EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 31, pp. 79–89, 2010 doi:10.1111/j.1460-9568.2009.07045.x

NEUROSYSTEMS

Impact of cocaine on adult hippocampal neurogenesis in an animal model of differential propensity to drug abuse

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Keywords: bLR/bHR rats, cell genesis, dentate gyrus, hippocampus, psychomotor sensitization

Abstract

Hippocampal plasticity (e.g. neurogenesis) likely plays an important role in maintaining addictive behavior and/or relapse. This study assessed whether rats with differential propensity to drug-seeking behavior, bred Low-Responders (bLR) and bred High-Responders (bHR) to novelty, show differential neurogenesis regulation after cocaine exposure. Using specific immunological markers, we labeled distinct populations of adult stem cells in the dentate gyrus at different time-points of the cocaine sensitization process; Ki-67 for newly born cells, NeuroD for cells born partway, and 5-bromo-2′-deoxyuridine for older cells born prior to sensitization. Results show that: (i) bHRs exhibited greater psychomotor response to cocaine than bLRs; (ii) acute cocaine did not alter cell proliferation in bLR/bHR rats; (iii) chronic cocaine decreased cell proliferation in bLRs only, which became amplified through the course of abstinence; (iv) neither chronic cocaine nor cocaine abstinence affected the survival of immature neurons in either phenotype; (v) cocaine abstinence decreased survival of mature neurons in bHRs only, an effect that paralleled the greater psychomotor response to cocaine; and (vi) cocaine treatment did not affect the ratio of neurons to glia in bLR/bHR rats as most cells differentiated into neurons in both lines. Thus, cocaine exerts distinct effects on neurogenesis in bLR vs. bHR rats, with a decrease in the birth of new progenitor cells in bLRs and a suppression of the survival of new neurons in bHRs, which likely leads to an earlier decrease in formation of new connections. This latter effect in bHRs could contribute to their enhanced degree of cocaine-induced psychomotor behavioral sensitization.

Introduction

Cocaine is a highly abused drug that poses an enormous health, social and economic toll on society. A major challenge of addiction research is to identify factors that predispose individuals' addiction in order to develop novel and better-tailored treatment options. Our laboratory has selectively bred Sprague–Dawley rats for high (High-Responder, bHR) and low (Low-Responder, bLR) novelty-seeking behavior, a trait that predicts a variety of differences in emotional reactivity (Perez *et al.*, 2009), including propensity to acquire cocaine self-administration (Piazza *et al.*, 1989; Piazza & Le Moal, 1996; Davis *et al.*, 2008) and behavioral sensitization (Hooks *et al.*, 1991, 1992).

Drugs of abuse produce neuroadaptations in particular brain areas implicated in reward and learning that likely underlie their long-lasting effects (Robinson & Kolb, 2004; Kalivas, 2007). It is well known that drugs of abuse such as cocaine are rewarding by virtue of their ability to increase dopamine concentrations in ventral tegmental area projections, such as the nucleus accumbens (NAc) (Gold *et al.*, 1989). However, beyond modulating specific transmitter systems, initial propensity to drug abuse (i.e. bLR vs. bHR rats) may have more to do with environmental reactivity and stress responsiveness at other anatomical levels. In particular, the hippocampus (HC), which is highly interconnected with limbic structures that regulate mood and cognition, can influence the learning component of addiction and is associated with many psychostimulant effects, including contextual conditioning (Shen *et al.*, 2006) and behavioral sensitization (Lodge & Grace, 2008). Indeed, neonatal lesions of the ventral HC produced enhanced cocaine sensitization in adulthood (Chambers & Taylor, 2004), providing a direct association between sensitization and altered hippocampal (HCal) circuits. Moreover, some of the known bLR/bHR neuroadaptations that may contribute to their drug-taking differences occur in the HC (Maccari *et al.*, 1991; Kabbaj *et al.*, 2000; Turner *et al.*, 2008).

Adult neurogenesis represents one form of HCal plasticity that may contribute to the brain's ability to process, adapt and respond to stimuli (e.g. brain dysfunction in drug addiction). Adult HCal neurogenesis is a highly dynamic process where mature functionally integrated granular neurons are generated from progenitor cells in the dentate gyrus (DG) (Ming & Song, 2005). Each stage of neurogenesis is demarcated by distinct molecular markers (von Bohlen Und Halbach, 2007) and is regulated by a variety of factors, including adverse environmental conditions (Schmidt & Duman, 2007) and exposure to addictive substances (Eisch & Harburg, 2006). Particularly, experimenter-administered cocaine impaired cell proliferation in adult rat HC (Yamaguchi *et al.*, 2004) without altering the survival and growth

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Received 16 June 2009, revised 29 October 2009, accepted 6 November 2009

of immature cells (Dominguez-Escriba *et al.*, 2006), suggesting that cocaine differentially modulates cells at different stages of neurogenesis. Thus, altered neurogenesis represents one drug-regulated form of neuroplasticity that may contribute to the maintenance of addictive behavior and/or relapse.

Against this background, the present study attempts to understand how experimenter-administered cocaine may alter the cellular composition of the HC in an animal model of differential propensity to drug abuse (bLR/bHR rats), and to ascertain which stages of adult HCal neurogenesis (e.g. cell proliferation, survival, differentiation) may be regulated by cocaine within each phenotype.

Materials and methods

Experimental design, animals and drug treatment

The first part of the study (Study I) characterized the bLR/bHR animal model of differential propensity to drug abuse to examine their behavioral responses to novelty (i.e. what defines the phenotype) and cocaine (i.e. different psychomotor response to cocaine). Once the animal model was well defined, the second part of the study (Study II) assessed the impact of cocaine on adult HCal neurogenesis in bLR/bHR rats. The experimental design, animals used and drug treatments are described in detail below.

Study I: characterization of bLR and bHR rats

bLR/bHR differences in locomotor response to novelty

For the last several years our laboratory has selectively bred rats (bLR and bHR lines) on the basis of locomotor reactivity to a novel environment, demonstrating reliable differences across multiple behavioral and neurobiological dimensions. A description of the breeding strategy and initial behavioral characterization of the bHR and bLR lines was previously published (Stead *et al.*, 2006) and has been extended in the present study (see Results; Fig. 1).

Each generation of male rats was screened for locomotor activity at about 60 days old (see Stead *et al.*, 2006). Note that Fig. 1A represents bLR and bHR rats' inborn basal locomotor differences from generations F1–F20, although the present studies only used rats from generations F15 and F20. Horizontal and rearing activity was monitored by computer in 5-min intervals over 60 min by placing animals into clear acrylic 43 × 21.5 × 25.5 cm (high) cages equipped with infrared photocell emitters mounted 2.3 and 6.5 cm above the grid floor in a different room from where the animals had been housed. The locomotion-testing rig and motion-recording software were created in-house at the University of Michigan. All testing was performed between 08:00 and 11:30 h. Final locomotion scores for each rat were calculated by adding the total number of horizontal and rearing movements. bHR animals whose scores fell one standard

