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ORIGINAL PAPER

Effects of Methamphetamine Exposure on Fear Learning and Memory in Adult and Adolescent Rats

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Abstract

Methamphetamine (meth) use is often comorbid with anxiety disorders, with both conditions predominant during adolescence. Conditioned fear extinction is the most widely used model to study the fear learning and regulation that are relevant for anxiety disorders. The present study investigates how meth binge injections or meth self-administration affect subsequent fear conditioning, extinction and retrieval in adult and adolescent rats. In experiment 1, postnatal day 35 (P35—adolescent) and P70 (adult) rats were intraperitoneally injected with increasing doses of meth across 9 days. At P50 or P85, they underwent fear conditioning followed by extinction and test. In experiments 2a–c, P35 or P70 rats self-administered meth for 11 days then received fear conditioning at P50 or P85, followed by extinction and test. We observed that meth binge exposure caused a significant disruption of extinction retrieval in adult but not adolescent rats. Interestingly, meth self-administration in adolescence or adulthood disrupted acquisition of conditioned freezing in adulthood. Meth self-administration in adolescence did not affect conditioned freezing in adolescence. These results suggest that intraperitoneal injections of high doses of meth and meth self-administration have dissociated effects on fear conditioning and extinction during adulthood, while adolescent fear conditioning and extinction are unaffected.

Keywords Methamphetamine · Self-administration · Fear · Extinction · Rats · Adolescence

Introduction

Methamphetamine (meth) is a highly addictive psychostimulant that is used world-wide. Its use is often linked with other mental health disorders [1, 2]. For example, anxiety is considered a common side effect of meth intoxication [3], and anxiety disorders are highly comorbid with meth dependence [2, 4]. Whether meth use causes anxiety or anxiety predisposes to meth use is poorly understood. Interestingly, findings in adults suggest that anxiety is likely a risk factor to meth dependence [4], whereas prospective studies beginning in adolescence show that high incidence of anxiety disorders in adolescent-onset meth users is likely a consequence of, and not a risk for meth use [5, 6]. Substance use disorder and anxiety disorders both emerge early in life [7], and it may be that meth exposure in adolescence leads to a greater chance of anxiety disorders compared to meth exposure in adulthood.

Animal research strongly suggests that meth exposure causes anxiety in adult and adolescent rodents. For example, adolescent and adult male mice spend less time in the centre during an open field test after a single intraperitoneal (IP) injection of meth (4 mg/kg) compared to saline [8, 9]. While acute exposure studies may model anxiety as an immediate side effect of meth use, chronic exposure studies are necessary to understand whether anxiety is a consequence of meth dependence. Indeed, twice-daily IP injections of meth (escalating 1–4 mg/kg over 7 days or escalating 1–10 mg/kg over 10 days) in adult rats significantly decreased open arm entries in elevated plus maze [10]. Similarly, daily oral gavage of 2 mg/kg meth for 5 days in adolescent rats resulted in less open arm entries in elevated plus maze and less time in the centre of an open field when tested during adulthood [11]. However, daily 3 mg/kg meth IP injections for 7 days in adult rats increased time spent in the open arm of the elevated plus maze after 3 weeks of withdrawal [12], indicating

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that further studies are needed. In addition, the impact of chronic meth exposure in adolescence on anxiety-relevant behaviours in adolescence has never been tested [13]. Further, all of those studies involve experimenter-administered meth, which limits their translational value in understanding humans, where meth intake is always voluntary. Therefore, the first aim of our study is to examine both experimenter-administered and self-administered meth on anxiety-relevant behaviors.

The second aim of the study is to use Pavlovian fear conditioning and extinction as a model to understand the impact of meth exposure on anxiety. Fear conditioning involves pairings of a discrete conditioned stimulus (CS), such as a tone, with an aversive unconditioned stimulus (US), such as a footshock. Initially, the CS is a neutral stimulus but when it is paired with the US, the CS starts to elicit conditioned responses on its own. Although this paradigm does not model all aspects of anxiety disorders, fear conditioning is the most utilised model for investigating anxiety disorders because it is not easily forgotten, and is readily acquired across a range of different ages in many different animals, including humans [14-17]. Extinction refers to the decrease in fear to the CS due to repeat or prolonged exposure to the CS without any US, and it measures how fear can be suppressed or regulated. Extinction models exposure therapy in the clinic, and extinction impairments can predict anxiety disorder severity [18], and it is considered the best translational model used in rodents and humans to understand anxiety disorders [14] including in adolescents [16, 19, 20]. Measure of inhibitory control such as fear extinction was chosen for this study because it is more advantageous than trait anxiety measures such as elevated plus maze and open field tests in comparing anxiety disorder relevant behaviours between adolescents and adults. Firstly, extinction deficits are consistent with the clinical observations that the majority of anxiety disorders involves impaired cognitive, emotional, and behavioral inhibition, all of which share similar neurocircuitry with anxiety disorders in adults and adolescents [14, 21]. Such evidence is yet unclear for trait anxiety measures. Secondly, exaggerated extinction deficits during adolescence is widely replicated [20, 22–28]. It is now a well-established model to understand mental disorders in adolescence. In contrast, trait anxiety measures in adolescent rodents have been shown to have opposite outcomes in open field test compared to elevated plus maze test [29]. Also, adolescent and adult show differences in trait anxiety measures at baseline, with inconsistent findings between different tests [30].

There is one study that has examined the effects of meth on conditioned fear [31]. In that study, adult rats received subcutaneous injections of escalating doses of meth (1.25–5 mg/kg twice a day every other day over 7 days). After 5 days of abstinence rats received footshocks in a novel context. Compared to saline, rats previously exposed to meth showed significantly increased freezing to the context [31]. However, neither adolescent rats nor fear extinction were assessed.

