affect activity of the master cytoskeletal regulatory protein cofilin in the OFC (Whyte et al., 2019). Considering the field as a whole, the dose is low, minimizing the likelihood of off-target effects (see tables in Urban and Roth, 2015).

Other drugs were administered *i.p.* daily in a volume of 1 ml/100 g from P39-47 with the exception of one treatment group, in which the administration period was P31-39 as a comparison. These drugs were: 5 mg/kg fluoxetine (FLX) in PBS (LKT Laboratories, St. Paul, MN) (Doosti et al., 2013); 10 mg/kg fasudil in PBS (LC Laboratories, Woburn, MA) (Swanson et al., 2017); 10 mg/kg RU38486 in 2% v/v EtOH and PBS suspension (Sigma-Aldrich, St. Louis, MO) (Swanson et al., 2013); 40 mg/kg spironolactone (Sigma-Aldrich) in 2% v/v EtOH and PBS suspension.

3.4.11 Behavioral assays.

3.4.11.1 Instrumental response training.

Mice were food-restricted to ~90-93% of their original body weights and trained to nose poke for grain-based food reinforcers (20 mg, Bio-Serv, Flemington, NJ) in Med-Associates operant conditioning chambers (St. Albans, VT). Chambers were equipped with two nose poke recesses and a separate food magazine. Responding was reinforced using a fixed ratio 1 (FR1) schedule wherein 30 pellets were available for responding on the 2 distinct nose poke recesses, resulting in 60 pellets/session. Here, one nose-poke action resulted in one food pellet outcome. This FR1 schedule of reinforcement was chosen to be able to detect whether groups showed differences in their ability to acquire action-outcome contingencies. At this point we also monitored response rates to verify that mice did not demonstrate nose poke aperture preferences. In initial experiments (fig.3-2), mice were reinforced with two separate pellets (*e.g.*, left nose poke resulted in a purified grain pellets, while right nose poke resulted in a chocolate pellet). In subsequent experiments (fig.3-3 and on), both nose pokes were reinforced with the same pellet (purified grain), and response rates are represented as total nose pokes/min. Mice acquired the responses within 5-7 70min sessions (1/day).

3.4.11.2 Instrumental contingency degradation.

A modified version of classical action-outcome contingency degradation was used (as per Swanson et al., 2013,2017; Gourley et al., 2012a). Following FR1 training, contingency degradation and a subsequent "early test" was conducted. Contingency degradation consisted of two sessions. In a 25-min "non-degraded" session, one nose poke aperture was occluded, and responding on the other aperture was reinforced using a variable ratio 2 schedule of reinforcement. Here, the variable ratio schedule was chosen to bias behavior towards goal-directed responding as ratio schedules facilitate this responding. The following day during a 25min "degraded" session, the opposite aperture was occluded, and reinforcers were delivered into the magazine at a rate matched to each animal's

reinforcement rate from the previous session. Responses produced no programmed consequences. In this case, only ~7% of pellets are delivered (by chance) within 2 sec following a response (Butkovich et al., 2015). Thus, the schedule of reinforcement associated with both responses changes, relative to FR1 training, but one response becomes significantly less predictive of reinforcement than the other. Sessions were counter-balanced between and within groups. The following day, both apertures were available during a 10min probe test conducted in extinction. Throughout this initial probe test is referred to as "early test". During probe tests preferential engagement of the response most likely to be reinforced is considered goal-directed, evidence of updating action-outcome expectancies.

In accordance with the model of Dias-Ferreira et al. (2009) and others, we mapped the development of habitual responding, which is outcome *insensitive*. Outcome insensitivity can develop over extended training sessions and in response to training sessions that operate on certain schedules of reinforcement. Ratio schedules deliver outcomes that are dependent on the number of actions performed, which facilitates action-outcome associations. This is in contrast to interval schedules of reinforcement which deliver an outcome only in response to the first nose poke that takes place following a certain interval of non-reinforced time. During the time in which actions are not being reinforced mice are still able to freely nose-poke, but these actions do not result in food pellet outcomes. While ratio schedules are known to bias toward goal-directed strategies, interval schedules bias behavioral responding towards habits (Yin et al., 2004, Dickinson et al., 1983). We implemented random interval schedules to bias behavior towards habits following the "early test" and again following the "test." Following the "early test", responding on both apertures was reinforced according to a random interval (RI) 30-sec schedule for 4 sessions. Here, the first nose-poke was reinforced with one food pellet outcome following an interval averaging 30 seconds. After these sessions the instrumental contingency degradation procedure was repeated and followed by a

subsequent 10min probe test ("test"). An RI-60-sec schedule was then implemented for 5 sessions, and contingency degradation was repeated followed by a subsequent probe test ("late test"). Results from the "late test" were used to determine whether or not all mice could eventually develop habitual response strantegies.

3.4.11.3 Extinction.

Given probe tests were conducted in extinction, we ran all mice through extinction sessions to verify that group differences observed during the probe tests could not be explained by an altered sensitivity to nonreinforcement. Following the final probe test shown for each respective cohort of mice, the same mice were placed in the conditioning chambers (Med-Associates) the next day for 15 or 75min, as indicated graphically, for 2 days. Decreased response rates were compred between groups.

3.4.11.4 Conditioned taste aversion and reinforcer devaluation.

Mice were initially trained to nose poke on an FR1 schedule to bias towards goal-directed responding for a food reinforcer. Then, mice were placed individually in clean cages with *ad libitum* access to the reinforcer pellets for 1 hour. Mice were then injected with 0.15M LiCl (40 ml/kg, *i.p.*) (Quinn et al., 2007) to induce conditioned taste aversion in order to devalue the food outcome. The process occurred 6 times (1 LiCl pairing/day). In a separate cohort when mice had been trained to acquire 2 unique pellets, we paired each mouse's preferred pellet with LiCl, and the other pellet was paired 6 times with NaCl (40 ml/kg, *i.p.*) as a control condition. Pairings were alternated (1/day).

The following day, mice were returned to the conditioning chambers, and responding during a probe test conducted in extinction was monitored. When a single reinforcer was used, response

rates were monitored for 10 min and compared to the final day of FR1 training. When 2 pellets were used, response rates were monitored for 15min and compared between LiCl and NaCl conditions.

3.4.11.5 Sucrose consumption.

A sucrose consumption protocol that allows for standard laboratory group housing was used (Gourley et al., 2013b). First, mice were water-restricted. Then, each mouse was housed alone and allowed access to a 1% w/v sucrose solution in its home cage while cagemates were housed in a separate clean cage in the same room. Each mouse had 1 hour access such that the *average* water deprivation period across the cage of mice was 19 hours. ANOVA using testing order as the independent measure confirmed no order effects ($F<1$).

3.4.11.6 Cocaine-induced locomotor activity.

Female mice were isolated during adolescence and treated with fasudil or vehicle from P39-47. At P60, isolated mice were housed with each other in groups of 8. (In other words, in this experiment assessing whether fasudil influences cocaine-induced locomotor sensitization, all mice were subject to isolation.) Mice were then injected daily with both cocaine (10 mg/kg, *i.p.*) and vehicle. Following each injection, mice were placed in locomotor monitoring chambers (Med-Associates) for 1 hour. Injections were separated by $\geq$3 hours. The order of injections was randomized between and within subjects. This protocol is sensitive to increases *or decreases* in cocaine-induced locomotor activity (Gourley et al., 2009).

3.4.11.7 Elevated plus maze.

Naïve socially-housed female mice were injected with fasudil or vehicle 30 min before test. The maze consisted of two open arms (50x6.5 cm) and 2 "closed" arms with walls (50x6.5x15 cm)

attached to a central platform (6.5x6.5 cm). The maze was elevated 65 cm from the floor. Mice were placed in the center of the maze under dim light, and exploratory behavior was recorded by a video camera suspended overhead for 6 min. Arm entries were calculated by a computer running Limelight software (Coulbourn, Whitehall, PA).

3.4.11.8 Open field test.

Naïve socially-housed female mice were injected with fasudil or vehicle 30 min before test. Mice were placed in the center of a rectangular field (41x20x20 cm). Exploratory activity was videotaped under dim light for 6 min, and time spent in a 6x6 cm center square was recorded.

3.4.12 Statistics.

Blood serum CORT, response rates, sucrose consumption, exploration time on the elevated plus maze, and locomotor activity counts were compared by 2- or 3-factor ANOVA with repeated measures when appropriate. Tukey's post-hoc comparisons were applied to all possible comparisons following interactions or main effects between >2 groups, and significant post-hoc comparisons are indicated graphically. Time spent immobile and in the center of the open field, as well as gland weights and PSD95 and CNPase quantification values, were compared by unpaired 2-tailed t-tests.

For dendritic spine analyses, dendritic spines were enumerated on 6-8 dendrites per mouse. Densities were first compared by ANOVA, with each animal contributing a single value (the mean density of all of its dendrites). In a secondary analysis, we first evaluated all dendrites in the control group and divided them into thirds based on their densities. In this case, the highest third had densities $\geq$1.38 spines/μm; these dendrites were termed "spine-rich." For each mouse, the proportion of spine-rich dendrites was then calculated. For instance, in a typical control mouse, a minority 33% of dendrites would be considered spine-rich. Group means were compared by 1-

sample t-test against the expected one-third proportion, again with each mouse contributing a single value to avoid artificial power inflation. Significant differences indicate that a given group had greater or fewer spine-rich dendrites than would typically be expected. Notably, categorizing dendrites based on the control population, as opposed to predetermined cut-offs, accommodates naturally-occurring variance between cohorts in tissue preparation and processing that can affect fluorescence.

Throughout all analyses, $p \leq 0.05$ was considered significant, and values >2 standard deviations outside of the mean were considered outliers and excluded. Group sizes were based on power analyses of existing datasets. SPSS and Sigmastat were used.

