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RESEARCH PAPER

Persistent downregulation of hippocampal CREB mRNA parallels a Y-maze deficit in adolescent rats following semi-chronic amphetamine administration

T Featherby[1,2], M van den Buuse[2,3], DI Lubman[4] and AJ Lawrence[1,2]

[1]*Brain Injury and Repair Group, Howard Florey Institute, University of Melbourne, Parkville, Victoria, Australia;* [2]*Centre for Neuroscience, University of Melbourne, Parkville, Victoria, Australia;* [3]*Behavioural Neuroscience Laboratory, Mental Health Research Institute of Victoria, Parkville, Melbourne, Victoria, Australia and* [4]*ORYGEN Research Centre, Department of Psychiatry, University of Melbourne, Melbourne, Victoria, Australia*

Background and purpose: We investigated possible differences in the impact of chronic amphetamine administration during adolescence and adulthood on aspects of behaviour and brain chemistry.
Experimental approach: Adult ($n = 32$) and adolescent ($n = 32$) male Sprague–Dawley rats were given either D-amphetamine sulphate (10 mg kg^{-1} daily, i.p.) or saline (1 mL kg^{-1}, i.p.) for 10 days. Rats were subsequently tested for anxiety-like behaviour, learning and memory, and sensorimotor gating. Nine weeks later, rats received saline (1 mL kg^{-1}) or acute amphetamine challenge (1.5 mg kg^{-1}) and the expression levels of mRNA for tyrosine kinase B (TrkB) or cAMP response element-binding protein (CREB) were measured in the hippocampus.
Key results: The adolescent amphetamine pretreated group revealed a deficit in exploration on the Y-maze during a 6 h retention test. The frequency of visits to the novel arm was 35% lower for the amphetamine group compared with controls. In parallel, a 43% decrease in hippocampal CREB mRNA, but not TrkB mRNA, was observed in periadolescent rats treated chronically with amphetamine 9 weeks earlier. None of the effects were detected in the adult treated cohort.
Conclusions and implications: Chronic amphetamine treatment during periadolescence resulted in altered behaviour on the Y-maze and persistent downregulation of hippocampal CREB mRNA expression. Given that this group had intact spatial learning and reference memory, it would appear that the deficits observed on the Y-maze reflect a dysfunction in response to novelty. Because no effects of amphetamine treatment were observed in the adult cohort, these data suggest idiosyncratic sensitivity of periadolescence to the long-term effects of psychostimulants.
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Keywords: Y-maze; periadolescent rats; chronic amphetamine; novelty exploration; CREB; hippocampus; CA1–3

Abbreviations: CREB, cAMP response element-binding protein; PND, postnatal day; PPI, prepulse inhibition; TrkB, tyrosine kinase B; PBS, phosphate-buffered saline; SSC, saline sodium citrate

Introduction

Psychostimulants (encompassing amphetamines, cocaine and ecstasy) are the second most commonly abused illicit substances, with the highest prevalence of use occurring among young adults (National Drug Strategy Household Survey, 2004). Experimentation with amphetamines typically occurs during late adolescence, with around 13% of British 16- to 19-year-olds (Ramsay *et al*., 2000) and 6.6% of Australian 14- to 19-year-olds reporting lifetime use (National Drug Strategy Household Survey, 2004). In Great Britain, monthly amphetamine use among adolescents and young adults (16- to 24-year-olds) is twice as prevalent as use in adults aged 25–34 years (Condon and Smith, 2003). Although early onset of drug use is associated with a wide range of adverse outcomes in adulthood, the neurobiological mechanisms that underpin these relationships are poorly understood (Lubman *et al*., 2007). In particular, despite growing rates of amphetamine use internationally (Parry *et al*., 2004; Roehr, 2005), the long-term behavioural consequences of amphetamine abuse during adolescence are largely unknown.

Correspondence: Professor A Lawrence and T Featherby, Brain Injury and Repair Group, Centre for Neuroscience, Howard Florey Institute, University of Melbourne, Royal Parade, Melbourne, Victoria 3010, Australia.
E-mails: andrew.lawrence@florey.edu.au and travis.featherby@florey.edu.au
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Adolescence is a key period of neural development, encompassing extensive reorganization within those regions of the brain that play prominent roles in executive function, learning and memory, and reward and motivation, for example, the prefrontal cortex (Zecevic *et al*., 1989; Rosenberg and Lewis, 1994), hippocampus (Swann *et al*., 1999), amygdala (Zehr *et al*., 2006) and nucleus accumbens (Teicher *et al*., 1995). Adolescence is also associated with certain characteristic behaviours, such as risk-taking, exploration, novelty and sensation-seeking, as well as peer-directed social interactions (Spear, 2000). It is therefore not surprising that the incidence of hazardous driving, unprotected sex and substance abuse among young people is generally high (Berndt, 1979; Arnett, 1992).

The developmental epoch most similar to adolescence in rats appears to range from postnatal days (PNDs) 28–42 (Spear, 2000). Lower levels of anxiety regarding the perception of harm and the motivation to seek higher levels of novelty receive some support from animal studies. Developmentally unique alterations in gene transcription in response to environmental manipulation have demonstrated distinctive responses to both stress and novelty in adolescence. Adolescent rats (PND 28) were found to show less stress-induced Fos-like immunoreactivity in cortical and amygdaloid nuclei than adult rats (Kellogg *et al*., 1998), but higher novelty-induced Fos in the hippocampus during this period of development (Waters *et al*., 1997). Correspondingly, studies have also reported greater novelty exploration in adolescent rats compared with their adult counterparts (Spear *et al*., 1980; Adriani *et al*., 1998). However, Heyser and colleagues (2004) demonstrated that chronic methylphenidate ($5\,mg\,kg^{-1}$) disrupted novelty exploration in periadolescent rats. Although adolescent subjects have generally been reported to be more disrupted by stressors than subjects in other stages of development (Spear, 2000), the effects of adolescent amphetamine exposure on general anxiety are not known. In adult rats, some confusion exists. Previous studies have shown that rats administered chronic amphetamine (Cancela *et al*., 2001) or cocaine (Sarnyai *et al*., 1995) can exhibit either anxiogenic-like responses or no effects (Basso *et al*., 1999; Lilly and Tietz, 2000; Russig *et al*., 2005). Requisite testing when animals are no longer under the direct influence of psychostimulants may provide some clarification.

There is some evidence that methamphetamine administered during early adulthood can result in impairment on the Morris water maze while learning acquisition trials (Vorhees *et al*., 2005). Nonetheless, there is limited research examining the effect of psychostimulants on the development of learning and memory processes during adolescence. The importance of the cAMP response element-binding protein (CREB) in the hippocampus in novelty exploration (Vianna *et al*., 2000; Winograd and Viola, 2004), and both hippocampal CREB family and tyrosine kinase (Trk) receptor signalling in long-term-potentiation-associated learning and memory (Kandel and Pittenger, 1999; Minichiello *et al*., 2002; Tyler *et al*., 2002; Purcell and Carew, 2003), is established. In contrast, the impact of chronic amphetamine treatment on these factors is poorly understood.

