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Methamphetamine decreases dentate gyrus stem cell self-renewal and shifts the differentiation towards neuronal fate

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Running title: Methamphetamine impairs dentate gyrus stem cell properties

Abstract

Methamphetamine (METH) is a highly addictive psychostimulant drug of abuse that negatively interferes with neurogenesis. In fact, we have previously shown that METH triggers stem/progenitor cell death and decreases neuronal differentiation in the dentate gyrus (DG). Still, little is known regarding its effect on DG stem cell properties. Herein, we investigate the impact of METH on mice DG stem/progenitor cell self-renewal functions. METH (10 nM) decreased DG stem cell self-renewal, while 1 nM delayed cell cycle in the G0/G1-to-S phase transition and increased the number of quiescent cells (G0 phase), which correlated with a decrease in cyclin E, pEGFR and pERK1/2 protein levels. Importantly, both drug concentrations (1 or 10 nM) did not induce cell death. In accordance with the impairment of self-renewal capacity, METH (10 nM) decreased $Sox2^{+}/Sox2^{+}$ while increased $Sox2^{-}/Sox2^{-}$ pairs of daughter cells. This effect relied on N-methyl-D-aspartate (NMDA) signaling, which was prevented by the NMDA receptor antagonist, MK-801 (10 μM). Moreover, METH (10 nM) increased doublecortin (DCX) protein levels consistent with neuronal differentiation. In conclusion, METH alters DG stem cell properties by delaying cell cycle and decreasing self-renewal capacities, mechanisms that may contribute to DG neurogenesis impairment followed by cognitive deficits verified in METH consumers.

Keywords: methamphetamine, neurogenesis, dentate gyrus, stem/progenitor cells, cell cycle, cell fate division

Abbreviations: bFGF2, basic Fibroblast growth factor 2; BrdU, 5-Bromo-2'-deoxyuridine; Cdk, Cyclin-dependent kinase; DCX, doublecortin; DG, Dentate gyrus; EGF, Epidermal growth factor; EGFR, Epidermal growth factor receptor; ERK,

Extracellular-signal-regulated kinases 1/2; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFAP, Glial fibrillary acidic protein; MAPK, Mitogen-activated protein kinase; MDMA, 3,4-Methylenedioxymethamphetamine; MEK1, MAPK/ERK kinase 1; METH, Methamphetamine; MK-801, (5R,10S)-(–)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cylcohepten-5,10-imine maleate; NMDA, N-methyl-D-aspartate; pEGFR, phospho-epidermal growth factor receptor; pERK1/2, phospho-extracellular-signal-regulated kinases 1/2; SGZ, Subgranular zone; Sox2, SRY (sex determining region Y)-box 2; SVZ, Subventricular zone; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick-end labeling.

1 Introduction

Methamphetamine (METH) is a highly addictive drug whose consumption has been increasing worldwide and turned to be a public health problem (Silva et al., 2010). Several studies have extensively described the negative effects of METH in the Central Nervous System (Gonçalves et al., 2014; Krasnova and Cadet, 2009), concluding that METH abusers exhibit smaller hippocampal volume, which was positively correlated with poorer memory performance (Thompson et al., 2004). Accordingly, animal studies have clearly demonstrated hippocampal neuronal dysfunction (Gonçalves et al., 2010), as well as cognitive deficits induced by this psychostimulant (Simões et al., 2007). Nevertheless, the mechanisms of METH-induced memory deficits are still poorly understood, but pieces of evidence show that neurogenesis is tightly related to memory. In fact, reduction in the number of immature neurons induces deficits in long-term retention of spatial cognitive functions (Deng et al., 2009), and ablation of hippocampal neurogenesis impairs memory performance related to pattern separation functions (Clelland et al., 2009).

The information available regarding the effect of METH on neurogenesis describes that, in the dentate gyrus (DG), cell proliferation is decreased in gerbils (postnatal day 30) administered once with the drug at postnatal day 14-20 (50 mg/kg) (Hildebrandt et al., 1999). On the other hand, a lower dose of METH (25 mg/kg) transiently decreased cell proliferation in the same region (Teuchert-Noodt et al., 2000). Furthermore, a chronic METH administration (1 mg/kg/day for 14 days) had no effect on the number of proliferating cells in mice DG (Maeda et al., 2007). Interestingly, Wistar rats self-administered with METH (0.05 mg/kg/infusion, 1 h intermittent access, 2x a week during 28 days) displayed an increase in DG cell proliferation as well as in neuronal differentiation, whereas both short (1 h/day) and long (6 h/day) access

decreased proliferation and differentiation followed by a reduced number of DG granule cell neurons (Mandyam et al., 2008). Furthermore, self-administration of METH (1 h/day access METH for 13 days) increased the number of radial glia-like cells (type 1 cells), but decreased the proportion of preneuronal neuroblasts (type 2a cells) (Yuan et al., 2011) showing that at different maturation stages cells respond differently to an external stimuli (Tashiro et al., 2007). Also, daily access to METH (6 h/day for 4 or 13 days) decreased the number of proliferating cells in the DG without changing, however, the length of S-phase of the cell cycle (Yuan et al., 2011). *In vitro* studies also point that METH reduced proliferation of rat hippocampal neural progenitor cells (Tian et al., 2009; Venkatesan et al., 2011). Additionally, our group recently verified that a nontoxic concentration of METH (1 nM for 7 days) decreased the number of mature neurons in DG-derived neurosphere cultures (Baptista et al., 2012). Concerning the subventricular zone (SVZ), we have also shown that METH decreases cell proliferation, neuronal differentiation and maturation of stem/progenitor cells (Bento et al., 2011).

Overall, it seems clear that METH interferes with hippocampal neurogenesis, but many questions remain unanswered. In fact, the direct effect of this drug on stem cell self-renewal capacities has never been addressed before. Herein, we show that METH delays cell cycle progression from G0/G1-to-S phase. This effect could be due to the down-regulation of cyclin E, a cyclin involved in the progression through the G1 phase and initiation of DNA replication in the S phase, and to the decrease of epidermal growth factor receptor (EGFR) and extracellular-signal-regulated kinases 1/2 (ERK1/2) phosphorylation, mediators in the MAPK signaling pathway involved in cell proliferation progression. Also, METH decreases DG stem cell self-renewal capacities, which seems to involve NMDA receptors. In conclusion, the present work reveals a

negative impact of METH on DG stem cell capacities that can contribute to memory deficits upon METH consumption.

2 Material and methods

2.1 Dentate gyrus neurosphere cultures

Post-natal 1-3-day-old C57BL/6J mice were sacrificed by decapitation and brains were placed in sterile saline solution. Afterwards, meninges were removed and DG fragments were dissected from 450 µm-thick brain coronal sections, digested in 0.025% trypsin and 0.265 mM EDTA (both from Life Technologies, Carlsbad, CA, USA), and single cells were obtained by gentle trituration. Then, cells were diluted in serum-free culture medium (SFM) composed of Dulbecco's modified Eagle's medium/Ham's (DMEM) F-12 medium GlutaMAX-I supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% B27 supplement, 5 ng/ml epidermal growth factor (EGF) and 2.5 ng/ml basic fibroblast growth factor (bFGF-2) (all from Life Technologies). Afterwards, cells were plated in uncoated Petri dishes and neurospheres were allowed to develop for 6 days in a 95% air-5% CO_2 humidified atmosphere at 37ºC. At 6 days, the neurospheres mean diameter was 90.22 ± 2.24 µm (measurements performed on 2 independent cultures).

Experimental procedures were performed according to the guidelines of the European Communities Council Directives (2010/63/EU) and the Portuguese law for the care and use of experimental animals (DL nº129/92). All efforts were made to minimise animal suffering and to reduce the number of animals.

2.2 Cell death assay

DG neurospheres were exposed to 10 or 100 nM METH (Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Portugal) for 24 h (Fig. 1A) and then dissociated with NeuroCult® chemical dissociation Kit (Stem Cell Technologies, Grenoble, France). Cells were adhered to SuperFrost Plus glass slides (Thermo Scientific, Menzel GmbH & Co KG, Braunscheweig, Germany) by centrifugation (360 x*g*, 5 min; Cellspin I, Tharmac GmbH, Waldsoms, Germany), and fixed in 4% paraformaldehyde (PFA). Afterwards, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed to label apoptotic nuclei, as previously described by us (Baptista et al., 2012). Briefly, cells were rinsed 3x 10 min with 0.01 M phosphate-buffered saline (PBS) and permeabilized in 0.25% Triton X-100 for 30 min at room temperature. Cells were then incubated with terminal deoxynucleotidyl transferase buffer (0.25 U/µl terminal transferase, 6 µM biotinylated dUTP, pH 7.5; Roche, Basel, Switzerland) for 1 h at 37ºC in a humidified chamber. Afterwards, cells were rinsed with a termination buffer solution (300 mM NaCl and 30 mM sodium citrate) for 15 min, followed by PBS for 5 min and incubated with Fluorescein (1:100; Vector Laboratories, Burlingame, USA) for 1 h. Additional SRY (sex determining region Y)-box 2 (Sox2) immunostaining was performed (as described in the immunocytochemistry section) to identify stem cells. Finally, nuclei were counterstained with 4 µg/ml Hoechst 33342 (Sigma-Aldrich) for 5 min and mounted in Dako fluorescence mounting medium (Dako, Glostrup, Denmark). Cells counts were obtained from 6 microscope fields of each coverslip, from 3 independent cultures performed in triplicate.