3.5 RESULTS

Throughout the majority of these experiments, we isolated female mice from P31-60, most of the adolescent period in rodents (Spear 2000; Green and McCormick, 2013). Control mice (*i.e.*, socially-housed mice) lived in groups of 6-8. At P60, we then re-housed all mice into new social groups to determine the manner in which a history of isolation – occurring only during adolescence – influences neurobehavioral outcomes in adulthood. Each cage contained 3-4 previously-isolated and 3-4 previously-socialized mice.

Although little is known regarding the long-term consequences of social isolation (specifically, those that manifest despite normalization of the social environment), important leads were reported by Makinodan and colleagues, revealing that social isolation durably simplifies oligodendrocyte morphology in the PFC (Makinodan et al., 2012). Thus, we initially validated our procedure by quantifying CNPase, an oligodendrocyte marker, throughout multiple cortico-striatal brain regions. During the social isolation period (at P39), CNPase levels did not differ between groups (all ps ≥ 0.067)(fig.3-1a). At P82, however, >3 weeks following social reintegration, CNPase

was diminished in several structures. These were: the ventrolateral OFC (t_{17}=-2.25,p=0.038); prefrontal cortical Cg1 (t_{17}=-2.67,p=0.016); the somatosensory cortex (t_{17}=-2.51,p=0.022); and dorsolateral striatum (t_{17}=-2.67,p=0.016)(fig.3-1b). Thus, social experience during late adolescence (after P39) appears to support cortico-striatal CNPase.

3.5.1 Social experience in adolescence optimizes action-outcome response updating in adulthood

CNPase patterns were notable because brain regions involved in both goal-directed response updating (*e.g.*, ventrolateral OFC) *and* habitual behavior (*e.g.*, dorsolateral striatum) were affected, even while others were spared (*e.g.*, prelimbic and infralimbic cortices). These patterns raised the possibility that social experience during adolescence could have long-term effects on the ability of mice to appropriately balance goal-oriented actions *vs.* habits. Action/habit decision making can be defined using instrumental contingency degradation (Balleine and O'Doherty, 2010)(fig.3-2a). Mice are first trained to generate two distinct nose poke actions for two distinct food reinforcers (*e.g.*, left nose poke results in purified grain pellets, while right nose poke results in chocolate pellets). Then, food pellets associated with one nose poke are delivered non-contingently (instrumental contingency degradation) in one session, while the action-outcome contingency associated with the other behavior remains intact in another session. Thus, one nose poke becomes significantly more predictive of reinforcement than the other. Next, mice have access to both nose poke recesses during a brief probe test conducted in extinction; the purpose of the probe test is to assess whether mice have updated action-outcome expectations, responding preferentially towards the intact action-outcome contingency (a goal-directed action). Meanwhile, non-specific responding is considered habitual.

A history of social isolation did not impact response rates during training [no group x session interaction $F_{(10,140)}$=1.28,p=0.25, no effect of group F<1], and pellet preferences were not detected [no group x pellet interaction $F_{(1,14)}$=2.5,p=0.14, no group x day x pellet interaction $F_{(10,140)}$=1.86,p=0.88, no effect of pellet F<1], indicating that mice could learn to nose poke for pellets, and both pellets were preferred equivalently (fig.3-2b). In a probe test following instrumental contingency degradation, all mice initially favored the behavior most likely to be reinforced ["early test," main effect of choice $F_{(1,14)}$=100.5,p<0.001, no effect of group or interactions Fs≤1](fig.3-2c), evidence of action-outcome response updating.

We then tested sensitivity to action-outcome contingencies following further training using RI schedules of reinforcement, which elevate response rates (fig.3-2b) and with time, can induce habitual behavior (Dickinson et al., 1983). A second contingency degradation test revealed that control mice retained sensitivity to action-outcome associations, inhibiting a nose poke when reinforcement was unlikely. By contrast, previously-isolated mice generated both responses equivalently during the probe test, despite instrumental contingency degradation ["test," interaction $F_{(1,14)}$=7.9,p=0.01](fig.3-2c). When we compared responding during both probe tests together, we identified a group x response choice x probe test 3-factor interaction [$F_{(1,14)}$=5.5,p=0.034], with post-hoc comparisons indicating that response strategies differed between groups, depending response experience: While mice exposed to social isolation during adolescence were capable of learning about and updating action-outcome associations, these mice to more readily deferred to habitual behaviors with response familiarity than control mice.

We also assessed response rates *during* the period of noncontingent pellet delivery. Both groups decreased responding over time [main effect of time $F_{(4,56)}$=6.8,p<0.001](fig.3-2d), but to a lesser degree in the mice with a history of social isolation [interaction $F_{(4,56)}$=3,p=0.026](fig.3-2d). This pattern cannot obviously be attributable to differences in sensitivity to nonreinforcement, given

that response extinction – tested following the final probe test – was unaffected (no group or interaction effects *F*s<1)(fig.3-2e).

The failure to select actions based on predicted outcomes is commonly associated with insensitivity to the *value* of an outcome (Balleine and O'Doherty, 2010). We thus next devalued one of two food reinforcers in separate mice subject to the same training procedures (FR1, followed by RI training). While response rates during training did not differ between groups (main effect, group x session, group x pellet, and group x pellet x session *F*s≤1)(fig.3-2f), rates associated with the chocolate pellet were modestly lower overall [$F_{(1,130)}$=10.93,p=0.006]. Mindful of possible individual differences in pellet preferences, we next paired each mouse's preferred pellet with LiCl, decreasing its value, while the other pellet was paired with NaCl, leaving its value intact. Pairings occurred in a separate environment relative to the training environment. LiCl pairings decreased intake over time as expected, while consumption of the NaCl-paired pellet did not change [session x pellet interaction $F_{(2,26)}$=19.65,p<0.001](fig.3-2g). A history of social isolation did not impact conditioned taste aversion [no group x session x pellet interaction $F_{(2,23)}$=2.73,p=0.11], though previously-isolated mice consumed modestly less overall [main effect $F_{(1,13)}$=6.32,p=0.026].

When returned to the conditioning chambers, a pellet x group interaction was detected [$F_{(1,13)}$=10.28,p=0.007](fig.3-2h). Control mice generated higher response rates associated with the valued *vs.* devalued reinforcer (post-hoc p=0.02). Meanwhile, response rates did not significantly differ between conditions in the previously-isolated mice (post-hoc p=0.058). Inspection of individual mice (fig.3-2h, right) revealed that all but 1 control mouse inhibited responding associated with the devalued pellet, while several previously-isolated mice generated *higher* response rates associated with the devalued pellet. This pattern is likely attributable to innate pellet preferences, given that we paired LiCl with each mouse's preferred pellet. Thus, social isolation during adolescence weakens behavioral sensitivity to outcome value in adulthood.

Mice can also be trained to respond on two separate nose pokes for a single food pellet (*i.e.*, both left and right nose pokes are reinforced with a grain pellet)(fig.3-3a). In this case, mice cannot use the distinct sensory properties of each unique reinforcer to help update response strategies. Using this procedure, we again found that a history of social isolation did not impact response rates during nose poke training (no group or interaction effects Fs<1)(fig.3-3b). Following instrumental contingency degradation, all mice again initially favored the nose poke most likely to be reinforced ["early test," main effect of choice $F_{(1,22)}$=17.5,p<0.001, no effects of group or interaction Fs≤1](fig.3-3c). A second test after RI training, however, revealed that previously-isolated mice failed to update action-outcome responding, generating both responses equivalently [group x response choice x probe test interaction $F_{(1,84)}$=4.4,p=0.04](fig.3-3c). This pattern duplicates our findings in the prior figure, and this single-reinforcer procedure was used for the rest of our instrumental contingency degradation experiments.

We took the opportunity to train these mice further using RI schedules to confirm that, with extensive experience, all mice would ultimately develop habitual behavior in this procedure, as would be expected ("late test," no group, response, and interaction effects Fs<1)(fig.3-3c). Again, response extinction did not differ between groups, even when tested over a more prolonged period of time than in our initial experiments in fig.3-2 (no group or interaction effects, Fs<1)(fig.3-3d).

We next trained separate mice to respond for a single reinforcer using an FR1 schedule, which is expected to bias responding towards goal-oriented strategies, then devalued the food reinforcer by pairing it with LiCl. LiCl induced conditioned aversion as expected, with no differences between groups (no group or interaction effects Fs<1)(fig.3-3e). When returned to the conditioning chambers, previously-isolated mice nevertheless generated the food-associated response, apparently insensitive to reinforcer value, before ultimately inhibiting responding [group x time bin interaction $F_{(2,19)}$=3.9,p=0.05](fig.3-3e, right). Thus, even using a schedule of reinforcement that would be

expected to bias responding towards goal-sensitive response strategies (*i.e.*, an FR1 schedule), mice with a history of isolation display habitual response tendencies before correcting them. Altogether, we thus use 4 assays (1- and 2-reinforcer instrumental contingency degradation and 1- and 2-reinforcer devaluation) to reveal that social isolation during adolescence weakens the ability of adult mice update action-outcome responding, causing them to favor habit-based behavior. Meanwhile, the formation of habitual behavior and response extinction appear to be intact.

To determine whether isolation-induced habit biases are developmentally sensitive, we repeated the instrumental contingency degradation experiment, except we delayed isolation until P60. Following social reintegration, all mice acquired the reinforced responses without group differences (no group or interaction effects Fs<1)(fig.3-3f) and were sensitive to changes in action-outcome associations ["early test," main effect of choice $F_{(1,10)}$=9.6,p=0.01, no effect of group or interactions Fs≤1; "test," main effect of choice $F_{(1,10)}$=5.5,p=0.04, no effect of group or interactions Fs≤1](fig.3-3g). Thus, social isolation in adulthood did not obviously bias response strategies.

