Development of working memory in the male adolescent rat

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A B S T R A C T

Working memory develops over the course of adolescence, and neuroimaging studies find development-associated changes in the activity of prefrontal cortical brain regions. Establishment of a rodent model of working memory development would permit more comprehensive studies of the molecular and circuit basis for working memory development in health and disease. Thus, in this study, working memory performance was compared between adolescent and adult male Sprague-Dawley rats using an operant-based, delay-match-to-sample working memory task. Adolescent and adult rats showed similar rates of learning the task and similar performance at a low cognitive load (delays ≤ 6 s). However, when the cognitive load increased, adolescents exhibited impaired working memory performance relative to adults, until postnatal day 50 when performance was not significantly different. Despite evidence that cannabinoids disrupt working memory, we found no effect of acute treatment with the cannabinoid receptor agonist, WIN55212,2, at either age. Moreover, expression of glutamate and GABA receptor subunits was examined in the prelimbic and infralimbic prefrontal cortex across development. NMDA receptor subunit GluN2B expression significantly decreased with age in parallel with improvements in working memory. Thus, we show evidence that rats can be used as a model to study the molecular underpinnings of working memory development.

1. Introduction

Adolescence is a unique developmental period in which social, emotional, and cognitive behaviors change dramatically (Casey and Jones, 2010; Doremus-Fitzwater et al., 2010; Spear, 2000). Behavioral changes often are attributed to the neurodevelopment that occurs during adolescence, including myelination, synaptic pruning, changes in receptor levels, and projection elaborations, particularly in the prefrontal cortical (PFC) regions regulating cognition and inhibitory control (Cruz et al., 2003; Cunningham et al., 2002; Doremus-Fitzwater et al., 2010; Eggan et al., 2010; Lewis et al., 2004; Sowell et al., 1999). Typically, striatal regions begin to mature early in adolescence, while PFC maturation is protracted, particularly PFC connections to subcortical regions like the striatum (Casey et al., 2010; Luna et al., 2015). Due to the imbalance in the connectivity of these brain structures, it has been hypothesized that adolescents are driven by striatal drive for reward without corresponding top-down cognitive control to limit risky decision-making.

Higher-order cognitive functions, including working memory, require the PFC (Luciana et al., 2005; Luna et al., 2010), and it is known that working memory improves over the course of human adolescence (Luciana and Nelson, 1998; Siegel and Ryan, 1989; Simmonds et al., 2017). Age-related working memory improvement is generally associated with increased activity in prefrontal and parietal cortices (Luna et al., 2010, 2015). However, working memory-associated PFC activity peaks in adolescence and declines in adulthood, suggesting that adolescents may be less efficient at engaging the PFC to complete the task (Luna et al., 2010, 2015). In addition, while children showed greater activity in the insula and striatum when completing a working memory task, adults recruited the inferior frontal gyrus in the same task, suggesting that different brain networks may subserve working memory across development, and that as these networks are refined, performance improves (Luna et al., 2010).

Given that exposure to alcohol, drugs, and stress during childhood and adolescence is associated with numerous negative health consequences, including poor cognitive function and academic achievement (Gershon et al., 2013; Grant et al., 2006; Salom et al., 2014; Taylor et al., 2004), an increased understanding of the molecular underpinnings of working memory development, and how those factors are impacted by environmental insults could help improve long-term outcomes. Therefore, an animal model of adolescent working memory development would help establish causal associations between human

imaging and behavioral results. Recently, a study found that working memory improves from adolescence to adulthood in the non-human primate (Zhou et al., 2016); however, to date, no one to our knowledge has thoroughly determined the course of working memory development in a rodent model. Rather, prior studies of working memory development have only assessed behavior at a few discrete time points or used tasks that rely on short-term memory or other innate behaviors (e.g. object recognition and delayed alternation) that do not mimic the types of working memory assessments used in humans (Castner et al., 2004; Green and Stanton, 1989). Thus, here we investigated the emergence of adult-like cognitive performance in a delayed-match-to-sample working memory task, and we examined whether age-related cognitive improvement was associated with changes in the expression of proteins regulating excitatory and inhibitory activity in the medial prefrontal cortex (mPFC), and if working memory was differentially sensitive to cannabinoid receptor activation based on age.

2. Materials and methods

2.1. Animals

A total of 72 male, Sprague Dawley rats (Harlan, Indianapolis, IN) were pair-housed on a 12 h light/dark cycle (lights on at 4:30am) in a climate-controlled room for all experiments. Rats had *ad libitum* access to food and tap water, except for periods of food restrictions described below. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

2.2. Experiment 1

In experiment 1, we aimed to determine if adolescent and adult rats delivered to our facility and trained identically in an operant-based delay-match-to-sample working memory task would show age-related differences in working memory performance. We further wanted to determine if performance differences would scale with cognitive load, if we could observe improvements in working memory performance across adolescence, and if performance deficits are independent of general learning ability (i.e., learning that does not require working memory). Rats were delivered on postnatal day (PND) 25 (adolescents; n = 12) or PND67 (adults; n = 12), and task training began on PND32 for adolescents and PND74 for adults (see Fig. 1 for experimental timeline). We defined rat adolescence based on the criteria outlined in Spear (2000), which suggests that adolescence begins roughly around PND28 (though some changes may begin earlier, particularly in

females) and can extend to PND60 or beyond (particularly in males). Relevant to the current study, prefrontal cortical development and maturation of working memory are thought to be later stage processes in humans, with improvements noted into the mid 20's, which likely corresponds to the P45-60 range described in the current study (Simmonds et al., 2017). Thus, rats were trained and tested until PND52 in this experiment, corresponding to late adolescence. We then tested the effects of cannabinoid receptor activation on performance (described below), followed by additional working memory tests, up to PND63, corresponding to early adulthood, and up to PND107 in adults.

2.3. Experiment 2

In experiment 2, we aimed to replicate the age-related difference in working memory performance observed in experiment 1 in a separate cohort of rats. In addition, we wanted to determine if we could observe age-related differences in impulsivity, and if punishing impulsive (premature) responses would alter performance in the working memory task. Rats were delivered on PND22 (adolescents; n = 12) or PND67 (adults; n = 12), and task training began on PND29 for adolescents and PND74 for adults. Rats were trained in the working memory task as described below. The differences in training timing noted between experiment 1 and experiment 2 were largely due to differences in the speed of acquisition in the two cohorts and the inclusion of impulsivity testing in experiment 2. Rats in this experiment were trained until late adolescence (PND50) and until PND95 for the adult group.