The capacity of dopamine-releasing agents like amphetamine to produce psychotic-like states is well documented (Joyce, 1993; Carlsson *et al*., 2001). In this respect, an apparent discrepancy between sensitivities at two age groups appears to exist. In adults, protracted disruptions in prepulse inhibition (PPI), or sensorimotor gating, have been documented for 6 and 70 days following amphetamine treatment (Peleg-Raibstein *et al*., 2006). In contrast, a relative short-term insensitivity to the effects of amphetamine on PPI has been reported in adolescents (Brunell and Spear, 2006). However, parallel treatment regimes are required to further assess this apparent discrepancy between age groups.

Thus, the aims of the current study were to examine the effect of chronic amphetamine treatment in adult and adolescent Sprague–Dawley rats on facets of affective behaviour, learning and memory, novelty exploration, startle and PPI. The effects of protracted withdrawal from amphetamine and amphetamine challenge, both acutely and after protracted withdrawal, on the expression level of hippocampal TrkB and CREB mRNA, were also examined due to the importance of these factors in both novelty exploration and learning and memory. Here, we report that the repeated administration of D-amphetamine during adolescence in rats leads to a deficit during a retention trial on the Y-maze, which may represent a dysfunction in the response to novelty. In parallel, periadolescent treatment with D-amphetamine results in persistent downregulation of hippocampal CREB mRNA expression. These behavioural and molecular alterations were unique to the adolescent group and provide evidence that the adolescent brain presents idiosyncratic sensitivities to the long-term effects of amphetamine that were not present in a parallel adult cohort.

Methods

All experiments were preformed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council of Australia's Code of Practice for the Care and Use of Animals for Experimental Purposes. Experiments were carried out on male Sprague–Dawley rats ($n=64$) obtained from ARC (Perth, WA, Australia). Rats were housed in pairs in the Integrative Neuroscience Facility at the Howard Florey Institute under a constant 12 h light/dark cycle (lights on at 0700 hours), and allowed access to food pellets (Ridley Agriproducts, Pakenham, VIC, Australia) and tap water *ad libitum*. The temperature was kept constant at 22 °C, with a relative humidity of 50–60%. Treatments began either during periadolescence, at PND 28 (weight 108–145 kg, $n=32$; Spear, 2000), or during adulthood, at PND 84 (weight 364–524 kg, $n=32$). Rats were randomly allocated and injected with either amphetamine ($10\,mg\,kg^{-1}$ D-amphetamine sulphate, i.p., $n=16$; Sigma, VIC, Australia) dissolved in saline, or saline ($1\,mL\,kg^{-1}$ of 0.9% NaCl, i.p., $n=16$; Delta West P/L), once daily for 10 consecutive days. As previously reported, the amphetamine dose used in the present study does not impact on the growth rate or cause neuronal cell loss (McPherson and Lawrence, 2006). After the last

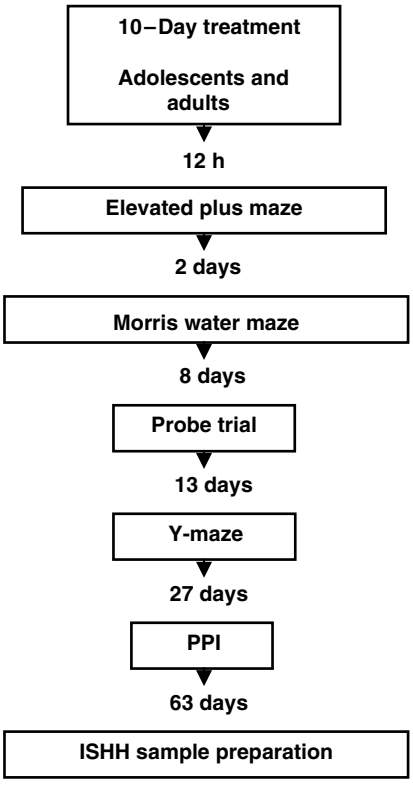


Figure 1 Schedule of treatment and testing on adult and adolescent rats. Behavioural testing began 12 h after the last injection. This was at postnatal days 38 and 94 for the adolescent and adult groups, respectively. Nine weeks (63 days) after drug treatment, chronically treated rats or controls received either saline (1 mL kg^{-1}) or acute amphetamine (amph) challenge (1.5 mg kg^{-1}). One hour later, rats were deeply anaesthetized using sodium pentobarbitone (80 mg kg^{-1}, i.p.) and decapitated for *in situ* hybridization histochemistry (ISHH). PPI, prepulse inhibition.

amphetamine injection, behavioural testing commenced according to the schedule depicted in Figure 1.

After behavioural testing and a total of 9 weeks of abstinence, each group was randomly and evenly divided to receive either amphetamine (1.5 mg kg^{-1}, i.p.) or saline (1 mL kg^{-1}, i.p.). The final groups are described related to their initial treatment and subsequent challenge: (i) chronic saline treatment–saline challenge (control), (ii) chronic saline treatment–amphetamine challenge (acute), (iii) chronic amphetamine treatment–saline challenge (withdrawal) and (iv) chronic amphetamine treatment–amphetamine challenge (amphetamine challenge). Sixty minutes following the final challenge, rats were deeply anaesthetized using sodium pentobarbitone (80 mg kg^{-1}, i.p.), decapitated and their brains removed and rapidly frozen over dry ice.

Elevated plus maze

Rats were assessed on the elevated plus maze as described previously (Lodge and Lawrence, 2003). The apparatus consisted of four arms, each arm 50 cm long and 10 cm wide, with a central platform of 10 cm × 10 cm. The plus maze was positioned 1 m off the ground. For testing, rats were placed on the central platform facing a closed arm of the maze and were observed for 5 min. The session was videotaped for subsequent rating using Ethovision, which included time spent and entries made into the open and closed arms. An entry was determined when all four paws of the rat had crossed the line defining the open or closed arm.

Morris water maze

The maze was a circular plastic tank of 143 cm (diameter) × 60 cm (height), with a movable, circular, plastic platform (10 cm diameter). The top surface of the platform was 24 cm above the floor of the pool, and the water level was always 2 cm above the platform. To obscure the platform's appearance, the water was rendered opaque before the learning trials. Lights were placed around the room to provide dim, even lighting. Temperature of the water was maintained at 25 ± 1 °C. Spatial cues were placed on the four walls of the room and the pool divided into four hypothetical quadrants of equal dimensions. The starting positions for learning trials corresponded to the four cardinal points (north, south, east and west). The edge of the pool was marked to ensure that rats were dropped at the same position. Experimental sessions were recorded on video and analysed by video tracking (Ethovision, Noldus Information Technology, The Netherlands).

The rats were examined on the Morris water maze as described previously (Russig *et al*., 2003). Four learning trials were completed per day. In each trial, the rat was released from one of four points where the latency to the platform was recorded. If at the 2 min mark, the platform was not found, the rats were guided to the platform. After 30 s on the platform, the rat was removed from the pool, dried and returned to its home cage. To maintain randomization between rats, the platform was moved around into the different quadrants for different rats; however, the platform remained in the same position for individual rats. In the current study, 6 days of training were employed. At this point, a plateau was observed in the learning curve. Subsequently, reference memory was examined in a probe trial during which the platform was removed.

