Repeated morphine treatment alters polysialylated neural cell adhesion molecule, glutamate decarboxylase-67 expression and cell proliferation in the adult rat hippocampus

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Abstract

Altered synaptic transmission and plasticity in brain areas involved in reward and learning are thought to underlie the long-lasting effects of addictive drugs. In support of this idea, opiates reduce neurogenesis [A.J. Eisch *et al.* (2000) *Proceedings of the National Academy of Sciences USA*, **97**, 7579–7584] and enhance long-term potentiation in adult rodent hippocampus [J.M. Harrison *et al.* (2002) *Journal of Neurophysiology*, **87**, 2464–2470], a key structure of learning and memory processes. Here we studied how repeated morphine treatment and withdrawal affect cell proliferation and neuronal phenotypes in the dentate gyrus-CA3 region of the adult rat hippocampus. Our data showed a strong reduction of cellular proliferation in morphine-dependent animals (54% of control) that was followed by a rebound increase after 1 week withdrawal and a return to normal after 2 weeks withdrawal. Morphine dependence was also associated with a drastic reduction in the expression levels of the polysialylated form of neural cell adhesion molecule (68% of control), an adhesion molecule expressed by newly generated neurons and involved in cell migration and structural plasticity. Polysialylated neural cell adhesion molecule levels quickly returned to normal following withdrawal. In morphine-dependent rats, we found a significant increase of glutamate decarboxylase-67 mRNA transcription (170% of control) in dentate gyrus granular cells which was followed by a marked rebound decrease after 1 week withdrawal and a return to normal after 4 weeks withdrawal. Together, the results show, for the first time, that, in addition to reducing cell proliferation and neurogenesis, chronic exposure to morphine dramatically alters neuronal phenotypes in the dentate gyrus-CA3 region of the adult rat hippocampus.

Introduction

Long-lasting neuroadaptations in brain areas involved in reward and learning are thought to participate in the effects of addictive drugs. Addiction has been associated with durable alterations of elementary forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (Williams *et al.*, 2001). Adult neurogenesis persists throughout adult life in mammals in the subventricular zone of lateral ventricles and the subgranular layer of the dentate gyrus (DG; Altman & Das, 1965; Eriksson *et al.*, 1998; Gould *et al.*, 1999b; Kornack & Rakic, 1999; Gage, 2002). Several pieces of evidence indicate a prominent role of adult neurogenesis in hippocampalmediated learning (Gould *et al.*, 1999a; van Praag *et al.*, 1999). In the adult rat hippocampus, neurogenesis is reduced in morphine-dependent animals (Eisch *et al.*, 2000) but the precise duration of this effect during opiate withdrawal has not been determined.

Polysialylated neural cell adhesion molecule (PSA-NCAM), a cell surface protein implicated in the regulation of cell interactions (Bruses & Rutishauser, 2001), is transiently expressed by newly generated neurons during development (Rutishauser *et al.*, 1988). In the DG of adult rats,

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PSA-NCAM is expressed by newly formed granule cells and has been proposed to 'contribute to the early developmental events of adult neurogenesis' (Seki, 2002). In addition, PSA-NCAM has been involved in hippocampal synaptic plasticity; both LTP and long-term depression are impaired in NCAM-deficient animals or after treatment with the PSA-removing enzyme endo-N-endosialidase (Muller *et al.*, 1996; Cremer *et al.*, 1998; Bruses & Rutishauser, 2001). In the adult rat hippocampus, nicotine self-administration decreases neurogenesis and PSA-NCAM expression and increases cell death (Abrous *et al.*, 2002).

Finally, the phenotype of granule cells of the DG and of their projecting fibers (the mossy fibers) has been shown to vary during maturation and under pathological or experimental conditions. For instance, while in adult rats the phenotype of granule cells is predominantly glutamatergic, these neurons also express GABA (Sandler & Smith, 1991), glutamate decarboxylase (GAD) (Sloviter *et al.*, 1996) and both vesicular and membrane GABA transporters (Frahm *et al.*, 2000; Lamas *et al.*, 2001). Accordingly, a GABAergic component of mossy fiber transmission has been identified in hippocampal slices (Walker *et al.*, 2001). This GABAergic phenotype is constitutively expressed during the first 3 weeks of development, is abruptly down-regulated on post-natal days 23–24 (Gutierrez *et al.*, 2003) and remains poorly detectable thereafter. Nevertheless, in the adult rat, the GABAergic phenotype of the granule cells can be 'restored'

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in pathological conditions, e.g. after kainic acid-induced seizures (Schwarzer & Sperk, 1995) or in electrically kindled rats (Lehmann *et al.*, 1996) and in response to LTP-like stimulations (Sloviter *et al.*, 1996; Gutierrez & Heinemann, 2001; Ramirez & Gutierrez, 2001; Maqueda *et al.*, 2003). How addictive drugs modulate the phenotype of hippocampal granule cells has never been reported.