The present study investigated the effects of experimenter-administered or self-administered meth on fear conditioning and extinction in adolescent and adult rats. The binge exposure schedule was based on Jayanthi et al. [32], which observed that meth can cause significant downregulation of the AMPA receptor subtypes glutamate receptor 1 and 2 [32, 33] relevant for fear extinction [34, 35]. Based on previous observations in rodents, we hypothesised that experimenter-injected meth would cause increased freezing during conditioning and/or extinction, with more dramatic effects observed in adolescents compared to adults. Considering that meth use in adolescence, but not in adulthood, may lead to anxiety in humans, we also hypothesised that self-administered meth would cause increased freezing during conditioning and/or extinction in adolescent, but not adult rats.

Materials and Methods

Animals & Surgery

Male Sprague-Dawley rats were bred in-house at the Florey Institute of Neuroscience and Mental Health. For experimentation during adolescence, postnatal day 21 (P21) rats were moved into 12:12 reversed light cycle room (lights off at 7:00 am) and housed in pairs in open top cages. If rats were tested during adulthood, they were moved into the same reversed light cycle room at P56 ± 2 and housed in pairs in open top cages. For experiments 2a, b and c, intravenous catheters were inserted into the jugular vein 4–6 days prior to commencement of self-administration and rats were individually housed immediately post-surgery. Housing conditions, handling, and surgery of adult and adolescent rats were as described previously [27]. Catheters were flushed daily with 0.05 mL of heparinised saline (50 IU/mL; Pfizer USA) consisting of 10% Fisamox antibiotic (amoxicillin sodium; Aspen Australia). All experiments were approved by the Florey Animal Ethics Committee and performed in accordance with the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Meth Administration

Adolescent rats were P35 ± 2 and adult rats were P70 ± 2 at the start of meth administration. In experiment 1, all rats received daily IP injections of saline or meth (methamphetamine hydrochloride, Sigma-Aldrich, Australia) dissolved in

saline (1 mL/kg injection volume). Chronic injection dose and schedule (Table 1) were based on previous studies [32, 33]. In experiments 2a, b, and c, self-administration of saline or meth dissolved in saline (0.03 mg/kg/infusion, 50 µl volume per infusion) occurred in standard operant chambers equipped with retractable levers (Med Associates, VT, USA) during 2-h daily sessions on a fixed ratio 1 schedule for 11 days. The concentration of the meth solution was calculated every 3 days based on the average weight of the rats per age group to maintain accurate mg/kg/infusion across days. Each infusion was paired with a 2.7 s illumination of a light cue located above the active lever, followed by a 17.3 s time-out period. Inactive lever pressing had no consequences.

Fear Conditioning

Adolescent rats were P50 ± 2 and adult rats were P85 ± 2 on the day of fear conditioning. Fear conditioning, extinction, and test sessions occurred in Contextual Near Infrared Video Freeze Chambers (31.8 × 25.4 × 26.7 cm) that allow automated scoring of freezing (Med Associates, VT, USA). Chambers were fitted with a low-profile contextual grid floor with 5 mm stainless steel grid rods, with 16 mm of spacing between the rods. A speaker on the right wall was present in all chambers, which produced a 5000 Hz; 80 dB tone. The chambers were placed in a sound attenuated cubicle to minimize external noise. Each cubicle was equipped with a fan, which produced a consistent background noise (~70 dB). Two different contexts placed in different rooms were used to distinguish conditioning and extinction/test contexts as described previously [36]. The use of these contexts was counterbalanced across all groups for all experiments.

On conditioning day, baseline freezing was recorded for 2 min, followed by the 10 s tone CS (80 dB) that co-terminated with a 1 s foot shock US (0.6 mA). In total, 3 CS-US pairings were presented and the inter trial interval (ITI) was 85 s between the first two pairings and 135 s between the second and third pairing. Rats remained in the chambers for 120 s following the last CS-US trial.

One day after conditioning, all rats received CS extinction in a different context to where conditioning occurred. Baseline freezing was recorded for 2 min, after which rats were exposed to sixty (experiment 1) or thirty (experiments 2a, 2b and 2c) 10 s CS-only presentations with a 10 s ITI. These extinction parameters were chosen based on pilot experimentation.

Extinction recall test occurred 24 h later and was conducted in the same context as CS extinction. Baseline freezing was recorded for 2 min, after which rats were exposed to five 10 s CS-only presentations with a 10 s ITI.

Table 1 Meth injection dosing (mg/kg) schedule for experiment 1

	Mon	Tue	Wed	Thu	Fri
Week 1					
10:00	0.5	1.0	1.0	1.5	2.0
12:00			1.0	1.5	2.0
14:00			1.0	1.5	2.0
16:00	0.5	1.0	1.0	1.5	2.0
Week 2					
10:00	1.5	2.0	2.5	3.0	
12:00	1.5	2.0	2.5	3.0	
14:00	1.5	2.0	2.5	3.0	
16:00	1.5	2.0	2.5	3.0	

Scoring and Analysis

Number of rats in each group is reported in Table 2. All statistical analyses were performed using SPSS statistics (IBM corp., New York, USA) using analysis of variance (ANOVA), repeated measures (RM) ANOVA, or *t* tests as appropriate. Automatically scored freezing data from Video Freeze software was obtained using < 50 pixel changes as the motion threshold for freezing for at least 30 frames (i.e., 1 s as the minimum freeze duration). These parameters show high concordance with manual scoring ($r > 0.9$) and work across adolescent and adult ages in rats [24]. For conditioning analyses, the first 9 s of freezing from CS onset was used to avoid including shock-induced movement. The rest of the sessions were based on 10 s of freezing for each CS trial. Extinction data were analysed in blocks of 5 CS presentations. When significant group differences were detected in baseline freezing, analysis of co-variance (ANCOVA) was used with the baseline freezing levels as the co-variate. This was to control for significantly different baseline freezing levels affecting CS-elicited freezing, as reported in previous publications [24, 37]. For self-administration analyses, RM ANOVA of the total number of active lever presses (including time-out responses) was used. Significant ANOVA/ANCOVA interactions were followed up by Bonferroni-corrected post hoc *t* tests. Significant RM ANOVA/ANCOVA interactions were followed up by ANOVA/ANCOVAs per day or trial as previously reported [38].