Y-maze

Rats were tested according to a previously described protocol (Nakagawa *et al*., 2004), with the following adjustments. The Y-maze had three arms, each 50 cm (length) × 16 cm (width) × 31 cm (height). Each of the three arms had a unique visual cue at the end. In the first trial, rats were exposed to two out of the three arms for 5 min. Bedding from the rats' cages was laid out in the Y-maze to prevent any foreign odours confounding the study. Between trials for rats from different cages, the maze was cleaned thoroughly. The 5 min retention trial (trial 2) began after a 6 h hiatus; during this trial, rats were given access to all three arms (home, familiar and novel). The duration and frequency of visits in all three arms were recorded.

Prepulse inhibition

Rats were assessed for startle and PPI responses as described previously (de Jong and van den Buuse, 2006). The PPI

experiments were performed using a four-unit automated SRLab startle system (San Diego Instruments, San Diego, CA, USA). Each unit consisted of a small Plexiglass cylinder on a platform under which a sensitive piezo-electric sensor was mounted. During the sessions, the animals were kept in the cylinders within a sound-attenuating cabinet where 70 dB of white background noise was delivered through speakers attached to the ceiling of the box. Responses to stimuli were measured using the SRLab software (San Diego Instruments).

A total of 100 trials were delivered with an average (non-constant) interval of 25 s. The first and last 10 trials involved 40 ms of 115 dB pulse-alone startle stimuli. These groups of 10 stimuli, together with the middle two groups of 10 pulse-alone stimuli, were used to measure response habituation in response to repeated delivery of startling stimuli. The middle 80 trials consisted of random delivery of 20 115 dB pulse-alone trials, 10 trials during which no stimuli were delivered and 50 prepulse trials. Prepulse trials consisted of a single 115 dB pulse preceded 100 ms earlier by a 20 ms non-startling stimulus prepulse of 2, 4, 8, 12 or 16 dB over baseline (that is, 72, 74, 78, 82 or 86 dB, respectively).

In situ *hybridization*

Coronal sections of cryostat-cut rat brain (14 μm) were thaw-mounted onto microscope slides, which had been prebaked overnight in an oven at 180 °C and coated with poly-L-lysine (0.01%). Slide-mounted tissue sections were then stored at −80 °C. Before hybridization, slides were allowed to warm to room temperature and fixed and delipidated as described previously (Lawrence *et al*., 1996; Cowen and Lawrence, 2001; Lodge and Lawrence, 2001). In brief, slides were placed in ice-cold 4% (w/v) depolymerized paraformaldehyde in phosphate-buffered saline (PBS (in mM): 7 Na_2HPO_4, 3 NaH_2PO_4 and 130 NaCl; pH 7.0) for 5 min, followed by 1 × PBS at room temperature for 3 min. Sections were then dehydrated through serial alcohol washes (70, 95 and 100% v/v), for 2 min each, before delipidation in chloroform for 20 min. Sections were finally placed in 100% alcohol and stored at 4 °C until use.

On the day of use, slides were allowed to equilibrate to room temperature. Aliquots (2 μL) of antisense oligonucleotides complementary to the mRNA for CREB (i) 5′-TGCTGC TTCCCTGTTCTTCATTAGACGAACCTCTCTCTTTCGTGCTG C-3′, (ii) 5′-TTGTAGAATGGTAGTACCCGGCTGAGTGGCA CCTGCATTGGTCATGGT-3′, (iii) 5′-ACTGTTTGGACTTGTG AATAACTGATGGCTGGGCCGCCTGG-3′ and the mRNA for TrkB (5′-TGCGACTGCGTCAGCTCGGTGGGCGGGTTCCC TCTGCCATCAGCACTGC-3′) were diluted to a working stock of 0.3 pmol μL^{-1}, and then the 3′-end was labelled with [α^{33}P]dATP in the presence of terminal transferase. The labelled probes were then applied to a Sephadex G25 column and centrifuged at 7500 *g* for 1 min to separate any unincorporated nucleotides from the probes. The labelled oligonucleotide probes (1pg μL^{-1}, 100 μL) were then applied to adjacent sections of rat brain in a hybridization buffer containing 50% formamide (v/v), 4 × saline sodium citrate (SSC: 0.6 M NaCl, 0.06 M $Na_3C_6H_5O_7$; pH 7.0) and 10% dextran sulphate (w/v). Nonspecific signal was determined in the presence of 100-fold molar excess of unlabelled (relative to labelled) oligonucleotide. As with autoradiography, four brain slices per rat were used to determine total hybridization and two slices to determine nonspecific hybridization. Strips of Parafilm were apposed to the slides to assist in covering the sections evenly with the hybridization buffer and to prevent the sections from drying out. Slides were then placed into Petri dishes that contained tissue paper soaked in 50% deionized formamide containing 4 × SSC to provide a humidified atmosphere. The Petri dishes were then sealed with Parafilm and placed in an oven that had been preheated to 42 °C overnight.

On the following day, the Parafilm strips were removed from the tissue sections and the tissue was rinsed in 1 × SSC for 30 s, washed in 1 × SSC for 1 h at 55 °C and again rinsed in 1 × SSC for 30 s at room temperature. Sections were then dehydrated in ethanol (70 and 95% (v/v), 30 s each) and allowed to air-dry. When dry, slides were apposed to Kodak Biomax MR X-ray film or X-ray film (X-omat AR; Eastman Kodak) with standard ^{14}C microscales (American Radiolabelled Chemicals Inc., St Louis, MO, USA). X-omat films were developed automatically with a 100 Plus Automatic X-ray film processor, whereas Biomax MR films were developed manually under darkroom conditions. Films were submerged in Kodak D19 (3 min), transferred to a stop solution (1 min), fixed in a 1:4 solution of Hypam Rapid Paper and Film fixer (3 min), washed in distilled water (1 min), rinsed in distilled water containing Photo-Flo (0.01%, 1 min) and dried.

Data and statistical analysis

Autoradiographic images on developed films were subsequently quantified using Image J 1.34 s (NIH, USA) by comparison of the optical densities of the autoradiographic images with those of the standard ^{14}C microscales (American Radiolabelled Chemicals Inc.). Autoradiogram images were captured under constant illumination using XC-77CE CD video camera (Sony, Tokyo, Japan) with an attached Micro-Nikkor 55 mm lens (Nikon, Tokyo, Japan). Brain regions were identified using a stereotaxic atlas (Paxinos and Watson, 1986). Signal intensity in any particular brain region was determined by densitometry, and the mean value of all sections of an individual region was calculated for either the saline or amphetamine group in both adolescent and adult pretreated rats.

