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Neuropeptide Y promotes neurogenesis and protection against methamphetamine-induced toxicity in mouse dentate gyrus-derived neurosphere cultures

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ABSTRACT

Methamphetamine (METH) is a psychostimulant drug of abuse that causes severe brain damage. However, the mechanisms responsible for these effects are poorly understood, particularly regarding the impact of METH on hippocampal neurogenesis. Moreover, neuropeptide Y (NPY) is known to be neuroprotective under several pathological conditions. Here, we investigated the effect of METH on dentate gyrus (DG) neurogenesis, regarding cell death, proliferation and differentiation, as well as the role of NPY by itself and against METH-induced toxicity. DG-derived neurosphere cultures were used to evaluate the effect of METH or NPY on cell death, proliferation or neuronal differentiation. Moreover, the role of NPY and its receptors (Y_1, Y_2 and Y_5) was investigated under conditions of METH-induced DG cell death. METH-induced cell death by both apoptosis and necrosis at concentrations above 10 nM, without affecting cell proliferation. Furthermore, at a non-toxic concentration (1 nM), METH decreased neuronal differentiation. NPY's protective effect was mainly due to the reduction of glutamate release, and it also increased DG cell proliferation and neuronal differentiation *via* Y_1 receptors. In addition, while the activation of Y_1 or Y_2 receptors was able to prevent METH-induced cell death, the Y_1 subtype alone was responsible for blocking the decrease in neuronal differentiation induced by the drug. Taken together, METH negatively affects DG cell viability and neurogenesis, and NPY is revealed to be a promising protective tool against the deleterious effects of METH on hippocampal neurogenesis.

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1. Introduction

Methamphetamine (METH) is an addictive psychostimulant drug of abuse highly toxic to different brain regions, including the hippocampus, and it was previously shown to induce working memory deficits (Simon et al., 2000; Thompson et al., 2004; Simões et al., 2007). Moreover, oxidative stress (Mirecki et al., 2004; Fitzmaurice et al., 2006), increase of glutamate release (Mark et al., 2004; Tata and Yamamoto, 2008), mitochondrial dysfunction (Jayanthi et al., 2004; Brown et al., 2005; Wu et al., 2007; Tian et al., 2009), neuroinflammation (Thomas et al., 2004; Gonçalves et al., 2008, 2010), hyperthermia (Bowyer et al., 1994) and disruption of the blood–brain barrier (BBB) (Bowyer et al., 2008; Ramirez et al., 2009; Silva et al., 2010; Martins et al., 2011) are some of the well-known neurotoxic features of this drug. However, the mechanisms underlying METH toxicity are still poorly understood, particularly its effect(s) on adult brain neurogenesis.

Adult hippocampal neurogenesis is an important modulator of brain plasticity (Prickaerts et al., 2004; Lledo et al., 2006) and plays a key role in learning and memory functions (Garthe et al., 2009; Clelland et al., 2009; Jessberger et al., 2009). This is a process that

Abbreviations: AraC, cytosine β-D-arabinofuranoside; BrdU, 5-Bromo-2′-deoxyuridine; DG, Dentate gyrus; METH, Methamphetamine; MK-801, (5R,10S)-(−)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cylcohepten-5,10-imine maleate; NeuN, Neuronal nuclei protein; NMDA, N-methyl-D-aspartate, NMDA; NPY, neuropeptide Y; PI, Propidium iodide; SGZ, Subgranular zone; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick-end labeling; Z-VAD, z-Val-Ala-DL-Asp (OMe)-fluoromethylketone.

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persists throughout life but several factors may impair the formation of new functional neurons. In fact, drugs of abuse generally have a negative impact on neurogenesis (Mandyam et al., 2008; Silva et al., 2010; Bento et al., 2011). Chronic exposure to cocaine decreases proliferation in the subgranular zone (SGZ), but has no effect on immature cell survival or cell death (Domínguez-Escribà et al., 2006). Furthermore, a binge alcohol administration to adolescent nonhuman primates decreases both proliferation and neurogenesis with an increase in neural degeneration (Taffe et al., 2010). On the other hand, 3,4-methylenedioxymethamphetamine (MDMA) administered to adolescent Sprague–Dawley rats increases proliferation, whereas neuronal differentiation is compromised (Catlow et al., 2010). Chronic administration of morphine and chronic self-administration of heroin impair cell proliferation and neurogenesis (Eisch et al., 2000). Concerning METH, the little information available points to a negative effect on cell proliferation (Tian et al., 2009; Teuchert-Noodt et al., 2000), neuronal differentiation, migration and survival of hippocampal neuronal cells (Mandyam et al., 2008). Additionally, we recently demonstrated that METH is toxic to stem/progenitor cells and decreases proliferation, neuronal differentiation and maturation in the subventricular zone (SVZ) (Bento et al., 2011).

Neuropeptide Y (NPY) is a 36 amino acid peptide widely distributed in both central and peripheral nervous systems (Dumont and Quirion, 2006). In fact, NPY regulates several physiological functions, such as feeding, anxiety, circadian rhythms, body temperature, sexual behavior and cognition (reviewed by Silva et al., 2005a), and has also an important neuroprotective role under pathological conditions (Woldbye et al., 2005; Silva et al., 2005b, 2007). Previously, Thiriet et al. (2005) demonstrated that METH-induced cell death in mouse striatum was prevented by NPY. Furthermore, this neuropeptide is able to increase proliferation and neuronal differentiation in the SGZ (Howell et al., 2003; Decressac et al., 2010), in the olfactory epithelium and in the SVZ through Y_1 receptors activation (Hansel et al., 2001; Agasse et al., 2008; Decressac et al., 2009; Thiriet et al., 2011).

The present study aimed to characterize the effects of METH on DG stem/progenitor cell survival, proliferation and neuronal differentiation, as well as to evaluate the protective role of NPY system. Overall, our results demonstrate that NPY is protective against METH-induced DG cell death and prevents the decrease of neurogenesis induced by this drug of abuse.

2. Materials and methods

2.1. Animals

Post-natal 1-3-day-old C57BL/6J mice were used in the present study. Experimental procedures were approved by the Institutional Review Board of Faculty of Medicine, University of Coimbra, and were performed according to the guidelines of the European Communities Council Directives (86/609/EEC) and the Portuguese law for the care and use of experimental animals (DL n°129/92). All efforts were made to minimize animal suffering and to reduce the number of animals.

2.2. Dentate gyrus-derived neurosphere cultures

Mice were sacrificed by decapitation and brains were removed and placed in sterile saline solution. DG fragments were dissected out from 450 μm-thick brain coronal sections, digested in 0.025% trypsin and 0.265 mM EDTA (both from Gibco, Rockville, MD, USA) and single cells were obtained by gentle trituration. Cells were diluted in serum-free culture medium (SFM) composed of Dulbecco's modified Eagle's medium/Ham's F-12 medium GlutaMAX-I supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1% B27 supplement, 10 ng/ml epidermal growth factor (EGF) and 5 ng/ml basic fibroblast growth factor (FGF-2) (all from Gibco). Then, 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 37 °C. Afterwards, neurospheres were plated onto poly-D-lysine (Sigma–Aldrich, St Louis, MO, USA) glass coverslips in SFM devoid of growth factors for 24 h in order to form a pseudo-monolayer.

2.3. Drug treatments

Methamphetamine [(+)-Methamphetamine hydrochloride, Sigma–Aldrich] was added to SFM devoid of growth factors at the concentrations of 1, 10, 100 or 1000 nM for 24 h, 48 h or 7 days to evaluate cell death, proliferation and differentiation, respectively. The concentrations of METH used were chosen in accordance with the physiological range found in blood, urine, or tissue samples, including the brain, of METH abusers (Takayasu et al., 1995; Kalasinsky et al., 2001; Klette et al., 2006; Melega et al., 2007), and with those that have been successfully used in prior studies (Ramirez et al., 2009; Bento et al., 2011; Lee et al., 2001; Tocharus et al., 2010; Aizenman et al., 2010).

