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Decreased Proliferation of Adult Hippocampal Stem Cells During Cocaine Withdrawal: Possible Role of the Cell Fate Regulator FADD

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The current study uses an extended access rat model of cocaine self-administration (5-h session per day, 14 days), which elicits several features manifested during the transition to human addiction, to study the neural adaptations associated with cocaine withdrawal. Given that the hippocampus is thought to have an important role in maintaining addictive behavior and appears to be especially relevant to mechanisms associated with withdrawal, this study attempted to understand how extended access to cocaine impacts the hippocampus at the cellular and molecular levels, and how these alterations change over the course of withdrawal (1, 14, and 28 days). Therefore, at the cellular level, we examined the effects of cocaine withdrawal on cell proliferation (Ki-67 + and NeuroD + cells) in the DG. At the molecular level, we employed a 'discovery' approach with gene expression profiling in the DG to uncover novel molecules possibly implicated in the neural adaptations that take place during cocaine withdrawal. Our results suggest that decreased hippocampal cell proliferation might participate in the adaptations associated with drug removal and identifies 14 days as a critical time-point of cocaine withdrawal. At the 14-day time-point, gene expression profiling of the DG revealed the dysregulation of several genes associated with cell fate regulation, highlighting two new neurobiological correlates (Ascl-1 and Dnmt3b) that accompany cessation of drug exposure. Moreover, the results point to Fas-Associated protein with Death Domain (FADD), a molecular marker previously associated with the propensity to substance abuse and cocaine sensitization, as a key cell fate regulator during cocaine withdrawal. Identifying molecules that may have a role in the restructuring of the hippocampus following substance abuse provides a better understanding of the adaptations associated with cocaine withdrawal and identifies novel targets for therapeutic intervention.

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INTRODUCTION

Cocaine is a highly abused drug that imposes an enormous health, social, and economic toll on society. One of the main problems in the treatment of substance abuse is the propensity of addicts to relapse even long after the discontinuation of drug use. It is therefore critical to achieve a better understanding of the neurobiological mechanisms as well as the neural adaptations that occur during the course of withdrawal, in order to develop better-tailored treatments. To accurately study cocaine withdrawal in the laboratory, it is essential to use an animal model of drug exposure that captures some of the key features of human cocaine addiction. Previous studies have shown that rats exposed to extended daily access cocaine self-administration (SA) procedures result in a pattern of drug-intake (ie, escalation of drug intake with session day) similar to that seen in human addicts (Bozarth and Wise, 1985; Ahmed and Koob, 1998) as well as a number of other symptoms characteristic of human addiction, including increased motivation for cocaine (Paterson and Markou, 2003) and deficits in fronto-cortical function and cognition (Briand *et al*, 2008). Moreover, extended daily access to drugs of abuse produces changes in neural plasticity in several brain regions implicated in addictive behavior (Ferrario *et al*, 2005) and therefore, represents an excellent rat model to study the neural adaptations that accompany the cessation of cocaine exposure.

A great deal is known about the impact of cocaine on the reward circuitry in the brain, which contains cell bodies in the ventral tegmental area that project to the nucleus accumbens and the prefrontal cortex (Gold *et al*, 1989). Recently, however, much interest has been generated in understanding the role of neural structures outside of the primary reward pathway, as these regions can critically modulate aspects of the cocaine experience beyond its

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Cell fate regulation during cocaine withdrawal
MJ García-Fuster *et al*
2304

immediate rewarding effects, such as contextual learning, memory, and the motivational value associated with drug stimuli. One region of interest, the hippocampus, is highly interconnected with the reward system (Everitt and Robbins, 2005: Dietz *et al*, 2009) and can influence the learning component of addiction. Indeed, previous work has associated different facets of hippocampal plasticity with psychostimulant effects on contextual conditioning (Shen *et al*, 2006) and behavioral sensitization (Lodge and Grace, 2008; García-Fuster *et al*, 2010). In a recent study, reduced hippocampal neurogenesis, a novel drug-induced hippocampal neuroadaptation, was shown to confer vulnerability in an animal model of cocaine addiction (Noonan *et al*, 2010).

The hippocampus is one of two known brain regions that continue to produce new neurons during adulthood (Altman and Das, 1965; Eriksson *et al*, 1998), representing a dramatic form of structural plasticity. Adult hippocampal neurogenesis is a highly dynamic process whereby mature, functional granular neurons are generated from newly born progenitor cells in the subgranular zone (SGZ) of the dentate gyrus (DG) (Ming and Song, 2005). These granular neurons are believed to integrate into existing circuitry (van Praag *et al*, 2002). Each stage of neurogenesis (ie, proliferation, differentiation and migration, maturation and survival) is demarcated by distinct molecular markers (von Bohlen Und Halbach, 2007) and is differentially regulated by a variety of factors, including adverse environmental conditions (Schmidt and Duman, 2007) and exposure to addictive substances (Eisch and Harburg, 2006). Previous reports have shown that experimenter-delivered cocaine decreased SGZ proliferation (Yamaguchi *et al*, 2004) in a dose- and time-dependent manner (Eisch and Nestler, 2002), without altering the survival and growth of immature cells (Domínguez-Escribà *et al*, 2006). Moreover, the decrease in cell proliferation after repeated administration of cocaine returned to normal levels following one week of withdrawal (Yamaguchi *et al*, 2005). When following a more clinically relevant paradigm of intravenous cocaine SA, similar to the one used in this study, 3 weeks of cocaine SA (4-h session per day) resulted in decreased cell proliferation in the adult SGZ (Noonan *et al*, 2008), without affecting cell death. Interestingly, following 4 weeks of withdrawal these deficits were normalized and the maturity of adult-generated hippocampal neurons was increased (Noonan *et al*, 2008). However, it is worth noting that the same study showed that a longer chronic paradigm of cocaine SA (7 weeks, 4-h session per day) did not change proliferation of Ki-67 + cells. Taken together, these data demonstrate that cocaine differentially modulates hippocampal cells at different stages of neurogenesis and highlights the importance of distinguishing between proliferation and neurogenesis. Therefore, a better understanding of the cellular and molecular changes that contribute to the restructuring of the hippocampus—such as proliferation of neural progenitor cells or hippocampal cell fate regulation—will be key to better understanding the neural adaptations that occur during the course of cocaine withdrawal.

The aim of this study was to understand how extended daily access to cocaine SA impacts the hippocampus at the cellular and molecular levels, and to ask how these alterations change over the course of withdrawal. To this end, we relied on two approaches: (1) to study the self-renewing capacity of the hippocampus by immunohisto-chemistry (IHC) analysis (Ki-67 + and NeuroD + cells in the DG); and, (2) to ascertain new candidate molecules likely involved in these processes by gene expression profile analysis in the DG.

MATERIALS AND METHODS

Rats

Forty-eight adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 225–250 g at the start of the experiment were used. Rats were housed in pairs and allowed to acclimatize to housing conditions for 1 week before any manipulations. Rats were housed in a temperature and humidity controlled room with a 12 : 12-h light–dark cycle, with water and food available *ad libitum*. Following catheterization surgeries, rats were individually housed for the remainder of the experiment. Rats were treated in accordance with the ethical guidelines of the University of Michigan Committee on the Use and Care of Animals.

Surgical Procedures

Rats were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) administered i.p. A silicone catheter was implanted in the jugular vein and passed subcutaneously to exit from the midscapular region of the animal's back. One rat died during surgery. The remaining 47 rats were allowed to recover from surgery for 1 week before drug SA. Catheters were flushed daily with 0.1 ml of gentamicin (10 mg/ml in 0.9% sterile bacteriostatic saline). The day before SA testing, catheter patency was assessed in the morning (around 1000 hours) by injecting 0.1–0.2 ml of thiopental sodium (20 mg/ml in sterile water) into the catheter. Rats that did not lose muscle tone within 2–3 s were excluded from the cocaine SA (LgA) group and utilized as no drug (ND) controls. Out of the 47 rats, 30 underwent cocaine SA procedures (LgA groups) and the remaining 17 were used as ND control groups (see experimental design, Figure 1a).