Here we determined the consequences of repeated exposure to morphine injection followed by 1, 2 and 4 weeks withdrawal on cell proliferation, cell death, PSA-NCAM expression and the GABAergic phenotype of granule cells in the adult rat DG.

Materials and methods

Animals and drug treatments

The adult male Sprague-Dawley rats (initial weight 240-250 g; Iffa-Credo, France) used for all experiments were housed in groups of three or four animals, under a 12-h light/dark cycle, at constant temperature and with ad libitum access to food and water. All the experiments were performed in accordance with European Communities Council Directives (86/609/EEEC) and authorized by the Direction départementale des services vétérinaires de la Gironde (no. 3300041). To decrease effects of handling and stress on cell proliferation, all rats were handled twice daily 5 days before starting morphine treatments. Sham rats were injected i.p. twice daily for 1 week with vehicle (NaCl 9%) and morphine-dependent rats were injected with morphine sulfate (20 mg/kg). For withdrawal conditions, rats received the same treatment and were kept alive, after the last injection, for 1, 2 and 4 weeks (1-, 2- and 4-week withdrawn rats). In all experiments and in all conditions tested, there was no significant difference between sham animals (1 week NaCl 9%, 1 week NaCl 9% and 1, 2 and 4 weeks of survival). Thus, all results are expressed compared with 1 week NaCl 9% -treated rats (control rats).

5-Bromo-2 -deoxyuridine injections and tissue fixation

To evaluate the effect of morphine injection on cell proliferation, 5-bromo-2'-deoxyuridine (BrdU, 150 mg/kg i.p. dissolved in NaOH, 0.1 N; Sigma-Aldrich) was injected into (i) control and morphine-treated rats at the end of the treatment (on day 8) and (ii) withdrawn rats 1, 2 and 4 weeks after the end of the morphine treatment. Three hours after the BrdU administration and under deep sodium pento-barbital anesthesia, animals were fixed by perfusion through the ascending aorta with 150 mL phosphate-buffered saline (PBS, pH 7.4) followed by 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Whole brains were dissected and fixed by immersion in the same fixative overnight at 4 °C.

Immunohistochemistry and histological staining

Serial coronal vibratome (50 μ m) sections through the hippocampus were collected and stored in PBS, pH 7.5, containing 0.1% sodium azide at 4 °C. Every tenth section was collected, rinsed in PBS and coded before processing for immunohistochemistry to ensure objectivity. All antibodies were diluted in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and 1% bovine serum albumin (Sigma-Aldrich).

For BrdU labeling, a pre-treatment with 2 N HCl for 30 min at room temperature (RT) was necessary. After being rinsed in PBS, sections were incubated for 72 h at 4 °C with primary antibodies: mouse anti-BrdU monoclonal antibody (1 : 100; Novocastra Laboratories, UK), mouse IgM anti-PSA-NCAM monoclonal antibody

(1 : 1000; kindly provided by Dr T. Seki, Japan) and mouse anti-GAD-67 monoclonal antibody (1 : 2000; Chemicon, USA). After four washes in PBS, sections were treated for 3 h at RT with either a Cy3labeled goat anti-mouse antibody (1 : 2000; Chemicon), an Alexa-Fluor 488-labeled goat anti-mouse IgM antibody (1 : 2000; Molecular Probes, USA) or an AlexaFluor 488-labeled goat anti-mouse IgG antibody (1 : 2000; Molecular Probes), then washed with PBS and placed on polylysine-coated slides (Calbiochem, USA) for 2 h at RT in darkness and mounted with Mowiol 4-88 (Calbiochem). Negative controls were carried out by omitting primary antibodies.

