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Circuits and behavior in context: the role of learning and puberty in sculpting adolescent brain function

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Circuits and behavior in context: the role of learning and puberty in sculpting adolescent brain function

by

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Abstract

Throughout the lifespan, the brain is sculpted by experience and by the developmental processes that prepare an animal to face the challenges of each life stage. Adolescence and young adulthood represent critical times in which humans and other animals face the risks and challenges associated with independence. During these times of exploration, specific learning experiences may shape neurodevelopmental trajectories and ultimately alter life outcomes. In Chapter 2, we focus on the idea that specific learning experiences in young adulthood may promote resilience in the face of environmental risk factors. We demonstrate that a brief cognitive training intervention in young adulthood can promote long-term resilience to cocaine-seeking behavior in mice. In Chapters 3 and 4, we shift to the role of developmental processes, particularly the changes in hormonal milieu associated with puberty, in shaping frontal circuits and behaviors associated with these circuits. The frontal cortex matures during adolescence and is critical for the decision-making processes that shape life trajectories into adulthood. In particular, the dorsomedial region of frontal cortex integrates sensory, limbic, and reward-related inputs to influence a variety of cognitive and affective behaviors. In Chapter 3, we show that pubertal hormones drive maturation of inhibitory neurotransmission in dorsomedial frontal cortex, which may have implications for the regulation of plasticity and learning across adolescent development. We also show that early exposure to pubertal hormones can induce an early reduction in flexible learning on a task dependent on the integrity of the dorsomedial frontal cortex, further supporting the idea that pubertal hormones may regulate shifts in learning and decision-making across adolescence. Chapter 4 extends these findings to affective behaviors, testing the role of pubertal hormones in the maturation of anxiety- and depression-related behavior, which both depend on interactions between frontal cortex and subcortical limbic structures. We find that gonadal hormones regulate anxiety-related behavior in a sex-specific manner during puberty in mice, while depression-related behavior increases with age regardless of gonadal hormone status. Our

findings demonstrate that pubertal hormones regulate aspects of neural and behavioral maturation that may be critical for successfully navigating this vulnerable life stage, indicating a need for further work on the implications of hormonal perturbation during puberty in humans. As we connect these results with the data and ideas presented in Chapter 2, we discuss adolescence as a critical time in which interventions may alter neurodevelopmental trajectories to promote emotional resilience and adaptive decision-making.

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Chapter 1. Introduction

Across the lifespan, humans and other animals progress through distinct life stages that require distinct types of learning (Ellis et al., 2009; Ellison et al., 2012; Hochberg and Belsky, 2013; Spear, 2000). Learning experiences that occur during development or in adulthood can sculpt neural circuits in measurable ways, a process termed experience-dependent plasticity (Fu et al., 2012; Holtmaat and Svoboda, 2009; Johnson et al., 2016b; Yang et al., 2009). Developmental processes also sculpt the brain, with different circuitry maturing at different life stages, potentially facilitating specific forms of learning-induced plasticity during the life stage in which this learning is most appropriate (Johnson et al., 2016a; Roberts et al., 2010; Suleiman and Dahl, 2017). This process, by which developmental processes prime specific circuits for distinct types of learning during appropriate life stages, is termed experience-expectant plasticity (Fahrbach et al., 1998; Greenough et al., 1987; Suleiman and Dahl, 2017). For example, the frontal cortex matures during adolescence and young adulthood, with distinct components of frontal circuits following distinct developmental trajectories (Anderson et al., 1995; Gogtay et al., 2004; Johnson et al., 2016a; Lewis, 1997; O'Donnell et al., 2005).

Through its interactions with subcortical structures and other cortical regions, the frontal cortex facilitates many of the cognitive, affective, and reward-related behaviors necessary for a successful transition to independence during adolescence and young adulthood (Bush et al., 2000; Felix-Ortiz et al., 2016; Johnson and Wilbrecht, 2011; Margulies et al., 2007). While many frontal cortex-mediated behaviors are adaptive for this important life stage transition, affective and reward-related behaviors that shift during the adolescent period can also produce deleterious outcomes. For example, adolescence and young adulthood are the times of greatest risk for illicit drug use (Fig. 1) and onset of substance use disorders (Kessler et al., 2005; Paus et al., 2008). Adolescence is also a time of increased risk for anxiety and depression, particularly in girls (Costello et al., 2011; Hayward and Sanborn, 2002; Paus et al., 2008; Silberg et al., 1999). Thus,

the dynamic nature of frontal circuits during adolescence and young adulthood may confer both vulnerability and opportunity during these critical life stages, as developing circuits may be sensitive to the influence of learning experiences that produce adaptive or maladaptive trajectories.

Experience-dependent plasticity: learning experiences as interventions during developmental windows of vulnerability

Adolescence and young adulthood are periods of high risk for illicit drug use (Fig. 1) and the onset of substance use disorders (Kessler et al., 2005; Paus et al., 2008). The remodeling of frontal circuits during these life stages (Anderson et al., 1995; Johnson et al., 2016a; Lewis, 1997) may alter decision-making and reward-related behaviors in ways that confer vulnerability to maladaptive drug-related behaviors (Brenhouse et al., 2008; Chambers et al., 2003). However, the dynamic nature of frontal circuits during adolescence and young adulthood may also render these circuits ripe for intervention, as learning experiences during these time periods may alter long-term neurodevelopmental trajectories.

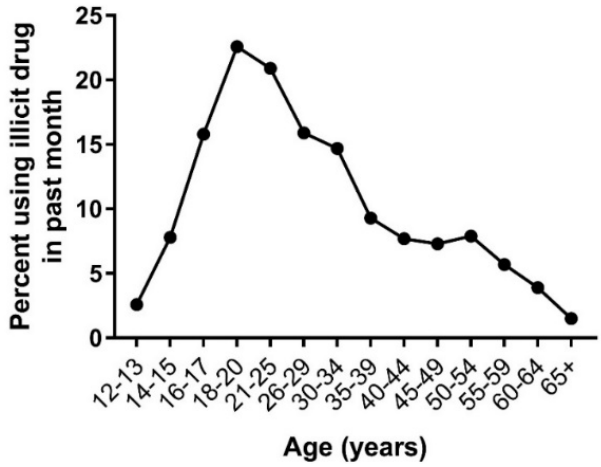

Figure 1. Risk of illicit drug use increases sharply during adolescence and young adulthood. Data obtained from the National Survey on Drug Use and Health (SAMHSA, 2014).

Learning experiences sculpt neural circuitry in both humans and animal models (Fig. 2; (Fu et al., 2012; Haut et al., 2010; Johnson et al., 2016b; Jolles et al., 2013; Mackey et al., 2013; Olesen et al., 2004; Subramaniam et al., 2012; Subramaniam et al., 2014; Takeuchi et al., 2010; Yang et al., 2009)). During periods of vulnerability to maladaptive behavior and psychiatric disease, this capacity for learning-induced plasticity may be leveraged through interventions that promote resilience (Bickel et al., 2014; Suleiman and Dahl, 2017; Verdejo-Garcia, 2016). In

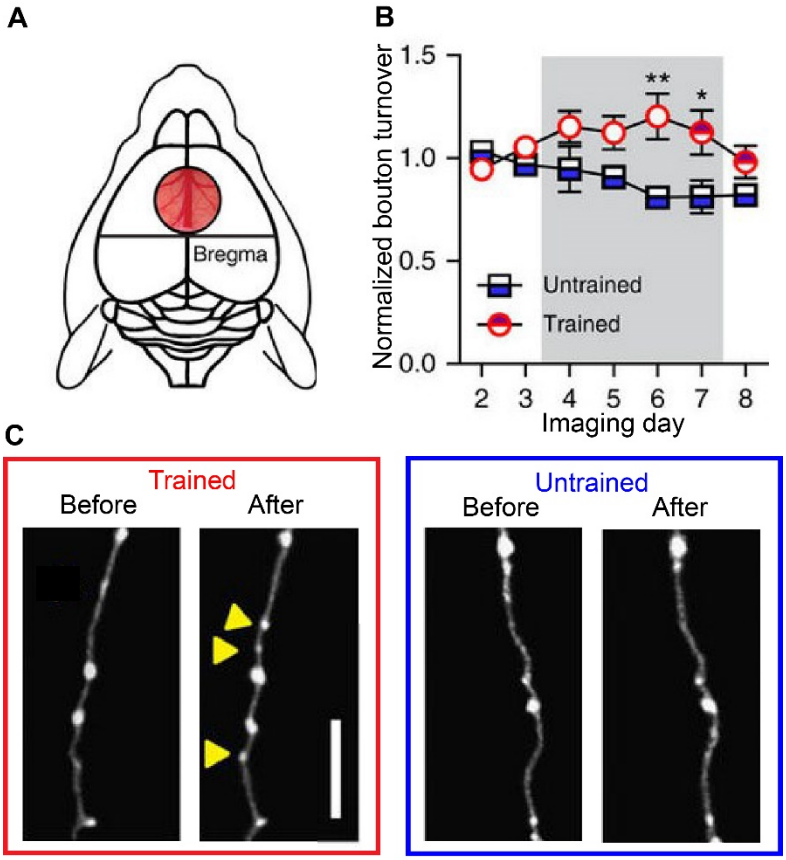

Figure 2. Learning experiences in young adulthood can sculpt frontal circuits. (A) Schematic of region imaged in mouse frontal cortex. (B) Turnover of boutons in projections from OFC to dorsomedial frontal cortex before and during a period of rule learning. (C) Sample boutons, with yellow arrows indicating new boutons gained after the first day of training. Scale bar represents 10μm. Adapted from (Johnson et al., 2016b).

Chapter 2, we test the effects of a cognitive training intervention during young adulthood, a time of vulnerability to illicit drug use (Fig. 1) and the onset of substance use disorders (Kessler et al., 2005), in a mouse model of cocaine-seeking behavior. We demonstrate that cognitive training in young adulthood can produce long-term reductions in cocaine conditioned place preference in mice, suggesting that specific types of learning can alter neurodevelopmental trajectories to promote resilience.

Experience-expectant plasticity in frontal cortex: puberty as an inflection point in the maturation of multiple systems in frontal cortex

Adolescence is a time of dynamic remodeling in frontal cortex (Anderson et al., 1995; Johnson et al., 2016a). The onset of puberty coincides with dramatic changes in multiple components of frontal circuits in non-human primates (Fig. 3; (Lewis, 1997)), and pubertal development correlates with some measures of cortical maturation in humans (Herting et al., 2015; Herting et al., 2014; Herting et al., 2012; Peper et al., 2009; Peper et al., 2008). However, it is unknown whether pubertal changes in gonadal hormone exposure are causally related to

frontal circuit maturation. The age of puberty is advancing to increasingly younger ages in girls in developed nations (Aksglaede et al., 2009; Herman-Giddens, 2006), and girls who experience puberty earlier than their peers show greater risk of negative educational and mental health outcomes (Graber, 2013). Thus, it is critically important to understand the role of pubertal hormones in circuit maturation. We therefore probed the effects of pubertal hormones on the maturation of frontal circuits during adolescence in mice. We demonstrate in Chapter 3 that gonadal hormones drive maturation of inhibitory neurotransmission in the frontal cortex at puberty onset in female mice and that earlier exposure to pubertal hormones can induce precocious adult-like performance on a frontal cortex-dependent task. Given the known role of inhibitory neurotransmission in regulating critical periods of plasticity in sensory regions of cortex (de Villers-Sidani and Merzenich, 2011; Southwell et al., 2010; Werker and Hensch, 2015), our results suggest that pubertal hormones may regulate transitions in learning and plasticity across adolescence. These findings raise concerns about the neurodevelopmental implications of the increasingly younger age of puberty in girls.

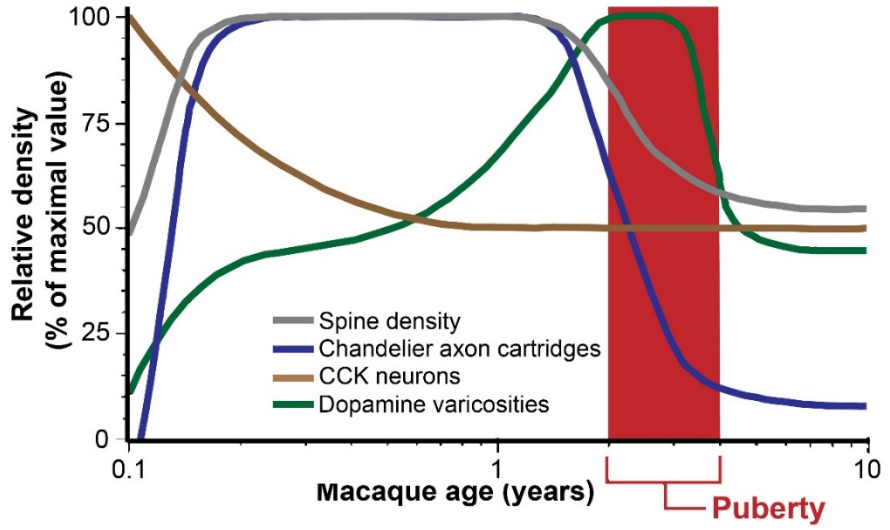

Figure 3. The frontal cortex matures during puberty, with multiple components of frontal circuits following distinct developmental trajectories. Figure adapted from Lewis, 1997. Data shown are from layer 3 of frontal cortex in rhesus macaques.

In addition to regulating learning and reward-related behaviors, frontal cortex interacts with subcortical limbic structures to influence anxiety- and depression-related behavior (Felix-Ortiz et al., 2016). Risk of anxiety and depression increases sharply at puberty onset, particularly in girls (Fig. 4; (Costello et al., 2011; Hayward and Sanborn, 2002; SAMHSA, 2012; Silberg et al., 1999)),

with girls who experience puberty earlier than their peers showing the highest rates of anxiety and depression (Graber, 2013). In humans, it is difficult to disentangle potential direct effects of pubertal hormones from the social and environmental factors that interact with pubertal development to influence mental health. Rodent models have demonstrated effects of gonadal hormones on anxiety- and depression-related behavior in adulthood, but little is known about the effects of these hormones during puberty. We therefore tested the effects of gonadal hormones on anxiety- and depression-related behavior during puberty in male and female mice. In Chapter 4, we demonstrate that in mice, depression-related behavior matures with age independent of gonadal hormone status, while anxiety-related behavior responds to gonadal hormone manipulation in a sex-specific manner during puberty.

Figure 4. Depression symptoms and diagnoses increase after puberty onset, particularly in girls. (A) Adapted from Silberg et al., 1999. (B) Data obtained from the National Survey on Drug Use and Health (SAMHSA, 2012).

Adolescence and young adulthood as windows of vulnerability and opportunity

The experiments described above focus on adolescence and young adulthood in part because the decisions animals make during these life stages can permanently influence their life outcomes. Rodents undertake substantial risks as they disperse to new territories and balance the need to forage while also avoiding predation, and humans make social, romantic, and career decisions that set the course of their adult lives. The dynamic nature of frontal circuits during these

life stages, including long-range projections known to underlie behaviors relevant to psychiatric disease (Felix-Ortiz et al., 2016; Johnson et al., 2016a), may render these circuits particularly susceptible to sculpting by experience. Adverse experience during adolescence is associated with alterations in frontal cortex structure, and adolescence and young adulthood are the times of greatest vulnerability to the onset of psychiatric disease and substance use disorders (Andersen et al., 2008; Kessler et al., 2005; Paus et al., 2008). However, the dynamic nature of frontal circuits during adolescence and young adulthood may also render these life stages windows of opportunity for intervention to improve mental health and promote resilience in the face of environmental risk factors (Crone and Dahl, 2012; Suleiman and Dahl, 2017). It is therefore critical to understand the developmental and experience-dependent factors that sculpt frontal circuits and influence decision-making during adolescence and young adulthood, as these factors may be leveraged to promote resilience.

Chapter 2. Brief cognitive training interventions in young adulthood promote long-term resilience to drug-seeking behavior

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Introduction

Environmental conditions of deprivation and stress confer vulnerability to substance use disorders in humans and drug-seeking behavior in animal models (Buu et al., 2009; Enoch, 2011; Gordon, 2002; Lu et al., 2003; Meaney et al., 2002; Nader et al., 2012; Pilowsky and Wu, 2006; Sinha, 2008). In contrast, experiencing control over one's environment can buffer the effects of adversity, shaping neural circuitry in ways that promote resilience to future challenges and reduce later responses to drugs of abuse (Amat et al., 2010; Amat et al., 2008; Amat et al., 2006; Christianson et al., 2008; Kubala et al., 2012; Rozeske et al., 2012; Rozeske et al., 2011; Varela et al., 2012). Interventions that provide a sense of mastery, stability and control over one's environment may therefore promote resilience to substance use disorders.

Cognitive training that allows subjects to engage in active learning processes, forming and testing hypotheses about their environment based on feedback they receive (Gureckis and Markant, 2012; Lagnado and Sloman, 2004; Markant and Gureckis, 2014; Sobel and Kushnir, 2006), may serve as an effective intervention for providing a sense of control and stability in the face of environmental stress or deprivation. Relatively brief periods of cognitive training have been shown to shape neural circuitry in both humans and animal models (Fig. 2; (Haut et al., 2010;

Johnson et al., 2016b; Jolles et al., 2013; Mackey et al., 2013; Olesen et al., 2004; Subramaniam et al., 2012; Subramaniam et al., 2014; Takeuchi et al., 2010)). We hypothesized that these changes in neural circuitry may influence animals' responses to future challenges. In particular, we hypothesized that a brief cognitive training intervention could promote resilience to drug-seeking behavior in mice raised in conditions of relative deprivation.

When rodents are removed from standard laboratory housing in which they have access to *ad libitum* food and water but have little opportunity for exploration and are placed in an enriched environment with greater cage size, playmates and toys, this enrichment has been shown to reduce vulnerability to drug-seeking behavior (Chauvet et al., 2012; Chauvet et al., 2009; Solinas et al., 2008; Solinas et al., 2009; Thiel et al., 2009). Upon returning to standard housing, however, animals with a history of enrichment show exacerbation of drug-seeking behavior (Nader et al., 2012) or no change in drug-seeking behavior (Chauvet et al., 2012) compared to animals with no history of enrichment. Permanent exposure to environmental enrichment is difficult to implement as a clinical intervention. We therefore tested whether a brief cognitive training intervention could promote long-term resilience to drug-seeking behavior even after animals returned to the relative deprivation of standard laboratory housing. We tested this intervention in young adult mice, as late adolescence and young adulthood are the times of greatest vulnerability to the onset of substance use disorders in humans (Kessler et al., 2005).

We employed a cognitive training paradigm in which mice learned arbitrary associations through trial and error, discriminating among multiple sensory stimuli in order to recover a buried food reward. Each trained mouse was paired with one yoked mouse, which explored an adjacent arena and received a food reward each time the trained mouse earned a food reward. An additional group of mice remained in their home cages without cognitive training or food rewards, experiencing the relative deprivation of standard housing. Importantly, all groups of mice remained in their home cages for 4 weeks after cessation of cognitive training before cocaine exposure, allowing us to test the long-term effects of cognitive training on drug-seeking behavior

after animals returned to standard housing. After this 4-week 'rest' period, mice underwent a cocaine conditioned place preference (CPP) protocol, which provided a measure of drug-seeking behavior. In our first experiment, we examined maintenance of drug-seeking behavior with weekly exposure to the CPP testing arena. In our second experiment, we examined extinction of drug-seeking behavior using daily exposure to the CPP testing arena (Mueller and Stewart, 2000).

All groups developed comparable preference for the cocaine paired chamber on the first CPP test day, but their behavior diverged with repeated exposure to the cocaine paired chamber. We found that cognitive training showed protective effects above and beyond the yoked control condition in weekly tests of maintenance of preference. In tests of extinction driven by daily exposure to the chamber, we found that all trained and yoked groups that left standard housing for an arena and reward showed significant extinction of cocaine CPP, while cage mates that were kept in standard housing for the same period did not extinguish CPP. These data suggest that in early adulthood, deprivation may confer vulnerability and that brief interventions may provide resilience to later substance abuse.

Methods

Experimental subjects

74 adult male C57BL/6 mice (Charles River Laboratories, bred in-house) were weaned at postnatal day (P)21 and housed with littermates in groups of 2 to 5. Cages contained plastic domes and nesting material and were kept on a reverse 12hr light/dark schedule. Mice were food restricted to 85-90% of their *ad libitum* body weights for 2 weeks during cognitive training but had *ad libitum* access to food and water at all other times. All animal procedures were approved by the Ernest Gallo Clinic and Research Center Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drugs

All subjects received the same drug exposure. Cocaine HCl (Sigma-Aldrich) (10mg/kg) and saline (in equivalent volume) were administered via intraperitoneal (i.p.) injection on alternating days for 8 days.

Procedures for Experiment 1: Effects of cognitive training on weekly CPP maintenance

Training groups

Littermates housed together were divided into 3 groups: trained, yoked to trained (YT), and standard housed (SH). Trained animals underwent the 9-day cognitive training protocol described below and in Fig. 5, while YT littermates explored separate training arenas and received cereal rewards on a schedule yoked to that of trained animals. SH animals were food restricted to 85-90% of their *ad libitum* body weight during the cognitive training period but did not receive any training or exposure to the training arena.

Cognitive training protocol

Mice were ages P55-P77 at the start of cognitive training. During training, mice learned to dig for cereal rewards (Honey Nut Cheerio pieces, General Mills, Minneapolis, MN) using odors, textures, and location as cues. The apparatus and task design exploited mice's natural foraging abilities to enable rapid learning of the task (Birrell and Brown, 2000; Johnson and Wilbrecht, 2011). The general training method and apparatus details are outlined in Johnson and Wilbrecht (2011) and are described more briefly here.

The apparatus included a start compartment, in which the mouse waited between trials, and digging compartments. Each digging compartment contained a digging pot filled with scented wood shavings (Hartz Mountain Corporation, Secaucus, NJ) and covered in a distinct texture.

Only one digging pot contained an accessible cereal reward (approximately 10mg) in each trial,

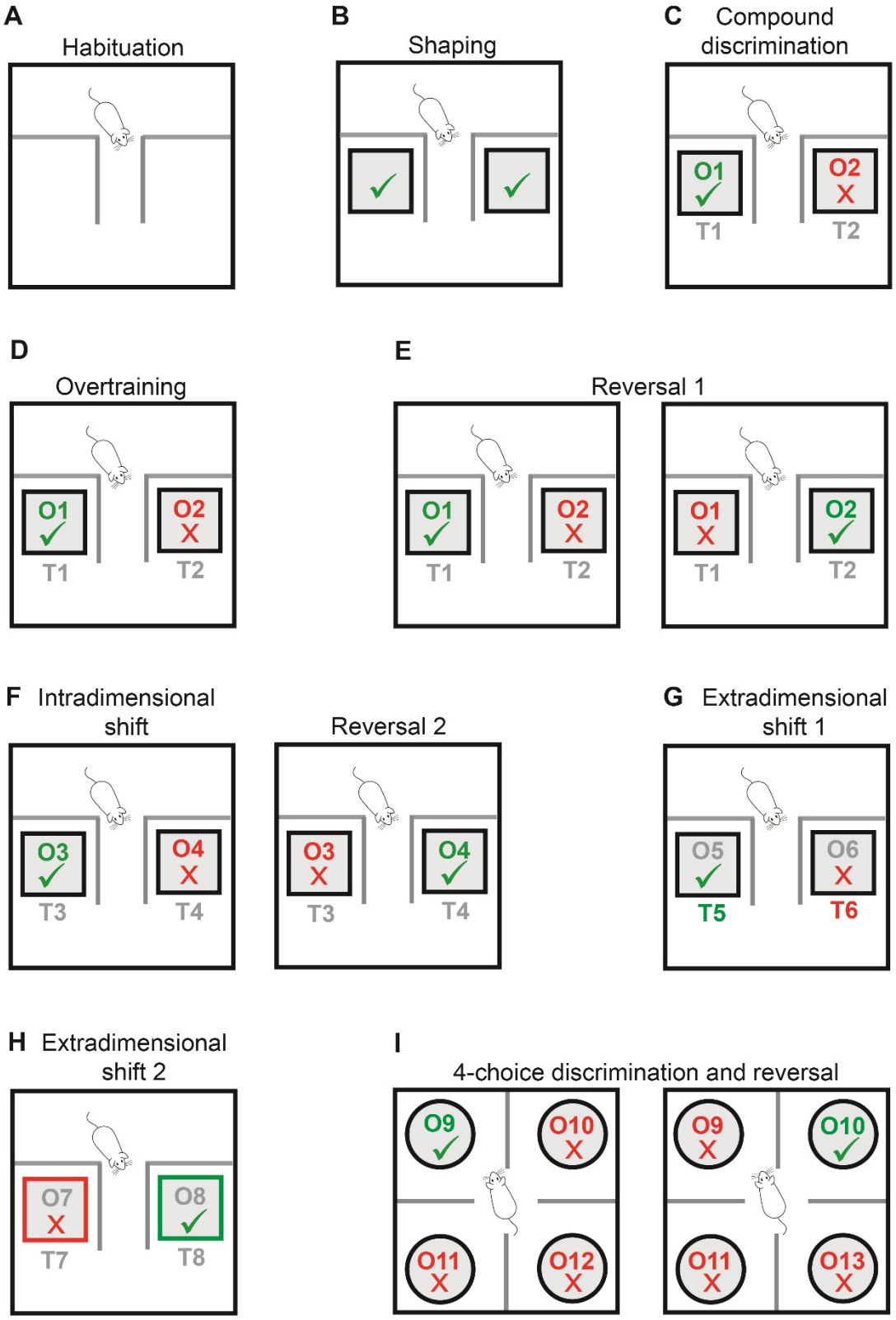


Figure 5. Schematic of cognitive training paradigm. Each subfigure (A-I) shows 1 day of training, with 9 total days. The mouse is shown in the start compartment of the training arena. Digging pots are shown by filled rectangles (B-H) or filled circles (I). The digging pot containing an accessible cereal reward is indicated by a check mark and green color on the relevant exemplar, while incorrect choices are indicated by an "x" and red color on the relevant exemplar. Irrelevant exemplars are shown in gray. O1-13 refer to the 13 odors used to scent the digging medium; T1-8 refer to the 8 textures used to cover the digging pots. To control for odor, all texture pairs were of the same material with discriminable textures on opposing sides (e.g. sand paper, reverse sandpaper). The location of each odor/texture combination was pseudorandomized between trials. (A) Day 1 (habituation): mice were exposed to cereal pieces in the arena. (B) Day 2 (shaping): mice learned to dig in wood shavings for cereal rewards. (C) Day 3 (compound discrimination): 1 of 2 odors was rewarded, while 2 textures were irrelevant (20 trials). (D) Day 4 (overtraining): the same rule used on Day 3 was repeated for 30 additional trials. (E) Day 5 (reversal 1): the same odors/textures were used as on days 3 and 4. The rule used on days 3 and 4 was repeated for the first 10 trials, after which the opposite odor was rewarded for 40 trials. (F) Day 6 (intradimensional shift and reversal 2): for the first 20 trials, an odor-based rule was employed with 2 novel odors and 2 novel textures. For last 35 trials, the opposite odor was rewarded. (G) Day 7 (extradimensional shift, texture): a texture-based rule was employed with 2 novel odors and 2 novel textures (30 trials). (H) Day 8 (extradimensional shift, spatial): a spatial rule was employed with 2 novel odors and 2 novel textures. One location (i.e. the digging pot on the left side) was rewarded, regardless of odor and texture (25 trials). (I) Day 9 (4-choice discrimination and reversal 3): 4 novel odors were used. One odor was rewarded for the first 20 trials, and a different odor was rewarded for the last 25 trials.

though all digging pots were pseudobaited with a whole Cheerio secured beneath a mesh screen on the bottom of the pot.

