



# Withdrawal from chronic amphetamine produces persistent anxiety-like behavior but temporally-limited reductions in monoamines and neurogenesis in the adult rat dentate gyrus

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## ABSTRACT

Acute amphetamine administration activates monoaminergic pathways and increases systemic corticosterone, both of which influence anxiety states and adult dentate gyrus neurogenesis. Chronic amphetamine increases anxiety states in rats when measured at 24 h and at 2 weeks of withdrawal. However, the effects of chronic amphetamine exposure and withdrawal on long term anxiety-like behavior and adult neurogenesis in the dentate gyrus are unknown. Adult male rats were administered amphetamine (2.5 mg/kg, ip.) daily for two weeks. Anxiety-like behaviors were increased markedly in amphetamine-treated rats following four weeks of withdrawal from amphetamine. Plasma corticosterone level was unaltered by amphetamine treatment or withdrawal. However, norepinephrine and serotonin concentrations were selectively reduced in the dentate gyrus 20 h following amphetamine treatment. This effect did not persist through the four week withdrawal period. In separate experiments, rats received bromodeoxyuridine to label cells in S-phase, prior to or immediately following amphetamine treatment. Newly generated cells were quantified to measure extent of progenitor cell proliferation and neurogenesis following treatment or withdrawal. Progenitor cell proliferation and neurogenesis were not significantly affected by amphetamine exposure when measured 20 h following the last amphetamine treatment. However, neurogenesis in the dentate gyrus was reduced after four weeks of withdrawal when compared to saline-pretreated rats. Overall, our findings indicate that withdrawal from chronic amphetamine leads to persistent anxiety-like behavior which may be maintained by reduced neurogenesis in the dentate gyrus at this protracted withdrawal time point. However, neurogenesis is unaffected at earlier withdrawal time points where anxiety states emerge, suggesting different mechanisms may underlie the emergence of anxiety states during amphetamine withdrawal.

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## 1. Introduction

Amphetamine and methylphenidate are the most commonly prescribed stimulants for treatment of attention-deficit/hyperactivity disorder (Berman et al., 2009). However, amphetamine has a high potential for abuse and is used illicitly by young adults (Teter et al., 2006). The acute pharmacological effects of amphetamine are to increase central monoamine neurotransmission by influencing the processes of release, re-uptake and metabolism, leading to increased extracellular levels of dopamine, serotonin and norepinephrine (Holmes and Rutledge, 1976), as well as inducing systemic release of corticosterone (Knych and Eisenberg, 1979). In

stimulating the release of monoamines, amphetamine is more efficient than methylphenidate (Kuczenski and Segal, 1997). Withdrawal from amphetamine use is accompanied by symptoms of anxiety and depression in both humans and animal models (Barr and Markou, 2005; Kitanaka et al., 2008; Vuong et al., 2010). In particular, we have recently demonstrated that rats exhibit anxiety-like behaviors at both 24 h and 2 weeks withdrawal from amphetamine (Vuong et al., 2010). Furthermore, increased anxiety states are associated with greater conditioned place preference for cocaine in rats (Pelloux et al., 2009), and negative affect during withdrawal is thought to be a critical factor leading to craving and relapse in humans (Koob et al., 2004).

The hippocampus has been implicated in the pathophysiology of affective disorders, fear and anxiety behavior (Kjelstrup et al., 2002; McHugh et al., 2004), and also plays a role in stimulant-induced behaviors and the relapse stage of addiction

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(Taepavarapruk and Phillips, 2003; Rogers and See, 2007; Fuchs et al., 2005). More specifically, the dentate gyrus of the hippocampus is important for the formation of context-specific memories associated with fear and drug reward (Hernandez-Rabaza et al., 2008) and is one of few regions of the brain that exhibit life-long neurogenesis (Altman and Das, 1965; Eriksson et al., 1998; Cameron and McKay, 1998). Experimental reductions of adult dentate gyrus neurogenesis are associated with an increase in the rewarding effects of drugs of abuse (Noonan et al., 2010), though, the effect of reduced neurogenesis on emotional behavior is less clear. For example, many studies show heightened anxiety following reduced neurogenesis (Revest et al., 2009; Earnheart et al., 2007; Bergami et al., 2008; Crupi et al., 2010) while others observed no effects of experimentally reduced neurogenesis in the dentate gyrus on anxiety states (Santarelli et al., 2003; Saxe et al., 2006; Surget et al., 2008; David et al., 2009). This suggests that altered neurogenesis may play an indirect role in the network involved in this emotional state. Therefore, changes in the levels of adult neurogenesis by drugs of abuse could have a direct or indirect influence on behaviors regulated by the hippocampus.

Repeated exposure to psychostimulants influences both the proliferative and maturation stages of neurogenesis. For example, cocaine has been shown to reduce progenitor cell proliferation in the dentate gyrus without influencing cell differentiation or cell survival, regardless of whether the drug was experimenter delivered (Yamaguchi et al., 2004; Dominguez-Escriba et al., 2006) or self-administered (Noonan et al., 2008). Also, amphetamine derivatives affect the neurogenic process. For example, 3, 4-methylenedioxymethamphetamine (MDMA) has been shown to affect proliferation and survival of newly generated cells depending upon injection schedule and timing of treatment (Hernandez-Rabaza et al., 2006; Cho et al., 2007). A single dose of methamphetamine can impact proliferation (Teuchert-Noodt et al., 2000) and more long term effects are dependent upon level of access when self-administered (Mandyam et al., 2008). With regard to amphetamine, no changes in progenitor cell proliferation or neurogenesis were observed after an acute injection (Mao and Wang, 2001). However, the effect of chronic amphetamine and withdrawal on the process of adult dentate gyrus neurogenesis has not been explored.

Alterations to neurogenesis by psychostimulants may be related to pharmacological actions of stimulants on monoamine and corticosterone systems. Serotonin (5-HT) and norepinephrine (NE) both enhance the production of new neurons in the dentate gyrus (Malberg et al., 2000; Malberg and Duman, 2003; Rizk et al., 2006). Conversely, corticosterone reduces neuronal production (Wong and Herbert, 2006) and interacts with monoamines in the regulation of neurogenesis (Huang and Herbert, 2005, 2006). Since amphetamine activates monoaminergic transmission and increases plasma corticosterone, both of which regulate the neurogenic process and emotive behaviors, we hypothesized that amphetamine withdrawal would be associated with altered plasma corticosterone, reduced monoamine levels in the hippocampus and disrupted adult neurogenesis in the dentate gyrus, and these measures would be associated with increased anxiety-like behavior during withdrawal. Due to the anatomical and functional differentiation of the hippocampus along its dorsoventral axis (Bannerman et al., 2002; Bertoglio et al., 2006), and the possible differential regulation of neurogenesis along this axis (Snyder et al., 2009b; Banasr et al., 2006; Ambrogini et al., 2000), we explored our hypothesis separately in the dorsal and ventral hippocampus.

## 2. Methods

### 2.1. Animals and treatments

The following procedures were approved by the Institutional Animal Care and Use Committee of South Dakota, and were carried out in accordance with the

National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Adult male Sprague–Dawley rats (8–10 weeks old, Animal Resources Center, The University of South Dakota) were housed in pairs at a constant room temperature (22 °C, 60% relative humidity) and with a reverse 12 h light: 12 h dark cycle (lights off at 10:00 am). Food and water were available *ad libitum*. Rats were injected with *D*-amphetamine (2.5 mg/kg, ip.) or an equivalent volume of saline during the dark cycle of the reverse photoperiod at the same time of day for 14 consecutive days. Specifically, injections occurred 1 h following onset of the dark cycle, and rats were placed back into their home cages following injection. This treatment regime results in behavioral sensitization to amphetamine (unpublished data) and greater anxiety-like behaviors at 24 h and 2 weeks of withdrawal (Vuong et al., 2010).

### 2.2. Assessment of anxiety-like behavior

To determine whether withdrawal from repeated amphetamine treatment alters anxiety-like behavior at the withdrawal time point when neurogenesis was assessed (4 weeks withdrawal), male rats (19 saline-; 22 amphetamine-treated) were tested for 5 min on the elevated plus maze (EPM) 4 weeks after the last injection. The maze included perpendicular, intersecting runways (12 cm wide × 100 cm long) connected by a central area. Two runways had high walls (40 cm high), the other two arms had no walls, and the entire maze was elevated 1 m from the floor (Noldus Information Technology, Wageningen, The Netherlands). The test consisted of placing a rat in the center of the apparatus (facing an open arm) and allowing it to freely explore for 5 min. All testing was conducted in the dark (active) phase of the light cycle using red light illumination. Rats were recorded from above the maze and scored by automated software (Ethovision 3.1; Noldus Technologies), with the latency to enter an open arm, the cumulative time spent in each arm, and total distance moved within the EPM recorded.

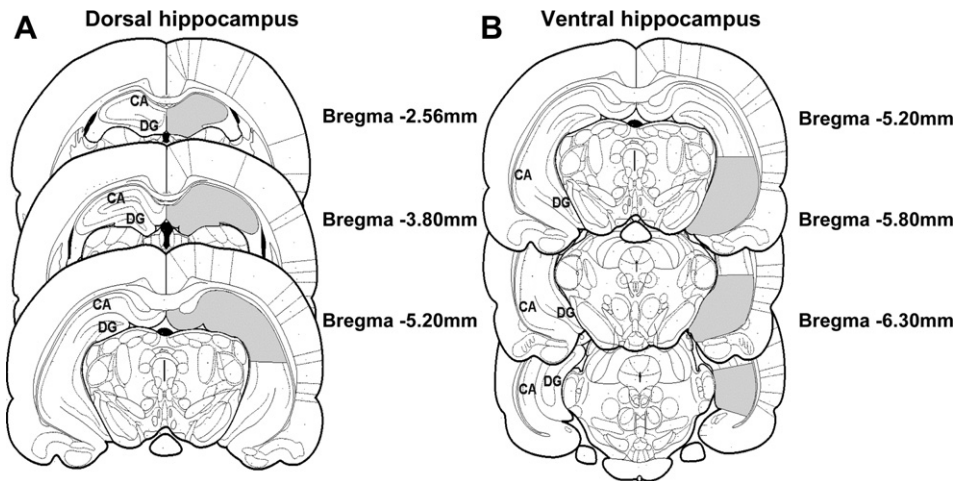
### 2.3. Plasma corticosterone assay and monoamine analysis

To assess the effects of amphetamine treatment and withdrawal on plasma corticosterone and hippocampal monoamine levels, amphetamine- and saline-treated rats were allocated into 20 h or 4 week withdrawal groups ( $n = 10$ /time period). Brains and plasma were collected in the last 3 h of the light phase of the light cycle (corresponding to 4 h prior to the next injection in the case of the 20 h withdrawal group). Animals in the 20 h withdrawal group were analyzed following the last amphetamine injection but before the next subsequent daily injection would take place to avoid protracted withdrawal. Rats were rapidly decapitated; trunk blood was collected, and centrifuged at 5000 rpm. Plasma was drawn off and frozen at  $-80$  °C until assayed. Brains were removed and stored at  $-80$  °C. Measurement of plasma corticosterone was performed using a corticosterone enzyme linked immunoassay kit (Assay Designs, Ann Arbor, MI), according to Forster et al. (2008). The level of non-specific binding was 2% and the sensitivity was 22 pg/mL.