To investigate the effect of NPY in cell proliferation and neuronal differentiation, DG-derived neurosphere cultures were incubated with 1 μM NPY (Bachem, Bubendorf, Switzerland) for 48 h and 7 days, respectively. Moreover, to study the protective role of NPY and its receptors, DG-derived neurosphere cultures were pre-incubated with 1 μM BIBP3226 (Y_1 receptor antagonist, Bachem) or 1 μM BIIE0246 (Y_2 receptor antagonist, Tocris, Bristol, UK,) for 15 min, followed by co-incubation with 1 μM NPY, 1 μM [Leu^{31},Pro^{34}]NPY, 300 nM NPY13-36 or 1 μM (Gly^{1},-$Ser^{3.22}$,$Gln^{4.34}$,Thr^{6},Arg^{19},Tyr^{21},$Ala^{23.31}$,Aib^{32})-PP (Y_1, Y_2 and Y_5 receptor agonists, respectively; all from Bachem) for 1 h, and then co-exposed with 10 nM METH for 24 h or 1 nM METH for 7 days for cell death and differentiation studies, respectively. In attempt to better clarify the cellular mechanisms involved in NPY-induced protection, DG-derived neurosphere cultures were pre-incubated with 1 μM NPY and the N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 (10 μM, Tocris), for 1 h and 15 min, respectively, and then co-exposed with METH (10 nM) for 24 h. Regarding the NPY system, the concentrations used in the present study were chosen based on previous works where we showed that NPY and its receptor agonists inhibited glutamate release from hippocampal synaptosomes (Silva et al., 2001) and intracellular Ca^{2+} concentration in cultured rat hippocampal neurons (Silva et al., 2003a), as well as prevented hippocampal excitotoxicity (Silva et al., 2003b).

To investigate the role of NPY on neuronal differentiation, DG-derived neurosphere cultures were co-incubated with 10 μM cytosine β-D-arabinofuranoside (AraC, Sigma–Aldrich) plus 1 μM NPY for 7 days. AraC is a nucleoside analogue which incorporates the C sites of the DNA strand, blocking the cells in the S phase of the cell cycle which results in the inhibition of cell proliferation (Azuma et al., 2001).

2.4. Immunocytochemistry

DG-derived neurosphere cultures were fixed in 4% paraformaldehyde (PFA), permeabilized in 1% Triton X-100, 3% bovine serum albumine (BSA; all from Sigma–Aldrich) in 0.1 M phosphate-buffered saline (PBS), followed by overnight incubation at 4 °C with mouse monoclonal anti-NeuN (1:100, MAB377, Chemicon, Temecula, CA), rabbit monoclonal anti-NPY (1:200, N9528, Sigma–Aldrich), sheep polyclonal anti-NPY Y_1 receptor (1:200, 6732-0150, AbD Serotec, Oxfordshire, UK), rabbit polyclonal anti-NPY Y_2 receptor (1:100, ANR-022, Alomone Labs, Jerusalem, Israel), mouse polyclonal anti-Tuj1 (1:500, MMS-435P, Covance, Emeryville, California, USA), mouse monoclonal anti-GFAP (1:500, #3670, Cell Signaling Technology, Danvers, MA) and mouse monoclonal anti-O4 (1:100, MAB345, Chemicon) in 0.1% Triton X-100, 0.3% BSA in PBS. Cells were rinsed and incubated for 1 h with the appropriate secondary antibodies: goat anti-mouse Alexa Fluor 488, donkey anti-rabbit 488, donkey anti-sheep 488 and goat anti-mouse 594 (1:200; all from Invitrogen). Nuclei were stained with 4 μg/ml Hoechst 33342 (Sigma–Aldrich) and slides were mounted in Dako fluorescence medium (Dako, Carpinteria, CA). To confirm antibody specificity, negative controls were performed for each immunocytochemical assay, and the antibodies used for the NPY system were also chosen based on previous studies (Ruscheweyh et al., 2007; Price et al., 2009; Ferreira et al., 2010; Thiriet et al., 2011). Fluorescence images for cell counts were recorded using an Axioskop 2 Plus fluorescent microscope, while representative images were recorded using a LSM 710 Meta confocal microscope (all from Carl Zeiss, Göttingen, Germany).

2.5. Cell death assays

To evaluate necrotic cell death, propidium iodide (PI; 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide; Sigma–Aldrich) was applied in DG-derived neurosphere cultures. PI is a polar non-toxic compound that in normal conditions does not cross the plasma membrane (Bernardino et al., 2005). Instead, PI only enters dying or dead cells, where membranes have lost their integrity, and then binds to DNA. In the present study, 3 μg/ml PI was applied to DG-derived neurosphere cultures for the last 40 min of the 24 h culture session. Afterwards, cells were fixed with 4% PFA. Then, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed to label apoptotic nuclei, as described previously (Bernardino et al., 2008; Bento et al., 2011). To confirm that METH triggers apoptosis in DG-derived neurosphere cultures, cells were also co-incubated for 24 h with 100 nM METH plus 25 μM z-Val-Ala-DL-Asp (OMe)-fluoromethylketone (Z-VAD; Calbiochem EMD4 Biosciences, Nottingham, UK), a general pan-caspase inhibitor that irreversibly binds to caspases. In control conditions, Z-VAD was also shown to decrease basal apoptosis in SVZ cultures as described previously (Bernardino et al., 2008; Bento et al., 2011).

To evaluate total cell death, the number of necrotic (PI-positive and TUNEL-negative) and apoptotic cells (TUNEL-positive/PI-negative and TUNEL-/PI-positive, representing cells in early and late phases of apoptosis, respectively) were counted and expressed as percentages of total cells stained with Hoechst 33342.

2.6. Cell proliferation studies

Cell proliferation was evaluated by 5-bromo-2′-deoxyuridine (BrdU, Sigma–Aldrich) incorporation based on our previous work (Bento et al., 2011). DG cells were treated for 48 h with METH (1–1000 nM) and 10 μM BrdU was added in the last 4 h of the culture session. According to Nowakowski et al. (1989), the estimated duration of the S-phase is 8 h, so the 4 h pulse with BrdU will allow the identification of cells that entered the S-phase of cell cycle.

Afterwards, cells were fixed in 4% PFA, rinsed in PBS and BrdU was unmasked following successive passages in 1% Triton X-100 for 30 min, ice-cold 0.1 M HCl for 20 min, and 2 M HCl for 40 min at 37 °C. Following the neutralization in sodium borate buffer (0.1 M $Na_2B_4O_7.10H_2O$, pH 8.5; Sigma–Aldrich) for 15 min, cells were rinsed in PBS and incubated in 3% BSA (Sigma–Aldrich), 0.3% Triton X-100 in PBS for 30 min. DG-derived neurosphere cultures were incubated with mouse monoclonal anti-BrdU antibody conjugated with Alexa Fluor 594 (1:100, B35132, Invitrogen) in PBS containing 0.3% Triton X-100 and 0.3% BSA, overnight at 4 °C. Nuclei were counterstained with Hoechst 33342 and cell preparations were mounted as previously described. Representative fluorescence images were recorded as described above.

2.7. Western blot analysis

Non-treated DG-derived neurosphere cultures 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). Afterwards, cells were centrifuged at 17000 × *g* for 15 min and protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific, Northumberland, UK). Then, 50 or 80 μg of protein samples were separated by electrophoresis in a 8% or 12% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Madrid, Spain) and blocked in 5% non-fat dried milk in PBS-0.5% Tween (PBS-T; Sigma–Aldrich) for 1 h. Afterwards, membranes were probed overnight at 4 °C with rabbit monoclonal anti-NPY (1:1000; Sigma–Aldrich), sheep polyclonal anti-NPY Y_1 receptor (1:10,000, AbD Serotec), rabbit polyclonal anti-NPY Y_2 receptor (1:200; Alomone Labs) or rabbit polyclonal anti-GluN1 (1:500; Tocris) antibodies and were re-probed with rabbit anti-β-actin (1:5000; Sigma–Aldrich) overnight at 4 °C. Membranes were rinsed in PBS-T and incubated for 45 min with alkaline phosphatase-conjugated secondary antibodies as follows: anti-rabbit IgG (1:20000, Amersham Biosciences, GE Healthcare Europe GmbH) and anti-sheep IgG, (1:1000, Sigma–Aldrich). Densitometric analysis was performed using the enhanced chemifluorescence (ECF) reagent (Amersham Biosciences) and visualized on the Typhoon 9000 system (GE Healthcare Europe GmbH). Band intensities were quantified using the ImageQuant 5.0 software.