Results

Conditioned Fear Following Experimenter-Injected Methamphetamine

Experiment 1 assessed the effects of experimenter-injected meth in P35 and P70 rats on conditioned fear at P50 and P85 (Table 1, Fig. 1a). Rats were weighed prior to each injection

Table 2 Mean (±SEM) levels of percent baseline freezing at conditioning, extinction and test

Experiment	Group	n	Conditioning	Extinction	Test
1	Adult-Saline	16	0.1 (±0.1)	9.2 (±5.6)$^{\#}$	6.2 (±2.3)
	Adult-Meth	17	0.07 (±0.07)	2.8 (±1.1)	7.6 (±2.8)
	Adolescent-Saline	14	0.08 (±0.08)	30.5 (±6.8)$^{\&\#}$	28.9 (±6.9)*
	Adolescent-Meth	14	0.8 (±0.4)*	9.5 (±2.9) $^{\&}$	10.4 (±3.7)
2a	Saline	16	0.0 (±0.0)	1.9 (±1.0)	5.0 (±3.3)
	Meth	15	0.2 (±0.2)	7.3 (±4.2)	1.4 (±1.1)
2b	Saline	10	1.1 (±1.2)	11.3 (±6.3)	2.8 (±3.0)
	Meth	9	0.0 (±0.0)	14.8 (±9.5)	8.9 (±9.1)
2c	Saline	11	0.1 (±0.1)	17.2 (±9.7)	0.1 (±0.1)
	Meth	12	1.2 (±1.0)	19.6 (±11.1)	6.5 (±3.4)

$^{\&}$denotes main effect of age, $^{\#}$denotes main effect of Drug, *denotes significant post hoc test difference driven by one group following Age × Drug interaction

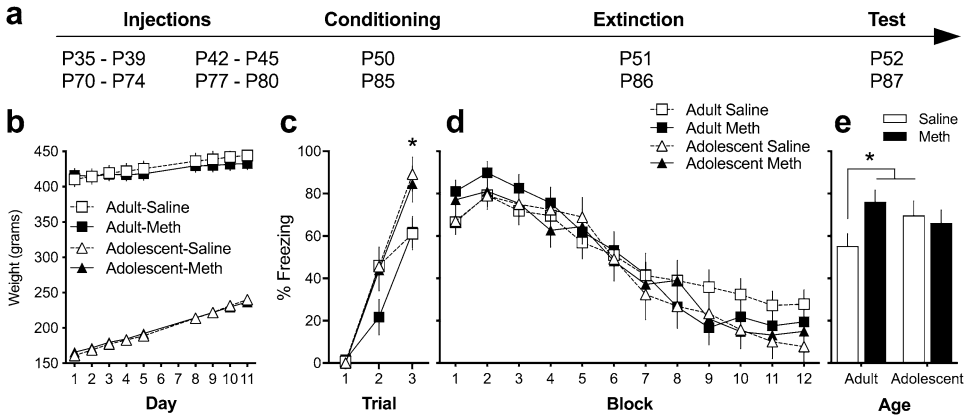

Fig. 1 **a** Experiment 1 timeline. **b** Weight gain during meth exposure. Baseline co-variate ANCOVA adjusted mean ± SEM CS-elicited freezing during fear **c** conditioning, **d** extinction, and **e** test following meth binge exposure. Adolescents reached higher levels of freezing than adults during conditioning. Freezing during extinction was similar across all groups. At test, adolescent saline and adult meth groups froze significantly more than adult-saline group. *significant post-hoc effects following significant interactions ($ps < 0.05$)

(Fig. 1b). Adult rats weighed more than adolescents (effect of Age, $F_{(1,59)} = 778.0$, $p < 0.001$) and all rats gained weight significantly over time (effect of Day, $F_{(8,456)} = 781.3$, $p < 0.001$). There was a Day × Drug interaction ($F_{(8,456)} = 8.5$, $p < 0.001$), however, post hoc tests did not reveal significant effects of Drug on any Day ($ps > 0.05$). This suggests that any effect of meth injection on each day was negligible. No other effects or interactions were observed ($ps > 0.05$).

Analysis of baseline freezing at conditioning (Table 2) showed no effect of Age ($F_{(1,57)} = 3.1$, $p = 0.09$) or Drug ($F_{(1,57)} = 2.9$, $p = 0.1$). However, an Age × Drug interaction was observed ($F_{(1,57)} = 4.3$, $p < 0.05$). Due to the significant interaction, post hoc tests were employed, which showed a significant effect of Age in meth treated rats ($p < 0.05$), with the adolescent rats freezing significantly more than adults (Table 2). No other differences were observed ($ps > 0.05$). Due to the Drug × Age interaction observed during baseline freezing, an ANCOVA was performed with baseline freezing as the co-variate to assess CS-elicited freezing during conditioning (Fig. 1c). We observed significant effects of Trial ($F_{(2,112)} = 114.2$, $p < 0.001$), Age ($F_{(1,56)} = 6.8$, $p < 0.05$), Trial × Age interaction ($F_{(2,112)} = 4.12$, $p < 0.05$), but no effects or interactions involving Drug ($ps > 0.05$). There was a post hoc Age effect at trial 3 ($p < 0.05$) but no other trials ($ps > 0.05$). These results suggest that while meth exposure did not affect freezing to the CS during conditioning, adolescent rats froze significantly more than adults by the end of conditioning.