3.5.2 Social experience during adolescence is necessary for age-typical glucocorticoid tone and dendritic spine densities

In adolescence, blood levels of the primary stress hormone CORT increase, then normalize as animals enter adulthood. We found that isolation induced corticosteroid insufficiency in late adolescence (starting at P39), such that blood CORT levels were low early in the active cycle (*i.e.*, when they should be high). Unlike decision-making behavior, however, CORT normalized upon social reintegration [day x group interaction $F_{(4,48)}$=2.9,p=0.03](fig.3-4a). Accordingly, adrenal and thymus gland weights were also typical at this time, corresponding to behavioral testing above (t_{11}=-0.5,p=0.6; t_{11}=-0.59,p=0.57)(fig.3-4a, inset).

GR occupancy is necessary for dendritic spine turnover during adolescence (Liston and Gan, 2011), which culminates in dendritic spine pruning and lower densities in adulthood than adolescence. To determine whether social experience impacted age-typical dendritic spine elimination, we enumerated dendritic spines on layer V neurons in the ventrolateral OFC. Typical adult mice aged P82 had lower spine densities than typical adolescent mice aged P39, as expected, given that dendritic spines in this region are eliminated during adolescence (Gourley et al., 2012b; Milstein et al., 2013; Shapiro et al., 2017). Meanwhile, adult P82 mice with a history of social isolation had elevated densities, suggestive of failures in age-appropriate spine pruning [main effect $F_{(2,15)}$=4.9,p=0.02](fig.3-4b).

The protein PSD95 is a marker of stable, functional synapses (Berry and Nedivi, 2017). PSD95 was elevated in adult mice with a history of social isolation, as measured using western blotting (t_{12}=3.44,p=0.005)(fig.3-4c) and in an independent cohort, immunostaining (t_{14}=-2.25,p=0.04)(fig.3-4d). This pattern suggests that over-abundant dendritic spines contained mature synapses.

3.5.3 Stimulating the ventrolateral OFC in healthy mice disrupts decision-making strategies

To explore the potential consequences of synaptic over-abundance in the OFC, we infused into typical, healthy mice viral vectors expressing Gs-coupled DREADDs and mCitrine (fig.3-5a-b). When activated, Gs-DREADDs artificially elevate neuronal excitability (Farrell and Roth, 2013). Infusions spanned the ventrolateral OFC in all mice, as intended (fig.3-5b). mCitrine was also detected in the medial OFC in a minority of mice, but this region is apparently not involved in sensitivity to instrumental contingency degradation (Bradfield et al., 2015).

Response training was not affected by DREADDs [no group effect $F_{(1,18)}$=1.14,p=0.3; no interaction effects Fs<1](fig.3-5c). CNO was then delivered to all mice, regardless of viral vector group, following instrumental contingency degradation. Thus, Gs-DREADDs were activated during a period of OFC-dependent action-outcome expectancy updating (see Zimmermann et al, 2018). Response preferences were then tested the next day. We detected a group x response interaction [$F_{(1,18)}$=7.29, p=0.02], with post-hoc comparisons indicating that control mice favored the intact action-outcome contingency, while Gs-DREADDs mice did not (fig.3-5d). To further understand response patterns, we also generated preference scores by simply calculating: non-degraded / degraded. In this case, values >1 indicate a preference for the intact action-outcome contingency. Again, groups differed (t_{16}=-2.94,p=0.01), with only control mice energizing the response that was likely to be reinforced (fig.3-5e). Thus, artificially activating the OFC is sufficient to impede action-outcome updating, causing mice to favor habit-based strategies.

3.5.4 Inhibiting ROCK improves action-outcome response updating and normalizes dendrite architecture

Our findings indicate that social isolation causes glucocorticoid insufficiency, dendritic spine excess, and biases towards habit-based behavior in adulthood. If these phenomena are connected, blocking GRs during adolescence should cause habit-based behaviors in adulthood. Also, normalizing dendritic spine densities in socially-isolated mice should correct decision-making behaviors. We next tested these predictions. First, we treated typical, socially-housed adolescent mice with the GR antagonist RU38486 from P39-47, corresponding to a period when CORT levels diverged in typical *vs.* isolated mice (see again, fig.3-4a). Control mice received a vehicle solution, the MR antagonist spironolactone, or RU38486 during an earlier adolescent period, starting at P31,

when isolation did not appear to impact CORT levels (see again, fig.3-4a) (experiment timeline in fig.3-6a).

As drug-free adults, mice were trained to nose poke for food reinforcers, with no significant differences between groups [interaction $F_{(18,222)}=1.5$,$p=0.08$, no effect of group $F<1$](fig.3-6b). In an initial test for sensitivity to instrumental contingency degradation, an interaction between group and response was detected ["early test" $F_{(3,36)}=3.5$,$p=0.02$](fig.3-6c). After additional training, an interaction effect was again detected ["test" $F_{(3,36)}=3.5$,$p=0.02$](fig.3-6c). Post-hoc comparisons at both probe tests revealed that mice exposed to the GR antagonist RU38486 starting at P39 failed to select actions based on their consequences, relying instead on inflexible habits. Mice exposed earlier in development or treated with the MR antagonist spironolactone were not obviously affected.

Next we tested whether facilitating dendritic spine elimination in adolescence conferred behavioral benefits. ROCK, particularly the ROCK2 isoform, is a neuronally-enriched cytoskeletal regulatory factor; its inhibition can expedite dendritic spine elimination in multiple contexts, including adolescent prefrontal cortical development (Shapiro et al., 2019). Thus, we administered the ROCK2-favoring inhibitor fasudil during the same P39-47 period, then euthanized mice as drug-free adults (fig.3-7a). Surprisingly, dendritic spine densities did not differ between any groups (ps$\geq$0.29; not shown), potentially due to repeated injection stress during adolescence. Nevertheless, we identified an important pattern: We first divided dendrites from control mice into thirds based on their densities. This highest third had densities $\geq$1.38 spines/μm, which we term "spine-rich." We then calculated the proportion of spine-rich dendrites for each mouse. In control mice, the mean proportion did not differ from one-third, as expected ($t_5=0.36$,$p=0.73$)(fig.3-7b). In mice with a history of social isolation, spine-rich dendrites made up roughly *half* of all dendrites, significantly higher than is typical ($t_5=2.8$,$p=0.04$)(fig.3-7b). Fasudil *reinstated typical proportions*, such that spine-rich dendrites again made up a minority of dendrites, not differing from the expected one-third

(t_5=0.37,p=0.72)(fig.3-7b). Fasudil did not obviously affect OFC dendrite architecture in socially-housed control mice (t_5=0.06,p=0.96)(fig.3-7b). Thus, fasudil normalized dendrite architecture in mice with a history of social isolation, ensuring that a minority of dendrites carried a large density of spines.

Next, we administered fasudil, again from P39-47, to a separate population of mice for behavioral testing (fig.3-8a). As a comparator, other mice received the selective serotonin reuptake inhibitor (SSRI) FLX, at a dose that has antidepressant-like effects in mature rodents. We were motivated by evidence that social poverty in adolescent humans is associated with depression later in life (Thapar et al., 2012).

As adults, mice were trained to respond for food reinforcers, and we assessed whether they could select actions based on their consequences. All groups initially engaged the action most likely to be reinforced following instrumental contingency degradation ["early test," main effect of response $F_{(1,64)}$=106,p<0.001; no effects of isolation, treatment, or interactions](fig.3-8b). Further response training caused failures in response updating in previously-isolated mice, as expected, which were blocked by fasudil ["test," isolation x drug x response interaction $F_{(2,64)}$=4.1,p=0.02](fig.3-8c). Meanwhile, post-hoc comparisons also indicated that FLX had no effects in previously-isolated mice and unexpectedly *induced* response updating failures in socially-housed mice (fig.3-8c).

We also tested sucrose consumption, an assay of anhedonic-like behavior. A history of social isolation decreased sucrose consumption, a depression-like behavior, and fasudil prevented this deficiency, an antidepressant-like effect [isolation x drug interaction $F_{(2,87)}$=7.05,p=0.001](fig.3-8d).

Interestingly, a history of fasudil appeared to increase food-reinforced response rates (fig.3-8c), which could conceivably be attributed to hyper-sensitivity to "reward." However, the same dose of fasudil from P39-47 did not influence cocaine-induced locomotor sensitization, a classical

measure of reward circuit plasticity (cocaine x fasudil and cocaine x fasudil x session Fs<1)(fig.3-8e). We also assessed the behavioral effects of the same dose of fasudil while mice were adolescents, revealing no effects on exploration of an elevated plus maze [arm x group interaction $F_{(1,12)}$=3.5, p=0.09](fig.3-8f), nor open field (p>0.05)(fig.3-8g). These patterns suggest that fasudil did not induce locomotor abnormalities or anxiety-like behavior.

3.5.5 Differential behavioral effects in male C57BL/6 mice

All of our experiments above utilized female C57BL/6 mice. Males of this strain are aggressive and largely do not engage in "pro-social" behavior beyond the early adolescent period (Simon 1979). For this reason, we anticipated different effects relative to females. Social isolation did not affect response rates during training [interaction $F_{(15,330)}$=1.59, p=0.075, no main effect of group $F\leq1$](fig.3-9a). During a first probe test following instrumental contingency degradation, a main effect of response choice indicated that all mice generated the response most likely to be reinforced [$F_{(1,22)}$=20.66, p<0.001, no interaction F<1](fig.3-9b). In a second test, both groups again preferentially generated the response likely to be reinforced (unlike in females), and further, isolated males generating *higher* response rates [interaction $F_{(1,22)}$=21.32, p<0.001; main effect $F_{(1,22)}$=209, p<0.001](fig.3-9b). Thus, social isolation does not obviously cause response updating failures in male C57BL/6 mice.