To assess cellular damage, sections adjacent to those processed for BrdU immunohistochemistry were stained with hematoxylin (Harris hematoxylin solution; Sigma-Aldrich) for 5 min after 3 days in PBStriton (0.1%) at 4 °C. After being washed in water for 3 min, slices were quickly incubated in acid-alcohol (HCl 1% in ethanol 70%), then washed (water, 2 min), dried (2 h, RT) and coverslipped. Pyknotic cells were identified by dark staining and pale or absent cytoplasm (Gould *et al.*, 1990) and counted under 100× magnification.

5-Bromo-2'-deoxyuridine quantification

The number of BrdU-immunoreactive (IR) cells was assessed in every tenth section through a rostro/caudal extent of 4000 µm through the hippocampus. The counting was performed with a DMR epifluorescent video-microscope (Leica) equipped with a Cool SNAP HQ camera (Photometrics) and driven by Metamorph software using a $100 \times / 1.32$ oil objective (Leica) in different hippocampal subregions, the hilus and subgranular layer. The subgranular layer was defined as a two-cell-thick layer along the inner border of the granular cell layer and the hilus; the hilus was defined by drawing straight lines from the tip of the two granular cell layer blades toward the tip of the CA3 layer. All BrdU-IR cells in the subgranular layer and hilus were counted, multiplied by 10 and reported as total number of cells per region (mean \pm SEM). Isolated proliferative cells were defined as one or two contiguous BrdU-IR cells while proliferative cell clusters were defined as three or more contiguous BrdU-IR cells (clusters of up to 11 cells were actually observed, data not shown). Contiguous cells and cells that were separated from another cell by a distance less than one cellular body were considered to belong to the same cluster.

Quantitative evaluation of polysialylated neural cell adhesion molecule and glutamate decarboxylase-67 labeling

Confocal image stacks were acquired on a DMR TCS SP2 AOBS confocal laser scanning microscope (Leica), using 20 ×/0.70 and 40×1.25 oil objectives (Leica), driven by confocal software (Leica). Working with a GreNe (green He/Ne) and an argon gas laser, two laser lines emitting at 488 and 543 nm were used for exciting Alexa 488- and Cy3-conjugated secondary antibodies, respectively. The exposure settings and gain were identical for each condition compared (note that images displayed in the figures are overexposed for a better visualization but do not correspond to the analysed images). The background noise of each confocal image was reduced by averaging two scans/line and two frames/image. Confocal analysis was restricted to the top of each section (i.e. where the penetration of all antibodies is optimum). The organization of the immunostained structures was studied on three successive confocal images, optically sliced in the z-plane with 1-µm interval, using Metamorph Imaging software (Universal Imaging Corporation). The numerical density of labeled cells in the subgranular layer of the DG and in the hilus was measured on such confocal images, collected from at least four

different sections per rat from at least four rats of the different groups examined. Acquired images were exported to Image Tools (UTHSCSA) and the surface of the DG and hilus was measured.