2.4. Delay-match-to-sample working memory task

Rats were trained in chambers (MedAssociates) equipped with five nosepoke apertures, a food dispenser, and a fan for background noise during 1-hr daily sessions as described previously (Kirschmann et al., 2017a,b). In experiment 1, rats were first given a pre-training session where sucrose pellets were freely given once a minute for 30 min into the magazine (i.e., food receptacle). This period was immediately followed by 15 min where all nosepokes were illuminated and a response in any aperture resulted in a sucrose pellet delivery on a fixed-ratio 1 (FR1) schedule. The following day, rats were placed in phase 1 of training where in a 1 h long session all nosepokes were illuminated and each response on any nosepoke resulted in sucrose pellet delivery. In experiment 2, rats simply had a minimum of two days of phase 1 training instead of the pre-training session. Rats remained in phase 1 until they acquired at least 10 reinforcers. Rats then went on to phase 2 of training where a single nosepoke was illuminated at random on each trial, and only responses in the lit aperture resulted in sucrose pellet delivery, while incorrect responses led to a 2 s timeout where the

Experiment 1

	WM Training Phases 1-3	WM Testing Low Cognitive Load	WM Testing Mod Cognitive Load	Pharmacological Testing (WIN)	WM Testing High Cognitive Load
Adolescent Age (PND)	32-38	39-42	43-52	54-55	53,60,63
Adult Age (PND)	74-80	81-84	85-93	95-96	94,102,107

Experiment 2

	WM Training Phases 1-3	WM + Impulsivity Testing	WM Testing Low Cognitive Load	WM Testing Mod Cognitive Load
Adolescent Age (PND)	29-38	39-43	44-45	46-50
Adult Age (PND)	74-83	84-88	89-90	91-95

Fig. 1. Timeline of experiments 1 and 2. The timeline shows at what ages in postnatal days (PND) adolescents and adults were trained and tested. WM = working memory, Mod = moderate, WIN = WIN55,212-2.

houselight and all other lights were turned off. Rats had to earn at least 30 reinforcers in phase 2 to move on to the next phase of training. In phase 3 of training, a response on a single lit "sample" aperture, randomly selected on each trial, resulted in immediate illumination of 3 "choice" apertures. A second response in the sample aperture resulted in sucrose reward. The choice apertures always neighbored the sample aperture such that three nosepokes in a row were always illuminated in the choice phase. After rats reliably responded twice in the correct aperture (> 70% accuracy), delays were introduced between the sample and the choice phases. Rats performed blocks of trials in which 7 delays (0.5–6 s) were presented in random order; each of the 7 delays occurred before a new block of trials began. Once rats reached training criterion (≥80% correct in 0.5 s delay), the range was increased (0.5–12 s; 0.5–24 s) in order to further assess working memory capacity. In experiment 2, initial training on the 0.5–6 s delays included a period where all rats were punished with a 2 s timeout if they responded on any nosepoke during the delay (prior to the illumination of the three choices) with the goal of preventing continued nosepoking from resulting in high accuracy, and to assess impulsive behavior. The rats were kept on this version of the task for 3–5 days before then switching to the standard version of the task where there is no punishment for premature responses, and training continued up to the 0.5–12 s delay phase. Some rats in both age groups failed to meet our training criteria at various points in training and were then eliminated from further analyses. These instances are described in the results section.

2.5. Pharmacological testing

Rats in experiment 1 were tested for the ability of the cannabinoid receptor agonist WIN55,212-2 (WIN) to alter the working memory performance of adolescents and adults in this task. Previous work has shown that cannabinoid receptor agonists, including WIN, can produce acute deficits in working memory as assessed by the radial arm maze (Lichtman et al., 1995), Morris water maze (Abush and Akirav, 2009; Ferrari et al., 1999), object recognition task (Kirschmann et al., 2017a), and delay non-match and match to sample tasks (Hampson and Deadwyler, 2000; Heyser et al., 1993), but it is unknown if acute cannabinoid receptor modulation affects working memory in this delay match to sample task, or if effects are age dependent. Thus, we tested the acute effects of WIN on working memory performance in both age groups. WIN (Tocris, Inc., Minneapolis, MN) was dissolved in Tween80 and brought to a 1 mg/mL concentration in saline (0.48% Tween80). The vehicle solution was made by dissolving 0.48% Tween80 in saline. Using a within-subjects design rats received an i.p. injection of 1 mg/kg WIN or Vehicle 15 min prior to working memory testing on two consecutive days. The order of testing was randomized across rats, and injection order was included in the statistical analysis to verify that there were no carryover effects on day 2 from receiving WIN on day 1.

2.6. Experiment 3

In experiment 3, a total of 24 rats were used to examine prefrontal protein changes from adolescence to adulthood where working memory was observed to mature (PND40-PND90). Rats (n = 6/group) arrived on PND32, PND46, PND53, and PND81 and were sacrificed 5–9 days later to assess ages PND40, PND52, PND60 and PND90 within one experimental cohort. For all rats, brains were flash-frozen in isopentane on dry ice and stored at −80 °C.

2.7. Western blot analysis

Working memory in rodents is known to rely on activity in the medial prefrontal cortex, which includes the prelimbic and infralimbic regions, and is thought to be homologous to the dorsal lateral prefrontal cortex that subserves working memory in humans and non-human primates (Bolkan et al., 2017; Dalley et al., 2004; Kirschmann et al.,

2017a). In particular, appropriate excitatory/inhibitory balance in the mPFC is thought be critical for optimal working memory performance (Bañuelos et al., 2014; Larsen and Luna, 2018; Starc et al., 2017). Thus, we sought to determine if the expression of receptors necessary for excitatory glutamatergic and inhibitory GABAergic signaling shifted from early adolescence into adulthood in these medial PFC regions. Prelimbic and infralimbic cortices were excised from ~1mm-thick sections, and were fractionated into membrane- and non-membrane-bound components based on published protocols (Bañuelos et al., 2014; Kirschmann et al., 2017a). Protein concentrations were determined using the bicinchoninic acid assay (BCA Protein Assay; Thermo-Scientific Pierce, Waltham, MA), according to the manufacturer's protocol. Samples were denatured and reduced in sample buffer [60 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 5% 2-β-mercaptoethanol], and heated to 90 °C for 5 min.

Membrane and soluble fractions (20 μg total protein) were resolved by SDS-PAGE (4–12% Tris-glycine gels; Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in blocking solution (5% nonfat dry milk in PBS containing 0.1% Tween20). Membranes were incubated overnight at 4 °C with specific primary antibodies against the following proteins: GABA receptor subunits $GABA_BR2$ (1:1000; Cell Signaling, Danvers, MA) and $GABA_AR1\alpha$ (1:10,000; Abcam, Cambridge, UK); AMPA receptor subunit GluA1 (1:1000; EMD Millipore); NMDA receptor subunit GluN2B (1:500; EMD Millipore); and loading control GAPDH (1:1000; EMD Millipore). Antigen binding was visualized with secondary fluorescent antibodies (IRDye 800CW anti-rabbit, 1:5000; IRDye 680CW anti-mouse, 1:5000). All primary and secondary antibodies were diluted in blocking solution (50% LI-COR Odyssey blocking buffer / 50% PBS). Protein expression was quantified using LICOR-Odyssey imaging software, with each sample normalized to its own GAPDH expression, and expression within a gel normalized to average levels of the PND90 age group.