3.6 DISCUSSION

The social environment during adolescence influences neurodevelopment. Investigations using rodents to study this phenomenon commonly isolate subjects, then assess neurobehavioral consequences while animals are still isolated. This approach precludes one from identifying critical periods when social experience molds the brain and behavior later in life. Makinodan et al. (2012)

made important in-roads addressing this issue, reporting that social isolation during the post-weaning period simplifies oligodendrocyte morphology in the PFC, despite social reintegration. Inspired by their report, we first measured CNPase, an oligodendrocyte marker, in mice exposed to social isolation during adolescence and socially reintegrated as young adults. CNPase was lower in multiple cortico-striatal regions involved in reward-related decision making. We then discovered that a history of social isolation weakened goal-directed action in mice, causing them to favor habit-based behaviors, a bias linked to long-term effects on OFC neurobiology.

3.6.1 Social interactions in adolescence optimize goal-directed behavior in adulthood

Social isolation in adolescence induces anxiety- and depression-like behavior and reduces myelination throughout cortico-limbic structures (Green and McCormick, 2013). Decision-making capacity remains relatively unexplored, despite evidence that early-life adversity causes biases towards habit-based behaviors in humans (Patterson et al., 2013). Social isolation here decreased oligodendrocyte marker levels in multiple brain regions, as expected. One region was the OFC, a structure conceptualized as building a cognitive map of "task spaces," allowing organisms to link behaviors and stimuli with anticipated outcomes (Wilson et al., 2014) and to update response strategies when expectations change (Sul et al., 2010).

To quantify decision-making behavior in mice with a history of social isolation, we utilized an instrumental contingency degradation procedure. Subjects are first trained to generate two reinforced behaviors. Then, the predictive relationship between one behavior and its outcome is violated by providing the associated food pellet noncontingently. Response inhibition is interpreted as evidence of response updating. We deployed two variants of the task – one using distinct reinforcers (two uniquely-flavored pellets associated with two unique nose pokes), and another using a single reinforcer (one pellet is linked with both actions). The first iteration allows mice to integrate

information regarding the sensory features of each outcome and connect them with the associated behavior. The second iteration is concentrated on action-outcome contingency, but response patterns can generalize, which may or may not be habit-based. Both iterations are amenable to repeated testing, allowing us to define the nature of decision-making abnormalities, if any. Throughout, mice with a history of social isolation were initially capable of forming action-outcome associations, inhibiting responding when one of the two trained behaviors was un-reinforced. They, however, failed to update expectancies when nose poking became more familiar, deferring to habits. Responding was also insensitive to changes in outcome value – another marker of habits. Thus, social isolation in adolescence weakens goal-sensitive behavior later in life, causing mice to favor habit-based response strategies despite social reintegration.

3.6.2 Neurobiological factors

During adolescence, circulating CORT increases (Goldsmith et al., 1978; Meaney al., 1985; Laviola et al., 2002; Silveri and Spear, 2004; Gunnar et al., 2009, Stroud et al., 2009; fig.3-4). Temporarily high CORT concentrations occupy low-affinity GRs and high-affinity MRs, coordinating high rates of cortical dendritic spine turnover and pruning during adolescence (Liston and Gan, 2011). We found that social isolation caused CORT insufficiency early in the wake cycle, when levels should be high (cf., Sanchez et al., 1998), and concurrently, previously-isolated adult mice retained adolescent-like spine densities. Further, PSD95, a marker of mature synapses, was elevated, suggesting that overabundant spines contained functional synapses (Berry and Nedivi, 2017).

To directly assess whether GR occupancy during adolescence affects decision making in adulthood, we treated group-housed mice with a GR inhibitor at the same adolescent age when

socially-isolated mice experienced low CORT (~sixth week of life), causing habit biases in adulthood. Thus, some minimal degree of GR occupancy in adolescence is necessary for optimal OFC function later in life, presumably acting by coordinating age-appropriate dendritic spine pruning (Liston and Gan, 2011). Naturally, other factors could be involved: Social isolation+reintegration triggers Brain-derived neurotrophic factor overexpression in the PFC (Murinova et al., 2017), which has been associated with spinogenesis (Shapiro et al., 2017) and weakened goal-directed action in the same task (Gourley et al., 2012a). Also, upon social reintegration, socially-housed mice appear to approach previously-isolated mice less than the previously-isolated mice approach them (Endo et al., 2018). These social imbalances normalize rapidly, however (1-2 days), so whether they would impact the long-term decision-making biases identified here is unclear.

We next hypothesized that artificially activating the OFC of typical mice would induce habit biases. Indeed, chemogenetic stimulation of excitatory CaMKII-expressing neurons in the ventrolateral OFC caused mice to fail to appropriately update response strategies; instead, they utilized habit-based behaviors. While this pattern was consistent with our expectations, it may seem unexpected to some readers, given that ventrolateral OFC inactivation also impedes response updating in this task in rats (Parkes et al., 2018), mice (Zimmermann et al., 2018), and nonhuman primates (Jackson et al., 2016). Importantly, repeated stimulation of the ventrolateral OFC causes compulsive-like behavior (Ahmari et al., 2013; Pascoli et al., 2018), thought to have a strong habit component (Gillan et al., 2016). We speculate that OFC stimulation intensifies connections that sustain compulsive behavior. Other possibilities are that stimulation is not causing compulsive-like behaviors, per se, but rather, interfering with essential task-related dendritic spine pruning (Whyte et al., 2019). Also (or alternatively), OFC function may adhere to an inverted U-shaped curve, in which "too much" or "too little" activity impedes optimal action-outcome decision making.

The actin cytoskeleton forms the structural lattice that supports the shape and plasticity of dendritic spines (Pontrello and Ethell, 2009). The protein ROCK influences cytoskeletal lability in large part by inhibiting cofilin (Maekawa et al., 1999; Koleske 2013). ROCK inhibitors can augment neuronal structural plasticity, enhancing dendritic spine elimination associated with memory formation (Swanson et al., 2017), glutamate-mediated plasticity (Schubert et al., 2006), and adolescent development (Shapiro et al., 2019). Consistent with these patterns, the brain-penetrant ROCK inhibitor fasudil normalized dendritic spine excess in socially-isolated mice here, decreasing the proportion of dendrites with high spine densities. Fasudil also corrected behavioral abnormalities, even compared to FLX, which elevates synaptic serotonin and is used to treat depression. Notably, FLX induced response updating failures. This outcome was surprising, given that serotonin has long been believed to sustain OFC function (Rogers et al., 1999a,b). Nevertheless, the enduring consequences of FLX in adolescence are not well understood; our findings warrant further investigation.

A history of social isolation also reduced sucrose intake, anhedonic-like behavior that was blocked by fasudil. Meanwhile, FLX had no consequences. Social isolation interferes with SSRI-induced serotonin signaling (Dankoski et al., 2014), which may account for this inefficacy. In humans, anhedonia, hypocortisolimia, and adolescent trauma are together associated with atypical depression (Gold 2015). Social isolation+reintegration of female mice may thus model aspects of depression, valuable given that other "social" models to study depression, like resident-intruder stress, do not have durable consequences in females (Snyder et al., 2015) or instead recapitulate aspects of PFC hypo-activity (Duman and Duman, 2015) and not hyper-activity (Akil et al., 2018; Riva-Posse et al., 2014).

3.6.3 Concluding remarks

Female mice were used throughout the majority of experiments, given that in male mice, particularly aggressive C57BL/6 and CD-1 strains, single housing may not be a major adversity (Goldsmith et al., 1978; Bartolomucci et al., 2003; Arndt et al., 2009), and instead, advantageous, eliminating competition for reproductive opportunities. Isolation did not occlude response updating in C57BL/6 males, as expected. Meanwhile, exogenous CORT impairs response updating in males and females in the same task (Gourley et al., 2012a; Barfield et al., 2017), together suggesting that single housing uniquely affects male and female C57BL/6 mice.

We did not monitor estrus cycle in order to avoid stressor-related confounds from vaginal cell sampling, particularly given that CORT regulates deep-layer cortical dendritic spines (Liu and Aghajanian, 2008; Swanson et al., 2013; Gourley et al., 2013b; Barfield et al., 2017). Meanwhile, deep-layer cortical spines are considered less sensitive to sex than those in other structures such as the hippocampus (Munoz-Cueto et al., 1991; Shors et al., 2001; see also Boivin et al., 2018). Additionally, our multi-day behavioral experiments were replicated in multiple cohorts, and brain tissues were collected from ~3 independent groups. Thus, it seems unlikely that rapid fluctuations in sex hormones due to the estrus cycle impacted our findings.

To conclude, social isolation during adolescence weakens age-typical dendritic spine elimination. Our findings are consistent with evidence that social play, other environmental stimuli, and stress hormones all contribute to neuron refinement during early-life critical periods (Zuo et al., 2005; Bell et al., 2010; Liston and Gan, 2011). Our finding that a ROCK inhibitory compound, delivered at a dose that normalizes dendritic spine excess, corrects deficiencies in action-outcome response updating provides empirical support for the supposition that neuronal refinement during adolescence is linked to complex behavior in adulthood. A challenge in future investigations will be to comprehensively identify specific cell populations, projections, and cognitive domains durably affected by social adversity and develop clinically-viable interventions to improve outcomes.