The following day, all rats underwent extinction. Baseline freezing ANOVA revealed a main effect of Drug ($F_{(1,57)} = 9.1$, $p < 0.05$) and Age ($F_{(1,57)} = 9.6$, $p < 0.05$), but no Drug × Age interaction ($F_{(1,57)} = 2.6$, $p = 0.11$). These results indicate that adolescents froze more than adults, and saline groups froze more than meth groups during extinction baseline (Table 2). For extinction of CS-elicited freezing, RM ANCOVA with the extinction baseline as a co-variate

revealed a main effect of extinction Block ($F_{(11,616)} = 4.0$, $p < 0.001$) but no effect of Age or Block × Age interaction ($ps > 0.05$). These results suggest that all rats froze similarly during extinction (Fig. 1d), despite age differences at trial 3 of conditioning in the previous day.

At test, baseline freezing ANOVA revealed significant effects of Drug ($F_{(1,57)} = 4.4$, $p < 0.05$), Age ($F_{(1,57)} = 9.8$, $p < 0.05$) and a Drug × Age interaction ($F_{(1,57)} = 5.9$, $p < 0.05$; Table 3.2). Post hoc tests showed all the effects were driven by adolescents-saline rats freezing significantly more than all the other rats ($ps < 0.05$). ANCOVA of CS-elicited freezing at test revealed no main effects ($ps < 0.05$) but a significant Drug × Age interaction ($F_{(1,56)} = 4.0$, $p = 0.05$). Post hoc tests showed that this significant interaction was driven by adult-saline group freezing significantly lower than adult-meth and adolescent-saline groups ($ps < 0.05$, Fig. 1e). No other differences were observed ($ps > 0.05$).

Overall, the results from experiment 1 suggest that experimenter-injected meth or saline can change baseline levels of freezing in adolescent rats. When these changes in baseline levels of freezing are accounted for, chronic meth exposure did not affect CS-elicited freezing during conditioning or extinction in adolescent and adult rats. However, meth exposure disrupted extinction recall in adults but not in adolescents.

Conditioned Fear Following Self-Administration of Methamphetamine in Adulthood

Experiment 2a assessed the effects of self-administered meth in P70 rats on conditioned fear at P85 (Fig. 2a). P70 rats self-administering meth responded more on the active lever than the inactive lever (Fig. 2b). RM ANOVA of active vs inactive lever presses revealed a significant effect of Day ($F_{(10,290)} = 14.6$, $p < 0.001$), Drug ($F_{(1,29)} = 26.6$, $p < 0.001$) and Lever type ($F_{(1,29)} = 59.1$, $p < 0.001$). Furthermore, interactions were observed for Day × Drug ($F_{(10,290)} = 9.8$, $p < 0.001$), Lever type × Drug ($F_{(1,29)} = 30.9$, $p < 0.001$), Day × Lever type ($F_{(10,290)} = 16.3$, $p < 0.001$) and Day × Drug × Lever type ($F_{(10,290)} = 11.7$, $p < 0.001$). Due to the significant interactions, post hoc tests were employed, which revealed that the Drug effect for the active lever responding was apparent from day 5 onwards ($ps < 0.05$). Consistent with the findings on the levers, adult rats self-administering meth significantly increased the amount of infusions over time, whereas saline-administering rats did not (data not graphed). RM ANOVA of the number of infusions revealed a main effect of Day ($F_{(10,290)} = 18.6$, $p < 0.001$), Drug ($F_{(1,29)} = 27.5$, $p < 0.001$) and a Day × Drug interaction ($F_{(10,290)} = 16.8$, $p < 0.001$). Due to the significant interaction, post hoc tests were employed, which revealed that the Drug effect was apparent from day 5 onwards ($ps < 0.05$). All rats significantly gained weight over time (effect of Day, $F_{(3,77)} = 70.7$, $p < 0.0001$), and there were no differences in overall or rate of weight gain between meth vs saline-administering rats ($ps > 0.05$, Table 3).

Baseline freezing was not different between groups at conditioning, extinction, or test ($ps > 0.05$, Table 2). RM ANOVA of CS-elicited freezing conditioning trials revealed a significant effect of Trial ($F_{(2,58)} = 14.6$, $p < 0.001$), Drug ($F_{(1,29)} = 13.0$, $p < 0.001$) and Trial × Drug interaction ($F_{(2,58)} = 7.7$, $p < 0.001$). Post hoc tests showed that the significant interaction was driven by the Drug effect at trials 2 and 3 ($ps < 0.05$; Fig. 2c). This suggests that meth-taking rats reached lower levels of freezing compared to saline-taking rats during conditioning. During extinction, all rats decreased CS-elicited freezing similarly with RM ANOVA showing a significant effect of extinction Block ($F_{(5,145)} = 26.3$, $p < 0.001$). There were no Drug or Block × Drug interaction ($ps > 0.05$; Fig. 2d). These results suggest that meth self-administration during adulthood did not affect fear extinction. For CS-elicited freezing at test, there was an effect of Drug ($t_{(29)} = 2.7$, $p < 0.05$), showing that meth self-administration during adulthood leads to