2.3 Immunocytochemistry

DG cell cultures or neurospheres were fixed in 4% PFA for 30 min, permeabilized in 1% Triton X-100 and 3% bovine serum albumin (BSA; all from Sigma-Aldrich, St Louis, MO, USA) in PBS, followed by overnight incubation at 4ºC with the following antibodies: goat polyclonal anti-Sox2 (1:200; Santa Cruz Biotechnology, Heidelberg, Germany), goat anti-doublecortin (DCX, 1:500; Santa Cruz Biotechnology) and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, 1:500; Sigma-Aldrich). Then, cells were rinsed with PBS and incubated for 1 h with the appropriate secondary antibodies: donkey anti-goat Alexa Fluor 594 or donkey anti-rabbit Alexa Fluor 488 (both 1:200; Life Technologies). Afterwards, nuclei were stained with 4 µg/ml Hoechst 33342 (Sigma-Aldrich) and slides were mounted in Dako. Fluorescence images for cells counts were recorded using a fluorescence microscopy (Leica DMIRE200, Wetzler, Germany).

2.4 Cell cycle analysis

Six-day-old DG primary neurospheres were exposed to 1 nM METH for 24 h or 72 h (Fig. 2A), dissociated and fixed in 70% ethanol for 20 min at 4ºC. Then, cells were centrifuged at 550 x*g* for 5 min and ressuspended in a solution containing 2% Fetal Bovine Serum (FBS; Life Technologies) in PBS. Afterwards, 10^6 cells/ml were incubated with 10 µM Vybrant® Dye Cycle Orange (Life Technologies), which is a DNA-selective and membrane permeant probe that binds stoichiometrically to DNA and becomes fluorescent upon binding, in the presence of 0.1% Triton X-100 for 45 min at 37ºC. Afterwards, cells in suspension were centrifuged at 550 x*g* for 5 min and resuspended in 2% FBS in PBS. Approximately 30,000 events were analyzed on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and cell cycle was analyzed using the ModFit software.

Cell quiescence was assessed in DG neurospheres treated with 1 nM METH for 24 h (Fig. 2A). Neurospheres were dissociated to single cells (NeuroCult®) and incubated for 15 min at 37ºC with 1 µg/ml Hoechst 33342 (Life Technolgies) and 1 µg/ml pyronin-Y (Sigma-Aldrich), a fluorescent probe that binds to RNA, in culture medium supplemented with 0,1% Triton. Then, cells were centrifuged for 5 min at 550 x*g*, resuspended in culture medium and analyzed on a FACS Aria III (Becton Dickinson). In detail, pyronin-Y was excited at 488 nm and red fluorescence was collected at 545/35 nm, and Hoechst 33342 was excited at 355 nm (UV) and blue fluorescence was recorded at 450/50 nm. A total of 10000 cells were analyzed per sample at a velocity of 400 events/second. Proper controls consisting of cells incubated with either Hoechst 33342 or Pyronin-Y were performed to calibrate the cytometer. Also, to exclude dead cells, DG cells were incubated with 2 µg/ml propidium iodide (Sigma-Aldrich). Data were analyzed using the flowJo software.

2.5 Western blot analysis

To evaluate cyclin A, D1 and E protein levels, 6-day-old neurospheres were exposed to 1 nM METH or to 10 nM METH for cyclin E protein levels determination during 24 h (Fig. 3A). Moreover, phospho-epidermal growth factor receptor (pEGFR), epidermal growth factor receptor (EGFR), phospho-fibroblast growth factor receptor 1 (pFGFR1), phospho-extracellular-signal-regulated kinases 1/2 (pErk1/2) and extracellular-signal-regulated kinases 1/2 (ERK1/2) protein levels were analyzed in DG neurospheres exposed to 1 or 10 nM METH for 6 or 24 h (Fig. 4A). Regarding DCX and GFAP protein levels, DG cells were exposed to 1 or 10 nM METH for 6 days (Fig. 7A) and the resulting neurospheres were harvested. Then, DG neurospheres were homogenized in RIPA buffer containing 150 mM NaCl, 5 mM EGTA, 50 mM Tris, 1% (v/v) Triton X-

100, 0.1% SDS and 0.5% sodium deoxycholate, supplemented with a protease inhibitor cocktail tablet (Roche, Amadora, Portugal) in the ratio of 1 tablet/10 ml RIPA buffer. Afterwards, cells were centrifuged at 17000 x*g* for 15 min and protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay (Thermo Fisher Scientific, Northumberland, UK). Then, 10 µg or 30 µg of protein samples were separated by electrophoresis in a 8% or 12% SDS-PAGE, respectively, transferred to a polyvinylidene dofluoride (PVDF) membrane (Millipore, Algés, Portugal) and blocked in a solution of 5% non-fat dried milk or 4% BSA in PBS-0.5% Tween (PBS-T; Sigma-Aldrich) for 1 h. Membranes were probed overnight at 4ºC with mouse monoclonal anti-cyclin A (1:200; Abcam, Cambridge, UK), mouse monoclonal anti-cyclin D1 (1:100), mouse monoclonal anti-cyclin E (1:100), rabbit polyclonal anti-p21 (1:200), mouse monoclonal anti-p27 (1:200) (all from Santa Cruz Biotechnology), mouse monoclonal anti-pEGFR (1:500; Millipore), rabbit polyclonal anti-EGFR (1:200; Abcam), rabbit monoclonal anti-pFGFR1 (1:200; Abcam), rabbit monoclonal anti-pERK1/2 (Thr202/Tyr204; 1:200; Cell Signaling Technology, Beverly, MA, USA), rabbit monoclonal anti-ERK1/2 (1:200; Cell Signaling Technology), rabbit polyclonal anti-GFAP (1:1000; Sigma-Aldrich), goat polyclonal anti-DCX (1:200; Santa Cruz Biotechnology), mouse monoclonal anti-β-actin (1:10000; Sigma-Aldrich) and rabbit polyclonal anti-GAPDH (1:500; Sigma-Aldrich). Then, membranes were rinsed in PBS-T and incubated for 45 min with alkaline phosphatase-conjugated secondary antibodies as follows: anti-rabbit IgG, (1:20000; GE Healthcare Europe GmbH, Freiburg, Germany), anti-mouse (1:10000, GE Healthcare Europe GmbH) and anti-goat IgG, (1:10000, Zymax, California, USA). Densitometric analysis was performed using the ECF reagent (GE Healthcare Europe GmbH), visualized on the Typhoon 9000 system

(GE Healthcare Europe GmbH), and band intensities were quantified using the ImageQuant 5.0 software.

2.6 Neurosphere self-renewal assay

Self-renewal capacity of DG stem/progenitor cells was assessed using the neurosphere assay. In detail, DG cells were seeded at clonal density of 10 cells/µl (Coles-Takabe et al., 2008; Pastrana et al., 2011) into uncoated 24-well plates and incubated with 1 or 10 nM METH for 6 days and the total number of primary neurospheres in each well was determined (Fig. 5A). Afterwards, primary neurospheres were dissociated (NeuroCult® chemical dissociation kit, Stem Cell Technologies) and cells were reseeded as aforementioned without treatments for 6 additional days (Fig. 4A). The total number of resulting secondary neurospheres was determined in each well. Results are expressed as percentage of the control (untreated) in both primary and secondary neurospheres from at least 3 independent cultures and performed in triplicate (3 wells per condition within the 24-well plate).

2.7 Cell-fate studies: cell pair assay

Uncommitted stem cells can divide symmetrically into two stem cells ($Sox2^+/Sox2^+$) or into two progenitor cells ($Sox2^-/Sox2^-$), or asymmetrically into one uncommitted cell and one committed progenitor cell ($Sox2^+/Sox2^-$). Taking advantage of these properties, we analyzed if METH interferes with cell-fate division. For that, cell pair assay was performed as previously described (Bernardino et al., 2012; Santos et al., 2012; Xapelli et al., 2013) with some modifications. Hence, stem cells were directly isolated from mice DG and 10000 cells (8840 cells/cm^2) were plated onto poly-D-lysine-coated (Sigma-Aldrich) glass coverslips. After seeding, cells were pre-incubated with 10 µM

(5R,10S)-(–)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cylcohepten-5,10-imine maleate (MK-801; Tocris) for 15 min and then co-exposed with 1 or 10 nM METH for 24 h (Fig. 6A). Then, immunocytochemistry to Sox2 was performed (section 2.3). A total of 40 pairs of daughter cells that resulted from the division of one stem cell was characterized according to its symmetric cell division towards self-renewal ($Sox2^+/Sox2^+$) and differentiation ($Sox2^-/Sox2^-$), or asymmetric cell division ($Sox2^+/Sox2^-$). Results are expressed as percentage of control (untreated) from at least 2 independent cultures performed in triplicate.

2.8 Data analysis

Statistical analysis was determined from at least two independent cultures and by using an analysis of variance (one-way ANOVA) followed by Dunn's multiple comparison or Mann Whitney post-hoc tests, as indicated in the figure legends. Data are expressed as mean + standard error of the mean (SEM) from at least 2 independent cultures in which each condition was performed in duplicate or triplicate, and statistical significance level was set for $P < 0.05$.

3 Results

3.1 Methamphetamine can induce dentate gyrus stem cell death

Several studies have previously shown that METH can be toxic to different brain cells (Deng et al., 2002; Genc et al., 2003; Mandyam et al., 2007), but the direct effect of this drug on DG stem cells has never been addressed before. Thus, in the present study we started by evaluating the toxic effect of METH on DG stem cells by quantifying the number of positive cells for TUNEL and Sox2 (Figs. 1B and 1C). We observed that 10

nM METH did not induce cell death to Sox2-positive cells (111.00 + 24.51% of control; Fig. 1C). However, 100 nM METH was toxic to DG neurospheres observed by the significant increase of Sox2- and TUNEL-positive cells (196.20 + 26.26% of control; $P < 0.01$; Fig. 1C).