3.7 ACKNOWLEDGEMENTS

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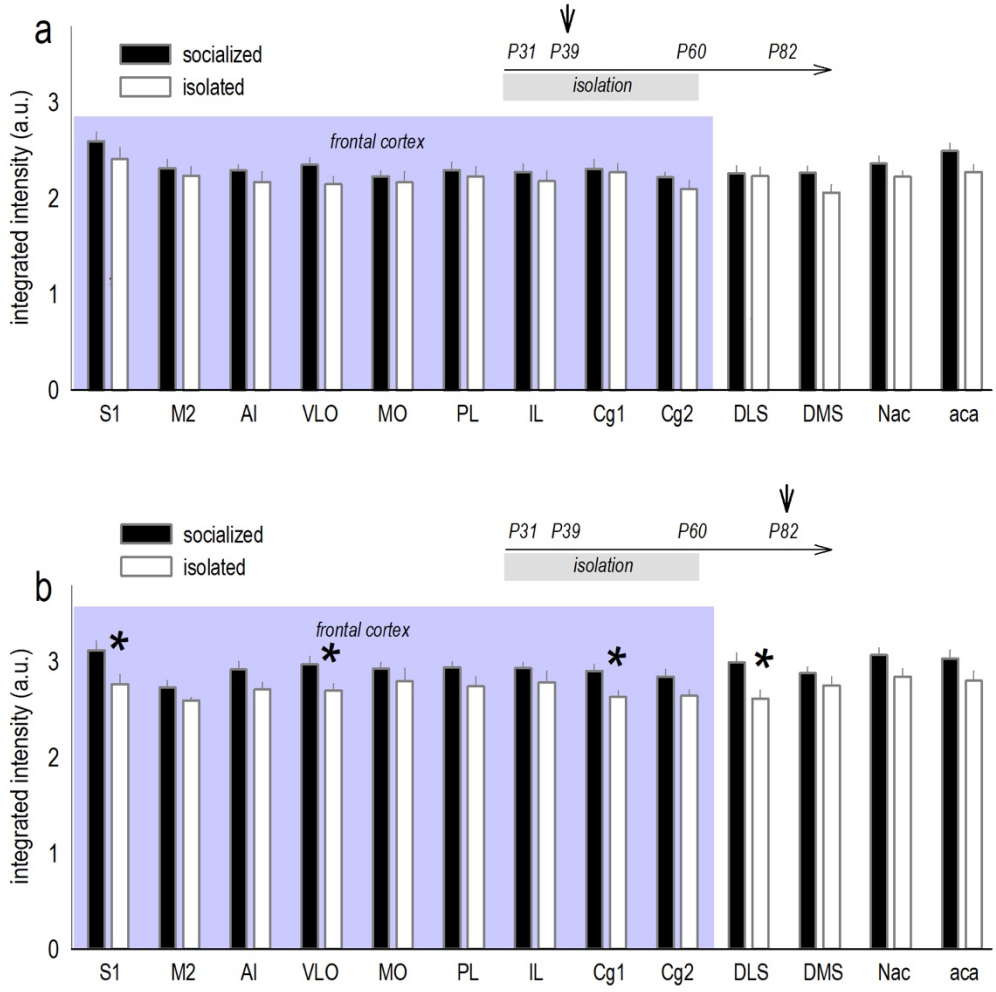

Figure 3-1: Effects of social isolation in adolescence on cortico-striatal CNPase during vs. after isolation. Experimental timelines are inset, and shading highlights cortical regions. (**A**) Mice were socially-housed or isolated starting at P31. CNPase was measured, given existing evidence that social isolation impoverishes oligodendrocyte morphology, and effects are persistent, despite social reintegration (Makinodan et al., 2012). At P39, CNPase did not differ between groups (n=8-9/group). (**B**) Isolated mice were reintegrated into social groups at P60. At P82, previously-isolated mice had decreased CNPase in multiple cortico-striatal subregions (n=7-12/group). Means+SEMs,*p<0.05 vs. socially-housed control.

Anatomical abbreviations: S1 – somatosensory cortex; M2 – secondary motor cortex; AI – agranular insula; VLO – ventrolateral orbitofrontal cortex; MO – medial orbitofrontal cortex; PL – prelimbic prefrontal cortex; IL – infralimbic cortex; Cg1 – primary cingulate cortex; Cg2 – secondary cingulate cortex; DLS – dorsolateral striatum; DMS – dorsomedial striatum; Nac – nucleus accumbens; aca – anterior commissure

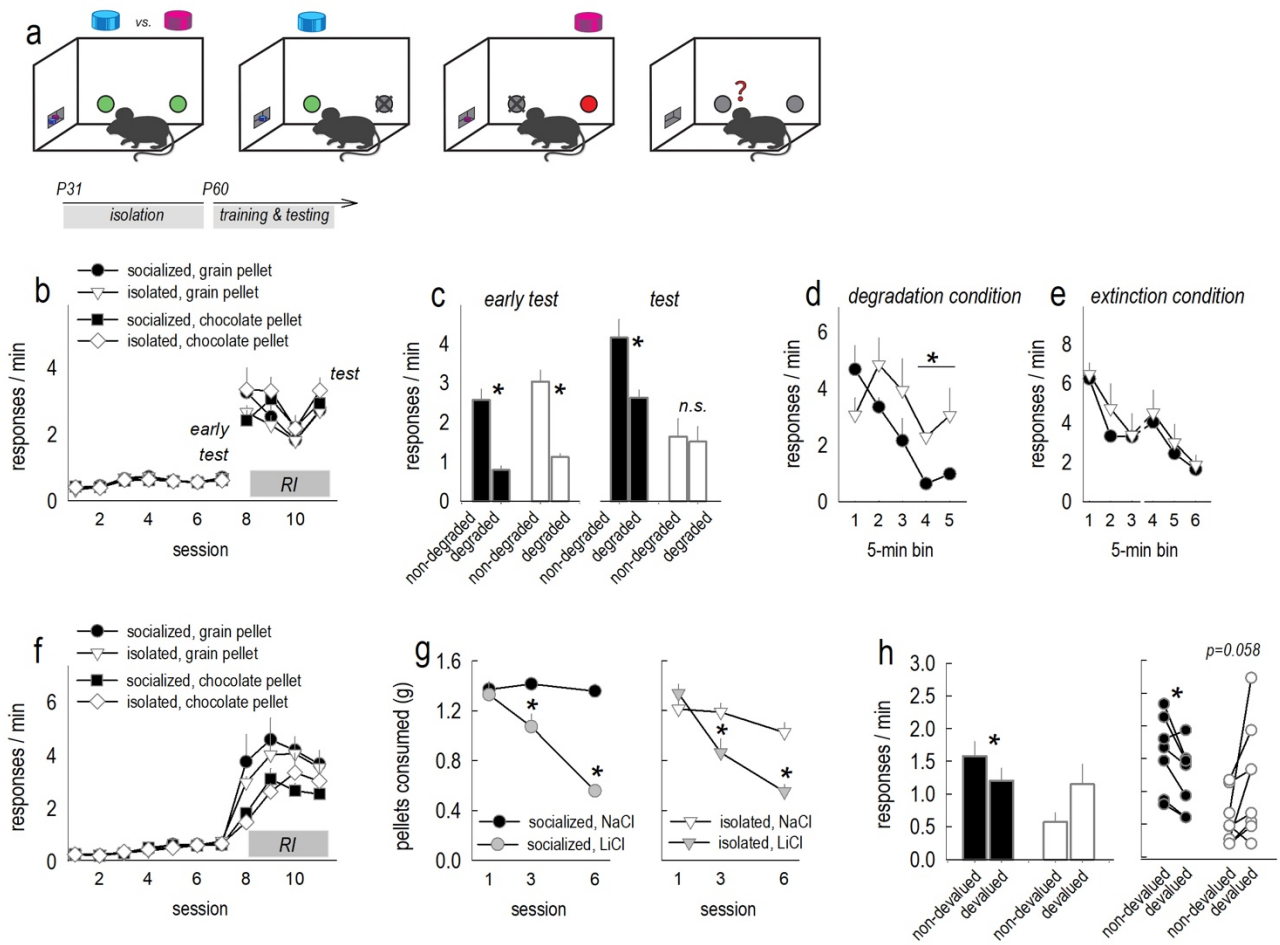

Figure 3-2: Social isolation in adolescence causes failures in action-outcome response updating in adulthood. (**A**) A task schematic is provided: Mice are trained to respond for two pellets. Then, the contingency between one nose poke and the associated pellet remains intact, while the other is "degraded" when that pellet is provided noncontingently. A brief probe test conducted in extinction gives mice the opportunity to demonstrate their response preference, if any, reflecting their ability to update responding based on action-outcome contingency. (**B**) Experimental timeline at top. Adult mice with a history of social housing or social isolation were trained to nose poke for two food reinforcers, with no group differences in response rates. Breaks in acquisition curves represent tests for sensitivity to instrumental contingency degradation. (**C**) In an "early test," all mice decreased responding when the action-outcome relationship associated with that response was violated ("degraded"). With additional training, however, previously-isolated mice failed to update

action-outcome expectancies deferred to habit-based behavior ("test"). (**D**) We also assessed response rates during the period of noncontingent pellet delivery. In this case, both groups decreased responding over time, but to a lesser degree in mice with a history of social isolation. (**E**) Meanwhile, responding in the absence of food pellets (extinction) was unaffected. The break in the x axis represents the passage of one day (n=8/group). (**F**) Separate mice were trained to nose poke for pellets. (**G**) LiCl was paired with each mouse's preferred pellet, inducing conditioned taste aversion, while leaving consumption of the other pellet intact. (**H**) When returned to the conditioning chamber, control mice reduced responding for the now-devalued pellet. Meanwhile, mice with a history of social isolation continued to respond, failing to update response strategies. Right: Response rates of each mouse are plotted (n=7-8/group). "RI" refers to random interval training. Bars and symbols represent means+SEMs or individual mice,*p<0.05.