For monoamine analysis, frozen brains were sliced (300  $\mu$ m) coronally in a cryostat at  $-10$  °C. The dorsal and ventral CA and dentate regions of the hippocampus were identified (Paxinos and Watson, 1997; see Fig. 1) and microdissected on a freezing plate (Physitemp Instruments, Inc, Clifton, NJ) using a 432  $\mu$ m diameter cannula. Brain samples were expelled into 60  $\mu$ l sodium acetate buffer (pH 5.0) containing the internal standard dihydroxybenzylamine and freeze-thawed. Prior to centrifugation at  $15,000 \times g$  for 3 min, 2  $\mu$ l of 1 mg/mL ascorbate oxidase was added to each sample. Supernatant (45  $\mu$ l) was injected into an HPLC system (Waters 717 Plus Autosampler, Milford, MA) and levels of NE and 5-HT were analyzed electrochemically using an LC-4C detector (BioAnalytical Systems, Inc., IN) with the electrode potential set at +0.6 V with respect to a Ag/AgCl reference electrode. The mobile phase consisted of 14 g citric acid, 8.6 g sodium acetate, 110 mg 1-octanesulfonic acid (sodium salt), 150 mg EDTA disodium salt and 100 mL methanol in 1 L deionized water (Renner and Luine, 1986; Ling et al., 2009). The tissue pellet was dissolved in 110 mL 0.1 N NaOH and protein content was assayed using the Bradford method (Bradford, 1976; Watt et al., 2007).

### 2.4. Proliferation, neurogenesis and cell death

To determine the effects of amphetamine treatment on progenitor cell proliferation in the dentate gyrus, the S-phase marker bromodeoxyuridine (BrdU, 200 mg/kg, ip.; Cameron and McKay, 2001; Taupin, 2007) was administered during a limited time-window (<24 h; three times at 6 h intervals) following the last amphetamine or saline injection, and rats were perfused (as described below) 2 h after the last BrdU injection (Fig. 2A). In a separate group of rats, the effect of amphetamine treatment on neuronal survival in the last two weeks of the process of neurogenesis was assessed. Injections of BrdU were administered two weeks prior to the two weeks of amphetamine or saline injections without confound of withdrawal (Fig. 2B). In this case, rats were perfused 20 h following the last amphetamine or saline treatment. Finally, the effects of amphetamine withdrawal on neurogenesis was assessed



**Fig. 1.** Representative sections of the hippocampus between 2.56 mm and 6.3 mm from bregma (Paxinos and Watson, 1997) illustrating the (A) dorsal and (B) ventral regions analyzed for monoamine levels and bromodeoxyuridine- labeled cells. CA = Cornu Ammonis, DG = Dentate gyrus.

by administering the BrdU injections within the 24 h period following the last amphetamine or saline injection, with the perfusions conducted 4 weeks following the BrdU injections (Fig. 2C).

For perfusions, rats were anesthetized with sodium pentobarbital (100 mg/kg, ip.) and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4; room temperature [RT]), followed by 4% paraformaldehyde (pH 7.4, 4 °C). Brains were post-fixed in 4% paraformaldehyde (4 °C), for 1 h then placed in 25% sucrose and stored for 2 days at 4 °C. The brain containing the entire hippocampus (bregma –1.8

to –6.3) was sectioned at 40  $\mu$ m using a sliding microtome. Sections were stored in 0.1% NaN<sub>3</sub> in PBS at 4 °C until processing.

For proliferation and neurogenesis studies, every ninth section was processed for BrdU and NeuN (neuron specific nuclear protein as a marker of mature neurons; Mullen et al., 1992) labeling. Sections were rinsed in 0.1 M PBS, incubated in 1 M HCl for 1 h at 60 °C to denature DNA, neutralized in 1 M sodium borate buffer (pH 8.5) and rinsed 3X in 0.1 M PBS for 5 min. Sections were then exposed to rat monoclonal anti-BrdU (1:100, Abcam, Cambridge, MA) and mouse anti-NeuN (1:750, Chemicon International Inc, Temecula, CA) at 4 °C for 20 h, rinsed 3X for 5 min in 0.1 M PBS and treated with secondary antibodies containing Cy3-conjugated goat anti-rat (1:200, Jackson ImmunoResearch, West Grove, PA) and Cy2-conjugated goat anti-mouse (1:200, Jackson ImmunoResearch) for 2 h at RT. Sections were mounted on gelatin-coated slides, air-dried, dehydrated, and cover slipped with cytochrome seal (Richard-Allan Scientific, Kalamazoo, Michigan). Immunofluorescence controls consisted of sections in which the primary antibody was omitted.

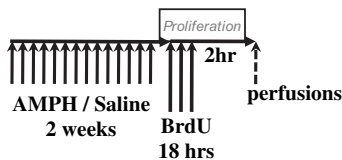
Since neurogenic regions exhibit high levels of apoptosis (Biebl et al., 2000), cells undergoing apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) were detected using an Apoptag red detection kit on separate sections (Chemicon International Inc, Temecula, CA). Control TUNEL reaction was performed without the TdT enzyme.

The effects of chronic amphetamine treatment on the number of BrdU-labeled cells was examined separately in the dorsal and ventral hippocampus similar to Banasr et al. (2006) with the dorsal hippocampus defined as hippocampal structures occurring above 6 mm on the dorsal-ventral scale (Fig. 1; bregma –1.8 to –6.3; Paxinos and Watson, 1997), and the ventral hippocampus was defined as occurring below 6 mm on the dorsal-ventral scale (Fig. 1; bregma –4.3 to –6.3; Paxinos and Watson, 1997). This is different than separation along rostrocaudal extent (anterior-posterior) where regions contain both dorsal and ventral structures. A counting profile was applied including the subgranular zone (SGZ; defined as a two cell body wide zone along the border of the granule cell layer and the hilus) and the granule cell layer. The numbers of BrdU- BrdU + NueN- and TUNEL-labeled cells were quantified by an investigator blind to treatment history using a previously described modified fractionator method (Gundersen et al., 1988; Guillery and Herrup, 1997; Cameron and McKay, 2001; Coggeshall and Lekan, 1996). Since the number of sections containing dorsal and ventral dentate gyrus was different, means for each region were obtained and adjusted to the total number of hippocampal sections containing either dorsal or ventral dentate gyrus. The BrdU + NeuN co-labeled cells in the dorsal or ventral dentate gyrus were analyzed in their entire z-axis with a 0.5  $\mu$ m step using an Olympus Fluoview 500 laser scanning confocal microscope (Olympus America, NY, USA) to exclude false double-labeling.

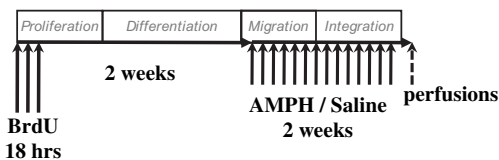
## 2.5. Data analysis

All analyses were performed using SigmaStat v.3.0. Plasma corticosterone levels and hippocampal monoamine levels were analyzed using two-way ANOVAs followed by Student-Newman-Keuls (SNK) *post-hoc* test. The number of TUNEL-positive, BrdU, and BrdU + NeuN double-labeled cells was analyzed using one-way ANOVA for the dorsal and ventral dentate gyrus. The effect of amphetamine on anxiety behaviors at 4 week withdrawal was determined by one-way ANOVA. The level of significance for all analyses was set at  $P < 0.05$ .

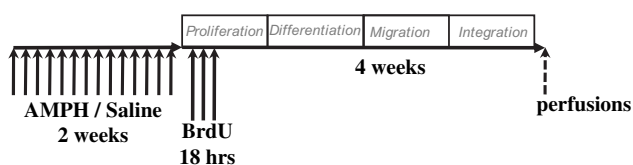
## A Effect of chronic amphetamine on proliferation



## B Effect of chronic amphetamine on neurogenesis



## C Effect of withdrawal from chronic amphetamine on neurogenesis



**Fig. 2.** Timeline of experiments. All animals received saline or amphetamine (2.5 mg/kg, ip.) daily for 2 weeks. (A) Following amphetamine or saline treatment, animals were given Bromodeoxyuridine (BrdU; 200 mg/kg, ip.) three times with 6 h intervals and perfused 2 h after the last BrdU injection to assess the level of cytogenesis and apoptosis. (B) Two weeks following BrdU (200 mg/kg, ip.) treatment, animals were given amphetamine or saline for two weeks and perfused 20 h after the last amphetamine or saline injection to assess the level of neurogenesis and apoptosis. (C) Following amphetamine or saline treatment, animals were given BrdU (200 mg/kg, ip.) three times with 6 h intervals and perfused 4 weeks after the last BrdU injection to assess the level of neurogenesis and apoptosis.

### 3. Results

#### 3.1. Long term withdrawal from amphetamine increases anxiety-like behavior

Anxiety-like behavior was assessed using the EPM, and testing was conducted four weeks after the last injection of amphetamine or saline to coincide with the same time frame in which

neurogenesis was measured during withdrawal (see below). Latency of open arm entry was increased by amphetamine treatment and withdrawal (Fig. 3A;  $F(1,25) = 4.333$ ,  $P < 0.05$ ). Also, time in open arms was significantly reduced in amphetamine-treated rats compared to saline controls (Fig. 3B;  $F(1,35) = 10.025$ ,  $P < 0.05$ ). There was no effect of treatment on locomotion as measured by total distance moved on the EPM ( $F(1,40) = 0.188$ ,  $P > 0.05$ ; Fig. 3C). Thus, altered general activity could not explain reduced time spent in open arms observed for amphetamine-treated rats.