The odors used to scent the wood shavings (Table 1 and Table 2) were purchased from Alfa Aesar (Ward Hill, MA), San Francisco Massage Supply Co. (San Francisco, CA), and McCormick & Company (Sparks, MD). The scented liquids were diluted 1:10 in mineral oil or 50% ethanol and mixed with wood shavings at a concentration of 0.02ml/g. The textures used to cover the acrylic digging pots (Table 1) were purchased from local discount stores.

Each trial began with opening of the start gate, after which the mouse was allowed to explore freely but only dig in one pot per trial. Digging was defined as moving the wood shavings with both front paws. If a correct choice was made, the mouse was allowed to consume the cereal reward before being guided back to the start compartment. If an incorrect choice was made, the mouse was guided back to the start compartment. If the mouse did not dig within three minutes,

Training phase	Odor A	Odor B	Texture A	Texture B	Rewarded exemplar
Compound discrimination	Vanilla	Cinnamon	Velvet	Reverse velvet	Vanilla
Overtraining	Vanilla	Cinnamon	Velvet	Reverse velvet	Vanilla
Reversal 1	Vanilla	Cinnamon	Velvet	Reverse velvet	Cinnamon
Intradimensional shift	Almond	Coconut	Diaper	Reverse diaper	Almond
Reversal 2	Almond	Coconut	Diaper	Reverse diaper	Coconut
Extradimensional shift (texture)	Lemongrass	Orange	Sandpaper	Reverse sandpaper	Sandpaper
Extradimensional shift (spatial)	Lavandin	Wintergreen	Pleather	Reverse pleather	Left side

Table 1. Exemplar combinations used during the 2-choice phase of training.

the trial was terminated. The number of trials per session was held constant for all mice independent of performance. Trial number was set for each phase (see Fig. 5 caption) based on previous experiments and approximated the average number of trials that P60 male mice take to make 8/10 correct choices (Johnson and Wilbrecht, 2011). The 9-day training protocol proceeded as shown in Fig. 5 and included 3 rule reversals, 2 extradimensional shifts, and 1 intradimensional shift. Reversals and set-shifts were included in the training in order to engage executive functions such as cognitive flexibility and inhibitory control of behavior (Birrell and Brown, 2000; Johnson and Wilbrecht, 2011; Kim and Ragozzino, 2005; McAlonan and Brown, 2003). The specific odor/texture combinations used in each phase of training are listed in Table 1 and Table 2.

For the yoked control condition (i.e. YT mice), each YT mouse was paired with one trained mouse and was placed in a separate training arena during the trained mouse's sessions. Each time the trained mouse received a cereal reward, a comparable cereal reward was dropped into the arena of the YT mouse.

Training phase	Odor A	Odor B	Odor C	Odor D	Rewarded odor
4-choice discrimination	Anise	Clove	Thyme	Litsea	Anise
Reversal 3	Anise	Clove	Eucalyptus	Thyme	Clove

Table 2. Exemplar combinations used during the 4-choice phase of training.

Measures of cognitive training performance

We calculated an overall performance score for each mouse using the fraction of trials resulting in a correct choice (correct trials/total trials) during sessions that involved rule changes (i.e. all sessions except compound discrimination and overtraining). The fraction of trials resulting in a correct choice was calculated for each session and then averaged across sessions. Performance scores (correct trials/total trials) were also calculated specifically for reversals (reversal 1, reversal 2, and reversal 3), extradimensional shifts (texture and spatial), and intradimensional shift (see Fig. 5).

To measure the mice's ability to inhibit responding to a previously rewarded cue, we measured perseverative errors, defined as errors before the first rewarded trial during reversal sessions (reversal 1, reversal 2, and reversal 3). To measure the mice's ability to adapt to a new contingency, we measured regressive errors, defined as errors after the first rewarded trial in any session involving a rule change (i.e. all sessions except compound discrimination and overtraining). Perseverative and regressive error indices were calculated as the fraction of trials resulting in each error type.

Conditioned Place Preference

Cocaine conditioning began 4 weeks after completion of cognitive training (Fig. 8A) and took place during the animals' dark cycle. The conditioned place preference (CPP) apparatus consisted of a plexiglass open field (Med Associates) divided into two chambers (27cm x 13cm x 20cm). The two chambers were distinguished by visual cues (horizontal vs. vertical bars on wall) and floor texture (pebbled vs. square pattern). Mouse movement was monitored by infrared beam breaks (Activity Monitor, Med Associates). Each animal was habituated to handling for 3 days

prior to the start of the experiment. Habituation and conditioning took place over 9 weekdays, with the first CPP test day on the 10th weekday.

Habituation. Mice were allowed to explore both chambers for 20 minutes, and their baseline preference for one of the two chambers was measured.

Conditioning. Mice received cocaine (10mg/kg) and saline injections on alternate days for 8 weekdays (4 injections of each drug). Immediately after the injections, mice were placed in one of the two chambers for 15 minutes with no access to the other side. Mice received saline in the initially preferred chamber (determined on habituation day) and cocaine in the non-preferred chamber.

CPP testing. Mice were next tested for chamber preference on post-conditioning days 1, 7, 14, 21, and 28, with day 1 being the first day after completion of cocaine/saline conditioning. We chose a weekly testing schedule based on previous literature showing that infrequent exposure to the drug-associated context can maintain CPP over long time periods in rodents (Mueller et al., 2002; Mueller and Stewart, 2000; Solinas et al., 2008). The weekly schedule allowed us to test long-term effects of cognitive training under conditions in which control animals would be expected to maintain cocaine CPP.

On each test day, mice received mock injections (i.e. handling with no needle puncture) and were allowed to freely explore either chamber for 20 minutes. CPP values were calculated as the number of seconds spent in the cocaine-paired chamber minus the number of seconds spent in the saline-paired chamber. All CPP values were normalized to habituation preference by subtracting each mouse's CPP score on habituation day from its own CPP values on all other days. Mice that failed to achieve normalized CPP levels of 100 seconds on test day 1 were excluded from the study (1 trained mouse, 3 YT mice, and 1 SH mouse) due to questions about the success of i.p. injections in these mice. Mice with CPP less than 100 seconds on test day 1 were excluded from all data analysis, including analysis of data collected before CPP was measured (i.e. training performance, locomotor sensitization, and habituation to CPP chambers).

Procedures for Experiment 2: Effects of cognitive training on daily CPP extinction

Cognitive training

Mice were divided into 4 groups: trained, single rule trained (SRT), yoked to single rule trained (YS), and standard housed (SH) (Fig. 6). Trained mice underwent the same 9-day training protocol described above (Fig. 5; Fig. 6D). SRT mice completed the first 3 days of training in the

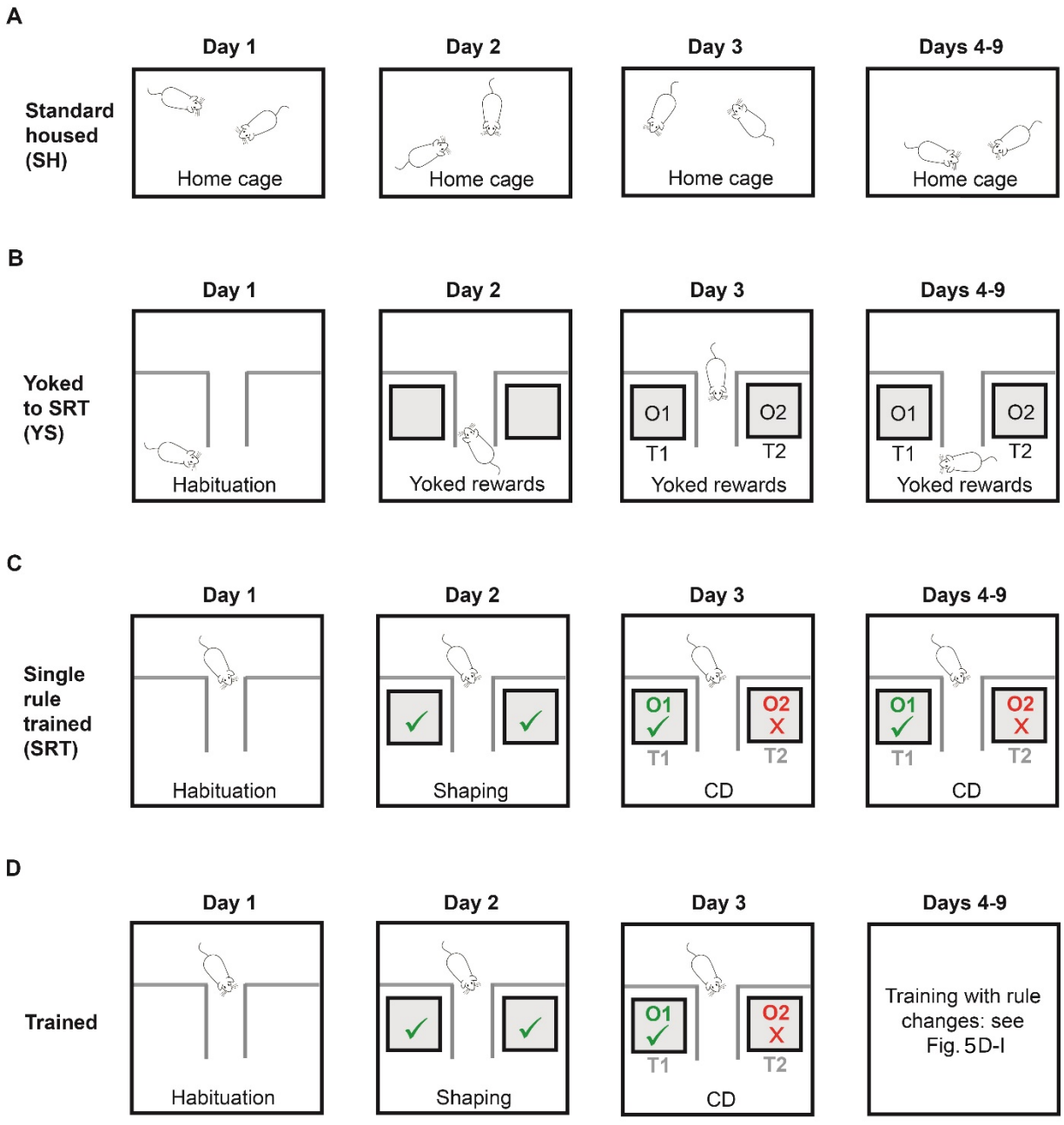


Figure 6. Schematic of experimental groups for Experiment 2. Mice are shown in the home cage (A) or in a training arena (B-D). Digging pots containing scented bedding are shown by filled rectangles. The digging pot containing an accessible cereal reward is indicated by a check mark and green color on the relevant exemplar, while incorrect choices are indicated by an "x" and red color on the relevant exemplar. Irrelevant exemplars are shown in gray. O1 and O2 refer to the 2 odors used to scent the digging medium; T1 and T2 refer to the 2 textures used to cover the digging pots. To control for odor, texture pairs were of the same material with discriminable textures on opposing sides (e.g. velvet, reverse velvet). (A) Standard Housed (SH) mice remained in their home cages with no training or cereal rewards. (B) Each yoked mouse was paired with one mouse that underwent single rule training (SRT). The yoked mouse explored an identical arena adjacent to the SRT mouse and received a cereal reward each time the SRT mouse earned a cereal reward. (C) On days 1-3 of training, single rule trained (SRT) mice underwent habituation, shaping, and compound discrimination (CD) as described in Fig. 5A-C. On days 4-9 of training, SRT mice repeated the compound discrimination task with no rule changes. The location of each odor/texture combination was pseudorandomized between trials. (D) Trained mice underwent the same procedures shown in Fig. 5A-I, including set-shifts and reversals. The location of each odor/texture combination was pseudorandomized between trials.

same manner as trained mice (Fig. 5A-C; Fig. 6C). SRT mice then repeated the compound discrimination session on days 4-9 rather than undergoing set-shifts and reversals (Fig. 6C).

Yoked mice were paired with SRT mice rather than trained mice in order to match the group with the highest number of cereal rewards. Each yoked mouse was paired with one SRT mouse and was placed in a separate training arena during the SRT mouse's sessions (Fig. 6B). Each time the SRT mouse received a cereal reward, a comparable cereal reward was dropped into the arena of the yoked mouse (Fig. 6B). SH mice were food restricted to 85-90% of their *ad libitum* body weight during the cognitive training period but did not receive any training or exposure to the training arena (Fig. 6A).

CPP

CPP habituation, conditioning and testing procedures for Experiment 2 were exactly the same as those for Experiment 1, with the exception that mice were tested for CPP daily on post-conditioning days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11. In contrast to the weekly schedule used in

Experiment 1, this schedule was designed to promote extinction of CPP (Mueller and Stewart, 2000).

On post-conditioning day 12, mice in Experiment 2 underwent a reinstatement session, in which they received a 5mg/kg priming injection of cocaine (intraperitoneal) followed by a 20-minute test for chamber preference.

Statistics

For analysis of CPP and locomotion, 2-way repeated measures ANOVAs were performed using time and training as factors. Post-hoc comparisons were performed using Holm-Sidak corrections for multiple comparisons. For analysis of reinstatement data in Experiment 2, a 1-way ANOVA was performed with Holm-Sidak post-hoc comparisons among the groups. For correlations between training performance and CPP levels, the slope coefficient of a linear regression curve fitted to CPP from days 1-14 (the time period in which we observed a difference between trained and yoked animals) was used as a measure of CPP for each mouse. Pearson correlations were performed between training performance and CPP slope coefficients; a non-parametric Spearman correlation was used for one dataset determined to have a non-normal distribution by a D'Agostino-Pearson omnibus test. Analysis and graphing were performed using GraphPad Prism (GraphPad, San Diego, CA).

Results

Experiment 1: Effects of cognitive training on weekly maintenance of cocaine CPP

Cognitive training performance

To test the effects of cognitive training on maintenance of cocaine CPP, we first trained mice on a 9-day cognitive training paradigm in which the mice employed odor-based, texture-

based, and spatial rules while digging in scented bedding for food rewards (Fig. 5 and Tables 1-2). The training included three reversals, one intradimensional shift, and two extradimensional shifts. As a control, yoked littermate mice explored identical training arenas while receiving cereal rewards at the same times as trained animals. Standard housed (SH) mice were food restricted in the same manner as trained and yoked mice but did not receive any training or exposure to the training arena. Cognitive training performance, as measured by the fraction of trials resulting in a correct choice in each phase of training, is shown in Fig. 7.

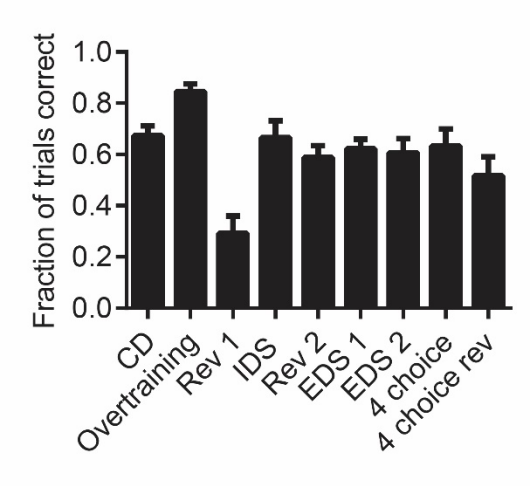


Figure 7. Performance during cognitive training. Fraction of trials resulting in a correct choice across training sessions in Experiment 1. N=12 trained mice. CD is compound discrimination; IDS is intradimensional shift; EDS is extradimensional shift; Rev is reversal. Bars represent means; error bars represent SEM.

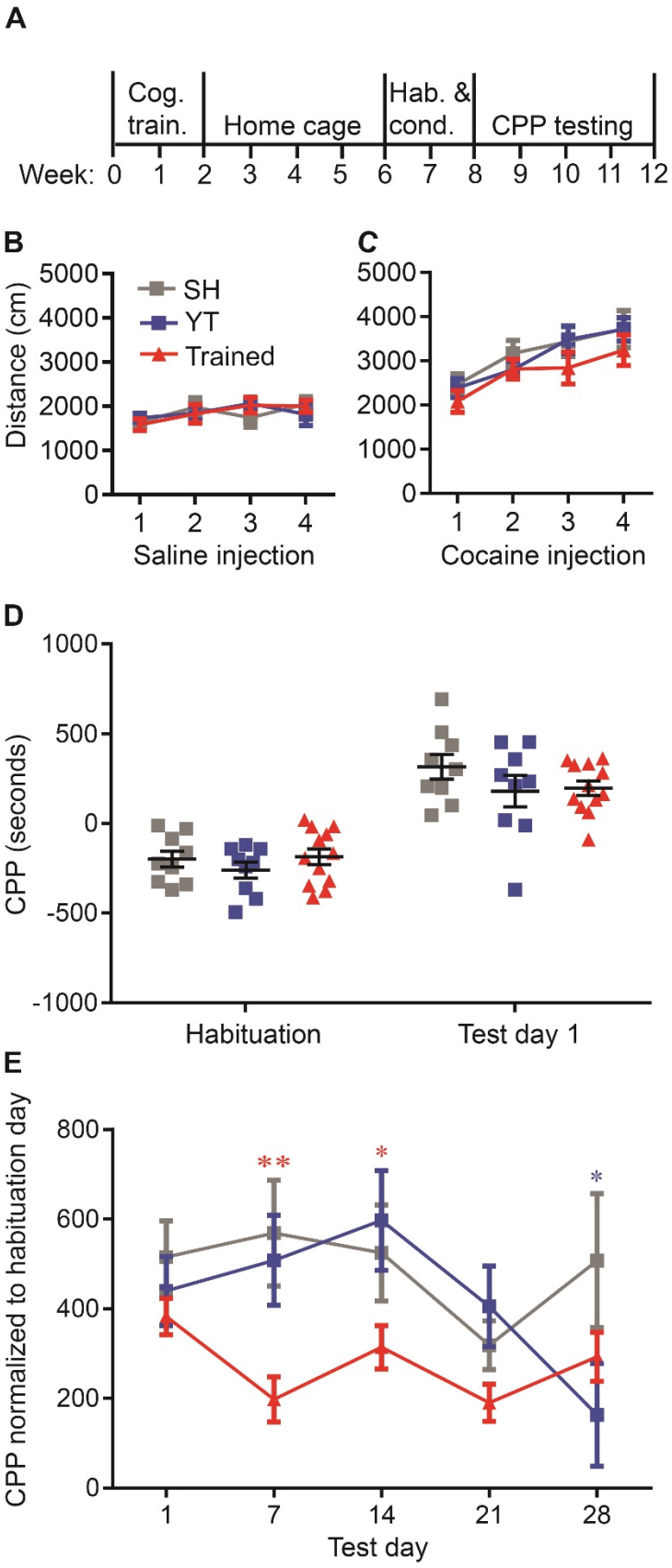


Figure 8. Cognitive training reduces maintenance of cocaine CPP without altering locomotor sensitization or initial CPP levels. (A) Experiment timeline. "Cog. train." is cognitive training, "Hab. & cond." is habituation and conditioning (i.e. habituation to CPP chambers followed by cocaine conditioning, see Methods). Mice were ages P55-P77 at the start of cognitive training (day 0 on the timeline). (B) Cognitive training did not alter locomotor activity recorded during 15-minute sessions after saline injections. N=11 trained mice (1 mouse excluded due to equipment failure), N=9 yoked to trained (YT) mice, N=9 standard housed (SH) mice. (C) Cognitive training did not alter locomotor activity recorded during 15-minute sessions after cocaine injections. All groups showed increased locomotor activity across successive cocaine injections, consistent with sensitization. N=12 trained mice, N=9 YT mice, N=9 SH mice. (D) Cognitive training did not alter the initial establishment of cocaine CPP, as measured by seconds in the cocaine-paired chamber minus seconds in the saline-paired chamber. CPP is shown before and after cocaine conditioning (i.e. on habituation day and on test day 1). N=12 trained mice, N=9 YT mice, N=9 SH mice. (E) Cognitive training reduced maintenance of CPP over a 28-day testing period. CPP values on test days were normalized to each mouse's CPP value on habituation day. N=12 trained, N=9 YT mice, N=9 SH mice. Red *p<0.05; **p<0.01 for trained compared to YT or SH animals. Blue *p<0.05 for YT compared to SH animals. All bars represent means; all error bars represent SEM.

Cognitive training does not affect locomotor sensitization to cocaine

Beginning 4 weeks after completion of cognitive training, we conditioned and tested the mice in a cocaine conditioned place preference (CPP) paradigm (Fig. 8A). During cocaine conditioning, we analyzed the animals' locomotor activity in response to cocaine injections. As expected, mice showed locomotor sensitization across successive cocaine injections (Fig. 8C, $F_{3,81}$=21.34, p<0.0001 for main effect of time; Holm-Sidak adjusted p=0.0002 for day 1 to day 4 in SH animals; Holm-Sidak adjusted p<0.0001 for day 1 to day 4 in yoked animals; Holm-Sidak adjusted p<0.0001 for day 1 to day 4 in trained animals). However, training had no effect on locomotor response to cocaine injections (Fig. 8C; $F_{2,27}$=0.98, p=0.39 for main effect of training; $F_{6,81}$=0.52, p=0.79 for training by time interaction). Trained and yoked mice also did not differ in locomotor activity after injections of saline (Fig. 8B; $F_{2,26}$=0.0021, p=1.00 for main effect of training; $F_{6,78}$=1.16, p=0.34 for training by time interaction).

Cognitive training does not affect initial CPP

After conditioning, we tested mice for their preference for the cocaine- and saline-paired contexts. All groups formed a significant preference for the cocaine-paired context (Fig. 8D, $F_{1,27}$=142.57, p<0.0001 for main effect of cocaine conditioning, Holm-Sidak adjusted p<0.0001 for habituation versus test day 1 within each group). Trained, yoked, and SH animals did not differ in their establishment of CPP on test day 1 (Fig. 8D; $F_{2,27}$=1.07, p=0.36 for main effect of training; $F_{2,27}$=1.10, p=0.35 for training by time interaction).

Cognitive training reduces maintenance of cocaine CPP during weekly testing

Next, to determine the long-term effects of cognitive training on maintenance of cocaine CPP, mice were exposed to the chambers weekly over a 28-day period. Over the course of this 28-day period, trained animals showed reduced CPP compared to yoked and SH controls (Fig. 8E; $F_{2,27}$=5.19, p=0.01 for main effect of training; $F_{8,108}$=2.16, p=0.04 for training by time

interaction; $F_{4,108}$=3.24, p=0.02 for main effect of time). In particular, post-hoc comparisons showed lower CPP in trained animals compared to yoked animals on test days 7 and 14 (Holm-Sidak adjusted p=0.02 for day 7, Holm-Sidak adjusted p=0.04 for day 14), as well as lower CPP in trained animals compared to SH animals on test day 7 (Holm-Sidak adjusted p=0.005). Yoked animals showed significantly lower CPP than SH animals on test day 28 (Holm-Sidak adjusted p=0.02), although yoked animals did not differ significantly from trained animals on test day 28 (Holm-Sidak adjusted p=0.26).

Within-group comparisons across time revealed significant reductions in CPP from test day 1 to test days 7 and 21 in trained animals (Holm-Sidak adjusted p=0.01 for day 1 compared to day 7; Holm-Sidak adjusted p=0.01 for day 1 compared to day 21). In contrast, yoked and SH animals did not show significant changes in CPP from day 1 to any later day (Holm-Sidak adjusted p=0.84, 0.54, 0.84, and 0.14 for days 7, 14, 21, and 28, respectively in yoked animals; Holm-Sidak adjusted p=0.97, 1.00, 0.50, and 1.00 for test days 7, 14, 21, and 28, respectively in SH animals). These data demonstrate that the 9-day cognitive training paradigm employed in these experiments reduced cocaine CPP during a weekly testing period that took place 6-10 weeks after cessation of cognitive training.