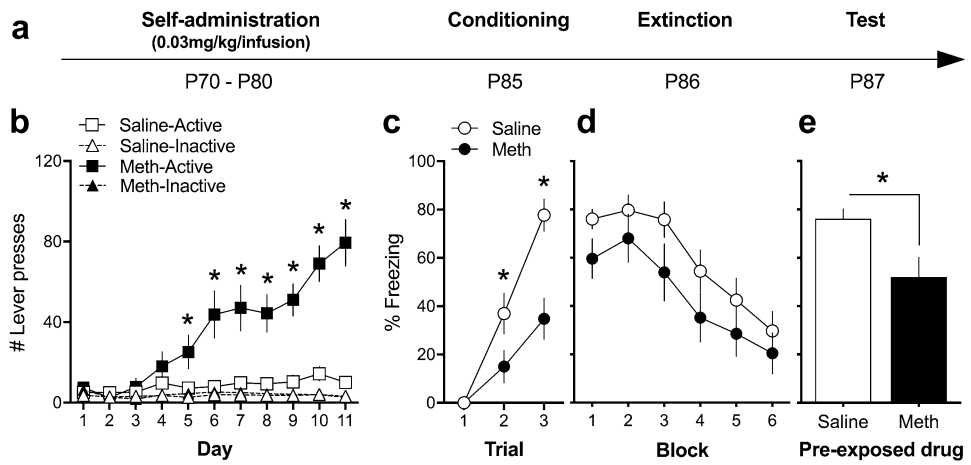

Fig. 2 **a** Experiment 2a timeline. **b** Mean ± SEM total lever presses during self-administration of meth or saline during adulthood. Mean ± SEM CS-elicited freezing during fear **c** conditioning, **d** extinction, and **e** test following meth self-administration. Meth self-administration during adulthood significantly reduced freezing during conditioning and test in adult rats. *significant *t* tests or post-hoc effects following significant interactions ($ps < 0.05$)

Table 3 Mean (±SEM) weight for experiments 2a, 2b and 2c during self-administration

Experiment	Group	Day 1	Day 4	Day 7	Day 10
2a	Saline	392 (±10.5)	398 (±13.6)	405 (±10.9)	418 (±13.2)
	Meth	412 (±11.3)	417 (±13.9)	417 (±12.1)	437 (±1.1)
2b	Saline	150 (±4.1)	177 (±4.3)	205 (±4.5)	232 (±3.9)
	Meth	149 (±10.5)	172 (±5.2)	203 (±5.9)	227 (±5.9)
2c	Saline	150 (±3.9)	178 (±4.1)	205 (±4.2)	232 (±3.7)
	Meth	147 (±2.9)	170 (±3.7)	200 (±4.4)	225 (±3.4)

reduced CS-elicited freezing at extinction recall (Fig. 2e). Together, these results suggest that a period of meth self-administration during adulthood reduces subsequent CS-elicited freezing during conditioning and test.

Adolescent Conditioned Fear Following Self-administration of Methamphetamine in Adolescence

Experiment 2b assessed the effects of self-administered meth in P35 rats on conditioned fear at P50 (Fig. 3a). Similar to adults, adolescents self-administering meth responded more on the active lever than the inactive lever (Fig. 3b). RM ANOVA revealed a significant effect of Day ($F_{(10,170)} = 2.8$, $p < 0.01$), Drug ($F_{(1,17)} = 7.6$, $p < 0.05$) and Lever type ($F_{(1,17)} = 9.2$, $p < 0.01$). Furthermore, interactions were observed for Day × Drug ($F_{(10,170)} = 2.8$, $p < 0.01$), Lever type × Drug ($F_{(1,17)} = 6.9$, $p < 0.05$), Day × Lever type ($F_{(10,170)} = 2.8$, $p < 0.01$) and Day × Drug × Lever type ($F_{(10,170)} = 2.7$, $p < 0.01$; Fig. 3b). Post hoc tests showed that the interaction was driven by the Drug effect on active lever responding on day 5 onwards ($ps < 0.05$). Similarly, meth infusions increased significantly over time, whereas saline infusions did not (data not graphed). RM ANOVA showed a significant effect of Day ($F_{(10,170)} = 4.1$, $p < 0.001$), Drug ($F_{(1,17)} = 12.3$, $p < 0.01$) and a Day × Drug interaction ($F_{(10,170)} = 4.0$, $p < 0.001$). Post hoc tests showed that the Drug effect was apparent from day 4 onwards ($ps < 0.05$). All rats significantly gained weight over time (effect of Day, $F_{(3,51)} = 83.1$, $p < 0.001$), and there were no differences in overall or rate of weight gain between meth vs saline-administering rats ($ps > 0.05$, Table 3).

Baseline freezing was not different between groups at conditioning, extinction, or test ($ps > 0.05$, Table 2). For conditioning, RM ANOVA of CS-elicited freezing revealed a significant effect of Trial ($F_{(2,34)} = 16.3$, $p < 0.001$) but there was no effect of Drug nor Drug × Trial interaction ($ps < 0.05$, Fig. 3c). CS-elicited freezing for extinction was similar with a significant effect of Block ($F_{(5,85)} = 23.1$, $p < 0.001$) but no effect of Drug nor an interaction of Drug × Block ($ps < 0.05$, Fig. 3d). CS-elicited freezing at test were not different between groups ($p < 0.05$, Fig. 3e). Together, these results show that meth self-administration during adolescence did not affect fear conditioning or extinction in late adolescence.