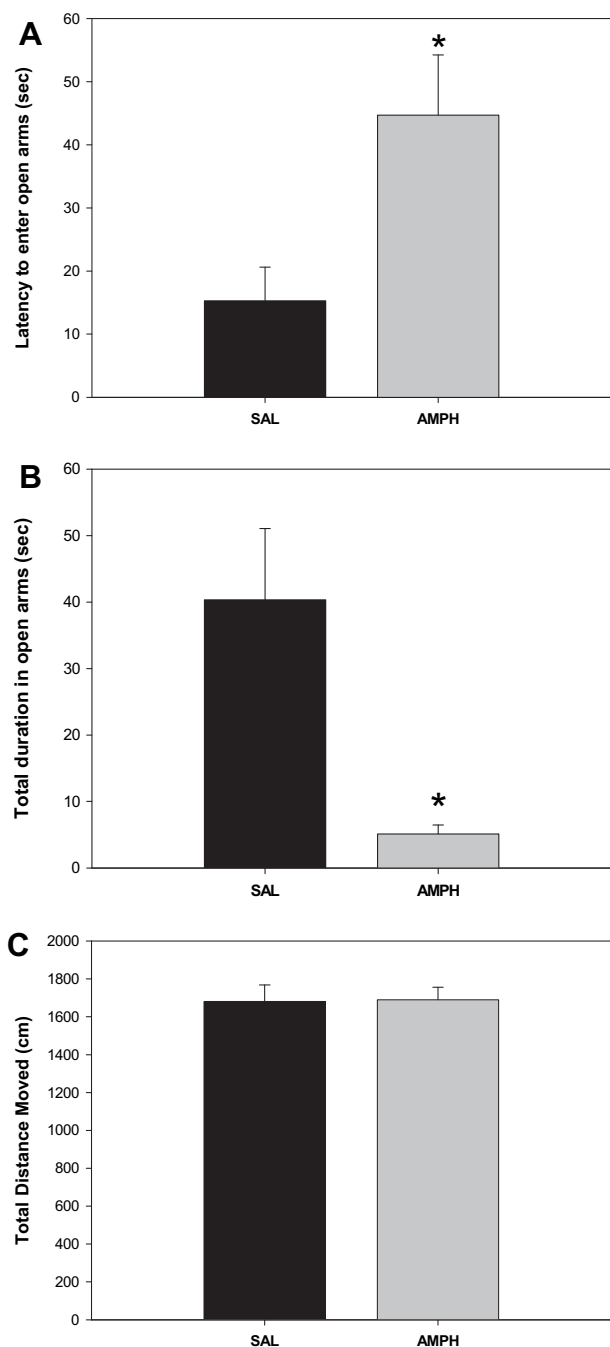
#### 3.2. Chronic amphetamine reduces dentate gyrus monoamine levels

As shown in Fig. 4, there were no significant effects of treatment ( $F(1,25) = 0.0223$ ,  $P > 0.05$ ) or withdrawal time ( $F(1,25) = 1.957$ ,  $P > 0.05$ ) nor an interaction ( $F(1,25) = 0.0789$ ,  $P > 0.05$ ) on basal plasma corticosterone levels. The effects of treatment ( $F(1,35) = 2.828$ ,  $P > 0.05$ ), withdrawal time ( $F(1,35) = 0.269$ ,  $P > 0.05$ ) and the interaction between treatment and withdrawal time ( $F(1,35) = 0.009$ ,  $P > 0.05$ ) on NE levels in the dorsal CA were also not significant (Fig. 5A). Similarly, 5-HT levels in the dorsal CA (Fig. 5B) were unaffected by treatment ( $F(1,30) = 0.004$ ,  $P > 0.05$ ) or withdrawal time ( $F(1,30) = 2.429$ ,  $P > 0.05$ ), and an interaction between the two was not observed ( $F(1,30) = 0.118$ ,  $P > 0.05$ ).

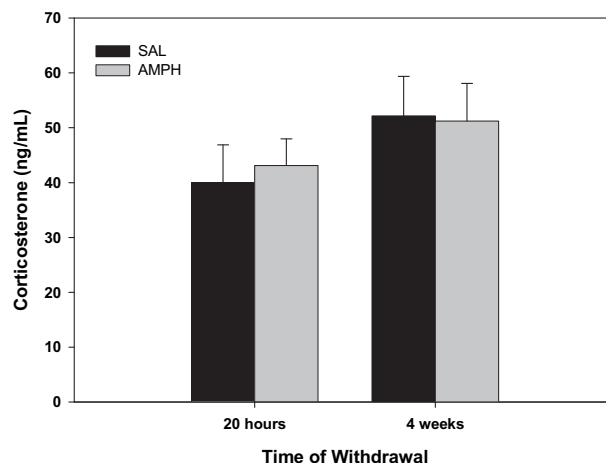
In the ventral CA, treatment ( $F(1,35) = 0.547$ ,  $P > 0.05$ ), withdrawal time ( $F(1,35) = 0.660$ ,  $P > 0.05$ ) or interaction ( $F(1,35) = 1.337$ ,  $P > 0.05$ ) for levels of NE were not significant (Fig. 5C). Also, there was no significant effect of treatment ( $F(1,36) = 0.218$ ,  $P > 0.05$ ) nor an interaction ( $F(1,36) = 0.037$ ,  $P > 0.05$ ) for levels of 5-HT (Fig. 5D). However, there was a significant effect of withdrawal time ( $F(1,36) = 29.225$ ,  $P < 0.001$ ). Both treatment groups had lower 5-HT levels following four weeks withdrawal compared to 20 h (SNK  $P < 0.001$ ; Fig. 5D).

In contrast, NE in the dorsal dentate gyrus showed a significant interaction between treatment and withdrawal time ( $F(1,33) = 3.593$ ,  $P < 0.05$ ) but no effect of treatment ( $F(1,33) = 1.564$ ,  $P > 0.05$ ), or withdrawal time ( $F(1,33) = 2.441$ ,  $P > 0.05$ ) alone (Fig. 6A). *Post hoc* analysis revealed that amphetamine-treated animals showed a significant reduction in NE at 20 h compared to saline controls (SNK  $P < 0.05$ ; Fig. 6A). However, there was no effect of treatment ( $F(1,34) = 0.002$ ,  $P > 0.05$ ), withdrawal time ( $F(1,34) = 0.419$ ,  $P > 0.05$ ), or interaction ( $F(1,34) = 0.469$ ,  $P > 0.05$ ) on 5-HT levels in this brain region (Fig. 6B).

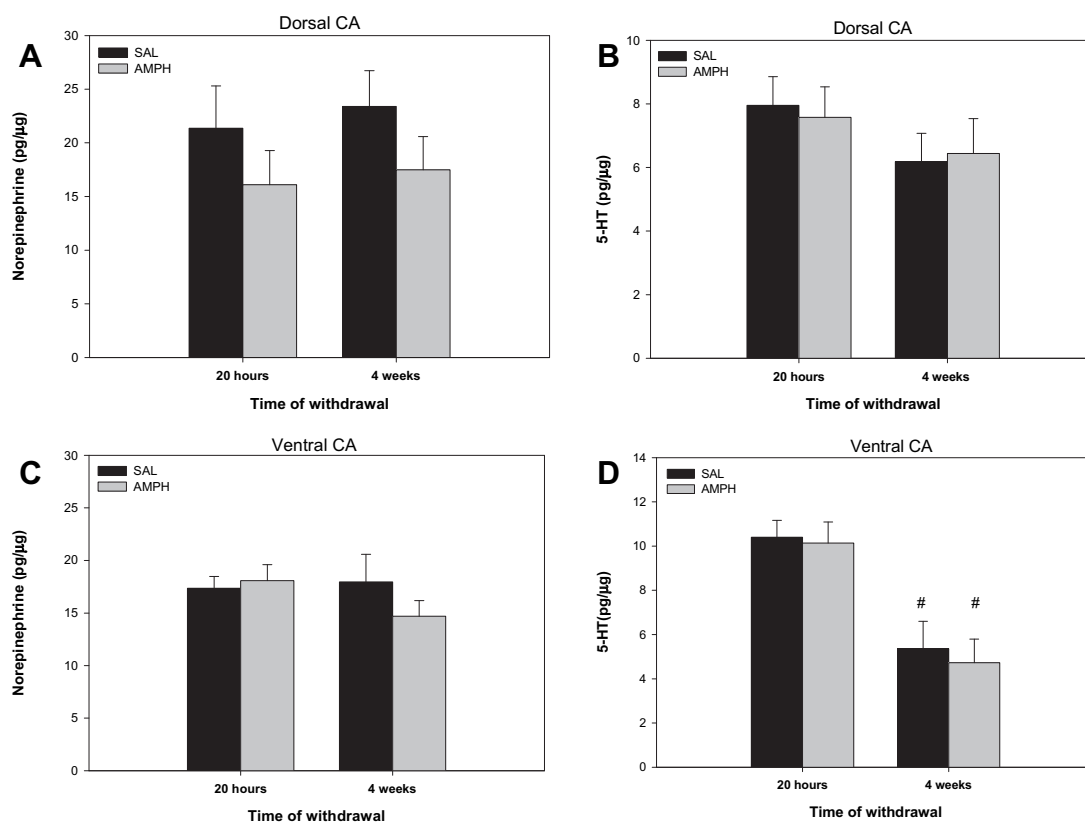
In the ventral dentate gyrus, NE levels differed between treatments ( $F(1,35) = 5.442$ ,  $P < 0.05$ ) but there was no effect of