2.8. High performance liquid chromatography

Levels of glutamate release from DG-derived neurosphere cultures exposed to 10 nM METH during several time points (30 min, 1 h, 6 h or 24 h) in the absence or presence of 1 μM NPY were analyzed by high performance liquid chromatography with electrochemical detection (HPLC-EC) using a Gilson instrument (Gilson, Inc., Middleton, WI, USA). For that, samples were diluted 1:1 in the following solution: 0.4 mM sodium disulfite, 0.9 mM EDTA and 0.4 N perchloric acid. Afterwards, samples were filtered (0.2 μm nylon filter, Costar, Corning, NY) and derivatized by adding 100 μl of NaOH 0.1N and 15 μl OPA (10 mg/mL of OPA, 45.4 M sodium sulphite, 4.5% absolute ethanol in 327 mM borate buffer at pH 10.4) to 50 μl of sample. Samples were allowed to react at room temperature in the dark for a period of 10 min. The mobile phase containing 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA and 20% methanol (pH 4.4) was filtered and degassed with an isocratic protocol (0.8 mL/min) passing through a Supercosil LC 18 (25 cm × 4.6 mm × 5 mm, Supelco, Bellefonte, PA, USA) analytical column. The potential was set at 0.85 V relative to an Ag/AgCl reference electrode. Concentrations of glutamate were calculated using a standard curve generated with a glutamate standard purchased from Sigma (St. Louis, MO, USA).

2.9. Data analysis

Cell quantifications were performed in the border of the neurospheres where migrating cells emerged, forming a pseudo-monolayer. All the experimental conditions were performed in three different wells obtained from at least two independent cultures. Number of TUNEL, PI, BrdU and NeuN were obtained as percentage of total Hoechst-positive cells from the total of five independent microscopic fields in each coverslip. Statistical analysis was determined by using an analysis of variance (one-way ANOVA) followed by Dunnett's, Bonferroni's or Mann Whitney post-hoc tests, as indicated in the figure legends. Data are expressed as mean ± SEM, and statistical significance level was set for $p < 0.05$.

3. Results

3.1. METH induces DG cell death

It is well established that METH induces neural dysfunction or/and death and that the hippocampus is particularly affected by this drug (Thompson et al., 2004; Simões et al., 2007; Gonçalves et al., 2010). To determine the toxic effect of METH on DG-derived neurosphere cultures, we evaluated total cell death (PI-positive and TUNEL-positive cells), and also discriminated between necrotic-like (PI-positive and TUNEL-negative cells) and apoptotic-like cell death (TUNEL-positive cells). No significant differences were observed in total cell death when cells were exposed to 1 nM METH (Fig. 1A; control: 26.18 ± 1.59%; 1 nM: 23.34 ± 2.70%). However, total cell death increased in the presence of 10, 100 or 1000 nM METH as follows: 45.36 ± 6.04%, $p < 0.05$; 49.95 ± 6.53%, $p < 0.001$; 59.37 ± 3.73%, $p < 0.001$, respectively (Fig. 1A). Moreover, the increase in total cell death was due to an increase in both necrotic- and apoptotic-like cell death (Fig. 1B and C, respectively). METH at 1 nM had no effect on the number of PI-positive cells (12.57 ± 1.40%) when compared to control (11.68 ± 1.02%; Fig. 1B) but did induce an increase when METH was applied at 10, 100 or 1000 nM (22.45 ± 1.41%, 29.38 ± 4.94% or 28.14 ± 2.22%, respectively; $p < 0.001$ for all concentrations; Fig. 1B). Similarly, the number of TUNEL-positive cells in DG-derived neurosphere cultures exposed to 1 nM METH (8.19 ± 1.10%) was not significantly different from control (8.12 ± 0.31%; Fig. 1C) but increased in

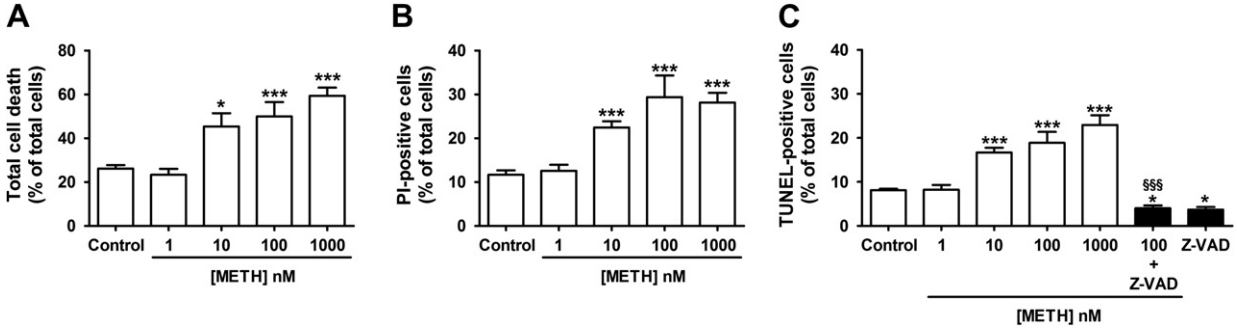

Fig. 1. Methamphetamine (METH) induces cell death to dentate gyrus (DG)-derived neurosphere cultures. (A) METH, at concentrations above 10 nM for 24 h, increases total cell death by both necrosis and apoptosis. (B, C) METH increases the number of (B) PI-positive cells (necrosis) and (C) TUNEL-positive cells (apoptosis). In order to confirm that METH triggers apoptosis, DG cells were incubated with the pan-caspase inhibitor, Z-VAD (25 μM), which prevents METH-induced increase of apoptosis. Data are expressed as mean percentage of total cells ± SEM, $n = 4$–14 coverslips. $^{*}p < 0.05$, $^{***}p < 0.001$, significantly different from control using Dunnett's post-hoc test. $^{§§§}p < 0.001$, significantly different when compared to 100 nM METH using Bonferroni's post-hoc test.

cultures exposed to 10, 100 or 1000 nM METH (16.67 ± 1.08%, 18.88 ± 2.47% or 22.94 ± 2.20%, respectively; $p < 0.001$ for all concentrations; Fig. 1C). Furthermore, in order to confirm that METH triggers apoptosis, DG-derived neurosphere cultures were co-incubated with the pan-caspase inhibitor, Z-VAD (25 μM), which completely prevented METH-induced apoptosis (100 nM METH + Z-VAD: 4.00 ± 0.16%; Z-VAD: 3.69 ± 0.59%, $p < 0.05$; Fig. 1C).

3.2. NPY is protective against METH-induced DG cell death by inhibiting glutamate release

It is well known that NPY is neuroprotective under several pathological conditions (Woldbye et al., 2005; Silva et al., 2005b, 2007). Thus, we aimed to evaluate a possible protective role of this neuropeptide in DG-derived neurosphere cultures exposed to a toxic concentration of METH (10 nM; Fig. 1). We concluded that NPY (1 μM), which is itself not toxic, completely prevented apoptotic cell death induced by 10 nM METH (control: 8.10 ± 0.32%; NPY: 8.49 ± 0.48%; METH: 16.67 ± 1.08%, $p < 0.001$; METH + NPY: 8.72 ± 0.70%, $p < 0.001$ *vs.* 10 nM METH; Fig. 2A). To identify which NPY receptor mediates this protective effect, selective antagonists for Y_1 and Y_2 receptors (1 μM BIBP3226 and 1 μM BIIE0246, respectively) were used. We observed that both antagonists were able to block the protective effect induced by 1 μM NPY under conditions of METH-induced apoptosis (METH + NPY + Y_1 receptor antagonist: 14.24 ± 1.78%, $p < 0.01$; METH + NPY + Y_2 receptor antagonist: 15.08 ± 1.16%, $p < 0.001$; Fig. 2A). Accordingly, the selective activation of Y_1 or Y_2 receptors by the respective agonists (1 μM [Leu^{31},Pro^{34}]NPY or 300 nM NPY13-36, respectively) completely prevented METH-induced apoptosis (control: 8.10 ± 0.32%; METH + Y_1 receptor agonist: 7.41 ± 0.39%; METH + Y_2 receptor agonist: 7.84 ± 0.46%, $p < 0.001$ *vs.* 10 nM METH; Fig. 2B). Moreover, our results demonstrate that the Y_5 receptor did not play any protective role since the number of TUNEL-positive cells were similar to the ones observed in the presence of METH alone (METH: 16.67 ± 1.08%; METH + Y_5 receptor agonist: 13.43 ± 0.90%, $p < 0.001$; Fig. 2B). Additionally, the Y_1, Y_2 or Y_5 receptor agonists by themselves did not affect apoptotic cell death (control: 8.10 ± 0.32; Y_1 receptor agonist: 6.75 ± 0.79%; Y_2 receptor agonist: 7.73 ± 0.63%; Y_5 receptor agonist: 7.59 ± 0.63%; Fig. 2B). We further evaluate if NPY could also prevent necrosis induced by METH. Interestingly, we observed that NPY (1 μM) did not protect DG-derived neurosphere cultures from METH-induced necrosis (Fig. 2C). Specifically, the number of PI-positive cells in DG-derived neurosphere cultures was as follows: control, 11.68 ± 1.02%; METH, 22.45 ± 1.41%, $p < 0.001$; NPY, 14.74 ± 0.92%; METH + NPY, 23.36 ± 1.31%, $p < 0.001$ (Fig. 2C).