Adult Conditioned Fear Following Self-administration of Methamphetamine in Adolescence

Experiment 2c examined whether the effects self-administered meth on fear conditioning in P85 rats observed in experiment 2a can still be observed in P85 rats when meth self-administration occurs at P35 (Fig. 4a). Adolescents administering meth responded more on the active lever than

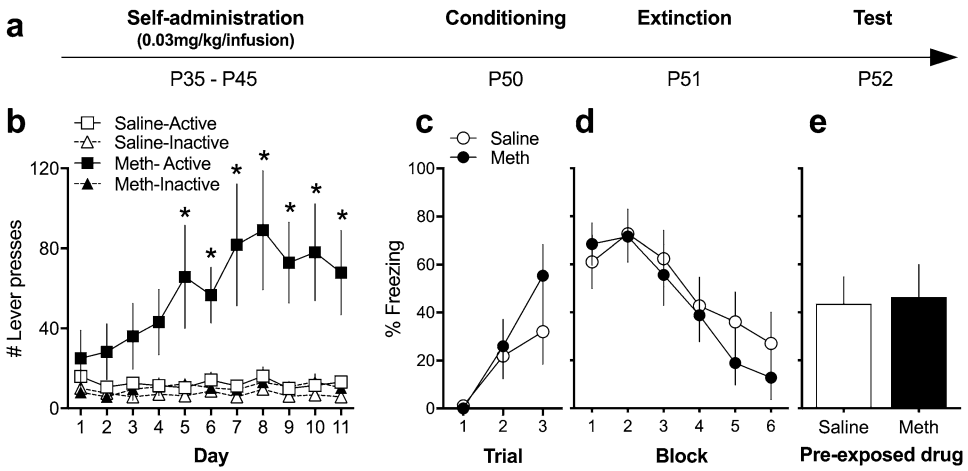

Fig. 3 **a** Experiment 2b timeline. **b** Mean ± SEM total lever presses during self-administration of meth or saline during adolescence. Mean ± SEM CS-elicited freezing during fear **c** conditioning, **d** extinction, and **e** test following meth self-administration. Meth self-administration during adolescence did not affect conditioned freezing during adolescence

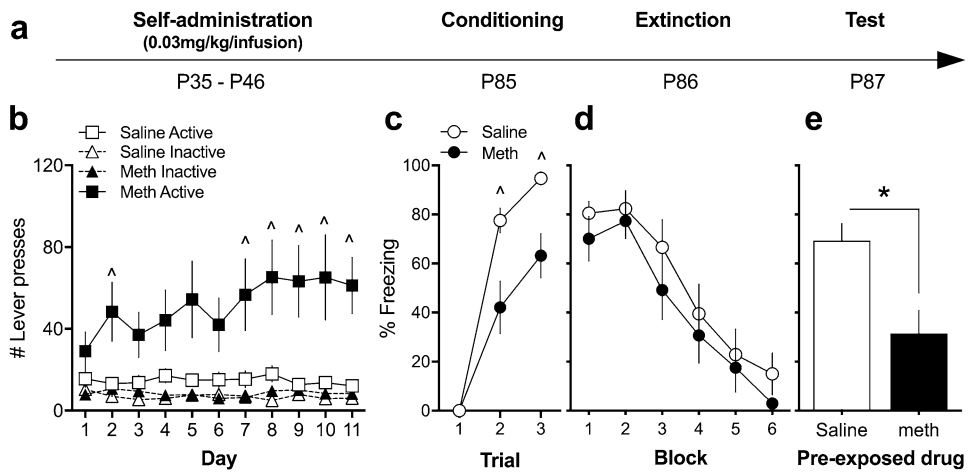

Fig. 4 **a** Experiment 2c timeline. **b** Mean ± SEM total lever presses during self-administration of meth or saline during adolescence. Mean ± SEM CS-elicited freezing during fear **c** conditioning, **d** extinction, and **e** test following meth self-administration. Meth self-administration during adolescence significantly reduced freezing during conditioning and test in adult rats. *significant *t* tests or post-hoc effects following significant interactions (*ps* < 0.05)

the inactive lever (Fig. 4b). Analysis of lever presses revealed a main effect of Lever type ($F_{(1,21)} = 21.5$, $p < 0.001$), Drug ($F_{(1,21)} = 7.8$, $p < 0.05$) and a Lever type × Drug interaction ($F_{(1,21)} = 10.4$, $p < 0.01$). Post hoc tests showed that the Drug effect was apparent on the active lever responding on days 2, 7, 8, 9, 10 and 11 (*ps* < 0.05). No effects of Day, Day × Drug, Day × Lever type or Day × Lever type × Drug interactions (*p*s > 0.05) were observed. Adolescent rats self-administering meth significantly increased the amount of infusions over time, whereas saline self-administering rats did not (data not graphed). RM ANOVA showed a trend of Day ($F_{(10,210)} = 1.8$, $p = 0.06$), a significant effect of Drug ($F_{(1,21)} = 9.2$, $p < 0.01$) and an interaction of Day × Drug ($F_{(10,210)} = 2.1$, $p < 0.05$). Post hoc tests showed that the Drug effect was apparent on days 2, 5, 7, 8, 9, 10 and 11 (*p*s < 0.05). The lack of significance in Day × Lever type × Drug interaction could be explained by the fact that the active lever presses on days 1–2 were relatively high compared to previous experiments, masking the increase in active lever pressing in meth self-administering rats. Nevertheless, the significant Day × Drug interaction for infusions suggests that rats having access to meth showed an increase in self-administration whilst saline self-administering rats did not. All rats significantly gained weight over time (effect of Day, $F_{(3,63)} = 85.7$, $p < 0.001$), and there were no differences in overall or rate of weight gain between meth vs saline-administering rats (*p*s > 0.05, Table 3).