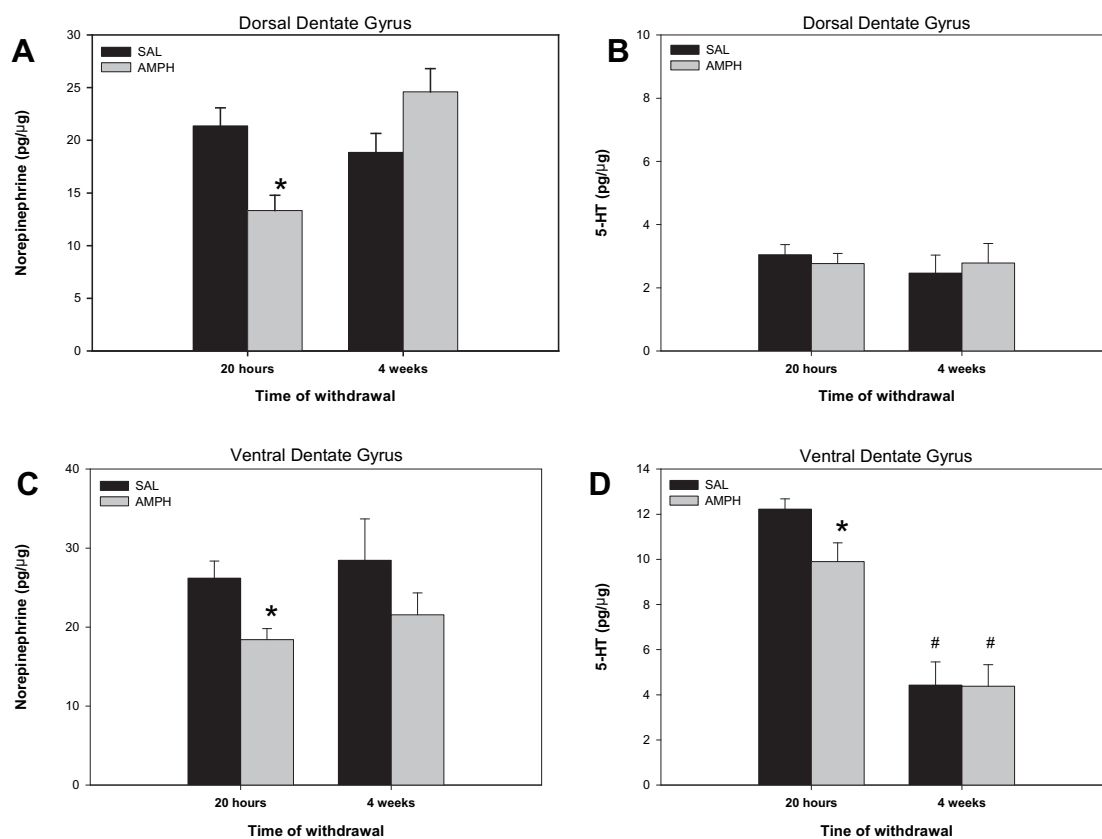
**Fig. 3.** Chronic amphetamine altered behavior during the 5 min elevated plus maze test when tested at four weeks withdrawal. (A) The latency to enter an open arm was significantly increased (B) and the duration of time spent in the open arms of the apparatus was significantly lower in amphetamine-treated rats, whereas (C) total locomotion during test was not different between treatment groups. ( $n = 18$  saline,  $n = 22$  amphetamine, mean  $\pm$  SEM). \* indicates a significant difference compared to the saline group.



**Fig. 4.** Plasma corticosterone concentrations 20 h and four weeks following two week treatment of amphetamine or saline ( $n = 10$  per group, mean  $\pm$  S.E.M.).



**Fig. 5.** Norepinephrine and serotonin concentrations in the dorsal (A–B) or ventral (C–D) CA for amphetamine and saline-treated rats 20 h or four weeks following treatment ( $n = 10$  per group, mean  $\pm$  S.E.M). # represents significantly different from 20 h time point. ( $n = 10$  per group, mean  $\pm$  S.E.M).



**Fig. 6.** Norepinephrine and serotonin concentrations in the dorsal (A–B) or ventral (C–D) dentate gyrus for amphetamine and saline-treated rats 20 h or four weeks following treatment. \* represents significantly different from saline-treated group. # represents significantly different from 20 h time point. ( $n = 10$  per group, mean  $\pm$  S.E.M).



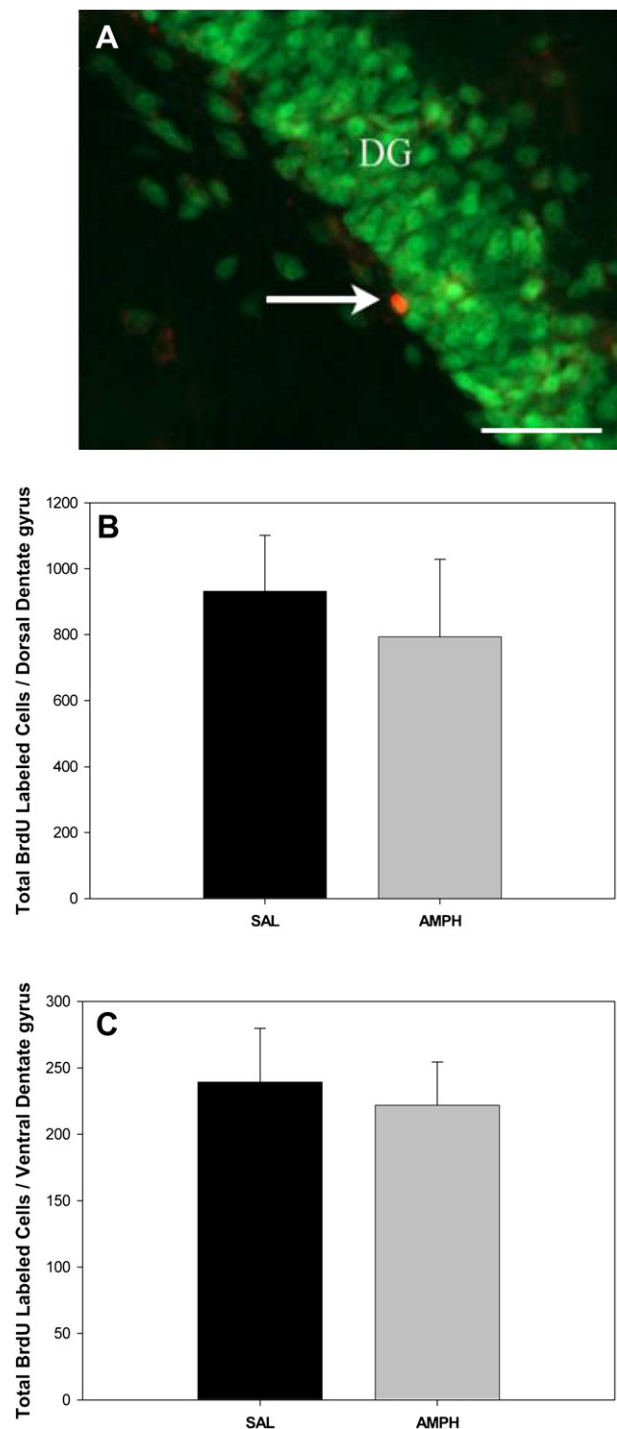
withdrawal time ( $F(1,35) = 0.741$ ,  $P > 0.05$ ) nor was there an interaction ( $F(1,35) = 0.019$ ,  $P > 0.05$ ; Fig. 6C). Amphetamine-treated animals had significantly less NE in the ventral dentate at 20 h withdrawal compared to saline controls (SNK  $P < 0.05$ ; Fig. 6C). When 5-HT levels were analyzed, an effect of withdrawal time ( $F(1,36) = 68.762$ ,  $P < 0.001$ ) was detected but there was no effect of treatment ( $F(1,36) = 2.184$ ,  $P > 0.05$ ) nor was there a significant interaction ( $F(1,36) = 1.990$ ,  $P > 0.05$ ; Fig. 6D). *Post-hoc* tests revealed that amphetamine-treated animals had significantly less 5-HT at 20 h compared to saline controls (SNK  $P < 0.05$ ; Fig. 6D) and both treatment groups had lower 5-HT levels following four weeks withdrawal compared to 20 h (SNK  $P < 0.001$ ; Fig. 6D).

### 3.3. Amphetamine withdrawal reduces survival of newly generated neurons in the dentate gyrus

To determine the impact of chronic amphetamine treatment and withdrawal on progenitor cell proliferation, the number of BrdU-positive cells in the dorsal and ventral subgranular zone (SGZ) of the dentate gyrus were quantified 20 h after the final amphetamine/saline injection (Fig. 7A). No co-labeling of BrdU with the mature neuronal marker NeuN was detected in the double-labeled tissue collected 2 h after the last BrdU injection. Compared to control animals, amphetamine-treated animals did not have significantly different levels of progenitor cell proliferation in the dorsal ( $F(1,14) = 0.610$ ,  $P > 0.05$ ; Fig. 7B) or ventral ( $F(1,14) = 0.129$ ,  $P > 0.05$ ; Fig. 7C) SGZ of the dentate gyrus. Levels of total number of BrdU-labeled cells were similar to previously published reports (Banar et al., 2006; Ueda et al., 2005). The greater level of progenitor cell proliferation observed in the dorsal (Fig. 7B) compared to the ventral (Fig. 7C) dentate gyrus is also consistent with previous results (Ferland et al., 2002; Dawirs et al., 1998).