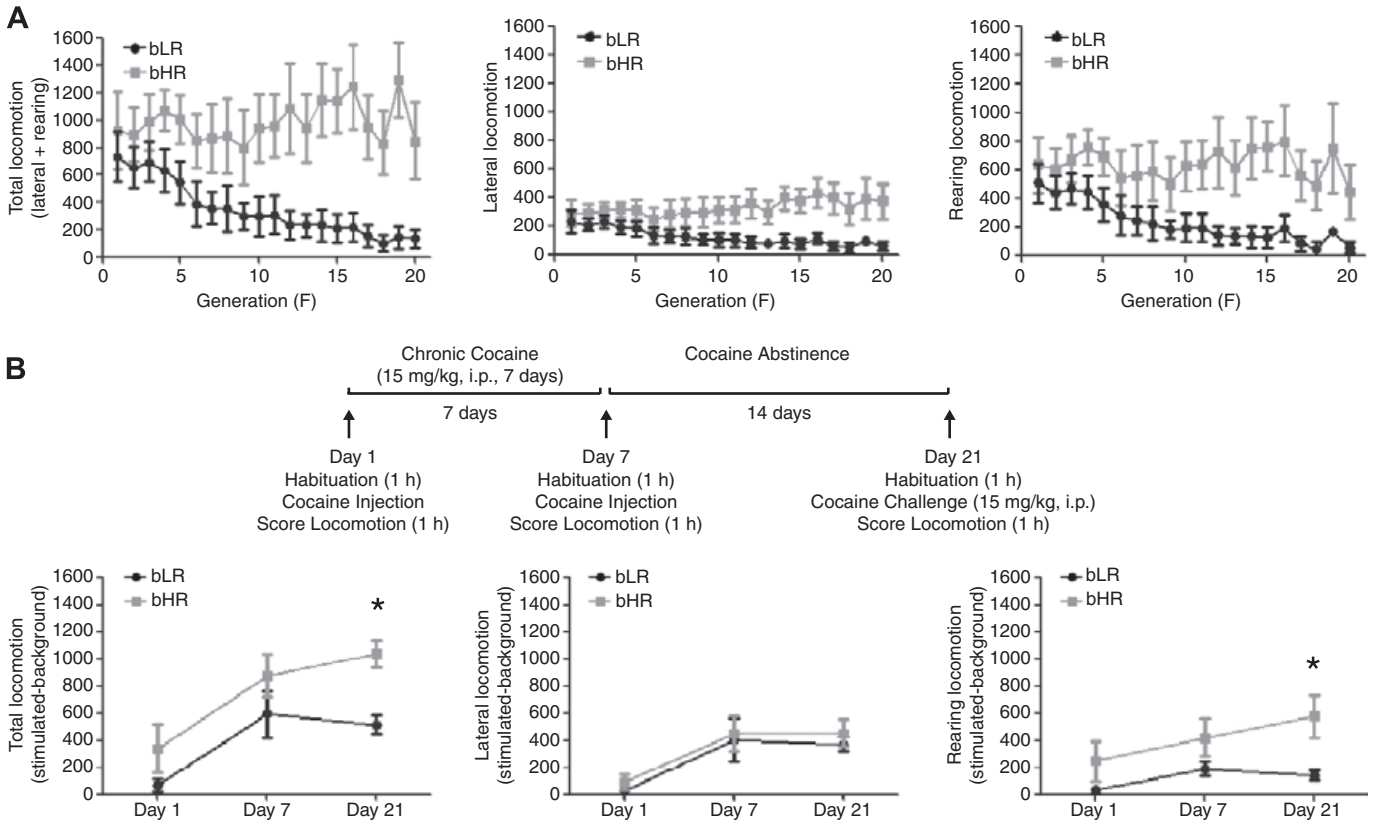

FIG. 1. Characterizing the bred Low-Responder (bLR)/bred High-Responder (bHR) rat phenotype: selective breeding for novelty-seeking trait. (A) bLR/bHR differences in locomotor response to novelty across generations (F1–F20). Differences in mean locomotion (total activity, which is the sum of lateral movement and rearing, lateral movement, rearing) between bLR and bHR animals are shown for 20 generations of selective breeding (F1–F20). Mean locomotion scores diverge dramatically between the two lines with increasing rounds of selection. All data are mean ± standard deviation. (B) bLR/bHR differences in psychomotor sensitization to cocaine (F20). Experimental design: effects of chronic cocaine administration (15 mg/kg, i.p., 7 days) on locomotor activity on Days 1, 7 and 21 of testing in bLR and bHR rats (7 bLR/bHR pairs). On testing days rats were habituated for 1 h to the locomotor cage before receiving cocaine. Locomotion (total, lateral, rearing) was calculated for each rat as the locomotion scores after cocaine injection (stimulated) minus the background (last 30 min of habituation; mean ± SEM; $*P < 0.05$).

deviation below the bHR group average and bLR animals whose scores fell one standard deviation above the group average were not used for the subsequent cocaine treatment study. The present series of studies used two sets of 25 bLR/bHR pairs from two different generations (F15 and F20). Animals were treated in accordance with the ethical guidelines of the University of Michigan Committee on the Use and Care of Animals (UCUCA).

bLR/bHR differences in psychomotor sensitization to cocaine

It is known that in commercially available outbred LR/HR rats, the animals' reactivity to novelty predicts the degree of cocaine sensitization, with HR rats showing a greater psychomotor response to cocaine than LRs (Hooks *et al.*, 1992). Thus, the idea behind this experiment was to extend these earlier findings showing that our selectively-bred bLR/bHR rats also show differences in psychomotor sensitization to cocaine.

For this purpose, following bLR/bHR phenotype characterization (described above), we used 7 pairs of adult bLR and bHR males from the 20th generation weighting 225–250 g (n = 7 per group). On the first day of testing, rats were transferred from the colony room and placed into the activity-monitoring chambers described above. Due to the known bLR/bHR differences in motor activity, animals were allowed to habituate for 1 h before receiving a cocaine injection (15 mg/kg, i.p.; Fig. 1B), followed by 1 h during which locomotion (horizontal and rearing activity) was scored. After the first day of testing rats were returned to their home cages. Once a day for the next 6 days rats were injected in an adjacent novel room with 15 mg/kg of cocaine (at about 10:00 h). On Day 7, rats were returned to the activity chambers where they were again allowed to habituate before receiving a single cocaine injection followed by 1 h behavioral scoring. After 14 days of abstinence, rats were transferred to the activity chambers; following 1 h habituation animals were challenged with 15 mg/kg dose of cocaine to assess its impact on locomotion for an additional hour.

For each rat we calculated locomotion scores (total, lateral and rearing activity) as the value following cocaine injection (stimulated) minus the background (defined as activity occurring during the last 30 min of habituation). This method allowed us to correct for the basal inborn differences in bLR/bHR rats and to compare both groups following cocaine treatments.

Study II: bLR/bHR differences in neurogenesis following experimenter-administered cocaine

Once we characterized animals according to their bLR/bHR phenotype, the aim of the next study was to investigate potential differences in adult HCal neurogenesis (recent cell proliferation, early and late cell survival, and cell differentiation) following cocaine treatments. This second study consisted of two sets of experiments detailed below. The initial pilot study assessed the effect of cocaine on cell proliferation in bLR/bHR rats, and later led to a second more complete study that included neurogenesis regulation at different stages.

bLR/bHR differences in cell proliferation following experimenter-administered cocaine

We used bLR and bHR rats (F15, n = 5 per phenotype per experimental group) to determine the effects of acute, chronic and early cocaine abstinence on adult HCal cell proliferation as measured by Ki-67 immunohistochemistry (IHC) in the DG (Fig. 2A). The chronic cocaine treatment is known to induce behavioral sensitization