Differences in signal intensities were determined by two-way ANOVA, with Tukey *post hoc* test for multiple comparisons. For behavioural data, two-way ANOVA (age × treatment) with Tukey *post hoc* test for multiple comparisons was used. An additional one-way ANOVA with repeated measures was performed to examine the effect of treatment on latency between learning trial days in the Morris water maze. Data are presented as mean ± s.e.mean. A significance level of $P<0.05$ was used throughout. All statistics were calculated using SigmaStat (Jandel, San Jose, CA, USA). All graphical representations of the data were created in GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Table 1 Effects of chronic amphetamine treatment on behaviour of adult and adolescent Sprague–Dawley rats

Elevated plus maze	*Open arms*		*Closed arms*	
	Saline	*AMPH*	*Saline*	*AMPH*
Adult				
Frequency	2.2 ± 0.6	3.9 ± 0.7	6.4 ± 0.7	5.9 ± 0.5
Duration	30.8 ± 8.4	67.3 ± 10.2	207.0 ± 13.9	179.3 ± 11.9
Adolescent				
Frequency	3.9 ± 0.6	5.5 ± 0.9	9.0 ± 0.4	9.7 ± 0.7
Duration	39.2 ± 7.4	47.8 ± 11.5	162.5 ± 12.0	152.1 ± 14.9

Rats were administered amphetamine (AMPH; 10 mg kg^{-1} for 10 days, i.p.) or saline (1 mL kg^{-1} for 10 days, i.p.) and tested on the elevated plus maze 12 h after the last injection. Duration (s) and frequency of entries into the open and closed arms of the maze were quantified. Data are presented as mean ± s.e.mean. Each group contained 14 or 15 rats. Two-way ANOVA revealed no significant differences between treatment groups or age groups for frequency ($F_{(1,50)} = 0.01$, $P = 0.92$) and duration ($F_{(1,50)} = 2.1$, $P = 0.15$) in the open arms.

Results

Effect of amphetamine on elevated plus maze behaviour

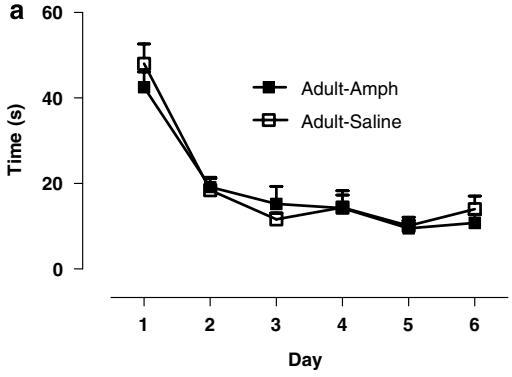
No significant effect of treatment was observed in either age group when tested on the elevated plus maze (Table 1). Comparisons between amphetamine- and saline-treated rats indicated no significant differences either in time spent in, or entries into, the open arms, revealing no differences in anxiety-like behaviour.

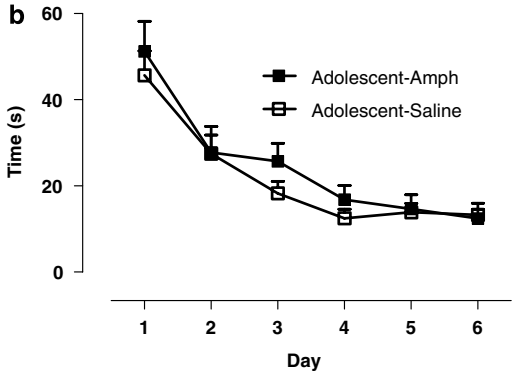
Effect of amphetamine on Morris water maze behaviour

In the Morris water maze, each group demonstrated spatial learning, as swimming time (latency) to the hidden platform was reduced over testing days 1–6 (adolescent saline: $F_{(5,70)} = 23.67$, $P < 0.001$; adolescent amphetamine: $F_{(5,70)} = 12.2$, $P < 0.001$; adult saline: $F_{(5,50)} = 30.0$, $P < 0.001$; adult amphetamine: $F_{(5,50)} = 18.16$, $P < 0.001$; Figures 2a and b). There were no significant differences in latency to reach the platform between the amphetamine and saline pretreatment groups, in either cohort. As shown in Figure 2c, there were no differences between pretreatment groups in adult or adolescent cohorts for either frequency of visits to, or time spent in, the home quadrant during a probe trial for reference memory.

Effect of amphetamine on Y-maze behaviour

Thirteen days after the last drug administration, the adolescent amphetamine pretreated group revealed a deficit in the exploration of the novel environment when compared with saline pretreated controls. Although there was no significant interaction between age and treatment ($F_{(1,46)} = 2.5$, $P = 0.12$), the frequency of visits to the novel arm was significantly lower (~35%) for the amphetamine group than the group pretreated with saline ($F_{(1,46)} = 4.437$, $P < 0.01$; two-way ANOVA, Tukey *post hoc*; Figure 3d). A similar tendency was observed for the duration of time spent in the novel arm; however, this difference did not reach

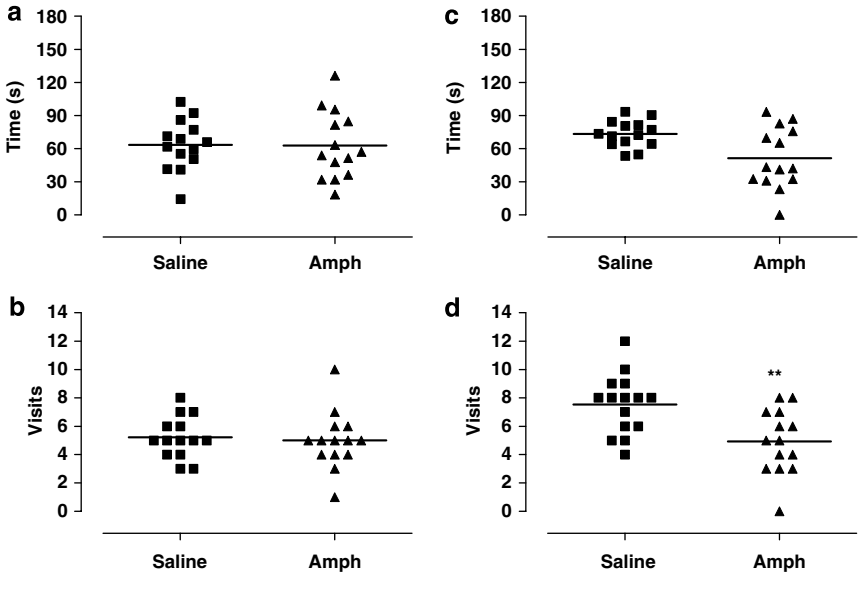




MWM – Probe Trial		Saline	Amph
Adult	Freq	5.0 ± 0.3	3.8 ± 0.6
	Duration	22.9 ± 2.3	21.3 ± 3.3
Adolescent	Freq	7.3 ± 0.9	6.3 ± 0.8
	Duration	24.1 ± 2.3	25.3 ± 1.8

Figure 2 Behavioural effects of chronic amphetamine (amph, 10 mg kg^{-1} for 10 days, i.p.) or saline (1 mL kg^{-1} for 10 days, i.p.) on adult (**a**) and adolescent (**b**) Sprague–Dawley rats tested during withdrawal in the Morris water maze, commencing 48 h after the completion of treatment. Learning was assessed on time (s) taken to reach the platform. (**c**) Memory was assessed on a probe trial in the absence of the platform, 8 days after amphetamine treatment. Frequency and duration (s) of visits in the home quadrant were quantified. All data are presented as mean ± s.e.mean. Each group contained 14or 15 rats. Two-way ANOVA revealed no significant differences between the treatment groups at any time point during learning trials (adolescence: $F_{(1,168)} = 1.45$, $P = 0.23$; adult: $F_{(1,120)} = 0.23$, $P = 0.63$) or in the probe trial (frequency: adolescence, $F_{(1,50)} = 1.87$, $P = 0.33$; adulthood, $F_{(1,50)} = 1.87$, $P = 0.34$; duration: adolescence, $F_{(1,50)} = 0.01$, $P = 0.93$; adulthood, $F_{(1,50)} = 0.01$, $P = 0.93$).