2.8. Statistical analyses

Differences in working memory accuracy were determined using a two-way repeated measures ANOVA (group x delay). Significant interactions were followed by Bonferroni's post-hoc test. Age comparisons of performance were made daily, such that task exposure remained constant for both age groups. In other words, we treated each day as an independent measure as we wanted to compare adult and adolescent performance at equivalent levels of training, in order to determine at what age range adult-like performance emerged. Other measures of performance were analyzed using two-sided t-test, and the percentage of rats meeting training criteria were plotted as survival curves and compared using the Log-rank Mantel Cox test. Protein expression in prelimbic and infralimbic cortices was analyzed by a one-way ANOVA followed by tests for linear trend over age. All statistical analyses were performed using Prism 7 software (GraphPad).

3. Results

3.1. Experiment 1: Comparison of task acquisition in adolescents versus adults

In the first experiment, the aim was to determine if working memory in adolescent rats is impaired relative to adults, and at what age working memory becomes adult-like. However, in order to assess working memory, rats first must be trained to make an operant response (nose poke) for a sucrose reinforcer. Thus, we were able to determine if there were any age-related differences in general, working memory-independent learning ability in the early phases of task training. In experiment 1, all adolescent rats and all but one adult rat met our acquisition criterion within 2 days of phase 1 training (i.e., 10 reinforcers earned). Rats then had to learn to respond in a single illuminated
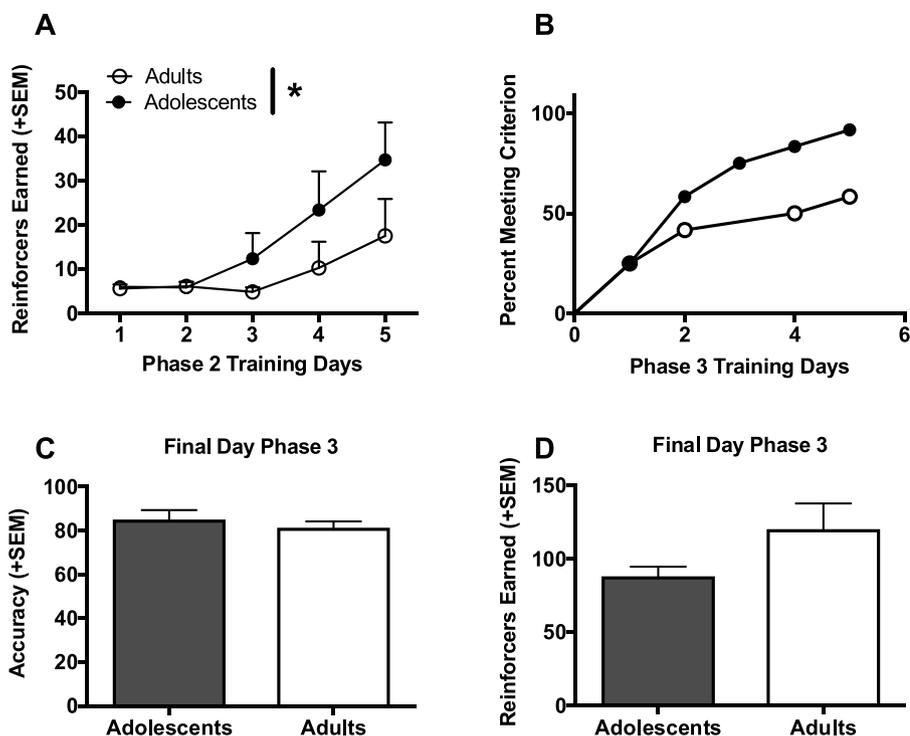
Fig. 2. Task acquisition in adolescents and adults. (A) Number of reinforcers earned during training to nosepoke for sucrose pellets. All rats earned an increasing number of reinforcers over days, but adolescents earned significantly more than adults. (B) Survival curve plotting the number of rats in each group meeting training criterion of 70% accuracy on phase 3 of training (2 correct nosepokes with zero delay). There was no significant difference between groups though adolescents appeared to acquire somewhat faster than adults. (C) Accuracy on the last day of phase 3 training was not different between age groups. (D) The number of reinforcers earned on the last day of phase 3 training was not different between groups. All data are presented as the mean + standard error of the mean (SEM), *p < 0.05 main effect of age.

aperture for a sucrose reinforcer (phase 2). Two-way rmANOVA revealed that all rats earned significantly more reinforcers across days of training [$F_{(4,108)} = 5.26$, $p = 0.0007$], and that adolescents earned significantly more reinforcers than adults [$F_{(1,108)} = 5.13$, $p = 0.026$], suggesting a faster rate of learning in adolescents (Fig. 2A). Data are only shown for 5 days of phase 2 training as 8 of the adolescents moved on to phase 3 by day 6; however, several adults were maintained on phase 2 for additional days to meet our training criterion. Next, rats had to learn to respond in the single lit aperture and then respond in that aperture again after the neighboring two apertures were illuminated in order to receive reinforcement (i.e., 2 responses in the correct nosepoke with zero delay; phase 3). Rats had to achieve 70% accuracy on this phase of the task to move on to working memory testing, though occasionally rats were kept on phase 3 for an extra day to help other rats catch up in training. Fig. 2B plots survival curves for the days of training required for each age group to achieve our training criterion. A comparison of the survival curves with the Log-rank Mantel Cox test did not find a significant difference in acquisition rate for this phase of the task [$\chi^2 = 2.79$, $p = 0.09$]. However, one adolescent and five adults did not make it through phase 3 of training, and adults appeared to be somewhat slower to learn the task. We ultimately excluded two adolescents and two adults that required more than 3 days of phase 3 training because we did not want the age range of our adolescent group to be too broad during subsequent stages of working memory testing, and we did not want large differences in training history to affect our results. Therefore, the remaining behavioral data are reported for n = 9 adolescent and n = 5 adult rats. By the end of phase 3 training, there was no significant difference in accuracy based on age group assessed by two-tailed, unpaired *t*-test [$t = 0.594$, $p = 0.56$], and there was not a significant effect of age on reinforcers earned at the end of training [$t = 2.06$, $p = 0.061$]; though there was a strong trend towards adults earning more reinforcers, potentially indicating greater motivation to perform the task. This discrepancy in number of reinforcers earned could be due to the adults' larger size and, thus, an ability to consume more sucrose pellets before becoming satiated (Fig. 2C–D).