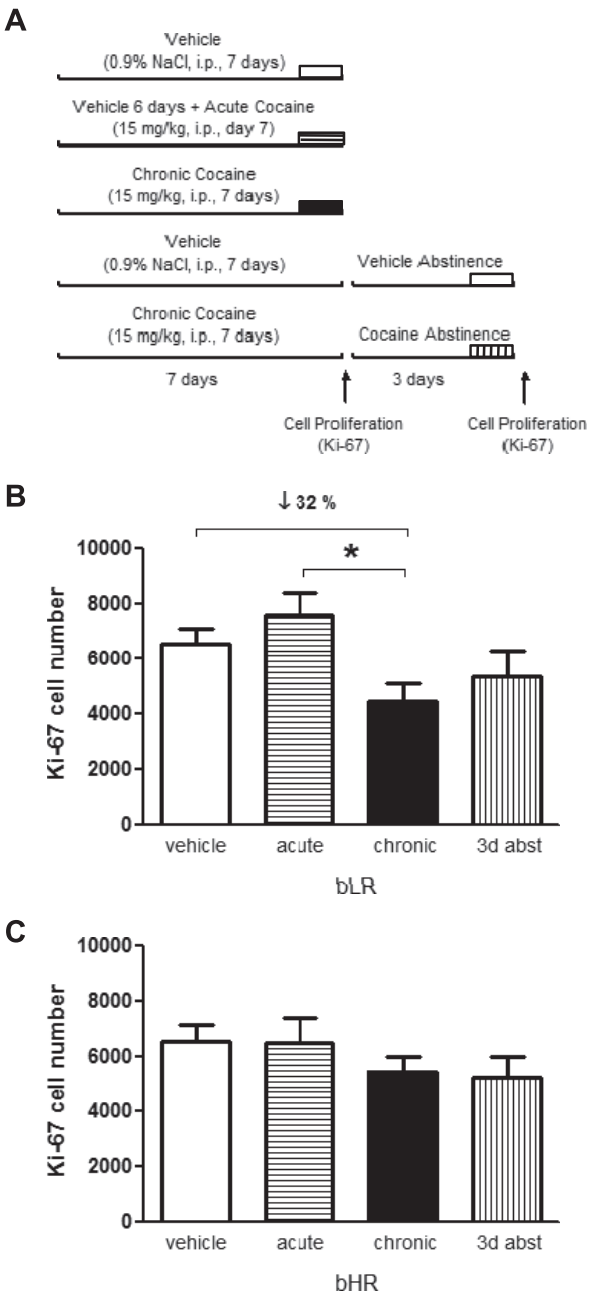

FIG. 2. Bred Low-Responder (bLR)/bred High-Responder (bHR) differences in cell proliferation following experimenter-administered cocaine. (A) bLR and bHR rats (F15, 25 pairs, n = 5 per group) were used to determine the effects of acute, chronic and early cocaine abstinence on adult HCal cell proliferation as measured by Ki-67 IHC in the DG. Groups of treatment: vehicle (0.9% NaCl, i.p., 7 days); acute cocaine (acute, 6 days of saline, and on Day 7 15 mg/kg of cocaine); chronic cocaine (15 mg/kg for 7 days); chronic cocaine followed by abstinence (3 days); and vehicle abstinence control group. The pool of Ki-67-positive cells being labeled is indicated by a rectangle coded for each treatment group and represents the cells recently born in the last 25 h at the time of killing the animals (within a cell cycle; Cameron & McKay, 2001). (B and C) Chronic cocaine decreased cell proliferation only in bLR rats. Columns are means ± SEM per group and expressed as Ki-67 cell number in the DG. The groups vehicle and vehicle abstinence were pooled as a single control group due to no significant Ki-67 cell number differences between them. *P < 0.05 acute vs. chronic cocaine. Note that there is a 32 ± 11% decrease in Ki-67 cell number when comparing vehicle with chronic cocaine, even though this effect did not reach statistical significance.

(Gosnell, 2005), and produces bLR/bHR differences in psychomotor response to the drug (Fig. 1B). Thus, one group of bLR/bHR rats received daily injections of 15 mg/kg cocaine (i.p.), whereas the control group received daily injections of saline for 7 consecutive days. The acute cocaine group received daily injections of saline for 6 days and an acute cocaine injection (15 mg/kg, i.p.) on Day 7. All animals were transferred to an adjacent room to receive their daily injections (at about 10:00 h) and were then returned to their colony room. On Day 7 of cocaine administration, animals were either killed 45 min or 3 days (early abstinence; Garcia-Fuster *et al.*, 2009) following the last cocaine injection.

bLR/bHR differences in neurogenesis following experimenter-administered cocaine

We used bLR/bHR rats (F20, $n = 6$ per phenotype per experimental group) to determine the effects of experimenter-administered cocaine on the different stages of adult HCal neurogenesis (Fig. 3A). The chosen cellular markers enabled us to look back in time from the day of the animal's death and study cells born at different stages along the sensitization process – i.e. new cells that proliferated immediately prior to death, young immature neurons born partway through the sensitization process, or mature neurons that were born prior to the first cocaine or saline administration and survived and differentiated during the course of the treatment. As depicted in Fig. 3, rats were pretreated for 3 days with 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg, i.p.; Calbiochem) delivered at 12-h intervals 4 days before the treatment started (Thomas *et al.*, 2007). This protocol avoids marker dilution with cell division (Dayer *et al.*, 2003) and labels newly generated cells that will differentiate, and after 28 days of survival (Brown *et al.*, 2003) be stably incorporated within the HC (Kempermann, 2002; Kempermann *et al.*, 2004). This enabled us to measure cocaine effects on late cell survival (BrdU) and cell differentiation [BrdU/NeuN/glial fibrillary acidic protein (GFAP)] separately from measures of recent cell proliferation (Ki-67) and early cell survival (NeuroD) that can be achieved with *post mortem* markers. Following the same paradigm as described earlier, some bLR/bHR rats received injections of 15 mg/kg cocaine (7 days, i.p.) whereas the control group received saline for seven consecutive days (Fig. 3B). All animals were transferred to an adjacent room to receive their daily injections (at about 10:00 h) and were then returned to their colony room. On Day 7 of cocaine administration, animals were either killed 45 min or 14 days (intermediate abstinence) following the last cocaine injection. In an attempt to minimize the number of animals used for the study, the control abstinence group was not run due to the fact that we had observed no differences in neurogenesis between the control group and the control abstinence group (see Fig. 2, Results of previous experiment).

Tissue collection and IHC studies

At the times indicated above, animals were killed by decapitation and the brains removed. The left half-brain was fast frozen and stored at −80°C until use for IHC experiments. For each animal, a series of every 8th section was cryostat cut at 30 μm and slide-mounted throughout the entire extent of the HC.

As mentioned earlier, we utilized molecular tools to examine at a given point in time the effects of experimenter-administered cocaine on cells at different stages of adult HCal neurogenesis within bLR vs. bHR rats. Distinct cellular markers signified date of birth, thus serving as an index of cocaine's effects on recent cell generation, early cell survival and late cell survival at the time of killing the animals.

To determine the rate of recent cell proliferation, we performed IHC labeling of Ki-67 (Figs 2 and 3), an intrinsic marker of ongoing cell proliferation present during the active phases of the cell cycle (G_1-M; Scholzen & Gerdes, 2000; Kee *et al.*, 2002). As described in detail by Perez *et al.* (2009), sections were postfixed in 4% paraformaldehyde (PFA) for 1 h, followed by 1 h incubation in 10% sodium citrate pH 6.0 at 90°C for epitope retrieval. Sections were then rinsed with phosphate-buffered saline (PBS), washed in 0.3% peroxide, blocked with bovine serum albumin (BSA) containing 1% goat serum and 0.05% Triton X-100, and incubated overnight with polyclonal rabbit anti-Ki-67 (1 : 40 000; University of Michigan). After PBS washes, sections were incubated in biotinylated anti-rabbit secondary antibody 1 : 1000 (Vector Laboratories, Burlingame, CA, USA) followed by Avidin/Biotin complex (Vectastain Elite ABC kit; Vectors Laboratories) and diaminobenzidine (DAB) as chromogen.

The survival of developing neurons (cells born during the course of the sensitization process) was visualized with the marker NeuroD (Fig. 3), a transcription factor with a transient expression restricted to cells as they become committed to a neuronal lineage during differentiation within the DG in the HC (Lee, 1997; Miyata *et al.*, 1999; Amrein *et al.*, 2004; Hevner *et al.*, 2006). Sections were postfixed in 4% PFA, incubated in endogenous peroxidase blocking reagent (0.1% peroxide) and blocked with BSA containing 1% donkey serum and 0.05% Triton X-100. Sections were then incubated overnight with goat polyclonal anti-NeuroD (1 : 2500; Santa Cruz Biotechnology). The next day, following PBS washes, sections were incubated in biotinylated anti-goat secondary antibody 1 : 1000 (Vector Labs) followed by Avidin/Biotin complex and DAB reaction with nickel chloride for visualization of signal.