After the last self-administration session, adolescent rats were left undisturbed for 39 days, so that fear conditioning occurred at P85. Baseline freezing was not different between groups at conditioning, extinction, or test (*ps* > 0.05, Table 2). For conditioning, RM ANOVA of CS-elicited freezing revealed effects of Trial ($F_{(2,42)} = 113.1$, $p < 0.001$), Drug ($F_{(1,21)} = 12.0$, $p < 0.01$), and a Drug × Trial interaction ($F_{(2,42)} = 6.3$, $p < 0.01$; Fig. 4c). Post hoc tests showed that there was an effect of Drug on trials 2 and 3 (*p*s < 0.05). During extinction, all rats decreased freezing to the tone similarly with RM ANOVA showing a significant effect of extinction Block ($F_{(5,105)} = 40.4$, $p < 0.001$). No Drug nor a Block × Drug interaction (*p*s > 0.05) was observed (Fig. 4d). On test day, there was a significant effect of Drug ($t_{(21)} = 3.2$, $p < 0.005$), with rats that self-administered meth during adolescence freezing less than rats that self-administered saline (Fig. 3e). Experiment 2c results are similar to the results observed in experiment 2a. That is, meth self-administration reduces CS-elicited freezing during conditioning and test in adulthood regardless of when meth is self-administered.

Discussion

The present study first showed that experimenter-injected meth had significant effects on baseline freezing in adolescent and adult rats. For CS-elicited freezing, meth binge exposure did not affect conditioning or extinction at any age. However, extinction recall was significantly impaired in meth-exposed adults but not in adolescents. Contrary to our hypotheses, meth self-administration also did not affect CS-elicited freezing in adolescents. Further, meth self-administration caused reduced levels of freezing during conditioning and test in adults. Interestingly, this effect was apparent regardless of when meth self-administration occurred (adolescence or adulthood). Taken together, meth affects conditioned fear to the CS in adulthood but not in adolescence, at least under the current conditions. Additionally, the route of administration appears to have opposite effects on retrieval of CS memory in adult rats. That is, adult rats that were exposed to meth by experimenter injections froze more during retrieval following extinction, whereas adults that self-administered meth froze less. Consistent with a previous study [10], we did not observe significant effects of meth on weight gain, which suggests that the present results are not confounded by any differences in body weight or appetitive motivation. Other chronic studies did

not report any weight data [8, 9, 11]. However, they used lower doses than experiment 1 of the present study, so it is unlikely weight gain was affected in those studies.

Methamphetamine's Effects on Baseline Freezing

After rats were subjected to repeated meth or saline injections by the experimenter, baseline freezing before CS onset in each session was significantly affected (Table 2). These baseline effects are not due to potential changes in locomotion because CS-elicited freezing was not affected similarly. In contrast, baseline freezing was unaffected following self-administration. While there was a substantial dose difference in the two models in the present study, with the total meth received at 57 mg/kg per rat in experiment 1 and 9 mg/kg per rat in experiments 2a–c, it was the saline injections that increased baseline freezing in experiment 1.

These findings suggest that chronic IP injections are stressful to rats, and affect baseline levels of freezing once rats are fear conditioned. Indeed, IP injections can significantly elevate heart rate in Sprague-Dawley rats [39]. The adolescents were particularly affected by chronic IP injections, which may indicate that adolescents are more likely to generalise conditioned fear across contexts (i.e. enhanced freezing to a novel context following conditioning). The generalisation of freezing observed in the present study is interesting because it is commonly observed following fear conditioning in humans [40], and is believed to underlie anxiety disorders without a discrete focus, such as in generalised anxiety disorder [41]. Inactivation of the medial prefrontal cortex (PFC) increases generalization of baseline freezing after fear conditioning [42]. The PFC undergoes a dramatic alteration during adolescence in rodents and humans, which is associated with its hypofunction [16, 19, 20, 25, 43–45]. This likely explains why the increase in baseline freezing was more pronounced in adolescents compared to adults in our study. Future studies should assess whether there are age differences in generalisation of fear following other types of stress (e.g., social isolation).

CS-Elicited Freezing During Conditioning

Consistent with the idea that chronic IP injections may be more stressful to adolescents than adults, we observed that adolescent rats froze more than adults to the second and third tone-shock trials during conditioning in experiment 1. When adolescent CS-elicited freezing was compared between experiment 1 (Fig. 1c) and 2b (Fig. 2c), it is clear that IP injections led to higher levels of freezing at conditioning compared to self-administration in adolescent rats. Meth vs saline exposure had no impact on these findings, suggesting that chronic IP injections of any substance may elevate CS-elicited freezing levels in adolescent rats.

While meth did not seem to have any effects on adolescent fear behaviour, meth self-administration reduced CS-elicited freezing during conditioning in adult rats (Figs. 2c and 4c). Reduced freezing during conditioning may indicate hyperlocomotion due to lasting effects of chronic meth self-administration [46]. If the effects are due to locomotion, the present results suggest that meth has greater effects on adult locomotion than adolescent locomotion. This is consistent with previous observations in mice [38]. However, it is then unclear why experimenter-injected meth did not affect freezing during conditioning in experiment 1. Further, meth reduced freezing even when conditioning occurred 39 days after the final day of self-administration (experiment 2c), and there is no evidence in the literature that any effects of meth on baseline locomotion last this long. Moreover, meth does not decrease freezing the day after conditioning (i.e., at extinction) in any experiment, which strongly suggests that meth effects during conditioning are not due to hyperlocomotion.

Reduced freezing during conditioning after meth self-administration in adults may reflect disruptions in attention to the CS. It has been shown that meth self-administration at 0.1 mg/kg/infusion for 21 consecutive days caused reduced performance on a visual attention task in adult rats [47]. In that study, visual attention deficits were observed up to 14 days after the last day of meth self-administration. The present results suggest that attention deficits may last at least 39 days following the last day of meth self-administration. No one has assessed the effects of meth on attention in adolescence, and the present results suggest that meth self-administration selectively affects attention in adults rather than adolescents. Interestingly, freezing levels at the last conditioning trial were not correlated with the amount of meth self-administered in any experiment (biggest $r^2 = 0.1$), suggesting that meth self-administration effects are not dose-dependent and any meth self-administration disrupts CS-elicited freezing.