Cognitive training performance does not correlate with CPP during weekly testing

Given that the cognitive training paradigm was designed to engage executive functions such as cognitive flexibility, which might in turn influence drug-seeking behavior, we hypothesized that mice showing the most flexible behavior during cognitive training might show the lowest levels of CPP during the maintenance period. To test this hypothesis, we ran correlation analyses to test whether performance during cognitive training predicted CPP maintenance, as measured by the slope of a linear regression curve fitted to CPP data for each mouse. Using a measure of overall performance, we found no correlation between the fraction of trials that resulted in correct choices across sessions and cocaine CPP (Fig. 9A, r=0.48, p=0.12). Considering each type of rule change

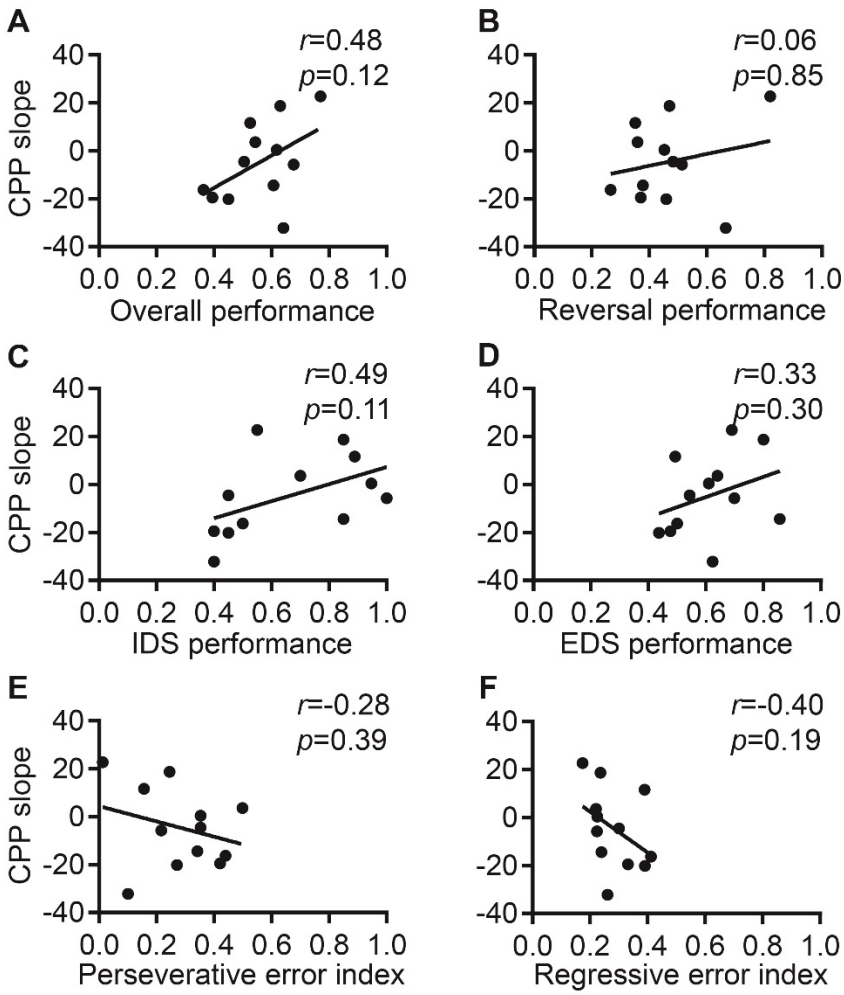


Figure 9. Performance during cognitive training does not predict CPP maintenance. CPP maintenance was defined as the slope coefficient of a linear regression curve for test days 1-14, the time period in which we observed an effect of training on CPP. (A) Overall performance (fraction of trials that resulted in a correct choice) did not correlate with CPP. (B) Performance (fraction of trials that resulted in a correct choice) during reversals did not correlate with CPP. (C) Performance (fraction of trials that resulted in a correct choice) during intradimensional shift (IDS) did not correlate with CPP. (D) Performance (fraction of trials that resulted in a correct choice) during extradimensional shifts (EDS) did not correlate with CPP. (E) Perseverative error index (fraction of trials that resulted in a perseverative error during reversals) did not correlate with CPP. (F) Regressive error index (fraction of trials that resulted in a regressive error during all rule changes) did not correlate with CPP. N=12 trained mice.

separately, we found no correlation between CPP and performance during reversals (Fig. 9B, r=0.06, p=0.85), intradimensional shift (Fig. 9C, r=0.49, p=0.11), or extradimensional shifts (Fig. 5D, r=0.33, p=0.30). Lastly, we considered 2 specific measures of executive function: the animals' ability to inhibit responding to a previously rewarded cue during reversals (perseverative errors), and the animals' ability to maintain responding to a new rule after the first rewarded trial (regressive errors). We found no correlation between either of these measures and CPP (Fig. 9E-F; r=-0.28 and p=0.39 for perseverative

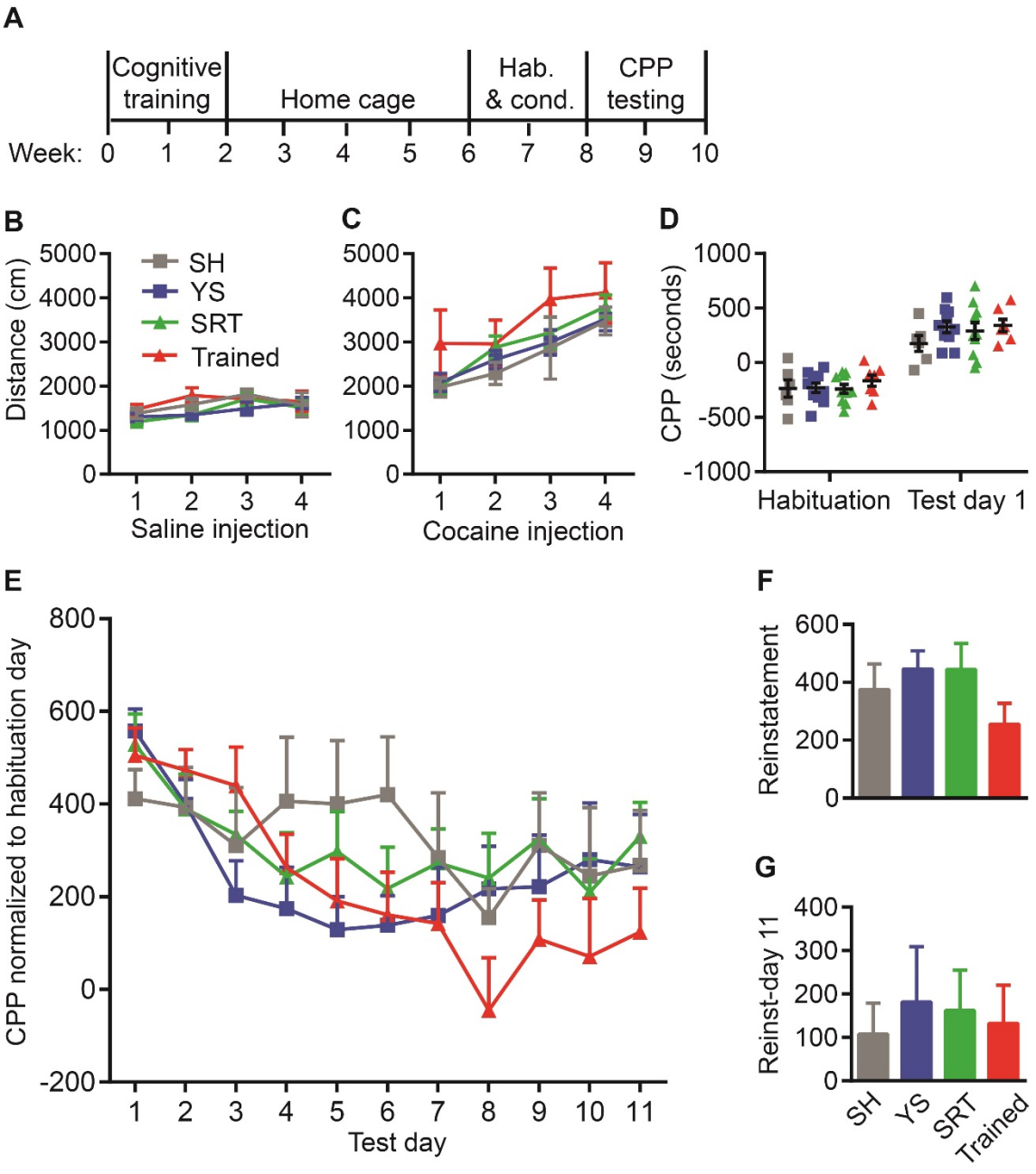
errors; r=-0.40 and p=0.19 for regressive errors).

Experiment 2: Effects of cognitive training on daily extinction of cocaine CPP

To test whether cognitive training could reduce cocaine CPP during a daily extinction testing period, we performed an additional experiment in which mice were tested for CPP on post-conditioning days 1-11. Given the lack of correlation between performance during rule changes and CPP in Experiment 1 (Fig. 9), we further investigated the role of rule changes by including a group of animals that received single rule training (SRT) with no set-shifts or reversals (Methods; Fig. 6C). In order to match the large number of cereal rewards received by the SRT mice, the yoked mice were paired with SRT mice rather than trained mice. The yoked group therefore differed in Experiments 1 and 2; yoked mice in Experiment 2 are designated YS (i.e. yoked to SRT) in Fig. 6 and Fig. 10.

Cognitive training does not affect locomotor sensitization to cocaine or initial establishment of CPP

In accordance with the results of Experiment 1, we observed locomotor sensitization to cocaine (Fig. 10C; $F_{3,87}$=21.95, p<0.0001 for main effect of time; Holm-Sidak adjusted p=0.003, p=0.0002, p<0.0001, and p=0.02 for injection 1 compared to injection 4 in SH, YS, SRT, and trained mice, respectively) but no effect of training on locomotor responses to cocaine ($F_{3,29}$=1.20, p=0.33 for main effect of training; $F_{9,87}$=0.46, p=0.90 for training by time interaction). Training also had no effect on locomotor responses to saline injections (Fig. 10B; $F_{3,29}$=0.79, p=0.51 for main effect of training; $F_{9,87}$=1.12, p=0.36 for training by time interaction). Similarly, we observed establishment of CPP in all groups (Fig. 10D; $F_{1,29}$=270.72, p<0.0001 for main effect of time; Holm-Sidak adjusted p<0.0001 for habituation to test day 1 within each group), with no effect of training on initial establishment of CPP ($F_{3,29}$=0.71, p=0.55 for main effect of training; $F_{3,29=}$0.97, $p_{=}$0.42 for training by time interaction).


A
Cognitive training
Home cage
Hab. & cond.
CPP testing
Week: 0 1 2 3 4 5 6 7 8 9 10
B
Distance (cm)
SH
YS
SRT
Trained
Saline injection
C
Cocaine injection
D
CPP (seconds)
Habituation
Test day 1
E
CPP normalized to habituation day
Test day
F
Reinstatement
G
Reinst-day 11
SH
YS
SRT
Trained
H
Test day 1 2 3 4 5 6 7 8 9 10 11
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SH ns ns ns ns ns ns ns ns ns ns
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Figure 10. Trained and yoked mice show extinction of CPP, while standard housed mice show no extinction of CPP. (A) Experimental timeline. "Hab. & cond." is habituation and conditioning (i.e. habituation to CPP chambers followed by cocaine conditioning). (B) Cognitive training did not alter locomotor activity recorded during 15-minute sessions after saline injections. "SH" is standard housed. "SRT" is single rule trained. "YS" is yoked to single rule trained. N=6 SH mice, N=10 YS mice, N=10 SRT mice, N=7 trained mice. (C) Cognitive training did not alter locomotor activity recorded during 15-minute sessions after cocaine injections. All groups showed increased locomotor activity with successive cocaine injections, consistent with sensitization. N=6 SH mice, N=10 YS mice, N=10 SRT mice, N=7 trained mice. (D) Cognitive training did not alter the initial establishment of cocaine CPP, as measured by seconds in the cocaine-paired chamber minus seconds in the saline-paired chamber. CPP is shown before and after cocaine conditioning (i.e. on habituation day and on test day 1). N=6 SH mice, N=10 YS mice, N=10 SRT mice, N=7 trained mice. (E) Trained, SRT, and YS animals showed significant within-group reductions in CPP across the 11-day CPP extinction period, while SH animals showed no reduction in CPP. Statistics for within-group comparisons across time are shown in H. N=6 SH mice, N=10 YS mice, N=10 SRT mice, N=7 trained mice (F) Cognitive training did not affect reinstatement, as measured by CPP on reinstatement day minus CPP on habituation day. N=6 SH mice, N=10 YS mice, N=9 SRT mice (1 outlier excluded), N=7 trained mice. (G) Cognitive training did not affect reinstatement, as measured by CPP on reinstatement day minus CPP on test day 11. N=6 SH mice, N=10 YS mice, N=9 SRT mice (1 outlier excluded), N=7 trained mice. (H) Trained, SRT, and YS animals showed extinction of CPP, as evidenced by significant reductions in CPP from day 1 to later test days. SH animals did not show extinction of CPP. Asterisks indicate significance levels for Holm-Sidak adjusted p values. N=6 SH mice, N=10 YS mice, N=10 SRT mice, N=7 trained mice. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. All bars represent means; all error bars represent SEM.

Trained and yoked mice show extinction of CPP, while standard housed mice show no extinction of CPP

In contrast to our weekly testing paradigm, we observed no main effect of training on CPP during the daily extinction testing period (Fig. 10E; $F_{3,29}$=0.46, p=0.71). We did, however, observe a training by time interaction ($F_{30,290}$=1.72, p=0.01). To investigate the nature of this interaction, we performed within-group comparisons of CPP from day 1 to each later day. SH mice showed no reduction in CPP from test day 1 to any later test day, while YS, SRT, and trained mice all showed reductions in CPP from test day 1 to later test days (Fig. 10H).

In order to test whether cognitive training altered reinstatement of CPP, we exposed the mice to the CPP chamber after a cocaine priming injection (5mg/kg i.p.) on post-conditioning day 12. Cognitive training had no effect on reinstatement, as measured by CPP on reinstatement day

normalized to habituation day (Fig. 10F; $F_{3,28}$=1.25, p=0.31) or as measured by CPP on reinstatement day minus CPP on test day 11 (Fig. 10G; $F_{3,28}$=0.09, p=0.97).

Discussion

Experiment 1: Effects of cognitive training on weekly CPP maintenance

Our results from Experiment 1 show that 9 days of cognitive training reduced maintenance of cocaine CPP compared to standard housed and yoked control conditions in a weekly testing paradigm (Fig. 8E). Locomotor sensitization during conditioning and initial CPP on test day 1 were comparable between the trained and control groups (Fig. 8B-D), but the groups differed in their behavior when the drug was no longer delivered. Importantly, the CPP testing period in which we saw a beneficial effect of training took place 6-10 weeks after cessation of the training, suggesting that brief cognitive training may carry long-term benefits for reducing drug-seeking behavior.

Exposure to drug-associated contexts can elicit both reconsolidation and extinction of drug-context associations. Previous literature shows that infrequent (i.e. biweekly) CPP testing supports long-term maintenance of CPP, potentially by allowing for reconsolidation to strengthen the drug-context association, while daily testing promotes extinction of CPP (Mueller and Stewart, 2000). In alignment with these previous findings, YT and SH animals in our weekly testing paradigm did not show significantly reduced CPP from test day 1 to any later test day. Cognitive training may have altered reconsolidation processes, leading to a weakening of the drug-context association during the testing period. Alternatively, cognitive training may have promoted extinction learning, allowing mice to more quickly form a new association between the CPP context and the absence of drug.

In addition to reconsolidation and extinction, previous literature shows increases in drug-seeking behavior over time, termed incubation, in abstinent rats with a history of cocaine self-administration (Grimm et al., 2001). Although we did not observe statistically significant increases

in CPP from day 1 to any later day, it is possible that cognitive training interfered with an incubation-like effect occurring along with reconsolidation and/or extinction during the weekly CPP testing period. Whether cognitive training primarily alters reconsolidation, extinction, or incubation, the results from Experiment 1 suggest that cognitive training may hold long-term benefits for reducing maintenance of drug-context associations.

Although the cognitive training paradigm involved brief periods of environmental enrichment, as animals explored an arena and received cereal rewards during training, YT control mice received this same enrichment. The beneficial effect of cognitive training on CPP maintenance is therefore not explained by a simple enrichment effect. Furthermore, our results diverge from environmental enrichment literature in that we observed a persistent effect of cognitive training during a testing period in which animals remained in relatively deprived housing conditions (i.e. standard laboratory housing) with no further cognitive training. This is in contrast to studies of environmental enrichment in which animals returning to standard housing after a history of enrichment show no reduction in drug-seeking (Chauvet et al., 2012) or exacerbation of drug-seeking (Nader et al., 2012) compared to animals that never experienced enrichment. For animals that experience relatively few opportunities for challenge or learning in their housing environments, engaging in cognitive training may induce long-term changes in neural circuitry underlying appetitive behaviors. These long-term changes may in turn influence later responses to future challenges such as exposure to drugs and drug-associated contexts.

Our results parallel environmental enrichment literature in that both training and enrichment reduce drug-seeking behavior specifically when the drug is no longer delivered, suggesting that both enrichment and cognitive training may alter animals' responses to drug-associated cues in the absence of the drug itself (Chauvet et al., 2012; Chauvet et al., 2009; Thiel et al., 2009). Interestingly, environmental enrichment also reduces sucrose-seeking behavior specifically when sucrose is no longer delivered, suggesting a potentially broad effect of

enrichment and/or cognitive training on animals' ability to respond adaptively when contingencies change (Grimm et al., 2008).

When we looked more deeply into performance in the trained group, quantifying individual differences in performance during the different phases of cognitive training, we did not find any relationship with individual differences in CPP scores during weekly maintenance tests (Fig. 9). This result suggests that engaging with cognitively challenging tasks, even with varying levels of success or prior ability, may hold benefits for reducing future maintenance of drug-context associations.

The learning paradigm we employed was complex, involving multiple sensory modalities and two distinct types of rule changes. Even before experiencing rule changes, mice had an opportunity to choose among multiple options, receive feedback, and learn an abstract rule based on cues from two sensory modalities. This active and complex learning experience contrasts starkly with standard laboratory housing conditions, in which mice experience few opportunities to learn new contingencies. One explanation for the beneficial effect of training is that set-shifts and reversals caused mice to develop executive functions such as cognitive flexibility and inhibitory control of behavior, which predict resilience to drug-seeking behavior in both humans and animal models (Aytaclar et al., 1999; Belin et al., 2008; Economidou et al., 2009; Perry et al., 2005; Tarter et al., 2003). Given the lack of correlation between performance during rule changes and CPP (Fig. 9), however, it is also possible that rule changes were unnecessary for the beneficial effect of training. The training paradigm included many opportunities to make choices and learn about different features of the task, and a simpler version of the task may have been sufficient to reduce maintenance of cocaine CPP. Future studies could investigate whether rule changes were necessary for the beneficial effects of cognitive training on CPP maintenance in Experiment 1.

Experiment 2: *Effects of cognitive training on daily CPP extinction*

In Experiment 2, we tested the effects of cognitive training and yoked rewards on CPP extinction using a daily testing schedule. All groups that had previous experience in which they had the opportunity to leave their home cages to receive rewards in a training arena showed significant extinction of CPP, while standard housed cage mates that remained in their home cages during the training period did not show extinction of CPP (Fig. 10H). This result suggests that brief interventions, involving either cognitive training or receipt of rewards in a novel context, may hold long-term benefits for the extinction of drug-context associations.

As in Experiment 1, the effect of interventions observed in Experiment 2 may have been mediated by extinction learning or reconsolidation. Mice that left their home cages for rewards in a training arena may have been better able to learn a new association between the CPP context and the absence of drug during the extinction period. Alternatively, the cognitive training and yoked interventions may have altered reconsolidation, such that mice with prior experiences of reward in a training arena engaged in less strengthening or more weakening of the drug-context association upon re-exposure to the CPP context.

In contrast to Experiment 1, the yoked control condition (YS, i.e. yoked to SRT) in Experiment 2 appeared to be just as beneficial as the trained and SRT conditions. Importantly, YS animals in Experiment 2 were paired with SRT rather than trained animals in order to match the higher number of rewards received by SRT animals. Due to the repetitive nature of the SRT task, which involved the same compound discrimination session on days 3-9, YS animals in Experiment 2 experienced a more predictable schedule of rewards than YT (i.e. yoked to trained) animals in Experiment 1. YS animals in Experiment 2 may therefore have learned associations between sensory stimuli associated with the SRT mouse's performance (e.g. the opening and closing of the start gate or the sounds of digging) and the delivery of cereal rewards. This predictable schedule of reward delivery, combined with potential Pavlovian learning related to task-relevant sensory stimuli, may have influenced YS mice's later formation of cue-reward associations in the CPP paradigm. Thus, although their receipt of rewards was not contingent on

learning an odor, texture, or spatial association, YS animals may still have experienced unintended contingency learning that influenced their later behavior in the CPP paradigm. Future experiments could elucidate the potential effects of predictable and unpredictable schedules of reward delivery on CPP extinction.

Although we observed a training by time interaction and within-group reductions in CPP during the extinction period (Fig. 10E, H), comparisons among the groups at each time point did not yield statistically significant differences. With 4 groups of mice tested at 11 time points, our statistical power was limited for detecting differences among the groups at individual time points. Future studies may help to clarify whether, for example, training with rule changes held benefits beyond those of single rule training.

After the CPP extinction period, we tested the 4 groups of mice for reinstatement of CPP in response to a cocaine priming injection. No benefits of training were observed in the reinstatement assay (Fig. 10F, G). However, the lack of CPP extinction in SH animals may have interfered with this measure. Further experiments will be needed to clarify whether training or predictable reward delivery affect reinstatement of cocaine CPP.

In addition to the relative deprivation of standard laboratory housing, SH animals experienced other potential stressors along with their trained cage mates during the training period. All groups of mice were food restricted, transported to and from the training room, and exposed to light during their dark cycle. Cognitive training or predictable reward delivery therefore may have counteracted not only the effects of environmental deprivation (i.e. standard laboratory housing), but also the effects of these additional stressors.

General Discussion

Conditions of deprivation, including standard laboratory housing, are known to promote drug-seeking behavior in animal models of substance use disorders. Here, we tested the long-term effects of brief interventions on drug-seeking behavior after animals returned to deprived

standard housing conditions. We found that cognitive training induced long-term reductions in CPP maintenance, above and beyond the effects of receiving yoked rewards in a training arena. In tests of CPP extinction, we found that a brief period of either learning rules or receiving predictable yoked rewards in a training arena supported CPP extinction. Taken together, these results suggest that brief interventions may buffer the effects of adversity and deprivation, promoting long-term resilience to drug-seeking behavior.

Future directions: developmental windows of vulnerability and opportunity

The cognitive training intervention described here took place in young adulthood, a life stage in which risk for onset of substance abuse is high (Kessler et al., 2005). Adolescence is another window of vulnerability to the onset of substance use disorders and other psychiatric diseases (Costello et al., 2011; Kessler et al., 2005). During adolescence and young adulthood, circuits in frontal cortex that are critical for executive function, decision-making, reward-related and affective behaviors are maturing (Felix-Ortiz et al., 2016; Gogtay et al., 2004; Johnson and Wilbrecht, 2011; Johnson et al., 2016a). These behaviors are critical for successfully navigating the transition to adulthood, and maturation of the circuitry underlying these behaviors may make adolescence and young adulthood sensitive periods for adverse experiences or positive interventions to alter neurodevelopmental trajectories (Suleiman and Dahl, 2017). It is therefore critical to understand the factors—both experience-dependent and developmental—that sculpt frontal circuits during these life stages. In the remaining chapters, we focus on the specific developmental factors that drive adolescent maturation of frontal circuits and behaviors associated with these circuits.

Chapter 3. Pubertal hormones drive circuit maturation in the dorsomedial frontal cortex of female mice

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Introduction

The onset of adolescence, initiated by the onset of puberty, is increasingly recognized as an inflection point for the development of frontal cortex (Piekarski et al., 2017b). Across mammals, adolescence is characterized by changes to cognitive and executive functions that coincide with large-scale reorganization of associative cortical regions, including the frontal cortex (Crone and Dahl, 2012; Huizinga et al., 2006; Paus et al., 2008). This period of development is also associated with declining plasticity in language-related circuits, declining capacity for recovery from cortical damage (Hertz-Pannier et al., 2002; Johnson and Newport, 1989; Kim et al., 1997; Lenneberg, 1967; Nemati and Kolb, 2010; Nemati and Kolb, 2012; Wright and Zecker, 2004), and increased risk of psychiatric disease (Paus et al., 2008; Silberg et al., 1999). While it remains unclear if these developmental changes are caused by the pubertal rise in gonadal hormones or are simply coincident (Piekarski et al., 2017b), it is clear that early puberty onset exacerbates risk of psychiatric illnesses connected to frontal functions (Deardorff et al., 2007; Graber, 2013; Whittle et al., 2012), suggesting a possible causal link. Gonadal steroid receptors are present across the neocortex (Kritzer, 2004; Shughrue et al., 1998; Simerly et al., 1990), and a number of anatomical changes in human cortex correlate with changes in hormone levels during puberty (Herting et al.,

2015; Herting et al., 2014; Herting et al., 2012; Peper et al., 2009; Peper et al., 2008). In rodent experiments that manipulate hormones, frontal cortex neuron density is sensitive to gonadectomy in females but not males (Koss et al., 2015), but it is still unknown if puberty onset impacts functional measures of frontal cortex circuit development, such as inhibitory and excitatory neurotransmission.

Nuclear estrogen receptors in the associative cortices are expressed almost exclusively in Parvalbumin (PV) positive fast spiking interneurons (Blurton-Jones and Tuszynski, 2002; Kritzer, 2004), suggesting that the pubertal rise in gonadal hormones may directly impact inhibitory neurotransmission. PV cells are implicated in regulation of cognition, plasticity, and neuropsychiatric illness (Berryer et al., 2016; Cho et al., 2015; Lewis and Gonzalez-Burgos, 2008; Sibille et al., 2011) and are thought to play an important regulatory role in brain development by adjusting the balance of excitation and inhibition (E/I) onto cortical pyramidal neurons. This shift in E/I balance is likely a key mechanism regulating sensitive period plasticity in primary sensory cortices (de Villers-Sidani and Merzenich, 2011; Southwell et al., 2010; Werker and Hensch, 2015). We have previously demonstrated a striking increase in the strength of inhibitory neurotransmission in deep layers of the frontal cortex during early adolescence (Vandenberg et al., 2015), which led us to develop a working model in which gonadal steroids drive frontal cortex maturation by increasing the strength of local inhibitory neurotransmission (Piekarski et al., 2017b).

In addition to the increase in inhibitory neurotransmission across adolescence (Vandenberg et al., 2015), we have observed a decrease in the density and turnover of dendritic spines in Layer 1 of frontal cortex from pre-pubertal to post-pubertal ages (Johnson et al., 2016a). As a site of integration of excitatory and inhibitory inputs, dendritic spines may relate critically to the balance of excitation and inhibition in the developing cortex (Chadderton et al., 2014; Chiu et al., 2013; Higley, 2014; Nimchinsky et al., 2002). Spine dynamics also correlate with behavioral outputs of specific circuitry in rodents (Fu et al., 2012; Munoz-Cuevas et al., 2013; Yang et al.,

2009), and a decline in spine turnover is associated with reduced capacity for flexible song learning across development in birds (Roberts et al., 2010). Thus, maturational changes in dendritic spine dynamics may be relevant to the maturation of flexible learning across pubertal development.

In the present report, we manipulated exposure to gonadal steroids in mice at peripubertal ages and measured the strength of excitatory and inhibitory inputs onto layer 2/3 (L2/3) pyramidal cells, intrinsic and spiking properties of PV cells, and dendritic spine dynamics in a subregion of frontal cortex implicated in goal-directed behavior, decision-making, and multiple forms of psychiatric disease. We found that intact gonads are necessary for the peripubertal increase in phasic inhibitory neurotransmission onto L2/3 pyramidal neurons. Because of the potential health consequences of early puberty onset in girls, we next developed a mouse model of early puberty in which we advanced the age of puberty onset by treating mice with gonadal hormones. Pre-pubertal hormone injections drove an early increase in inhibitory neurotransmission in the frontal cortex, but not somatosensory cortex, suggesting that earlier puberty can advance cortical maturation in a regionally specific manner. Electrophysiological properties of PV cells and dendritic spine dynamics were both unaffected by hormone treatment over the same time window in which we observed hormone-induced changes in inhibitory neurotransmission, suggesting that gonadal hormones drive some but not all aspects of frontal circuit maturation. Finally, pre-pubertal hormone injections induced early maturation of performance on a frontal cortex-dependent task, indicating that gonadal hormones can induce changes in flexible learning across adolescence. Extrapolating from rodents to humans, our findings may enhance concerns about the risks of increasingly earlier puberty onset in modern society.

Methods

Animals

All experiments except electrophysiological recording of PV cells were conducted in female C57BL/6J mice from the Thy-1-YFP-H line bred in our animal facility. For electrophysiological recording of PV cells, cre+ female mice resulting from crosses of heterozygous PV-cre mice and homozygous Ai14 mice were used. All mice were weaned at P21 and housed in groups of 2-3 same-sex siblings on a 12:12 reverse light:dark cycle (lights on at 10PM). All procedures were approved by the Animal Care and Use Committee of the University of California, Berkeley and conformed to principles enunciated in the NIH guide for the use and care for laboratory animals.

Assessment of puberty onset

To collect normative data from our colony on the age at pubertal milestones, we determined the age at vaginal opening and first estrus. Age at vaginal opening was determined by visual examination of the vagina beginning on P24. To determine age at first estrus, starting on the day of vaginal opening, vaginal lavages were collected by placing a small drop of distilled water at the vaginal opening to collect vaginal cells. The first day in which cornified epithelials represented a majority of cells was recorded as first estrus. The mice used for vaginal lavages were not used for collection of any other data (i.e. behavior or electrophysiology) in order to avoid potential confounds related to the additional handling required for vaginal lavage. Visual assessment of vaginal opening was performed to confirm pubertal status of mice used in electrophysiology and behavior studies, but this one-time visual assessment (approximately 2 seconds) did not require any additional handling beyond what would otherwise be required to transfer mice to a behavior apparatus or anesthetize mice for perfusion/electrophysiology.

Hormone Manipulations

To advance age at puberty onset, gonadally intact females were injected with 17 beta-estradiol (0.01mg/kg subcutaneous) or vehicle at P24. At P26, mice were injected with

progesterone (20mg/kg subcutaneous) or vehicle. This treatment advances first peripubertal exposure to gonadal steroids and is sufficient to induce endogenous puberty (Ramirez and Sawyer, 1965; Smith and Davidson, 1968).