The survival of cells born just prior to the sensitization process (rate of late cell survival) was determined by labeling of BrdU (Fig. 3), a synthetic thymidine analog that was injected before cocaine administration. BrdU is incorporated during the S-phase of the cell cycle into newly synthesized DNA strands of actively proliferating cells (Wojtowicz & Kee, 2006), and the number of these new cells that survive throughout the treatment can then be measured at the end of the study. Tissue was postfixed in 4% PFA for 1 h, rinsed in PBS and washed in 0.3% peroxide. Sections were then incubated in 50% formamide-50% 2× SSC at 65°C for 2 h followed by rinses in 2× SSC. Slides were placed for 30 min in 2N HCl at 37°C for DNA denaturation and 10 min in 0.1 M boric acid at room temperature followed by rinsing in PBS and blocking with BSA. Sections were incubated overnight with polyclonal anti-BrdU (1 : 20 000; University of Michigan) followed by anti-rabbit secondary antibody 1 : 1000 (Vector labs), Avidin/Biotin complex amplification and DAB reaction for visualization of signal.

For all antibodies, controls were performed by blocking the specific signal with the respective blocking peptide (data not shown). Cresyl violet staining was performed for Ki-67 and BrdU protocols. All sections were dehydrated through graded alcohols followed by immersion in xylene and then coverslipped with Permount® mounting medium.

For determining the effects of cocaine treatments on cell differentiation (phenotype of surviving cells) in the DG of the HC, we used NeuN antibody to identify mature neurons (Mullen *et al.*, 1992) and GFAP antibody to label mature glial cells. Briefly, for fluorescent triple labeling, following the same protocol as for BrdU labeling we first incubated the sections overnight with a combination of polyclonal anti-BrdU (1 : 20 000; University of Michigan) with monoclonal anti-GFAP (1 : 1000; Millipore Bioscience Research Reagents). The next day the sections were incubated with fluorescent secondary antibodies: Alexa 594 goat anti-rat and Alexa-647 goat anti-mouse (Invitrogen).

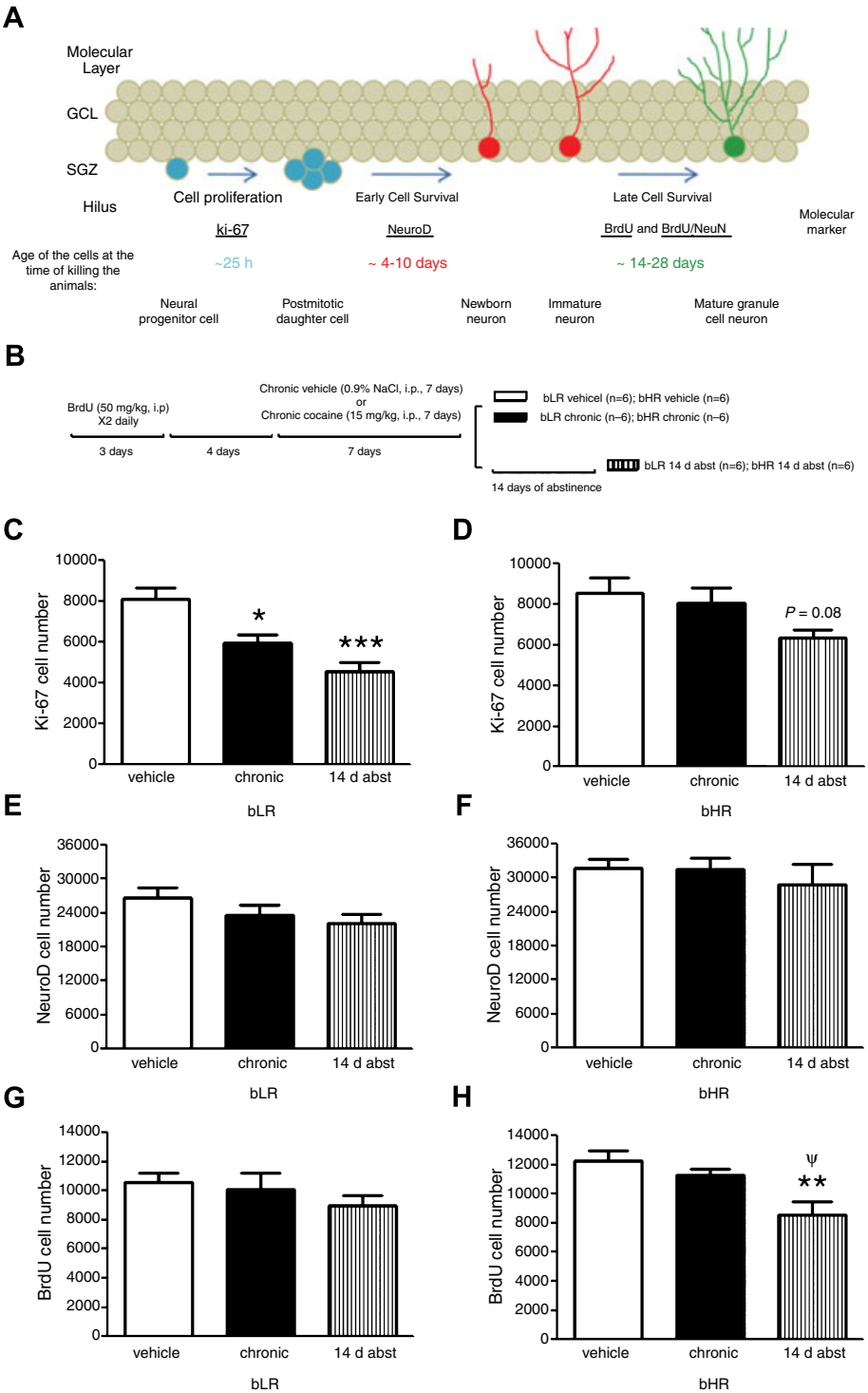

FIG. 3. Bred Low-Responder (bLR)/bred High-Responder (bHR) differences in adult HCal neurogenesis following experimenter-administered cocaine. (A) Stages of adult HCal neurogenesis showing the molecular markers used to label each pool of cells: recent cell proliferation (Ki-67, cells born in the last 25 h); early cell survival (NeuroD, approximately 4–10-day-old immature neurons); late cell survival and cell differentiation [5-bromo-2′-deoxyuridine (BrdU), approximately 14–28-day-old mature neurons]. Figure adapted from Schmidt & Duman (2007). (B) Experimental design: bLR/bHR rats (F20, 18 pairs, $n = 6$ per group) were pretreated for 3 days with BrdU delivery (50 mg/kg, i.p.) at 12-h intervals 4 days before the treatment began. Groups of treatment: vehicle (0.9% NaCl, i.p., 7 days); chronic cocaine (15 mg/kg for 7 days); chronic cocaine followed by 14 days of abstinence. (C) Quantitative analysis of Ki-67-positive cells revealed a decrease in cell proliferation following chronic cocaine in bLR rats. This effect persisted during the course of abstinence. (D) bHR rats showed no significant change in cell proliferation following chronic cocaine. Note that 2 weeks of cocaine abstinence decreased cell proliferation by 26 ± 5% in the DG, although it did not reach statistical significance. (E and F) Quantitative analysis of NeuroD-positive cells revealed no significant change in the number of short-term surviving cells in the DG following cocaine treatments. (G) Quantitative analysis of BrdU-positive cells revealed no significant change in the number of long-term surviving cells in the DG following cocaine treatments in bLR rats. (H) bHR rats showed cocaine abstinence-specific changes as measured by a decrease in the number of BrdU-positive cells (28 days old) in the DG. Data are presented as the mean ± SEM. $^{**}P < 0.01$ vs. vehicle; $^{\psi}P < 0.05$ vs. chronic cocaine. GCL, granule cell layer; SGZ, subgranular zone.