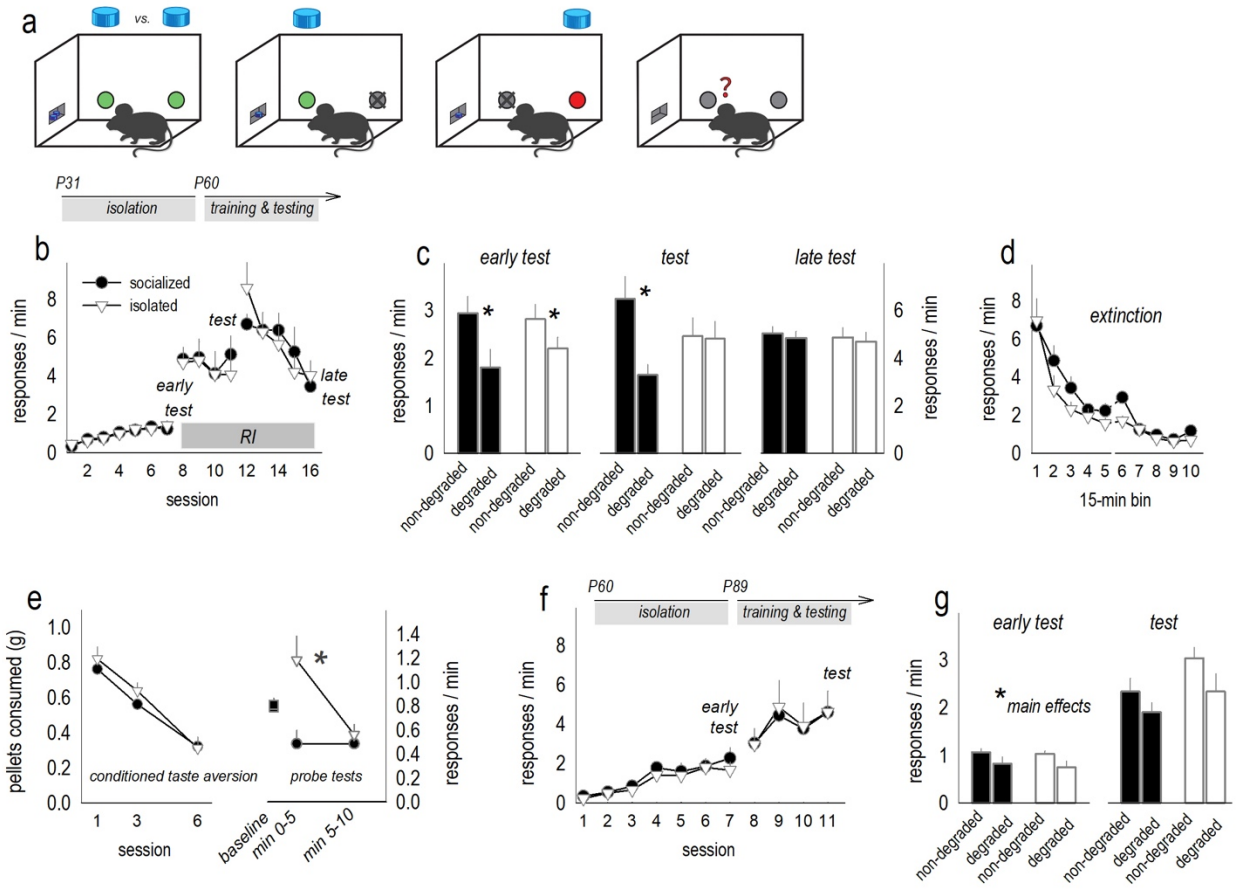

Figure 3-3: Social isolation in adolescence, but not adulthood, causes failures in action-outcome response updating later in life. (**A**) A task schematic is provided: An instrumental contingency degradation task was used as in figure 3-2, except both nose pokes resulted in the same type of pellet (represented here as a blue pellet). (**B**) Experimental timeline at top. Adult mice with a history of social housing or social isolation were trained to nose poke on two recesses for a single food reinforcer, with no group differences in response rates. Breaks in acquisition curves represent tests for sensitivity to instrumental contingency degradation. (**C**) In an "early test," all mice decreased responding when the action-outcome relationship associated with that response was violated ("degraded"). As in figure 3-2, however, previously-isolated mice failed to update expectations with additional training, deferring to habitual behavior ("test"). In a "late test" following extended training, all mice developed habit-based strategies (non-specific responding) as

expected. (**D**) Response extinction was unaffected. The break in the x axis represents the passage of one day (n=12/group). (**E**) In a separate experiment, we decreased reinforcer value by pairing the food reinforcers with LiCl. LiCl induced conditioned taste aversion, as indicated by less food consumed over the course of multiple pairing sessions (left). When placed in the conditioning chambers, previously-socialized mice inhibited responding relative to their pre-aversion baseline (before LiCl). By contrast, previously-isolated mice initially responding robustly despite diminished outcome value before ultimately inhibiting their responding (right) (n=10-11/group). (**F**) Next, separate mice with a history of social isolation *in adulthood* were trained to nose poke for food reinforcers. (**G**) Unlike mice with a history of social isolation during adolescence, mice with a history of social isolation during adulthood retained goal-directed response strategies (n=6/group). "Main effects" refers to main effects of response preference, regardless of group (no interactions). "RI" refers to random interval training. Means+SEMs,*p<0.05.

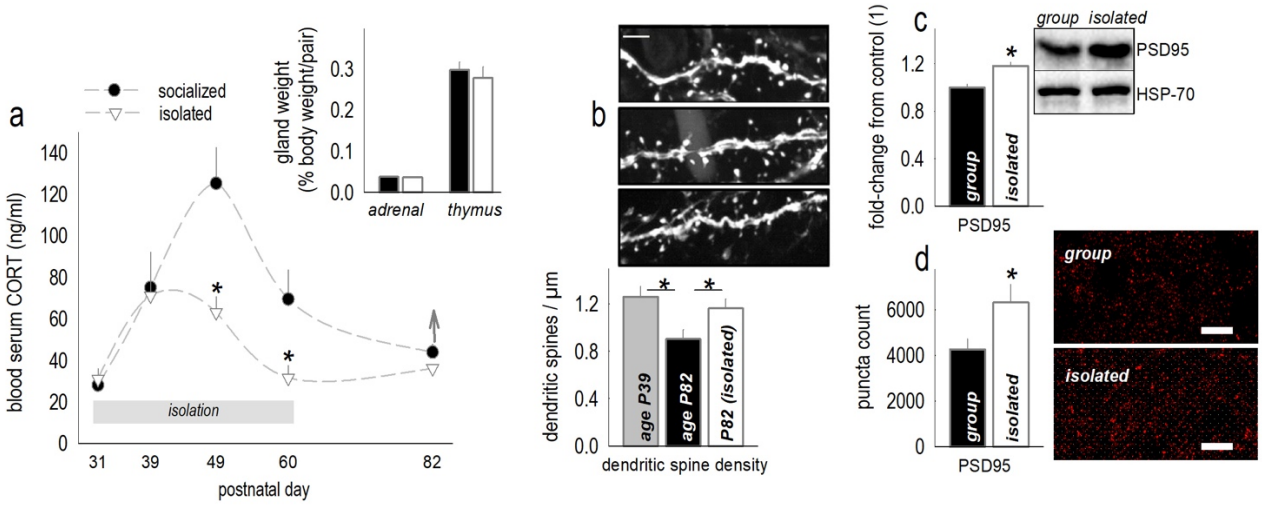

Figure 3-4: Social isolation induces glucocorticoid insufficiency in adolescence and dendritic spine and PSD95 excess in adulthood. (**A**) Blood serum CORT was measured during and after social isolation in adolescence. Social isolation blunted blood serum CORT, collected early in the active cycle when levels should be high. CORT normalized with social reintegration, such that adrenal and thymus gland weights were indistinguishable between groups by P82 (**inset**) (*n*=5-7/group). (**B**) Dendritic spine densities on excitatory neurons in deep-layer OFC were elevated following adolescent isolation. These densities, collected at P82, did not differ from those in typical socially-housed adolescent (P39) mice, suggestive of a failure in dendritic spine pruning during adolescence (*n*=6/group, with each mouse contributing a single value). Representative spines are above in the same order. Scale=3 µm. (**C**) A history of social isolation also elevated OFC PSD95, as determined by western blot, and in an independent cohort of mice, (**D**) immunostaining (*n*=6-8/group, with each mouse contributing a single value). Representative bands and images are adjacent. Scale=10 µm. Means+SEMs,*$p<0.05$.