statistical significance ($F_{(1,47)} = 2.4$, $P = 0.13$; Figure 3c). No differences were observed between treatment groups within the adult cohort (frequency: $F_{(1,46)} = 4.44$, $P = 0.74$; duration: $F_{(1,47)} = 2.4$, $P = 0.13$; Figures 3a and b). The deficit recorded was specific to the novel arm as there were no significant differences in the duration or frequency of visits into the other two arms for either trial 1 (frequency in familiar arm: $F_{(1,50)} = 1.21$, $P = 0.28$; duration in familiar arm: $F_{(1,50)} = 0.54$, $P = 0.47$; frequency in home arm: $F_{(1,50)} = 3.66$, $P = 0.06$; duration in home arm: $F_{(1,50)} = 3.46$, $P = 0.07$) or the retention trial (frequency in familiar arm: $F_{(1,50)} = 2.02$, $P = 0.16$; duration in familiar arm: $F_{(1,49)} = 0.11$, $P = 0.74$; frequency in home arm: $F_{(1,51)} = 0.61$, $P = 0.44$; duration in home arm: $F_{(1,51)} = 1.2$, $P = 0.74$; Figure 3e). Moreover, there were no significant differences between treatment groups for



e

			Familiar arm		Home arm		Total arm entries
			Freq	Duration	Freq	Duration	
Trial 1	Adult	Saline	8.8±0.9	114.1±11.2	10±1.3	137.5±10.2	18.8±0.8
		Amph	9.9±0.7	117±9.3	10.4±0.8	119.4±9.9	20.3±0.5
	Adolescent	Saline	10.6±0.5	117.5±4.7	10.4±0.6	123.8±5	21±0.4
		Amph	10.2±0.5	108.5±7.5	9.4±0.7	134.2±6.1	19.6±0.4
Trial 2 retention	Adult	Saline	4.3±0.5	88.2±18.9	5.5±0.7	79.4±15.2	15±3
		Amph	5.4±0.6	79.3±12.3	5.8±0.7	83.2±12.2	16.2±0.3
	Adolescent	Saline	5.1±0.6	82.9±11	5.0±0.6	85.6±16.9	17.6±0.4
		Amph	5.2±0.4	83.3±12.9	5.5±0.6	78.2±17.6	15.6±0.3

Figure 3 Behavioural effects of chronic amphetamine (amph, 10 mg kg^{-1} for 10 days, i.p.) or saline (1 mL kg^{-1} for 10 days, i.p.) on adult (**a** and **b**) and adolescent (**c** and **d**) Sprague–Dawley rats tested on the Y-maze at withdrawal day 13. The graphs are as follows: (**a**) adult duration (s), (**b**) adult frequency, (**c**) adolescent duration (s) and (**d**) adolescent frequency. The table (**e**) represents duration (s) and frequency of visits, as well as total arm entries into the home and familiar arms during trials 1 and 2 (retention). All data are presented as mean ± s.e.mean, and each group contained 14 or 15 rats. A two-way ANOVA was performed with age × treatment as factors. Amphetamine treatment during adolescence was found to influence the Y-maze retention trial: $F_{(1,46)} = 4.437$, $^{**}P < 0.01$ compared with the saline group for frequency of visits to the novel arm (Tukey *post hoc* analysis). No significant differences were detected between treatment groups in the home and familiar arms, nor were there differences in total arm entries (**e**).

total arm entries, indicating that the deficit observed did not arise as a consequence of reduced activity in the amphetamine pretreated group (Figure 3e).

Effect of amphetamine on PPI and startle

In both cohorts, there was overall habituation of the magnitude of startle responses (data not shown), but no significant difference in startle between treatment groups, in either adult or adolescent cohorts ($F_{(1,44)} = 0.34$, $P = 0.57$; Figures 4a and c). For PPI, both cohorts yielded no significant differences between treatment groups at any of the five prepulses tested (adult: $F_{(4,110)} = 0.73$, $P = 0.58$; adolescent: $F_{(4,70)} = 0.73$, $P = 0.57$; Figures 4b and d).

Effect of amphetamine on hippocampal TrkB mRNA

The expression level of TrkB mRNA in all regions of the hippocampus studied (Figure 5) was unchanged in all rats receiving chronic amphetamine (10 mg kg^{-1} for 10 days, i.p.) with subsequent withdrawal or amphetamine challenge (1.5 mg kg^{-1}, i.p.). Sprague–Dawley rats treated acutely with amphetamine (1.5 mg kg^{-1}, i.p.), regardless of treatment age, showed no change in hippocampal TrkB mRNA (dentate gyrus: $F_{(3,163)} = 0.42$, $P = 0.74$; CA1–3: $F_{(3,479)} = 0.61$, $P = 0.61$).

Effect of amphetamine on hippocampal CREB mRNA

Analysis of the assays for CREB mRNA by two-way ANOVA revealed a statistically significant interaction between treatment and age for the CA1–3 region of the hippocampus ($F_{(3,425)} = 7.0$, $P < 0.001$). Expression levels of the mRNA encoding CREB in CA1–3 increased significantly (~15%) in saline pretreated adolescent rats given amphetamine acutely compared with saline controls ($P < 0.05$; Figures 6d, 7a and b). In contrast, adolescent rats that received chronic amphetamine and were then withdrawn for 9 weeks showed a significant reduction (~43%) in CREB mRNA in the CA1–3 region of the hippocampus ($P < 0.001$; Figures 6d and 7c). Similarly, chronically treated and acutely challenged adolescent rats also featured a marked reduction (~48%) in CREB mRNA ($P < 0.001$; Figures 6d and 7d). The changes observed were specific to the CA1–3 region, as no significant