For NeuN labeling, we used a primary monoclonal mouse anti-NeuN antibody tagged with an Alexa 488 fluor (Millipore Bioscience Research Reagents).

Cell counting

To ensure that a blind observer performed all cell counts for quantifying Ki-67-, NeuroD- and BrdU-positive cells, slides were initially coded and the code was not broken until counts were analyzed. Thus, immunostained positive cells were counted with a Leica DMR light microscope within the DG focusing through the thickness of the section using a 63× oil objective. We used a modified unbiased stereological procedure (Malberg & Duman, 2003) in which every 8th section was counted throughout the extent of the HC, and the sum was multiplied by the sampling factor 8 to provide an estimate of the total number of positive cells per animal. In triple labeling experiments, in order to determine the percentage of BrdU-positive cells that co-labeled with NeuN or GFAP, we based our quantification methodology on previous studies that followed the same procedures and obtained reliable measures (Warner-Schmidt & Duman, 2007; Perez *et al.*, 2009) and examined at least 30 BrdU-positive cells per subject using a laser-scanning confocal microscope (Olympus BX62). Laser scans of 0.5-μm serial Z-section planes were visualized using a 63× oil objective. To obtain an estimate of the total number of new neurons and new glial cells in the DG of each animal, the percentage of BrdU-positive cells co-labeled with NeuN, GFAP or neither was calculated and multiplied by the total number of BrdU-positive cells for each animal.

Data and statistical analysis

All data were analyzed with GraphPad Prism, Version 5. Results are expressed as mean values ± standard error of the mean (SEM). One-way or two-way ANOVA followed by a multiple comparison test (Bonferrroni) and Student's two-tailed *t*-test were used for the statistical evaluations. The level of significance was chosen as $P = 0.05$.

Drugs and chemicals

Cocaine-HCl was obtained from Mallinckrodt (St Louis, MO, USA), and BrdU was purchased from Calbiochem. Other materials were purchased from Santa Cruz Biotechnology, Sigma-Aldrich and Vector Laboratories.

Results

Study I: characterization of bLR and bHR rats

bLR/bHR differences in locomotor response to novelty

As shown in Fig. 1A, selective breeding across several generations (F1–F20) produced a line of bHR rats, relative to bLR rats, that show high levels of locomotion activity (lateral movement, rearing and total activity, which is the sum of lateral and rearing movement) when placed in a novel environment. By the 10th generation of breeding we could predict with over 95% certainty the bLR or bHR classification of our animals based on an animals' parentage (i.e. ≥ 95% of bLR offspring behaved reliably as bLRs, and ≥ 95% of bHR offspring showed typical bHR behavior). Baseline differences in novelty-induced locomotor activity dramatically diverged with increasing rounds of selection (Fig. 1A). Note that for the present set of experiments, we used bLR/bHR rats from generation 15 and 20 (F15, F20), which clearly show marked basal differences in novelty-induced motor activity, both lateral movement and rearing.

bLR/bHR differences in psychomotor sensitization to cocaine

As mentioned earlier, following bLR/bHR phenotype characterization (Fig. 1A), we used seven pairs of adult bLR and bHR males from the 20th generation to ascertain bLR/bHR differences in psychomotor sensitization to cocaine. Figure 1B shows the effects of repeated cocaine administration and 14 days of cocaine abstinence on locomotor activity on test days 1, 7 and 21 in bLR and bHR rats. To analyze bLR and bHR differences in psychomotor response to cocaine we first calculated the background levels of total locomotion (last 30 min of habituation) for bLR (Day 1: 29 ± 9; Day 7: 14 ± 9; Day 21: 56 ± 5) and bHR (Day 1: 494 ± 53; Day 7: 390 ± 64; Day 21: 530 ± 79) rats. We then used these background locomotion values to correct the values post-cocaine stimulation for each phenotype, providing the graphs shown in Fig. 1B. The same was done for lateral (bLR, Day 1: 11 ± 4; Day 7: 4 ± 2; Day 21: 20 ± 3; and bHR, Day 1: 150 ± 23; Day 7: 120 ± 25; Day 21: 145 ± 26) and rearing locomotion (bLR, Day 1: 18 ± 6; Day 7: 10 ± 7; Day 21: 36 ± 4; and bHR, Day 1: 345 ± 38; Day 7: 270 ± 47; Day 21: 385 ± 63).

When analyzing total locomotion, two-way ANOVA followed by Bonferroni's *post hoc* test revealed a significant effect of day ($F_{2,30} = 12.05$, $P = 0.0001$) and group ($F_{1,30} = 11.36$, $P = 0.0021$), with no interaction day × group ($F_{2,30} = 0.54$, $P = 0.589$). *Post hoc* comparisons revealed a significant difference between groups after the challenge dose at Day 21 ($P < 0.05$), with bHR rats showing increased total locomotion compared with bLR rats. When lateral and rearing locomotion were analyzed separately, two-way ANOVA revealed the following differences: lateral locomotion (effect of day, $F_{2,30} = 9.09$, $P = 0.0008$; no group effect, $F_{1,30} = 0.66$, $P = 0.424$; no interaction, $F_{2,30} = 0.01$, $P = 0.989$); and rearing locomotion (no day effect, $F_{2,30} = 2.23$, $P = 0.124$; effect of group, $F_{1,30} = 11.10$, $P = 0.0023$; no interaction, $F_{2,30} = 0.63$, $P = 0.538$). *Post hoc* comparisons revealed a significant difference between groups only for rearing locomotion after the challenge dose at Day 21 ($P < 0.05$), with bHR rats showing increased rearing locomotion compared with bLR rats. These results indicate that although both groups of animals exhibited a certain degree of psychomotor sensitization to cocaine (e.g. Day 1 vs. Day 21 for each phenotype when analyzing total and lateral locomotion), the enhanced drug response seen in bHRs is mainly driven by differences in rearing locomotion, a hallmark of sensitization development (Szumlinski *et al.*, 2003).

Study II: bLR/bHR differences in neurogenesis following experimenter-administered cocaine

bLR/bHR differences in cell proliferation following experimenter-administered cocaine

The effects of experimenter-administered cocaine on recent cell proliferation (within a cell cycle time of 25 h; Cameron & McKay, 2001) as measured by Ki-67-positive cells in the DG was ascertained in bLR/bHR rats (F15, $n = 5$ per phenotype per experimental group; Fig. 2A). Note that two groups – Vehicle and Vehicle Abstinence – were pooled as a single control group due to the lack of significant differences in Ki-67-positive cell number between them (*t*-test: bLR, $F_{1,8} = 10.46$, $P = 0.19$, n.s.; bHR, $F_{1,8} = 1.76$, $P = 0.26$, n.s.). As shown in Fig. 2B and C, one-way ANOVA detected a significant effect of cocaine treatment only in bLR rats (ANOVA followed by Bonferroni's test: LR rats: $F_{3,21} = 3.247$, $P = 0.04$; HR rats: $F_{3,21} = 0.855$, $P = 0.480$, n.s.). Acute cocaine treatment did not alter cell prolifer-

ation (bLR rats: 16 ± 12% increase, n.s.; bHR rats: 1 ± 14% change, n.s.). However, after chronic cocaine treatment, cell proliferation was decreased for both phenotypes, although it reached significance only in the bLR animals (bLR rats: 32 ± 11% decrease, $P < 0.05$ when compared with acute cocaine; bHR rats: 16 ± 8% decrease, n.s.). This effect, although it lost significance, persisted after 3 days of abstinence (bLR rats: 18 ± 14% decrease, n.s.; bHR rats: 20 ± 12% decrease, n.s.). The results suggest that bLR and bHR animals exhibit different patterns of cell proliferation as measured by Ki-67 cells in the DG, with bLR rats being more reactive to the effects of cocaine on recent cell proliferation.

bLR/bHR differences in neurogenesis following experimenter-administered cocaine

bLR/bHR rats from the second cohort of animals (F20, $n = 6$ per phenotype per experimental group) were used to examine the effects of chronic cocaine exposure and 14 days cocaine abstinence on adult HCal neurogenesis. As shown in Fig. 3 and described above, the three measures quantified were: (i) cells that were recently born at the time of killing the animals (in the last 25 h; recent cell proliferation); (ii) cells born midway through the cocaine sensitization process (early cell survival); and (iii) cells born at the beginning of the experiment (approximately 14–28 days old; late cell survival).