In situ hybridization

On day 8, control and morphine-dependent rats were anesthetized with pentobarbital and killed by decapitation. The same protocol was used 5 for 1- and 4-week-withdrawn rats, with killing on days 15 and 36, respectively. Brains were dissected on an ice-cooled plate, immediately frozen in -45 °C isopentane and stored at -80 °C until use. A previously described cDNA clone corresponding to a 2730-bp insert of the rat GAD-67 (Julien et al., 1987) in the plasmid pST18 (Pharmacia) was used. Anti-sense riboprobe was prepared by in vitro transcription from 100 ng of linearized plasmid (HindIII restriction enzyme) using [35S]UTP (> 1000 Ci/mmol) and SP6 RNA polymerase; after alkaline hydrolysis the probe was reduced to a final length of 0.50 kb. The [³⁵S]-labeled probe was purified by elution on a sephadex G50 column, precipitated with two volumes ethanol and 1/10 volume sodium acetate (3 M) and rehydrated. Cryostat coronal serial sections (14 µm, -18 °C) were thaw-mounted on gelatin-coated slides and processed for in situ hybridization. For pre-hybridization, slices were fixed by 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2, 10 min), then rinsed twice in $4 \times$ standard sodium citrate (SSC) and placed in 0.25% acetic acid anhydride in 0.1 M triethanolamine/ $4 \times SSC$ (pH 8.0, 10 min); slices were dehydrated in graded ethanol solutions (2 min each). Hybridization was performed overnight at 50 °C with 10⁶ cpm of [³⁵S]-labeled probes in 50 µL of hybridization buffer, pH 8.0 (20 mM Tris HCl, 50% formamide, 0.3 M NaCl, pH 8.0, 1 mM EDTA, 10% dextran sulfate, 1 × Denhardt's solution, 250 µg/mL yeast tRNA, 100 mg/mL salmon sperm DNA, 0.2% sodium dodecyl sulfate). Post-hybridization was performed with washes in $4 \times SSC$ (RT, 5 min, twice), RNAse A in RNAse buffer (4 M NaCl, 2 M Tris-HCl, pH 8.0, 0.25 M EDTA; 20 µg/mL, 20 min at 37 °C), $2 \times SSC$, $1 \times SSC$, $0.5 \times SSC$ (RT, 5 min each), $0.1 \times SSC$ (65 °C, 30 min, twice) and 0.1 × SSC (RT, 5 min). Slices were then dehydrated in graded alcohols. For revelation, slides were first exposed in contact with Biomax film (Kodak) for 4 days, then dipped into silver emulsion (Ilford K5 emulsion), dried overnight and exposed in the dark at 4 °C for 3 weeks. After revelation and fixation, the sections were counterstained with Mayer's hemalun and mounted.

Quantitative evaluation of glutamate decarboxylase-67 mRNA-labeled cells

For quantitative analysis, all sections from all conditions were processed for *in situ* hybridization in a single batch to ensure identical experimental conditions. Images were acquired on an IX71 inverted microscope using a $10 \times / 0.25$ objective (Olympus), equipped with a Cool SNAP HQ camera (Photometrics), driven by Metamorph software (Universal Imaging Corporation), working with transmitted light. The exposure settings were identical for each compared condition. The numerical density of labeled cells in the subgranular layer of the DG and in the hilus was estimated on such images, collected from four rats per condition and four sections per rat. Acquired images were exported to Image Tools (UTHSCSA) and the surface of the DG and hilus was measured.

Statistics

Statistical analysis was performed using ANOVA followed, if significant, by Tuckey multiple comparison test (Kyplot 2.0 β 13).

Results

Alteration of cell proliferation in the subgranular layer of the dentate gyrus in morphine-dependent and -withdrawn rats

In their pioneering study, Eisch et al. (2000) reported that, in both morphine pellet-implanted and heroin self-administering rats, cell proliferation and neurogenesis in the subgranular cell layer (but not in the hilus) are reduced in dependent animals. We decided to measure cell proliferation by using a different morphine treatment protocol; rats were injected i.p. with morphine (20 mg/kg) twice daily for 1 week and BrdU was injected at the end of the morphine treatment, 3 h before the fixation. The number of BrdU-IR cells was significantly altered by morphine treatment ($F_{4,21} = 37.78, P < 0.001$). We found that, in morphine-dependent rats, the number of BrdU-IR cells was reduced by 45.5% (54.4 \pm 3.4% of control, n = 8, compare Fig. 1A and B, summary data Fig. 1F). The inhibition of cell proliferation was restricted to the subgranular layer of the DG as the number of BrdU-IR cells in the hilus was similar to that observed in control rats. In dependent animals (n = 8) the number of isolated cells (defined as one or two contiguous BrdU-IR cells, see Materials and methods) decreased by $60.2 \pm 12.6\%$ (compared with control) and by only $34.1 \pm 16.0\%$ (compared with control) in clustered cells (defined as three or more contiguous BrdU-IR cells; data not shown). Although these differences did not reach statistical significance the present data are consistent with the idea that the decrease in the number of BrdU-IR cells may principally be due to a decrease in the number of 'isolated proliferative cells'.

These data validate our drug treatment protocol and support the idea that the inhibitory effects of opiates on hippocampal cell proliferation can be induced with a variety of drug regimens.

Although opiate addiction involves long-lasting neuronal modifications that outlive the presence of the drug, the effects of morphine withdrawal on hippocampal cell proliferation remained to be elucidated. To address this question, cell proliferation was quantified in rats treated for 1 week with morphine and withdrawn for 1, 2 or 4 weeks. As shown in Fig. 1C and F, the marked reduction of cell proliferation found in morphine-dependent animals was followed by a rebound increase after 1 week withdrawal (156.65 \pm 11.36% of control, n = 4).