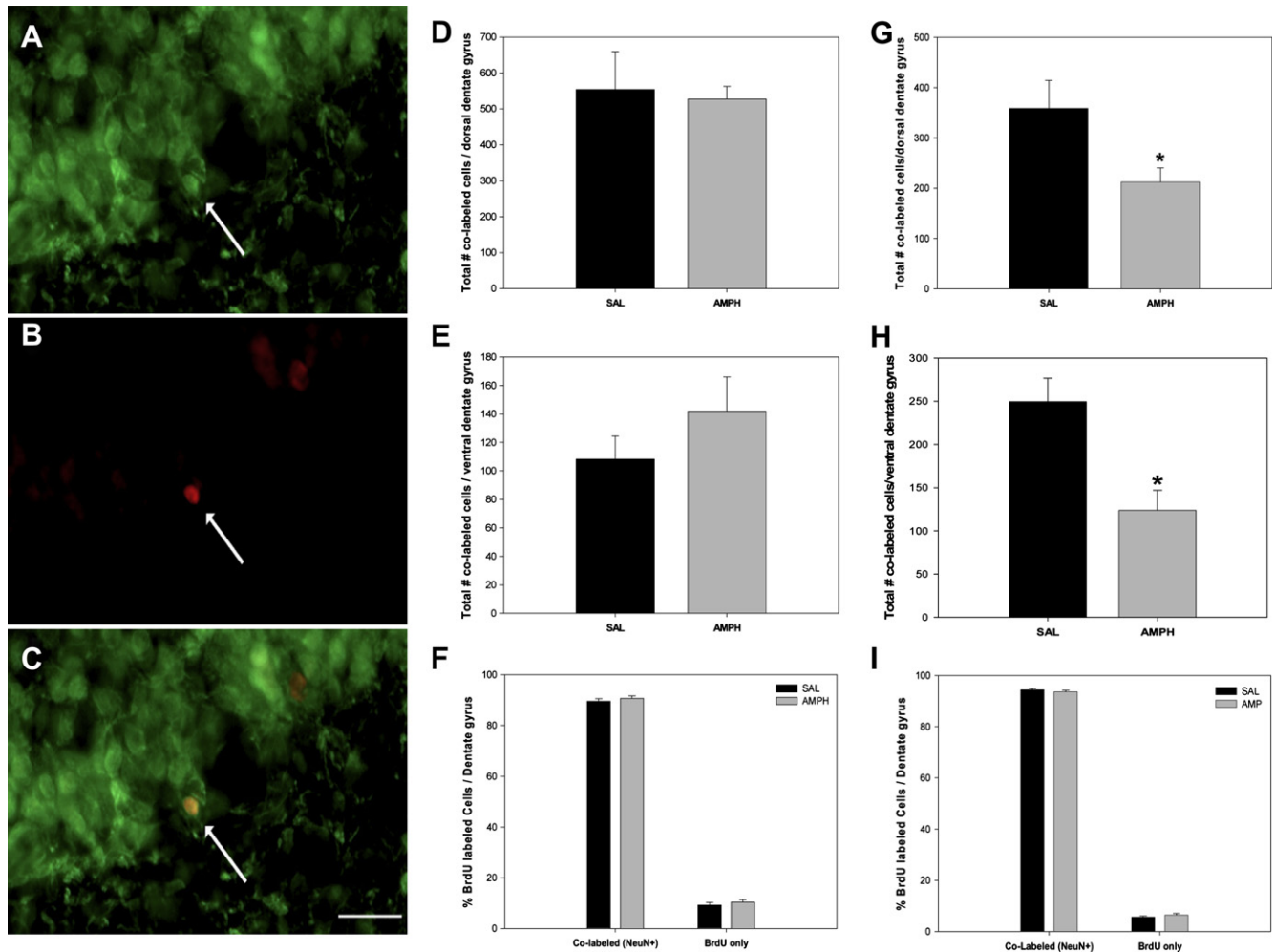
When rats were allowed to survive 4 weeks after BrdU injections, co-labeling of cells with BrdU and NeuN was apparent in the dentate gyrus, indicating the presence of newly generated mature neurons or neurogenesis (Fig. 8A–C). Amphetamine treatment had no effect on neurogenesis (co-labeled BrdU + NeuN cells 4 weeks after BrdU injections) in the dorsal ( $F(1,12) = 2.532$ ,  $P > 0.05$ ; Fig. 8D) or ventral ( $F(1,12) = 0.513$ ,  $P > 0.05$ ; Fig. 8E) dentate gyrus when amphetamine treatment began two weeks following BrdU labeling of cells (Fig. 1B). However, at four weeks following amphetamine treatment, amphetamine-treated animals had significantly decreased neurogenesis compared with saline-treated animals in both the dorsal ( $F(1,14) = 5.510$ ,  $P < 0.05$ ; Fig. 8G) and ventral ( $F(1,14) = 11.319$ ,  $P < 0.05$ ; Fig. 8H) dentate gyrus. The percentage of NeuN + BrdU-labeled cells ( $F(1,16) = 0.612$ ,  $P > 0.05$ ;  $F(1,14) = 1.144$ ,  $P > 0.05$ ) and singly labeled cells (NeuN-,  $F(1,16) = 0.612$ ,  $P > 0.05$ ;  $F(1,14) = 1.144$ ,  $P > 0.05$ ) did not differ between groups 20 h after amphetamine treatment or after four weeks of withdrawal, respectively (Fig. 8F, I), suggesting that chronic amphetamine did not affect differentiation. The singly labeled cells most likely represent astrocytes (Kempermann et al., 1998, 2003) as gliogenesis also takes place in the dentate gyrus.

Apoptosis was as measured by number of TUNEL-positive cells in the SGZ of the dentate gyrus. A large portion of TUNEL-positive cells did not express NeuN (Fig. 9A), possibly due to DNA breakdown during the final stages of apoptosis which would eliminate NeuN staining due to a reduction in protein synthesis, as suggested by Biebl et al. (2000). The number of TUNEL-positive cells were similar in both amphetamine- and saline-treated groups in the dorsal (Fig. 9B) and ventral (Fig. 9C) SGZ of the dentate gyrus at both 20 h ( $F(1,10) = 1.262$ ,  $P > 0.05$ ;  $F(1,10) = 0.429$ ,  $P > 0.05$ ) and



**Fig. 7.** Assessment of progenitor cell proliferation in the rat dentate gyrus. (A) Photomicrograph depicting a BrdU-labeled cell (red) in the subgranular zone (SGZ) of the dentate gyrus 2 h after last BrdU injection. The granule cell layer of the dentate gyrus (DG) is labeled with the mature neuronal marker NeuN (green). Chronic amphetamine treatment did not alter progenitor cell proliferation, as assessed by BrdU-labeled cell counts 20 h following last amphetamine treatment in the dorsal (B) or ventral (C) dentate gyrus. Data shown as mean  $\pm$  S.E.M.  $n = 8$  per group. Scale bar = 10  $\mu$ m.

four weeks post-treatment ( $F(1,10) = 0.001$ ,  $P > 0.05$ ;  $F(1,10) = 0.0769$ ,  $P > 0.05$ ), suggesting that neither daily exposure to amphetamine or amphetamine withdrawal altered the level of apoptosis in the dentate gyrus at these time points.



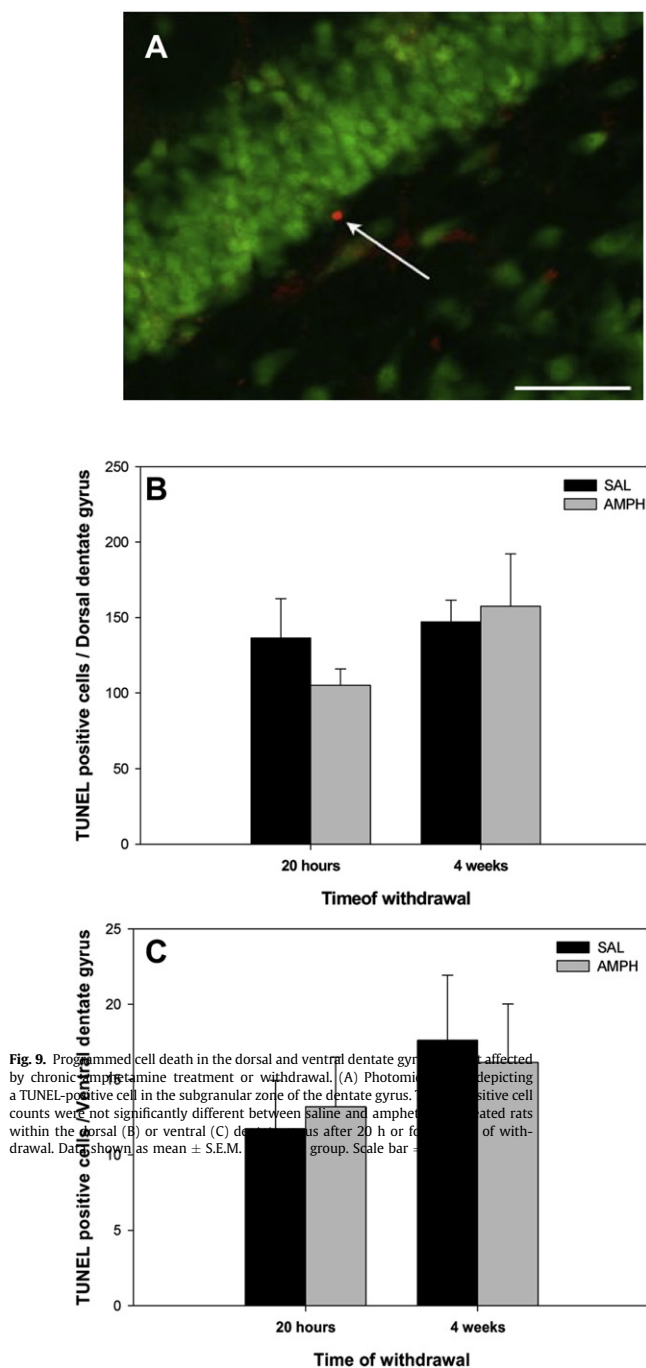
**Fig. 8.** Assessment of neurogenesis in the rat dentate gyrus. BrdU-labeled cells in amphetamine and saline-treated rats were examined for co-localization with NeuN, by confocal microscopy, as a marker of newly generated mature neurons. (A) Photomicrograph depicting a cell positive for the neuronal marker NeuN (green). (B) BrdU (red) positive cell. (C) Co-labeled cell (NeuN + BrdU). (D–E) Amphetamine-treated rats exhibited similar levels of co-labeled (BrdU + NeuN) cells when treatment followed BrdU incorporation (G, H) Amphetamine-treated animals had fewer co-labeled cells in the dorsal and ventral dentate gyrus at four weeks withdrawal. (F, I) For both amphetamine and saline-treated rats, the majority of the BrdU-positive cells were co-localized with NeuN suggesting that amphetamine treatment and withdrawal did not alter differentiation of cells. Data shown as mean  $\pm$  S.E.M.  $n = 8$  per group. \* represents significant differences from the saline-treated group.

#### 4. Discussion

The major findings of this study are that long term withdrawal from chronic amphetamine increased anxiety behavior and reduced neurogenesis in the dentate gyrus. The reduction in neurogenesis was independent of alterations to progenitor cell proliferation measured 4 weeks earlier. Also, cells undergoing later stages of neurogenesis were unaltered by amphetamine treatment in the absence of withdrawal.

Interestingly, amphetamine withdrawal-induced decreases in neurogenesis were observed in the dorsal dentate gyrus, which is important for spatial learning and memory, as well as the ventral dentate gyrus, which has a greater role in fear-related and anxiety behavior (Moser and Moser, 1998; Bannerman et al., 2002, 2003; Kjelstrup et al., 2002). This is unlike methylphenidate, which appears to affect newly generated neurons only in the ventral dentate gyrus (Lagace et al., 2006). Our findings, combined with previous reports, suggest that psychostimulant exposure and withdrawal are associated with alterations to cognitive and emotive behaviors associated with dorsal and ventral hippocampal function (Mandillo et al., 2003; Castner et al., 2005; Wood and Anagnostaras, 2009).