Extended Access Cocaine SA Procedures

During the training sessions, rats from the LgA groups (30 rats) were transported from their home cage to an operant chamber for 6 consecutive days, where they were allowed to nose-poke for cocaine (0.5 mg/kg per infusion in 50 μl of saline administered over 1.6 s) for 1-h sessions on a fixed ratio schedule of reinforcement (FR1) with a time-out of 20 s. One response in the 'active' nose-poke hole resulted in delivery of a single drug infusion. Responding in the 'inactive' hole had no consequences, but was recorded. Given the dose of cocaine used, the fact that these animals were not food-deprived or pre-trained for food responding, and that they were trained and tested during their light cycle, 33% of the rats tested did not acquire stable SA behavior (ie, at least five infusions each day for 3 consecutive days) and were removed from the study. After the initial training
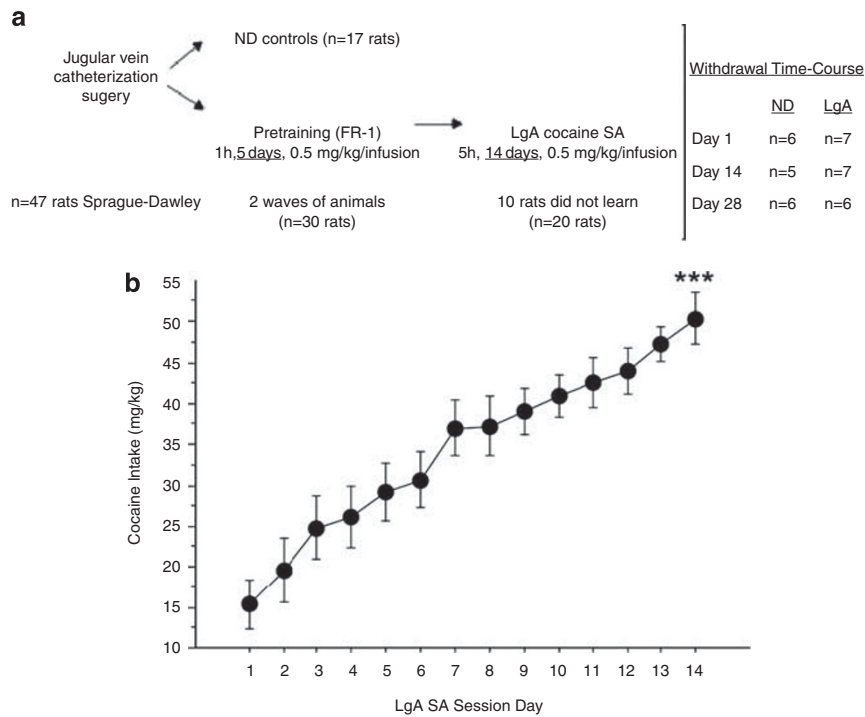
Figure 1 Escalation of cocaine intake during LgA cocaine SA procedures. (a) Experimental design (see Materials and methods section). (b) Data represent mean ± SEM amount of cocaine intake (0.5 mg/kg cocaine per infusion) over the entire daily sessions (5-h session per day of cocaine SA) for 14 days in adult male Sprague–Dawley rats (LgA, $n = 20$). Rats progressively increased their daily cocaine intake starting at an average of 15 mg/kg on the first day of SA and finishing at an average intake of about 50 mg/kg on the final SA session day (day 1 vs day 14; ***$p < 0.0001$).

period, rats from the LgA groups were switched to extended access (5-h session per day) cocaine SA at 0.5 mg/kg per infusion of cocaine for a total of 14 sessions (5 days per week). Rats were separated into two waves, which were balanced according to the amount of drug intake during the initial training period. The first wave was run from 0700 to 1200 hours and the second wave from 1300 to 1800 hours. Rats were checked for catheter patency once a week (1000 hours on a day with no LgA session) throughout the SA paradigm and immediately following the last day of testing. Only those rats that passed the catheter patency tests were included in the analyses. Twenty rats successfully completed the experiment for the LgA groups. Following the last SA session, LgA rats were counterbalanced by drug intake and wave and underwent withdrawal for 1, 14, or 28 days (see experimental design, Figure 1a).

ND control rats ($n = 17$) were also divided into two waves, transported daily to the same testing room, and placed in holding chambers similar to the SA chambers for the same amount of time as LgA rats on each day of training or extended access exposure. Following the last session, these rats were also divided into three groups ND-Day 1, ND-Day 14, ND-Day 28 and were left in their home cage for 1, 14, or 28 days, respectively.

Tissue Collection

Rats were killed by rapid decapitation and their brains removed at the conclusion of the assigned withdrawal period: 1, 14, or 28 days following the final SA session. Brains were processed in such a way as to allow the

investigation of hippocampal cell fate regulation using different approaches. The left half-brain was quickly frozen in a $-30\,^{\circ}\mathrm{C}$ isopentane solution and stored at $-80\,^{\circ}\mathrm{C}$ until further processing. For each animal, 30 μm sections were cryostat cut and slide-mounted throughout the entire extent of the HC (-1.72 to -6.80 mm from Bregma; Paxinos and Watson, 1997) and kept at $-80\,^{\circ}\mathrm{C}$ until further analysis. The following analyses were conducted on this tissue from the left hemisphere: (1) cell proliferation markers (ie, Ki-67 + and NeuroD + cells in the DG) by IHC; (2) laser capture microdissection (LCM) of the DG followed by gene expression profile analysis using Illumina microarray platform and RT-PCR validation; and (3) mRNA analysis in the DG by in situ hybridization (ISH). The right half of the brain was used to dissect the hippocampus, which was fast frozen on dry ice, and kept at $-80\,^{\circ}\mathrm{C}$ until use for western blot (WB) experiments (ie, protein content of cell fate markers). Proliferation of adult hippocampal stem cells (ie, Ki-67 + and NeuroD + cells) was assessed in ND and LgA groups at the three time points of withdrawal (Day 1, Day 14, and Day 28). However, given that the main changes in cell proliferation occurred following 14 days of withdrawal, further analysis (ie, microarray gene expression and validation) were ascertained only at this time-point (ND-Day 14 vs LgA-Day 14).

Immunohistochemistry Analysis

The rate of cell proliferation was determined in the left hemisphere DG by performing IHC labeling of Ki-67, an intrinsic marker of ongoing cell proliferation, which is expressed

in all dividing cells (Scholzen and Gerdes, 2000; Kee et al, 2002). Following a standardized protocol (see Perez et al, 2009; García-Fuster et al, 2010), hippocampal tissue sections (30 μm) were post-fixed for 1 h in 4% paraformaldehyde (PFA) and incubated for epitope retrieval (10% sodium citrate pH 6.0 at 90 °C for 1 h). Sections were then rinsed with PBS, washed in 0.3% peroxide, blocked with 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) followed by avidin–biotin complex (Vectastain Elite ABC kit; Vectors Laboratories) and diaminobenzidine (DAB) as chromogen. Sections were counterstained with cresyl violet before dehydration through graded alcohols, xylene immersion and coversliping (Permount mounting medium).

The proliferation of neural progenitors was analyzed in the left hemisphere DG by performing IHC labeling of NeuroD, a transient transcription factor restricted to developing neurons (Lee, 1997; Miyata et al, 1999; Hevner et al, 2006; Gao et al, 2009). As previously described (García-Fuster et al, 2010), sections were post-fixed for 1 h in 4% PFA and incubated in endogenous peroxidase blocking reagent (0.1% peroxide). Sections were then blocked with BSA (1% donkey serum and 0.05% Triton X-100) and incubated overnight with goat polyclonal anti-NeuroD (1:2500; Santa Cruz Biotechnology). The next day, 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. Finally, tissue was dehydrated in graded alcohols, immersed in xylene and cover-slipped using Permount mounting medium.

To ensure an unbiased count, all slides were randomly coded by a non-experimenter before quantification. Codes were broken after all slides were quantified and analyzed. The number of immunostained positive (+) cells was counted unilaterally in every eighth section throughout the extent of the left DG (−1.72 to −6.80 mm from Bregma) with a Leica DMR light microscope using a 63× oil objective lens and focusing through the thickness of the section (approximately 30 μm). For each rat, multiplication of raw cell counts by eight provided a final estimate of the total number of positive cells for one hippocampi. This method is based on a modified unbiased stereological procedure (Malberg and Duman, 2003) and has been used previously in our lab (see Perez et al, 2009; García-Fuster et al, 2010).

In addition to estimating the total number of Ki67+ and NeuroD+ cells, the number of cells was also quantified within the anterior (−180 to −4.52 mm from Bregma) and posterior (−4.52 to −6.80 mm from Bregma) hippocampal demarcation (see Guzmán-Marín et al, 2003; Noonan et al, 2008). A more detailed anatomical analysis in relation to the distance from Bregma was conducted for the groups in which the overall number of Ki-67+ and NeuroD+ cells was decreased (ND-Day 14 and LgA-Day 14). For this analysis, data are presented as total number of positive cells in the DG per region at both the anterior and posterior levels (Paxinos and Watson, 1997).