3.2. Experiment 1: Comparison of working memory performance in adolescents versus adults

Next, rats began working memory testing, starting with relatively short delays (0–6 s), and thus a low cognitive load (phase 4). As expected, both groups showed a significant, delay-dependent reduction in accuracy [$F_{(6,72)} = 26.8$, $p < 0.0001$], but there was no effect of age on performance [$F_{(1,12)} = 0.108$, $p = 0.748$], indicating that PND41-42 adolescents did not differ from adults when delays are relatively short (Fig. 3A). However, one adolescent rat from this point forward consistently only completed about half of the number of trials of the other rats and did not maintain high (> 70%) accuracy at zero delay, and was thus eliminated from further analysis, leading to a final n = 8 for the adolescent group and n = 5 for the adult group in subsequent tests. After 2–3 days on phase 4, rats were moved to delays ranging to 12 s, which we consider to be a "moderate" working memory load. Here, we found a significant effect of delay and a main effect of age with PND43 and PND44 adolescents performing significantly worse than adults (PND43:[$F_{(1,11)} = 5.96$, $p = 0.033$]; PND44: [$F_{(1,11)} = 6.19$, $p = 0.030$]; Fig. 3B). Beginning on PND45, adolescent performance was no longer significantly different from adults, but the accuracy curves of adolescents did not completely overlap with adults until PND48 (see Table 1 for F and p values for the main effect of age; Fig. 3C). The rats continued to be tested at delays up to 12 s until PND52. Interestingly, while performance between the age groups was equal until PND50, on days PND51 and PND52 the behavior of the adolescents and adults began to diverge again. In particular, on PND52, two-way rmANOVA revealed a significant effect of delay [$F_{(6,66)} = 18.18$, $p < 0.0001$], a trend toward effect of age [$F_{(1,11)} = 3.50$, $p = 0.088$], and a significant interaction between age and delay [$F_{(6,66)} = 3.46$, $p = 0.005$], with Bonferroni's post-hoc test indicating significantly better performance in adults at the 8 and 12 s delays (Fig. 3D). However, the effect seemed to primarily be driven by a large increase in performance accuracy in the adults, suggesting a practice/developmental effect that either did not occur or occurred to a lesser degree in adolescents.

On PND53, we increased the difficulty (i.e., cognitive load) of the task by increasing the delay range to 0–24 s, where delays beyond 20 s
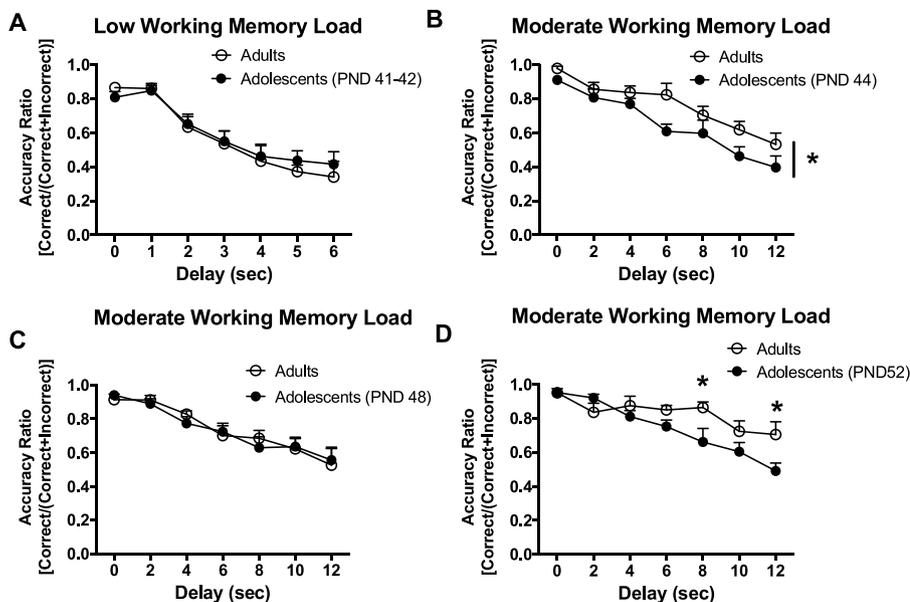
Fig. 3. Working memory performance with low-to-moderate cognitive loads. (A) Working memory performance plotted as an accuracy ratio (proportion correct) across increasing delays. Performance accuracy decreases with delay, but there was no difference based on age at this low cognitive load (0–6 s). Working memory performance at a moderate cognitive load (0–12 s) was significantly better in adults relative to adolescents at postnatal day (PND)44 (B), but not at PND48 (C). (D) With more days of training, adult performance was significantly better than adolescents at 8 and 12 s delays. Chance performance would be a ratio of 0.33. All data are presented as the mean + standard error of the mean (SEM), *p < 0.05 main effect of age in (B) and significant interaction between age and delay (D).

Table 1
Statistical Comparison of Adolescent vs. Adult Working Memory Performance.

Age (Postnatal Day)	F-value (effect of age)	p-value (effect of age)
45	1.81	0.205
46	2.17	0.169
47	2.17	0.169
48	0.026	0.874
49	0.031	0.864
50	0.550	0.474
51	1.409	0.260

are thought to be beyond normal working memory capacity, even in non-human primates (Kojima and Goldman-Rakic, 1982). Indeed, at these delays, both age groups performed roughly at chance levels (33% accuracy) at delays of 20–24 s, with an overall significant effect of delay on accuracy [$F_{(6,66)}$ = 53.91, p < 0.0001]. In addition, we observed a significant effect of age [$F_{(1,11)}$ = 8.89, p = 0.013], with adolescents performing significantly worse than adults (Fig. 4A). Rats were tested for working memory performance again on PND60 and PND63 to determine if the performance of the late adolescents would "catch-up" to adults. Indeed, there were no significant differences between adolescents and adults on these test days, but there were strong trends towards effects of age and age x delay interactions, suggesting that even as late as PND63, working memory performance at high cognitive loads is not completely mature (Fig. 4B & C; PND60: main effect of age [$F_{(1,11)}$ = 3.85, p = 0.076]; age x delay interaction [$F_{(6,66)}$ = 1.97,

p = 0.082]; PND63: main effect of age [$F_{(1,11)}$ = 4.38, p = 0.06]; age x delay interaction [$F_{(6,66)}$ = 1.97, p = 0.082]).

3.3. Experiment 1: Effect of cannabinoid receptor activation on working memory

On PND54 and PND55, rats underwent two days of testing the effect of cannabinoid receptor activation on working memory performance. Acute injection of the synthetic cannabinoid receptor agonist WIN55,212-2 (WIN) had no significant effect on working memory, relative to vehicle injection, on performance in adolescents, with a two-way rmANOVA across both delay and treatment identifying a main effect of delay [$F_{(6,42)}$ = 53.68, p < 0.0001], but no effect of treatment [$F_{(1,7)}$ = 0.240, p = 0.640], or treatment x delay interaction [$F_{(6,42)}$ = 1.03, p = 0.420] (Fig. 5A). Similarly, acute WIN had no significant effect on adult working memory performance; though there was a slight tendency for a WIN-induced impairment in adults, with a main effect of delay [$F_{(6,24)}$ = 25.32, p < 0.0001], and trend toward effect of treatment [$F_{(1,4)}$ = 4.86, p = 0.092], but no treatment x delay interaction [$F_{(6,24)}$ = 0.337, p = 0.911] (Fig. 5B). There was no evidence of lingering effects of prior WIN injection on working memory performance; nevertheless, we did not test working memory again until PND60, as described above.