Previously, experimentally reduced neurogenesis in the dentate gyrus has been associated with heightened anxiety behavior in transgenic mouse models (Revest et al., 2009; Earnheart et al., 2007; Bergami et al., 2008; Crupi et al., 2010). We demonstrate for the first time that anxiety states are elevated during protracted withdrawal at the same time point as reduced hippocampal neurogenesis. However, using the exact methodology as described here, heightened anxiety-like behaviors have been reported within 24 h of the final amphetamine treatment (Vuong et al., 2010), a time point where the current study failed to observe a reduction in hippocampal neurogenesis. Additionally, other experimental evidence suggests impaired neurogenesis alone is insufficient to have an impact on emotional behavior. For example, reductions of neurogenesis induced by x-irradiation did not affect behavior in the novelty suppressed feeding latency test, forced swim test, grooming test and open field (Santarelli et al., 2003; Surget et al., 2008; David et al., 2009). However, most experiments investigating the effect of reduced neurogenesis on emotional behavior are in mice. Snyder et al. (2009a) have demonstrated species differences in the process of neurogenesis, with newly generated neurons having a greater role in fear



**Fig. 9.** Programmed cell death in the dorsal and ventral dentate gyrus affected by chronic amphetamine treatment or withdrawal. (A) Photomicrograph depicting a TUNEL-positive cell in the subgranular zone of the dentate gyrus. (B) TUNEL-positive cell counts were not significantly different between saline and amphetamine-treated rats within the dorsal (B) or ventral (C) dentate gyrus after 20 h or 4 weeks of withdrawal. Data shown as mean  $\pm$  S.E.M.

neurogenesis does not appear to be related to the onset or expression of heightened anxiety states during early withdrawal, but reduced dentate gyrus neurogenesis may represent an important underlying mechanism for the maintenance of withdrawal symptoms during protracted withdrawal. Future studies should examine whether a direct relationship exists between amphetamine withdrawal-induced reductions in neurogenesis in the dorsal or ventral dentate gyrus and cognitive or affective deficits.

The finding that reduction of neurogenesis occurred during amphetamine withdrawal without an apparent effect of treatment and/or withdrawal on differentiation of newly generated cells, suggests that a greater number of newly generated neurons underwent cell death during maturation. Therefore, amphetamine withdrawal may affect the post-mitotic phase of neurogenesis, which is a heavily regulated stage of neurogenesis (Tashiro et al., 2007; Epp et al., 2007). Most new neurons undergo cell death during this phase and surviving neurons integrate into the existing hippocampal circuitry (Dayer et al., 2003; Gould et al., 1991; Hastings and Gould, 1999; Ge et al., 2006). Although changes in apoptosis have been correlated with changes in progenitor cell proliferation or neurogenesis (Heine et al., 2004; Kuhn et al., 2005; Sun et al., 2004) we found no alteration in cell turnover at either time point. Future measures of apoptosis within the integration stage of neurogenesis (2–3 weeks from BrdU injection) during amphetamine withdrawal may be valuable in elucidating the time point when new neurons are reduced below control levels.

The possibility that amphetamine withdrawal has a negative impact on maturing stages of neurogenesis is in contrast to Noonan et al. (2008), who found that cocaine self administration resulted in a greater number of immature (DCX expressing) neurons in the posterior hippocampus regardless of whether withdrawal occurred or administration continued. This raises the possibility that there may be long term adaptations to the neurogenic process in response to drugs of abuse other than an impact on discrete populations of newly generated cells. The present study measured the effects of amphetamine on proliferation and neurogenesis on a single population of cells following treatment using the S-phase marker BrdU. Additional studies are needed to determine the effects of amphetamine treatment and withdrawal on proliferating cells in other phases of the cell cycle and immature neurons in the dentate gyrus.

Alterations to progenitor cell proliferation or neurogenesis were not observed 20 h following amphetamine treatment, but NE and 5-HT were reduced at this same time point specifically in the dentate gyrus of amphetamine-treated rats. Previous studies have shown that NE re-uptake inhibitors and  $\alpha_1$ -adrenergic receptor agonists enhance progenitor cell proliferation (Malberg et al., 2000; Hiramoto et al., 2006) and noradrenergic lesions decrease progenitor cell proliferation (Kulkarni et al., 2002). Similarly, serotonergic re-uptake inhibitors and 5-HT<sub>1A</sub> receptor agonists enhance progenitor cell proliferation (Malberg et al., 2000; Santarelli et al., 2003) and serotonergic lesions decrease progenitor cell proliferation (Brezun and Daszuta, 1999). However, our results suggest that amphetamine-induced reductions of monoamine concentrations in the dentate gyrus do not appear related to progenitor cell proliferation in this region.

The reduced monoamine concentrations in the dentate gyrus at 20 h of amphetamine withdrawal may instead relate to decreased neurogenesis as revealed at 4 weeks withdrawal. Increased noradrenergic activity is associated with increased survival of newly generated neurons in the dentate gyrus (Rizk et al., 2006) and NE regulates brain-derived neurotrophic factor (BDNF) expression and signaling (Garcia et al., 2003; Chen et al., 2007; Chen and Russo-Neustadt, 2009). Therefore, the initial reduction

memory in rats than mice. Conversely, suppression of the generation of new neurons in rats using methylazoxymethanol acetate (MAM) for 14 days did not affect contextual fear conditioning or exploration in the elevated plus maze (Shors et al., 2002). The disparity in these studies may be due to method of reduction (x-irradiation, MAM, transgenic mechanisms), species, behavioral test or time of testing in relation to reduction/maturation of new neurons. In regards to the current study, reduced



in dentate gyrus NE concentration by chronic amphetamine as observed in the current study may decrease neurotrophic support in the dentate gyrus and contribute to reduced survival of immature neurons (Sairanen et al., 2005) as revealed by reduced number of newly matured neurons at 4 weeks withdrawal. Related, the amphetamine derivative MDMA produces a time dependent decrease in BDNF expression in the hippocampus (Martinez-Turrillas et al., 2006), and amphetamine can alter the expression of the BDNF receptor TrkB (Meredith and Steiner, 2006), which is expressed by immature neurons and is necessary for survival of maturing neurons (Donovan et al., 2008; Bergami et al., 2008). Therefore, future work should explore the role of amphetamine-mediated decreases in dentate monoamines in altering neurotrophic factor levels or signaling in the hippocampus.

Circulating corticosterone is a potent regulator of adult dentate gyrus neurogenesis (Cameron and Gould, 1994), and acute exposure to drugs of abuse activates the hypothalamic-pituitary-adrenal (HPA) axis (Knynch and Eisenberg, 1979; Sinha, 2008). However, we found that chronic amphetamine treatment and withdrawal did not alter basal circulating corticosterone. Our study is one of many that suggest that drugs of abuse alter the process of adult dentate gyrus neurogenesis independent of their effect on corticosterone (Eisch et al., 2000; Eisch and Harburg, 2006; Mandyam et al., 2008). While drug-elicited HPA activity may not affect adult neurogenesis, drug-induced reductions in dentate gyrus neurogenesis may underlie the increased HPA response to stress observed following chronic psychostimulant treatment (Schloesser et al., 2009; Mantsch et al., 2007a,b).

Interestingly, greater 5-HT levels were observed in both the dentate gyrus and CA of the ventral hippocampus 20 h following the last injection (compared to 4 weeks withdrawal) regardless of treatment, or sub-region. This is consistent with previous results showing increased 5-HT concentrations in the ventral hippocampus following repeated stress (Storey et al., 2006). Serotonergic activity in the hippocampus is important for adaptation to chronic stress (Guimaraes et al., 1993; Joca et al., 2003), and higher 5-HT levels in the ventral hippocampus following repeated injections may represent adaptation to this stressor. Animals receiving amphetamine had reduced 5-HT concentration in the dentate gyrus compared to saline-treated rats at this time point, suggesting a reduced stress-related serotonergic adaptation in this sub-region of the ventral hippocampus.

Overall, it is clear that the mechanisms underlying reduced neuronal survival during amphetamine withdrawal requires further examination beyond the monoaminergic analysis presented by this report. However, it is possible that reduced monoamine levels in the dentate gyrus following chronic amphetamine treatment triggers a cascade of events that results in reduced survival of newly generated neurons. Therefore, pharmaceutical or environmental manipulations that increase dentate gyrus neurogenesis such as antidepressants (Malberg et al., 2000), neurotrophins (Li et al., 2008) or exercise (van Praag et al., 1999), may function to reverse the effects of amphetamine withdrawal on dentate gyrus neurogenesis.