Gene Expression Profile Analysis

To ascertain the possible molecular adaptations that might accompany the cellular changes observed at the level of cell proliferation in the DG (ie, decreased Ki-67+ and NeuroD+ cells), we conducted gene expression profile analysis of the left hemisphere DG taken after 14 days of withdrawal (ND-Day 14 vs LgA-Day 14). The tissue used for this experiment was taken from consecutive slides adjacent to those used to assess proliferating markers. Moreover, it is important to note that while proliferation mainly occurs in a small percentage of cells in the SGZ of the DG, for the gene profiling analysis all cells in the DG were dissected. Following a standardized lab protocol (see Kerman et al, 2006), four DGs (SGZ and GCL) were laser capture microdissected (LCM) from selected slides for each rat. Each slide contained a total of eight cryostat-cut sections (approximately 30 μm thick) from the left half of the brain. Given the changes in Ki-67+ and NeuroD+ cells were observed throughout the whole hippocampal extent, both within the anterior (−1.80 to −4.52 mm from Bregma) and the posterior (−4.52 to −6.80 mm from Bregma) demarcation, the samples for this analysis were taken from tissue between −3.60 and −5.80 mm to Bregma. Before LCM, the slides were removed from −80°C, thawed for 30 s at room temperature, dehydrated, and defatted (Kerman et al, 2006). After LCM, during which tissue was captured and placed onto caps, all samples underwent RNA isolation (PicoPure RNA Isolation Kit; Molecular Devices, Sunnyvale, CA) as previously described (Bernard et al, 2010), and RNA concentration and quality was assessed by Agilent BioAnalyzer (Agilent Technologies, Palo Alto, CA). Total cellular RNA samples were subjected to two rounds of amplification (RiboAmp Plus). Before biotinylation, a portion of amplified double-stranded complementary DNA (cDNA) was saved for validation of candidate genes by RT-PCR (ie, TaqMan Array analysis, see below). Biotinylated amplified RNA concentration was calculated for each rat using a Nanodrop ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE), yielding amounts in the range of 800–1600 ng/μl for most of the samples, except for three samples: 3-ND (265 ng/μl), 12-LgA (162 ng/μl), and 48-LgA (351 ng/μl). Equal amounts of biotinylated amplified RNA was hybridized to Illumina microarrays (RatRef-12 Beadchips) and processed according to the manufacturer's instructions.

Gene Expression Profile Validation

Gene expression microarrays yielded a list of candidate genes altered in our samples when comparing LgA-Day 14 with their respective controls (ND-Day 14). In an attempt to validate some of these changes through RT-PCR, we performed gene quantification with custom ordered TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA). A portion of previously saved amplified cDNA from ND-Day 14 and LgA-Day 14 samples obtained from the LCM dissected left DG (see above) were prepared in a 50 μl volume containing 30 ng of cDNA. Following mixture with 50 μl of TaqMan Universal PCR Master Mix 2×, samples were centrifuged twice at 1200 r.p.m. for 1 min and then loaded to the Array containing the

pre-designed probes and primer sets (based on 5′ nuclease chemistry). Samples were then run on the Applied Biosystems 7900HT Fast Real-Time PCR System (University of Michigan Core Facility). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), in which $2^{-\Delta\Delta Ct}$ yields fold change in gene expression of the gene of interest normalized to the internal control gene expression and relative to a calibrator. The threshold cycle (Ct) is defined by the number of cycles to reach threshold of detection. $\Delta\Delta Ct = $ (Ct gene–Ct GAPDH)–(mean Ct gene control group–mean Ct GAPDH control) was determined for each reaction. Normalization to a housekeeping gene (GAPDH) is valid under experimental conditions where the housekeeping gene is not altered by the experimental treatments, as occurs with GAPDH in the present experimental treatments (data not shown).

In Situ Hybridization Analysis

Since Fas-Associated protein with Death Domain (FADD) gene expression was seen as altered by microarray but was not detectable by the TaqMan PCR procedure, ISH was used to monitor the changes in FADD mRNA levels in the DG (Bregma −1.80 to −5.80 mm) of rats exposed to 14 days of withdrawal following cocaine SA (LgA-Day 14) and their respective controls (ND-Day 14). FADD mRNA analysis was conducted for each rat in two slides containing eight tissue-sections each, starting from the beginning of the DG (Bregma −1.80 mm) until approximately Bregma −5.80 mm. Briefly, before probe hybridization, slide-mounted tissue sections (approximately 30 μm) from the left-half brain were fixed for 1 h in 4% PFA at room temperature, rinsed with salt buffers, and dehydrated with graded alcohols. After air-drying, the sections were hybridized with a ^{35}S-labeled cRNA probe previously cloned from cDNA fragments with specific primers using standard *in vitro* transcription methodology (FADD: 542 nucleotide fragment directed against the rat FADD mRNA coding region, nucleotides 355–898; see García-Fuster *et al*, 2009). The probes were labeled by incorporation of ^{35}S-UTP and ^{35}S-CTP and hybridized to tissue overnight at 55°C. The next day, sections were washed with increasing stringency, dehydrated with graded alcohols, air-dried, and exposed to film for 2 weeks. The specificity of the hybridization signal was confirmed with a sense probe control (data not shown). Digital images were scanned and integrated optical density (IOD) was measured for all the DGs per rat using an image analysis system (Image J, Version 1.43u). For the overall analysis, percent changes in LgA-Day 14 IOD with respect to control samples (ND-Day 14, 100%) were calculated. In addition to estimating the overall change in FADD mRNA, quantitative analysis was shown in relation to distance to Bregma in ND- and LgA-Day 14 rats. For this analysis, data are presented for each group as the mean IOD in the DG at the specific Bregma level.

Western Blot Analysis

The regulation of FADD protein was assessed in the right-half hippocampus of ND- and LgA- (Day 1, 14, and 28) rats by WB analysis. To do so, brain tissue samples were prepared in the presence of various protease inhibitors. Aliquots of total homogenate of known protein concentration (BCA Protein Assay, Pierce, IL) were mixed with equal volumes of electrophoresis loading buffer, denatured, and stored at −20 °C until use. Hippocampal proteins (40 μg) were separated under non-reducing conditions on 10% SDS-PAGE minigels (Bio-Rad Laboratories, Hercules, CA), followed by standard immunoblotting procedures (García-Fuster *et al*, 2003). The primary antibodies used (overnight incubation at 4 °C) were selected based on previous experiments and characterization in rat brain (see García-Fuster *et al*, 2009): anti-FADD (dilution 1 : 2500; sc-5559; Santa Cruz); and anti-β-actin (dilution 1 : 10 000; clone AC-15; Sigma). The secondary antibody (anti-rabbit or -mouse) was incubated for 1 h at room temperature at a 1 : 5000 dilution in blocking solution. The immunoreactivity was detected with an ECL detection system (Amersham International, Buckinghamshire, UK) and visualized by exposure to Hyperfilm ECL (Amersham) for 30 s to 60 min. The films were quantified by densitometric scanning (IOD) using Image J software. The amount of hippocampal target proteins was compared in the same gel between the control group (ND) and the drug group (LgA) at each specific time point of withdrawal. This procedure was repeated several times in different gels (each gel with different samples from ND-control and LgA rats). Percent changes in immunoreactivity with respect to control samples ND (100%) were calculated in various gels and the mean value was used as a final estimate. The content of β-actin was quantified as a loading control.

Assay for Apoptotic Cell Death

The induction of abnormal cell death was monitored in the right-half hippocampus (ND-Day 14 *vs* LgA-Day 14) by measuring the cleavage of nuclear enzyme poly(ADP-ribose)-polymerase (PARP) mediated by executioner caspase-3 (116 kDa PARP to 85 kDa fragment), which is a hallmark of apoptosis (see García-Fuster *et al*, 2007, 2009). The kit is designed to detect PARP cleavage by WB analysis (anti-PARP: dilution 1 : 800; Calbiochem) as described above.

Data and Statistical Analysis

SA behavior was analyzed with linear mixed effects model analysis (PASW 18.0) followed by Bonferroni corrected *post hoc* comparisons. Microarray data were analyzed using Illumina BeadStudio Software (version 3.1). Two samples from LgA-Day 14 group were excluded from further analysis because of poor quality (BeadStudio Gene Expression Module, version 3). Ingenuity Pathway Analysis (Ingenuity Systems, version 8.0, Redwood City, CA) was used to filter microarray data as described in Bernard *et al* (2010) to only include genes with Student's *t*-test *p*-values of ⩽0.1. These relaxed parameters were used to maximize the number of candidate genes for downstream analysis. A specific gene was considered significantly altered if (1) the *p*-value was ⩽0.05, and (2) the fold-change was ⩾1.2. The rest of the data were analyzed with GraphPad Prism, version 5. Results are expressed as mean values ± SEM. One-way or two-way ANOVAs followed by a multiple comparison test and

Student's one or two-tailed *t*-test were used for statistical evaluations. Pearson's correlation coefficients were calculated to test for possible association between variables. The level of significance was chosen as $p \leqslant 0.05$.