One of the well known mechanisms of METH-induced neurotoxicity is the abnormal increase of glutamate release (Mark et al., 2004), which leads to the hyperactivation of ionotropic glutamate receptors that may culminate in cell death (Schinder et al., 1996; reviewed by Riddle et al., 2006). Furthermore, we demonstrated that NPY inhibits glutamate release in rat hippocampal cultures (Silva et al., 2001, 2003b). Thus, we hypothesized that the protective role of NPY in DG-derived neurosphere cultures could involve the modulation of glutamatergic system. To clarify this issue, we started by showing that these cultures express the GluN1 subunit of the NMDA receptors, since this is an obligatory subunit of these receptors (Fig. 3A). Our results also show that METH (10 nM) did not interfere with GluN1 protein levels (Fig. 3A). Then, we analyzed the involvement of NMDA receptors in METH-induced DG-derived neurosphere cultures apoptosis, and we concluded that the blockade of NMDA receptors by MK-801 (10 μM) completely prevented cell death induced by METH (control: 5.06 ± 0.54; MK-801: 5.36 ± 0.42; METH + MK-801: 5.57 ± 0.60%; Fig. 3B). Interestingly, this protective effect was very similar to that induced by NPY (METH + NPY: 5.03 ± 0.56%) or in the presence of both MK-801 + NPY (4.94 ± 0.40%; Fig. 3B). Thus, since NMDA receptors are involved in METH-induced cell death and NPY is able to prevent this toxic effect, we further evaluated the modulation of glutamate release by both METH and NPY. In fact, we concluded that 10 nM METH increases glutamate release by DG-derived neurosphere cultures at all time points analyzed (30 min: 134.60 ± 12.76%; 1 h: 121.70 ± 3.30%; 6 h: 157.40 ± 21.33%; 24 h: 124.0 ± 9.89% of control; Fig. 3C). Regarding the effect of NPY, we observed that at 30 min this peptide did not interfere with the levels of glutamate release (133.40 ± 5.12% of control; Fig. 3C). However, NPY prevented METH-induced increase of glutamate release at 1 h, 6 h and 24 h after METH exposure (1 h: 84.79 ± 8.62%, $p < 0.05$; 6 h: 100.10 ± 3.63%, $p < 0.01$; 24 h: 87.42 ± 9.46%, $p < 0.01$; Fig. 3C).

As abovementioned, NPY proved to protect DG-derived neurosphere cultures from METH-induced cell death. Thus, we further evaluated the expression of NPY as well as both Y_1 and Y_2 receptors by western blot (Fig. 4A–C) and immunocytochemistry (Fig. 4D–L) in DG-derived neurosphere cultures. In fact, NPY-positive cells

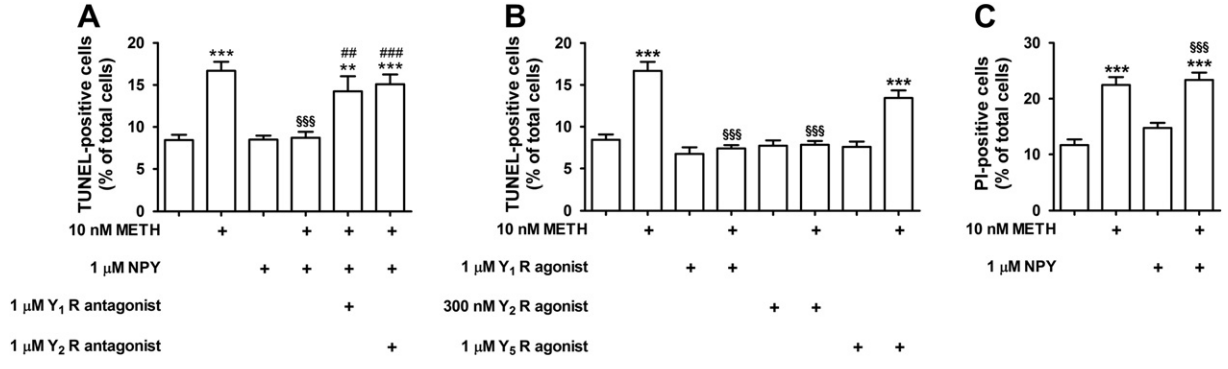

Fig. 2. Neuropeptide Y (NPY) prevents METH-induced apoptosis through activation of both Y_1 and Y_2 receptors. (A) Pre-incubation with NPY (1 μM; 1 h) followed by co-exposure to METH (10 nM) for 24 h prevents drug-induced increase of TUNEL-positive cells in DG-derived neurosphere cultures, and 1 μM BIBP3226 or 1 μM BIIE0246 (Y_1 or Y_2 receptor antagonists, respectively) inhibit the protective effect of NPY. (B) Pre-incubation (1 h) with 1 μM [Leu^{31},Pro^{34}]NPY or 300 nM NPY13-36 (Y_1 and Y_2 receptor agonists, respectively) followed by co-exposure with METH (10 nM) for 24 h reduces the increase of apoptotic DG cell death induced by METH. However, NPY Y_5 receptor agonist (1 μM Gly^1,-Ser^{3,22},Gln^{4,34},Thr^6,Arg^{19},Tyr^{21},Ala^{23,31},Aib^{32})-PP) did not protect DG cells from METH-induced cell death. (C) NPY (1 μM) did not prevent the increased number of PI-positive cells induced by 10 nM METH. Data are expressed as mean percentage of total cells ± SEM, $n = 5$–14 coverslips. $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different when compared to control using Dunnett's post-hoc test. $^{§§§}p < 0.001$, significantly different when compared to 10 nM METH using Bonferroni's post-hoc test. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, significantly different when compared to 10 nM METH plus 1 μM NPY using Bonferroni's post-hoc test.

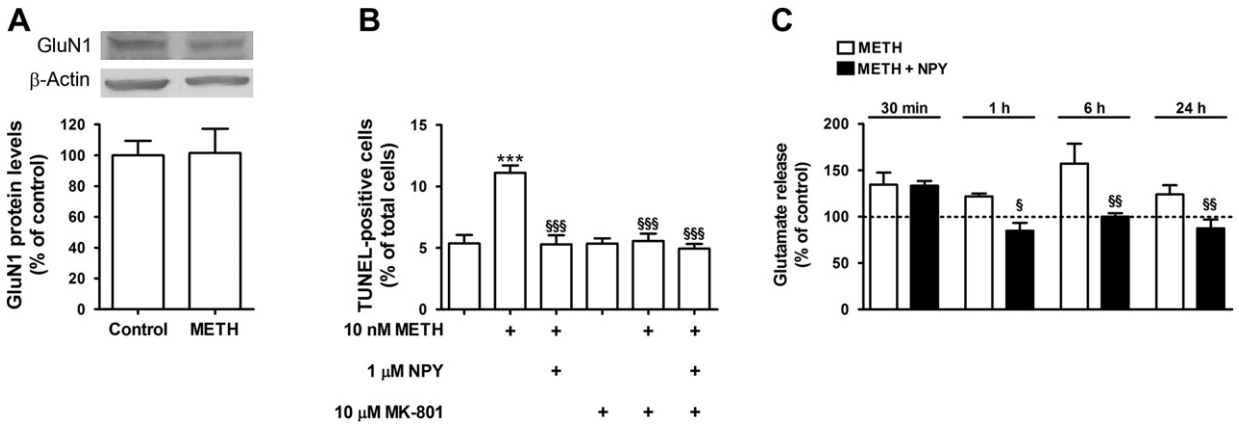

Fig. 3. Methamphetamine (METH) induces apoptotic cell death through activation of N-methyl-D-aspartate (NMDA) receptors and increases glutamate release, which is prevented by neuropeptide Y (NPY). (A) DG-derived neurosphere cultures express the GluN1 subunit (120 kDa) of NMDA receptors, and exposure to METH (10 nM) for 24 h had no effect on its protein levels. (B) Inhibition of NMDA receptors by MK-801 (10 μM) completely prevents METH-induced increase of TUNEL-positive cells. (C) METH (10 nM) increases glutamate release at all time points analyzed (30 min, 1 h, 6 h and 24 h), and NPY (1 μM) inhibits this increase at 1 h, 6 h and 24 h post-drug exposure. Data are expressed as mean percentage of total cells ± SEM or percentage of control ± SEM, $n = 4–9$. $^{***}p < 0.001$, significantly different when compared to control using Dunnett's post-hoc test. $^{§}p < 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.001$, significantly different when compared to 10 nM METH using Bonferroni's or Mann Whitney post-hoc tests.