To eliminate circulating gonadal steroids, gonadectomies were performed before puberty onset (P24 or P25). Prior to surgery, mice were injected with 0.05mg/kg buprenorphine and 10mg/kg meloxicam subcutaneously. During surgery, animals were anesthetized with 1-2% isoflurane. The incision area was shaved and scrubbed with ethanol and betadine. Ophthalmic ointment was placed over the eyes to prevent drying. A 1 cm incision was made with a scalpel in the lower abdomen across the midline to access the abdominal cavity. For ovariectomies, the ovaries were clamped off from the uterine horn with locking forceps and ligated with sterile sutures. After ligation, ovaries were excised with a scalpel. For castrations, the blood supply to each testis was clamped with locking forceps, after which the testes were ligated with sterile sutures and excised with a scalpel. The muscle and skin layers were then sutured, and wound clips were placed over the incision for 7-10 days to allow the incision to heal. An additional injection of 10mg/kg meloxicam was given 12-24 hours after surgery. Sham control surgeries were identical to ovariectomies and castrations except that the ovaries/testes were simply visualized and were not clamped, ligated or excised. Mice were allowed to recover on a heating pad until ambulatory and were post-surgically monitored for 7-10 days to check for normal weight gain and signs of discomfort/distress.

Slice Electrophysiology

Mice were deeply anesthetized with an overdose of ketamine/xylazine solution and perfused transcardially with ice-cold cutting solution containing (in mM): 110 choline-Cl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, 11.6 Na-ascorbate, 3 Na-pyruvate, 1.25 NaH_2PO_4, and 25 D-glucose, and bubbled in 95% O_2/ 5% CO_2. 300 µm thick coronal sections were cut in ice-cold cutting solution before being transferred to ACSF containing (in mM): 120 NaCl, 2.5 KCl, 1.3

MgCl2, 2.5 CaCl2, 26.2 NaHCO3, 1 NaH2PO4 and 11 Glucose. Slices were bubbled with 95% O2/ 5% CO2 in a 37°C bath for 30 min, and allowed to recover for 60 min at room temperature before recording.

All recordings were made using a Multiclamp 700 B amplifier and were not corrected for liquid junction potential. Data were digitized at 10 kHz and filtered at 1 or 3 kHz using a Digidata 1440 A system with PClamp 10.2 software (Molecular Devices, Sunnyvale, CA, USA). Only cells with access resistance of <25 MΩ were retained for recording of mPSCs and <30 MΩ for recording of evoked spikes. Access resistance was not corrected. Cells were discarded if parameters changed more than 20%. Data were analyzed using MiniAnalysis (Synaptosoft, INC, GA), PClamp, or R (R 3.3.1; R Foundation for Statistical Computing, Vienna, AT).

Miniature inhibitory and excitatory post synaptic currents (mIPSCs and mEPSCs, respectively) were recorded in L2/3 pyramidal cells in the anterior cingulate cortex. Whole cell mEPSCs were recorded at -70mV and mIPSCs at +10mV. The bath contained 32°C aCSF with 0.5 µM tetrodotoxin to block action potentials. Recording pipettes had 2-5 MΩ resistances and were filled with internal solution (in mM): 115 Cs-methanesulfonate, 10 HEPES, 10 BAPTA, 10 Na2-phosphocreatine, 5 NaCl, 2 MgCl2, 4 Na-ATP, 0.3 Na-GTP. An integrated measure of the total inhibitory charge transfer was calculated using the equation: $Q=f \times Q_{mPSCs}$, where f is the frequency (Hz), and Q_{mPSCs} is the average charge transfer for each mPSC (Ataka and Gu, 2006).

To calculate paired pulse ratio, layer 2/3 pyramidal cells in the anterior cingulate cortex (ACC) were whole-cell patch clamped, and inhibitory currents were isolated using a high chloride internal solution (in mM): 115 CsCl, 10 HEPES, 10 Na2-phosphocreatine, 5 NaCl, 2 MgCl2, 4 Na-ATP, 0.3 Na-GTP, 0.02 EGTA and 15 KCl. The bath solution contained 5 µM NBQX and 25 µm APV to block AMPA/Kainate and NMDA currents, respectively. A bipolar stimulating electrode was placed ~300 uM from the cell body, and stimulation intensity ranged between 1 and 2 µA. Paired pulse ratio was calculated by injecting 0.1µS stimulations at either 50 ms, 100 ms or 500 ms inter stimulus intervals and dividing the second pulse by the first pulse. With short inter-

stimulus intervals, the second pulse overlapped with the decay phase of the first pulse; thus, all traces were baseline subtracted.

For recording of intrinsic and spiking properties in PV cells, tdTomato-expressing cells in L2/3 and L5 of cingulate cortex in PV-Ai14 mice were whole cell patch clamped. The bath contained 32°C ACSF with no drugs. Recording pipettes had 2-5 MΩ resistances and were filled with internal solution (in mM): 140 K Gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl2, 4 MgATP, 0.3 Na2GTP, and 10 Na2 Phosphocreatine. Current was injected in 500ms pulses from -100pA to 450pA, in 50pA increments. All intrinsic and spiking properties were analyzed using pClamp software (Molecular Devices, Sunnyvale, CA). Resting membrane potential was measured immediately before the first current injection. Input resistance and membrane time constant were measured in response to -100pA current. Spiking properties were measured in response to current injection 100pA above spiking threshold, where spiking threshold was defined as the first current injection to elicit an action potential. Spike amplitude and width were measured for the first spike during the current injection 100pA above spiking threshold. Spike width was defined as the width (in ms) at half-maximal amplitude. Adaptation ratio was defined as the ratio between the first and last interspike intervals during the current injection 100pA above spiking threshold.

In order to avoid oversampling from individual mice, a maximum of 4 cells per mouse were included for analysis across all electrophysiology experiments.

Cranial window surgery

Cranial window surgeries were performed in YFP-H mice at P26 or in young adulthood (age range P54-P63). Detailed surgical procedures have been described previously (Holtmaat et al., 2009). Briefly, mice were anesthetized with isoflurane anesthesia. A craniotomy (diameter 2.5mm) was made over the dorsomedial frontal cortex, centered over the midline, with the caudal edge of the craniotomy at bregma. The dura was left intact during this procedure. The craniotomy was covered with a thin layer of agarose solution (0.7% in ACSF) and sealed with a glass

coverslip. Mice were given subcutaneous injections of Meloxicam (10mg/kg) during surgery and 24 hours after surgery.

Dendritic spine imaging

After 1 day of recovery from cranial window surgery, mice were imaged once per day for 2 consecutive days. Imaging took place at P27 and P28 for vehicle-and hormone-treated mice and between P55 and P65 for OVX and sham mice. Detailed *in vivo* imaging procedures have been described previously (Holtmaat et al., 2009). Briefly, apical dendrites of YFP-expressing pyramidal cells were imaged using a Mai Tai HP laser (950nm, Spectra Physics, Santa Clara, CA), Ultima IV *in vivo* laser-scanning microscope (Bruker, Middleton, WI), and a 40x 0.8N objective (Olympus, Center Valley, PA). 40μm segments of dendrite located within 100μm of the surface (i.e. in layer 1) were imaged at a resolution of 0.08μm/pixel. Image stacks encompassing the entire dendritic segment were acquired with a step size of 1μm.

Image processing and analysis

Images used for analysis were median-filtered 3-dimensional z stacks. Dendritic spines were scored according to established criteria (Holtmaat et al., 2009) using custom Matlab software (Mathworks, Natick, MA). Dendritic spines were scored if they protruded laterally more than 0.4μm from the dendritic shaft. To calculate spine density, the total number of spines for each mouse was divided by the total length of analyzed dendrite in μm. To calculate the fraction of spines gained for each mouse, the number of new spines present on the second day of imaging (P28) was divided by the total number of spines present on the first day of imaging (P27). To calculate the fraction of spines lost, the number of spines lost between the first and second day of imaging was divided by the total number of spines present on the first day of imaging (P27).

One measure of density, fraction gained, and fraction lost was calculated for each mouse, such that n for analysis was the number of mice.

For image presentation (Fig. 17), the relevant sections of the 3-dimensional z stack were projected onto a 2-dimensional image. The featured section of dendrite was cropped from the full-size image, and the cropped image was Gaussian filtered and contrasted for presentation.

Behavior

A detailed description of the behavioral task has been published (Johnson and Wilbrecht, 2011). Briefly, mice were trained in a 4-choice discrimination and reversal task. Prior to training, mice were food restricted to 85% of *ad libitum* body weight, adjusting for expected weight gain with growth in young animals. On day 1, mice were habituated to the testing arena, on day 2 were taught to dig for cheerio rewards in a pot filled with wood shavings, and on day 3 underwent a 4-choice odor discrimination and reversal task.

During the discrimination phase, the mouse learned to discriminate among four different pots differentiated only by the scent of the wood shavings, to learn that a cereal reward was hidden beneath only the anise-scented shavings. The stimulus presentation was pseudo-randomized so that an odor only repeated in a quadrant once per 4 trials in order to prevent mice from using a spatial strategy to solve the task. Each trial was considered complete when the mouse indicated a “decision” by digging in a pot. If the chosen pot contained a reward, the mouse was allowed to finish eating the cereal reward, but if the pot did not contain a cereal reward, the mouse was immediately placed back in the starting cylinder to prevent multiple digging choices. If a mouse did not dig in a pot within 3 minutes of the start of the trial, the mouse was placed back in the starting cylinder and the trial was scored an omission. In the unusual circumstance of 3 consecutive omissions, a pot with unscented shavings was placed in the start cylinder with the mouse in order to reinitiate digging. The discrimination phase ended after the mouse had successfully chosen the correct pot on eight out of the last ten choices.

Upon reaching criterion, the reversal phase was immediately started. All shavings were replaced with new shavings, and the thyme scented pot was replaced with a new pot filled with eucalyptus scented shavings. During this phase, the cereal reward was now located in the clove scented pot. The reversal phase was considered complete once the mouse reached criterion of eight out of the last ten choices correct. Animals typically completed both discrimination and reversal phases within 3 hours.

Statistical analysis

All data were analyzed blind to the experimental group. Statistical analyses were conducted in R (R 3.3.1; R Foundation for Statistical Computing, Vienna, AT) and GraphPad Prism (GraphPad, San Diego, CA). Additional statistical details are located in the results and figure legends. Because electrophysiology measures included multiple cells from the same brain, these data were analyzed by fitting a linear mixed model using residual maximum likelihood in the lme4 package (version 1.1.12; (Bates et al., 2015)). Residuals were verified to be normally distributed. P-values for these analyses were determined using the car package (version 2.1.3; (Fox and Weisberg, 2011)). Group assignment was included as the fixed effect and subject as the random effect. Student's t tests were used to analyze pubertal milestones. Analysis of variance (ANOVA) was used to analyze behavior and dendritic spine data. When required, Tukey's HSD test, Sidak's multiple comparisons, or Dunn's multiple comparisons were used to control for error-rate in post-hoc analyses. All results are reported as the mean ± standard error of the mean (S.E.M). Differences were considered significant if $p<0.05$.

Results

Inhibitory neurotransmission onto L2/3 pyramidal neurons in the cingulate cortex increases during puberty

Because the strength of inhibition onto pyramidal neurons is thought to regulate sensitive period maturation of the visual and auditory cortices, we first investigated if inhibitory neurotransmission increases in the anterior cingulate cortex during development. To this end, we measured miniature inhibitory and excitatory post synaptic currents (mIPSCs and mEPSCs) onto visually identified L2/3 pyramidal cells of prepubertal (P24) and late-pubertal mice (P40-45; Fig. 11A). We focused on female mice in this study due to public health concerns surrounding early-onset puberty specifically in girls (Deardorff et al., 2007; Graber, 2013; Whittle et al., 2012). We found that mIPSC total charge transfer increased between P24 and P40 (553.86 ± 72.04 [6 brains, 19 cells] vs 901.36 ± 60.91 [7 brains, 21 cells] pA/s; $p<0.001$; Fig. 11B), and this change was driven by a significant increase in mIPSC frequency (3.60 ± 0.38 vs 5.10 ± 0.26 Hz; $p=0.009$; Fig. 11C) and amplitude (23.99 ± 1.04 vs 27.45 ± 0.50 pA; $p=0.0015$; Fig. 11D). In a subset of cells, mEPSC parameters were also collected. No changes in mEPSC total charge transfer (163.68 ± 38.31 [6 brains, 12 cells] vs 118.55 ± 19.99 [6 brains, 11 cells] pA/s; $p=0.29$; Fig. 11E), frequency (3.76 ± 0.88 vs 2.78 ± 0.50 Hz; $p=0.32$; Figure 11F), or amplitude (14.99 ± 1.34 vs 15.26 ± 0.77 pA; $p=0.69$; Fig. 11G) were observed. These data suggest there was a change in the balance of excitation and inhibition onto L2/3 pyramidal neurons from P24 to P40 driven by changes in inhibitory neurotransmission.

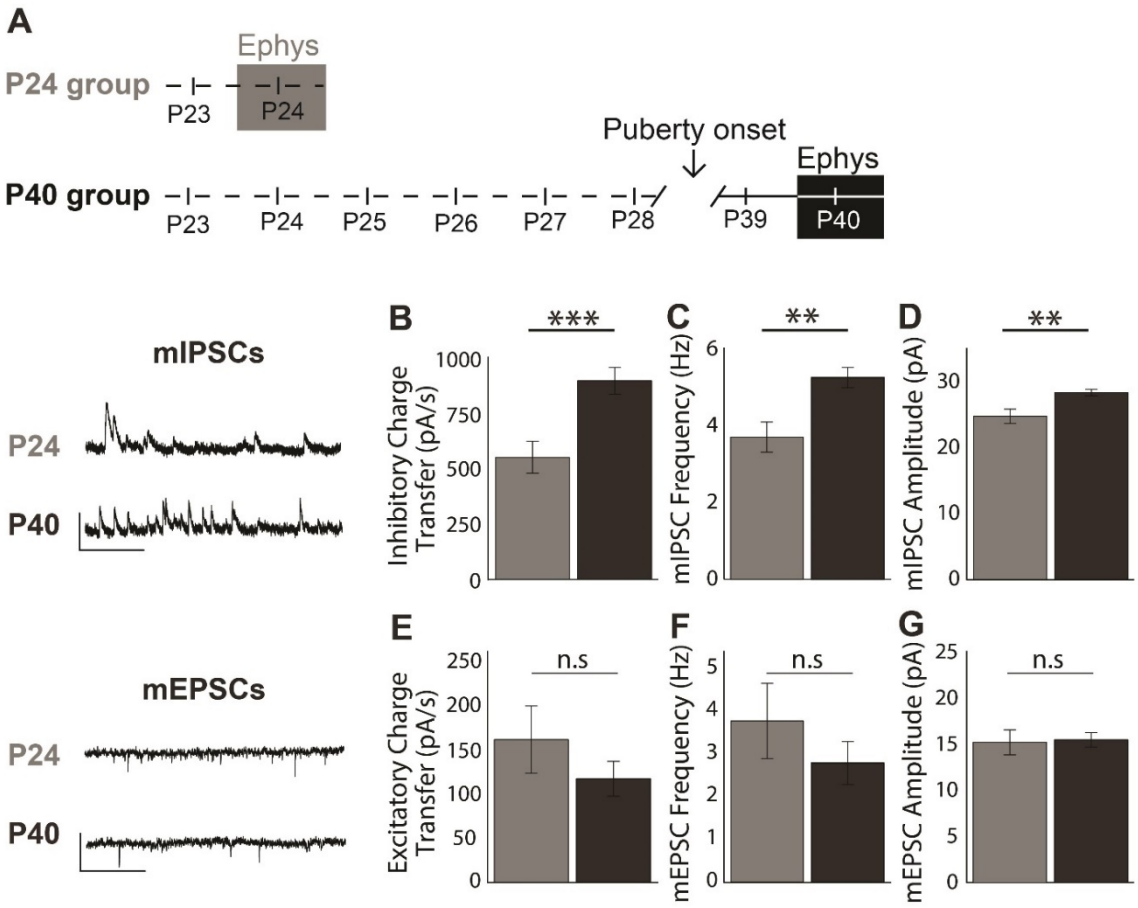

Figure 11. Inhibitory neurotransmission increases in strength during pubertal development in layer 2/3 of the cingulate cortex, but excitatory neurotransmission does not. (A) We made whole cell recordings from L2/3 pyramidal cells in the anterior cingulate cortex before puberty (P24-25) and during late puberty (P40-45). mIPSC charge transfer (B), frequency (C), and amplitude (D) were significantly higher in late-pubertal mice (7 brains, 21 cells) compared to pre-pubertal mice (6 brains, 19 cells). Neither mEPSC charge transfer (E), frequency (F), nor amplitude (G) was different between pre-pubertal (6 brains, 12 cells) and late-pubertal (6 brains, 11 cells) age groups. These data suggest a developmental change in the balance of excitation and inhibition onto these cingulate neurons driven primarily by changes in inhibitory neurotransmission. All graphs are mean ± S.E.M. Hypothesis tests were conducted using linear mixed models. **p<0.01, ***p<0.001; n.s., not significant. Scale bar: 50pA, 500ms.

Intact gonads are necessary for peripubertal maturation of mIPSC frequency in cingulate cortex

To test if intact gonads are necessary for the observed rise in mIPSC frequency, we performed ovariectomy (OVX) at P24 to prevent puberty onset and then collected mIPSC data at P40-45 (4 brains, 16 cells; Fig. 12A). A control group underwent sham OVX (6 brains, 19 cells),

while a third group was ovariectomized at P24, then injected with estradiol and progesterone at P40 and P42 (5 brains, 15 cells) in a replacement regimen that is sufficient to induce puberty in intact mice (Piekarski et al., 2017a). The three groups differed significantly in total inhibitory charge transfer (sham OVX, 912.44 ± 86.31; OVX, 593.81 ± 57.30; OVX + Hormone, 992.72 ± 89.83; pA/s; p=0.036; Fig. 12B) and mIPSC frequency (sham OVX, 5.68 ± 0.51; OVX, 3.98 ± 0.28; OVX + Hormone, 5.60 ± 0.36 Hz; p=0.039; Fig. 12C). Using planned comparisons, we found

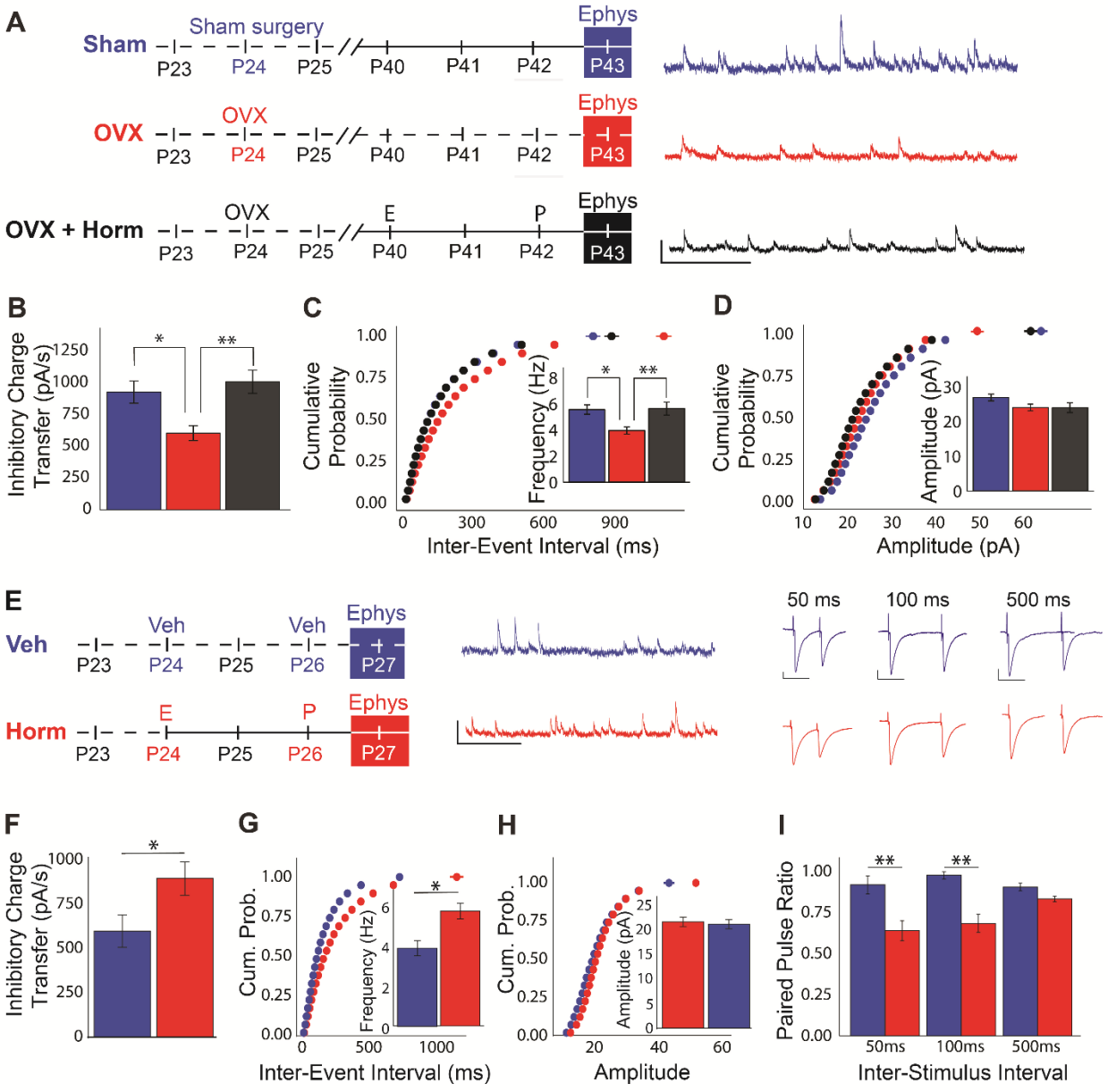

Figure 12. Pubertal hormones drive maturation of inhibitory neurotransmission onto L2/3 pyramidal cells in cingulate cortex. (A) Mice were either ovariectomized (OVX) or sham ovariectomized (Sham) prior to puberty. One group of OVX mice received hormone replacement at P40 and P42 (OVX + Horm). L2/3 pyramidal cells were patched between P40 and P45 for the OVX and Sham OVX groups, and within two days of progesterone injection in the OVX + Horm group. Pre-pubertal OVX (4 brains, 16 cells) resulted in significantly decreased inhibitory charge transfer (B), and mIPSC frequency (C) but no differences in mIPSC amplitude (D) compared to both sham controls (6 brains, 19 cells) and OVX+ Horm mice (5 brains, 15 cells; planned comparisons). All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models. *p<0.05; **p<0.01; n.s, not significant. Scale bar: 50pA, 500ms. (E) To model earlier puberty, intact female mice were injected with estradiol at P24 and progesterone at P26, while controls were injected with vehicle at both ages. This hormone treatment was timed to occur before an expected rise in gonadal steroids and to drive a leftward (earlier) shift in the age at puberty onset. We recorded mPSCs at P27-P28, when the groups differed with respect to pubertal status but not age. At this time, the hormone treatment group (9 brains, 17 cells) showed higher total inhibitory charge transfer (F) and higher mIPSC frequency (G) compared to controls (8 brains, 17 cells). mIPSC amplitude (H) did not differ between groups. (I) In a follow up experiment, we investigated presynaptic function at P27-28 using a standard paired pulse inhibition paradigm. Measuring electrically evoked currents in Layer 2/3 pyramids, we found that hormone treated mice (4 brains, 7 cells) exhibited more depressing inhibitory synaptic transmission at 50 and 100 ms inter-stimulus intervals compared to vehicle treated mice (6 brains, 11 cells). Post hoc tests in paired pulse ratio experiment (I) were conducted using Tukey's HSD. *p<0.05; **p<0.01. Scale bar: 50 pA, 500 ms for mini traces, 50 pA, 50ms for paired pulse ratio.

that Sham OVX and OVX + Hormone groups both had significantly higher mIPSC frequency than the OVX group (p=0.015; p=0.008, respectively), suggesting that gonadal hormones are necessary and sufficient to increase mIPSC frequency. mIPSC amplitude did not vary between groups (p=0.49; Fig. 12D). Prepubertal OVX did not affect mEPSC charge transfer, frequency, or amplitude (data not shown).

These data demonstrate that intact gonads are necessary for the peripubertal increase in inhibitory neurotransmission onto cingulate cortex L2/3 pyramidal cells, establishing a causal role for pubertal hormones in the maturation of frontal inhibitory neurotransmission.

A mouse model of earlier puberty shows early increases in mIPSC frequency and altered probability of GABA release in the cingulate cortex

The age of puberty onset is advancing in girls (Aksglaede et al., 2009; Biro et al., 2013; Herman-Giddens, 2006; Marshall and Tanner, 1969; Rosenfield et al., 2009), and early puberty is associated with increased risk for adolescent-onset psychopathology (Deardorff et al., 2007; Graber, 2013; Whittle et al., 2012) and may reduce plasticity (Lenneberg, 1967; Piekarski et al., 2017b; Wright and Zecker, 2004). To understand how earlier puberty affects the developing cortex, we developed a mouse model of earlier puberty. This model advances first peripubertal exposure to gonadal steroids by injecting estradiol and progesterone at P24 and P26, respectively (Fig. 12E). Control sibling cage-mates were injected with oil vehicle on both days (Fig. 12E). This treatment significantly advanced vaginal opening (P26.13 ± 0.13 vs P29.13 ± 0.85; n=8 per group; t_{14}=3.47, p<0.01; data not illustrated), which is the first external indicator of puberty onset in female mice. Hormones also advanced first estrus (P27.7 ± 0.18 vs P33.88 ± 1.63; t_{13}=3.50, p<0.01; data not illustrated), which agrees with previous literature showing that gonadal hormone injections disinhibit the hypothalamic-pituitary-gonadal axis to induce endogenous puberty (Ramirez and Sawyer, 1965; Smith and Davidson, 1968). This treatment therefore yields an approximately 3-4 day time period during which age-matched, co-housed siblings vary only with respect to hormone exposure, allowing us to causally separate age- from hormone-dependent maturation of brain and behavior.

To determine if a rise in gonadal steroids can induce frontal cortex inhibitory neurotransmission development, we compared hormone treated (9 brains, 17 cells) and vehicle treated (8 brains, 17 cells) mice at P27-28, when cage mates are age-matched but differ with respect to hormone exposure. The hormone treatment group showed significant increases in inhibitory charge transfer (854.84 ± 80.52 vs 521.77 ± 73.04 pA/s; p=0.021; Figure 12F), and mIPSC frequency (5.78 ± 0.37 vs 3.93 ± 0.39 Hz; p=0.018; Fig. 12G) but not mIPSC amplitude (21.46 ± 0.94 vs 20.99 ± 0.93 pA; p=0.71; Fig. 12H). There were no differences in mEPSC parameters (data not shown).

A change in mIPSC frequency could result from an increase in the number of synapses and/or enhanced probability of GABAergic vesicle release. To test this latter possibility, we conducted a paired pulse ratio experiment in hormone (4 brains, 7 cells) and vehicle (6 brains, 11 cells) treated animals at P27 with interstimulus intervals of 50, 100 and 500ms. There was a main effect of hormone treatment ($p<0.001$; Fig. 12I), and interstimulus interval ($p=0.006$), and an interaction ($p=.001$). Simple comparisons revealed that hormone treated mice had significantly more depressing inhibitory synapses than vehicle treated mice at 50ms (0.64 ± 0.06 vs. 0.87 ± 0.35; $p<0.001$) and 100ms (0.68 ± 0.05 vs. 0.97 ± 0.023; $p<0.001$). The groups did not differ at the 500ms interstimulus interval after adjusting for multiple comparisons (0.83 ± 0.02 vs. 0.90 ± 0.03; $p=0.044$ unadjusted). These data suggest that hormones enhance release probability of GABAergic vesicles.