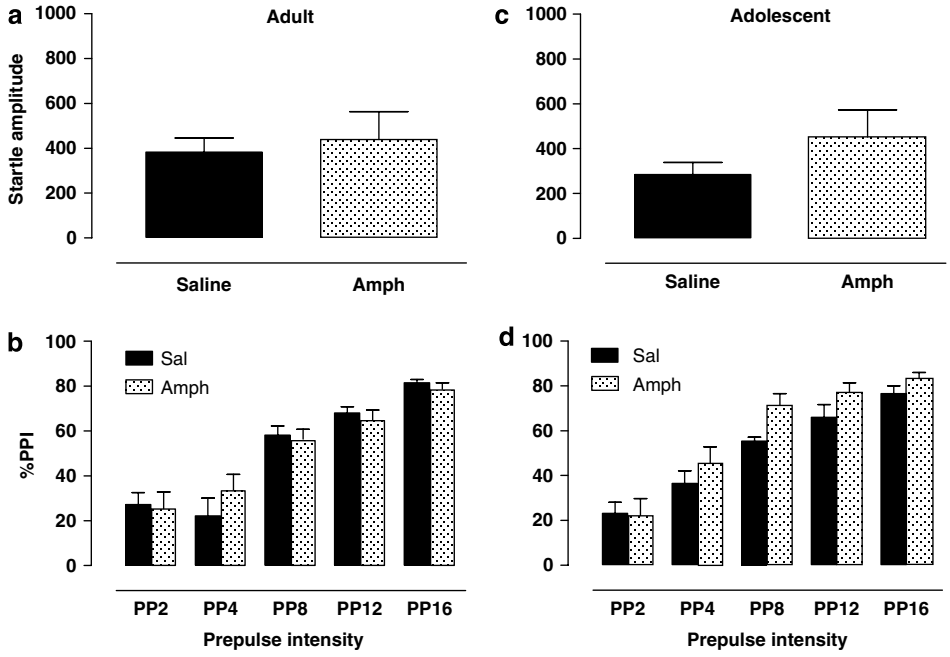


Figure 4 Behavioural effects of chronic amphetamine (amph, 10 mg kg^{-1} for 10 days, i.p.) or saline (sal, 1 mL kg^{-1} for 10 days, i.p.) on PPI (prepulse inhibition) and startle in adult (**a** and **b**) and adolescent (**c** and **d**) Sprague–Dawley rats at withdrawal day 27. The graphs are as follows: (**a**) adult startle, (**b**) adult PPI, (**c**) adolescent startle and (**d**) adolescent PPI. Startle responses (**a** and **c**) are measured in arbitrary units, and are represented as the median of the 40 pulse-alone responses recorded among a total of 100 trials. Percentage PPI (**b** and **d**) values were measured at prepulses (PP) 2, 4, 8, 12 and 16 db above baseline. All data are presented as median ± s.e.mean, and each group contained 8–12 rats. Two-way ANOVA revealed no significant differences between the groups.

differences were detected in the dentate gyrus. No changes were detected in the adult cohort irrespective of treatment (Figures 6a and b).

Discussion

Here, we report that periadolescent treatment with D-amphetamine results in a specific deficit during a retention trial on a Y-maze 13 days after cessation of amphetamine treatment. This deficit was reported exclusively in the novel arm, as no significant differences were found between the treatment groups in the other arms. Given that these rats had intact spatial learning and reference memory, it would appear that this finding likely represents a deficit in novelty seeking. Thus, adolescent rats treated with amphetamine during development were significantly less likely to explore the novel environment on a Y-maze than were a cohort treated during adulthood.

These data provide support for and extend previous research by Heyser and colleagues (2004), who demonstrated that chronic methylphenidate (5 mg kg^{-1}) disrupted novelty exploration in periadolescent rats 30 min after the last injection. The persistent disruption may be dependent on the dose, as another study found that amphetamine disrupted novelty exploration at 4 mg kg^{-1}, but not at lower doses of 1.5 and 2 mg kg^{-1} (Nicholls *et al*., 1992). To gain further insight into the nature of this deficit, future studies should investigate different groups of rats tested at different retention intervals on either side of the 6 h epoch used in the present experiment (for example, 2, 4, 8, 12 and 24 h). Moreover, it would also be instructive to vary the window between amphetamine treatment and behavioural testing to determine the degree of persistence of this drug-induced behavioural phenotype. In parallel, we also found that periadolescent treatment with D-amphetamine resulted in persistent downregulation of hippocampal CREB mRNA expression. Indeed, there was a specific interaction between treatment and age for modulation of CREB gene expression, where rats treated in adulthood were resistant but those treated during adolescence were sensitive. Collectively, these data provide behavioural and molecular evidence that the periadolescent brain reveals idiosyncratic sensitivities to psychostimulants.

Twelve hours into the withdrawal period, amphetamine pretreated rats did not show evidence of enhanced anxiety when tested on the elevated plus maze. Previous studies on the effect of acute and chronic administration of amphetamine have produced conflicting results in adult rats. Studies have shown that acute amphetamine administration may result in anxiogenic (Lapin, 1993; Lin *et al*., 1999) or anxiolytic (Dawson *et al*., 1995) responses, or have no effect (Lister, 1987) at all. Similarly, both chronic amphetamine (Cancela *et al*., 2001) and cocaine (Sarnyai *et al*., 1995) treatment have been found to produce anxiogenic-like responses, or no overall effect (amphetamine; Russig *et al*., 2005; cocaine, Basso *et al*., 1999; Lilly and Tietz, 2000). These inconsistent results may be due to differences in withdrawal time points. Other conflicts may be related to discrepancies in administration schedules, or strain and species differences. Extended withdrawal time points were not assessed in the present study, and it is not known whether our treatment

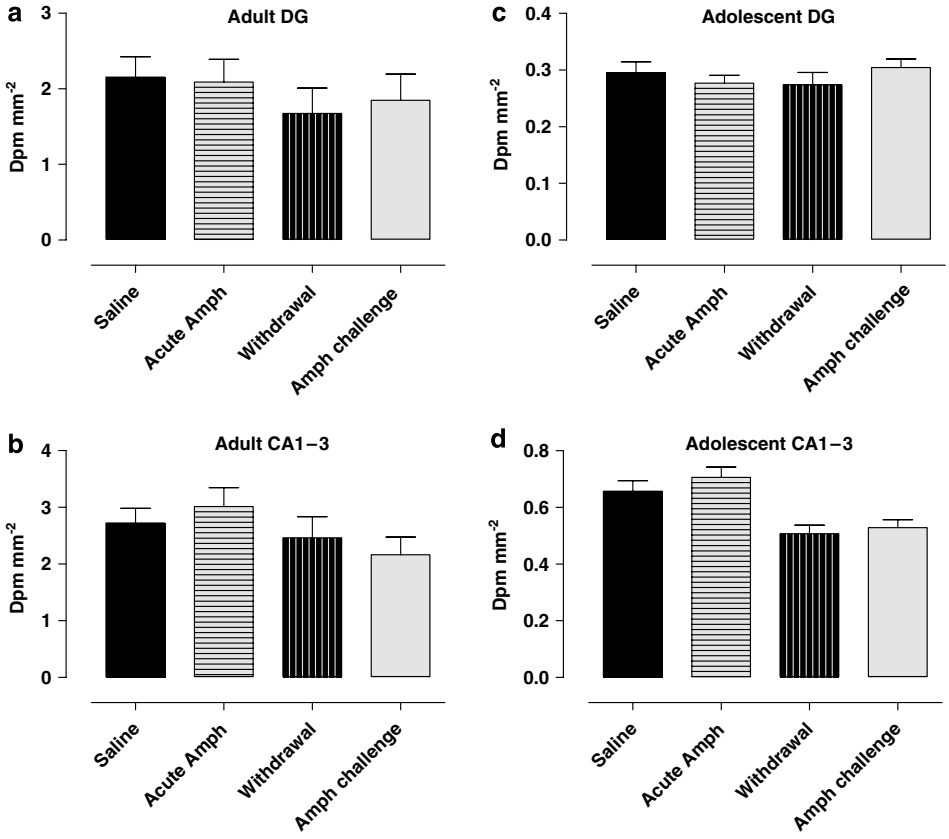


Figure 5 Neurochemical effects on TrkB mRNA in the dentate gyrus (DG) (**a** and **c**) and CA1–3 (**b** and **d**) regions of the hippocampus at challenge day, 9 weeks after treatment. Half of each treatment group received either saline (1 mL kg^{-1}, i.p.) or were challenged with amphetamine (amph, 1.5 mg kg^{-1} i.p.). Treatment groups are defined by their chronic treatment followed by their challenge; control (saline–saline), acute (saline–amph), withdrawal (amph–saline) and amphetamine challenge (amph–amph). Dpm (disintegrations per minute) (mm^{-2}) was compared within treatment groups and between adult (**a** and **b**) and adolescent (**c** and **d**) cohorts. All data are presented as mean ± s.e.mean, and each group contained 5 or 6 rats. Two-way ANOVA revealed no significant differences.

regime would cause anxiety to emerge at a later time point. However, the potential importance of emergent anxiety 10 weeks after methyldioxymethamphetamine administration has been documented (McGregor *et al*., 2003). Nonetheless, in a comparison within and between adult and adolescent cohorts, no effects on elevated plus maze performance were found at the 12 h withdrawal time point in the present study. Furthermore, previous studies demonstrating emergent anxiety have focussed largely on adult rats (McGregor *et al*., 2003), and in our study, adult rats did not show a deficit on the Y-maze that could be explained by altered anxiety levels.