An increase in the number of BrdU-IR cells in clusters seems to be mostly responsible for the rebound increase; 1-week-withdrawn rats (n = 4) gained 60.9% of cells in clusters and 51.9% of isolated cells compared with control rats (data not shown). Interestingly, cell proliferation levels were back to control after 2 weeks withdrawal (95.8 ± 6.6% of control, n = 4, Fig. 1D and F) and remained at control level thereafter (up to 4 weeks of withdrawal was tested, 98.4 ± 4.7%, n = 4). The present results further confirm the powerful inhibitory actions of opiates on cell proliferation in the subgranular layer of the DG (Eisch *et al.*, 2000) and show that this profound inhibition is followed by a marked rebound increase after 1 week withdrawal and a progressive return to control levels.

Repeated morphine treatment and withdrawal do not modify the number of pyknotic cells in the rat hippocampal subgranular layer

Throughout life, the continuous generation of new neurons in the subgranular layer of the DG is normally accompanied by cell death (Biebl *et al.*, 2000). Thus, the modification of cell proliferation observed in morphine-dependent animals as well as the following rebound increase in morphine-withdrawn rats may result from

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FIG. 1. Alteration of cell proliferation in the subgranular layer of the dentate gyrus (DG) in morphine-dependent and -withdrawn rats. (A–E) Coronal sections of hippocampal DG showing the typical distribution of 5-bromo-2'-deoxyuridine (BrdU)-immunoreactive (IR) cells (arrows) in control animals (A), dependent rats (B) and after 1 (C), 2 (D) and 4 (E) weeks withdrawal. (F) Bar graph (mean \pm SEM) of the number of BrdU-IR cells in the DG (left bar) and hilus (right bar) in control, morphine-dependent (dep) and 1-, 2- and 4-week-withdrawn (wd) rats. Chronic morphine treatment results in a significant decrease of BrdU-IR cells in the DG of morphine-dependent rats and a significant increase in 1-week-withdrawn rats relative to control animals. For each condition, the quantificant of BrdU-IR cells in the subgranular layer of the DG is illustrated by the left column and in the hilus by the right column. There was no significant difference in the number of BrdU-IR cells in the hilus of the different conditions tested. *P < 0.02; **P < 0.01. Scale bar, 200 µm. The numbers on the abscissa are the numbers of rats per condition.

corresponding changes in cell death. To directly address this question, brain slices adjacent to those used for the BrdU assay were processed with hematoxylin and the number of pyknotic cells was evaluated based on the following criteria: dark/condensed chromatin and reduced or absent cytoplasm (see Fig. 2A inset). In perfect agreement with the study of Eisch *et al.* (2000), the strong reduction of cell proliferation observed in morphine-dependent animals was not accompanied by an increased number of pyknotic cells (Fig. 2B). Moreover, we found that the rebound increase in cell proliferation apparent after 1 week withdrawal was not correlated to a decrease in cell death (Fig. 2B). Thus, the morphine-induced changes in cell proliferation are not related to changes in cell death in the subgranular layer of the DG.

Polysialylated neural cell adhesion molecule expression in the hippocampus is markedly altered in morphine-dependent and -withdrawn rats

The relationships between PSA-NCAM, neurogenesis and synaptic plasticity (see Introduction) prompted us to test the possibility that the morphine-induced changes in hippocampal cell proliferation could be accompanied by alterations of PSA-NCAM expression. In agreement with previous studies (Seki & Arai, 1993), PSA-NCAM immunostaining was associated with cell bodies located in the subgranular layer of the DG, dendritic processes extending throughout the granule cell layer and axonal fibers projecting throughout the mossy fiber region (Fig. 3A and inset). We found that the PSA-NCAM immunostaining was markedly reduced in morphine-dependent rats. The quantification of the images revealed that the number of PSA-NCAM-IR cellular bodies was markedly reduced within the DG of morphine-dependent rats ($F_{4,17} = 4.45$, P < 0.05). The PSA-NCAM labeling was back to control levels after 1 week withdrawal (Fig. 3C) and remained stable thereafter (2 and 4 weeks withdrawal, Fig. 3D and E; summarized

data, Fig. 3F). This is the first demonstration that morphine exposure alters the expression of PSA-NCAM in the hippocampal DG.