Before analyzing the effects of cocaine on different stages of neurogenesis, the basal rates of cells marked for each phenotype were compared between vehicle-treated bLR and bHR groups. It should be noted that as previously described in Perez *et al.* (2009), bLR/bHR rats showed similar basal rates of cell proliferation as measured by Ki-67 IHC (6 ± 9% difference; bLR: 8088 ± 547 vs. bHR: 8544 ± 748; $P = 0.637$), and a non-significant tendency to exhibit different basal rates of early (19 ± 7% difference; bLR: 26 648 ± 1901 vs. bHR: 31 653 ± 1766; $P = 0.08$) and late cell survival (16 ± 7% difference; bLR: 10 547 ± 675 vs. bHR: 12 229 ± 705; $P = 0.116$). The combination of data from the Ki-67, NeuroD and BrdU markers (Fig. 3C–H) suggests that bLR and bHR lines tend to have some basal differences in the turnover of adult-born cells in the HC, with bLRs showing similar birth rates but lower rates of survival (Perez *et al.*, 2009).

When studying the effects of cocaine treatments on recent cell proliferation in bLR rats, Ki-67 immunostaining revealed that chronic cocaine reduced the number of Ki-67-positive cells in the DG by 26 ± 5% relative to the bLR vehicle treatment group, and this effect was even greater (44 ± 6% decrease) after 14 days of abstinence (ANOVA: $F_{2,15} = 13.52$, $P = 0.0004$; Fig. 3C). For bHR rats, one-way ANOVA did not reveal significant changes ($F_{2,15} = 3.072$, $P = 0.08$; Fig. 3D) in Ki-67 cell number in the DG following chronic cocaine (1 ± 12% change) when compared with the bHR vehicle treatment group. However, although it did not reach statistical significance, following 2 weeks of cocaine abstinence, recent cell proliferation was decreased by 26 ± 5% in the DG of bHR rats. These results strengthen the previous findings (F15 generation; Fig. 2B) and suggest that following chronic cocaine treatments, bLR and bHR animals exhibit different patterns of recent cell proliferation as measured by Ki-67 cell number in the DG, with bLR rats showing a greater effect of cocaine on the rate of cell birth (decreased after chronic cocaine and abstinence). Taken together, the results show that chronic cocaine exposure has a negative impact on recent cell proliferation in the DG of bLR rats.

To study the effects of experimenter-administered cocaine on the survival of cells born midway through the sensitization process (Fig. 3A), we examined NeuroD, a neuronal marker transiently expressed by immature neurons (approximately 4–10 days of age). As shown in Fig. 3E and F, quantitative analysis of NeuroD-positive cells revealed no significant change in the number of young immature neurons in the DG following cocaine treatments in bLR/bHR rats (ANOVA followed by Bonferroni's test: bLR rats: $F_{2,15} = 1.6$, $P = 0.234$, n.s.; bHR rats: $F_{2,15} = 0.350$, $P = 0.71$, n.s.). Therefore, these results suggest that experimenter-administered cocaine did not alter the survival of cells that were already born (young immature neurons) at the time of killing the animals. This lack of effect suggests that less mature cells may not respond to cocaine treatment because they are a resistant cell population.

In order to study the effects of cocaine treatments on the survival of cells born at the beginning of the experiment (late phase of cell survival), BrdU (50 mg/kg, i.p., twice a day for 3 days) was administered under basal conditions before the animals were exposed to vehicle or cocaine (Fig. 3B; based on Thomas *et al.*, 2007). The animals were killed either 14 or 28 days after BrdU pretreatment. Thus, determination of BrdU-positive cells in the DG yields a measure of the impact of chronic exposure to cocaine, with or without subsequent abstinence, on the survival rate of the cells born at the time of BrdU labeling.

For bLR rats, Fig. 3G shows that BrdU incorporation in the DG did not differ significantly between treatment groups ($F_{2,15} = 0.858$, $P = 0.444$), suggesting that in bLRs cocaine did not compromise the viability of the cells that were generated in the DG prior to treatment and then exposed to cocaine. Interestingly, for bHR rats, we found that the number of surviving BrdU-positive cells was reduced by 30 ± 8% after 2 weeks of abstinence (28 days after BrdU labeling) following chronic cocaine treatments ($F_{2,15} = 7.256$, $P < 0.01$ vs. vehicle, $P < 0.05$ vs. chronic cocaine). Thus, bLR and bHR animals exhibited different patterns of cell survival as measured by BrdU cell in the DG, with bHR rats showing a main effect on the late phase of survival of cells that were in the final stages of maturation during the course of cocaine abstinence.

Given the differential response in late cell survival following cocaine abstinence for bLR and bHR rats, we evaluated the pattern of cell differentiation of the surviving BrdU-labeled cells and obtained an estimate of the total number of neurons and astrocytes generated in the DG. As detailed in Fig. 4A, injections of BrdU were administered 28 days before animals were killed, providing us with a measure of the survival rate of the cells born at the time of BrdU labeling and showing us possible effects of cocaine abstinence on basal neurogenesis. Such a protocol allows the follow-up of newborn cells with the analysis of their maturation dynamics and the process of phenotype acquisition. Figure 4B illustrates that the large majority of newborn BrdU-positive cells in the DG expressed the neuronal marker NeuN (bLR, 91.7 ± 2.6%; bHR, 92.9 ± 1.4%), whereas a minority was either double labeled with GFAP, an astroglial marker (bLR, 3.8 ± 1.7%; bHR, 4.6 ± 1.4%), or not identified (bLR, 4.6 ± 1.5%; bHR, 2.5 ± 0.9%). These ratios were not significantly different between groups (NeuN, $F_{1,10} = 1.088$, $P = 0.766$; GFAP, $F_{1,10} = 1.440$, $P = 0.585$; Neither, $F_{1,10} = 2.531$, $P = 0.215$). These results show that the difference in late cell survival shown by bLR and bHR rats following cocaine abstinence did not correlate with a change in their maturation dynamics, as most of the surviving cells expressed the neuronal phenotype. It is therefore tempting to speculate that the decrease in late cell survival (mature cells, 28 days old) observed for bHR rats might translate in a decrease in mature neurons in the DG.