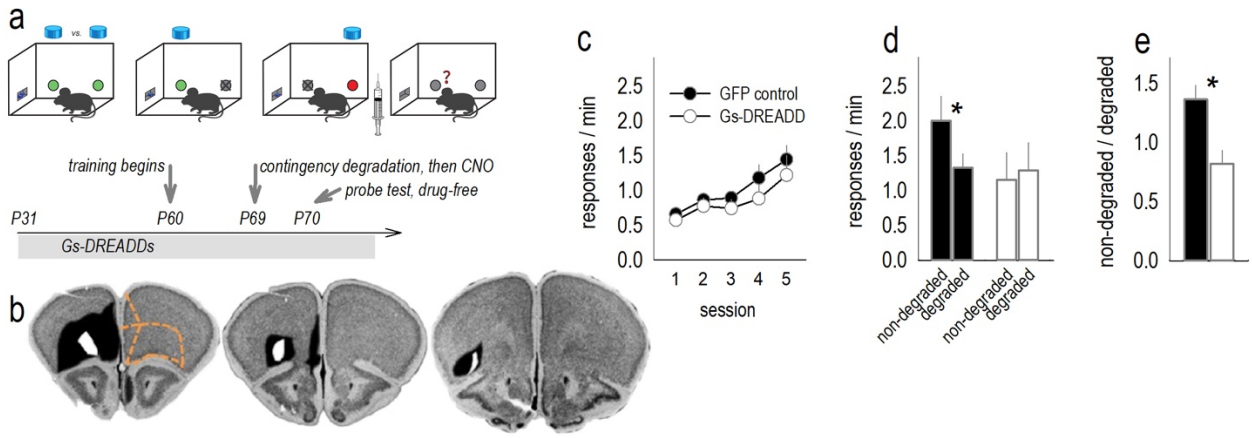

Figure 3-5: Artificial activation of the OFC causes failures in action-outcome response updating. (**A**) The task used here was identical to that used in figure 3-3. Experimental timeline: Mice were infused with either a Gs-DREADD-expressing viral vector or a control viral vector into the OFC. Mice were trained to nose poke for food reinforcers. Next, one action-outcome contingency was degraded. Immediately following, the presumed DREADDs ligand CNO was delivered to all mice. Response preferences were tested the next day, when mice were drug-free. (**B**) Histological representation of viral vector spread is represented at right on images from the Mouse Brain Library (Rosen et al., 2000), with black representing the largest spread and white the smallest. Orange lines on the rostral-most section outline the prelimbic cortex (medial, top), the medial OFC (medial, bottom), and the ventrolateral and lateral OFC. Viral vector spread was largest in rostral sections, and more selective to the ventrolateral OFC in more caudal sections. (**C**) Mice were trained to nose poke for food reinforcers, with no differences between groups. (**D**) Gs-DREADDs mice failed to update expectations, deferring to habitual behavior. (**E**) Response preferences were also calculated, with values >1 representing preference for the intact action-outcome contingency. Again, Gs-DREADD mice failed to prefer the intact contingency. One value per group was considered an outlier and excluded (n=12 control; n=8 DREADDs). Bars and symbols represent means+SEMs, *p<0.05.

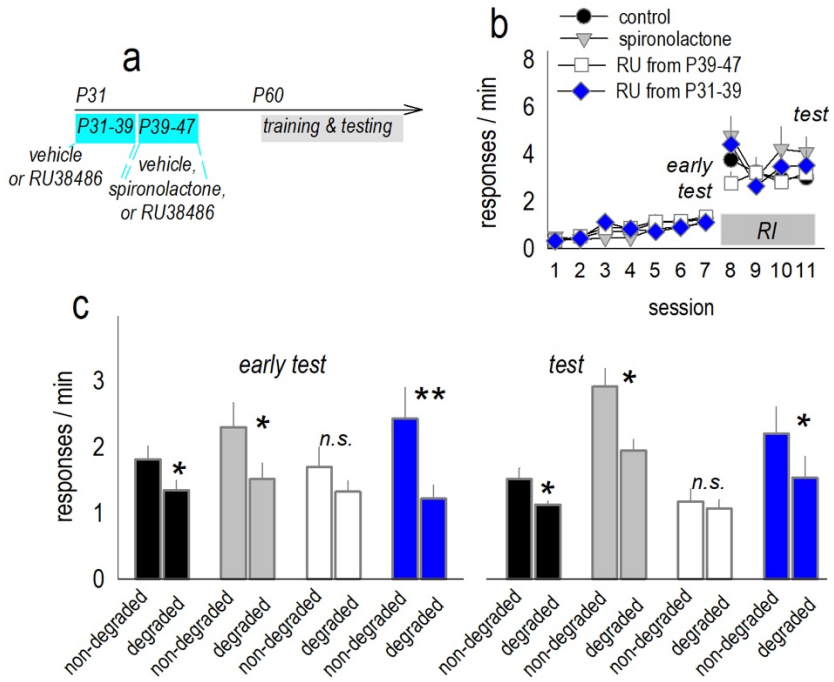

Figure 3-6: Identifying a sensitive period when GR binding in adolescence is necessary for response updating in adulthood. (**A**) Experimental timeline: To determine whether and when CORT insufficiency in adolescence was associated with response updating abnormalities in adulthood, typical, group-housed mice were treated with the GR antagonist RU38486 (RU) or the MR antagonist mifepristone during the same adolescent period when isolated mice experienced low CORT. A separate group of mice was treated with RU at an earlier period as a comparator. Mice were then tested for sensitivity to instrumental contingency degradation in adulthood, indicated by breaks in the response acquisition curve. (**B**) Groups did not differ during response training, however (**C**) mice exposed to the GR antagonist RU from P39-47 failed to update action-outcome response strategies, indicated by insensitivity to instrumental contingency degradation across 2 tests (n=15 total control; n=7-9/drug group). "RI" refers to random interval training. Means+SEMs,*p<0.05,**p<0.001.

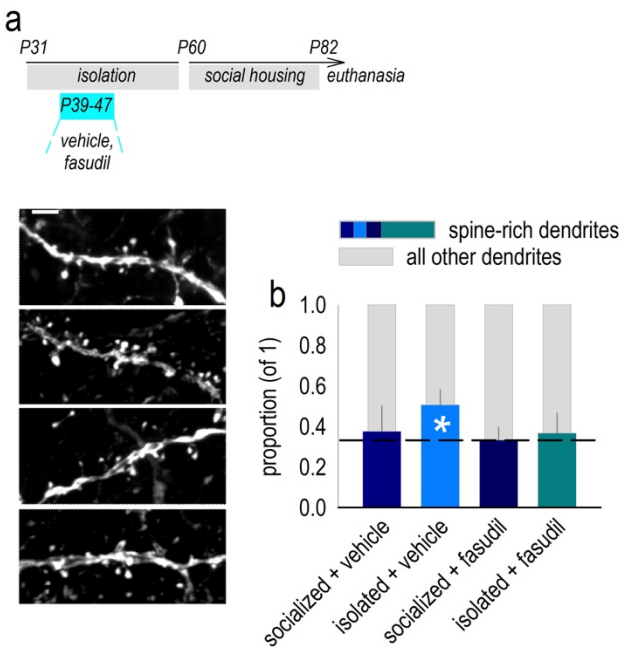

Figure 3-7: ROCK inhibition normalizes dendritic spine patterning in socially isolated mice. (**A**) Timeline: Mice were socially-housed or isolated in adolescence (P31-60) and treated (from P39-47) with vehicle or fasudil. Mice were euthanized at P82, corresponding with the timing of behavioral experiments. (**B**) In typical mice, the proportion of "spine-rich" dendrites – colored in darker, blue tones – is smaller than the rest of the dendrite population, constituting 1/3 of all dendrites (dashed line). In mice with a history of social isolation, the proportion of spine-rich dendrites increased, such that they made up roughly half of all dendrites. Fasudil *reinstated typical proportions*, such that spine-rich dendrites again made up a minority of dendrites. Fasudil in typical, healthy mice did not appear to affect these proportions. Representative dendrites are adjacent in the same order (n=6 mice/group, with each mouse contributing a single value). Scale=3 μm. Means+SEMs,*p<0.05 *vs.* one-third.