Drugs and Chemicals

Cocaine–HCl was obtained from Mallinckrodt Inc (St Louis, MO). PARP Cleavage Detection Kit was obtained from Calbiochem (Darmstadt, Germany). Other materials were purchased from Santa Cruz Biotechnology, Sigma-Aldrich, and Vector Laboratories.

RESULTS

Escalation of Cocaine Intake during Cocaine SA Procedures

Before the experiments reported in this paper, we conducted a pilot dose-finding study using a separate group of purchased Sprague–Dawley rats to determine the optimal dose of cocaine to elicit escalation of drug intake during LgA cocaine SA procedures (three doses tested: 0.5, 1, and 2 mg/kg per infusion, 5-h session per day, 14 days; data not shown). Our preliminary studies showed that a dose of 0.5 mg/kg per infusion resulted in a profound increase in drug intake from the first 5-h SA session (day 1) to the last (day 14) (data not shown). Consequently, for the present results, we followed a similar paradigm (0.5 mg/kg per infusion, 5-h session per day, 14 days; see Figure 1a for experimental design) with a new batch of rats. As shown in Figure 1b, LgA cocaine SA procedures (0.5 mg/kg per infusion, 5-h session per day, 14 days) elicited a profound increase in drug intake from the first SA session (day 1) to the last (day 14) (effect of session: $F(13, 100) = 26.09$, $p < 0.0001$). Rats progressively increased their daily cocaine intake starting at an average of 15 mg/kg on the first day of SA and finishing at an average intake of about 50 mg/kg on the final SA session day (Bonferroni *post hoc* comparison session 1 *vs* session 14, ***$p < 0.0001$). When examining the motivational aspects of cocaine intake by analyzing the first hour of each of the 5-h sessions there was no difference in the pattern of intake when compared with the entire session—average intake of 3.5 mg/kg and 12.5 mg/kg on the first and last day of SA respectively—(data not shown). The present behavioral data provided a good animal model to further ascertain the cellular and molecular adaptations that occur in the hippocampus following cocaine exposure.

Decreased Proliferation of Adult Hippocampal Stem Cells

As depicted earlier, the brains from rats that underwent cocaine SA procedures (LgA) and their respective controls (ND, see Figure 1a) were used to examine hippocampal proliferation with different cell markers. Figure 2 shows the effects of the time-course of withdrawal (ie, Day 1, Day 14, and Day 28) following cocaine SA on: (1) Ki-67 + cells (proliferation of all dividing cells, within a cell cycle time of 25 h; Cameron and McKay, 2001), and (2) NeuroD + cells

(proliferation of neural progenitor cells) in the DG (see García-Fuster *et al*, 2010).

Measures of Ki-67 + (Figure 2a) and NeuroD + (Figure 2b) cells at the different time points of analysis revealed that: (1) chronic exposure to cocaine, as measured 24 h following the last 5-h SA session (LgA-Day 1), did not alter the number of Ki-67 + or NeuroD + cells in the DG relative to ND-Day 1 control group (Ki-67: $p = 0.95$, NS; NeuroD: $p = 0.31$, NS); (2) 14 days of withdrawal following the last 5-h SA session led to a significant decrease in the number of Ki-67 + and NeuroD + cells in the DG relative to ND-Day 14 control group (Ki-67: **$p < 0.01$; NeuroD: *$p < 0.05$); and (3) 28 days of withdrawal following the last 5-h SA session did not change the number of Ki-67 + or NeuroD + cells in the DG when compared with the ND-Day 28 control group (Ki-67: $p = 0.65$, NS, NeuroD: $p = 0.90$, NS).

Interestingly, if only comparing the LgA groups (LgA-Day 1, LgA-Day 14, and LgA-Day 28), Day 28 samples have similar levels of Ki-67 + and NeuroD + cells compared with those observed at Day 14 (see groups LgA-Day 28 in Figures 2a and b). However, as the control groups also had lower values on these measures (see ND-Day 28 both for Ki-67 + and NeuroD + cells), these comparisons did not reach statistical significance. In fact, there were no significant differences between the control groups at any time point of withdrawal (ND-Day 1, ND-Day 14, and ND-Day 28) as measured by a one-way ANOVA followed by Tukey's multiple comparison test (Ki-67: $F(2, 14) = 1.861$, $p = 0.19$, NS; NeuroD: $F(2, 14) = 1.474$, $p = 0.26$, NS).

When splitting the analysis by anterior (-1.80 to -4.52 mm from Bregma) and posterior (-4.52 to -6.80 mm from Bregma) hippocampal demarcation (Guzmán-Marín *et al*, 2003; Noonan *et al*, 2008), the posterior had more positive cells than the anterior part (Ki-67: ***$p < 0.001$, Figure 2c; NeuroD: **$p < 0.01$, Figure 2d). The results showed a similar pattern as the overall cell quantification, with decreased Ki-67 + (Figure 2c) and NeuroD + (Figure 2d) cells in LgA-Day 14 both in the anterior and posterior demarcation (Ki-67: *$p < 0.05$; NeuroD: *$p < 0.05$) when compared with ND-Day 14. Moreover, if the analysis was split by anatomical level (Figures 2e and f), a two-way ANOVA followed by Bonferroni's *post hoc* test revealed an effect of group (ND-day 14 *vs* LgA-Day 14; Ki-67, $F(1, 120) = 15.82$, $p = 0.0001$; NeuroD, $F(1, 119) = 14.77$, $p = 0.0002$) and an effect of anatomical level (Bregma -1.80 to -6.80; Ki-67, $F(11, 120) = 13.09$, $p < 0.0001$; NeuroD, $F(11, 119) = 14.82$, $p < 0.0001$). However, no interaction between group and anatomical level was found (Ki-67, $F(11, 120) = 1.41$, $p = 0.175$; NeuroD, $F(11, 119) = 0.88$, $p = 0.566$). *Post hoc* analysis revealed that changes in Ki-67 + cells occurred at the more posterior level of the hippocampus (distance from Bregma: -6.30 mm, **$p < 0.01$).