3.2 Methamphetamine delays dentate gyrus cell cycle

Based on our previous results (Fig. 1C) and in order to select drug concentrations more similar to those frequently present in the brain of METH users, we decided to use 1 or/and 10 nM of METH in the following studies. Noteworthy, both concentrations did not induce stem cell death. Thus, we analyzed the effect of METH (1 nM) on cell cycle progression and, after 24 h, there was an increase in the population of cells in the G0/G1 phase (61.59 + 2.77%, $P < 0.01$) when compared to the control (48.91 + 2.12%; Fig. 2B). Furthermore, METH induced a decrease in the percentage of cells in the S phase (control: 36.86 + 2.81%; METH: 27.72 + 1.69%, $P < 0.01$; Fig. 2B). Regarding the G2/M phases, no differences were observed between METH and control conditions (control: 15.04 + 0.77%; METH: 13.42 + 1.08%; Fig. 2B). To further clarify if METH induces cell cycle inhibition or delay, neurospheres were exposed to 1 nM METH for 72 h (Fig. 2A). We concluded that METH no longer interfered with G0/G1 (control: 86.82 + 0.57%; METH: 85.41 + 0.92%), S (control: 9.37 + 0.16%; METH: 10.94 + 0.83%) or G2/M phases (control: 3.81 + 0.72%; METH: 3.64 + 0.42%; Fig. 2C), indicating that METH delays cell cycle progression rather than inhibiting it.

To disclose whether delay in the cell cycle is due to entry in quiescence (G0), DG neurospheres were exposed to 1 nM METH for 24 h (Fig. 2A) and then incubated with Hoechst 33342 and pyronin Y to label DNA and mRNA, respectively. Quiescent cells possess less mRNA than actively cycling cells and therefore display low levels of

pyronin Y-emitted fluorescence. As represented in Fig. 2D, METH increased in about 20% the cell population that rests in G0 (control: 16.5%; METH: 36.4%).

After demonstrating that METH impairs the progression of DG stem cells from G0/G1 to S phase of the cell cycle, we further investigated possible alterations of cyclins D1, E and A, since these proteins are involved in the cell cycle progression. It was possible to conclude that METH (1 nM) did not induce alterations of cyclin D1 (control: 100.00 + 17.06%; METH: 94.40 + 10.10% of control; Fig. 3B) or cyclin A protein levels (control: 100.00 + 12.04%; METH: 104.60 + 13.93% of control; Fig. 3E). On the other hand, the expression of cyclin E was down-regulated by this drug (control: 100.00 + 4.36%; METH: 90.20 + 1.52% of control; $P < 0.05$; Fig. 3C). A similar down-regulation of cyclin E was observed with 10 nM METH (control: 100.00 + 12.74%; 10 nM METH: 49.46 + 8.18% of control; $P < 0.05$; Fig. 3D). Afterwards, we assessed the protein levels of both p21 and p27, the main inhibitors of the complexes cyclin D1/Cdk4/6 and cyclin E/Cdk2, respectively. Thus, METH neither altered the protein levels of p21 (control: 100.00 + 13.38%; METH: 102.60 + 7.34% of control; Fig. 3F) nor of p27 (control: 100.00 + 11.64%; METH: 101.40 + 5.93% of control; Fig. 3G).

Additionally, since activation of ERK1/2 is highly involved in cell proliferation through the upstream activation of EGFR (Gampe et al., 2011) or FGFR1 (Xiao et al., 2007), we determined the effect of METH on the phosphorylation levels of FGFR1, EGFR and ERK1/2. Indeed, METH (1 nM) did not change the protein levels of pFGFR1 (control: 100.00 + 9.44%; METH: 86.02 + 12.00% of control; Fig. 4B) at 6 h of drug exposure, whereas decreased protein levels of pEGFR (control: 100.00 + 9.77%; METH: 48.60 + 13.37% of control; $P < 0.05$; Fig. 4C) and pERK1/2 (control: 100.00 + 3.95%; METH: 82.02 + 2.11% of control, $P < 0.05$; Fig. 4D) in DG neurospheres at 6 h post-METH exposure. However, after 24 h of METH (1 nM) treatment, pERK1/2

protein levels were similar to control (101.60 + 5.52% of control; Fig. 4D). The same effect was observed with 10 nM METH (control: 100.00 + 2.68%; METH 6 h: 77.68 + 5.66% of control, $P < 0.05$; METH 24 h: 123.70 + 13.78% of control; Fig. 4E).

3.3 Methamphetamine decreases dentate gyrus neurosphere self-renewal

DG stem cells have the ability to self-renew and this capacity was assessed using the neurosphere assay (Coles-Takabe et al., 2008; Pastrana et al., 2011). Thus, METH (1 nM) decreased the number of primary neurospheres (control: 100.00 + 5.29%; METH: 76.77 + 5.29% of control, $P < 0.01$; Fig. 5B). However, no differences in the number of secondary neurospheres were observed (control: 100.00 + 6.05%; METH: 96.79 + 8.56% of control; Fig. 5B) indicating that 1 nM METH did not interfere with self-renewal capacity of DG stem/progenitor cells. In parallel, a higher concentration of METH (10 nM) also decreased the number of primary neurospheres (72.37 + 2.06% of control, $P < 0.05$; Fig. 5C). Interestingly, the number of secondary neurospheres was also decreased (control: 100.00 + 4.97%; METH: 76.47 + 3.57% of control, $P < 0.01$; Fig. 5C) showing that METH at 10 nM decreases self-renewal capacity.

3.4 Methamphetamine shifts cell fate towards differentiation *via* activation of NMDA receptors

DG stem cells can self-renew, *i.e.*, one stem cell can divide and give rise at least to one identical cell to itself and/or generate progenitors that undergo differentiation (Gage, 2000). Herein, we observed that 1 nM METH had no effect on DG symmetric cell division towards self-renewal ($Sox2^+/Sox2^+$: 87.01 + 6.60% of control; Fig. 6B). Similarly, no effect was verified on symmetric cell division towards differentiation ($Sox2^-/Sox2^-$: 110.90 + 4.63% of control) or on asymmetric cell division ($Sox2^+/Sox2^-$:

99.07 + 7.00% of control; Fig. 6B). However, 10 nM METH decreased the number of $Sox2^+/Sox2^+$ pairs of cells (53.74 + 4.69% of control; $P < 0.01$), and the blockade of NMDA receptors with MK-801, completely prevented the effect induced by METH (99.34 + 10.52% of control, $P < 0.05$ compared to METH alone; Fig. 6C). Furthermore, METH induced an increase of $Sox2^-/Sox2^-$ pairs of cells and MK-801 also prevented this effect (METH: 191.10 + 8.20%, $P < 0.01$; METH + MK-801: 78.57 + 9.82% of control, $P < 0.001$ compared to METH alone; Fig. 6C). On the other hand, 10 nM METH did not affect asymmetric cell division ($Sox2^+/Sox2^-$; Fig. 6C).

3.5 Methamphetamine increases doublecortin expression in DG neurospheres

As METH directs cell division towards differentiation, we further evaluated neuronal and astroglial differentiation in DG cells treated for 6 days with METH (Fig. 7A). Protein levels of DCX and GFAP, a marker for immature neurons and astrocytes, respectively, were determined by western blot. We observed that 1 nM METH did not interfere with both DCX (105.60 + 7.69% of control; Fig. 7B) and GFAP (94.14 + 14.08% of control; Fig. 7C) protein levels. However, at a higher concentration (10 nM) METH was able to up-regulate DCX expression (133.70 + 6.83% of control; $P < 0.05$; Fig. 7B and D), whereas no changes were observed in GFAP protein levels (93.05 ± 4.29% of control, Fig. 7C and D). Overall, these results demonstrate that 10 nM METH directs DG stem/progenitor cell differentiation towards the neuronal fate.

4 Discussion

The present work addresses the effect of the drug of abuse METH on DG stem cell properties. Indeed, our results show that METH at nontoxic concentrations impaired stem cell properties, specifically by decreasing self-renewal capacity, delaying cell

cycle progression and directing cell fate division towards differentiation. Firstly, we verified that METH (100 nM) induced cell death of $Sox2^+$ DG stem/progenitor cells from free floating neurospheres, having no effect at a lower concentration (10 nM). Interestingly, we have previously shown that exposure to 10 nM METH for 24 h induced cell death on plated DG-derived neurosphere cultures (Baptista et al., 2012). Thus, we may conclude that neurospheres plated on poly-D-lysine and in a medium devoid of growth factors initiate differentiation and are more sensitive to METH toxicity as compared to free floating $Sox2^+$ neurospheres cultured in the presence of growth factors. This difference may rely on differential abilities to repair DNA damages (Fernando et al., 2011), to counteract oxidative stress (Le Belle et al., 2011) or/and METH-induced apoptosis mechanisms displayed by stem/progenitor cells *versus* committed progenitors.