Pubertal hormones do not strengthen inhibitory neurotransmission in barrel cortex

To test if hormone-driven increases in inhibitory neurotransmission are specific to the frontal cortex, we treated mice with either hormone (7 brains, 15 cells) or vehicle (4 brains, 10 cells) before puberty (Fig. 13A) and collected data at p27-28 from somatosensory barrel cortex. Hormone treatment did not affect mIPSC inhibitory charge transfer (790.93 ± 119.41 hormone vs 1044.63 ± 281.66 vehicle, pA/s; $p=0.33$; Fig. 13B) or frequency (4.31 ± 0.53 hormone vs 4.71 ± 1.00 vehicle, Hz; $p=0.69$; Fig. 13C). However, hormone treated mice had significantly smaller mIPSC amplitudes (23.15 ± 0.88) compared to vehicle controls (26.09 ± 1.35 pA; $p=0.046$; Figure 13D). These data suggest hormonal effects on the maturation of inhibition during adolescence are regionally specific.

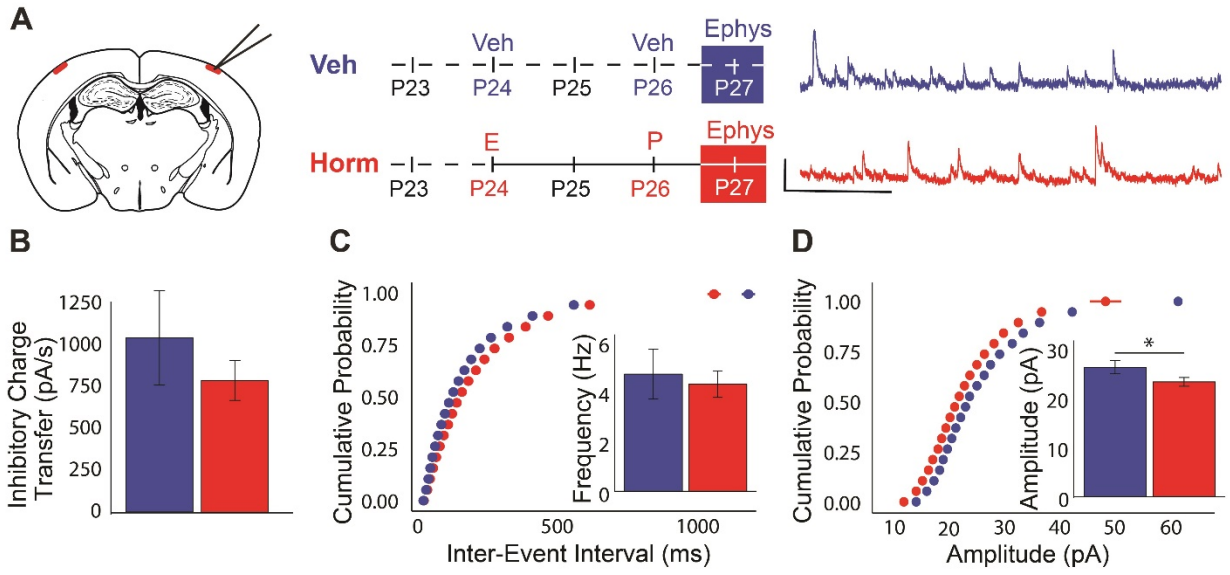

Figure 13. Early exposure to gonadal steroids does not increase inhibitory neurotransmission in somatosensory cortex. (A) Mice were injected with either gonadal steroids (7 brains, 15 cells) or vehicle (4 brains, 10 cells) at P24 and P26. Layer 2/3 pyramidal cells were patched at P27 or P28 in barrel cortex. We observed no significant differences in inhibitory charge transfer (B) or mIPSC frequency (C), but the hormone treatment group did show significantly smaller mIPSC amplitude (D) compared to vehicle. All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models. *p<0.05. Scale bar: 50 pA, 500ms.

Intrinsic and spiking properties of PV+ interneurons across natural puberty and in response to pre-pubertal hormone treatment

Given that estrogen receptors in frontal cortex are found primarily on PV+ interneurons (Blurton-Jones and Tuszynski, 2002; Kritzer, 2004), these cells may be directly responsive to gonadal hormone exposure during puberty. We therefore tested intrinsic and spiking properties of PV+ interneurons in L2/3 and L5 of cingulate cortex across natural puberty and in response to pre-pubertal hormone exposure. We first measured intrinsic properties in response to -100pA current injections in cells from vehicle-treated mice at P27 (i.e. pre-pubertal), hormone-treated mice at P27 (i.e. mice in which early puberty was induced), and hormonally unmanipulated mice at P40 (i.e. during late puberty) (Fig. 14A). Resting membrane potential did not differ among the

groups in L2/3 (vehicle, -73.70 ± 2.13; hormone, -76.44 ± 1.38; P40, -77.54 ± 0.92; mV; p=0.25; Fig. 14B) or L5 (vehicle, -74.64 ± 1.11; hormone, -74.64 ± 1.93; P40, -75.72 ± 1.44; mV; p=0.89; Fig. 14B). Input resistance also did not differ among the groups in L2/3 (vehicle, 146.0 ± 5.67; hormone, 146.7 ± 11.02; P40, 140.9 ± 6.13; mΩ; p=0.71; Fig. 14C) or L5 (vehicle, 146.2 ± 9.88; hormone, 155.4 ± 8.64; P40, 152.2 ± 6.67; mΩ p=0.76; Fig. 14C). Membrane time constant did

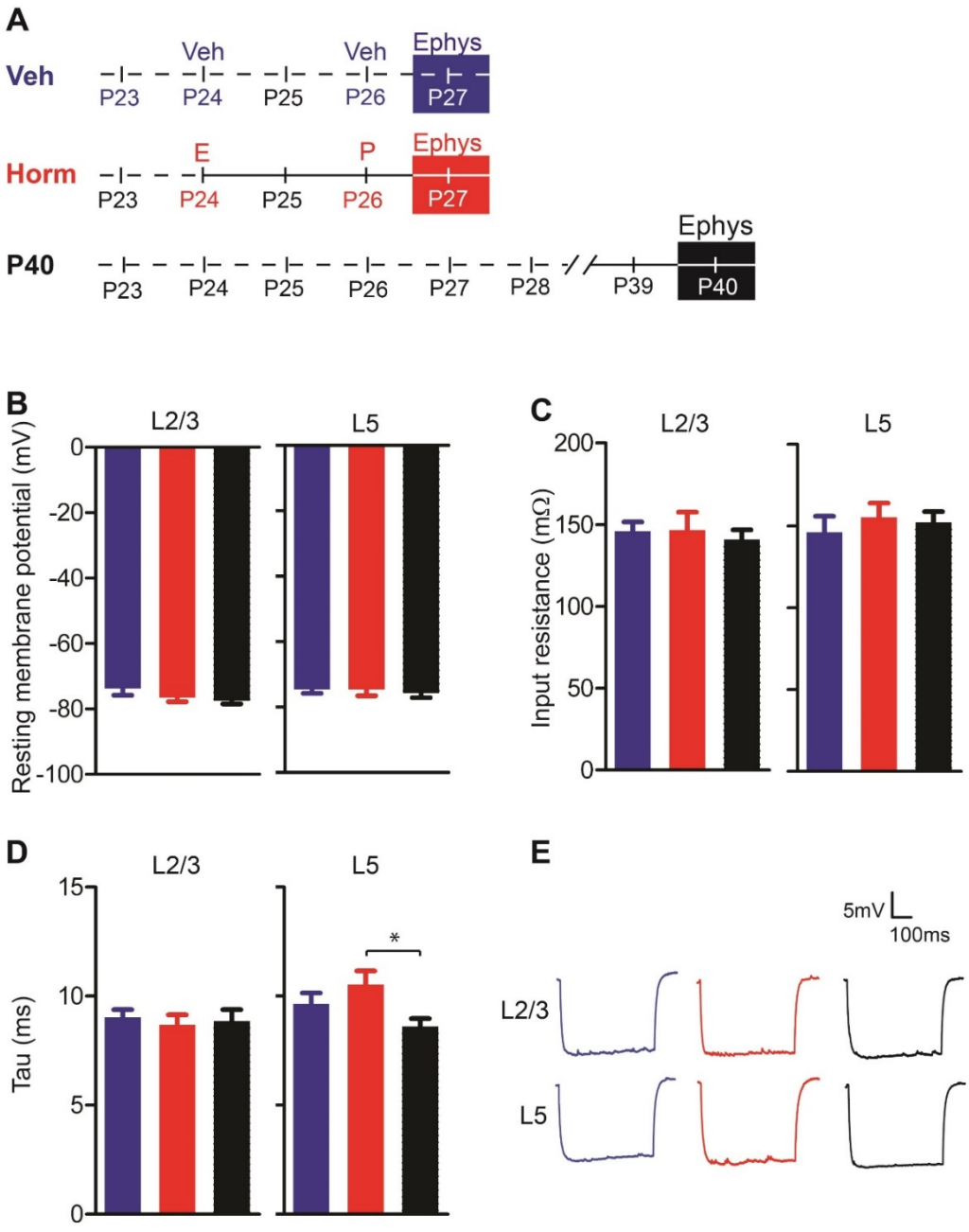

Figure 14. Neither natural puberty nor pre-pubertal hormone injections alter intrinsic properties of PV cells. (A) To model earlier puberty, intact female mice were injected with estradiol at P24 and progesterone at P26 (5 brains, 12 cells for L2/3; 4 brains, 12 cells for L5), while controls were injected with vehicle at both ages (5 brains, 13 cells for L2/3; 6 brains, 17 cells for L5). Data were collected from vehicle- and hormone-treated mice at P27, when vehicle-treated mice were still pre-pubertal. A second group of mice was allowed to undergo natural puberty (6 brains, 14 cells for L2/3; 6 brains, 16 cells for L5), and data were collected from these mice at P40. PV cells in L2/3 and L5 were whole cell patch clamped, and intrinsic properties were measured in response to a -100pA, 500ms current pulse. Resting membrane potential (B) and input resistance (C) did not differ among the 3 groups in L2/3 or in L5. Membrane time constant, tau, did not differ among the groups in L2/3 but did differ among the groups in L5 (D), with P40 mice showing lower tau than hormone-treated mice. There was, however, no difference between vehicle-treated (i.e. pre-pubertal) and P40 (i.e. late-pubertal) mice, and no difference between vehicle- and hormone-treated mice, suggesting that neither natural puberty nor hormone injections alter tau when compared to pre-pubertal vehicle-treated mice. (E) Sample traces from -100pA current injections. All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models. Post-hoc tests were conducted using Tukey's HSD. *adjusted $p<0.05$.

not differ among the groups in L2/3 (vehicle, 9.01 ± 0.36; hormone, 8.68 ± 0.47; P40, 8.86 ± 0.52; ms; p=0.95; Fig. 14D). Membrane time constant differed among the groups in L5 (vehicle, 9.64 ± 0.50; hormone, 10.53 ± 0.63; P40, 8.61 ± 0.36; ms; p=0.022; Fig. 14D). Tukey post-hoc comparisons revealed higher tau in hormone-treated compared to P40 mice (adjusted p=0.017; Fig. 14D) but no difference between vehicle- and hormone-treated mice (adjusted p=0.41; Fig. 14D) or between vehicle-treated and P40 mice (adjusted p=0.24; Fig. 14D).

To test whether hormone treatment would increase excitability or alter spiking properties in PV cells, we measured evoked spikes in response to current injections (see Methods). Treatment groups were the same as described above and included vehicle-treated mice at P27, hormone-treated mice at P27, and hormonally unmanipulated mice at P40 (Fig. 15A). Spiking threshold, i.e. the first current step that evoked a spike, did not differ among the groups in L2/3 (vehicle, 184.6 ± 19.92; hormone, 212.5 ± 24.71; P40, 200 ± 13.87; pA; p=0.70; Fig. 15C) or in L5 (vehicle, 208.8 ± 16.74; hormone, 179.2 ± 14.38; P40, 171.9 ± 12.88; pA; p=0.18; Fig. 15C). The number of spikes evoked during a 500ms current step 100pA above spiking threshold also did not differ among the groups in L2/3 (vehicle, 28.79 ± 0.82; hormone, 30.11 ± 1.80; P40, 28.29 ± 1.02; p=0.57; Fig. 15D) or in L5 (vehicle, 28.18 ± 0.86; hormone, 31.36 ± 1.64; P40, 28.67 ±

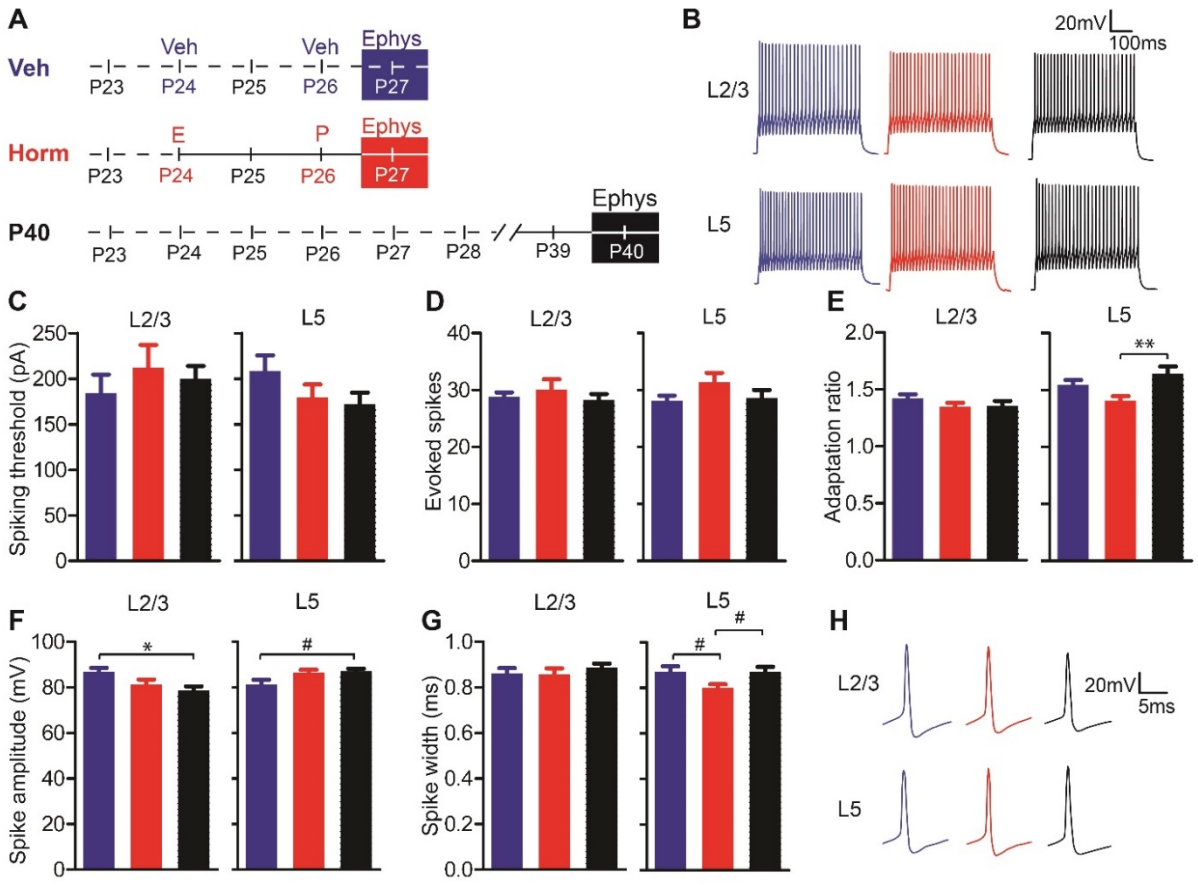

Figure 15. Spike amplitude changes from pre-pubertal to late-pubertal ages, but neither natural puberty nor hormone treatment alter other spiking properties. (A) Treatment groups and N's were the same as those described above for Fig. 10. PV cells in L2/3 and L5 were whole cell patch clamped, and spiking properties were measured in response to a 500ms current pulse 100pA above spiking threshold, where spiking threshold was defined as the first current step that evoked an action potential (see Methods). (B) Sample spike trains during 500ms current pulses 100pA above spiking threshold. Spiking threshold (C) and the number of evoked spikes (D) did not differ among the groups. (E) Adaptation ratio did not differ among the groups in L2/3 but did differ among the groups in L5, with hormone-treated mice showing lower adaptation ratio than P40 mice. (F) Spike amplitude was lower in vehicle-treated (i.e. pre-pubertal) mice compared to P40 (i.e. late-pubertal) mice in L2/3, while spike amplitude was higher in vehicle-treated compared to P40 mice in L5. (G) Spike width did not differ among the groups in L2/3 but did differ among the groups in L5, with a trend toward hormone-treated mice showing shorter spike width than vehicle-treated and P40 mice. (H) Sample traces of single spikes recorded during current pulses 100pA above spiking threshold. All graphs are mean ± S.E.M. Hypothesis tests conducted using linear mixed models. Post-hoc tests were conducted using Tukey's HSD. *adjusted $p<0.05$. #adjusted $p<0.1$.

1.36; p=0.17; Fig. 15D). Adaptation ratio measured 100pA above spiking threshold also did not differ among the groups in L2/3 (vehicle, 1.42 ± 0.037; hormone, 1.35 ± 0.037; P40, 1.36 ± 0.042; p=0.44; Fig. 15E). Adaptation ratio differed among the groups in L5 (vehicle, 1.55 ± 0.043;

hormone, 1.41 ± 0.038; P40, 1.65 ± 0.063; p=0.005; Fig. 15E). Tukey post-hoc comparisons revealed higher adaptation ratio in P40 mice compared to hormone-treated mice (adjusted p=0.003; Fig. 15E) but no difference between vehicle- and hormone-treated mice (adjusted p=0.14; Fig. 15E) or between vehicle-treated and P40 mice (adjusted p=0.29; Fig. 15E). Spike amplitude, measured from the first spike evoked 100pA above spiking threshold, differed among the groups in L2/3 (vehicle, 86.79 ± 1.85; hormone, 81.34 ± 2.15; P40, 78.72 ± 1.76; mV; p=0.025; Fig. 15F) and in L5 (vehicle, 81.36 ± 2.05; hormone, 86.56 ± 1.16; P40, 87.16 ± 1.17; mV; p=0.048; Fig. 15F). Tukey post-hoc comparison revealed higher amplitude in vehicle-treated compared to P40 mice in L2/3 (adjusted p=0.021; Fig. 15F) and a trend toward lower amplitude in vehicle-treated compared to P40 mice in L5 (adjusted p=0.052; Fig. 15F). Tukey post-hoc comparisons revealed no difference between vehicle- and hormone-treated mice in L2/3 or L5 (adjusted p=0.17 in L2/3; adjusted p=0.18 in L5; Fig. 15F). Tukey post-hoc comparisons also revealed no difference between hormone-treated and P40 mice in L2/3 or in L5 (adjusted p=0.73 in L2/3; adjusted p=0.93 in L5; Fig. 15F). Spike width, measured for the first spike evoked 100pA above spiking threshold, did not differ among the groups in L2/3 (vehicle, 0.86 ± 0.022; hormone, 0.86 ± 0.027; P40, 0.89 ± 0.018; ms; p=0.80; Fig. 15G). Spike width differed among the groups in L5 (vehicle, 0.87 ± 0.022; hormone, 0.80 ± 0.015; P40, 0.87 ± 0.022; ms; p=0.039; Fig. 15G). Tukey post-hoc comparisons revealed a trend toward lower spike width in hormone-treated compared to vehicle-treated mice (adjusted p=0.055; Fig. 15G) and a trend toward lower spike width in hormone-treated compared to P40 mice (adjusted p=0.068; Fig. 15G) but no difference between vehicle-treated and P40 mice (adjusted p=1.00; Fig. 15G).

Spine pruning and stabilization across adolescence in Layer 5 cells are age-dependent and not hormone-dependent processes

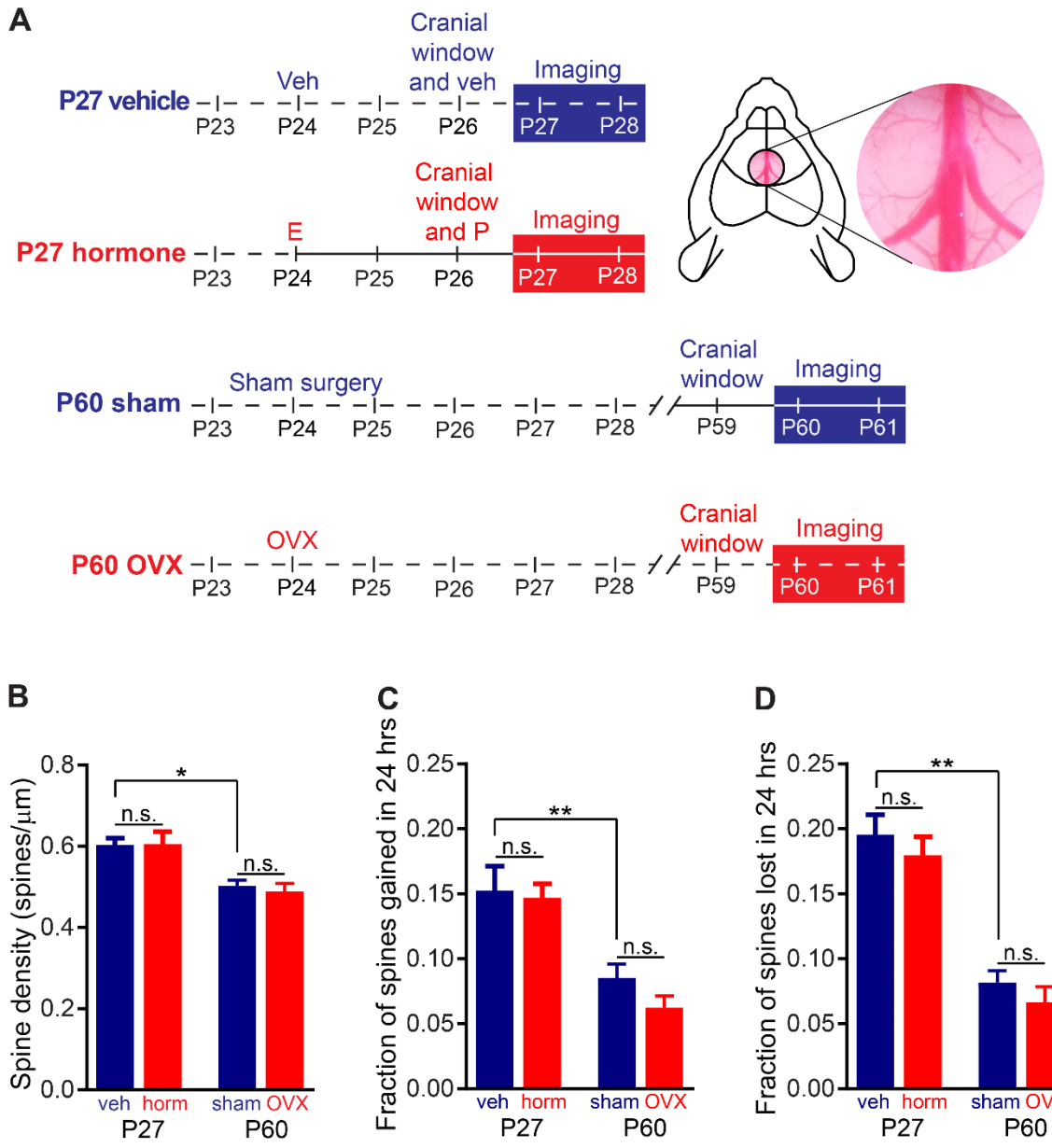

Figure 16. Pruning and stabilization of spines on layer 5 cells across adolescence are age-dependent and not hormone-dependent processes. (A) To model earlier puberty, intact female mice were injected with estradiol at P24 and progesterone at P26 (N=12 mice), while controls were injected with vehicle at both ages (N=12 mice). To prevent pubertal exposure to gonadal hormones, female mice were ovariectomized (OVX) or received sham surgery at P24 (N=8 OVX mice and 8 sham mice). A cranial window was placed over dmPFC anterior to bregma as shown in the schematic. The inset is a photograph of a representative window taken at the end of cranial window surgery. Apical dendrites in L1 of dorsomedial frontal cortex were imaged at P27-P28 in vehicle- and hormone-treated mice and in young adulthood (age range P55-65, labeled as P60) in OVX and sham mice. Spine density (B), spine gains (C), and spine losses (D) decreased from P27 to P60 in control (i.e. vehicle-treated or sham) mice. Neither hormone injections nor ovariectomy altered spine density (B), spine gains (C), or spine losses (D). All graphs are mean ± S.E.M. *Sidak's adjusted $p<0.05$, **Sidak's or Dunn's adjusted $p<0.01$. Sample images are shown below in Fig. 17.

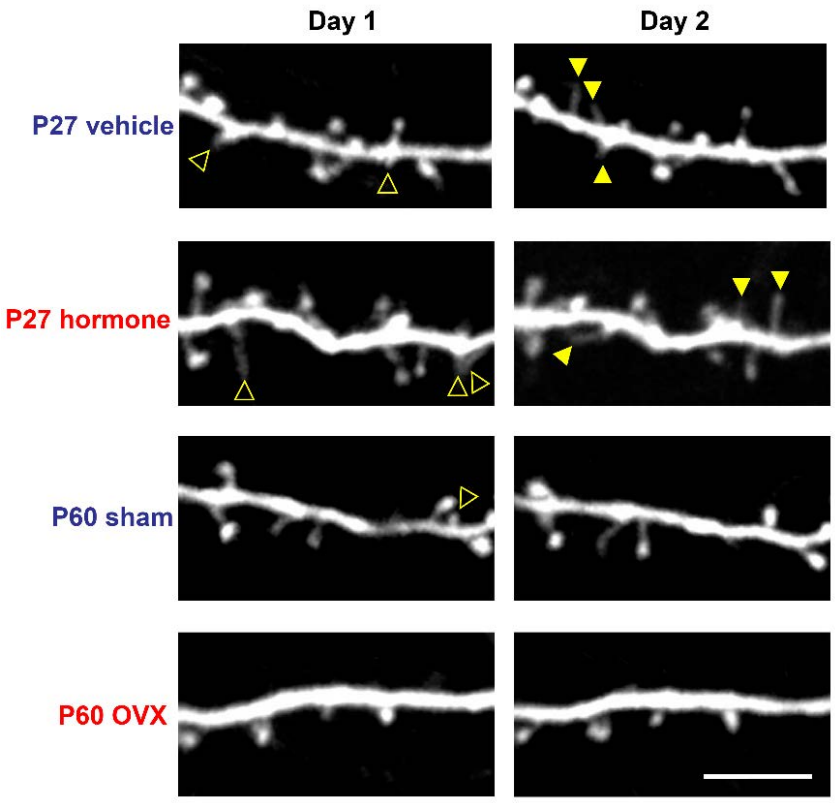

Figure 17. Sample images of spines analyzed for Fig. 16. Open triangles mark spines that were lost between imaging days 1 and 2; filled triangles mark spines that were gained between imaging days 1 and 2. One spine that was not analyzed appears on the right side of the P27 hormone image for day 1; the primary protrusion of this spine was in the z plane. Scale bar represents 5μm.