High Working Memory Load
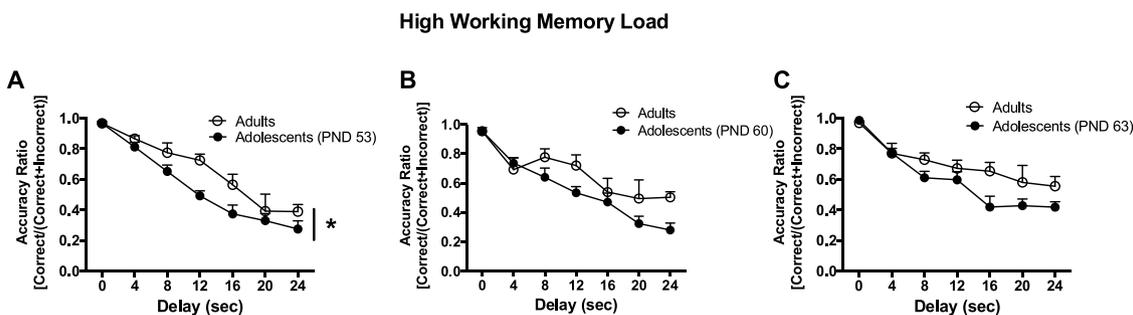
Fig. 4. Working memory performance with high cognitive load. (A) Working memory performance plotted as an accuracy ratio (proportion correct) across 0–24 s delays, representing a high working memory load. Adults performed significantly better than adolescents at PND53. Adolescent working memory performance at a high working memory load was not statistically different from adults on PND60 (B) or PND63 (C), though adults tended to be more accurate. Chance performance would be a ratio of 0.33. All data are presented as the mean + standard error of the mean (SEM), *p < 0.05 main effect of age.
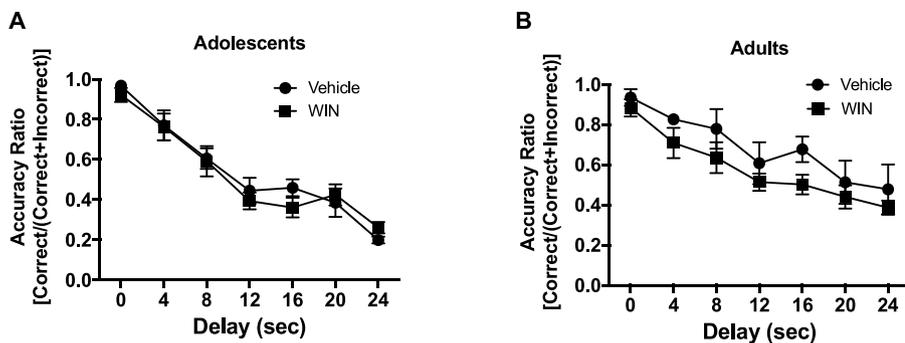
Fig. 5. Effect of cannabinoid receptor activation on working memory performance. The effect of acute injection of 1 mg/kg WIN55,212-2 (WIN) was assessed using a within-subjects design in adolescents (A) and adults (B). No statistically significant differences were found for WIN treatment on working memory performance in either age group.

3.4. Experiment 2: Comparison of task acquisition in adolescents versus adults

As in experiment 1, we observed no major age differences in task acquisition parameters. All but one adult rat met our acquisition criterion within 2 days of phase 1 training, and all were moved on to single nosepoke responding. As in experiment 1, adolescents earned significantly more reinforcers than adults over the days of training, indicating a faster learning rate. Two-way rmANOVA revealed a significant effect of age [$F_{(1,108)} = 13.04$, $p = 0.0005$], effect of day [$F_{(4,108)} = 18.69$, $p < 0.0001$], and only a trend toward an interaction between the two [$F_{(4,108)} = 2.22$, $p = 0.071$] (data not shown). All but one adolescent met our training criterion within 6 days of training on phase 2 of the task; however, as before, several adults were maintained on phase 2 for additional days to meet our training criterion. Once rats were on phase 3 of training (2 responses on the sample nosepoke with no delay), adolescent and adult rats acquired our training criterion (70% accuracy) within a similar amount of time, with a Log-rank Mantel Cox test finding no significant difference in acquisition rate for this phase of the task [$\chi^2 = 1.03$, $p = 0.31$]. Ultimately, two adolescents required either too many days of phase 2 or phase 3 training to be within a close enough age range to be included in the analysis. Similarly, four adults did not acquire the task quickly enough to be included in the analyses. Therefore, the remaining behavioral data are reported for n = 10 adolescent and n = 8 adult rats. By the end of phase 3 training, there was no significant difference in accuracy based on age group assessed by two-tailed, unpaired *t*-test [$t = 0.007$, $p = 0.99$]. However, there was a significant effect of age on reinforcers earned at the end of training [$t = 2.85$, $p = 0.012$] (data not shown), with adults earning significantly more than adolescents, similar to the trend-level effect observed in experiment 1. Therefore, all training measures replicated across experiments, with adolescents generally meeting acquisition criteria more rapidly than adults, but groups obtaining similar levels of accuracy, and adults potentially being more motivated to obtain reinforcers.

3.5. Experiment 2: Comparison of measures of impulsivity and working memory performance in adolescents versus adults under conditions of premature response punishment

In experiment 2, rats were initially tested on a different version of the working memory task, where the delays were short (0.5–6 s), but a response on any nosepoke prior to the illumination of the three nosepokes in the choice phase, led to a timeout and the initiation of a new trial. Thus, we obtained measures of impulsive, premature responding, in addition to working memory performance. Overall, as delay increased, both adolescents and adults made increasing numbers of premature responses, making analysis of working memory unreliable, as the number of trials completed at the longer delays was quite low. Fig. 6A illustrates trials completed as a function of delay length on the last day of training on this version of the task. Two-way rmANOVA revealed a significant effect delay length reducing the trials completed

[$F_{(6,96)} = 50.32$, $p < 0.0001$], but there was no effect of age [$F_{(1,16)} = 2.59$, $p = 0.127$] or age x delay interaction [$F_{(6,96)} = 0.871$, $p = 0.52$]. Working memory performance was also measured on this day, but in some cases there were missing values due to no trials being completed at a particular delay. Thus, missing points were replaced with the mean value for that delay within age group, and rmANOVA revealed no effects of age [$F_{(1,112)} = 0.162$, $p = 0.69$] or interaction between age and delay [$F_{(6,112)} = 1.9$, $p = 0.087$] on performance, though significant effects of delay were observed, as expected [$F_{(6,112)} = 14.96$, $p < 0.0001$] (Fig. 6 B). Despite the difficulty in assessing working memory by including timeouts for premature responses, the overall pattern of effect was similar to experiment 1, where no age differences were observed with a low cognitive load. Next, we compared the number of premature responses made on the last day of training on this phase, both in total number and as a percent of all trials initiated. We observed significantly greater premature responses [$t = 2.79$, $p = 0.013$] and percent of trials ending in a premature response [$t = 2.35$, $p = 0.032$] in adolescents compared to adults (Fig. 6C–D). Thus, we found evidence for increased impulsivity in adolescents, which is similar to reports in human adolescents (Casey and Jones, 2010; Steinberg et al., 2009; van Duijvenvoorde et al., 2015; Van Leijenhorst et al., 2010).