Alteration of glutamate decarboxylase-67 mRNA and protein level in the subgranular layer of the dentate gyrus after repeated morphine treatment

The GABAergic phenotype of adult hippocampal granule cells is drastically altered in response not only to pathological conditions such as seizures (Schwarzer & Sperk, 1995) or kindling (Lehmann et al., 1996) but also to LTP-like stimulations (Sloviter et al., 1996; Gutierrez & Heinemann, 2001; Ramirez & Gutierrez, 2001; Magueda et al., 2003). Considering the effects of morphine treatment on mossy fiber LTP (Harrison et al., 2002) and epileptic seizures (Snead & Bearden, 1982; Snead, 1986; Velisek et al., 2000; Cano-Martinez et al., 2001), the expression and transcription of the GABA-synthesizing enzyme GAD-67 in response to morphine treatment were quantified. The number of GAD-67 mRNA-positive cells in the subgranular layer of the DG was found to differ between control, morphine-dependent and withdrawn rats ($F_{3,12} = 26.21$, P < 0.001). There was a significant increase in the percentage of GAD-67 mRNA-labeled cells in the subgranular layer of morphine-dependent rats compared with control (Fig. 4A, B and E). This enhancement was followed by a significant decrease in the number of GAD-67 mRNA-positive cells in 1-weekwithdrawn rats (Fig. 4C and E). GAD-67 mRNA levels were back to control level after 4 weeks withdrawal (Fig. 4D and E).

We then tested the possibility that morphine alters the expression of GAD-67 in the DG. The immunohistochemical detection of GAD-67 in control rats showed the presence of this enzyme in two types of neurons, granule cells and interneurons. In agreement with the study of Gutierrez *et al.* (2003), GAD-67-IR granule cells were distinguished from GAD-67-IR interneurons based on their morphology (round cell body, dendritic projections toward the granule cell layer

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FIG. 2. Chronic morphine treatment and withdrawal do not modify the number of pyknotic cells in the subgranular layer of the dentate gyrus (DG). (A) Illustration of hematoxylin-stained cells in the subgranular layer of the DG of a control rat. (B) Bar graph showing no significant difference in the number of pyknotic cells (mean \pm SEM) in the different groups of rats. Scale bar, 200 μ m, inset, 20 μ m. The numbers on the abscissa are the numbers of rats per condition. dep, dependent; wd, withdrawn.



FIG. 3. Polysialylated neural cell adhesion molecule (PSA-NCAM) expression is decreased in the subgranular layer of morphine-dependent (dep) rats. (A–E) Confocal images of hippocampal dentate gyrus showing the typical distribution of PSA-NCAM-immunoreactive (IR) cells in control (A), morphine-dependent (B), 1- (C), 2- (D) and 4- (E) week-withdrawn (wd) rats. (F) Bar graph showing a significant decrease of PSA-NCAM-IR cells (mean \pm SEM) in the subgranular layer of morphine-dependent (n = 4) relative to control (n = 5) rats. PSA-NCAM expression was indistinguishable from control level after 1 (n = 6), 2 (n = 4) and 4 (n = 3) weeks of withdrawal. Note that PSA-NCAM expression was also modified in stratum lucidum, the projection area of the mossy fibers (inset). *P < 0.05. Scale bar, 50 µm; inset, 10 µm. The numbers on the abscissa are the numbers of rats per condition.

and the putative axonal projection toward the hilus) and their orientation in the subgranular layer of the DG. The number of GAD-67-IR granule cells was enhanced in the subgranular layer of morphine-dependent rats (Fig. 5A, B and E), decreased in 1-week-withdrawn rats (Fig. 5C and E) and came back to control level after 4 weeks withdrawal (Fig. 5D and E) but these differences did not reach statistical significance. No difference was observed in the number of GAD-67-IR interneurons (data not shown). Together, the data indicate that, in adult rats, repeated *in vivo* morphine exposure strongly enhances the 'GABAergic' phenotype of dentate granule cells, reminiscent of what has been previously observed following epileptic-like activity (Schwarzer & Sperk, 1995; Lehmann *et al.*, 1996).