In an attempt to ascertain the relationships between the different variables that account for the changes in Ki-67 + and NeuroD + cells during cocaine withdrawal we carried out several correlational analyses. There was a significant positive correlation between Ki-67 + and NeuroD + cells ($r = 0.76$; $n = 37$; ***$p < 0.00001$) in the DG when all treatment and control groups were collapsed (Figure 3a). If the analysis was conducted separately for each treatment group (ie, basal effect in the ND controls and drug effect in the LgA groups), the correlation
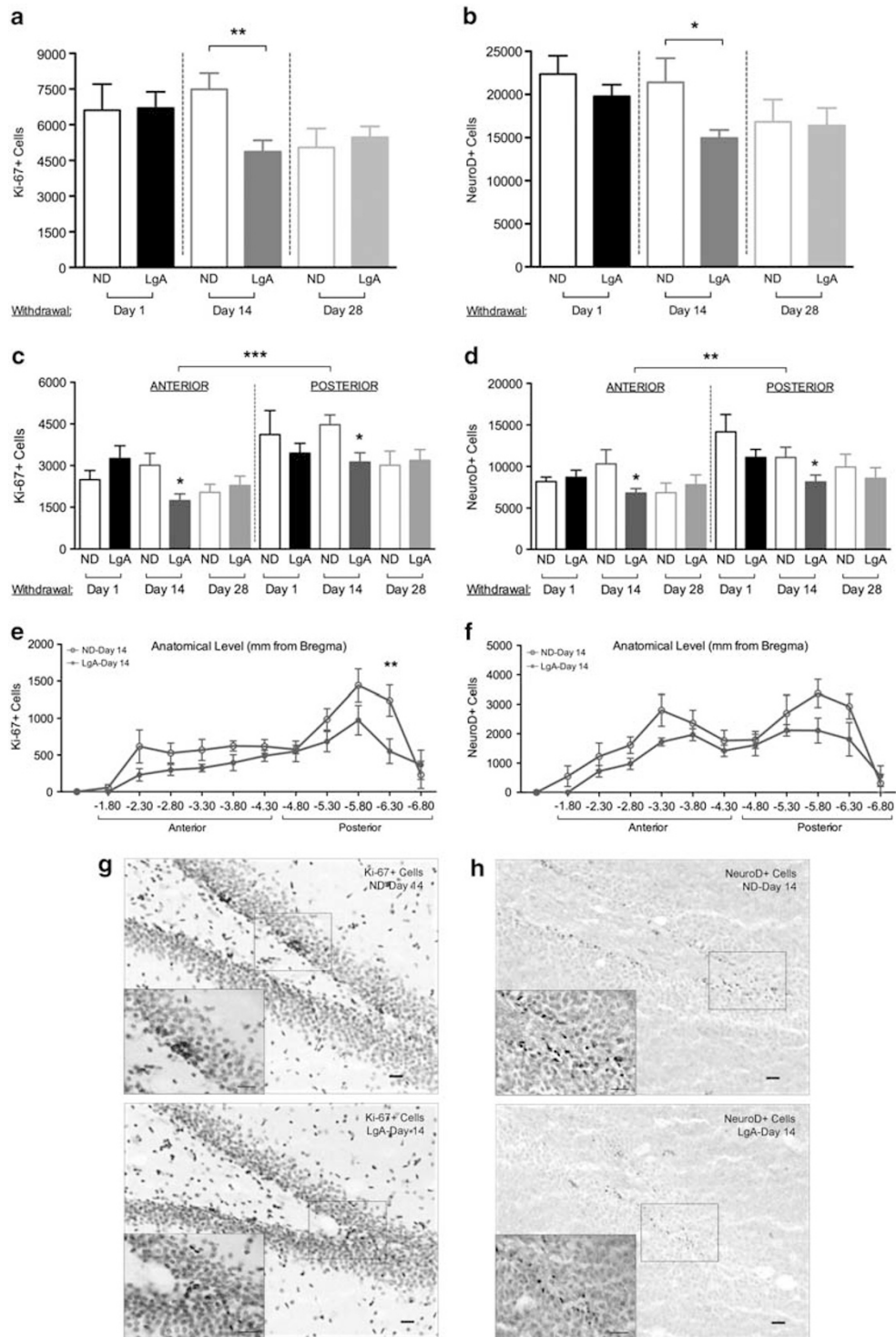
Figure 2 Decreased proliferation of adult hippocampal stem cells. (a, b) Quantitative analysis of Ki-67 + (a) and NeuroD + (b) cells in the left hemisphere DG revealed a decrease in cell proliferation at Day 14 of withdrawal following LgA cocaine SA. **$p < 0.01$, *$p < 0.05$ when compared with their respective control (ND-Day 14). (c, d) Analysis split by anterior (-1.80 to -4.52 mm from Bregma) and posterior (-4.52 to -6.80 mm from Bregma) hippocampal demarcation. The posterior DG had more Ki-67 + and NeuroD + cells than the anterior DG. ***$p < 0.001$, **$p < 0.01$. Ki-67 + and NeuroD + cells were decreased both in the anterior and posterior demarcation (Ki-67: *$p < 0.05$; NeuroD: *$p < 0.05$) in LgA-Day 14 when compared with ND-Day 14. (e, f) Quantitative analysis of Ki-67 + (e) and NeuroD + (f) cells in relation to distance from Bregma in ND-Day 14 and LgA-Day 14 groups. (g, h) Representative IHC of Ki-67 + (g, brown labeling) and NeuroD + (h, dark blue labeling) comparing ND vs LgA at Day 14. The bigger images were taken in a light microscope using a $20 \times$ objective lens to illustrate the anatomy of the DG. For each image, the left-bottom corner shows a representative IHC image taken in a light microscope using a $63 \times$ oil objective lens. Scale bar: 25 µm. The color reproduction of this figure is available at the *Neuropsychopharmacology* journal online.
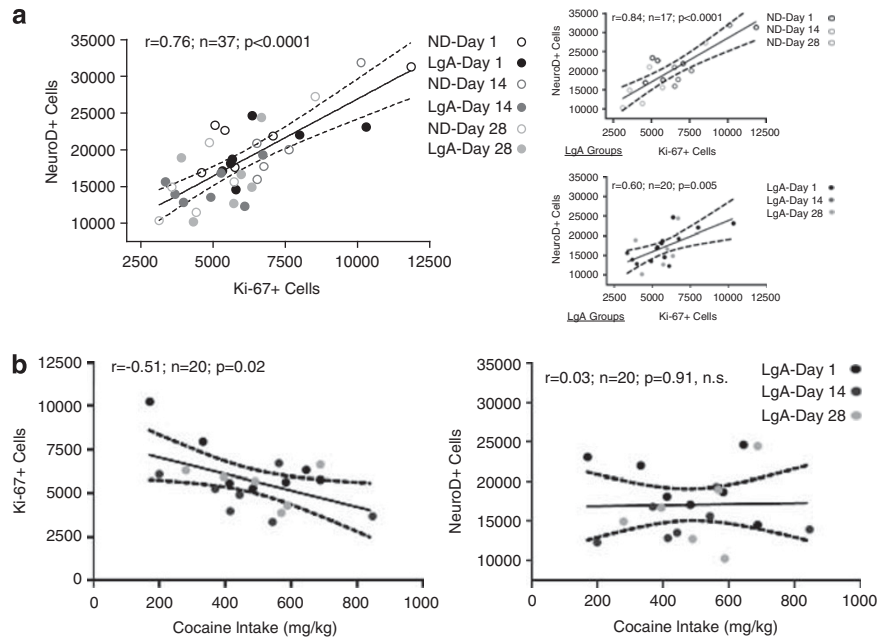
Figure 3 Correlation analysis. (a) Scatterplot depicting a significant positive correlation between the number of Ki-67 + and Neuro + cells in the DG of rats independently of their treatment. Each symbol represents a different rat. The solid line is the best fit of the correlation ($r = 0.76$, $n = 37$, $p < 0.0001$). The dotted curves indicate the 95% confidence interval for the regression line. To note that this correlation is still there for each treatment group individually (ND groups: $r = 0.84$, $n = 17$, $p < 0.0001$; LgA groups: $r = 0.60$, $n = 20$, $p = 0.005$). (b) Scatterplot depicting a significant negative correlation between the number of Ki-67 + cells and the amount of cocaine intake (mg/kg) in the DG of LgA groups rats. Each symbol represents a different rat. The solid line is the best fit of the correlation ($r = 0.51$, $n = 20$, $p = 0.02$). The dotted curves indicate the 95% confidence interval for the regression line. To note that there was no correlation when comparing the number of NeuroD + cells with the amount of cocaine intake ($r = 0.03$, $n = 20$, $p = 0.91$).

remained for both groups (ND groups, $r = 0.84$; $n = 17$; ***$p < 0.0001$; LgA groups: $r = 0.60$; $n = 20$; **$p = 0.005$). However, the area under the curve for the LgA groups is smaller, indicating that cocaine exposure reduced the number of Ki-67 + and NeuroD + cells in the DG of drug-exposed animals.

Figure 3b illustrates that the magnitude of decrease in cell proliferation as measured by Ki-67 + cells in the DG was correlated with the total amount of cocaine intake (mg/kg) ($r = -0.51$, $n = 20$, *$p = 0.02$). However, there was no correlation when comparing the number of NeuroD + cells with the amount of cocaine intake ($r = 0.03$, $n = 20$, $p = 0.91$, NS).

Thus, this study provides a specific time-point of cell proliferation regulation during withdrawal (ie, 14 days) at which both Ki-67 + (ie, proliferation of all dividing cells) and NeuroD + cells (ie, proliferation of cells that are fated to become neurons) were decreased. Since the only significant effects were found at Day 14 of withdrawal, the remaining experiments and analyses were conducted at this time-point.

Gene Expression Profile Analysis

The analysis comparing the left DG of ND *vs* LgA at Day 14 of withdrawal revealed the top networks by rank order and their associated network functions. The results uncovered differential regulation of several genes associated with cell fate regulation. In particular, the top bio functions associated with gene expression changes included molecular and cellular functions such as cell death, cell-to-cell signaling and inter-

action, cell morphology, cellular assembly and organization, and cellular development (see Table 1). Given the decrease in Ki-67 + and NeuroD + cells at 14 days of withdrawal following LgA cocaine SA, we decided to further analyze the list of candidate genes rendered in the top bio function (ie, cell death). The list provided 28 genes, 10 of which were genes of known sequences. A complete list of altered genes for the associated top three network functions can be found in the Supplementary Material (Supplementary Figure I).

As mentioned above, given that cell proliferation occurs mainly in the SGZ, which represents a fraction of the total DG, evaluation of gene expression profiles in the entire DG may dilute the effects taking place in the SGZ (Ki-67 + and NeuroD + cell counts). Indeed, NeuroD gene expression was not found altered in the microarray analysis when comparing ND and LgA groups at Day 14 of withdrawal. Moreover, among the genes found to be altered in a gene expression profile analysis some will be false positives and some false negatives.