Next, rats were shifted to the standard working memory procedure that did not punish premature responses so that we could increase the number of trials completed for more accurate working memory assessment. Fig. 7A illustrates that the number of trials completed is equivalent across delays, with no significant difference based on delay length [$F_{(6,96)} = 2.0$, $p = 0.069$], however adults completed significantly more overall trials than adolescents [$F_{(1,16)} = 4.97$, $p = 0.041$]. Similar to what was observed on PND43, adolescents did not differ from adults in working memory performance at low cognitive load over two days of testing (Fig. 7B; effect of age PND44: [$F_{(1,18)} = 0.09$, $p = 0.767$; PND45: $F_{(1,18)} = 0.00009$, $p = 0.998$]).

The rats were then shifted to 0–12 s delays (moderate working memory load). Here we observed a significant interaction between age and delay [$F_{(6,96)} = 2.57$, $p = 0.024$], effect of delay [$F_{(6,96)} = 51.57$, $p < 0.0001$], but no main effect of age [$F_{(1,16)} = 0.134$, $p = 0.72$]. Post-hoc analyses indicated that PND46 adolescents performed significantly worse than adults at the 12 s delay, and there was a strong trend towards worse performance at the 10 s delay ($p = 0.053$). At PND48 there was still a significant interaction between age and delay, though adolescents were only worse than adults at the 8 s delay (data not shown). However, on PND50, adolescent performance was not significantly different from the adults. Thus, we again found that with a moderate working memory load, adolescents do exhibit impaired working memory performance relative to adults, but that it improves over the course of late adolescence, maturing from PND40 to PND50.

3.6. Experiment 3: Ontogeny of prefrontal cortex protein levels

In experiment 3, we analyzed the expression of glutamatergic and GABAergic receptor proteins in the prefrontal cortex in adolescent, late
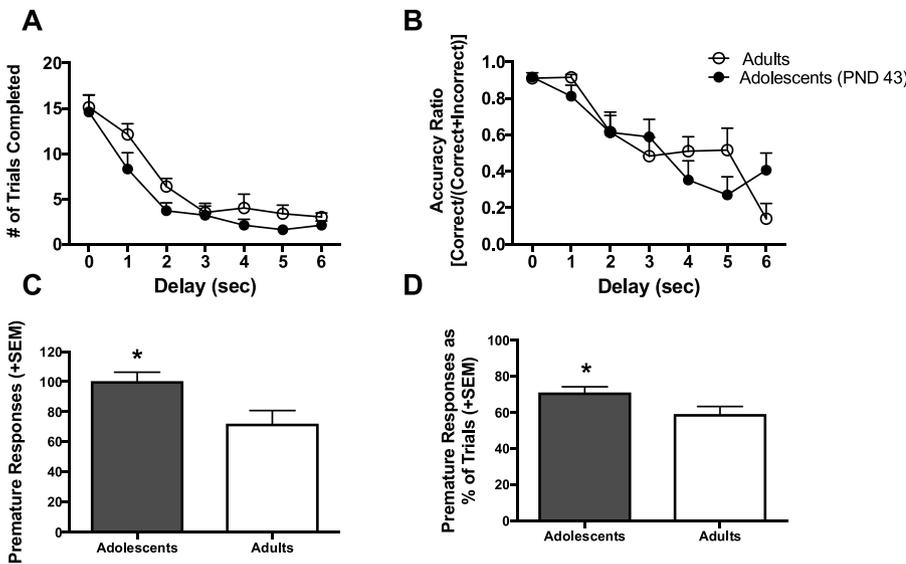
Fig. 6. Task performance during impulsivity testing. (A) The number of trials completed at each delay on the last day of training is plotted based on age group. With punishment of premature responses, all rats performed significantly fewer trials at the longest delays, indicating increased premature responses with increasing delay. (B) Working memory performance at low cognitive load assessed with inclusion of premature response punishment. No differences between PND43 adolescents and adults was observed. (C) The number of premature responses made on the last day of impulsivity testing was significantly greater in adolescents than adults. (D) The number of premature responses plotted as a percent of trials completed was also significantly greater in adolescents versus adults. All data are presented as the mean + standard error of the mean (SEM), *p < 0.05, t-test.

adolescent, and adult rats to determine if there were any shifts in protein expression that might explain differences in working memory performance. We specifically dissected the prelimbic and infralimbic cortices and analyzed them separately, as they are reported to mediate different behavioral effects in a number of tasks (Barker et al., 2013; Coutureau and Killcross, 2003; Hitchcott et al., 2007; Peters et al., 2009; Sierra-Mercado et al., 2011). Analysis of membrane fractions revealed few effects of age on receptor expression. However, in the prelimbic cortex, the GluN2B subunit of the NMDA receptor appeared to decrease from adolescence into adulthood. Analysis of protein expression by one-way ANOVA only revealed a trend toward a significant effect $[F_{(3,20)} = 2.40, p = 0.099]$, but given the expected variance across development, we conducted a test for linear trend to determine if there is evidence of a significant progressive change in protein expression over development. This test found a significant negative slope $[p = 0.016]$ indicating a decrease in expression from PND40 to PND90 (Fig. 8A). There was no change in GluN2B expression over age in the infralimbic cortex $[p = 0.40;$ Fig. 8B]. We performed similar analyses for the AMPA receptor subunit GluA1 and observed no significant differences in the prelimbic cortex $[p = 0.19]$, but an almost significant negative linear trend in the infralimbic cortex $[p = 0.057,$ Fig. 8C–D]. We also examined expression of the obligate subunits of the GABAB receptor and the GABAA receptor. However, we found no significant differences in expression in either brain region over development [GABABR2 prelimbic: p = 0.20; GABABR2 infralimbic: p = 0.10; GABAAR1 prelimbic: p = 0.83; GABAAR1 infralimbic: p = 0.21; Fig. 8E–H].

4. Discussion

In the present series of experiments, we utilized an operant-based, spatial, delay-match-to-sample working memory task in order to assess changes in working memory performance as a function of cognitive load (delay length) in rodent adolescence. We compared adolescent and adult rats with equivalent training experience on the task and found that by PND41 adolescents and adults performed equally well at a low cognitive load (delays up to 6 s). However, when the cognitive load was increased (delays up to 12 s) adolescents performed worse than adults, particularly at 8–12 s delays, until about PND45-47. Moreover, when the cognitive load was further increased (up to 24 s), late adolescent rats (PND53) again showed deficits relative to adults. Thus, working memory does appear to develop into late adolescence in rodents, as in other species (e.g., Luciana and Nelson, 1998; Zhou et al., 2016).