We also did not detect any disruptions in acoustic startle or PPI after chronic amphetamine treatment. Although these findings are not wholly in keeping with previous research, this is likely to be due to differences in administration schedules of amphetamine, withdrawal time points and/or strain differences. Peleg-Raibstein *et al*. (2006) showed that disruptions in adult rats persisted to withdrawal day 70; however, treatment in this instance was applied 3 times per day in contrast to the current study, which used only single administration of drug per day. A greater proportion of time spent under the influence of amphetamine, due to a greater frequency of administration, may perhaps be the reason for the discrepancy observed.

In the adolescent pretreated cohort, significant increases in CREB mRNA in the CA1–3 region were observed after acute amphetamine administration at 9 weeks. Although to our knowledge this is the first time this has been reported, some consistency is seen with previous research documenting acute increases in phosphorylated CREB in the hippocampus following chronic treatment with amphetamine (Yin *et al*., 2006). Additionally, the chronic administration of toluene at an abusive exposure level has also been found to increase CREB mRNA levels acutely (Ahmed *et al*., 2007). However, significant reductions were observed in CREB mRNA levels in the CA1–3 region 9 weeks after amphetamine administration in the adolescent withdrawal and amphetamine challenge groups. In contrast, CREB mRNA in the adult cohort was not influenced by any of these treatment regimes.

The hippocampal region is an important component of the circuitry that responds to the detection of new stimuli (Knight, 1996; Grunwald *et al*., 1998). Biochemical and plastic changes associated with novelty may be detected in the hippocampus (Xu *et al*., 1998; Giovannini *et al*., 2001; Winograd and Viola, 2004), and CREB activation is an important component of novelty recognition within the hippocampus (Viola *et al*., 2000; Winograd and Viola, 2004; Moncada and Viola, 2006). Increases in phosphorylated

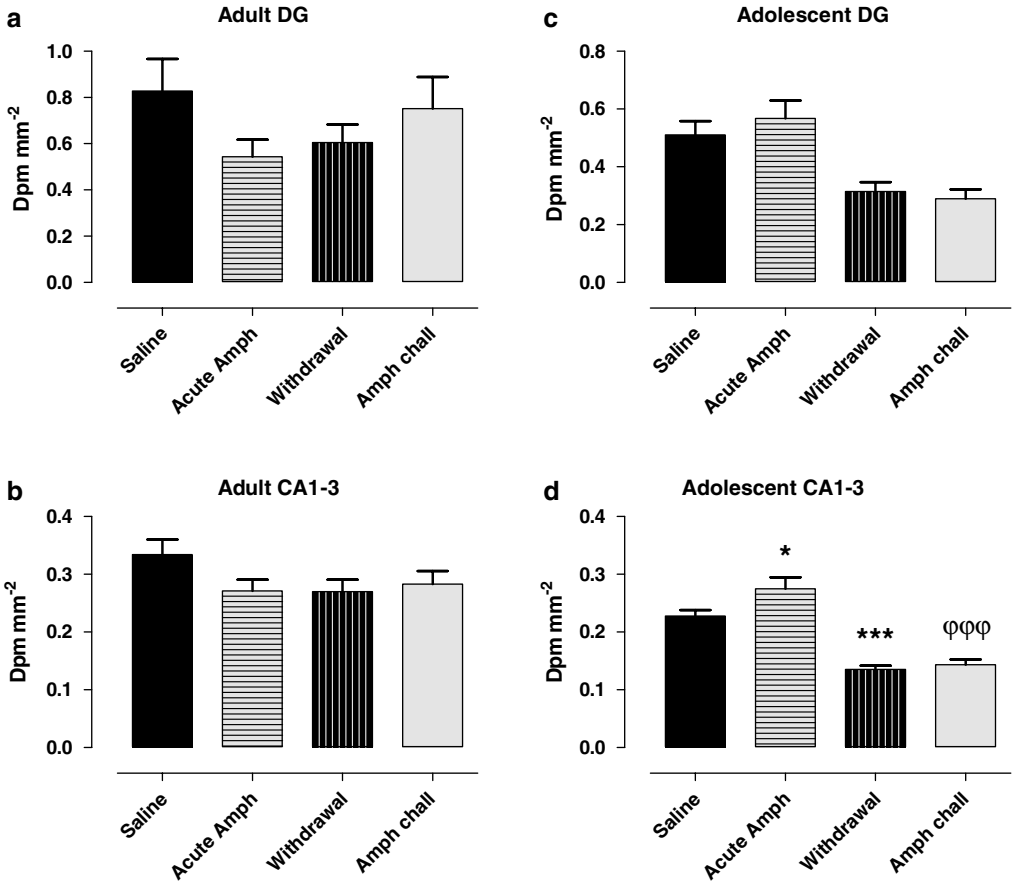


Figure 6 Neurochemical effects on cAMP response element-binding protein mRNA in the dentate gyrus (DG) (**a** and **c**) and CA1–3 (**b** and **d**) regions of the hippocampus at challenge day, 9 weeks after treatment. Half of each treatment group received either saline (1 mL kg^{-1}, i.p.) or were challenged with amphetamine (amph, 1.5 mg kg^{-1}, i.p.). Treatment groups are defined by their treatment followed by their challenge: control (saline–saline), acute (saline–amph), withdrawal (amph–saline) and amphetamine challenge (amph–amph). All data are presented as mean ± s.e.mean. Dpm (disintegrations per minute) (mm^{-2}) was compared within treatment groups and between adult (**a** and **b**) and adolescent (**c** and **d**) cohorts. A two-way ANOVA (Tukey *post hoc*) was performed with age × treatment as factors, and each group contained 5 or 6 rats. There was a statistically significant interaction between treatment and age for CA1–3 in the group pretreated during adolescence: $F_{(3,425)} = 6.99$, $^{**}P < 0.01$. In the CA1–3, acute amph vs saline, $^{*}P < 0.05$; $^{***}P < 0.001$ saline vs withdrawal, $^{\varphi\varphi\varphi}P < 0.001$; acute amph vs amph chall.