Gene Expression Profile Validation

To validate some of the changes observed in gene expression by RT-PCR, we performed gene quantification in samples from the left hemisphere DG with custom ordered TaqMan Arrays. The results showed that two genes reached statistical significance, Achaete-scute complex homolog 1 (Ascl-1) and DNA (cytosine-5)-methyltransferase 3 beta (Dnmt3b), both previously described to have a role in cell fate regulation. However, FADD gene expression fell below the gene-card threshold level, and thus was undetectable by this method. Two factors could account for

Table I This Table Illustrates the Results of the Microarray Experiment and Further Validation by RT-PCR or ISH Performed in ND- and LgA-Day 14 Samples from the Left Hemisphere DG

Top networks

Associated network functions *by rank order* (p < *0.001*)	# Molecules	Score
1. Cellular growth and proliferation, embryonic development, molecular transport	22	42
2. Cellular assembly and organization, behavior, organ morphology	23	40
3. Neurological disease, genetic disorder, cancer	17	30
4. Cellular development, nutritional disease, organismal injury and abnormalities	15	25
5. Cellular compromise, cell signaling, molecular transport	14	22

Top bio functions

Molecular and cellular functions	# Molecules	*p*-Value
Cell death	28	0.0003–0.0472
Cellular development	28	0.0015–0.0498
Cellular assembly and organization	20	0.0015–0.0498
Cell-to-cell signaling and interaction	14	0.0011–0.0498
Cell morphology	14	0.0015–0.0498

Cell death	# Molecules	*p*-Value
ABCC5, ASCL1, CD200, DICER1, DMD, DNASE1, DNMT3B, F2R, FADD, FAF1, JAK3, KIAA1967, LDHA, LGALS1, MAOB, MT1F, NTRK3, NUF2, PHIP, PLCD1, PPP2R2B, PPT1, PTPRE, RECK SCG5, SMAD2, SNCG, SOD2	28	0.0361

Gene name	Symbol	Microarray results		Gene expression validation	
		F-change	*p*-Value	F-change	*p*-Value
Achaete-scute complex homolog 1 (Drosophila)	ASCL1	−1.35	0.036	−3.03	0.010
Deoxyribonuclease I	DNASE1	−1.32	0.036		
DNA (cytosine-5-)-methyltransferase 3 beta	DNMT3B	−1.57	0.030	−4.01	0.059
Coagulation factor II (thrombin) receptor	F2R	−2.36	<0.001		
Fas (TNFRSF6)-associated via death domain	FADD	1.31	0.022	1.35	0.014
Janus kinase 3	JAK3	−1.47	0.020		
Phospholipase C, delta 1	PLCD1	−1.35	0.047		
Protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform	PPP2R2B	1.34	0.018		
Palmitoyl-protein thioesterase 1	PPT1	1.30	0.046		
Synuclein, gamma (breast cancer-specific protein 1)	SNCG	−1.68	0.022		

Only genes with known sequences
F-change > 1.2, *p* < 0.05

The microarray data were filtered to only include genes with Student's *t*-test *p*-values of ≤0.1. These relaxed parameters were used to maximize the number of candidate genes for downstream analysis. The table includes the results from the analysis and includes the top network functions and the top bio functions. Cell death was the function with more molecules dysregulated (28 genes). For further analysis, a specific gene was considered significantly altered if (1) the *p*-value was ≤0.05, and (2) the fold-change was ≥1.2.

this issue: (1) the initial limited amount of cDNA material in our samples, and (2) the fact that this transcript might have relatively low abundance in the DG. Therefore, considering FADD has been described as a key molecule regulating apoptotic and/or proliferative events (Tourneur and Chiocchia, 2010) involved in cocaine addiction (García-Fuster *et al*, 2009), and was significant in the microarray analysis (F-change = 1.31, *p* = 0.022), further validation was done at the mRNA level by ISH and at the protein level by WB analysis.

Possible Role of the Cell Fate Regulator FADD

As depicted in Figure 4a, FADD mRNA was increased in the left hemisphere DG of rats exposed to 14 days of withdrawal following LgA cocaine SA procedures when compared with their respective controls (*p* = 0.014). Moreover, if the analysis was split by anatomical level (see Figure 4b), a two-way ANOVA followed by Bonferroni's *post hoc* test revealed an effect of group (ND-Day 14 *vs* LgA-Day 14; $F(1, 145) = 20.62$, $p < 0.0001$), an effect of anatomical level
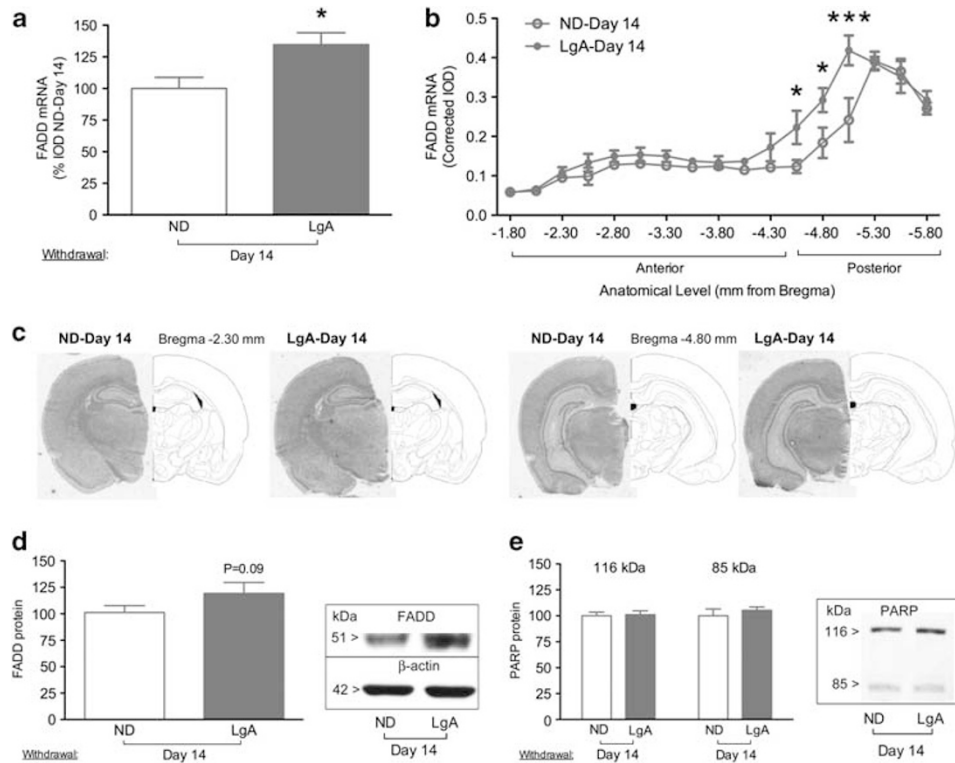
Figure 4 Possible role of the cell fate regulator FADD. (a) ISH analysis: data represents mean ± SEM mRNA level of LgA-Day 14 rats when compared with the percentage of the ND-control group (100%) at 14 days of withdrawal (ND-Day 14) in the left hemisphere DG determined by ISH analysis (*$p = 0.014$). (b) ISH analysis: FADD mRNA across the anatomical level of the DG analyzed (−1.80 to −5.80 mm from Bregma) (*$p < 0.05$, ***$p < 0.001$ vs ND-Day 14). (c) ISH analysis: representative X-ray images of FADD mRNA for each treatment group at two levels of analysis (anterior DG: −2.30 mm and, posterior DG: −4.80 mm from Bregma) (d) WB analysis: data represent mean ± SEM protein level (LgA-Day 14), and are expressed as percentage of the ND-control group (100%) at 14 days of withdrawal (ND-Day 14) in dissected right hippocampus. Student's *t*-test did not detect significant changes between groups. FADD: nonsignificant 19% increase, $p = 0.09$. The figure also illustrates representative immunoblots of the corresponding proteins. β-Actin was used as a loading control. (e) Assay for apoptotic cell death. ND-Day 14 and LgA-Day 14 showed similar levels of basal 116 kDa PARP cleavage (∼85 kDa fragment) indicating similar rates of basal induction of apoptotic cell death in the right hemisphere hippocampus. The figure also illustrates a representative immunoblot of the corresponding protein.

(Bregma −1.80 to −5.80 mm; F(16, 145) = 38.52, $p < 0.0001$), and an interaction between group and anatomical level (F(16, 145) = 2.31, $p < 0.01$). *Post hoc* analysis revealed that changes in FADD mRNA occurred at the most posterior level of the DG (distance from Bregma: −4.55 mm, *$p < 0.05$; −4.80 mm, *$p < 0.05$; −5.05 mm, ***$p < 0.001$). These results validated the data observed in the microarray analysis and pointed out the importance of the abundance of the gene and tissue limitations when choosing a method to validate the results.