Herein, we also explored for the first time the impact of METH on the cell cycle of DG stem cells. It was possible to observe a delay in the transition from G0/G1 to the S phase, an increase of cell population in the quiescence state (phase G0), and a down-regulation of cyclin E protein, which forms a complex with cyclin-dependent kinase 2 (Cdk2) being involved in the progression through the G1 phase and initiation of DNA replication in the S phase (Mazumder et al., 2004). Accordingly, to confirm that METH delays cell cycle progression, the doubling time for cell division was assessed and we observed that DG cells undergo two cell divisions in 24 h (data not shown). On the other hand, METH did not induce any alterations in both cyclin D1 and cyclin A protein levels, which lead us to conclude that METH specifically impairs G1-to-S transition. In fact, little information is available regarding the effect of METH in cell cycle. Nevertheless, Yuan and collaborators (2011) assumed that hippocampal proliferating cells may be arrested in the G1 phase by METH (self-administration of 0.05

mg/kg/injection, 6 h/daily for 13 days) since they observed not only a decreased number of cells in the S phase, but also of cells that enter and exit the S phase without changing, however, its length. In fact, other psychostimulants like cocaine (10 or 100 µM, nontoxic concentrations) also interfere with the transition from G1-to-S phase in AF5 progenitor cells (a rat mesencephalic cell line), confirmed by the down-regulation of cyclin A2 (Lee et al., 2008). The authors further demonstrated that this effect was triggered by oxidative endoplasmic reticulum stress (Lee et al., 2008). Additionally, Hu and colleagues (2006) observed that cocaine inhibited proliferation in human fetal neural progenitor cells with simultaneous increase of the cyclin-dependent kinase inhibitor, p21. Moreover, very recently Blanco-Calvo and collaborators (2014) showed that acute administration of cocaine (10 mg/kg) decreases the number of proliferating cells in the subgranular zone, which was prevented by the inhibition of cannabinoid CB1 or CB2 receptors. Cannabinoid receptor blockade was also able to prevent hippocampal-dependent contextual memories induced by cocaine (Blanco-Calvo et al., 2014).

On the other hand, with the present study we verified that METH did not alter protein expression of cyclin inhibitors, p21 and p27, which suggests the involvement of other mechanisms in METH-induced cyclin E down-regulation. In fact, Heo and collaborators (2006) showed that blockade of MAPK pathway with PD-98059 (MEK1 inhibitor) decreased both cyclins D1 and E protein levels. These authors investigated the involvement of MAPK pathway activation in cell proliferation by stimulating mouse embryonic stem cells with epidermal growth factor (EGF), which induced an increase in the phosphorylate state of ERK1/2 and cyclin E proteins (Heo et al., 2006). Furthermore, homocysteine, a sulfur-containing intermediate of methionine metabolism, decreased phosphorylation of ERK1/2 and cyclin E protein expression in mice SVZ

cells incubated with FGF-2 (Rabaneda et al., 2008). Based on our results, we may suggest that METH acts upstream to ERK1/2 by decreasing the phosphorylation levels of EGFR, which will interfere with the MAPK pathway through down-regulation of pERK1/2 followed by a decrease in cyclin E expression, that in turn will affect cell cycle progression and resulting in the impairment of DG stem cell self-renewal. In fact, little is known regarding the effect of METH on EGFR, but it was previously demonstrated that neonatal EGF administration increased METH-induced locomotor responses (Mizuno et al., 2004). Still, it was shown that EGFR plays an active role in promoting cell proliferation and neuronal differentiation in the SGZ, as well as in rescuing neurogenesis in aged rats (Jin et al., 2003). Additionally, in a Parkinson's disease animal model, EGFR expression is down-regulated in proliferating cells in the SVZ (Hö glinger et al., 2004). Furthermore, it was demonstrated that EGFR expression and signaling was reduced in the SVZ of aged mice, which resulted in a decrease of cell proliferation and neurogenesis (Enwere et al., 2004). Consistent with this finding, we suggest that METH decreases the expression of pEGFR, which down-regulates MAPK signaling as verified by a transient decrease of ERK1/2 phosphorylation, which could lead to down-regulation of cyclin E and, consequently, a decrease in DG stem cell proliferation and self-renewal.

Although MAPK pathway can mediate cell proliferation, it can also be involved in memory performance. Indeed, depending on the frequency of METH exposure, an acute (Cao et al., 2013) or chronic (Ito et al., 2007) administration can improve or decline memory performance, accompanied with an increase or decrease in the phosphorylation levels of ERK1/2, respectively. Furthermore, ERK1/2 activation leads to the activation of the downstream transcription factor cyclic adenosine monophosphate response element-binding protein (CREB) followed by increased

expression of c-fos, which in the DG identifies neurons processing spatial information (Sweatt, 2001). Noteworthy, activation of ERK1/2 is necessary to generate long-term potentiation in DG immature neurons (Darcy et al., 2014). Interestingly, Kee and collaborators (2007) verified that immature neurons present increased c-fos expression, which strongly suggest that these cells are recruited to integrate spatial memory circuitries. Accordingly, in the present study we demonstrate that METH transiently decreased pERK1/2 protein levels in DG stem cells and increased immature neurons, which allow us to hypothesize that these neurons can be synaptically active and though participate in memory processes.

Other important characteristic of neural stem cells is the ability to self-renew. Thus, we further explored for the first time the effect of METH on DG stem cell self-renewal. METH, at nontoxic concentrations (1 nM or 10 nM), was able to decrease the number of primary neurospheres, showing that this drug has a negative effect on stem cell proliferation. In fact, the findings obtained in cell cycle analysis can be correlated with the decreased number of primary neurospheres. Specifically, METH delayed G0/G1-toS phase progression which decreased the proliferative capacity of DG stem cells and, consequently, could decrease the number of neurospheres. Also, at the highest nontoxic concentration (10 nM), METH decreased the number of secondary neurospheres proving that it affects DG stem cell self-renewal. Some studies addressed the effect of METH on DG neurogenesis and focused mainly on cell proliferation. In detail, it has been described that METH (25 mg/kg) transiently decreases the number of BrdU-positive cells in gerbil dentate gyrus (Teuchert-Noodt et al., 2000). Moreover, Mandyam and collaborators (2008) observed that this drug (0.05 mg/kg/infusion) administered 1 h/day and 6 h/day decreases the number of proliferating cells, characterized by the decreased number of Ki-67-positive cells, a protein expressed

during the active phases of the cell cycle. Also, METH induces long-term effects in SGZ stem/progenitor cell proliferation, as described by Schaefers and colleagues (2009) showing that a single METH administration (50 mg/kg) to 14-day-old gerbils decreases the number of BrdU-positive cells, 45 days post-administration. In addition, our group demonstrated that 100 µM METH (48 h of exposure) reduced proliferation in SVZ cultures (Bento et al., 2011).

As METH impairs self-renewal capacity, we also explored the influence of METH in DG stem cell-fate division, a suitable method to characterize the phenotype of cells that are derived from the division of one DG stem cell. Indeed, a stem cell can undergo symmetric cell division towards self-renewal, where the two resulting cells are both stem cells ($Sox2^+/Sox2^+$ pairs of daughter cells), and towards differentiation, resulting two cells that are committed to differentiate ($Sox2^-/Sox2^-$ pairs of daughter cells). Moreover, one stem cell can divide asymmetrically, originating one pair of cells consisting in one $Sox2^+$ and one $Sox2^-$ cell. Accordingly, we found that METH (10 nM) decreased self-renewal symmetric cell division ($Sox2^+/Sox2^+$ pairs of daughter cells), directing division towards $Sox2^-/Sox2^-$ pairs of daughter cells consistent with cell commitment. In fact, an animal study showed that Sox2-positive cells in the SGZ are able to undergo symmetric or asymmetric cell division, in which one Sox2-positive cell can give rise to one neuron and one astrocyte, or to one neural stem cell and one neuron, respectively (Suh et al., 2007). Also, we observed that these alterations could be, in part, due to the activation of NMDA receptor signaling because inhibition of these receptors prevented the shift of cell division towards differentiation. Moreover, we had previously demonstrated that METH induces an increase of glutamate release from DG neurospheres and the inhibition of NMDA receptors protects DG cells from METH toxicity (Baptista et al., 2012). In fact, several brain injuries, such as status epilepticus

(Sugaya et al., 2010), cerebral ischemia (Choi et al., 2012) and traumatic brain injury (Zheng et al., 2013) can increase neurogenesis, and glutamate seems to play a central role upon activation of NMDA receptors (Arvidsson et al., 2001; Urbach et al., 2008). Indeed, Nacher and colleagues (2003) observed that a single injection of NMDA receptor antagonist (CGP-43487, 5 mg/kg) prevents age-induced decrease of SGZ cell proliferation and neurogenesis in both middle-aged (10 month-old) and aged (20 month-old) Fisher F344 rats. Moreover, administration of NMDA (30 mg/kg) to Sprague-Dawley rats decreased the population of proliferating cells in the DG, whereas administration of MK-801 or CGP37849 resulted in the opposite effect (Cameron et al., 1995). Also, MK-801 administration (3 mg/kg) to NMDA-infused (2 mg/ml) Wistar rats increases the phosphorylated levels of ERK in newly generated neurons (Okuyama et al., 2004), which strengths the involvement of NMDA receptors in decreasing cell proliferation through regulation of the MAPK pathway. Moreover, Deisseroth and collaborators (2004) demonstrated that NMDA receptors play an active role in neuronal differentiation, showing that excitatory stimuli induced by NMDA receptor activation enhances expression of NeuroD, a downstream regulator of neuronal differentiation, and consequently increases neurogenesis in neural progenitor cells culture.