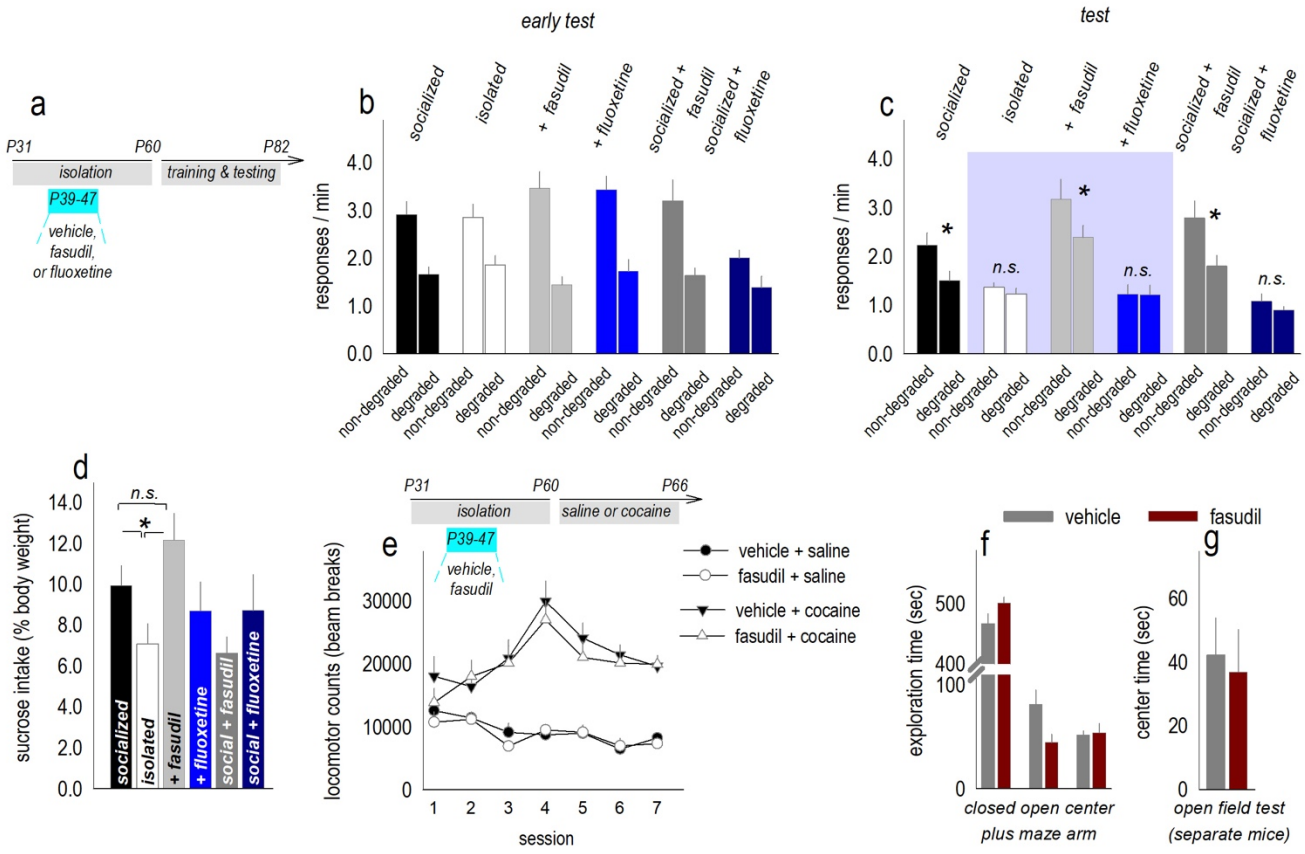

Figure 3-8: ROCK inhibition normalizes action-outcome response updating following social isolation. (**A**) Timeline: Mice were socially-housed or isolated in adolescence (P31-60) and treated (from P39-47) with vehicle, fasudil, or fluoxetine (FLX). (**B**) As adults, mice were trained to nose poke for food reinforcers, and as in prior figures, all mice were initially sensitive to action-outcome contingencies, inhibiting responding when it was unlikely to be reinforced (*n*=19 total group control; *n*=17 total isolation-housed control; other groups *n*=8-10). (**C**) Subsequently, adult mice exposed to isolation during adolescence failed to update action-outcome response strategies as expected ("test"). ROCK inhibition by fasudil rescued response strategies, while adolescent FLX *promoted* failures in response updating. Key comparisons are highlighted. (**D**) ROCK inhibition by fasudil also restored sucrose consumption in previously-isolated mice, an antidepressant-like effect, while FLX-treated mice differed from neither control nor previously-isolated mice (*n*=29 total group control; *n*=20 total isolation-housed control; other groups *n*=8-13). (**E**) In a separate series of experiments, fasudil

did not obviously impact the development of cocaine-induced locomotor sensitization (n=8/group; timeline above). (**F**) Exploration of an elevated plus maze and (**G**) open field were also not affected (n=7/group, tested in separate cohorts of mice). Means+SEMs,*p<0.05.

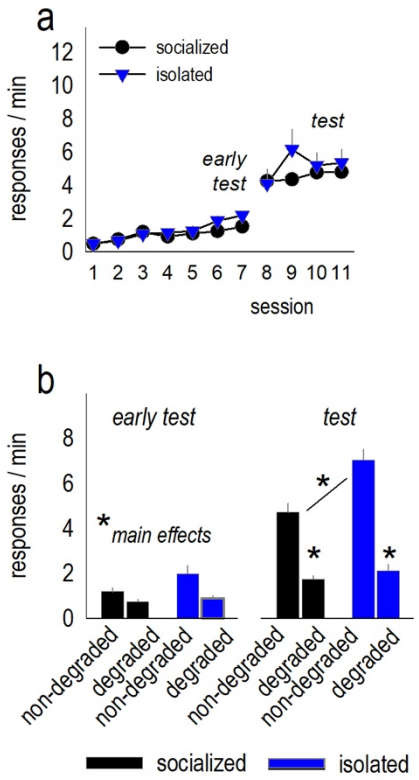

Figure 3-9: Intact action-outcome response updating in male C57BL/6 mice subject to social isolation. (**A**) As in females, socially isolated males acquired the nose poke responses. (**B**) Unlike in females, males were able to select actions based on the likelihood of reinforcement, despite social isolation (n=13 group; n=11 isolated). Means+SEMs,*p<0.05.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 ABSTRACT

While social isolation during development is a commonly used rodent model to emulate early-life social adversity, the majority of the findings to date result from experiments conducted while animals remain in a current state of isolation and therefore do not address long-term neurobehavioral consequences of isolation. In order to better address this question, animals can be re-housed into social groups, or "resocialized" prior to testing. Here, findings from studies implementing resocialization have been reviewed and synthesized with findings reported in Chapters 1-3. The following are recommendations meant to inform future experiments and include considerations to make when interpreting results of isolation-resocialization studies.

4.2 CONSIDERATIONS FOR FUTURE ISOLATION-RESOCIALIZATION PROTOCOLS

4.2.1 Adolescent social isolation timelines

The onset and duration of adolescent social isolation varies across studies reviewed here and could conceivably contribute to inconsistencies in reported outcomes. While the focus of this review is on long-term consequences of social isolation, prior literature reviews of acute effects of post-weaning isolation make this point particularly in terms of variations in isolation onset across studies (*e.g.* Lukkes et al., 2009b, Burke et al., 2017). For example, studies implementing an isolation timeline that initiates immediately after weaning and extends into adolescence are often criticized on the basis that this design precludes one from interpreting the effects due to isolation during the post-weaning period, isolation during adolescence, or both periods.

While studies may constrict the isolation period to adolescence alone, the adolescent period itself can be segmented into multiple developmental phases. Rather than abbreviating the isolation period to determine whether effects are due to early, middle, or late adolescent isolation, studies

often implement isolation periods that span these multiple developmental periods within adolescence. Again, these examples highlight critiques brought forth in prior literature reviews that discuss limitations worth noting when comparing results across studies. However, these prior critiques focus heavily on the parameters of adolescence and adolescent developmental periods governed in large part by age. Instead, how isolation overlaps with the timing of neurobiological maturation periods taking place throughout adolescence should be more carefully considered. Importantly, the timing of these trajectories that have been discussed in the context of the mPFC may not be generalizable to all regions or between subregions. For example, processes such as dendritic spine refinement and associated changes in levels of cytoskeletal regulator factors between the prefrontal cortical subregions occur at different timepoints during adolescence (Shapiro et al., 2017).

In addition to dendritic spine and synapse refinement, other prominent trajectories such as myelination have been reviewed here and should also be considered when designing isolation protocols. Again, instead of implementing isolation during specific adolescent stages (early, mid, late), it may be more informative to implement isolation specifically during the initiation, maintenance, and stabilization periods of these maturation events. In summary, I argue that the intersection of isolation with the timing of maturation periods within a region of interest should be more carefully considered than the intersection of isolation and a particular age category.

4.2.2 Resocialization may influence outcomes in a sex-dependent manner

The practice of resocialization itself may influence neurobehavioral outcomes in a sex-dependent manner. Housing of unfamiliar mature male rats (or mice) together can lead to aggressive encounters within the home cage and reduce huddling typically observed during the inactive period (Tulogdi et al., 2014). While certain aggressive behaviors in males attenuate within five days

following resocialization, previously-isolated males still exhibit more defensive behaviors in reaction to non-aggressive behavior of cage mates and environmental stimuli such as noises outside the home cage (Tulogdi et al., 2014). This consideration should be taken into account when interpreting results from experiments using male subjects – especially those in which neurobehavioral testing occurs immediately following resocialization (when aggressive encounters are likely frequent).

As a case in point, Leussis and Andersen (2008) reported greater effects of isolation on depressive-like behaviors in males compared to previously-isolated females, however testing was conducted within 24hrs of resocialization. In this case, it is unclear whether the difference in depressive-like behaviors represents an actual sex difference or discrepancies in home cage social environments. Considering the stressful nature of aggressive encounters present in males but not resocialized females (Lukkes et al., 2013), females might be better suited to certain isolation-resocialization investigations, depending on the purpose of the investigation.

Notably, short-term isolation is commonly used to evoke territoriality in male rodents and thereby facilitate aggression prior to resident-intruder encounters. Tulogdi et al., (2014) matched subjects in terms of levels of aggression exhibited by isolated males prior to re-housing in an attempt to control for aggression levels, but this investigation was the only study, to the best of our knowledge, to do so. Attempting to account for individual differences in aggression levels would likely benefit future investigations.

4.2.3 Post-isolation social environment

Cage composition following resocialization should also be considered when designing experiments and interpreting results. In terms of our own experiments using females (Hinton et al., 2019, in prep.; Chapters 2-3), we resocialized mice such that each cage contained equal or roughly equal numbers of previously-isolated and previously group-housed mice. However, many studies

have resocialized mice such that isolated mice are re-housed only with cage mates that were also exposed to prior isolation; meanwhile, control mice remain with familiar conspecifics. One obvious concern is that the acute stress associated with novel housing conditions is experienced only by previously-isolated mice.

Recently, cage composition effects have been explicitly studied in male subjects. Generally, re-housing isolated mice with previously-socialized mice facilitates the recovery of effects of isolation to a greater degree than when isolated mice are re-housed with other isolated mice (Table 1-1). For example, re-housing isolated mice with previously group-housed cage mates facilitates social exploration, mPFC myelin thickness, and mPFC mRNA expression of immediate early genes cFos, Arc, and Npas4 in response to social interaction (Makinodan et al., 2017). These findings further support the notion that balancing cage composition, or at least considering cage composition in experimental design, is critically important. Further studies are needed to determine whether the post-isolation social environment affects neurobehavioral outcomes in females, and the timeline within which these affects may subside — or not.

4.3 CONCLUDING REMARKS

In conclusion, the findings presented in this dissertation follow decades of preclinical social isolation research in rodents. These studies have attempted to investigate how neurobehavioral consequences of prior social isolation linger in adulthood. However, this prior research body largely excludes females and assesses outcomes while animals remain in a state of isolation, precluding an understanding of lasting consequences that are present despite correcting the social environment. The findings put forth in this dissertation implicate disrupted mPFC and OFC function in the enduring neuropsychiatric-related behavioral deficits present following a history of isolation. Disruption of ongoing maturational processes during adolescence and associated impairments in

mPFC- and OFC-related behaviors may constitute a mechanism by which social isolation experienced during adolescence becomes a risk factor for the occurrence of certain neuropsychiatric-related disorders in adulthood.

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