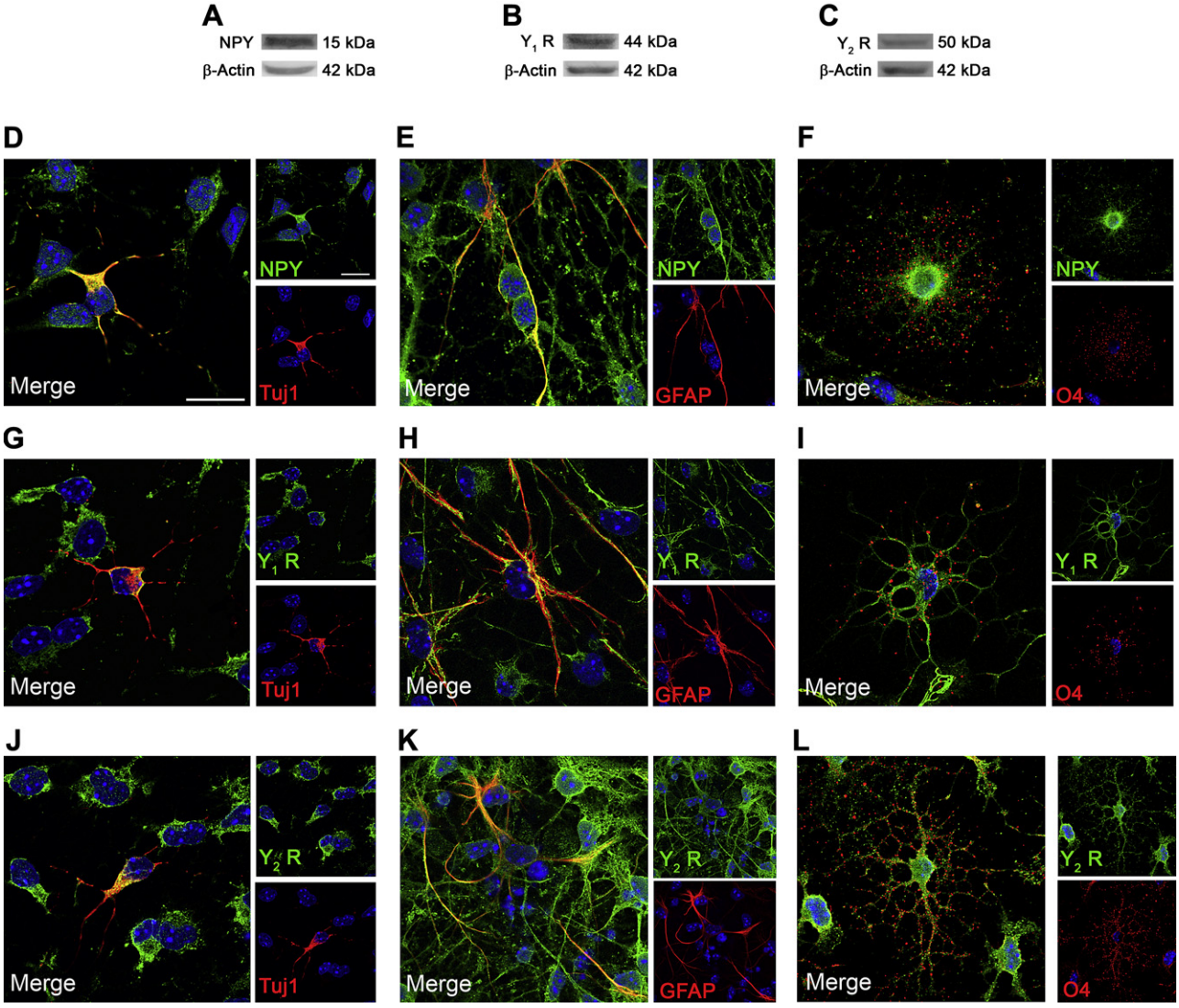

Fig. 4. Cells from DG-derived neurosphere cultures express (A, D–F) NPY, (B, G–I) Y_1 and (C, J–L) Y_2 receptor subtypes. The expression of (A) NPY (15 kDa), (B) Y_1 (44 kDa) and (C) Y_2 (50 kDa) receptor proteins in DG neurosphere-derived cultures was evaluated by western blot. Accordingly, representative fluorescence images of (D–F) NPY, (G–I) Y_1 and (J–L) Y_2 receptors (all green) show a clear co-localization with Tuj1, GFAP and O4 (all red) showing that neurons, astrocytes and oligodendrocytes, respectively, express these proteins. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(green, Fig. 4D–F) were found in DG cells, including in Tuj1 − (Fig. 4D), GFAP − (Fig. 4E) and O4-positive cells (Fig. 4F), as referring to neurons, astrocytes and oligodendrocytes, respectively (red; Fig. 4D–F). Furthermore, both Y_1 (green, Fig. 4G–I) and Y_2 receptors (green, Fig. 4J–L) are also expressed in neurons (Fig. 4G and J), astrocytes (Fig. 4H and K) and oligodendrocytes (Fig. 3I, L) from DG-derived neurosphere cultures.

3.3. METH does not affect DG cell proliferation

We have previously shown that METH inhibits SVZ progenitor cell proliferation (Bento et al., 2011). Here, we also investigated the concentration-dependent effect of METH (1–1000 nM) on proliferation in DG-derived neurosphere cultures by assessing BrdU incorporation. At low concentrations, METH had no effect on the number of BrdU-positive cells (control: 4.52 ± 0.18%; 1 nM: 4.28 ± 0.54%; 10 nM: 4.61 ± 0.44%; Fig. 5). However, exposure to 100 and 1000 nM METH decreased the number of BrdU-positive cells to 2.28 ± 0.17% ($p < 0.001$) and 2.63 ± 0.40% ($p < 0.01$), respectively (Fig. 5). Since METH decreases BrdU-labeled cells at the same concentrations that increases cell death (100 and 1000 nM), we further evaluated BrdU incorporation under co-exposure to 100 nM METH and Z-VAD (25 μM). In the presence of Z-VAD, the number of BrdU-positive cells was similar to control (Z-VAD: 3.71 ± 0.27%; METH+Z-VAD: 4.08 ± 0.47%, $p < 0.01$ *vs.* 100 nM METH; Fig. 5) indicating that decrease in BrdU incorporation was due to cell death rather than to the inhibition of cell proliferation.

3.4. NPY is proproliferative, proneurogenic and prevents METH-induced decrease of neuronal differentiation

Several studies have demonstrated that NPY increases proliferation and neuronal differentiation in SGZ (Howell et al., 2003; Decressac et al., 2010) and SVZ (Agasse et al., 2008; Decressac et al., 2009) cell cultures. In the present study, we evaluated the proproliferative effect of NPY on DG-derived neurosphere cultures and found that NPY (1 μM) increased proliferation as assessed by counting BrdU-positive cells (control: 3.64 ± 0.10%; NPY: 5.30 ± 0.25%; $p < 0.001$; Fig. 6A–F). Moreover, the proproliferative effect of NPY was completely blocked by the Y_1 receptor antagonist

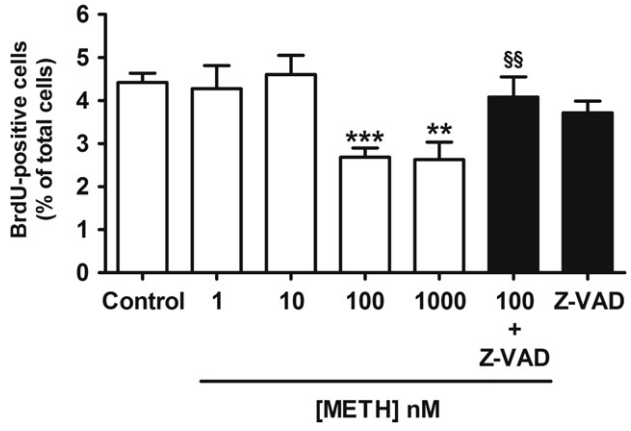

Fig. 5. Methamphetamine (METH) decreases BrdU incorporation in dentate gyrus (DG) cultures. Bar graph representing BrdU incorporation in DG-derived neurosphere cultures treated with METH (1–1000 nM) for 48 h, showing a decrease in the number of BrdU-labeled cells in cultures exposed to 100 and 1000 nM METH. Co-incubation with Z-VAD (25 μM) prevented METH-induced decrease in the number of BrdU-positive cells. Data are expressed as mean percentage of total cells ± SEM, $n = 4–12$ coverslips. $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different when compared to control using Dunnett's post-hoc test. $^{§§}p < 0.01$, significantly different when compared to 100 nM METH using Bonferroni's post-hoc test.