Discussion

We found that repeated morphine treatment and withdrawal have profound cellular, morphological and phenotypical effects on the adult rat hippocampus. The primary observation is that repeated morphine treatment induces a decrease in cell proliferation without altering cell death in the DG region of the hippocampus, paralleled with a decrease in the number of PSA-NCAM-IR granule cells and an increase in the number of GABAergic neurons detected in the DG. Rebound adaptations of cell proliferation and the 'GAD-67 phenotype' were apparent after 1 week of withdrawal; cell proliferation was markedly increased (without change in cell death) while the number of granule cells containing GAD-67 mRNA was decreased. With prolonged withdrawal (2 and 4 weeks), cell proliferation, the number of GAD-67 mRNA-positive cells, came back to control level. In contrast, the number of PSA-NCAM-IR cells came back to control level after 1 week withdrawal.

Our results showing that i.p. morphine injections reduce cell proliferation in the DG of dependent rats confirm the work of Eisch *et al.* (2000) who first observed this effect in both morphine pellet-implanted and heroin self-administering rats. Our data further suggest that morphine treatment has different consequences depending on the

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FIG. 4. Alteration of the number of glutamate decarboxylase (GAD)-67 mRNA-labeled cells in the subgranular layer of the dentate gyrus (DG) in morphinedependent (dep) and -withdrawn (wd) rats. (A–D) Representative illustration of the distribution of GAD-67 mRNA-labeled cells in the subgranular layer of the DG in control (A), morphine-dependent (B), 1- (C) and 4- (D) week-withdrawn rats. (E) Bar graph (mean ±SEM) of the number of GAD-67 mRNA-positive cells in the DG (left bar) and hilus (right bar) in control, morphine-dependent, 1- and 4-week-withdrawn rats. Note the significant increase of GAD-67 mRNA-labeled cells in the subgranular layer of morphine-dependent (n = 4) relative to control (n = 4) rats. The number of GAD-67 mRNA-labeled cells is significantly decreased in 1week-withdrawn rats (n = 4) and came back to control level after 4 weeks withdrawal (n = 4). There was no significant difference in the number of GAD-67 mRNA-positive cells in the hilus of the different conditions tested. *P < 0.05, **P < 0.01. Scale bar, 200 µm. The numbers on the abscissa are the numbers of rats per condition.



FIG. 5. Alteration of glutamate decarboxylase (GAD)-67 expression in the subgranular layer of the dentate gyrus (DG) in morphine-dependent (dep) and -withdrawn (wd) rats. (A–D) Confocal images of hippocampal DG showing GAD-67-immunoreactive (IR) cells in control (A), morphine-dependent (B), 1- (C) and 4- (D) week-withdrawn rats. (E) Bar graph (mean \pm SEM) of the number of GAD-IR cells in the DG (left bar) and hilus (right bar) in control, morphine-dependent, 1- and 4-week-withdrawn rats. When compared with control, the variations of GAD-67-IR cells in the subgranular layer in response to the various morphine treatments did not reach statistical significance but followed a similar trend as the *in situ* hybridization data. Scale bar, 100 μ m; inset, 10 μ m. The numbers on the abscissa are the numbers of rats per condition.

arrangement of proliferative cells (i.e. clusters vs. individual cells), the cells in clusters being less sensitive than isolated cells to the effect of repeated morphine exposure. We also describe for the first time the time-course of the morphine-induced alteration of cell proliferation. It was noteworthy that a rebound increase of cell proliferation was observed after 1 week morphine withdrawal, suggesting that compensatory mechanisms are engaged during withdrawal in order to counteract the reduction of the number of newly generated neurons

induced by the chronic morphine treatment. Two weeks of morphine withdrawal were necessary for the pattern of cell proliferation to return to control in terms of number of BrdU-IR cells (Fig. 1) and of clusters vs. isolated cells.

To date, we do not know the mechanisms responsible for the alteration of DG cell proliferation following morphine treatment and during withdrawal. Hippocampal neurogenesis is modulated by a large variety of factors including growth factors (Okano *et al.*, 1996;

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Yoshimura *et al.*, 2001), neurotransmitters (Gould, 1999; Cameron *et al.*, 1995) and corticosteroid hormones (Cameron *et al.*, 1993). Interestingly, morphine is known to strongly stimulate the release of corticosteroids which have potent inhibitory effects on DG neurogenesis (Bryant *et al.*, 1991). However, Eisch *et al.* (2000) have elegantly demonstrated that the morphine-induced decrease in cell proliferation was not dependent on circulating corticosteroids. Further studies should investigate the effects of morphine treatment and withdrawal on the local production of growth factors and/or on the functional modifications of the different neuronal systems afferent to the DG.