Discussion

The present findings provide a detailed characterization of cocaine's effect on adult HCal neurogenesis in rats selectively bred for low (bLR) vs. high (bHR) novelty-seeking behavior. In general, cocaine

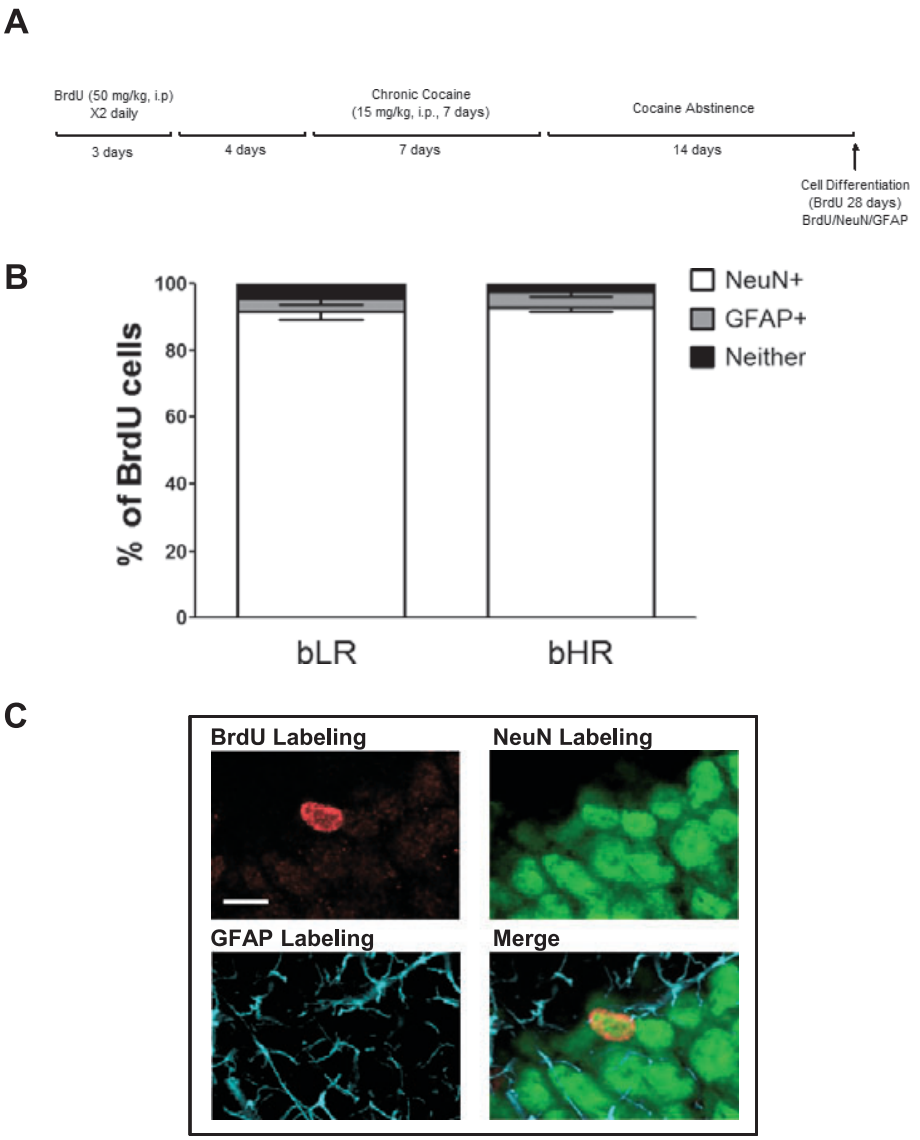

FIG. 4. Effect of cocaine abstinence on cell differentiation of adult-born cells in the DG of bred Low-Responder (bLR)/bred High-Responder (bHR) rats. (A) Experimental design. (B) Data are presented as the mean ± SEM. The large majority of the newborn 5-bromo-2′-deoxyuridine (BrdU)-positive cells in the DG expressed the neuronal marker NeuN (bLR, 91.7 ± 2.6%; bHR, 92.9 ± 1.4%), whereas a minority was either double labeled with glial fibrillary acidic protein (GFAP), an astroglial marker (bLR, 3.8 ± 1.7%; bHR, 4.6 ± 1.4%) or not identified (bLR, 4.6 ± 1.5%; bHR, 2.5 ± 0.9%). These ratios were not significantly different between groups. (C) Representative confocal images of BrdU (red labeling), NeuN (green labeling) and GFAP (blue labeling). The merged image, BrdU-labeled cell co-labeled with NeuN (merge) was confirmed in the *x*, *y*/*x*, *z* axis (scale bar, 10 μm), and included eight optical sections. Voxel size (x = 0.265 μm/pixel; y = 0.265 μm/pixel; z = 0.44 μm/pixel).

decreased HCal neurogenesis in both groups but at different control points. bLR rats primarily showed an effect of chronic cocaine and cocaine abstinence on cell proliferation, with no apparent impact on the survival or differentiation of preexisting adult stem cells. Moreover, this effect appears to become amplified over time, exhibiting a steady decrease in proliferation from acute to chronic cocaine exposure, and from chronic exposure to abstinence. By contrast, bHR rats exhibited abstinence-specific changes in the late survival of cells that were born under basal conditions before the cocaine treatment was initiated.

Specifically, the results for Study I regarding the characterization of the bLR/bHR animal model showed that: (i) selective breeding for the novelty-seeking trait across generations produced a line of bHR rats that show high levels of locomotion compared with their bLR counterparts; and (ii) the enhanced psychomotor response to cocaine seen in bHR rats is mainly driven by differences in rearing locomotion, a hallmark of sensitization development. For Study II, regarding the differential effects of cocaine on adult HCal neurogenesis in bLR/bHR rats, we showed the following. (i) Acute cocaine administration did not alter recent cell proliferation as measured by Ki-67-positive cells in the DG of either bLR or bHR rats. (ii) Repeated cocaine administration induced a decrease in cell proliferation, an effect only seen in bLR rats, which persisted and became amplified through the course of abstinence (14 days). This effect was replicated in two separate generations of bLR/bHR rats (F15 and F20) in separate sets of experiments. (iii) Neither chronic cocaine administration nor cocaine abstinence affected the survival of cells born midway through the sensitization process and expressing a marker of immature neurons (NeuroD) both in bLR and bHR rats. (iv) Cocaine abstinence was associated with decreased survival of adult mature neurons

specifically in bHR rats. These cells were born under basal conditions before the cocaine treatment started and were 28 days old when the animals were killed. (v) The course of treatment did not affect the ratio of neurons to glia in either bLR or bHR, with most of the surviving cells acquiring the neuronal phenotype.

The LR–HR rat model provides a useful tool to examine potential molecular mechanisms that underlie their differential behavioral response to cocaine although, notably, the LR/HR trait does not appear to predict the transition to compulsive drug use (see Belin *et al.*, 2008). Among other differences in drug-seeking behavior, commercially available (non-selectively bred) HR rats will self-administer psychostimulants more readily than their LR counterparts (Piazza *et al.*, 1989; Piazza & Le Moal, 1996) and will display exaggerated behavioral sensitization to these drugs (Hooks *et al.*, 1991, 1992). Consistent with these studies in commercially available LR/HR rats, selective breeding has amplified many of the differences seen in commercially available LR/HR animals that appear directly relevant to the enhanced differences in initial propensity to drug abuse. In this line, bHRs acquired cocaine self-administration more rapidly than bLR rats (Davis *et al.*, 2008). Moreover, a recent study from our lab (Waselus *et al.*, 2009) examined multiple measures indicative of behavioral sensitization (i.e. locomotion, darting, immobility, left–right head movements and rearing) to identify differences between cocaine-treated bHR and bLR rats. Similar to our results, the study demonstrated that while both phenotypes showed evidence of sensitization, bHRs exhibited an enhanced drug response. Along these lines, our differences in motor response were mainly driven by changes in rearing locomotion, which has been described as a landmark of cocaine sensitization (Szumlinski *et al.*, 2003).

After demonstrating the bLR/bHR differences in cocaine-induced psychomotor sensitization we examined adult HCal neurogenesis as a potential molecular mechanism differentially altered by cocaine within each phenotype. Note that individual correlations between the behavioral (i.e. psychomotor response to cocaine) and the molecular data (i.e. neurogenesis) were not assessed because these experiments were performed in separate groups of rats. However, the experiments were run in parallel to ensure that brains assessed for possible neurogenesis changes were exposed to an identical cocaine treatment regimen known to evoke psychomotor differences in bLR/bHR rats.