(3.17 ± 0.40%, $p < 0.001$ *vs.* 1 μM NPY; Fig. 6F), whereas the Y_2 receptor antagonist had no effect (5.01 ± 0.13%, $p < 0.01$; Fig. 6F). By themselves, the Y_1 or Y_2 receptor antagonists did not affect proliferation (3.29 ± 0.19%, or 3.33 ± 0.24%, respectively; Fig. 6F).

We also evaluated the effect of the selective activation of different NPY receptor subtypes, and concluded that 1 μM [Leu^{31},-Pro^{34}]NPY increased the number of BrdU-positive cells (control: 3.64 ± 0.10%; Y_1 R agonist: 6.55 ± 0.66%, $p < 0.001$; Fig. 6G), an effect blocked by the Y_1 receptor antagonist (3.69 ± 0.24%, $p < 0.001$ *vs.* 1 μM Y_1 receptor agonist; Fig. 6G). Moreover, the selective activation of Y_2 or Y_5 receptors with 300 nM NPY13-36 or 1 μM (Gly^{1},-$Ser^{3.22}$,$Gln^{4.34}$,Thr^{6},Arg^{19},Tyr^{21},$Ala^{23.31}$,Aib^{32})-PP, respectively, had no effect on the number of BrdU-positive cells when compared to control (3.91 ± 0.40% or 4.67 ± 0.21%, respectively; Fig. 6G).

Rats administered with METH have been reported to show a decrease in neuronal differentiation (Mandyam et al., 2008). Consistent with this, our fluorescent images clearly show that METH decreased the number of NeuN-labeled cells in DG-derived neurosphere cultures (Fig. 7A and B). In contrast, NPY was seen to be proneurogenic (Fig. 7C and H), increasing the number of NeuN-positive cells, and was also able to prevent the decrease in the number of NeuN-stained cells induced by 1 nM METH (Fig. 7D, J and K). Specifically, DG-derived neurosphere cultures incubated with 1 μM NPY showed an increase in NeuN-positive cells (control: 6.85 ± 0.12%; NPY: 11.64 ± 0.72%, $p < 0.001$; Fig. 6H). Furthermore, the proneurogenic effect of NPY was inhibited by the Y_1 receptor antagonist (6.34 ± 0.57%, $p < 0.001$ *vs.* 1 μM NPY; Fig. 7H), but the blockade of the Y_2 receptor had no effect (11.09 ± 0.87%, $p < 0.05$; Fig. 7H). The Y_1 and Y_2 receptor antagonists by themselves did not interfere with the number of NeuN-positive cells (5.52 ± 0.93% or 6.16 ± 0.62%, respectively; Fig. 7H). In addition, to eliminate the proproliferative effect of NPY in neuronal differentiation, an antimitotic agent (AraC) was used. We first concluded that AraC was not toxic to DG cells since there was no effect on PI- and TUNEL-positive cells when compared to control (data not shown). Furthermore, AraC had no effect on the number of NeuN-positive cells relative to control (6.38 ± 0.41%, Fig. 7H). Moreover, NPY in the presence of AraC, was able to increase the number of NeuN-positive cells (10.32 ± 0.72%), which allow us to conclude that NPY increases both cell proliferation and neuronal differentiation (Fig. 7H). Also, the selective activation of Y_1 receptors increased the number of NeuN-positive cells (1 μM [Leu^{31},Pro^{34}]NPY: 13.07 ± 0.94%, $p < 0.001$) when compared to control (7.12 ± 0.15%), whereas the activation of Y_2 or Y_5 receptors had no significant effect on neuronal differentiation (8.42 ± 0.49% or 7.75 ± 0.62%, respectively; Fig. 7I).

To investigate the effect of METH on neuronal differentiation, DG-derived neurosphere cultures were exposed to METH (1–1000 nM). A decrease in the number of NeuN-positive cells was observed at all concentrations tested (control: 6.96 ± 0.10%; 1 nM: 3.83 ± 0.18%; 10 nM: 4.24 ± 0.61%; 100 nM: 3.50 ± 0.31%; 1000 nM: 2.70 ± 0.48%; $p < 0.001$ for all concentrations; Fig. 7J). It was previously described that METH increases cell death at concentrations above 10 nM (Fig. 1), which suggest that the decrease in the number of NeuN-positive cells observed at 10, 100 and 1000 nM is probably due to cell death rather than to an inhibition of neuronal differentiation. However, at the non-toxic METH concentration (1 nM), there was a significant decrease in the number of NeuN-positive cells, which demonstrates that METH also inhibits neuronal differentiation in this system (Fig. 7A, B and J). Also, we verified that METH had no effect on GFAP protein expression in DG-derived neurosphere cultures exposed to 1 nM METH (data not shown).

Since NPY has an important protective role under METH exposure (Thiriet et al., 2005), we further investigated its effect in

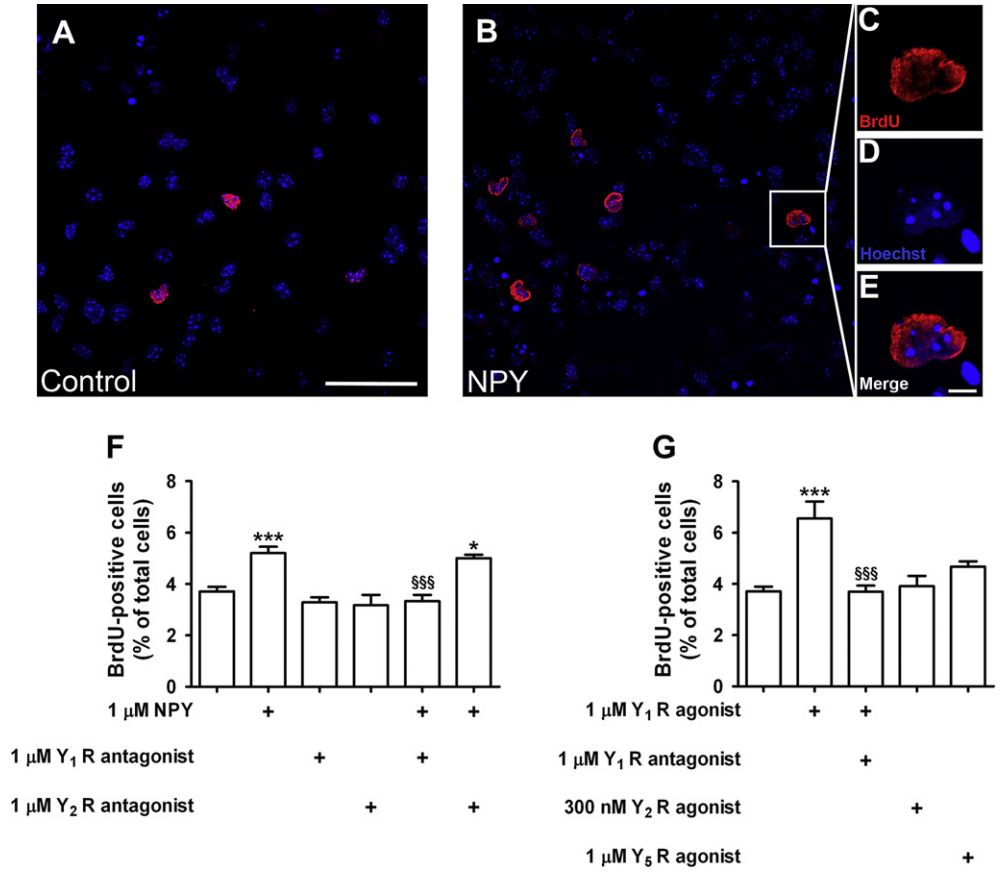

Fig. 6. Neuropeptide Y (NPY) increases dentate gyrus (DG) cell proliferation through the activation of NPY Y_1 receptor subtype. (A–E) Representative fluorescence images of DG-derived neurosphere cultures immunolabeled for BrdU (red) in (A) control conditions or (B–E) in the presence of 1 μM NPY for 48 h. Nuclei were identified by Hoechst 33342 staining (blue). Scale bars: 50 μm (A and B) and 5 μm (C–E). (F) The number of BrdU-positive cells in DG-derived neurospheres cultures is increased by 1 μM NPY (48 h exposure), which was prevented by the Y_1 receptor antagonist (1 μM BIBP3226). (G) Selective activation of Y_1 receptor by [Leu^{31},Pro^{34}]NPY (1 μM) increases the number of BrdU-positive cells, while activation of Y_2 or Y_5 by NPY13-36 (300 nM) or (Gly^{1},$Ser^{3.22}$,$Gln^{4.34}$,Thr^{6},Arg^{19},Tyr^{21},$Ala^{23.31}$,Aib^{32})-PP (1 μM), respectively, have no effect on DG cell proliferation. Data are expressed as mean percentage of total cells ± SEM, $n = 6–12$ coverslips. $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different when compared to control using Dunnett's post-hoc test. $^{§§§}p < 0.001$, significantly different when compared to (B) 1 μM NPY or (C) 1 μM Y_1 receptor agonist using Bonferroni's post-hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