Polysialylated neural cell adhesion molecule is highly expressed in newly generated immature neurons where it plays a major role in structural plasticity, including cell migration, axonal guidance and synapse formation (Bruses & Rutishauser, 2001). In the adult DG, newborn granule cells transiently express high levels of PSA-NCAM (Seki, 2002). As morphine reduces DG neurogenesis (Eisch *et al.*, 2000), we tested the hypothesis that morphine treatment could impact on PSA-NCAM expression in the hippocampus. We found a 32% reduction in the number of PSA-NCAM-IR cells in the subgranular layer of the DG of morphine-dependent animals (Fig. 3). The decrease in the number of PSA-NCAM-IR cells in the DG that was found following repeated morphine treatment is similar to that reported in nicotine self-administrating rats (Abrous *et al.*, 2002), suggesting a prominent role of the reduction of PSA-NCAM expression in the neuro-adaptations induced by addictive drugs.

Although glutamate is generally considered as the major neurotransmitter of granule cells, the presence of GABA and GAD has been described in both the mossy fibers and granule cell bodies, both in basal (Sandler & Smith, 1991; Sloviter et al., 1996) and pathological conditions where GABA and GAD-67 contents were markedly increased (Schwarzer & Sperk, 1995; Lehmann et al., 1996; Sloviter et al., 1996; Gutierrez & Heinemann, 2001; Ramirez & Gutierrez, 2001; Maqueda et al., 2003). For instance, Makiura et al. (1999) showed that, in adult mice, treatment with kainic acid modifies the neurochemical content of excitatory granule cells that transiently adopt a double inhibitory phenotype, GABA and NPY. Moreover, electrophysiological evidence showed that seizures (Gutierrez, 2000; Gutierrez & Heinemann, 2001) and synaptic potentiation (Gutierrez, 2002) induce simultaneous glutamatergic and GABAergic transmission. Our data suggest that the GABAergic phenotype of the granule cells is up-regulated in morphine-dependent rats; the number of cells containing GAD-67 mRNA significantly increased (and GAD-67 immunoreactivity followed a similar trend). At present, we do not know if the effects of morphine on cell proliferation are related to its effects on the phenotype of granule cells. Interestingly, newly generated neurons of the adult DG were found to express the GABAergic phenotype only when they acquired the expression of NeuN, a marker of neuronal maturity (Belachew et al., 2003). It is thus possible that morphine exposure accelerates the maturation process of newborn neurons, leading to an increased occurrence of identified GABAergic neurons in the DG. Such an effect on accelerated maturation of newborn neurons may also partly account for the decreased occurrence of PSA-NCAM after morphine treatment, as the expression of PSA-NCAM decreases with neuronal maturation (Seki & Arai, 1999). The present data focused on cell proliferation and additional studies will be necessary to formally identify the consequences of the morphine treatment on the phenotypes of BrdU-IR cells once they have reached their full maturation (e.g. 4 weeks after BrdU injection and withdrawal from chronic morphine, BrdU-IR cells could be colabeled with specific markers of adult neurons or glia and with glutamatergic or GABAergic markers).

Several of the most addictive drugs have been shown to dramatically alter cell proliferation and neurogenesis in the hippocampus; morphine (Eisch *et al.*, 2000), alcohol (Herrera *et al.*, 2003) and nicotine (Abrous *et al.*, 2002) have been tested. Additionally, nicotine can also decrease PSA-NCAM expression (Abrous *et al.*, 2002). Together with the present report, these data point towards a major role of alterations of the neurotransmitter system, cell proliferation and structural plasticity in the adaptations of the hippocampal circuitry in response to addictive drugs.

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Abbreviations

BrdU, 5-bromo-2'-deoxyuridine; DG, dentate gyrus; GAD, glutamate decarboxylase; IR, immunoreactive; LTP, long-term potentiation; PBS, phosphatebuffered saline; PSA-NCAM, polysialylated neural cell adhesion molecule; RT, room temperature; SSC, standard sodium citrate.

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