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## References

- Altman, J., Das, G.D., 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* 124, 319–335.
- Ambrogini, P., Cuppini, R., Cuppini, C., Ciaroni, S., Cecchini, T., Ferri, P., Sartini, S., Del Grande, P., 2000. Spatial learning affects immature granule cell survival in adult rat dentate gyrus. *Neurosci. Lett.* 286, 21–24.
- Banasr, M., Soumier, A., Hery, M., Mocaer, E., Daszuta, A., 2006. Agomelatine, a new antidepressant, induces regional changes in hippocampal neurogenesis. *Biol. Psychiatry* 59, 1087–1096.
- Bannerman, D.M., Deacon, R.M., Offen, S., Friswell, J., Grubb, M., Rawlins, J.N., 2002. Double dissociation of function within the hippocampus: spatial memory and hyponeophagia. *Behav. Neurosci.* 116, 884–901.
- Bannerman, D.M., Grubb, M., Deacon, R.M., Yee, B.K., Feldon, J., Rawlins, J.N., 2003. Ventral hippocampal lesions affect anxiety but not spatial learning. *Behav. Brain Res.* 139, 197–213.
- Barr, A.M., Markou, A., 2005. Psychostimulant withdrawal as an inducing condition in animal models of depression. *Neurosci. Biobehav. Rev.* 29, 675–706.
- Bergami, M., Rimondini, R., Santi, S., Blum, R., Gotz, M., Canossa, M., 2008. Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15570–15575.
- Berman, S.M., Kuczenski, R., McCracken, J.T., London, E.D., 2009. Potential adverse effects of amphetamine treatment on brain and behavior: a review. *Mol. Psychiatry* 14, 123–142.
- Bertoglio, L.J., Joca, S.R., Guimaraes, F.S., 2006. Further evidence that anxiety and memory are regionally dissociated within the hippocampus. *Behav. Brain Res.* 175, 183–188.
- Biebl, M., Cooper, C.M., Winkler, J., Kuhn, H.G., 2000. Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neurosci. Lett.* 291, 17–20.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Brezun, J.M., Daszuta, A., 1999. Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience* 89, 999–1002.
- Cameron, H.A., Gould, E., 1994. Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. *Neuroscience* 61, 203–209.
- Cameron, H.A., McKay, R., 1998. Stem cells and neurogenesis in the adult brain. *Curr. Opin. Neurobiol.* 8, 677–680.
- Cameron, H.A., McKay, R.D., 2001. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J. Comp. Neurol.* 435, 406–417.
- Castner, S.A., Vosler, P.S., Goldman-Rakic, P.S., 2005. Amphetamine sensitization impairs cognition and reduces dopamine turnover in primate prefrontal cortex. *Biol. Psychiatry* 57, 743–751.
- Chen, M.J., Nguyen, T.V., Pike, C.J., Russo-Neustadt, A.A., 2007. Norepinephrine induces BDNF and activates the PI-3K and MAPK cascades in embryonic hippocampal neurons. *Cell. Signal.* 19, 114–128.
- Chen, M.J., Russo-Neustadt, A.A., 2009. Running exercise-induced up-regulation of hippocampal brain-derived neurotrophic factor is CREB-dependent. *Hippocampus*.
- Cho, K.O., Kim, S.K., Rhee, G.S., Kwack, S.J., Cho, D.H., Sung, K.W., Kim, S.Y., 2007. Chronic 3,4-methylenedioxymethamphetamine treatment suppresses cell proliferation in the adult mouse dentate gyrus. *Eur. J. Pharmacol.* 566, 120–123.
- Coggeshall, R.E., Lekan, H.A., 1996. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J. Comp. Neurol.* 364, 6–15.
- Crupi, R., Cambiaghi, M., Spatz, L., Hen, R., Thorn, M., Friedman, E., Vita, G., Battaglia, F., 2010. Reduced adult neurogenesis and altered emotional behaviors in autoimmune-prone B-cell activating factor transgenic mice. *Biol. Psychiatry* 67, 558–566.
- David, D.J., Samuels, B.A., Rainer, Q., Wang, J.W., Marsteller, D., Mendez, I., Drew, M., Craig, D.A., Guiard, B.P., Guilloux, J.P., Artymyshyn, R.P., Gardier, A.M., Gerald, C., Antonijevic, I.A., Leonardo, E.D., Hen, R., 2009. Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron* 62, 479–493.
- Dawirs, R.R., Hildebrandt, K., Teuchert-Noodt, G., 1998. Adult treatment with haloperidol increases dentate granule cell proliferation in the gerbil hippocampus. *J. Neural. Transm.* 105, 317–327.
- Dayer, A.G., Ford, A.A., Cleaver, K.M., Yassaee, M., Cameron, H.A., 2003. Short-term and long-term survival of new neurons in the rat dentate gyrus. *J. Comp. Neurol.* 460, 563–572.
- Dominguez-Escriba, L., Hernandez-Rabaza, V., Soriano-Navarro, M., Barcia, J.A., Romero, F.J., Garcia-Verdugo, J.M., Canales, J.J., 2006. Chronic cocaine exposure impairs progenitor proliferation but spares survival and maturation of neural precursors in adult rat dentate gyrus. *Eur. J. Neurosci.* 24, 586–594.
- Donovan, M.H., Yamaguchi, M., Eisch, A.J., 2008. Dynamic expression of TrkB receptor protein on proliferating and maturing cells in the adult mouse dentate gyrus. *Hippocampus* 18, 435–439.
- Earnheart, J.C., Schweizer, C., Crestani, F., Iwasato, T., Itoharu, S., Mohler, H., Luscher, B., 2007. GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. *J. Neurosci.* 27, 3845–3854.

- Eisch, A.J., Barrot, M., Schadt, C.A., Self, D.W., Nestler, E.J., 2000. Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 97, 7579–7584.
- Eisch, A.J., Harburg, G.C., 2006. Opiates, psychostimulants, and adult hippocampal neurogenesis: insights for addiction and stem cell biology. *Hippocampus* 16, 271–286.
- Epp, J.R., Spritzer, M.D., Galea, L.A., 2007. Hippocampus-dependent learning promotes survival of new neurons in the dentate gyrus at a specific time during cell maturation. *Neuroscience* 149, 273–285.
- Eriksson, P.S., Perfilieva, E., Björk-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., Gage, F.H., 1998. Neurogenesis in the adult human hippocampus. *Nat. Med.* 4, 1313–1317.
- Ferland, R.J., Gross, R.A., Applegate, C.D., 2002. Increased mitotic activity in the dentate gyrus of the hippocampus of adult C57BL/6J mice exposed to the flurothyl kindling model of epileptogenesis. *Neuroscience* 115, 669–683.
- Forster, G.L., Pringle, R.B., Mouw, N.J., Vuong, S.M., Watt, M.J., Burke, A.R., Lowry, C.A., Summers, C.H., Renner, K.J., 2008. Corticotropin-releasing factor in the dorsal raphe nucleus increases medial prefrontal cortical serotonin via type 2 receptors and median raphe nucleus activity. *Eur. J. Neurosci.* 28, 299–310.
- Fuchs, R.A., Evans, K.A., Ledford, C.C., Parker, M.P., Case, J.M., Mehta, R.H., See, R.E., 2005. The role of the dorsomedial prefrontal cortex, basolateral amygdala, and dorsal hippocampus in contextual reinstatement of cocaine seeking in rats. *Neuropsychopharmacology* 30, 296–309.
- Garcia, C., Chen, M.J., Garza, A.A., Cotman, C.W., Russo-Neustadt, A., 2003. The influence of specific noradrenergic and serotonergic lesions on the expression of hippocampal brain-derived neurotrophic factor transcripts following voluntary physical activity. *Neuroscience* 119, 721–732.
- Ge, S., Goh, E.L., Sailor, K.A., Kitabatake, Y., Ming, G.L., Song, H., 2006. GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature* 439, 589–593.
- Gould, E., Woolley, C.S., McEwen, B.S., 1991. Adrenal steroids regulate postnatal development of the rat dentate gyrus: I. Effects of glucocorticoids on cell death. *J. Comp. Neurol.* 313, 479–485.
- Guillery, R.W., Herrup, K., 1997. Quantification without pontification: choosing a method for counting objects in sectioned tissues. *J. Comp. Neurol.* 386, 2–7.
- Guimaraes, F.S., Del Bel, E.A., Padovan, C.M., Netto, S.M., de Almeida, R.T., 1993. Hippocampal 5-HT receptors and consolidation of stressful memories. *Behav. Brain Res.* 58, 133–139.
- Gundersen, H.J., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Møller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., et al., 1988. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Apmis* 96, 857–881.
- Hastings, N.B., Gould, E., 1999. Rapid extension of axons into the CA3 region by adult-generated granule cells. *J. Comp. Neurol.* 413, 146–154.
- Heine, V.M., Maslam, S., Zareno, J., Joels, M., Lucassen, P.J., 2004. Suppressed proliferation and apoptotic changes in the rat dentate gyrus after acute and chronic stress are reversible. *Eur. J. Neurosci.* 19, 131–144.
- Hernandez-Rabaza, V., Dominguez-Escriba, L., Barcia, J.A., Rosel, J.F., Romero, F.J., Garcia-Verdugo, J.M., Canales, J.J., 2006. Binge administration of 3,4-methylenedioxymethamphetamine (“ecstasy”) impairs the survival of neural precursors in adult rat dentate gyrus. *Neuropharmacology* 51, 967–973.
- Hernandez-Rabaza, V., Hontecillas-Prieto, L., Velazquez-Sanchez, C., Ferragud, A., Perez-Villaba, A., Arcusa, A., Barcia, J.A., Trejo, J.L., Canales, J.J., 2008. The hippocampal dentate gyrus is essential for generating contextual memories of fear and drug-induced reward. *Neurobiol. Learn. Mem.* 90, 553–559.
- Hiramoto, T., Ihara, Y., Watanabe, Y., 2006. Alpha-1 adrenergic receptors stimulation induces the proliferation of neural progenitor cells in vitro. *Neurosci. Lett.* 408, 25–28.
- Holmes, J.C., Rutledge, C.O., 1976. Effects of the d- and l-isomers of amphetamine on uptake, release and catabolism of norepinephrine, dopamine and 5-hydroxytryptamine in several regions of rat brain. *Biochem. Pharmacol.* 25, 447–451.
- Huang, G.J., Herbert, J., 2005. The role of 5-HT<sub>1A</sub> receptors in the proliferation and survival of progenitor cells in the dentate gyrus of the adult hippocampus and their regulation by corticoids. *Neuroscience* 135, 803–813.
- Huang, G.J., Herbert, J., 2006. Stimulation of neurogenesis in the hippocampus of the adult rat by fluoxetine requires rhythmic change in corticosterone. *Biol. Psychiatry* 59, 619–624.
- Joca, S.R., Padovan, C.M., Guimaraes, F.S., 2003. Activation of post-synaptic 5-HT (1A) receptors in the dorsal hippocampus prevents learned helplessness development. *Brain Res.* 978, 177–184.
- Kempermann, G., Kuhn, H.G., Gage, F.H., 1998. Experience-induced neurogenesis in the senescent dentate gyrus. *J. Neurosci.* 18, 3206–3212.
- Kempermann, G., Gast, D., Kronenberg, G., Yamaguchi, M., Gage, F.H., 2003. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* 130, 391–399.
- Kitanaka, J., Kitanaka, N., Takemura, M., 2008. Neurochemical consequences of dysphoric state during amphetamine withdrawal in animal models: a review. *Neurochem. Res.* 33, 204–219.
- Kjelstrup, K.G., Tuvnes, F.A., Steffenach, H.A., Murison, R., Moser, E.I., Moser, M.B., 2002. Reduced fear expression after lesions of the ventral hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10825–10830.
- Knych, E.T., Eisenberg, R.M., 1979. Effect of amphetamine on plasma corticosterone in the conscious rat. *Neuroendocrinology* 29, 110–118.
- Koob, G.F., Ahmed, S.H., Boutrel, B., Chen, S.A., Kenny, P.J., Markou, A., O'Dell, L.E., Parsons, L.H., Sanna, P.P., 2004. Neurobiological mechanisms in the transition from drug use to drug dependence. *Neurosci. Biobehav. Rev.* 27, 739–749.
- Kuczenski, R., Segal, D.S., 1997. Effects of methylphenidate on extracellular dopamine, serotonin, and norepinephrine: comparison with amphetamine. *J. Neurochem.* 68, 2032–2037.
- Kuhn, H.G., Biebl, M., Wilhelm, D., Li, M., Friedlander, R.M., Winkler, J., 2005. Increased generation of granule cells in adult Bcl-2-overexpressing mice: a role for cell death during continued hippocampal neurogenesis. *Eur. J. Neurosci.* 22, 1907–1915.
- Kulkarni, V.A., Jha, S., Vaidya, V.A., 2002. Depletion of norepinephrine decreases the proliferation, but does not influence the survival and differentiation, of granule cell progenitors in the adult rat hippocampus. *Eur. J. Neurosci.* 16, 2008–2012.
- Lagace, D.C., Yee, J.K., Bolanos, C.A., Eisch, A.J., 2006. Juvenile administration of methylphenidate attenuates adult hippocampal neurogenesis. *Biol. Psychiatry* 60, 1121–1130.
- Li, Y., Luikart, B.W., Birnbaum, S., Chen, J., Kwon, C.H., Kernie, S.G., Bassel-Duby, R., Parada, L.F., 2008. TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressant treatment. *Neuron* 59, 399–412.
- Ling, T.J., Forster, G.L., Watt, M.J., Korzan, W.J., Renner, K.J., Summers, C.H., 2009. Social status differentiates rapid neuroendocrine responses to restraint stress. *Physiol. Behav.* 96, 218–232.
- Malberg, J.E., Duman, R.S., 2003. Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology* 28, 1562–1571.
- Malberg, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S., 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J. Neurosci.* 20, 9104–9110.
- Mandillo, S., Rinaldi, A., Oliverio, A., Mele, A., 2003. Repeated administration of phencyclidine, amphetamine and MK-801 selectively impairs spatial learning in mice: a possible model of psychotomimetic drug-induced cognitive deficits. *Behav. Pharmacol.* 14, 533–544.
- Mandyam, C.D., Wee, S., Crawford, E.F., Eisch, A.J., Richardson, H.N., Koob, G.F., 2008. Varied access to intravenous methamphetamine self-administration differentially alters adult hippocampal neurogenesis. *Biol. Psychiatry*.
- Mantsch, J.R., Cullinan, W.E., Tang, L.C., Baker, D.A., Katz, E.S., Hoks, M.A., Ziegler, D.R., 2007a. Daily cocaine self-administration under long-access conditions augments restraint-induced increases in plasma corticosterone and impairs glucocorticoid receptor-mediated negative feedback in rats. *Brain Res.* 1167, 101–111.
- Mantsch, J.R., Taves, S., Khan, T., Katz, E.S., Sajan, T., Tang, L.C., Cullinan, W.E., Ziegler, D.R., 2007b. Restraint-induced corticosterone secretion and hypothalamic CRH mRNA expression are augmented during acute withdrawal from chronic cocaine administration. *Neurosci. Lett.* 415, 269–273.
- Mao, L., Wang, J.Q., 2001. Gliogenesis in the striatum of the adult rat: alteration in neural progenitor population after psychostimulant exposure. *Brain Res. Dev. Brain Res.* 130, 41–51.
- Martinez-Turrillas, R., Moyano, S., Del Rio, J., Frechilla, D., 2006. Differential effects of 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) on BDNF mRNA expression in rat frontal cortex and hippocampus. *Neurosci. Lett.* 402, 126–130.
- McHugh, S.B., Deacon, R.M., Rawlins, J.N., Bannerman, D.M., 2004. Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav. Neurosci.* 118, 63–78.
- Meredith, G.E., Steiner, H., 2006. Amphetamine increases tyrosine kinase-B receptor expression in the dorsal striatum. *Neuroreport* 17, 75–78.
- Moser, M.B., Moser, E.I., 1998. Functional differentiation in the hippocampus. *Hippocampus* 8, 608–619.
- Mullen, R.J., Buck, C.R., Smith, A.M., 1992. NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116, 201–211.
- Noonan, M.A., Choi, K.H., Self, D.W., Eisch, A.J., 2008. Withdrawal from cocaine self-administration normalizes deficits in proliferation and enhances maturity of adult-generated hippocampal neurons. *J. Neurosci.* 28, 2516–2526.
- Noonan, M.A., Bulin, S.E., Fuller, D.C., Eisch, A.J., 2010. Reduction of adult hippocampal neurogenesis confers vulnerability in an animal model of cocaine addiction. *J. Neurosci.* 30, 304–315.
- Paxinos, G., Watson, C., 1997. *The Rat Brain in Stereotaxic Coordinates*, third ed. Academic Press, New York.
- Pelloux, Y., Costentin, J., Duterte-Boucher, D., 2009. Anxiety increases the place conditioning induced by cocaine in rats. *Behav. Brain Res.* 197, 311–316.
- van Praag, H., Christie, B.R., Sejnowski, T.J., Gage, F.H., 1999. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc. Natl. Acad. Sci. U.S.A.* 96, 13427–13431.
- Renner, K., Luine, V., 1986. Analysis of temporal and dose-dependent effects of estrogen on monoamines in brain nuclei. *Brain Res.* 366, 64–71.
- Revest, J.M., Dupret, D., Koehl, M., Funk-Reiter, C., Grosjean, N., Piazza, P.V., Abrous, D.N., 2009. Adult hippocampal neurogenesis is involved in anxiety-related behaviors. *Mol. Psychiatry*.
- Rizk, P., Salazar, J., Raisman-Vozari, R., Marien, M., Ruberg, M., Colpaert, F., Debeir, T., 2006. The alpha2-adrenoceptor antagonist dexefaroxan enhances hippocampal neurogenesis by increasing the survival and differentiation of new granule cells. *Neuropsychopharmacology* 31, 1146–1157.
- Rogers, J.L., See, R.E., 2007. Selective inactivation of the ventral hippocampus attenuates cue-induced and cocaine-prime reinstatement of drug-seeking in rats. *Neurobiol. Learn. Mem.* 87, 688–692.