preventing the decrease of neuronal differentiation in DG-derived neurosphere cultures. Interestingly, we demonstrated that NPY prevented METH-induced decrease in the number of NeuN-positive cells (8.57 ± 0.52%, $p < 0.001$ *vs.* 1 nM METH; Fig. 7K). Also, the Y_1 receptor antagonist was able to inhibit the protective effect of NPY (3.66 ± 0.29%, Fig. 7K). Thus, our results allow us to conclude that NPY preventive effect was mediated by the activation of Y_1 receptor.

4. Discussion

The present study demonstrates that METH is toxic and decreases neurogenesis in DG-derived neurosphere cultures without affecting cell proliferation. Furthermore, we also show that NPY displays effective proproliferative, proneurogenic and protective roles against METH-induced toxicity.

Several studies have shown that METH is toxic to different cell types, such as striatal neurons (Deng et al., 2002), astrocytes (Mandyam et al., 2007), microglia (Tocharus et al., 2010) and oligodendrocytes (Genc et al., 2003). The neurogenic niches have been overlooked, but recently we showed for the first time that METH triggers SVZ cell death (Bento et al., 2011). Accordingly, here we demonstrate that DG cells are highly sensitive to METH since it induced DG cell death by both necrosis and apoptosis, even at low concentrations. To clarify the underlying mechanisms of METH toxicity in DG-derived neurosphere cultures, we looked for the involvement of the glutamatergic system. Glutamate is the major excitatory neurotransmitter in the CNS and is responsible for many physiological functions, such as cognition and sensation (reviewed by O'Connor et al., 2010), but can also be involved in several pathological conditions. This neurotransmitter may act *via* different receptors, and NMDA ionotropic glutamate receptors have been shown to have a crucial role in many brain conditions (reviewed by Hardingham and Bading, 2010; Kitayama et al., 2004). Thus, in the present work, we demonstrated that DG cells express the GluN1 subunit, which is an obligatory subunit of NMDA receptors; however, METH did not induce alterations of GluN1 protein levels. Consistent with this, our group has also shown no alterations in GluN1 protein levels in the hippocampus of rats acutely administrated with 30 mg/kg METH (Simões et al., 2007). Additionally, we concluded that inhibition of NMDA receptors in our cultures by MK-801 prevented apoptotic cell death induced by METH. Afterwards, we demonstrated that METH increases glutamate release from DG-derived neurosphere cultures until, at least, 24 h post-drug exposure. In fact, our work corroborates other studies showing that METH provokes an increase of extracellular glutamate in the rat lateral striatum (Mark et al., 2004; Tata and Yamamoto, 2008) and in the hippocampus (Rocher and Gardier, 2001), and that inhibition of NMDA receptors results in blockade of METH-induced neurotoxicity (Farfel et al., 1992; Gross et al., 2011). Nevertheless, here we address for the first time the involvement of NMDA receptors in

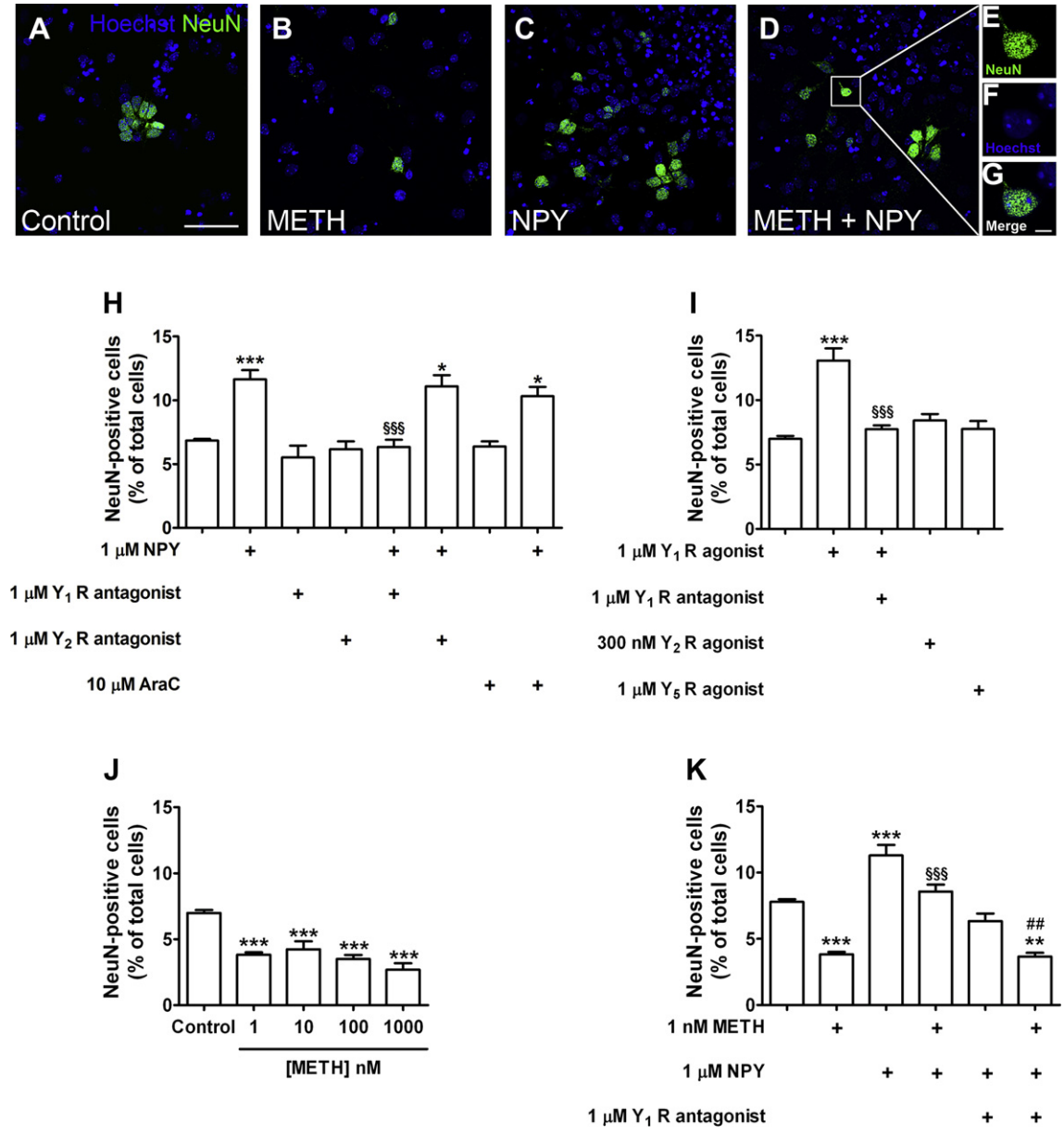

Fig. 7. Neuropeptide Y (NPY) increases neuronal differentiation through the activation of NPY Y_1 receptor subtype and prevents the decrease of neuronal differentiation induced by METH. (A–G) Representative fluorescence images of DG cells immunolabeled for NeuN (green) and nuclei staining by Hoechst 33342 staining (blue) in (A) control conditions, (B) exposed to 1 nM METH, (C) 1 μM NPY or (D) exposed to METH plus NPY all for 7 days. (E–G) Higher magnification of a (E) NeuN-positive cell (green), (F) stained with Hoechst (blue) and (G) merge from cultures exposed to METH plus NPY for 7 days. Scale bar: 50 μm (A–D) and 5 μm (E–G). (H) NPY (1 μM) triggers neuronal differentiation through the activation of Y_1 receptor subtype, even in the presence of the anti-mitotic agent, AraC (10 μM). (I) NPY Y_1 receptor subtype activation triggers neuronal differentiation in DG-derived neurosphere cultures. (J) METH decreases neuronal differentiation in DG-derived neurosphere cultures at all concentrations tested (1–1000 nM), including the non-toxic concentration of 1 nM (K) NPY prevents METH-induced decrease in neuronal differentiation *via* activation of Y_1 receptor subtype. Data are expressed as mean percentage of total cells ± SEM, $n = 3$–28 coverslips. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different when compared to control using Dunnett's post-hoc test. $^{§§§}p < 0.001$, significantly different when compared to (H) 1 μM NPY, (I) 1 μM [Leu^{31},Pro^{34}]NPY or (K) 1 nM METH using Bonferroni's post-hoc test. $^{\#\#}p < 0.01$, significantly different when compared to 1 μM NPY plus 1 nM METH (K) using Bonferroni's post-hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

METH-induced toxicity in DG-derived neurosphere cultures, which may have interesting implications for the understanding of the impact of drugs of abuse on brain neurogenic niches.