Dendritic spine density and turnover in dmPFC decrease from pre- to post-pubertal ages in male mice (Johnson et al., 2016a), and spine density in cortical pyramidal cells can be decreased by estrogen receptor activation in adult male mice (Tan et al., 2012), suggesting that spine dynamics may respond to pubertal hormone exposure. We therefore assayed spine dynamics in Layer 1 apical dendrites of Layer 5 pyramidal cells in hormone- and vehicle-treated female mice at P27-28 and in young adult mice (age range P55-65, labeled as P60 in Figs 16-17) that underwent OVX or sham surgery before puberty (Fig. 16A). All imaged mice were from the YFP-H line, which labels a subset of Layer 5 pyramidal cells. We hypothesized that hormonally unmanipulated female mice would show spine pruning and stabilization from pre-pubertal (P27) to young adult (P60) ages, as previously observed in male mice (Johnson et al., 2016a). We also hypothesized that hormone treatment would induce a precocious reduction in spine density and turnover in P27 mice and that pre-pubertal OVX would prevent the developmental reduction in spine density and turnover in mice imaged at P60. We found that the 4 treatment groups differed in spine density (Fig. 16B; $F_{3,36}=6.99$, $p=0.0008$), spine gains (Fig. 16C; $F_{3,36}=9.55$, $p<0.0001$), and spine losses

(Fig. 16D; H=23.66, p<0.0001). To determine whether hormonally unmanipulated female mice show spine pruning and stabilization across adolescence, we compared P27 vehicle-treated mice to P60 sham mice. P60 sham mice showed lower spine density (Fig. 16B; Sidak's adjusted p=0.015), lower spine gains (Fig. 16C; Sidak's adjusted p=0.0068), and lower spine losses (Fig. 16D; Dunn's adjusted p=0.0021) compared to P27 vehicle-treated mice. Gonadal hormone exposure during puberty was not necessary for this developmental reduction in spine density and turnover; P60 OVX mice did not differ from P60 sham mice in spine density (Fig. 16B; Sidak's adjusted p=0.98), gains (Fig. 16C; Sidak's adjusted p=0.68), or losses (Fig. 16D; Dunn's adjusted p>0.99). Pre-pubertal hormone treatment did not alter spine density or turnover; hormone-treated mice did not differ from vehicle-treated mice in spine density (Fig. 16B; Sidak's adjusted p>0.99), gains (Fig. 16C; Sidak's adjusted p=0.99), or losses (Fig. 16D; Dunn's adjusted p>0.99). These results indicate that spine pruning and stabilization occur across adolescence in female mice and that in the Layer 5 neurons investigated here, these processes do not depend on gonadal hormone exposure during puberty.

Exposure to gonadal hormones alters performance in a cognitive task sensitive to the integrity of the dorsomedial frontal cortex

The above results suggest that pubertal hormone exposure rapidly induces changes in some, but not all, aspects of cingulate cortex maturation. We next sought to determine if pubertal hormones alter behavior dependent on this brain region. We previously established that excitotoxic lesions centered on the cingulate disrupt performance in an odor-based 4-choice reversal task in both juveniles and adults (Johnson and Wilbrecht, 2011). Performance in this task also changes significantly with development such that mice at P26 show more efficient reversal learning compared to mice P40 or older (Johnson and Wilbrecht, 2011; Piekarski et al., 2017b). We predicted that early hormone exposure would prematurely induce adult-like performance in the reversal phase of the task, indicated by an increase in trials to criterion.

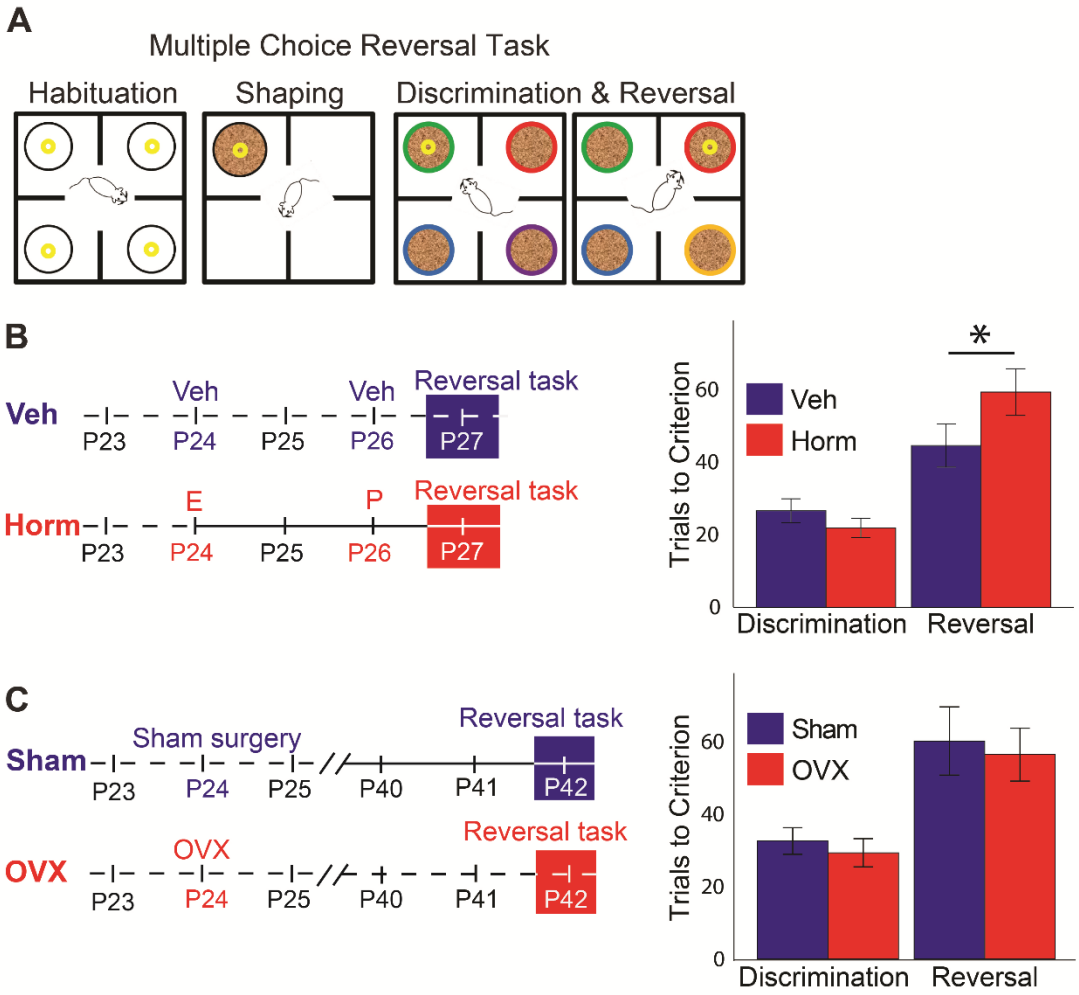

Figure 18. Hormone exposure induces early adult-like performance in a 4-choice odor based reversal learning task. (A) A schematic of the three-day protocol used for the behavior test measuring discrimination learning, in which mice learn to choose 1 out of 4 odors, and reversal learning, in which they have to update this rule when the reward contingency is changed to a new odor. (B) Littermate mice housed in pairs were either treated with gonadal steroids or vehicle at P24 and P26 (n=11 each group). Mice were habituated to the food reward on P25, shaped to dig in bowls on P26, and run in both the discrimination and reversal phase of the task on P27. There were no differences between groups during the discrimination phase, but hormone treated mice required significantly more trials to reach criterion in the reversal phase. This shift in behavioral performance occurs in a direction consistent with what would be expected to occur with development by P40 (Johnson and Wilbrecht, 2011; Piekarski et al., 2017a). (C) Prepubertal OVX or sham OVX groups did not differ on either discrimination or reversal when tested between P40 and P45. Given that maternal effects can impact performance on this this task (Thomas et al., 2016), groups were compared using F-tests controlling for litter/cage. All graphs are mean ± S.E.M. *p<0.05.

Mice were treated at P24 and P26 with either hormone or vehicle (as above) and tested on the 4-choice discrimination and reversal task at P27-28 (Fig. 18A). As predicted, hormone

treated mice required more trials to reach criterion during the reversal phase than littermate vehicle controls (controlling for cage; $F_{1,10}$=5.50; p=0.041; Fig. 18B). Hormone and vehicle pairs did not differ in the discrimination phase ($F_{1,10}$=1.33; p=0.28; Fig. 18B), suggesting that more basic aspects of task performance were comparable.

In order to determine if hormones were necessary for the developmental increase in trials to criterion in this task, we also performed OVX or sham OVX at P24 and tested mice under the same paradigm between P40 and P45 (Fig. 18C). OVX and sham groups did not differ in performance in the discrimination ($F_{1,9}$=0.27; p=0.62; Fig. 18C) or reversal phase ($F_{1,9}$=0.09; p>0.77; Fig. 18C).

These data suggest hormones can prematurely induce adult-like reversal learning performance, but that maturation of reversal learning can still occur in the absence of intact gonads.

Discussion

In the present report, we provide evidence that the rise in gonadal steroids at puberty is necessary for a developmental increase in phasic inhibitory neurotransmission in anterior cingulate cortex during adolescence. These data demonstrate that puberty itself, not just age, plays a role in frontal cortex maturation, supporting a broad literature of correlational studies that suggest both puberty and its timing play an important role in the development of associative neocortex function, plasticity, and associated pathology.

Our current study included only females, however, we previously found that inhibitory neurotransmission matures similarly between P25 and P40 in layer 5 in males and females (Vandenberg et al., 2015). Androgen and estrogen receptor expression is also comparable between sexes in rats (Kritzer, 2004), and aromatase can convert androgens to estrogens,

enabling signaling via estrogen receptors in either sex. Future studies in males will be needed to determine if the present results replicate in males.

Understanding the full cascade of pubertal changes—from gonad to cortical circuit—will require continued investigation

Changes to mIPSC frequency likely reflect changes to the presynaptic cell, while changes to mIPSC amplitude are thought to reflect the number of GABA receptors on the postsynaptic membrane. Because we primarily observe an effect of gonadal steroids on mIPSC frequency, our data suggest that gonadal steroids may primarily affect presynaptic inhibitory cells. An increase in mIPSC frequency could be caused by a larger number of synapses, higher probability of vesicle release, or an increase in the number of docked synaptic vesicles per bouton. We found that early hormone exposure caused inhibitory synapses to become more depressing, suggesting that the release probability in GABAergic terminals was increased, which would enhance measures of mIPSC frequency and total inhibitory charge transfer. However, increased number of synapses or docked vesicles could still contribute.

Because all inhibitory inputs onto patched pyramidal cells were recorded, it remains unknown what interneuron subtypes mediate the increase in phasic inhibition, but a strong candidate cell type is the PV+ fast-spiking interneuron. In the rodent frontal cortex, estrogen receptor beta is expressed primarily in these interneurons (Blurton-Jones and Tuszynski, 2002; Kritzer, 2004), providing a mechanism for pubertal estrogens to act specifically on these cells. Although we did not see hormone-induced changes in intrinsic or spiking properties of PV cells themselves (Figs. 14-15), future experiments directly testing inputs from PV cells onto L2/3 pyramidal cells may reveal hormone effects. For example, the hormone-induced increase in mIPSC frequency we observed could result from an increase in the probability of GABAergic vesicle release (an idea supported by the PPR result in Fig. 12I) and/or an increase in the number of GABAergic synapses onto L2/3 pyramidal cells. If PV cells are the presynaptic cell type

responsible for these changes, then these changes should be observable through simultaneous whole-cell patch clamp of synaptically connected PV and pyramidal cells.

Increases in inhibition have been implicated in regulating sensitive periods in neocortex

Studies in sensory cortices have identified numerous sensitive periods that vary by cortical region and input (de Villers-Sidani et al., 2007; Fox, 1992; Lu et al., 2001). A common mechanism linking many of these identified sensitive periods is a change in E/I balance, driven largely by

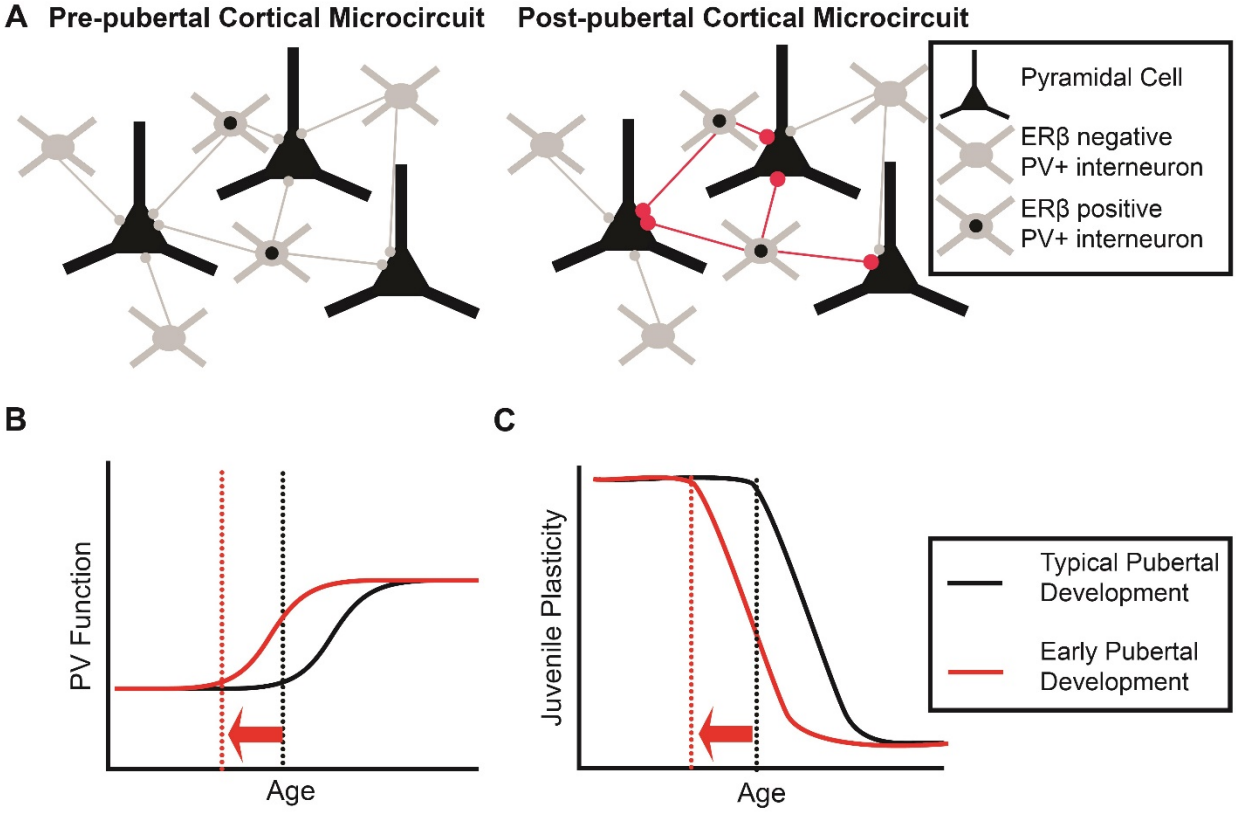

Figure 19. Model for the mechanism by which pubertal hormones may increase inhibition in the frontal cortex and regulate juvenile and adolescent plasticity. This model is adapted from work conducted in primary sensory cortices implicating PV-expressing interneurons in regulation of sensitive period plasticity. (A) Before puberty, circulating gonadal steroids are low. At puberty onset, circulating gonadal steroids rise, and estradiol (either produced in the ovaries or locally synthesized from circulating testosterone) binds to estrogen receptor beta (ERβ), which is expressed in a subset of PV+ interneurons. ERβ then acts as a transcription factor to increase these cells' inhibitory output (red axons and terminals). Because of their dense connectivity, increased inhibition from a minority of PV+ interneurons could affect all local pyramidal cells. (B) In this model, early puberty results in earlier increases in PV function and (C) premature closure of juvenile plasticity. Figure adapted from (Piekarski et al., 2017b).

increased inhibitory neurotransmission. For example, in the literature on ocular dominance in the visual cortex, pharmacologically increasing inhibition can prematurely open (Fagiolini and Hensch, 2000) and close (Iwai et al., 2003) sensitive period plasticity, while reducing inhibition in adulthood can reopen sensitive period plasticity (Harauzov et al., 2010). Changes in mIPSCs in the visual cortex also track shifts in ocular dominance plasticity, such that an increase in mIPSC frequency onto L2/3 pyramidal neurons occurs with age (from week 3 to week 5) and is blocked by dark rearing, an environmental manipulation that delays sensitive period onset (Morales et al., 2002).

The present data demonstrate that pubertal hormones can be causally linked to an increase in phasic inhibition in the frontal cortex, providing a potential mechanism by which puberty could regulate E/I balance and putative sensitive periods for plasticity in this region (Fig. 19). Importantly, hormone treatment did not strengthen inhibitory neurotransmission in the somatosensory cortex, demonstrating regional specificity of the effect. It remains unclear how many regions of cortex are affected by pubertal hormones, but it is possible that associative cortex may be particularly sensitive to puberty. If pubertal hormones regulate sensitive periods of plasticity and learning in frontal cortex in the human brain, the timing of educational and psychiatric interventions in relation to puberty may be critically important for their effectiveness (Blakemore, 2010; Wright and Zecker, 2004).

Early maturation of a frontal cortex dependent behavior can be elicited by hormonal treatment, but maturation was not blocked by ovariectomy

It is impossible to investigate human language learning and many education relevant variables in mice, but we can probe simpler functions of the frontal cortex in mouse models. We have previously established that performance in an odor-based reversal learning task in mice is dependent upon the integrity of the cingulate and surrounding frontal regions (Johnson and Wilbrecht, 2011) and matures between P26 and P40 (Johnson and Wilbrecht, 2011; Piekarski et

al., 2017b). Here, our mouse model of earlier puberty showed more adult-like performance at P27, but prepubertal ovariectomy did not prevent adult-like performance at P40. We speculate that capacity for learning or plasticity surrounding the time of puberty onset may be altered, but puberty-independent and/or compensatory processes may be sufficient to eventually produce adult-form behavioral performance in ovariectomized mice.

Hormone effects on some, but not all, aspects of circuit maturation suggest that developmental mismatches may occur with hormone perturbation

Our results suggest that hormones drive maturation of inhibitory neurotransmission onto Layer 2/3 pyramidal cells, while excitatory neurotransmission in Layer 2/3 and dendritic spine dynamics in Layer 5 cells were unaffected by hormone treatment over the same time course that we observed hormone effects on inhibitory neurotransmission in Layer 2/3. We have not yet tested if hormonal manipulation alters the density and turnover of dendritic spines in Layer 2/3 neurons. Layer 2/3 and Layer 5 of the cortex are generated and mature at different times, and cell types within these regions may also develop differently. In the YFP-H mouse line we used for spine imaging, only a subset of Layer 5 neurons are labeled. Inhibitory inputs change over the adolescent period only on YFP-negative cells (Vandenberg et al., 2015), which are enriched by the intra-telencephalic (IT) cell type that projects within the neocortex, similar to Layer 2/3 pyramidal neurons. We speculate that future studies isolating these IT cell types may still reveal hormone sensitive adolescent maturation of spine dynamics, due to greater evidence of maturation of inhibition on these neurons over adolescence. As we increase our knowledge, we speculate that we will find that some developing systems are sensitive to pubertal hormones while others are not, and the timing of puberty onset may alter the phase-relationship between a number of intricately interconnected neural systems, resulting in different neural, emotional, and behavioral outcomes mediated by age at pubertal onset.

Puberty onset is advancing to earlier ages, and early puberty is associated with disadvantage and greater disease burden

Adolescence is a paradoxical life stage in which physical health is strong, but there are increasing risks for wide ranges of psychiatric disorders and other negative behavioral outcomes (Graber, 2013; Paus et al., 2008; Silberg et al., 1999; Whittle et al., 2012). Earlier than normal puberty onset is associated with increased risk of psychiatric disease and lower educational achievement (Burt, 2006; Copeland et al., 2010; Ge et al., 2003; Graber et al., 2004; Kaiser and Gruzelier, 1996, 1999; Obeidallah et al., 2009; Tschann et al., 1994; Whittle et al., 2012; Zehr et al., 2007). In recent decades, the age at puberty onset has significantly advanced, particularly in girls (Aksglaede et al., 2009; Herman-Giddens, 2006; Marshall and Tanner, 1969; Rosenfield et al., 2009), with the youngest ages observed in urban poor, African American, and Hispanic populations (Biro et al., 2013). It is difficult to determine if negative psychiatric and educational outcomes associated with early puberty are mediated by direct effects of pubertal hormones on the brain, indirect effects of psychosocial difficulties associated with early puberty, or complex interactions between pubertal timing and psychosocial factors. However, the frontal cortex is implicated in many of these psychiatric and behavioral outcomes, and our data demonstrate that maturation of this region is directly impacted by gonadal hormones.

Conclusion

Our data suggest that pubertal hormones are critical for the maturation of inhibitory neurotransmission in the frontal cortex. Further work is required to understand the mechanisms by which gonadal maturation impacts neuronal development and how inhibitory neurotransmission alters plasticity of frontal cortical networks. Given that inhibitory neurotransmission regulates sensitive periods in other cortical regions and is implicated in psychopathology, we conclude that pubertal timing likely plays a powerful role in frontal cortex development. To fully understand normative and pathological development of brain and behavior,

measures of puberty onset and changes in circulating gonadal hormones are important variables to consider in future investigations of adolescent brain maturation.

Future directions: pubertal development and affective behavior.

In this chapter, we have shown that pubertal hormones influence the development of frontal circuitry and a specific behavior that depends on the integrity of this circuitry: flexible learning in a 4-choice reversal task. Frontal circuits, through their interaction with subcortical limbic structures, are also critical for anxiety- and depression-related behavior (Felix-Ortiz et al., 2016). The peripubertal period is a time of intense vulnerability to anxiety and depression symptoms in humans (Costello et al., 2011). It is therefore critical to understand the mechanisms by which adolescent maturation alters anxiety- and depression-related behavior. In the next chapter, we focus on the roles of age, sex, and gonadal hormones in the maturation of anxiety- and depression-related behavior during puberty.

Chapter 4. Age, sex, and gonadal hormones differently influence anxiety- and depression-related behavior during puberty in mice

These results have been submitted for publication in the following manuscript:

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*contributed equally

This project was performed jointly with David Piekarski, with behavior testing assistance from Jessica Wahlberg.

Introduction

Anxiety and depression symptoms increase in early adolescence, particularly in girls (Altemus et al., 2014; Burke et al., 1990; Costello et al., 2011; Ge et al., 2001; Hayward and Sanborn, 2002; Kessler et al., 2005; Kessler et al., 1994; Silberg et al., 1999). Gonadal hormones and sexual maturation of the body are hypothesized to play a role in this increase, but the causal route by which this effect occurs is debated (Graber, 2013). Multiple studies have found that pubertal status in girls is a better predictor of anxiety and depression symptoms than age (Angold et al., 1999; Angold et al., 1998; Brooks-Gunn and Warren, 1989; Reardon et al., 2009), and girls who start puberty earlier than their peers have higher risk of various negative mental health outcomes, including anxiety and depression symptoms (Blumenthal et al., 2011; Boden et al., 2011; Copeland et al., 2010; Crockett et al., 2013; Deardorff et al., 2007; Deng et al., 2011; Ge et al., 2006; Graber et al., 1997; Graber et al., 2004; Hayward et al., 1997; Kaltiala-Heino et al., 2003a; Kaltiala-Heino et al., 2003b; Mendle et al., 2007; Negriff et al., 2008; Stice et al., 2001). Data in boys is less consistent, with some studies demonstrating associations between pubertal status/timing and mental health outcomes, but the direction of these relationships differs across

studies (Crockett et al., 2013; Deardorff et al., 2007; Graber, 2013; Graber et al., 1997; Graber et al., 2004; Mendle and Ferrero, 2012; Reardon et al., 2009). Importantly, in both boys and girls, social and environmental factors interact with pubertal status and timing to impact mental health outcomes, making it difficult to distinguish direct effects of hormones from other factors (Bamaca-Colbert et al., 2012; Brooks-Gunn and Warren, 1989; Caspi and Moffitt, 1991; Deardorff et al., 2013; Ge et al., 2002; Ge et al., 1996, 2001; Hayward et al., 1999; Lynne et al., 2007; Obeidallah et al., 2004; Rudolph and Troop-Gordon, 2010; White et al., 2013).

Given the difficulty of disentangling multiple variables in humans, it is valuable to turn to animal models. In rodents, gonadal hormones can be manipulated through gonadectomy and hormone injection to test for causal relationships between hormones and anxiety- and depression-related behavior. In most studies in adult female rodents, estradiol, progesterone, and androgens reduce anxiety- and depression-related behavior (Bernardi et al., 1989; Bitran et al., 1995; Estrada-Camarena et al., 2003; Frye, 2011; Frye and Wawrzycki, 2003; Hilakivi-Clarke, 1996; Koonce and Frye, 2013; Lund et al., 2005; Marcondes et al., 2001; Martinez-Mota et al., 1999; Mora et al., 1996; Nomikos and Spyraki, 1988; Okada et al., 1997; Rachman et al., 1998; Walf and Frye, 2005a, b; Walf et al., 2009; Walf et al., 2008; Walf et al., 2004). A smaller number of studies, again in adult female rodents, have found anxiogenic effects of progesterone (Galeeva and Tuohimaa, 2001) and estradiol (Morgan and Pfaff, 2001, 2002). In adult male rodents, both androgens and estrogens reduce anxiety- and depression-related behavior (Chen et al., 2014; Frye et al., 2008; Frye and Wawrzycki, 2003; Hilakivi-Clarke, 1996; Wainwright et al., 2016).

Despite the large number of studies on the role of gonadal hormones in anxiety- and depression-related behavior in adult animals, data in peripubertal animals is lacking. It is critical to test hormone effects specifically during the developmental window surrounding puberty, because gonadal hormones can elicit starkly different effects at different ages (Sisk and Zehr, 2005). For example, certain gonadal hormone metabolites can influence anxiety-related behavior in opposite directions during puberty compared to adulthood (Shen et al., 2007), suggesting that

results from adult animals cannot simply be extrapolated to pubertal animals. Anxiety- and depression-related behavior also change across adolescence in male mice, but the role of gonadal hormones and potential sex differences in this process are unknown (Hefner and Holmes, 2007). Finally, puberty is thought to be a sensitive period for hormone-related circuit reorganization (Byrne et al., 2016; Cunningham et al., 2002; Peper and Dahl, 2013; Piekarski et al., 2017a; Piekarski et al., 2017b; Romeo, 2003; Schulz et al., 2009; Sisk and Zehr, 2005), underscoring the importance of understanding how pubertal hormones interact with age and sex to influence the development of brain and psychopathology.

In the current project, our goal was to determine if gonadal hormones cause the development of anxiety- and depression-related behavior at puberty in both males and females. To this end, we manipulated peripubertal gonadal hormone exposure and measured anxiety-related behavior, depression-related behavior, and repetitive/compulsive behavior with the elevated plus maze (EPM), open field test, forced swim test (FST), and the marble burying test. We found that gonadal hormones alter anxiety-related behavior in a sex-specific manner during puberty. Males castrated before puberty showed greater anxiety-related behavior than intact males, while females were unaffected by pre-pubertal ovariectomy or hormone injections in all assays except the marble burying test. In contrast, depression-related behavior increased from pre-pubertal to late-pubertal ages but was unaffected by pre-pubertal gonadectomy and did not differ between sexes. Interestingly, despite the sex-specific effect of gonadectomy on anxiety-related behavior during puberty, we found no sex differences in intact male and female mice tested in young adulthood. This pattern indicates that a similar adult behavioral phenotype may be achieved by different mechanisms in male and female mice (De Vries and Panzica, 2006). In conclusion, our data show that an increase in depression-related behavior at puberty can be modeled in mice. Furthermore, our mouse models suggest anxiolytic effects of testicular hormones during puberty but do not support a causal role for ovarian hormones in the etiology of

anxiety and depression symptoms during puberty. We discuss alternate factors that may explain sex differences observed in humans.