Moreover, the results showed that, although it did not reach statistical significance, FADD protein was increased (20%, $p = 0.09$) in the right hemisphere hippocampus of LgA-Day 14 rats when compared with ND-Day 14 group (see Figure 4d). At the other withdrawal time-points, there was no change in FADD protein content (ND- *vs* LgA-Day 1 and ND- *vs* LgA-Day 28) (data not shown). β-Actin was used as a loading control and therefore was not altered by the treatment. To note that for WB analysis we freshly dissected the whole right hippocampus, while for gene expression analysis we focused on the left hemisphere DG and did so in an anatomical context. Thus, some of the protein changes occurring at the level of the DG could be masked or diluted when examining the whole hippocampus. Moreover, some studies suggested that cell proliferation (Czéh *et al*, 2007) and

gene expression profiles (Stansberg *et al*, 2007) in rat brain are different and differently regulated between the left and right hemisphere and along front and back of the DG.

Assay for Apoptotic Cell Death

PARP enzyme, which is involved in DNA damage following DNA nicks, was investigated in the right hippocampus as a molecular marker of cell death (Cagnol *et al*, 2006). As shown in Figure 4e, ND-Day 14 and LgA-Day 14 showed similar levels of basal 116 kDa PARP cleavage (∼85 kDa fragment) indicating similar rates of basal induction of apoptotic cell death.

DISCUSSION

This study examined changes in hippocampal cell fate regulation in an animal model that mimics some of the key features manifested during the transition to cocaine addiction in humans (eg, escalation of drug intake). Previous work from our lab had examined the effects associated with experimenter-administered cocaine on alterations in neural plasticity (see García-Fuster *et al*, 2009, 2010). In this study, we utilized a rat model with better

clinical validity in which to study the hippocampal adaptations associated with withdrawal after cocaine abuse.

Using Sprague–Dawley rats we demonstrated that 5-h of extended daily access to cocaine for 14 days (LgA) can elicit a profound increase in drug intake from the first SA session to the last, providing an excellent model to study the neural adaptations that accompany cessation of cocaine exposure. Our findings demonstrate that LgA cocaine SA leads to alterations at various levels of hippocampal cell fate regulation at a specific time point during the course of withdrawal (ie, Day 14). Specifically, we showed that: (1) proliferation of adult hippocampal stem cells was decreased—Ki-67+ mitotic progenitor cells, and NeuroD+ neural progenitor cells; (2) several ensembles of genes were significantly altered, generally revolving around various facets of cellular morphology, cell development or cell death; (3) specific genes associated with cell fate regulation were validated as being dysregulated during withdrawal, including Ascl-1 and Dnmt3b, two potential novel targets for the treatment of cocaine withdrawal; and (4) FADD, a molecular marker previously associated with the propensity to substance abuse and cocaine sensitization (García-Fuster et al, 2009), was upregulated, which suggests this is a key molecular candidate in mediating hippocampal cell fate regulation during cocaine withdrawal.

Decreased Proliferation of Adult Hippocampal Stem Cells

The chronic administration of cocaine following LgA SA procedures (Day 1) did not alter proliferation of adult hippocampal stem cells, as measured by the number of Ki-67+ cells 24 h after the last SA session. Using a similar experimental design, Noonan et al (2008) reported a decrease in cell proliferation as measured by BrdU+ cells 24 h following the last cocaine SA session (0.5 mg/kg per infusion, 4-h session per day, 3 weeks). However, differences in the experimental design (ie, food-restriction during pre-training, amount of cocaine intake in 4- vs 5-h of daily SA session) and in the marker used to quantify cell proliferation (ie, BrdU only labels the % of proliferating cells that entered S-phase) could account for this discrepancy and adds to the complexity of comparing data across labs. Even though BrdU+ and Ki-67+ cell numbers are often used as interchangeable indices of proliferation (Kee et al, 2002; Wojtowicz and Kee, 2006), cells in S-phase may be particularly susceptible to certain stimuli (Arguello et al, 2008). The same group (Lagace et al, 2010) recently described discrepancies in how a specific treatment in mice affected cells that entered S-phase (BrdU labeling) vs all proliferating cells (Ki-67 labeling), as they found a decrease in BrdU+ and no change in Ki-67+ cell number 24 h after treatment. This interpretation is limited because of the fact that cell cycle length is about half as long in the mouse than in the rat. However, in another study conducted in rats and using BrdU and Ki-67 as mitotic makers to measure changes in cell proliferation (Domínguez-Escribà et al, 2006), Ki-67+ cell counts failed to reach statistical significance between the groups because of variability of the data. In any case, Noonan et al (2008) have also shown that a longer chronic paradigm of cocaine SA (7 weeks, 4-h session per day) did not alter cell

proliferation as measured by Ki-67+ cells. Moreover, the present results show that chronic administration of cocaine (ND- vs LgA-Day 1) did not have an impact on the number of the proliferating cells that are fated to become neurons (NeuroD+ cells). Similarly, cocaine SA did not alter the number DCX+ cells (immature neurons) (Noonan et al, 2008).

During the course of withdrawal (Day 14), proliferation of mitotic (Ki-67+) and neural progenitor (NeuroD+) cells was decreased. To our knowledge there are no studies looking at these measures at Day 14 of withdrawal after extended daily access to cocaine SA in rats. Following a similar anterior–posterior cell proliferation pattern as the one presented in this study, Lagace et al (2006) showed that the posterior part of the DG had more proliferating cells than the anterior part. The same pattern was described for DCX+ cells (Noonan et al, 2010). The decrease in Ki-67+ and NeuroD+ cells occurred throughout the DG, both at the anterior and posterior demarcations (Figures 2c and d). However, when comparing ND- and LgA-Day 14 by anatomical level (Figures 2e and f), the decrease in Ki-67+ cells was observed only at the most posterior part of the DG. Similarly, previous studies showed differences in proliferation across the entire longitudinal axis; yet the more posterior part of the hippocampus seems to be the preferential site of regulation of proliferation by restraint stress (Kim et al, 2005) and by the antidepressant drug agomelatine (Banasr et al, 2006). Decreased cell proliferation might be a reflection of enhanced cell death. However, the present results showed similar levels of basal induction of apoptotic cell death when comparing ND-Day 14 and LgA-Day 14.

At Day 28 of withdrawal there was no effect on the number of Ki-67+ and NeuroD+ cells (ND- vs LgA-Day 28). Previous results found that withdrawal from cocaine normalized the deficits in cell proliferation and increased the number of immature neurons (DCX+ cells) in the DG (Noonan et al, 2008). The normalization seen at Day 28 could be due to a lower rate of cell proliferation in the ND-Day 28 control groups. However, there were no significant differences in the number of positive cells between the control groups (Day 1, Day 14, and Day 28) for Ki-67 ($F_{(2, 14)} = 1.861$, $p = 0.192$) or for NeuroD ($F_{(2, 14)} = 1.474$, $p = 0.262$). Moreover, basal corticosterone levels were measured in trunk blood at the time of killing and there were no differences when comparing the control groups (one-way ANOVA followed by Tukey's test: $F_{(2, 14)} = 1.812$, $p = 0.120$, data not shown). Nevertheless, we question whether differences at Day 28 could be masked because of a floor effect and whether a number of variables inherent in the experimental design might obfuscate the results. For example, the age of the animals at this later time-point (ie, approximately 140 days old when killed) and the fact that the rats were socially isolated (ie, individually housed) for a prolonged period of time and withdrawn from experimental handling could all be potential stressors impacting interpretation of the results.