The observation of working memory performance improvements in
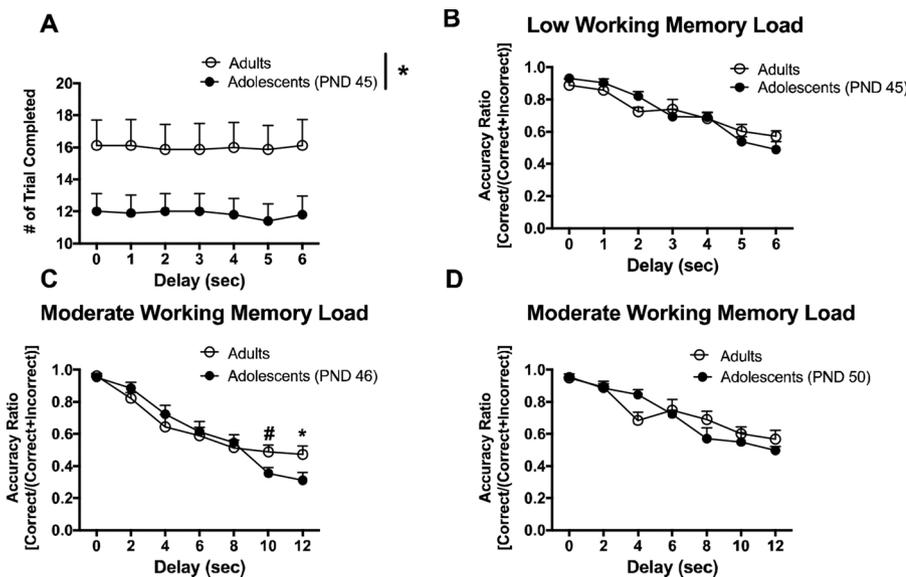
Fig. 7. Working memory performance in experiment 2. (A) The average number of trials completed at each delay was compared between adolescents and adults. An average of 12 trials in adolescents and 16 trials in adults was completed at all delays when premature response punishment was removed. Adults performed significantly more trials. (B) Working memory performance plotted as an accuracy ratio (proportion correct) across increasing delays at a low working load was not different between PND45 adolescents and adults. (C) Working memory performance at a moderate working memory load was significantly better in adults relative to PND46 rats at 10 and 12 sond delays. (D) By PND50, there was no difference between adolescent and adult working memory performance at a moderate working memory load. All data are presented as the mean + standard error of the mean (SEM), *p < 0.05 main effect of age in (A) and significant interaction between age and delay (C).
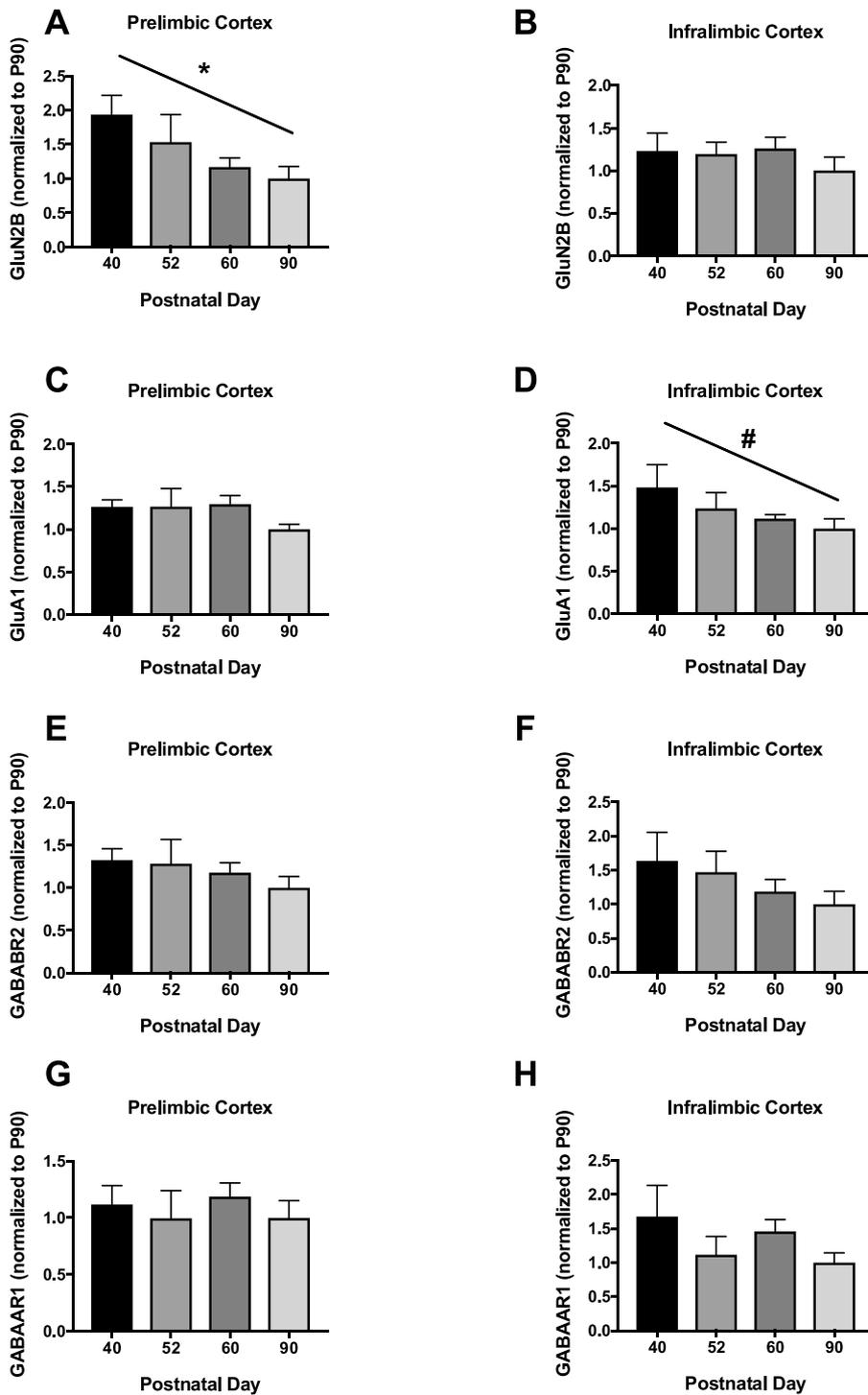
Fig. 8. Effects of age on prefrontal cortex protein expression. Membrane expression of prefrontal cortex proteins was assessed in the prelimbic (left side) and infralimbic (right side) cortices. A significant decrease in GluN2B expression was observed over age in the prelimbic (A) but not infralimbic (B) cortex. AMPA receptor subunit GluA1 expression did not change in prelimbic cortex with age (C), but there was a strong trend for a decrease in infralimbic cortex with increasing age (D). The expression of GABA receptor subunits, GABABR2 (E, F) and GABAAR1 (G, H) did not change with age. All data are presented as the mean + standard error of the mean (SEM), all values are normalized to PND (P) 90, *p < 0.05, #p < 0.06, test for linear trend.

rodent adolescence required the use of a task that allowed systematic modulation of cognitive load, which has been absent in prior studies of cognitive development in rodents. For example, prior studies have examined the development of delayed alternation behavior in the T-maze and object/spatial recognition memory, two tasks often used to study "working memory" in rodents. First, it should be noted that in novel object/spatial location tasks, the period of time over which information needs to be remembered is on the order of minutes, which corresponds to short-term, rather than working, memory. In addition, these ontogenetic studies tested the emergence of the capacity to perform these tasks, not the improvement of working memory ability over time. The ability to perform these tasks emerges quite early in rodent

development, often by PND21 (Green and Stanton, 1989; Westbrook et al., 2014). Our present studies support these past findings, as adolescent rats were capable of learning and performing the delay-match-to-sample task in early adolescence and performed equally to adults when the cognitive load was low. Only when adolescents were challenged with increasing cognitive load was it possible to observe deficits relative to adults and to determine the timeframe during which working memory performance reached adult-like levels.