CREB have been shown to last up to 2 h after brief novelty exploration, whereas no change was detected after rats explored familiar environments (Vianna *et al*., 2000; Winograd and Viola, 2004). In the present study, both adolescent withdrawal and amphetamine rechallenge groups revealed a reduction in hippocampal CREB mRNA; this suggests expression levels were persistently reduced and may have contributed to the deficit in novelty exploration in amphetamine-treated adolescent rats. As the activation of CREB is an important component of novelty detection (Viola *et al*., 2000; Winograd and Viola, 2004; Moncada and Viola, 2006), reduced levels in the amphetamine-treated adolescent rats may have affected their performance under retention conditions on the Y-maze. While a persistent reduction in hippocampal CREB mRNA may have resulted in reduced CREB protein available for phosphorylation, further protein-based studies would need to be performed to clarify the current findings. Nonetheless, as novelty detection plays an important role in both adapting to environmental changes and avoiding dangers, amphetamine administration during a stage of development characterized by existing vulnerability (Grant and Dawson, 1997; Wagner and Anthony, 2002) may have significant consequences in adulthood. Although a persistent depression of hippocampal CREB mRNA was observed, there was no effect on learning and memory in adolescent rats as tested in a Morris water maze. Previous data showing the effects of hippocampal CREB deletion in a dominant negative mouse have shown no change in latency to find a hidden platform during early spatial learning, and subtle effects on probe trial quadrant occupancy, tested on a Morris water maze (Pittenger *et al*., 2002).

Neurotrophic factors and their associated Trk receptors are essential to learning and memory (Tyler *et al*., 2002; Purcell and Carew, 2003). The disruption of an amphetamine-induced, context-dependent behaviour with intradentate gyrus infusions of the Trk inhibitor K-252a during the acquisition of drug/context associations suggests that the hippocampal Trk system is critical for this type of learning (Shen *et al*., 2006). Additionally, amphetamine induced both

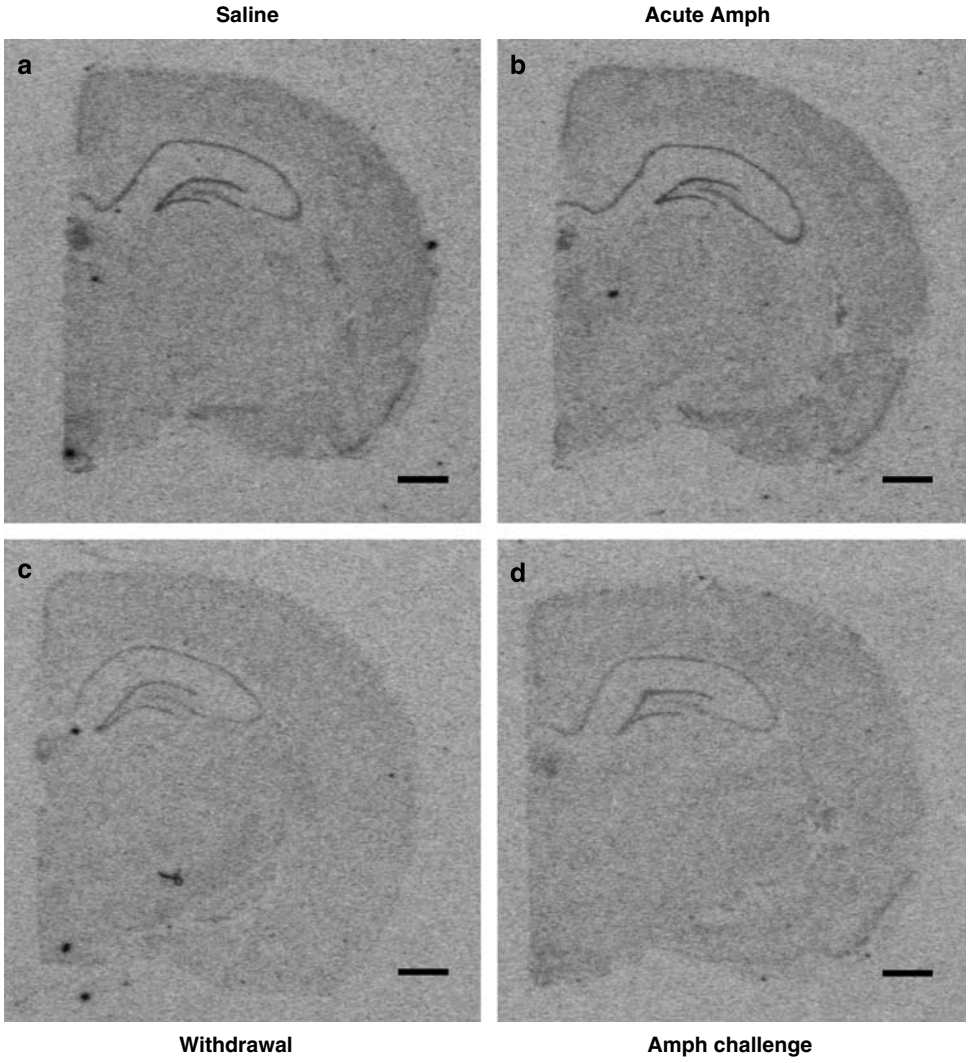


Figure 7 Autoradiograms representing expression of mRNA encoding cAMP response element-binding protein in the CA1–3 region of the hippocampus in the treatment groups (**a**) saline (saline–saline), (**b**) acute (saline–amphetamine (amph)), (**c**) withdrawal (amph–saline) and (**d**) amphetamine challenge (amph–amph). Treatment groups are defined by their treatment followed by their challenge. Scale bar = 1 mm.

a conditioned place preference and corresponding increases in hippocampal immunoreactivity for TrkB 96 h later, as well as increases in TrkB mRNA 2 h later in adult rats (Shen *et al.*, 2006). Although the hippocampal region is an important component of the limbic–cortical network that responds to the detection of new stimuli (Knight, 1996; Grunwald *et al.*, 1998), and TrkB receptors are important in drug-induced associative learning, no significant changes were found in hippocampal TrkB mRNA in the current study. This suggests that these receptors might play a less-prominent role following chronic amphetamine treatment. Accordingly, it may be concluded that any changes that may occur in hippocampal TrkB mRNA in a short term are not seen in either age group 9 weeks following chronic amphetamine treatment. To clarify whether the current findings relate to changes at the protein level, further immunohistochemical studies are required.

In conclusion, the findings of the current study confirm that periadolescent rats treated with chronic amphetamine show deficits on the Y-maze, at least 13 days after treatment cessation. As spatial learning and reference memory remained intact when tested on a Morris water maze, these deficits likely reflect a dysfunction in response to novelty. Chronic periadolescent treatment with D-amphetamine also resulted in a persistent downregulation of hippocampal CREB, but not TrkB, mRNA expression after 9 weeks of withdrawal. Notably, and distinct from the adolescent cohort, the adult groups were not affected by any of these treatment regimes. Collectively, these data provide behavioural and molecular evidence that the periadolescent brain reveals idiosyncratic sensitivities to the long-term effects of psychostimulants. As novelty detection is particularly important to the adaptation to environmental changes and avoiding dangers, amphetamine abuse during adolescence may substantially affect a young person's ability to master key developmental challenges. The impact of periadolescent amphetamine treatment on future drug-seeking is not yet known and will be the focus of future studies.

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Conflict of interest

The authors state no conflict of interest.

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