Relatively little is known about the effect of METH on proliferation of neural progenitor cells. Tian et al. (2009) observed a decreased number of proliferating cells in rat hippocampal neural progenitor cell (rhNPC) cultures exposed to METH (300 μM) for 24 h, and this effect was due to reactive oxygen species production. Similarly, Venkatesan et al. (2011) recently showed that METH decreases BrdU-positive cells in a concentration-dependent manner. Moreover, Teuchert-Noodt et al. (2000) demonstrated that a single dose of METH (25 mg/kg; i.p.) decreases proliferation, in a transient manner, in the SGZ of adult gerbils. On the other hand, chronic METH administration (1 mg/kg for 14 days, s.c.) had no effect on DG proliferation in mice (Maeda et al., 2007). Concerning self-administration procedures, 0.05 mg/kg/infusion METH (i.v.) taken 1 h/daily, 2 days per week, increases cell division, whereas 1 h/day and 6 h/day decreases cell proliferation (Mandyam et al., 2008). Here, we report that METH does not affect cell proliferation since at 1 nM (non-toxic concentration) and 10 nM (toxic concentration) there were no changes on the number of BrdU-positive cells. Moreover, at higher concentrations there was

a decrease in the number of BrdU-positive nuclei, but this effect was prevented by the caspase inhibitor, Z-VAD. These observations strongly indicate that the decreased number of BrdU-stained cells was due to the significant increase of cell death induced by METH. In contrast, we previously demonstrated that METH decreases SVZ cell proliferation (Bento et al., 2011). Thus, according to the neurogenic niche and METH concentration, this drug can have opposite effects on cell proliferation.

METH was found to decrease the number of mature neurons in DG-derived neurosphere cultures at non-toxic concentrations showing a direct and negative effect of METH on neurogenesis. We previously observed the same effects in SVZ cultures (Bento et al., 2011). On the other hand, Venkatesan et al. (2011) showed no effect in neuronal and glial differentiation, which can be justified by the fact that the authors used a dramatically higher METH (250 μM) concentration and different experimental conditions (24 h exposure with METH). *In vivo* studies have also demonstrated that 1 h/day (2 days per week) access to METH (0.05 mg/kg/infusion, i.v.) for 49 days increases differentiation and maturation of hippocampal progenitors, consistent with the increased numbers of proliferating cells (Mandyam et al., 2008). However, 1 or 6 h/day access to METH decreased neuronal differentiation, maturation and survival of the progenitor cells (Mandyam et al., 2008).

After identifying that METH is toxic to cells in DG-derived neurosphere cultures, we further aimed to look for a possible therapeutic strategy. We showed that NPY prevented METH-induced apoptotic cell death in DG-derived neurosphere cultures and that this protection is mediated *via* activation of Y_1 and Y_2 receptors both expressed by DG cells. Interestingly, by inhibiting only one of the receptors, NPY completely loses its protective function, suggesting that the pathways triggered by Y_1 and Y_2 receptor activation are similar and both confer cell protection. In fact, little is known about the role of NPYergic system against METH-induced toxicity, but in accord with our findings, Thiriet et al. (2005) observed that NPY (10 μg, i.c.v.), as well as the selective activation of Y_1 or Y_2 receptors, decreased the number of TUNEL-positive cells in mice striatum administered with METH (4 × 10 mg/kg every 2 h, i.p.). Furthermore, we aimed to uncover the mechanism(s) responsible for NPY protection in DG-derived neurosphere cultures. We clearly show that NPY blocked the glutamate release triggered by METH. NPY's protective effect was not potentiated in the presence of the NMDA receptor antagonist (MK-801), suggesting that METH induces DG apoptotic cell death by increasing glutamate release that in turn overactivates NMDA receptors. Thus, cell death was completely abolished either by inhibiting glutamate release with NPY or directly blocking NMDA receptors. Consistent with this, we have previously shown that NPY inhibits KCl-evoked glutamate release in rat hippocampal synaptosomes *via* Y_2 receptors (Silva et al., 2003a), and that activation of both Y_1 and Y_2 receptors is neuroprotective against rat hippocampal excitotoxicity (Silva et al., 2003b; Xapelli et al., 2007). Additionally, our results show that NPY did not prevent the increase in necrosis induced by METH. As reviewed by Barros et al. (2001), necrosis involves energy depletion by concurrent ATP hydrolysis by ion pumps and defective ATP production, resulting in cell swelling and lysis. This metabolic depletion leads to Ca^{2+} deregulation, which activates degradative enzymes occurring mitochondrial disruption. Despite the fact that NPY is able to prevent neuronal cell death by both apoptosis and necrosis in other pathological experimental models (Silva et al., 2003b; Xapelli et al., 2007), the only paper available in the literature regarding METH insult (Thiriet et al., 2005) reports only protection against apoptosis. Thus, in the present work we suggest that the pathways that mediate necrosis and apoptosis can be divergent, where we observed that NPY had no protective effect on METH-induced necrosis.

In addition to its protective effect, NPY also induces an increase in proliferation and neuronal differentiation *via* Y_1 receptors. These findings are consistent with previous studies performed in SGZ cell cultures (Howell et al., 2003, 2005) where incubation with 1 μM NPY for 3 days increased cell number, BrdU incorporation, nestin and class III β-tubulin immunoreactive cells. Also, NPY (1 μM) is a proproliferative factor for neuroblasts in the DG through the activation of ERK1/2 (Howell et al., 2005). Agasse et al. (2008) demonstrated that SVZ cells exposed to 1 μM NPY for 48 h or 7 days showed an increase in proliferation or neuronal differentiation, respectively, *via* Y_1 receptor activation and MAPK pathway. Others verified that administration of 2.5 pmol/μl NPY (i.c.v.) for 48 h or 7 days to in C57BL/6 adult mice induces an increase in proliferation or neuronal differentiation, respectively, and that this increase is due to the activation of Y_1 receptor subtype (Decressac et al., 2010).

It is well accepted that memory deficits are closely related to neuronal dysfunction or/and loss (Simon et al., 2000; Thompson et al., 2004). In fact, our group has previously evaluated the effect of METH on working memory and revealed mnemonic deficits in Sprague–Dawley rats injected with an acute subcutaneous dose of 30 mg/kg METH (Simões et al., 2007). Likewise, Simon et al. (2000) verified that METH abusers display cognitive deficits, which was correlated with a significant decrease in hippocampal volume (Thompson et al., 2004). Furthermore, it is known that deficits in brain adult neurogenesis can contribute to cognitive deficits. Indeed, Madsen et al. (2003) verified that the impairment of neurogenesis in rats subjected to fractionated brain irradiation, lead to cognitive deficits. Thus, to prevent the impairment of neurogenesis induced by METH, we also challenged DG-derived neurosphere cultures with NPY and we observed that it completely prevented the decrease of neurogenesis induced by METH. Also, we observed that the selective activation of Y_1 receptor completely prevented this effect. Importantly, taking into consideration the results reported here and by others showing that NPY is a protective, proneurogenic and proproliferative factor, we still can not unambiguously determine if this preventive effect under conditions of METH-induced decrease of neuronal cells is due to mechanisms of protection, proliferation or/and neuronal differentiation. However, it is clear that NPY, *via* Y_1 receptors, is able to prevent the toxic effect of METH on DG cells.

Taken together, the present work allows us to better understand how methamphetamine affects the cell dynamics in hippocampal neurogenesis. Moreover, it was also demonstrated that the NPYergic system plays an important protective role against the destructive effects of this drug of abuse on hippocampal neurogenesis.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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