When studying neurogenesis, it is critical to establish adequate protocols to appropriately distinguish dividing cells such that cell proliferation and cell survival can be clearly differentiated. The present study evaluated the impact of cocaine on cells born at different time points, i.e. cells at different stages of maturation along the neurogenesis process when the animals were killed. Ki-67 was used to mark recently divided cells (Scholzen & Gerdes, 2000; Kee *et al.*, 2002); NeuroD indicated immature young neurons (von Bohlen Und Halbach, 2007); and BrdU demarcated mature cells (Dayer *et al.*, 2003; Thomas *et al.*, 2007) that differentiated into glial, neuronal or an undefined cell type. Remarkably, it has been recently described that Ki-67 and NeuroD do not colocalize in the same population of cells (Larsen *et al.*, 2007), and that Ki-67 and BrdU only colocalize from 2 h until 4 days post-BrdU injection (Dayer *et al.*, 2003). This indicates that new BrdU-labeled daughter cells are added throughout this period of approximately four cell cycles (see Fig. 3 for experimental design), and that cell proliferation and survival can be clearly differentiated using these two molecular markers. Moreover, as no co-localization of NeuroD was observed following 14 and 28 days of BrdU injections (Kawai *et al.*, 2004), these molecular markers allowed us to study the effects of early and late cell survival separately.

Our results showed that for bLR rats, repetitive but not single administration of cocaine decreased cell proliferation as measured by Ki-67-positive cells in adult rat HC without influencing cell survival and cell differentiation. Thus, experimenter-administered cocaine in bLR rats affected different cell populations depending on their stage of maturity along the neurogenesis process; specifically, cocaine appears to decrease the rate of cell birth and proliferation without altering the maturation of cells that were already born at the time of killing the animals (i.e. NeuroD-positive immature neurons and BrdU-positive mature neurons). Similar results were previously described for commercially available Sprague–Dawley rats (Yamaguchi *et al.*, 2004; Dominguez-Escriba *et al.*, 2006). Indeed, newborn cells could be undergoing plasticity changes before they reach full maturity, and their functions may well be different from those of mature granule neurons (Canales, 2007). It is believed that the adult DG only has a limited supply of dividing progenitor cells (von Bohlen Und Halbach, 2007); thus, decreased cell proliferation might reflect a direct action of cocaine on the progenitor cell population (Eisch & Harburg, 2006), leading to a cessation, reduction or otherwise alteration of cell cycle division, or perhaps enhanced cell death. Furthermore, decreased cell proliferation might also lead to a delayed reduction of cell survival, i.e. less cell birth giving rise to a lesser number of mature neurons. Or, as described by Noonan *et al.* (2008), the effects of cocaine administration on cell proliferation may become evident in a later change in their maturity. Future experiments will address these hypotheses by administering BrdU immediately before the end of the abstinence period and assessing cocaine effects on cell maturation and cell survival (14–28 days later).

On the other end, for bHR rats, experimenter administration of cocaine (acute and chronic) did not affect the rate of recent cell proliferation (cell birth during the last cell cycle, 25 h), and did not influence the survival of cells born halfway (immature neurons) or at the beginning of the sensitization process (mature neurons). However, during the course of abstinence, there were specific changes in bHR rats, i.e. a 26% non-significant decrease in the rate of cell birth but, most importantly, a decrease in the number of mature surviving neurons when compared with vehicle or chronic cocaine groups. Notably, the survival of immature cells labeled with NeuroD was not compromised following cocaine abstinence. It has previously been shown that distinct populations of cells in the adult DG will respond to certain stimulus depending on the cell's age at the time of exposure to the stimulus (Cameron & Gould, 1996). An important thing to remember when studying the regulation of neurogenesis is that the generation of new neurons is the end product of a series of steps consisting of cell proliferation, survival, migration, differentiation and establishment of functional connections with other neurons (Lledo *et al.*, 2006). Having this in mind, the results showed that during abstinence to chronic cocaine, bHR rats appeared to have a critical window of neurogenesis regulation from immature neurons to mature integrated neurons in which the number of surviving mature neurons is decreased, possibly due to more cell death. The critical time-point in postnatal cell genesis when the decision toward neuronal differentiation is made and the crucial restriction 'point of no return' is reached remains unclear (von Bohlen Und Halbach, 2007). Our results suggest that in the context of cocaine sensitization, in bHR rats, the critical window of neurogenesis regulation occurs during abstinence at the level of decreasing the number of functionally mature neurons. Interestingly, this window of decreased neurogenesis regulation parallels the time when bLR and bHR rats show distinct psychomotor responses to cocaine, with bHR rats exhibiting greater drug-induced rearing locomotion. Thus, it is reasonable to hypothesize that the regulation of adult HCal neurogenesis may be involved in the emergence of certain symptoms manifested during psychomotor sensitization. More importantly, our results support the idea previously

described by Chambers & Taylor (2004), which associated an impairment of HCal function with a higher behavioral sensitization to cocaine. In the present study, the impairment of HCal function was manifested as a decrease in the number of mature neurons (lesser connections in the circuitry) in conjunction with a greater psychomotor response to cocaine in bHR rats. Other studies also showed that adult neurogenesis plays an important role in HCal function (Cameron & McKay, 2001), and specifically in drug-taking and drug-seeking behaviors via its projections to the reward-related limbic regions (Taepavarapruk *et al.*, 2000; Canales, 2007).

To conclude, the present results suggest that bLR and bHR rat brains react differently to drugs of abuse, with each selectively-bred line exhibiting a distinct psychomotor response to cocaine, and showing different patterns and time-points of neurogenesis regulation following experimenter-administered cocaine. Indeed, cocaine targets different populations of cells in bLR/bHR rats: the progenitor neural stem cells causing decreased birth of new cells in bLR rats; and the number of mature neurons in bHR rats. Several factors could account for this differential regulation. For example, the absence of bLR/bHR behavioral differences after chronic cocaine and their obvious difference in cell proliferation could be due to a disconnection between these factors and a connection between other factors known to differ in bLR/bHR (e.g. Perez *et al.*, 2009) and the manifestation of changes in cell proliferation. Another important aspect to consider involves a matter of timing; less cell birth in bLR rats will eventually lead to a decreased number of mature neurons, suggesting a delayed effect over neurogenesis regulation, and reaffirming the delayed or blunted response to drugs of abuse apparent for bLR vs. bHR animals. The exact role of new neurons in adult HCal function remains unknown, but it is tempting to speculate that the earlier decrease in new functional connections in bHR rats could contribute, at least in part, to their enhanced psychomotor response following chronic cocaine administration. These results support the growing literature regarding altered neurogenesis as one drug-regulated form of plasticity. Moreover, the use of selective breeding highlights the importance of identifying the neuroadaptations that might confer enhanced drug responses, because they might help develop novel and better-tailored treatment options. Thus, in future studies it will be of great interest to ascertain how neurogenesis is differentially regulated in bLR and bHR rats when exposed to a paradigm of long-term addiction (i.e. extended access cocaine self-administration).

Acknowledgements

This study was supported by NIDA: 5P01DA021633-02; NIMH Conte Center Grant #L99MH60398; Office of Naval Research (ONR) N00014-02-1-0879 to H.A. and S.J.W.; and The Pritzker Neuropsychiatric Research Foundation. The authors would like to thank Sharon Burke, Jennifer Fitzpatrick and James Beals for excellent technical assistance.

Abbreviations

bHR, bred High-Responder; bLR, bred Low-Responder; BrdU, 5-bromo-2′-deoxyuridine; BSA, bovine serum albumin; DAB, diaminobenzidine; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; HC, hippocampus; HCal, hippocampal; IHC, immunohistochemistry; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

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