Methamphetamine Does Not Affect CS-Elicited Freezing During Extinction

Even though the freezing levels at the end of conditioning varied across experiments, the retrieval of fear memory during extinction was unaffected for all rats across experiments. This discrepancy in CS-elicited freezing between conditioning and extinction is not uncommon [48–50], although the mechanism for the effect remains unclear. Lack of meth effects during extinction is inconsistent with the results of Tsuchiya et al. [31]. In that study, adult rats subcutaneously injected with 25 mg/kg of meth across 1 week showed increased freezing at test, whereas rats that received 50 mg/kg or 100 mg/kg of meth across 1 week froze similarly to saline groups. It may be that meth increases freezing only

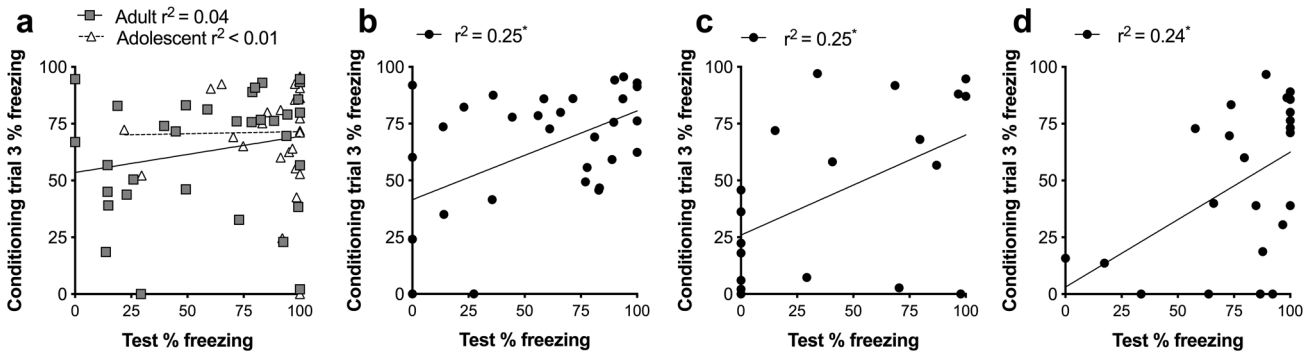

Fig. 5 Correlation between % freezing at the last trial of conditioning and test in adult and adolescent rats in **a** experiment 1, **b** 2a, **c** 2b, and **d** 2d. Conditioning trial 3 freezing levels did not correlate with test freezing levels after experimenter-injected meth, whereas the correlation was significant in all experiments involving meth self-administration. *significant correlation ($ps < 0.05$)

when the fear memory is formed with strong levels of foot shock because conditioning involved 30 s 2.5 mA foot shocks every minute over a 30 min period on each day for 2 days in Tsuchiya et al. [31]. In comparison, the present study used 1 s 0.6 mA foot shocks given three times.

Methamphetamine's Effects on CS-Elicited Freezing During Test

At test, experimenter-injected meth increased freezing in adult rats, suggesting that meth exposure in adulthood impairs extinction retrieval. This finding is consistent with cognitive deficits observed in adult rats that were exposed to a binge exposure paradigm [9, 10]. In contrast, adolescent animals froze similarly regardless of drug exposure on test day in experiment 1. Adolescents receiving saline did freeze significantly more than adults receiving saline at test, which is consistent with previous observations showing an adolescent extinction deficit [25, 26]. Because adults froze less than adolescents during conditioning by trial 3, we assessed whether their difference in freezing at test was due to their freezing levels at conditioning trial 3 using Pearson's correlation test (Fig. 5a). Conditioning trial 3 CS-elicited freezing was not correlated with test CS-elicited freezing in adults ($p = 0.24$) or adolescents ($p = 0.9$). This lack of correlation highlights that test effects are not due to conditioning effects in experiment 1. Taken together, our results suggest that experimenter-injected meth disrupts extinction recall in adult but not adolescent rats.

In contrast, meth self-administration reduced conditioned freezing at test in adults. While these results counter-intuitively suggest that meth self-administration may facilitate extinction recall, we propose that these test effects are due to meth-induced reduction in conditioning freezing levels. Specifically, conditioning trial 3 CS-elicited freezing was significantly correlated with test CS-elicited freezing in experiments 2a ($p < 0.001$, Fig. 5b), 2b ($p < 0.05$, Fig. 5c), and 2c ($p < 0.05$, Fig. 5d). Therefore, the effects observed at test following self-administration appear to be primarily driven by meth disrupting CS-US associative learning during conditioning. However, CS memory retrieval was the same, as shown by similar levels of freezing during extinction. Extinction learning also appeared equal, with all rats showing a similar rate in the decrease of freezing. Therefore, the reduced freezing at test implies that at test, the CS-US memory formed during conditioning is failing to compete with CS-no US memory created during extinction.

The current experiments highlight the different consequences of different preclinical models. It appears that experimenter-injected binge exposure of meth affected the extinction neurocircuitry, whereas self-administration affected the conditioning neurocircuitry. It is also important to note that the behavioural effects following the different types of meth exposure may be due to the differences in total meth received. While all the ages at conditioning, extinction, and tests were matched, administration schedule and dose differences between the two models may contribute to their different effects on conditioned fear.

In conclusion, prior meth exposure has effects on subsequent fear conditioning and extinction in adults, but not adolescents. Meth self-administration reduced conditioned freezing, which suggests that meth may not necessarily cause anxiety disorders. This was surprising, given the high co-morbidity between anxiety and meth use in clinical populations. Future studies may investigate whether anxiety is rather a risk factor, for example by assessing how fear conditioning early in life may change meth self-administration later in life in rodents. Together with the current study, this would provide important insight into the association between these disorders.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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