Methods

Animals

Male and female C57BL/6N mice (Charles River Laboratories, Wilmington, MA) were bred in our animal facility and were housed on a 12h/12h reverse light-dark cycle (lights on at 10pm). Mice were weaned at postnatal day (P) 21 and housed in groups of 2-5 same-sex siblings with nesting material and a paper hut. All mice had *ad libitum* access to food and water in their home cages. All procedures were approved by the UC Berkeley Animal Care and Use Committee.

Experimental groups

In the first set of experiments, peripubertal changes in anxiety- and depression-related behavior were studied in separate groups of male and female mice tested either before puberty (first day of testing on P24) or during late puberty (first day of testing between P40 and P47) (Fig. 20A).

To test if gonadal hormones mediate peripubertal changes in anxiety- and depression-related behavior, male and female mice were gonadectomized or sham gonadectomized prior to puberty (details below) and were tested for anxiety- and depression-related behavior during late pubertal ages (first day of testing between P40 and P47) (Fig. 21A).

To test if early-onset puberty could alter anxiety- and depression-related behavior, female mice were injected with estradiol and progesterone or oil vehicle to induce early puberty onset (details below) and were tested for anxiety- and depression-related behavior at ages when vehicle control animals were still pre-pubertal (first day of testing on P27) (Fig. 22A).

To test whether sex differences in anxiety- and depression-related behavior would emerge or persist into early adulthood, male and female mice with intact gonads were tested in young adulthood (first day of testing between P69 and P83) (Fig 23A).

Gonadectomies

Surgeries took place on P24 or P25, before puberty onset. Prior to surgery, mice were injected with 0.05mg/kg buprenorphine and 10mg/kg meloxicam subcutaneously. During surgery, animals were anesthetized with 1-2% isoflurane. The incision area was shaved and scrubbed with ethanol and betadine. Ophthalmic ointment was placed over the eyes to prevent drying. A 1 cm incision was made with a scalpel in the lower abdomen across the midline to access the abdominal cavity. For ovariectomies, the ovaries were clamped off from the uterine horn with locking forceps and ligated with sterile sutures. After ligation, ovaries were excised with a scalpel. For castrations, the blood supply to each testis was clamped with locking forceps, after which the testes were ligated with sterile sutures and excised with a scalpel. The muscle and skin layers were then sutured, and wound clips were placed over the incision for 7-10 days to allow the incision to heal. An additional injection of 10mg/kg meloxicam was given 12-24 hours after surgery. Sham control surgeries were identical to ovariectomies and castrations except that the ovaries/testes were simply visualized and were not clamped, ligated or excised. Mice were allowed to recover on a heating pad until ambulatory and were post-surgically monitored for 7-10 days to check for normal weight gain and signs of discomfort/distress. No mice were eliminated from study due to surgical complications.

Mouse model of early female puberty

To advance age at puberty onset, gonadally intact females were injected with 17 beta-estradiol (0.01mg/kg subcutaneous) or vehicle at P24. At P26, mice were injected with progesterone (20mg/kg subcutaneous) or vehicle (Piekarski et al., 2017a). This treatment

advances first peripubertal exposure to gonadal steroids and is sufficient to induce endogenous puberty (Ramirez and Sawyer, 1965; Smith and Davidson, 1968).

Vehicle- and hormone-treated mice were visually assessed for vaginal opening, an indicator of puberty onset in female mice, after the last behavior test was completed on P28. All hormone-treated mice had undergone vaginal opening on P28, while all but one vehicle-treated mouse had not yet undergone vaginal opening at P28. The one vehicle-treated mouse that had undergone vaginal opening by P28 was excluded from all analyses.

Behavioral test battery

Mice were gently handled for 1 minute each day for 2 days before the start of behavioral testing to habituate them to handling.

All groups of mice experienced the same behavior test battery. Behavior testing took place over 2 consecutive days. On the first day, mice were tested on the elevated plus maze (EPM) and then immediately transferred to the open field test. On the second day, mice were tested on the marble burying test and then immediately transferred to the forced swim test (FST). All testing took place during the last 3 hours of the light cycle (7am-10am). On each testing day, mice were allowed to habituate to the testing room in their home cages for 30 minutes before testing began.

Elevated plus maze (EPM)

The mouse was placed in the center of an elevated plus maze and allowed to explore freely for 10 minutes. The EPM was made of opaque white acrylic consisting of 2 open arms (30cm long by 6cm wide), 2 closed arms (30cm long by 6cm wide, with 20.5cm high walls on the sides and end of each arm), and a center square (6cm by 6cm). The closed arms of the EPM were attached to a stable platform raised 66cm from the floor. The number of entries into each zone (i.e. center, open, or closed) and the total time spent in each zone was analyzed using

EthoVision software (Noldus; Sacramento, CA). The chamber was cleaned with 70% ethanol and allowed to dry between mice. The EPM was performed with room lights on (260 lux).

Open field test

Immediately after finishing the elevated plus maze, the mouse was transferred to a clear acrylic open field arena (42cm by 42cm floor dimensions, with 4 walls that were each 30.5cm high, and no ceiling) for 15 minutes. The acrylic open field arena was located inside a sound-attenuated chamber (Med Associates; Fairfax, VT) with lights on (40 lux inside the chamber). Locomotion was monitored using infrared beam breaks (Versamax, AccuScan Instruments; Columbus, OH). Total distance covered and percent of time spent in the center (defined as >7.875cm from the edges of the chamber, i.e. 3 grid squares in Versamax analysis software) were analyzed. The chamber was cleaned with 70% ethanol and allowed to dry between mice. Mice were returned to the home cage immediately after the open field test.

Marble burying test

After 30 minutes of habituation to the testing room, each mouse was moved separately from its home cage to a clean cage containing 3cm deep fresh bedding and 20 marbles arranged in 5 evenly spaced rows on top of the bedding. A lid was placed over the cage, and the mouse was left undisturbed for 30 minutes. The test was performed with room lights on (45 lux inside the cage with the lid on). The number of buried marbles was recorded by the experimenter. “Buried” was defined as >50% covered by bedding. A fresh cage with fresh bedding was used for each mouse.

Forced swim test

Immediately after completing the marble burying test, each mouse was placed in a 2L glass beaker (13cm diameter, 18cm height) containing 10cm deep, room temperature water (21

degrees C). The beaker was located inside a sound-attenuated chamber (Med Associates; Fairfax, VT) with lights on (40 lux inside the chamber). The mouse was left in the beaker for 6 minutes and was monitored by video (Microsoft LifeCam; Redmond, WA). After 6 minutes, the mouse was removed from the water, gently patted with paper towel, and placed in a warmed cage lined with paper towel until dry. Glass beakers were cleaned with 70% ethanol and allowed to dry between mice. Fresh room temperature (21 degrees C) water was used for each mouse. Time spent immobile, defined as the absence of movements except those necessary for balancing the mouse and keeping its head above water (e.g. paddling of a paw for balance) was calculated for the last 4 minutes of each 6-minute video. Each video was scored manually by 2 experimenters blind to the mouse's experimental group. The 2 raters maintained an inter-rater correlation of R=0.96 and a mean difference of 12.45 +/- 8.16 seconds. The mean of the 2 raters' scores was used for each mouse.

Statistical comparisons

All statistical comparisons were performed using GraphPad Prism (GraphPad, San Diego, CA). Data were tested for normality using a D'Agostino and Pearson omnibus normality test, and groups that were directly compared to each other were tested for equal variance using an F test. For comparisons between 2 groups, a t test was used when data were normally distributed, and Welch's correction was applied when variance was unequal. Groups that were not normally distributed were compared using a Mann Whitney U test. When more than 2 groups were considered simultaneously (Fig. 20), a 2-way ANOVA was performed with age and sex as factors. Post-hoc comparisons were then performed as described above (i.e. using a t test or Mann Whitney U test) with Bonferroni corrections for multiple comparisons.

For gonadally intact P40 mice, unmanipulated animals (i.e. those that received no surgery) did not differ from sham-operated mice in any measure tested (EPM: t_{27}=0.43, p=0.67 for females; t_{21}=0.87, p=0.39 for males. Open field: t_{27}=0.88, p=0.39 for females; t_{21}=0.33, p=0.74 for males.

FST: U=78.00, p=0.45 for females; t_{21}=0.22, p=0.83 for males. Marble burying test: U=64.50, p=0.16 for females; t_{21}=1.39, p=0.18 for males. n=19 surgically unmanipulated females; n=16 surgically unmanipulated males; n=10 sham-operated females; n=7 sham-operated males). Unmanipulated and sham animals were therefore combined into a single gonadally intact P40 male group and a single gonadally intact P40 female group for all analyses.

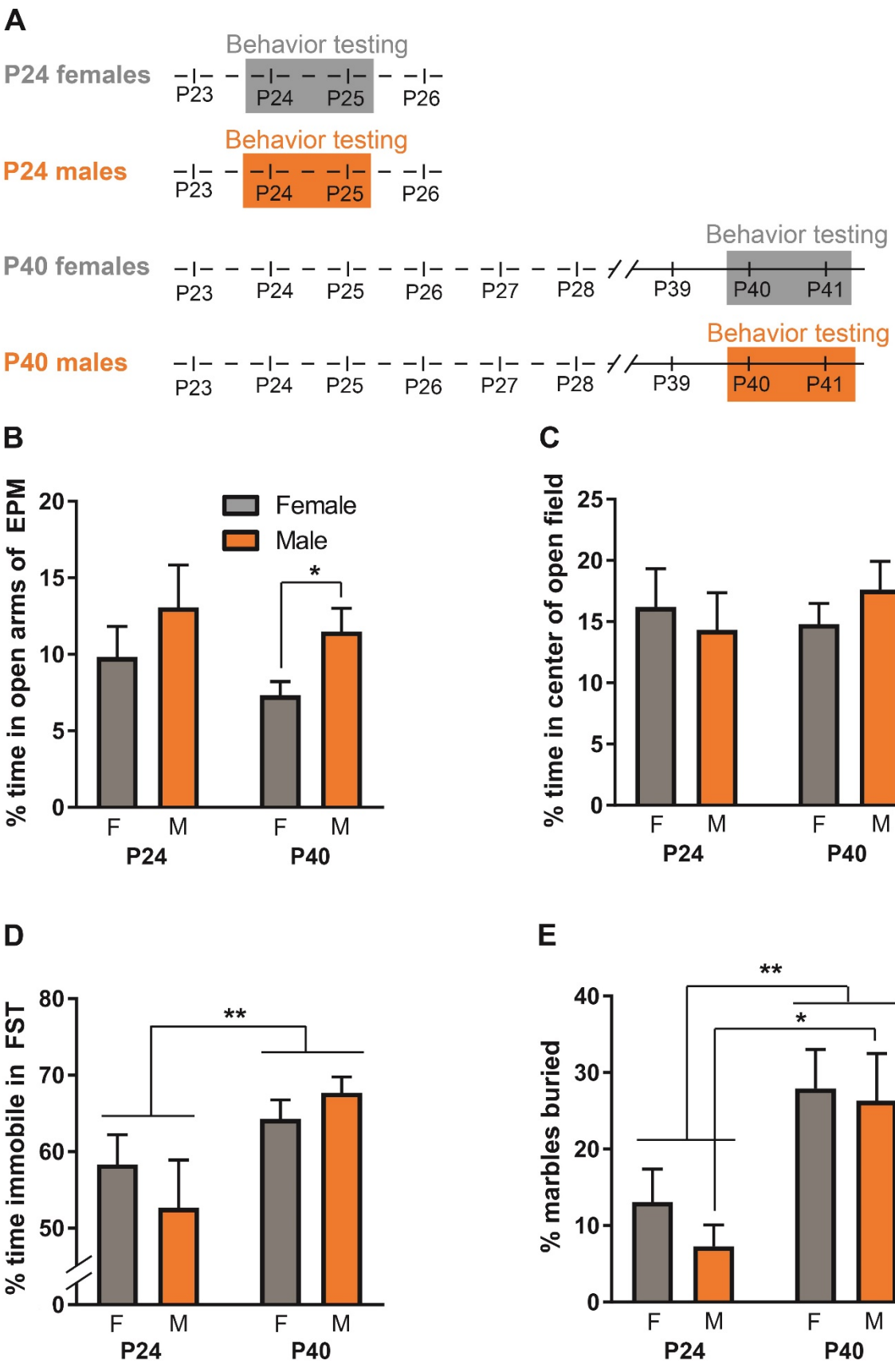
For the analysis shown in Fig. 20, planned comparisons were performed to ask 3 *a priori* questions: 1) Does castration alter anxiety- and depression-related behavior in males, 2) Does ovariectomy alter anxiety- and depression-related behavior in females, and 3) Does the effect of gonadectomy differ between males and females. As these were separate *a priori* questions, multiple comparisons corrections were not applied to these tests.

One animal was excluded from open field analysis due to equipment failure that resulted in data loss. One animal was excluded from EPM analysis due to failure to record the video. One female mouse was excluded from all analyses due to precocious puberty onset, as described above.

Results

Peripubertal changes in anxiety- and depression-related behavior in mice undergoing natural puberty

We first asked whether anxiety- and depression-related behavior change with age from pre-pubertal (P24) to late-pubertal (P40) ages in male and female mice undergoing natural puberty. To this end, separate groups of mice were tested at P24 or P40 on the elevated plus maze (EPM), open field, forced swim test (FST), and marble burying test (Fig. 20A). We found a main effect of sex ($F_{1,72}$=4.92, p=0.03) but no main effect of age ($F_{1,72}$=1.49, p=0.23) or age by sex interaction ($F_{1,72}$=0.07, p=0.79) for the percent of time spent in the open arms of the EPM (Fig. 20B). Post-hoc comparisons revealed that males spent more time in the open arms than females at P40 (t_{37}=2.36, uncorrected p=0.02) but not at P24 (U=51, uncorrected p=0.25). For the


A
Behavior testing
P24 females
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P39
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B
% time in open arms of EPM
Female
Male
*
F
M
F
M
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P40
C
% time in center of open field
F
M
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M
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P40
D
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**
F
M
F
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P24
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E
% marbles buried
**
*
F
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P24
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Figure 20. Peripubertal changes in anxiety- and depression-related behavior. All bars are mean ± SEM. A) Separate groups of male and female mice were tested for anxiety- and depression-related behavior prior to puberty (P24-25) and during late puberty (P40-47). n=13 P24 females; n=11 P24 males; n=29 P40 females; n=23 P40 males. B) Time spent in the open arms of the EPM did not change with age, but P40 males spent more time in the open arms than P40 females. C) Time spent in the center of the open field did not change with age or differ between males and females. D) P40 mice spent more time immobile in the FST compared to P24 mice, with no difference between males and females. E) P40 mice buried more marbles than P24 mice, with no difference between males and females. **p<0.01 for main effect of age. *p<0.05 after Bonferroni correction for multiple comparisons.

percent of time spent in the center of the open field (Fig. 20C), we found no main effect of sex ($F_{1,72}$=0.03, p=0.86), age ($F_{1,72}$=0.14, p=0.71), or interaction ($F_{1,72}$=0.85, p=0.36). In the FST (Fig. 20D), P40 mice spent more time immobile than P24 mice ($F_{1,72}$=9.32, p=0.003), but male and female mice did not differ from each other ($F_{1,72}$=0.11, p=0.74), and there was no age by sex interaction ($F_{1,72}$=1.76, p=0.19). Post-hoc comparisons of P24 to P40 within each sex did not remain significant after Bonferroni corrections for multiple comparisons (t_{12}=2.29, uncorrected p=0.04 for males; U=140, uncorrected p=0.19 for females). Similarly, in the marble burying test (Fig. 20E), P40 mice buried more marbles than P24 mice ($F_{1,72}$=7.60, p=0.007), but there was no effect of sex ($F_{1,72}$=0.37, p=0.55) and no interaction ($F_{1,72}$=0.12, p=0.74). Post-hoc comparisons revealed that P40 males buried more marbles than P24 males (t_{29}=2.80, uncorrected p=0.009), while a comparison of P40 to P24 females did not reach significance (U=136, uncorrected p=0.15). Effects of age or sex on performance in the EPM, FST, and marble burying test were not explained by generalized locomotor differences, as there was no effect of age, sex, or age by sex interaction in locomotor distance covered in the open field (data not illustrated; $F_{1,72}$=0.33, p=0.57 for age; $F_{1,72}$=1.28, p=0.26 for sex; $F_{1,72}$=1.80, p=0.18 for interaction).

Effects of pre-pubertal gonadectomy on anxiety- and depression-related behavior at late-pubertal ages

We next asked whether gonadal hormone exposure during puberty affects anxiety- and depression-related behavior. Male and female mice underwent gonadectomy or sham gonadectomy before puberty onset (P24) and were tested for behavior at P40, when gonadally intact mice were in late puberty (Fig. 21A). Analysis was designed to answer 3 *a priori* questions: 1) Does pre-pubertal castration alter anxiety- and depression-related behavior in P40 male mice, 2) Does pre-pubertal ovariectomy alter anxiety- and depression-related behavior in P40 female mice, and 3) Does the effect of gonadectomy on anxiety- and depression-related behavior differ between males and females. To enable direct comparison of the effect of gonadectomy in males to that in females, values for each sex were normalized to the mean intact value for that sex (Fig. 21).

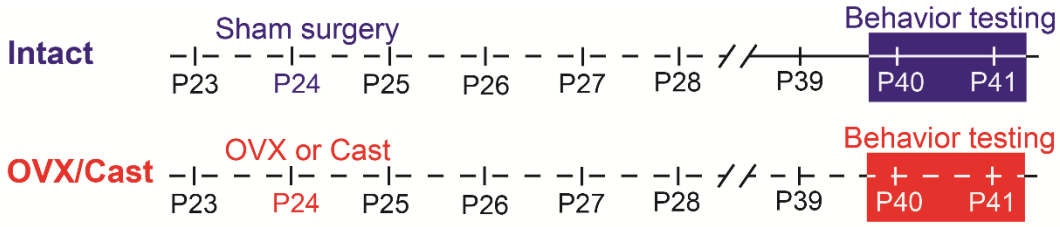
Effects of castration

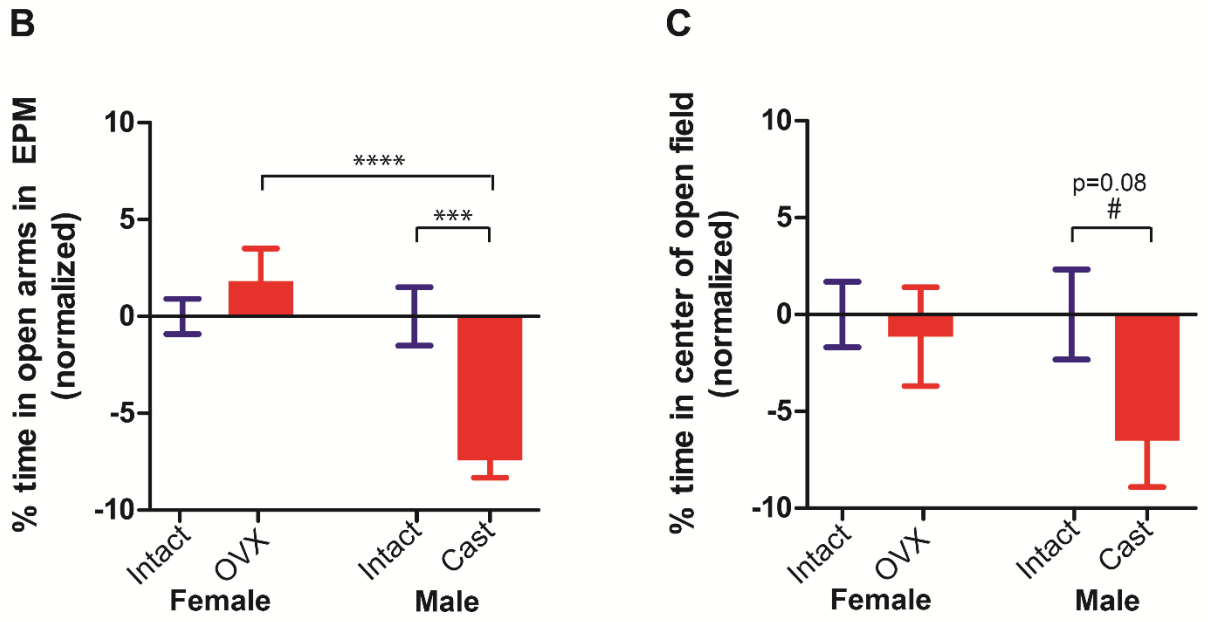
Compared to intact males, castrated males spent less time in the open arms of the EPM (Fig. 21B; t_{32}=4.23, p=0.0002) and showed a trend toward spending less time in the center of the open field (Fig. 21C; t_{33}=1.78, p=0.08). Effects of castration on performance in the EPM and open field were not explained by generalized locomotor effects, because castrated males did not differ from intact males in locomotor distance covered in the open field (data not illustrated; U=103, p=0.23). Castration did not affect the percent of time spent immobile in the FST (Fig. 21D; U=94.00, p=0.13) or the percent of marbles buried in the marble burying test (Fig. 21E; t_{33}=0.34, p=0.73).

Effects of ovariectomy:

Ovariectomized (OVX) females did not differ from intact females in the percent of time spent in the open arms of the EPM (Fig. 21B; t_{42}=1.06, p=0.30) or in the center of the open field (Fig. 21C; t_{42}=0.38, p=0.70). OVX also did not affect the percent of time spent immobile in the FST (Fig. 21D; U=210.0, p=0.86). In the marble burying test, OVX females buried fewer marbles

than intact females (Fig. 21E; U=128.5, p=0.02). This effect cannot be explained by generalized





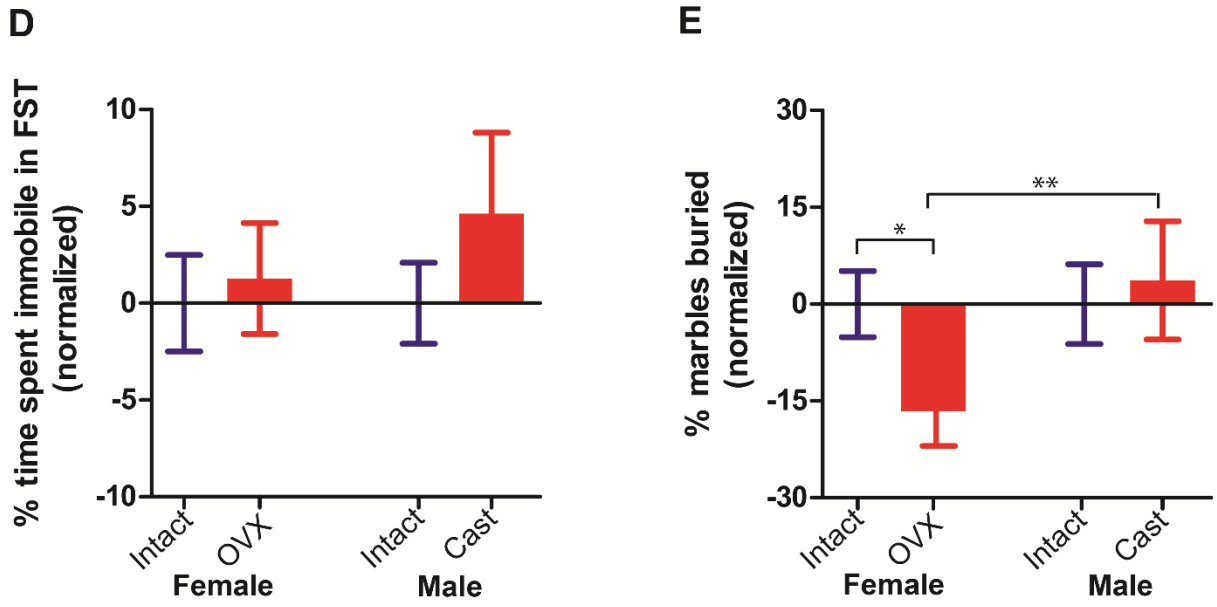


Figure 21. Effects of pre-pubertal gonadectomy on anxiety- and depression-related behavior in late-pubertal mice. All bars are mean ± SEM. A) Male and female mice underwent gonadectomy or sham surgery at P24, before puberty, and were tested for anxiety- and depression-related behavior at P40-47, during late puberty. n=15 ovariectomized (OVX) females; n=29 intact females; n=12 castrated (CAST) males; n=23 intact males. B) OVX did not affect EPM performance, but CAST males spent less time in the open arms compared to intact males, which resulted in a significant difference in the effect of gonadectomy between males and females. C) OVX did not affect open field performance, but CAST males showed a trend toward spending less time in the center of the open field compared to intact males. D) Gonadectomy did not affect FST performance in either sex. E) OVX decreased marble burying in females, while CAST had no effect, resulting in a significant difference in the effect of gonadectomy between males and females. #p<0.1; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

locomotor effects of ovariectomy, as OVX females did not differ from intact females in locomotor distance covered in the open field (data not illustrated; t_{42}=0.46, p=0.65).

Comparison of gonadectomy effect in males versus females:

Normalizing the values for gonadectomized mice to those of intact mice within each sex enabled direct comparison of the effect of gonadectomy between males and females. The effect of gonadectomy differed between males and females in the EPM (Fig. 21B; t_{20}=4.88, p<0.0001) and marble-burying test (Fig. 21E; U=37.00, p=0.0089), but the effect of gonadectomy did not differ between males and females in the open field test (Fig. 21C; t_{25}=1.51, p=0.14) or the FST (Fig. 21D; U=66.00, p=0.25).

Effects of pre-pubertal hormone exposure in females

Due to the association between early-onset puberty and anxiety and depression in girls (see Introduction), we used a mouse model of early puberty (Piekarski et al., 2017a) to ask if earlier exposure to gonadal hormones alters anxiety- and depression-related behavior in females. Female mice were injected with 0.01mg/kg 17 beta-estradiol (subcutaneous) on P24 and 20mg/kg progesterone (subcutaneous) on P26 (Fig. 22A). This treatment regimen advances first peripubertal exposure to gonadal steroids and is sufficient to induce endogenous puberty

(Ramirez and Sawyer, 1965; Smith and Davidson, 1968). Control littermates were injected with equivalent volumes of vehicle on P24 and P26. Hormone- and vehicle-treated mice were then tested on the EPM, open field, FST, and marble-burying test on P27-P28 (Fig. 22A). Hormone- and vehicle-treated mice did not differ in performance on the EPM (Fig. 22B; t_{25}=0.28, p=0.78), open field (Fig. 22C; t_{24}=0.02, p=0.98), FST (Fig. 22D; t_{25}=0.0074, p=0.99), or marble-burying test (Fig. 22E; t_{25}=0.06, p=0.95).