The present study also reveals that METH (10 nM) enhanced differentiation into immature neurons in DG neurospheres, verified by the increase of DCX protein levels. This effect correlates with the fact that METH increases the pairs of cells that did not express Sox2 ($Sox2^{-}/Sox2^{-}$ cell pairs), *i.e.*, differentiating cells. Furthermore, cell cycle alterations induced by METH suggest a differentiation shift. In fact, the length of G1 phase may influence the decision for a neural stem cell to proliferate or differentiate as reviewed by Salomoni and Calegari (2010). Furthermore, METH did not induce any alterations in GFAP expression, indicating that immature neurons are preferentially

generated rather than astrocytes. In accordance to our findings, it was described by Mandyam and collaborators (2008) that METH self-administration (0.05 mg/kg/infusion, 1 h/day, 2 days/week for 49 days) increases neuronal differentiation and maturation of hippocampal progenitor cells.

5 Conclusions

Our results show that METH interferes with DG stem cell proliferation, an effect due to a delay on cell cycle G0/G1-to-S transition involving down-regulation of pEGFR, cyclin E and and pERK1/2. Additionally, METH impairs DG cell self-renewal capacity *via* NMDA signaling. These effects could be useful to elucidate the impairment of hippocampal neurogenesis and memory deficits in METH abusers.

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Author contributions

Sofia Baptista designed the experiments and performed, conceived and analyzed most of the data and wrote the article. Charlène Lasgi performed the quiescence (G0 phase) experiments and analyzed the data. Caroline Benstaali performed some cell cultures.

Nuno Milhazes and Fernanda Borges were responsible for methamphetamine synthesis. Carlos Fontes-Ribeiro provided administrative support. Fabienne Agasse and Ana Paula Silva designed the experiments, analyzed the data, reviewed the article and provided administrative and financial supports.

Author Disclosure Statement

The authors declare no potential conflicts of interest.

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Figure legends

Figure 1 METH can induce DG stem/progenitor cell death. (A) Schematic representation of the experimental design to assess DG cell death. DG neurospheres were exposed to METH (10 or 100 nM) for 24 h and the number of TUNEL- and Sox2-positive cells was determined to evaluate apoptotic cell death of stem cells. (B) Representative fluorescence images of a TUNEL-positive nucleus (green) of a Sox2-positive stem cell (red) from a control condition. Scale bar: 10 µm. (C) Quantification of TUNEL- and Sox2-positive cells in the presence of 10 or 100 nM METH. Only the highest concentration was toxic to stem cells. Data are expressed as % of control + SEM from at least 2 independent cultures where each condition was performed in duplicate. $^{**}P < 0.01$, significantly different from control, using Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test.

Figure 2 METH delays DG neurosphere cell cycle in the transition from G0/G1 to S phase and induces stem/progenitor cell quiescence. (A) Schematic representation of the experimental protocol for cytometry analysis. (B, C) Quantification of cells present in different phases of the cell cycle after (B) 24 h or (C) 72 h of METH exposure. (B) METH increases the population of cells in the G0/G1 phase while decreasing the S phase. (C) In contrast, METH does not induce any alterations in cell cycle at 72 h exposure. Data are expressed as % of cells + SEM from at least 2 independent cultures and each condition was performed in duplicate. $^{**}P < 0.01$, significantly different from control using Mann Whitney post-hoc test. (D) METH (1 nM for 24 h) increases the number of cells in quiescent phase (G0) as showed by the representative dot plots (representative experiment of 3 independent cultures).

Figure 3 METH down-regulates cyclin E protein levels in DG neurospheres. (A) Schematic representation of the experimental design for western blot studies. METH (1 nM) does not induce alterations in (B) cyclin D1 (37 kDa) or (E) cyclin A (60 kDa) protein expression, but (C) down-regulates cyclin E (53 kDa) expression, which is also observed at (D) 10 nM METH. Additionally, METH (1 nM) does not interfere with (F) p21 (21 kDa) and (G) p27 (27 kDa) expression, the main inhibitors of the complexes cyclin D1/Cdk4/6 and cyclin E/Cdk2, respectively. Above the bars, representative western blot images of the different proteins are shown, including the housekeeping gene β-actin (42 kDa) or GAPDH (36 kDa). Data are expressed as % of control + SEM from at least 4 independent cultures. $^{*}P < 0.05$, significantly different from control using Mann Whitney post-hoc test.

Figure 4 METH down-regulates the phosphorylation form of EGFR and ERK1/2. (A) Schematic representation of the experimental design for western blot studies. Exposure to METH (1 nM) for 6 h does not induce any effect in (B) pFGFR1 (130 kDa), but (C) down-regulates the phosphorylation levels of EGFR (175 kDa). In addition, (D) 1 nM or (E) 10 nM METH decreases the phosphorylation form of ERK1/2 (42/44 kDa) after 6 h of drug exposure, but no alterations were observed after 24 h. Data are expressed as % of control + SEM from at least 4 independent cultures. $*P < 0.05$, significantly different from control using Mann Whitney or Kruskal-Wallis test followed by Dunn's Multiple comparison post-hoc tests. $^{§§}P < 0.01$, significantly different from METH 6 h-exposure using Kruskal-Wallis test followed by Dunn's Multiple comparison post-hoc test.

Figure 5 METH decreases DG neurosphere self-renewal. (A) Schematic representation of the experimental design to evaluate the impact of METH on self-renewal capacity of DG stem/progenitor cells. (B) METH (1 nM) decreases the number of primary neurospheres, but does not induce any effect on the number of secondary neurospheres. (C) However, 10 nM METH is able to decrease both the number of primary and secondary neurospheres. Data are expressed as % of control + SEM from 3 independent cultures and each condition was performed in triplicate. $*P < 0.05$; $**P < 0.01$, significantly different from control using Mann-Whitney post-hoc test.

Figure 6 METH triggers DG cell-fate division towards differentiation *via* activation of NMDA receptors. (A) Schematic representation of the experimental design to evaluate how METH interferes with cell fate. (B) METH (1 nM) does not induce any alterations in DG cell-fate division. (C) Nevertheless, a higher concentration of the drug

(10 nM METH) decreases the number of $Sox2^+/Sox2^+$ pairs of daughter cells, which was completely prevented by the NMDA receptor inhibitor, MK-801 (10 μM), and increases the number of $Sox2^-/Sox2^-$ pairs of cells without altering asymmetric cell division ($Sox2^+/Sox2^-$). Data are expressed as % of control + SEM from at least 2 independent cultures where each condition was performed in triplicate. $^{**}P < 0.01$, significantly different from control using Kruskal-Wallis test followed by Dunn´s Multiple comparison test. $^{\S}P < 0.05$; $^{\S\S\S}P < 0.001$, significantly different from 10 nM METH using Kruskal-Wallis test followed by Dunn´s Multiple comparison test. (D) Representative fluorescence images showing $Sox2^+/Sox2^+$ (symmetric self-renewing cell division), $Sox2^+/Sox2^-$ (asymmetric cell division), and $Sox2^-/Sox2^-$ (symmetric differentiation division) pairs of cells. Scale bar: 5 μm.

Figure 7 METH increases immature neurons in DG neurospheres. (A) Schematic representation of the experimental design of DG cells to evaluate the effect of METH on DG neurospheres growth. (B) METH (10 nM) increases DCX (40 kDa) protein levels, but without an effect on (C) GFAP (50 kDa) expression. Data are expressed as % of control + SEM from at least 2 independent cultures. $^{*}P < 0.05$, significantly different from control using Kruskal-Wallis test followed by Dunn´s Multiple comparison test. (D) Representative fluorescence images of DG neurospheres showing increased DCX (red) immunoreactivity in DG cells exposed to 10 nM METH, without inducing significant alterations in GFAP (green) expression. Nuclei were stained with Hoechst 33342 (blue). Scale bar: 50 μm.