- Sairanen, M., Lucas, G., Ernfors, P., Castren, M., Castren, E., 2005. Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. *J. Neurosci.* 25, 1089–1094.
- Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301, 805–809.
- Saxe, M.D., Battaglia, F., Wang, J.W., Malleret, G., David, D.J., Monckton, J.E., Garcia, A.D., Sofroniew, M.V., Kandel, E.R., Santarelli, L., Hen, R., Drew, M.R., 2006. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17501–17506.
- Schloesser, R.J., Manji, H.K., Martinowich, K., 2009. Suppression of adult neurogenesis leads to an increased hypothalamo-pituitary-adrenal axis response. *Neuroreport* 20, 553–557.
- Shors, T.J., Townsend, D.A., Zhao, M., Kozorovitskiy, Y., Gould, E., 2002. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus* 12, 578–584.
- Sinha, R., 2008. Chronic stress, drug use, and vulnerability to addiction. *Ann. NY Acad. Sci.* 1141, 105–130.
- Snyder, J.S., Choe, J.S., Clifford, M.A., Jeurling, S.I., Hurley, P., Brown, A., Kamhi, J.F., Cameron, H.A., 2009a. Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. *J. Neurosci.* 29, 14484–14495.
- Snyder, J.S., Radik, R., Wojtowicz, J.M., Cameron, H.A., 2009b. Anatomical gradients of adult neurogenesis and activity: young neurons in the ventral dentate gyrus are activated by water maze training. *Hippocampus* 19, 360–370.
- Storey, J.D., Robertson, D.A., Beattie, J.E., Reid, I.C., Mitchell, S.N., Balfour, D.J., 2006. Behavioural and neurochemical responses evoked by repeated exposure to an elevated open platform. *Behav. Brain Res.* 166, 220–229.
- Sun, W., Winseck, A., Vinsant, S., Park, O.H., Kim, H., Oppenheim, R.W., 2004. Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene Bax. *J. Neurosci.* 24, 11205–11213.
- Surget, A., Saxe, M., Leman, S., Ibarguen-Vargas, Y., Chalon, S., Griebel, G., Hen, R., Belzung, C., 2008. Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. *Biol. Psychiatry* 64, 293–301.
- Taepavarapruk, P., Phillips, A.G., 2003. Neurochemical correlates of relapse to d- amphetamine self-administration by rats induced by stimulation of the ventral subiculum. *Psychopharmacology (Berl)* 168, 99–108.
- Tashiro, A., Makino, H., Gage, F.H., 2007. Experience-specific functional modification of the dentate gyrus through adult neurogenesis: a critical period during an immature stage. *J. Neurosci.* 27, 3252–3259.
- Taupin, P., 2007. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. *Brain Res. Rev.* 53, 198–214.
- Teter, C.J., McCabe, S.E., LaGrange, K., Cranford, J.A., Boyd, C.J., 2006. Illicit use of specific prescription stimulants among college students: prevalence, motives, and routes of administration. *Pharmacotherapy* 26, 1501–1510.
- Teuchert-Noodt, G., Dawirs, R.R., Hildebrandt, K., 2000. Adult treatment with methamphetamine transiently decreases dentate granule cell proliferation in the gerbil hippocampus. *J. Neural. Transm.* 107, 133–143.
- Ueda, S., Sakakibara, S., Yoshimoto, K., 2005. Effect of long-lasting serotonin depletion on environmental enrichment-induced neurogenesis in adult rat hippocampus and spatial learning. *Neuroscience* 135, 395–402.
- Vuong, S.M., Oliver, H.A., Scholl, J.L., Oliver, K.M., Forster, G.L., 2010. Increased anxiety-like behavior of rats during amphetamine withdrawal is reversed by CRF(2) receptor antagonism. *Behav. Brain Res.* 208, 278–281.
- Watt, M.J., Forster, G.L., Korzan, W.J., Renner, K.J., Summers, C.H., 2007. Rapid neuroendocrine responses evoked at the onset of social challenge. *Physiol. Behav.* 90, 567–575.
- Wong, E.Y., Herbert, J., 2006. Raised circulating corticosterone inhibits neuronal differentiation of progenitor cells in the adult hippocampus. *Neuroscience* 137, 83–92.
- Wood, S.C., Anagnostaras, S.G., 2009. Memory and psychostimulants: modulation of Pavlovian fear conditioning by amphetamine in C57BL/6 mice. *Psychopharmacology (Berl)* 202, 197–206.
- Yamaguchi, M., Suzuki, T., Seki, T., Namba, T., Juan, R., Arai, H., Hori, T., Asada, T., 2004. Repetitive cocaine administration decreases neurogenesis in adult rat hippocampus. *Ann. N. Y. Acad. Sci.* 1025, 351–362.