Changes in Ki-67+ and NeuroD+ cells were positively correlated when all treatment groups were collapsed. Our results indicate that 14 days of cocaine withdrawal decreased cell proliferation (Ki-67+ cells) and the proliferation of cells that are fated to become neurons (NeuroD+

cells). There appears to be some controversy as to whether Ki-67 and NeuroD colocalize in the same population of cells. It has been described that about 75% of all proliferating type-2 cells co-express NeuroD (type 2b/3 progenitors; see Steiner *et al*, 2006). In mice NeuroD has been described to colocalize with Ki-67 only in a small subset of progenitors (Gao *et al*, 2009). In contrast, another study showed that Ki-67 and NeuroD do not colocalize in the same population of cells (Larsen *et al*, 2007), and therefore label distinct groups of cells. In any case new cells are born every day throughout life, leading to the situation where many different maturation stages exist in parallel and with close proximity to each other. It seem reasonable to hypothesize, therefore, that at least some of the reduction in NeuroD+ cells could be a consequence of the decreased proliferation of Ki-67+ cells. Further, our data show that both markers label different number of cell pools: Ki-67 labels all dividing cells during the 25-h length of the cell cycle, about 6000–9000 cells (see Figure 2a); while NeuroD labels neural precursors from different pools of proliferating cells, about 20 000–25 000 cells (Figure 2b). This could explain why our findings linked decreased number of Ki-67+ cells to the increased amount of drug administered and no relationship between NeuroD+ cells and drug intake was observed. Similarly, previous data reported that decreases in Ki-67 were attributable to the total amount of drug administered based on use of differential paradigms (ie, intermittent short-access SA, short-access SA, and long-access SA) (Mandyam *et al*, 2008). Moreover, the same study reported that doublecortin (DCX), a marker of young neurons, was not correlated with the amount of drug intake.

What might be the functional significance of these observed changes in the hippocampus? A reduction of adult hippocampal neurogenesis (DCX+ cells) has recently been shown to enhance vulnerability to addiction and relapse (Noonan *et al*, 2010). The present results support the view that decreased cell proliferation of neural precursors (NeuroD+ cells) might participate in the restructuring of the hippocampus during cocaine withdrawal and identifies 14 days as a critical time-point of regulation. Withdrawal to drugs of abuse in general, and cocaine in particular, has been associated with increased propensity to develop a depression-like phenotype. Since alterations in hippocampal cellular composition have been implicated in depression and the mode of action of antidepressants (Malberg *et al*, 2000; Jacobs *et al*, 2000), one could speculate that the decreased number of mitotic (Ki-67+) and neural progenitors (NeuroD+) cells observed at 14 days of withdrawal might contribute to increased propensity for negative affect during withdrawal. For example, withdrawal from chronic SA enhances startle-induced ultrasonic distress vocalizations in the rat (Barros and Miczek, 1996; Mutschler and Miczek, 1998). A recent study using mice reported a significant negative correlation between rates of cell proliferation and cell survival and depression-like behavior at 14 days of withdrawal from alcohol. Moreover, the behavior was reversed by chronic antidepressant treatment during withdrawal (Stevenson *et al*, 2009). Therefore, changes in the cellular composition (ie, proliferation) of the DG could be involved in some of the alterations underlying cocaine withdrawal (ie, affective components) and warrants further investigation.

Gene Expression Profile Analysis and Validation

A fundamental understanding of the DG cellular adaptations induced by cocaine withdrawal requires investigating its molecular basis in order to develop novel targets for its regulation. To this end, we relied on a discovery approach by using gene expression profile analysis in the DG of ND-Day 14 *vs* LgA-Day 14 rats where the greatest changes in Ki-67+ and Neuro+ cells were observed. Given that cell proliferation mainly occurs in the SGZ, which represents a fraction of the total DG, evaluation of gene expression profiles in the entire DG may dilute the effects taking place in the SGZ (Ki-67+ and NeuroD+ cell counts). Therefore, given the number of cells analyzed (SGZ *vs* the whole DG) and the dilution factor derived from the analysis, unraveling effects on cell proliferation from transsynaptic effects on other hippocampal cells should be evaluated carefully. The results revealed highly significant dysregulation in a number of networks and molecular and celullar functions (Table 1). Interestingly, the dysregulation of 'cell death' emerged as the top bio function with highly significant changes in 28 genes. We went on to validate 3 of the 10 transcripts by RT-PCR or ISH.

One such gene, Dnmt3b, was downregulated in the DG of rats that underwent 14 days of withdrawal following LgA cocaine SA. Epigenetic mechanisms, such as DNA methylation, were initially described for their ability to promote differentiation and drive cell fate in embryonic and early postnatal development (reviewed in Hsieh and Eisch, 2010). In particular, Dnmt3b is a cellular methyltransferase that adds methyl groups *de novo* to unmethylated DNA and has been previously shown to participate in various stages of neural fate (Feng *et al*, 2005) including the regulation of synaptic plasticity in the hippocampus (Levenson *et al*, 2006). Dnmt3a and Dnmt1, which are two other key methyltransferases, were not found altered in the microarray analysis (data not shown). Thus, these data suggest a specific role for Dnmt3b on cell fate during cocaine withdrawal and one that deserves further investigation.

A second candidate gene, Ascl-1, also called as Mash-1, was decreased in the DG of rats with decreased number of Ki-67+ and NeuroD+ cells. Mash-1 is a member of the basic helix-loop-helix transcription factor group and contributes to the production of neuronal precursor cells (Johnson *et al*, 1990; Jessberger *et al*, 2008), thus having a role in the regulation of the restructuring of the hippocampus. Therefore, a decrease in the pro-neural gene Ascl1 goes along with the decrease observed at the level of proliferation of adult hippocampal stem cells (Ki-67+ and NeuroD+ cells), and also deserves further investigation.

Possible Role of the Cell Fate Regulator FADD

The present data showed an increase in FADD by gene expression profile analysis in the DG during the course of cocaine withdrawal (Day 14). This change was confirmed by ISH, with increased gene expression at the more posterior level of the DG. Interestingly, the differential response of the anterior and posterior demarcation, also observed by changes in cell proliferation, might be related to the distinct anatomic connections and functions of these regions. The

ventral and posterior DG receives many limbic projections, and is more implicated in emotion-like behaviors relative to the dorsal and anterior DG, which has a greater role in spatial processing (Bannerman et al, 2004; Sahay and Hen, 2007). Therefore, it is reasonable to hypothesize that changes in the cellular/molecular composition (ie, proliferation, FADD gene expression) of the more posterior part of the DG during cocaine withdrawal may be critical in understanding the neural adaptations that accompany drug cessation. Moreover, the hypothesis that FADD regulation in the DG parallels changes in cell proliferation (Ki-67 + and NeuroD + cells) at Day 14 of withdrawal is strengthened by the fact that there was no change in FADD protein content nor in cell proliferation markers either at Day 1 or Day 28 between ND and LgA groups.

FADD, among the many cell fate mediators, is a unique regulator of cell life and death and has a critical role in many essential cellular processes (Tourneur and Chiocchia, 2010). FADD, initially described to be part of the apoptotic machinery, has since been shown to control a variety of intracellular processes that regulate cell fate toward cell proliferation, cell growth, cell survival, and cell death. Thus, this molecule is pivotal for maintaining cellular function and homeostasis. Importantly, FADD has also been implicated in the neural adaptations associated with cocaine (García-Fuster et al, 2009) and morphine sensitization (Ramos-Miguel et al, 2010) as well as with responsiveness to opiate (García-Fuster et al, 2007) and cannabinoid drugs (Alvaro-Bartolome et al, 2010). Moreover, animals selectively bred for differential propensity to substance abuse (high- vs low-responder rats) showed basal differences in the expression of FADD (García-Fuster et al, 2009), suggesting this gene might be involved in conferring vulnerability to developing addiction. However, to our knowledge, its role in an animal model that better mimics the transition to cocaine addiction and resembles some of the key features of human addictive behavior has not previously been described. The fact that FADD emerged as one of the most altered transcripts in the context of gene expression profiling converges with the previous hypothesis-driven studies of its role, and underscores its importance in the sequelae of drugs of abuse and withdrawal on brain structure and function. Further experiments will attempt to better understand the molecular mechanisms behind FADD activation during cocaine administration and withdrawal and its specific role in hippocampal cell fate regulation. Moreover, the specific role of FADD in the regulation of newly proliferating cells in the SGZ will be evaluated in detail.

CONCLUSION

This study identified specific hippocampal adaptations during cocaine withdrawal at the cellular and molecular levels in an animal model that mimics some aspects of human addiction. In particular, the proliferation of adult hippocampal stem cells was decreased in the SGZ of the DG at several stages of regulation (Ki-67 + cells and NeuroD + cells) following 14 days of cocaine withdrawal. Moreover, several molecules were dysregulated in the DG of rats with impaired proliferation rates. FADD, a protein previously

linked to cell fate dysregulation (balance proliferation/apoptosis) in response to cocaine abuse (García-Fuster et al, 2009), was increased following 14 days of cocaine withdrawal. Moreover, this study identified two additional key molecules, Ascl-1 and Dnmt3b, which appear to be involved in the structural changes that accompany cocaine withdrawal. We suggest that the decrease in the self-renewal capacity of the DG together with the altered expression of some cell fate markers are key to better understanding the restructuring of the hippocampus during cocaine withdrawal. These discoveries, therefore, offer novel candidate targets for further investigation into the neural adaptations underlying cocaine withdrawal.

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)