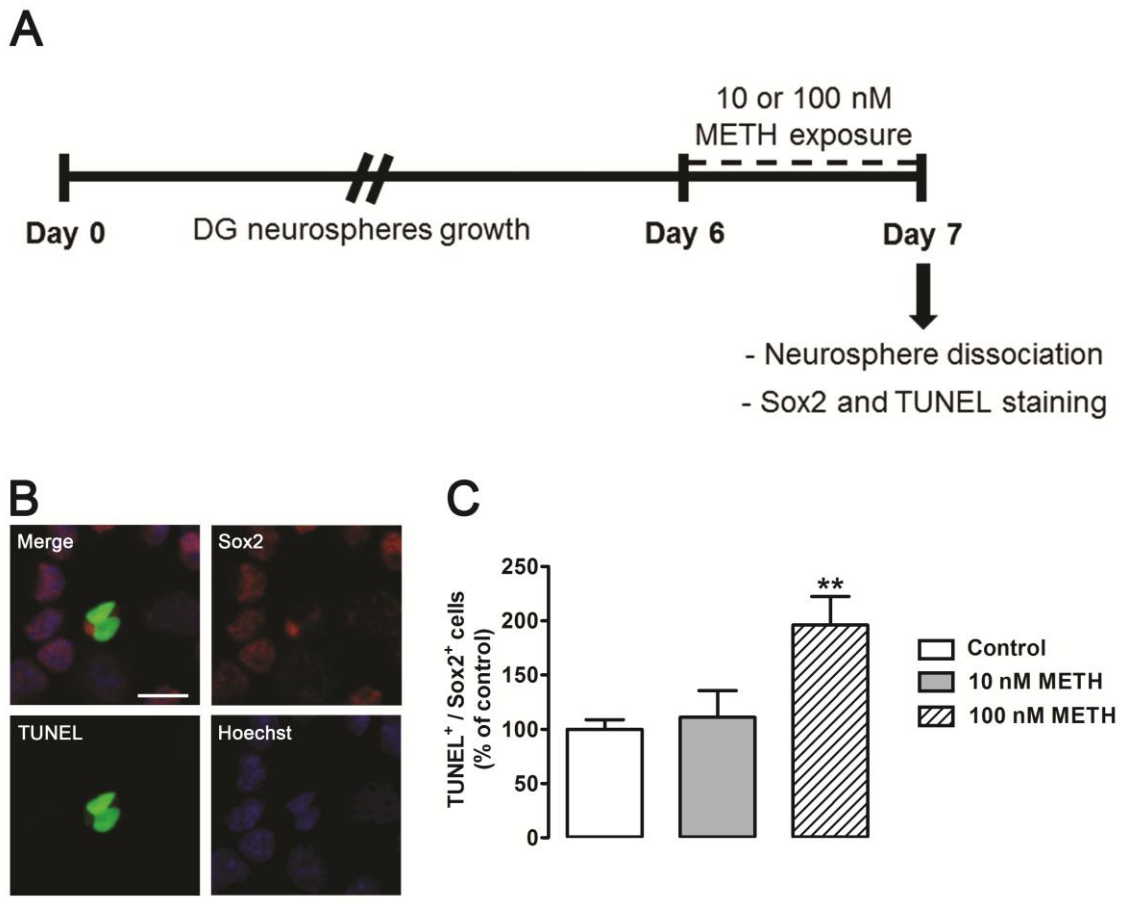


Figure 1

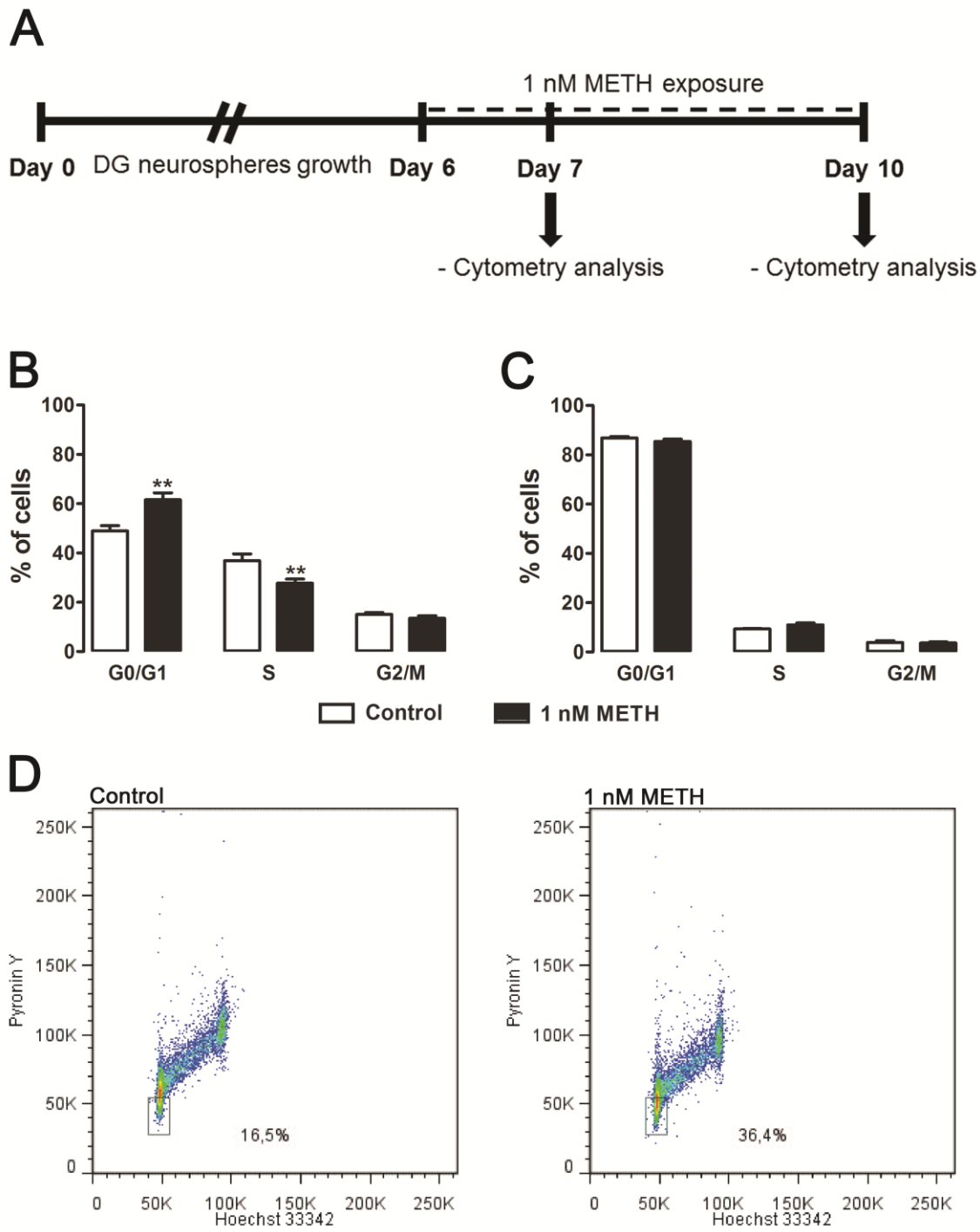


Figure 2

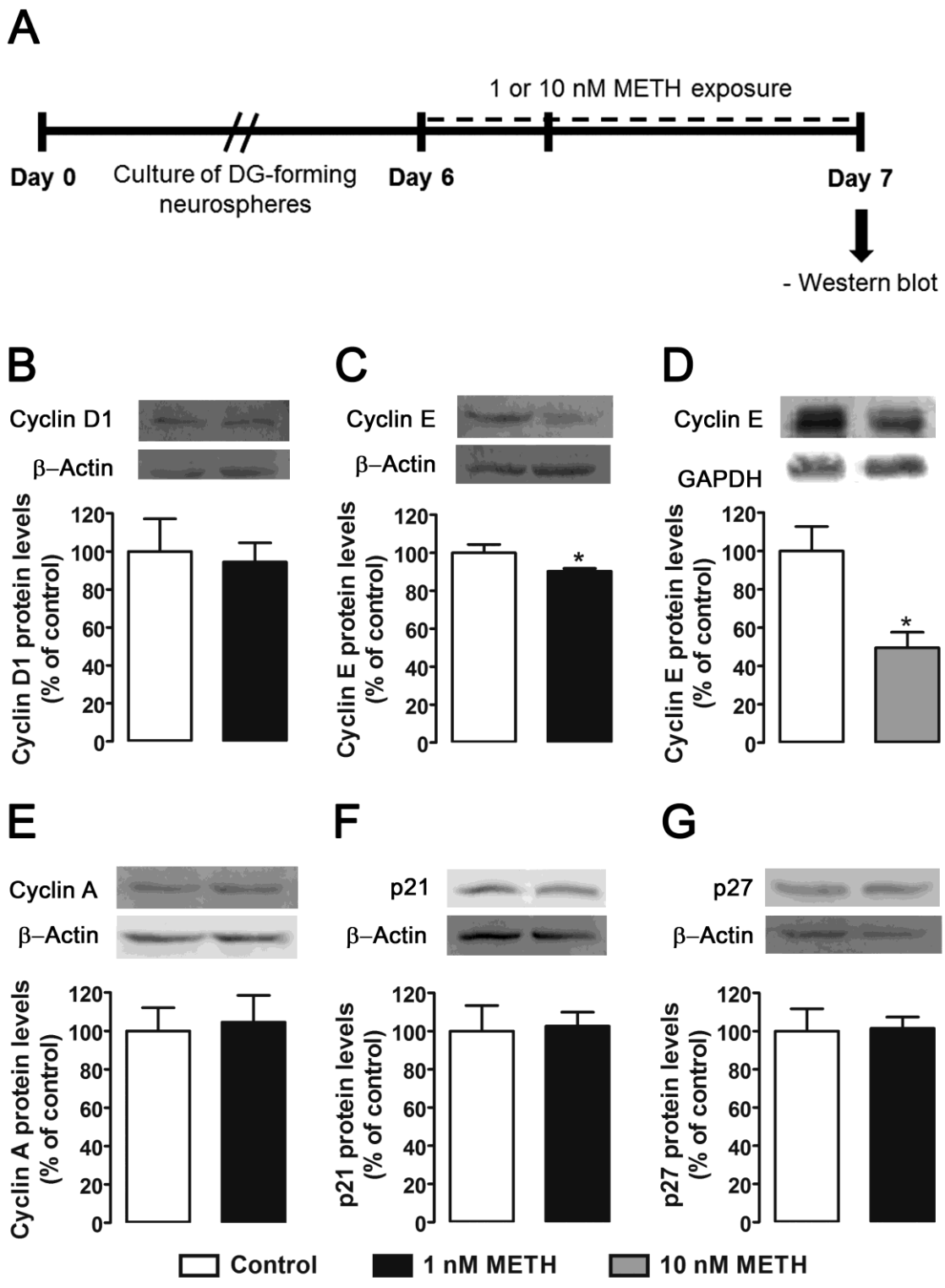


Figure 3

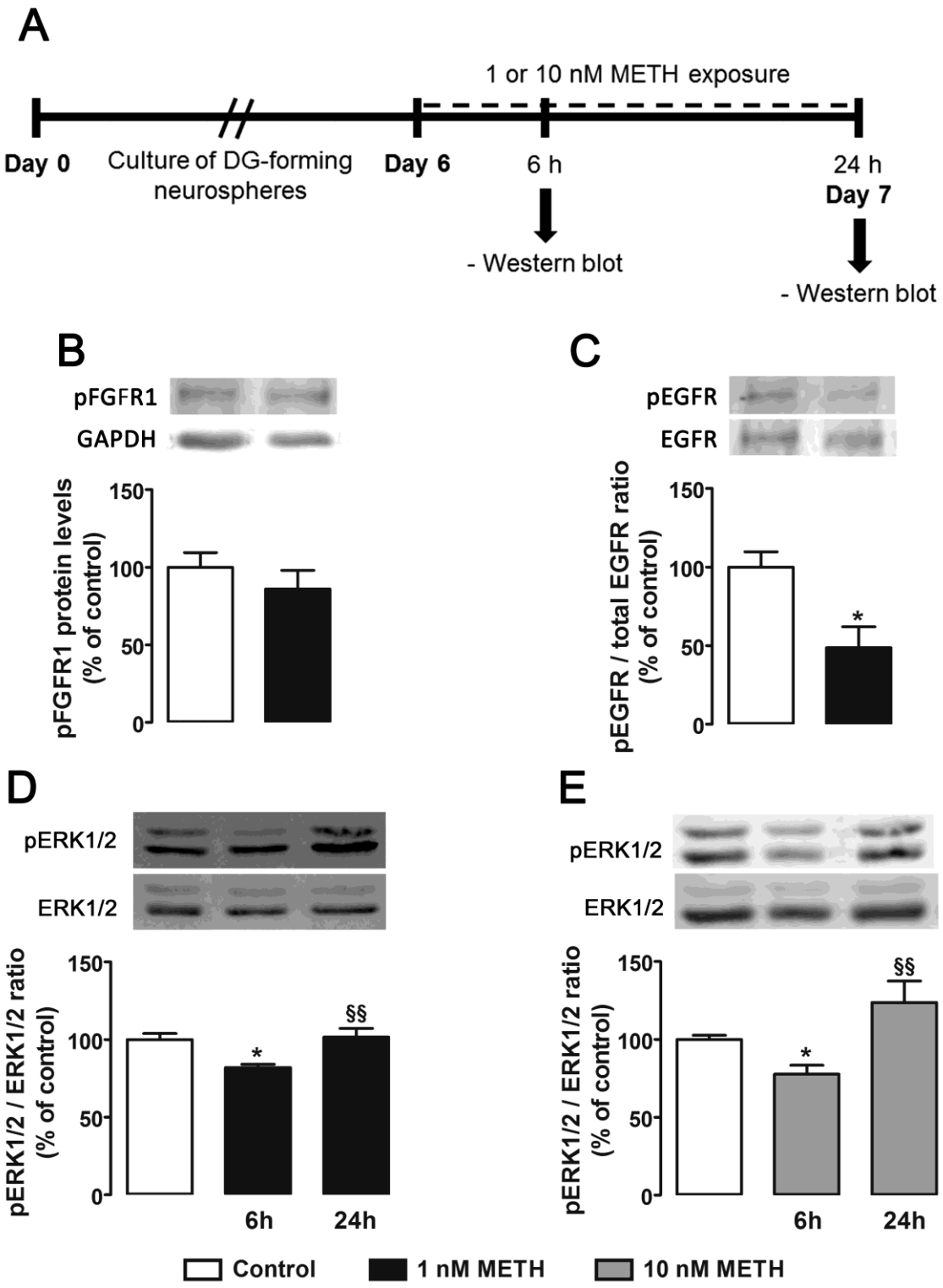

A
1 or 10 nM METH exposure
Day 0
Culture of DG-forming neurospheres
Day 6
6 h
24 h
Day 7
- Western blot
- Western blot
B
pFGFR1
GAPDH
pFGFR1 protein levels (% of control)
150
100
50
0
C
pEGFR
EGFR
pEGFR / total EGFR ratio (% of control)
150
100
50
0
*
D
pERK1/2
ERK1/2
pERK1/2 / ERK1/2 ratio (% of control)
150
100
50
0
*
§§
6h
24h
E
pERK1/2
ERK1/2
pERK1/2 / ERK1/2 ratio (% of control)
150
100
50
0
*
§§
6h
24h
Control
1 nM METH
10 nM METH