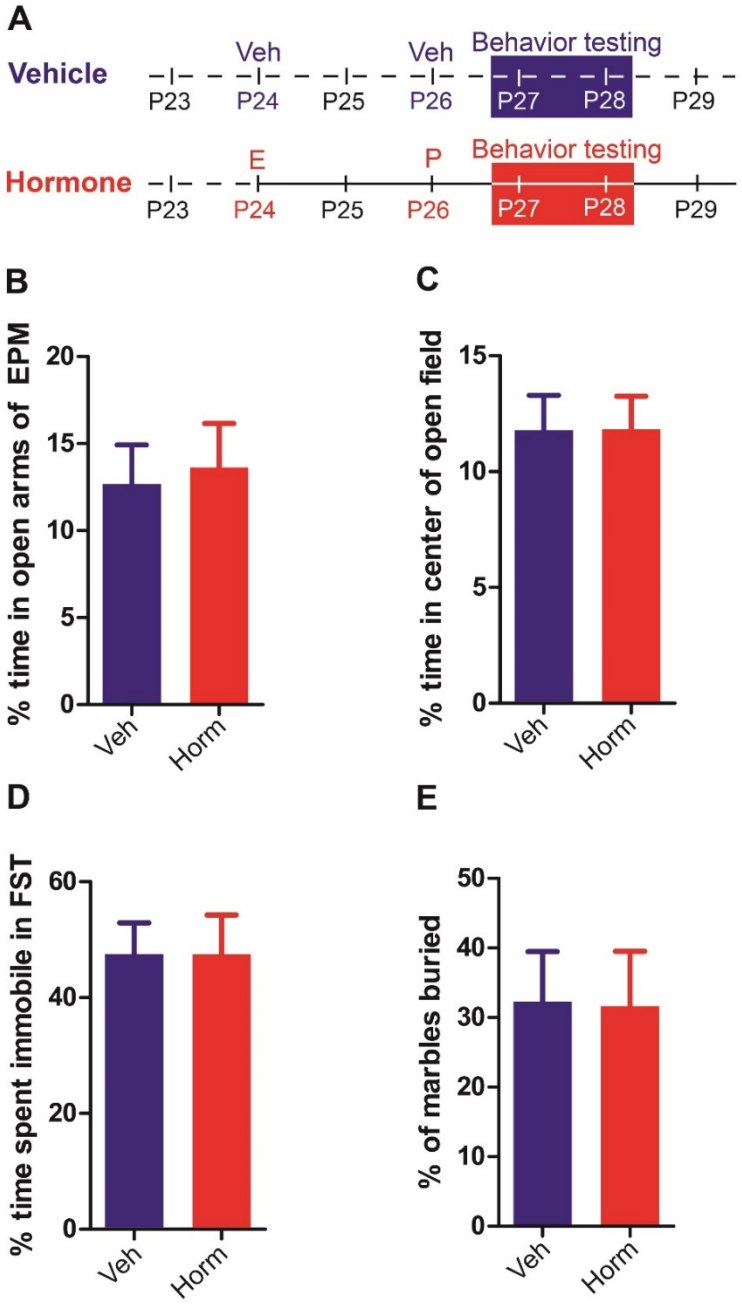


Figure 22. Induction of early-onset puberty in female mice does not alter anxiety- and depression-related behavior. All bars are mean ± SEM. A) Female mice were injected with estradiol or vehicle on P24 and progesterone or vehicle on P26, a treatment that disinhibits the HPG axis and induces endogenous ovarian hormone release (Ramirez and Sawyer, 1965; Smith and Davidson, 1968). Hormone- and vehicle-treated mice were tested for anxiety- and depression-related behavior at P27-28, an age when vehicle-treated mice were still pre-pubertal. n=15 vehicle-treated mice; n=12 hormone-treated mice. Hormone treatment did not alter performance on the EPM (B), open field (C), FST (D), or marble burying test (E).

Sex comparison in young adult animals

To test whether the sex difference we observed in the EPM performance of gonadally intact mice at P40 (Fig. 20B) would persist into adulthood, and to test whether additional sex differences would emerge post-pubertally, we tested gonadally intact male and female mice in young adulthood (P70) (Fig. 23A). We looked not only for a difference in means, but also for a difference in variance between the sexes, hypothesizing that females would show greater variance based on previous literature showing fluctuations in anxiety- and depression-related behavior across estrous cycle in rodents under some conditions (Contreras et al., 1998; Diaz-Veliz et al., 1997; Frye et al., 2000; Frye and Walf, 2002; Galeeva and Tuohimaa, 2001; Koonce et al., 2012; Marvan et al., 1996; Marvan et al., 1997; Mora et al., 1996; Walf et al., 2009). We found no difference between young adult male and female mice in their performance on the EPM (Fig. 23B; U=57.00, p=0.46), open field (Fig. 23C; t_{23}=0.33, p=0.74), FST (Fig. 23D; t_{23}=0.78, p=0.44), or marble burying

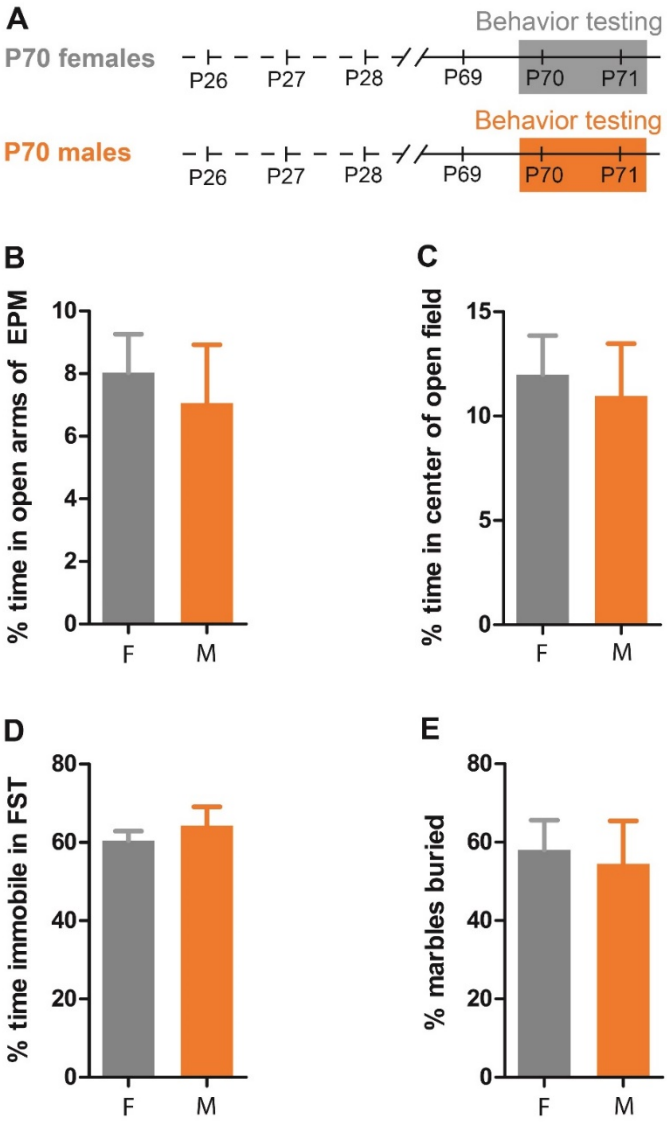


Figure 23. Young adult males and females do not differ in their performance on any behavior tested. All bars are mean ± SEM. A) Gonadally intact, hormonally unmanipulated mice were tested for anxiety- and depression-related behavior post-pubertally (P69-83). n=15 females; n=10 males. Young adult male and female mice did not differ in their performance on the EPM (B), open field (C), FST (D), or marble burying test (E).

test (Fig. 23E; t_{23}=0.27, p=0.79). There were also no sex differences in the amount of within-group variance on the EPM ($F_{9,13}$=1.62, p=0.41), open field ($F_{9,14}$=1.19, p=0.74), FST ($F_{9,14}$=2.52, p=0.12), or marble burying test ($F_{9,14}$=1.37, p=0.58).

Discussion

We were motivated by age and sex differences in human mental health to use a mouse model to test for direct effects of gonadal hormones on anxiety- and depression-related behavior during puberty. Based on a striking increase in anxiety and depression symptoms in girls just after puberty onset (Graber, 2013), we were particularly interested in testing the hypothesis that the increase in ovarian hormones at puberty onset plays a causal role in increasing anxiety- and depression-related behavior. Our data do not support this hypothesis. We found that depression-related behavior did increase in mice from pre-pubertal to late-pubertal ages (Fig. 20D) but that this increase did not depend on gonadal hormones (Fig. 21D) and did not show a significant sex effect (Fig. 20D). In contrast, gonadal hormones did alter anxiety-related behavior in a sex-specific manner during puberty. Males castrated before puberty showed greater anxiety-related behavior on the EPM and open field during late puberty compared to intact males, while ovariectomy had no effect on these tests but decreased marble burying in females (Fig. 21). These results are consistent with anxiolytic effects of testicular hormones during puberty in males but are not consistent with a causal role for ovarian hormones in anxiety- and depression-related behavior during puberty in females. Injection of ovarian hormones to elicit earlier puberty in females did not affect anxiety- and depression-related behavior (Fig. 22). Finally, we observed no sex difference in anxiety- and depression-related behavior in gonadally intact animals tested in young adulthood (Fig. 23), suggesting that a similar adult behavioral phenotype may be achieved by different mechanisms in male and female mice (De Vries and Panzica, 2006).

Testicular hormones are anxiolytic during puberty

Our results suggest that testicular hormones are anxiolytic during puberty. Anxiolytic effects of testicular hormones during puberty may be critical for promoting the approach/exploratory behaviors necessary for foraging and navigating the transition to adult social behaviors. Future work could test whether puberty in males is a sensitive period for the anxiolytic effects of testicular hormones, or whether hormone replacement in adulthood can rescue the effect of prepubertal castration. It is possible, for example, that testicular hormones influence learning processes related to approach/avoidance behaviors throughout puberty rather than simply having an acute, activational effect on anxiety-related behavior at the time of testing. This outcome would support the hypothesis that there is a sensitive period for gonadal hormones to influence the development of limbic circuitry (Cunningham et al., 2002; Peper and Dahl, 2013; Piekarski et al., 2017b; Romeo, 2003; Schulz et al., 2009; Sisk and Zehr, 2005).

Effects of pubertal ovarian hormones

Ultimately, both male and female mice must take risks during puberty to successfully navigate the transition from the natal nest to independent foraging and adult social behavior. While our results suggest that testicular hormones may facilitate exploratory and approach behaviors, as shown by reduced exploration of the 'risky' open arms of the EPM and center of the open field after castration, no change in these behaviors was observed in females after ovariectomy or injection of ovarian hormones. It is possible that in female mice, androgens also influence behavior, but the source of these androgens in females may be adrenal rather than gonadal. To examine this possibility, future studies could test the effects of adrenalectomy and androgen administration on anxiety-related behavior in pubertal female mice.

While OVX did not alter performance on the EPM or open field, OVX did reduce marble burying in late-pubertal females (Fig. 21E). Marble burying models some aspects of anxiety and/or compulsive behavior (Njung'e and Handley, 1991; Thomas et al., 2009), but interpreting changes

in marble burying is complex. While the OVX-induced decrease in marble burying could be interpreted as a reduction in anxiety-related or compulsive behavior, it is noteworthy that 9 out of the 15 OVX mice in our study buried zero marbles (compared to 8 out of 29 intact P40 females; p=0.04 for chi-square test for zero vs non-zero burying). An absence of burying, as opposed to a reduction in burying, may indicate an avoidant phenotype that is distinct from the reduction in marble burying observed during proestrus (i.e. a high estradiol/progesterone state) and with SSRI treatment in previous literature (Schneider and Popik, 2007). We speculate that both extremes of performance on the marble burying test, i.e. burying no marbles or burying all marbles, may represent anxiety-like phenotypes of either avoidant or compulsive responses to the marbles. In this interpretation, OVX may have induced an avoidant phenotype in our study, and ovarian hormones may promote active/approach responses to the potentially anxiogenic marbles. It is interesting to note, however, that ovarian hormone injection that elicited earlier puberty onset in females had no significant effect on marble burying (Fig. 22E).

Hormone-independent age effects and hormone by age interaction should both be considered

In our dataset, gonadectomy altered anxiety-related behavior, while depression-related behavior increased with age independent of gonadal hormone status. These results suggest that age and pubertal hormones may have dissociable effects on anxiety versus depression-related behavior at puberty.

However, we should be careful about concluding no effect of gonadal hormones at any stage in depression-related behavior. In adult male and female rodents, gonadectomy increases depression-related behavior in the FST (Bernardi et al., 1989; Frye and Wawrzycki, 2003; Hilakivi-Clarke, 1994, 1996; Okada et al., 1997). The contrast between our results in peripubertal animals and those in adults could reflect age differences in the effects of gonadal hormones. There are multiple examples in the literature of hormones affecting neural or behavioral outputs differently during puberty compared to adulthood (Schulz et al., 2009; Shen et al., 2007; Sisk and Zehr,

2005). Age differences in the effects of hormones in animal models are underscored by human studies showing different risk of anxiety and depression symptoms depending on the timing or tempo of puberty (Graber, 2013). Collectively, these data emphasize the importance of considering age, sex, and the current and 'historical' hormonal/pubertal status when considering the role of gonadal hormones in psychiatric disease states.

Interactions between biological and environmental factors: early puberty and mental health risk

In contrast to the human literature showing greater risk of anxiety and depression in girls who experience early puberty (Blumenthal et al., 2011; Boden et al., 2011; Copeland et al., 2010; Crockett et al., 2013; Deardorff et al., 2007; Deng et al., 2011; Ge et al., 2006; Graber, 2013; Graber et al., 1997; Graber et al., 2004; Hayward et al., 1997; Kaltiala-Heino et al., 2003a; Kaltiala-Heino et al., 2003b; Mendle et al., 2007; Negriff et al., 2008; Stice et al., 2001), we saw no effect of pre-pubertal hormone treatment on anxiety- and depression-related behavior in female mice (Fig. 22). Several human studies have highlighted the importance of environmental and social factors that mediate or moderate the relationship between pubertal timing and mental health outcomes. For example, recent stressful life events, pre-existing psychiatric symptoms, and harsh family/neighborhood conditions amplify the degree to which early puberty predicts psychiatric/behavioral symptoms (Caspi and Moffitt, 1991; Deardorff et al., 2013; Ge et al., 2002; Ge et al., 2001; Obeidallah et al., 2004; Rudolph and Troop-Gordon, 2010). In some studies, early puberty does not confer risk of psychiatric and behavioral symptoms unless specific environmental conditions are met. For example, in a racially diverse sample of adolescent girls, early puberty conferred risk of externalizing behavior only in subjects living in neighborhoods characterized by high concentrated disadvantage (Obeidallah et al., 2004). Similarly, in two illuminating studies, associating primarily with other girls rather than mixed-sex peer groups abolished the relationship between early puberty and later psychiatric/behavioral problems (Caspi and Moffitt, 1991; Ge et al., 1996). Our studies in rodents suggest that the relationship between

early puberty and psychiatric symptoms in girls is not likely to be deterministically driven by gonadal hormones alone. Future rodent studies examining interactions between environmental stressors and gonadal hormones during puberty might reveal effects of pre-pubertal hormone exposure not shown in our data. However, we speculate that negative mental health outcomes associated with early puberty in girls may also be related to the complex, gendered adversity that girls and women navigate.

Interactions between biological and environmental factors: sex differences in post-pubertal mice and humans

While rates of anxiety and depression are greater in women than in men (Hayward and Sanborn, 2002; Kessler et al., 2005), our study and the majority of previous rodent studies do not show greater anxiety- and depression-related behavior in adult females compared to males (Fig. 23) (Alonso et al., 1991; Diaz-Veliz et al., 1997; Frye et al., 2000; Frye and Walf, 2002; Frye and Wawrzycki, 2003; Gioiosa et al., 2007; Johnston and File, 1991; Lucion et al., 1996; Marcondes et al., 2001; Zimmerberg and Farley, 1993). Despite sex-specific effects of gonadal hormones on anxiety-related behavior during puberty (Fig. 21), males and females in our study converged on the same behavioral phenotype in young adulthood. It is possible that a latent sex difference in young adult mice could be revealed by the addition of specific environmental factors (McCarthy et al., 2012). For example, stress amplifies fluctuations in FST performance across the estrous cycle in adult female rats (Marvan et al., 1997), and similar interactions between stress and gonadal hormones could occur in relation to sex differences in anxiety- and depression-related behavior. As in the case of mental health risks associated with early puberty, it is possible that human sex differences in anxiety and depression are not an inevitable result of the differing hormonal milieu between males and females, but may depend on gender socialization and environmental conditions that are modifiable.

Relevance to public health: hormone manipulation during puberty in humans

Several human phenomena highlight the importance of understanding the role of pubertal hormones in adolescent brain maturation. For example, the prevalent use of hormonal contraception by adolescent girls calls for research on the neural effects of these alterations in hormonal milieu during a potential sensitive period of brain maturation. Similarly, the growing practice of gender-affirming hormone treatment in transgender adolescents calls for research on the neurodevelopmental implications of delaying pubertal hormone exposure and/or administering exogenous hormones during puberty. Finally, the advancing age of puberty in girls (Aksglaede et al., 2009; Biro et al., 2013; Herman-Giddens, 2006; Marshall and Tanner, 1969; Rosenfield et al., 2009) calls for research on the neurodevelopmental implications of gonadal hormone exposure at ages that are typically pre-pubertal, particularly in light of the negative mental health outcomes associated with early puberty in girls. Animal research can not only reveal direct effects of hormones on brain maturation, but can also help to disentangle interactions between environmental stressors and gonadal hormone exposure.

Conclusion

To better understand how gonadal hormones at puberty onset impact depression and anxiety in humans, we manipulated gonadal hormones and tested male and female mice on anxiety- and depression-related behavior assays. We were particularly interested in determining if ovarian hormones enhanced anxiety- and depression-related behavior after puberty onset in a standard laboratory environment where exposure to stressors was controlled. We found no evidence to support an independent, causal role for ovarian hormones in anxiety- and depression-related behavior at puberty.

We did observe an increase in depression-related behavior from pre-pubertal to late-pubertal ages, but this increase did not depend on gonadal hormones during puberty or show a main effect of sex. In contrast, anxiety-related behavior was sensitive to gonadal hormones during

puberty in a sex-specific manner. Testicular hormones were anxiolytic during puberty on classic tests for anxiety-related behavior, while pubertal ovarian hormones did not affect performance on any test except marble burying. Interestingly, gonadally intact males and females converged on the same performance in all behaviors by young adulthood despite sex-specific effects of prepubertal gonadectomy.

Our findings highlight the importance of investigating the effects of gonadal hormones in both males and females specifically during puberty, where results may differ from those obtained from adult animals. Our results also invite further investigation of potential interactions between environmental stressors and pubertal development, as these interactions may be critical for shaping mental health outcomes in human adolescents.

Chapter 5: Conclusions and Discussion

The results presented in this dissertation highlight multiple factors that interact to influence the maturation of behavior and neural circuitry in mice: gonadal hormone exposure, age, sex, and learning experiences. These studies were motivated by public health literature indicating dramatic differences in mental health risk based on pubertal status, age, sex, and experiences of adversity (Graber, 2013; Hayward and Sanborn, 2002). Animal models can help to disentangle the separate influences of these multiple interacting factors, leading to greater understanding of the maturational processes and environmental factors that confer vulnerability or resilience to psychiatric disease. In Chapter 2, we used a mouse model to show that learning experiences can combat the effects of environmental deprivation and confer resilience to drug-seeking behavior. In Chapter 3, we conducted a series of experiments to show that pubertal hormones drive maturation of inhibitory neurotransmission and flexible learning in female mice, a result that raises concerns about the neurodevelopmental implications of the increasingly earlier age of puberty in girls. Finally, in Chapter 4, we show differing effects of age, sex, and gonadal hormone status on adolescent maturation of anxiety- and depression-related behavior. These results indicate that the influence of sex, age, gonadal hormone status, and life experience can be disentangled in animal models and that these factors all influence the maturation of circuits and behavior. Although our results only scratch the surface of the complexity of these influences on brain maturation, we emphasize the importance of considering circuits and behavior within the context of these multiple interacting factors that sculpt them.

Although learning and plasticity are possible throughout the lifespan, there may be multiple sensitive periods for distinct types of learning during the life stages in which this learning is most appropriate (Fig. 24). For example, pre-pubertal humans and other animals develop foundational skills necessary for functioning in their natal environment, while adolescents and young adults must attune to new social cues as they assert increasing independence and learn to adopt adult social roles (Crone and Dahl, 2012; Ellison et al., 2012). Our data suggest that puberty may

regulate transitions in these stages of developmentally appropriate plasticity and learning. Our results in Chapter 3 demonstrate that gonadal hormones drive maturation of inhibitory neurotransmission, which is known to regulate critical periods for plasticity in sensory regions of cortex. We also show that inducing earlier puberty onset can induce early adult-like performance on a task requiring flexible learning. Together, these results suggest that pubertal hormones may regulate a transition in sensitive periods for distinct types of plasticity, learning, and decision-making during the transition from juvenile to adolescent life stages (Fig. 24).

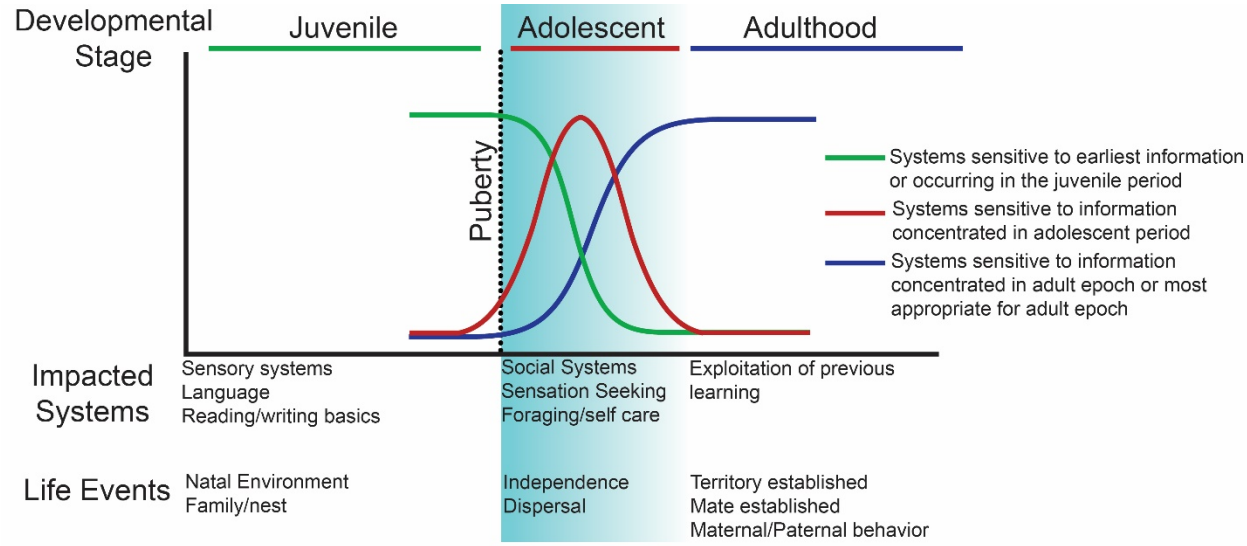


Figure 24. Heuristic model of three distinct periods of development: juvenile, adolescent and adult. In this model, the juvenile period (green line) is when an animal develops the foundations of sensory and cognitive/associational architectures in the brain. To facilitate this process, they display enhanced experience dependent plasticity, which serves to organize neural circuits to be best adapted to their natal environment. In humans, this period is also when the basic educational foundations are established, such as language skills. At the start of puberty (dotted line), which marks the onset of adolescence (red line), independence is exerted, social networks expand, and sensation seeking and the salience of social feedback increases. This period is important for humans and animals to explore their social world and learn how to survive independently. Finally, adolescents transition into adults (blue line) and exploit the knowledge they gained during childhood and adolescence, to successfully survive, mate, maintain territory, and raise young. These characteristic life stages are conserved across many mammalian species. Figure adapted from (Piekarski et al., 2017b).

Our results point to a need for deeper understanding of the neurodevelopmental implications of hormonal perturbation during puberty in humans. Puberty is advancing to

increasingly younger ages, particularly in girls (Aksglaede et al., 2009; Herman-Giddens, 2006), and early puberty is associated with greater risk of a variety of negative mental health outcomes (Graber, 2013). Medical interventions that alter hormonal milieu during puberty, such as the use of hormonal contraception in girls and the growing practicing of gender-affirming hormone treatment for transgender adolescents, also point to a need for increased understanding of the neurodevelopmental effects of pubertal hormones. We suggest that animal models provide an invaluable complement to epidemiological data in this respect, as animal studies enable control of environmental variables and probing of neural mechanisms that would be impossible to assay in humans.

A primary concern related the increasingly earlier age of puberty in girls is risk of anxiety and depression, which spikes at puberty onset and is highest in girls who experience puberty earlier than their peers (Graber, 2013). The results described in Chapter 4 highlight several factors that interact to influence anxiety- and depression-related behavior during puberty in mice: age, sex, and gonadal hormone exposure. While depression-related behavior increases with age regardless of sex or gonadal hormone status, anxiety-related behavior responds to pubertal hormone manipulation in a sex-specific manner in mice. Our results in mice do not, however, fully capture the etiology of anxiety and depression symptoms across adolescence in humans. In humans, a dramatic sex difference in anxiety and depression symptoms emerges after puberty onset, with girls showing greater risk than boys (Costello et al., 2011; Hayward and Sanborn, 2002; Silberg et al., 1999). Human studies have highlighted the critical importance of environmental stressors in influencing anxiety and depression symptoms during puberty, and animal work underscores this data with evidence that the effects of environmental stress interact with gonadal hormone exposure to influence anxiety- and depression-related behavior (Graber, 2013; Marvan et al., 1997). In humans, it is also important to consider that anxiety and depression phenotypes may depend on specific social stressors, as adolescents assign particularly strong salience to social cues as they navigate their complex roles during this transitional life stage

(Crone and Dahl, 2012). Thus, circuits that are primed for adolescent forms of social learning (Fig. 24) may also be vulnerable to deleterious effects of social stressors on mental health. Combining the data presented in Chapter 4 with public health literature, we suggest that the striking increase in anxiety and depression symptoms at puberty onset in girls may not be deterministically driven by gonadal hormone exposure, but may depend on social and environmental factors that are modifiable and ripe for intervention.

Interventions targeting social and environmental factors may be most effective during time periods in which the neural circuitry these interventions engage is in a process of dynamic remodeling, primed for the influence of learning experiences (Fig. 24; (Suleiman and Dahl, 2017)). In Chapter 3, we showed that specific aspects of circuit function in the dorsomedial frontal cortex undergo striking maturation driven by gonadal hormones at the onset of puberty. The dorsomedial frontal cortex interacts with subcortical limbic circuitry and other cortical regions to influence many of the cognitive and affective outputs that are critical for successfully navigating adolescence and the transition to adulthood (Felix-Ortiz et al., 2016; Johnson and Wilbrecht, 2011; Johnson et al., 2016b). Thus, this circuitry—which is dynamically remodeled during adolescence and is directly influenced by gonadal hormones at puberty onset—may be an effective target for intervention.

In Chapter 2, we demonstrated that a specific intervention involving exploration and contingency learning in young adulthood induced long-term reductions in maintenance of cocaine conditioned place preference. Young adulthood is a time of vulnerability to the onset of substance use disorders in humans, and both human and animal data indicate that frontal circuits underlying reward-related behaviors undergo extensive remodeling throughout late adolescence and young adulthood (Gogtay et al., 2004; Johnson and Wilbrecht, 2011; Johnson et al., 2016a). While the specific neural mechanisms of action of the cognitive training intervention in Chapter 2 remain to be tested, previous data show that the training paradigm used in Chapter 2 sculpts long-range projections to frontal cortex in young adult animals (Fig. 2; (Johnson et al., 2016b)). Thus, young

adulthood may represent an additional window of opportunity in which interventions targeting circuits that are remodeling may alter neurodevelopmental trajectories and improve life outcomes.

Data from animal models can inform our understanding of the maturational processes and environmental factors that render adolescence and young adulthood times of great vulnerability to anxiety, depression, and substance use disorders. These data can also, however, provide insight into the mechanisms by which targeted interventions can most effectively engage specific dynamic circuits during these developmental inflection points that offer windows of opportunity for positive change (Suleiman and Dahl, 2017). Ultimately, developmental insights gleaned from animal models can contribute to interventions that leverage these developmental processes to create positive spirals of adaptive decision-making and emotional resilience.

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