However, there are a few caveats to the experimental design that should be considered when interpreting the results. First, adolescents and adults were shipped at different ages, which could theoretically lead to differential effects of stress on behavior and developmental

processes. We think that the influence of shipping likely had minimal effect as shipping occurred several weeks before working memory testing at moderate to high cognitive loads and the rats had substantial handling experience prior to training. Moreover, the adolescents, if anything, learned the basic task faster than adults, yet still exhibited worse working memory performance, suggesting that there were not systematic effects of shipping stress on behavior. In addition, the nature of task training and testing in rodents requires the animals to be exposed to working memory testing over several sessions leading to the potential for both practice effects and actual improvements in working memory ability/capacity. Our results from testing at a moderate cognitive load in Fig. 3B and 5 C, suggest better performance in adults when practice levels were lower, and that adolescents equaled adults in the late PND40 s. However, as training continued, both age groups showed substantial increases in accuracy, even at the longest delays, suggesting that practice itself can lead to improvement (Fig. 3D). Intriguingly, when these practice effects appear to emerge, the performance of the adults was again better than the adolescents, suggesting that there may also be age related effects on the ability of task repetition to improve performance. However, there is no clear way to disentangle the effects of age and task experience when using complex cognitive tasks that require extensive training.

Next, we tested whether or not there were age differences in the ability of acute cannabinoid receptor activation to impair working memory, given that several studies have found that cannabinoid receptor agonists disrupt working memory using a variety of tasks (Abush and Akirav, 2009; Hampson and Deadwyler, 2000). However, we observed no effects of acute injection of WIN in either adolescents or adults. There are a number of factors that could influence these divergent results. First, several aspects of the tasks used differ. Our task involved three different nosepoke choices as opposed to 2 lever choices in the Hampson and Deadwyler (2000) study, and our rats were not required to make a different response in between the sample and choice phases. Thus, our task may involve different neural circuits that are less affected by cannabinoid exposure than the non-match to sample task or spatial water maze. Indeed, prior studies have strongly implicated disruption of hippocampal neuron activity in the effects of cannabinoids on memory in these tasks, but this has not yet been established for our task (Abush and Akirav, 2009; Hampson et al., 2011; Hampson and Deadwyler, 2000). Rather, we have found that prelimbic prefrontal cortical activity is required for accurate performance (Kirschmann et al., 2017a). In addition, our study used a different strain of rats (Sprague-Dawley versus Long Evans) and we used a higher dose of WIN, leading to the possibility that there is a U-shaped dose effect curve, though what mechanism would lead to a loss of effect at higher doses is unclear. Thus, we conclude that acute cannabinoid receptor activation does not disrupt all measures of working memory performance.

In addition to working memory, we also tested the ability of rats to perform a variant of the task that punished premature responses (i.e., responses between the sample and choice phases). We found that all rats were likely to emit a premature response when the delay between sample and choice phases was several seconds long, reducing the number of trials completed at long delays. However, this effect was larger in adolescents as they emitted significantly more premature responses. Thus, these results suggest that adolescents have an increased tendency to be impulsive, which is also consistent several studies indicating that human adolescents are more impulsive than adults (Casey and Jones, 2010; Steinberg et al., 2009; van Duijvenvoorde et al., 2015; Van Leijenhorst et al., 2010).

Finally, we examined whether the expression of receptors regulating excitatory glutamatergic or inhibitory GABAergic signaling in the mPFC changed along the same time course as working memory improvements. The only receptor that showed a significant change over time was the GluN2B subunit of the NMDA receptor, specifically in the prelimbic cortex region of the mPFC. This result is intriguing as it is well established that GluN2B expression is prominent early in

development relative to GluN2A containing NMDA receptors (Dumas, 2005; Yashiro and Philpot, 2008). Signaling through GluN2B is required for critical period plasticity in numerous sensory cortical regions. Moreover, the timing of the shift from predominant GluN2B to predominant GluN2 A expression varies across brain regions, with regions like the hippocampus and cerebellum developing much later than somatosensory and auditory cortices (Dumas, 2005). However, in all cases, this switch in NMDAR signaling occurs by the third week of life in rodents, prior to the onset of adolescence (Dumas, 2005; Yashiro and Philpot, 2008). Our results suggest the intriguing possibility that the prelimbic cortex has a more protracted development, with a critical period that is open well into adolescence. This is consistent with prominent theories of human cognitive development (Casey and Jones, 2010) and recent findings of protracted development of other critical period markers in the prefrontal cortex across species (Larsen and Luna, 2018). Thus, taken together, our data suggests that working memory development is associated with similar shifts in NMDA receptor subunit expression that occur in the early postnatal period in other brain regions. Our data also suggest that this phenomenon, at least in rats, is limited to the prelimbic PFC, as no changes in GluN2B expression were observed in the infralimbic PFC. It may be that the critical period in the infralimbic cortex occurs earlier in development and was already closed by postnatal day 40. Future studies could investigate infralimbic development and confirm that the ratio of GluN2 A/2B increases in the prelimbic cortex over the course of adolescence, which we were not able to do in the current study. It should also be noted, that another study of medial prefrontal cortex development has shown input-specific increases in GluN2B signaling with age in layer 5 neurons, raising the possibility that even within a brain region, there is differential development of specific circuits (Flores-Barrera et al., 2014).

5. Conclusions

Overall, in concordance with studies in humans (Luciana and Nelson, 1998; Siegel and Ryan, 1989; Simmonds et al., 2017) and non-human primates (Verrico et al., 2014; Zhou et al., 2016), we observed an age-related improvement in working memory performance across rodent adolescence in the male Sprague-Dawley rat. We also observed increased impulsive responding in adolescents, again, consistent with human studies (Steinberg et al., 2009; van Duijvenvoorde et al., 2015; Van Leijenhorst et al., 2010). Thus, it appears that the protracted development of executive functions, such as working memory and inhibitory control, is conserved across species. The establishment of a rodent model of adolescent working memory development will allow further exploration of the molecular and circuit underpinnings of cognitive maturation, given that invasive brain studies are more tractable in rodent models. As a first step in this direction, we investigated the expression of proteins in the medial prefrontal cortex that regulate glutamatergic and GABAergic signaling across late adolescent development and into adulthood. We observed a selective decline in expression of the NMDAR subunit GluN2B in the prelimbic cortex over the course of adolescence, suggestive of a critical period of development during this time. Therefore, future studies will be able to investigate how disruptions in prelimbic cortex development may contribute to cognitive deficits observed in a number of neuropsychological disorders.

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