Figure 4

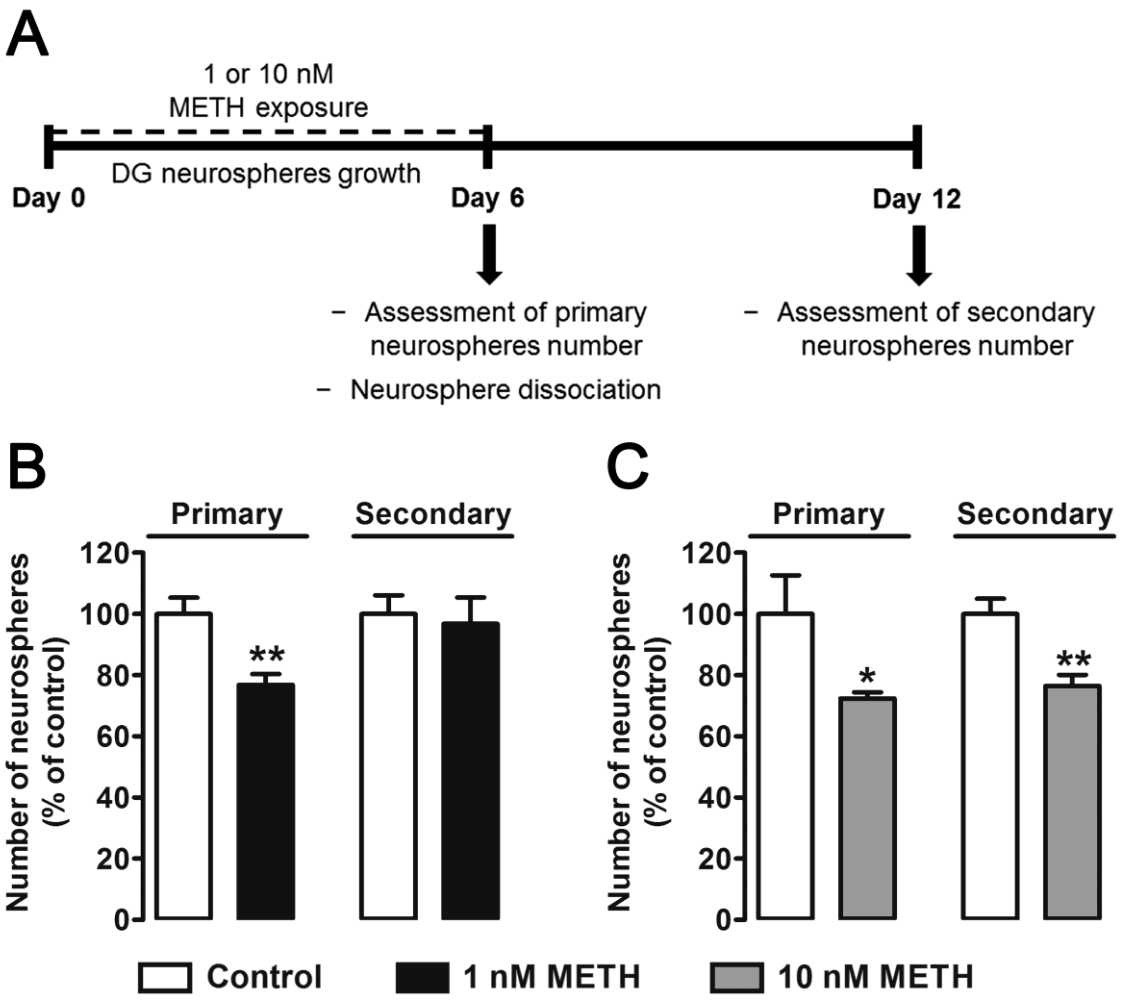


Figure 5

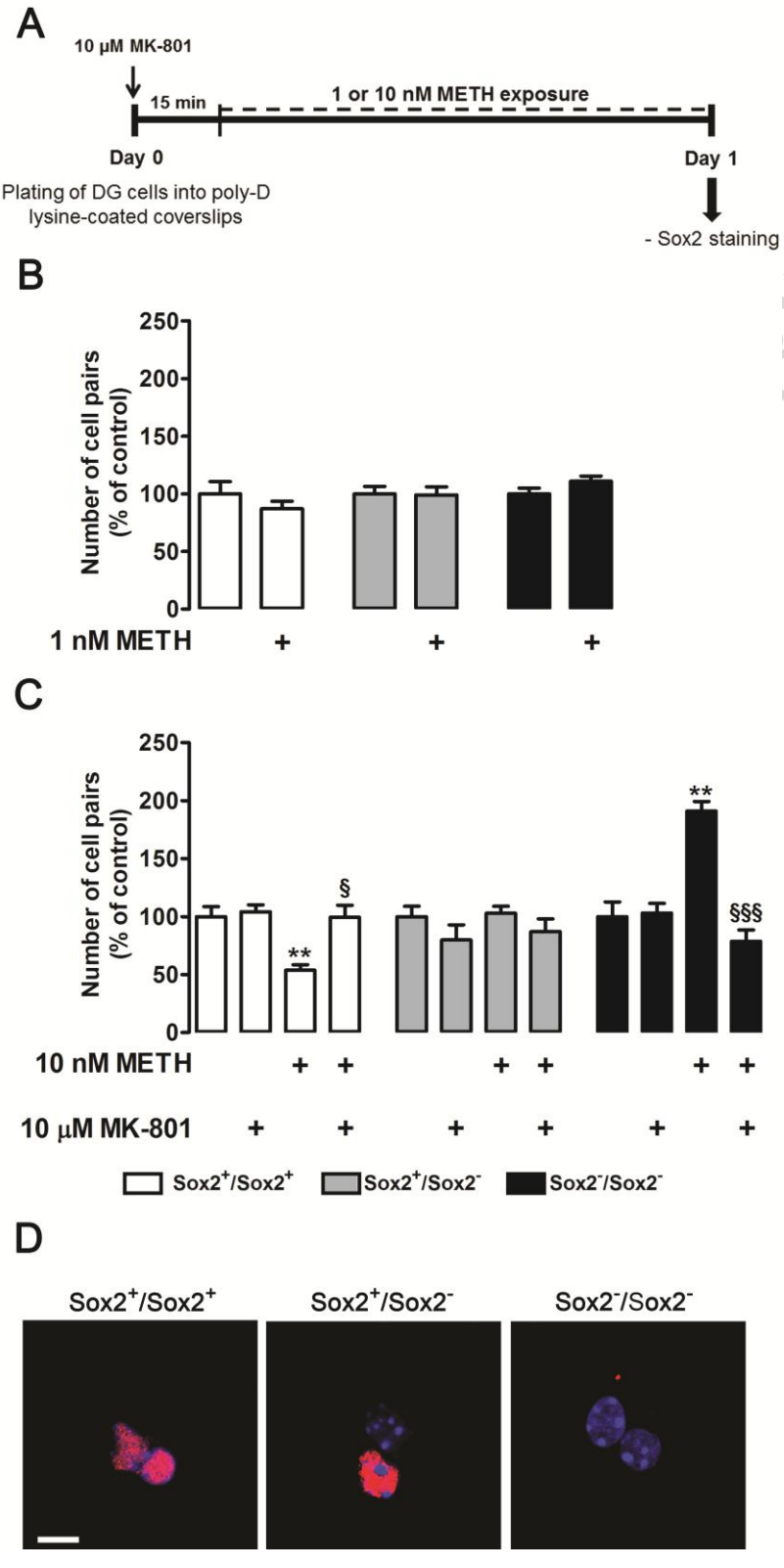


Figure 6

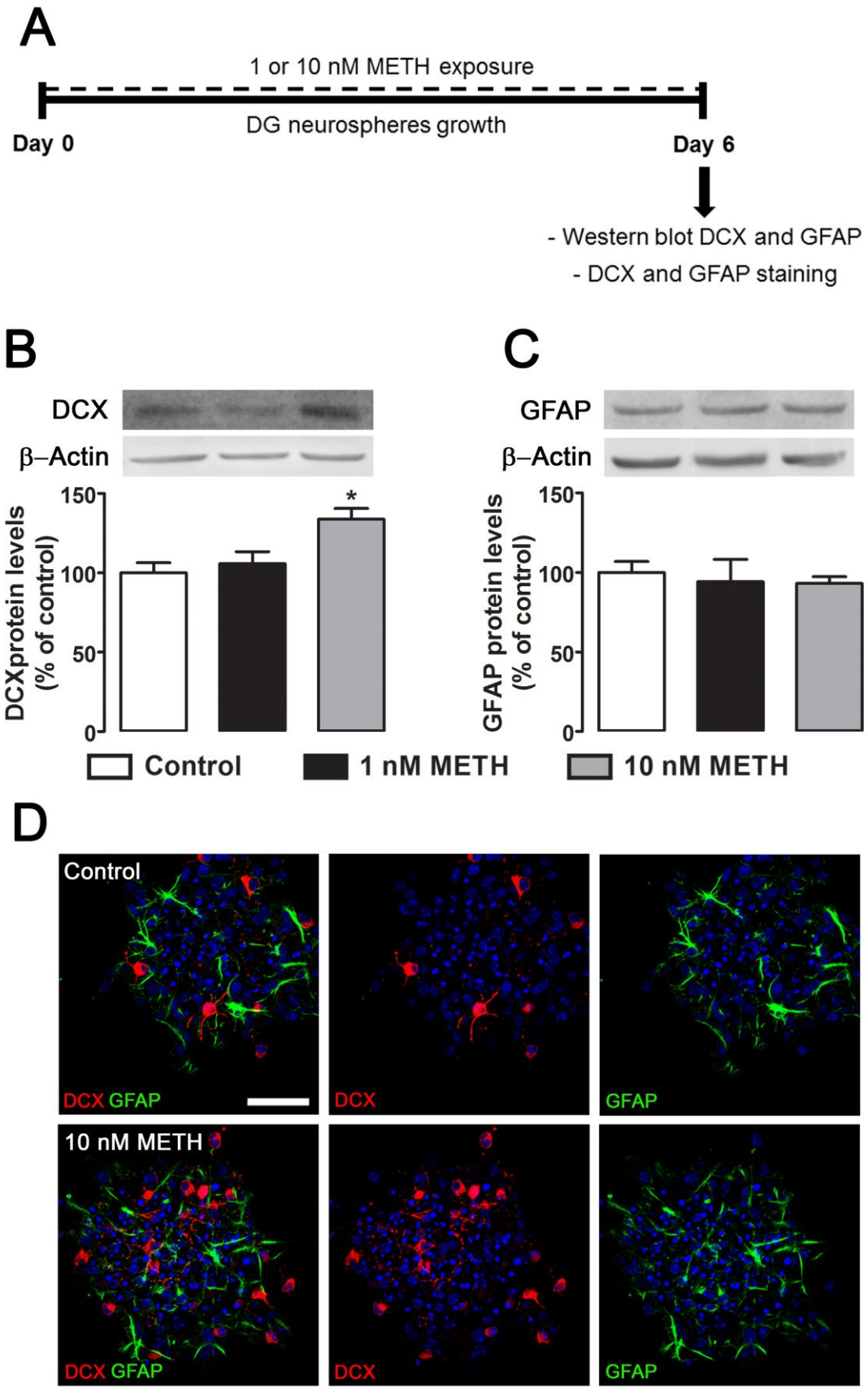
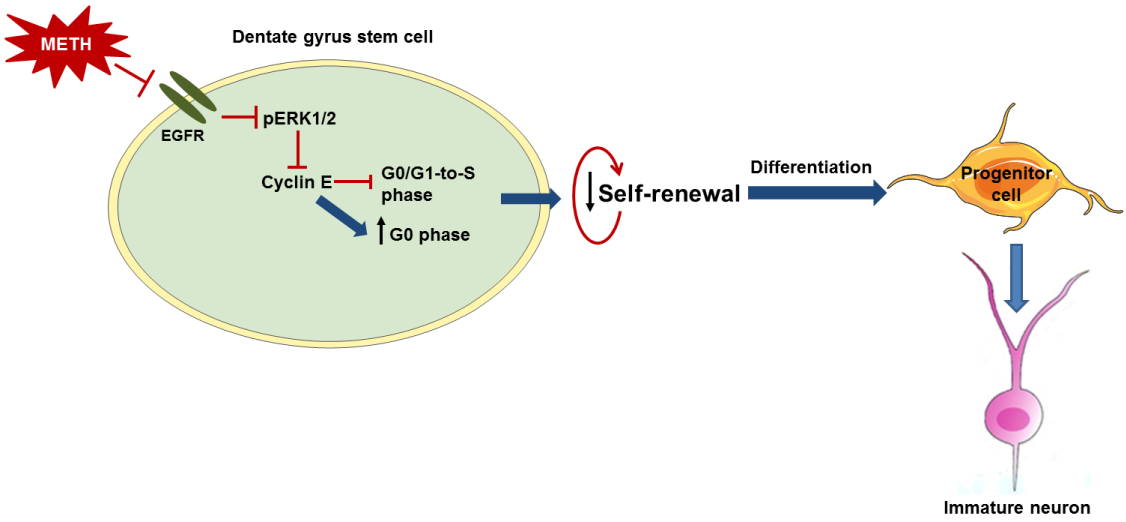

A
1 or 10 nM METH exposure
DG neurospheres growth
Day 0
Day 6
- Western blot DCX and GFAP
- DCX and GFAP staining
B
DCX
β–Actin
DCXprotein levels (% of control)
150
100
50
0
*
C
GFAP
β–Actin
GFAP protein levels (% of control)
150
100
50
0
Control
1 nM METH
10 nM METH
D
Control
DCX GFAP
DCX
GFAP
10 nM METH
DCX GFAP
DCX
GFAP

Figure 7



Graphical abstract

Graphical abstract Schematic representation of the effect of methamphetamine in dentate gyrus stem cell properties. METH down-regulates the phosphorylation levels of EGFR and ERK1/2, compromising DG cell proliferation as observed by down-regulation of cyclin E levels. As a result, METH increases the population of quiescent cells and delays the transition from G0/G1 to S phase of the cell cycle. These effects may result in the decrease of DG stem cell self-renewal under METH exposure. The impairment of self-renewal can direct DG stem cells to differentiate as observed by the increase of DCX expression in neurospheres.

Highlights

METH decreases the phosphorylation levels of EGFR and ERK1/2

METH delays cell cycle in G0/G1-to-S phase transition and decreases cyclin E levels

Methamphetamine (METH) decreases DG stem cells self-renewal

METH shifts cell division towards differentiation via activation of NMDA receptors

METH enhances neuronal differentiation in DG neurospheres