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RAPID COMMUNICATION

Exogenous Neuropeptide Y Promotes In Vivo Hippocampal Neurogenesis

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ABSTRACT: Adult neurogenesis mainly occurs in two brain regions, the subventricular zone and the dentate gyrus (DG) of the hippocampus. Neuropeptide Y (NPY) is widely expressed throughout the brain and is known to enhance in vitro hippocampal cell proliferation. Mice lacking either NPY or the Y1 receptor display lower levels of cell proliferation, thereby suggesting a role for NPY in basal in vivo neurogenesis. Here, we investigated whether exogenous NPY stimulates DG progenitors proliferation in vivo. We show that intracerebroventricular administration of NPY increases DG cell proliferation and promotes neuronal differentiation in C57BL/6 adult mice. In these mice, the proliferative effect of NPY is mediated by the Y1 and not the Y2 receptor, as a Y1 ([Leu^{31},Pro^{34}]), but not a Y2 (NPY$_{3–36}$), receptor agonist enhanced proliferation. In addition, no NPY-induced DG cellular proliferation is observed following NPY injection when coadministered with a Y1 antagonist or in the Y1 receptor knockout mouse. These results are in line with data obtained in Y1$^{-/-}$ mice, demonstrating that NPY regulates in vivo hippocampal neurogenesis. © **2010 Wiley-Liss, Inc.**

KEY WORDS: neuropeptide Y; dentate gyrus; neurogenesis

Neurogenesis persists in the adult mammalian forebrain, mainly within two restricted regions, the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965; Ming and Song, 2005). In the DG, neural stem cells proliferate within the subgranular zone and give rise to neuroblasts that migrate toward the outer layers of granular zone where they differentiate into granular mature neurons.

Adult hippocampal neurogenesis is regulated by not only endogenous factors such as growth factors, hormones, neurotransmitters or aging, but also exogenous factors such as exercise, environmental enrichment, and psychotropic drugs (Abrous et al., 2005; Ming and Song, 2005), thereby suggesting that DG neurogenesis is highly regulated under physiological conditions. Among these factors, neuropeptide Y (NPY), a 36 amino acid peptide widely expressed in the central nervous system (Tatemoto et al., 1982; Adrian et al., 1983; Allen et al., 1983), has been shown to exert a neuroproliferative effect in different regions of the adult brain. NPY promotes cellular proliferation in the SVZ, the olfactory epithelium, and the retina (Hansel et al., 2001; Agasse et al., 2008; Alvaro et al., 2008; Stanic et al., 2008; Decressac et al., 2009).

In the hippocampus, in vitro studies have shown that NPY exerts a neuroproliferative effect on neuronal precursors proliferation, both in the postnatal and adult DG (Howell et al., 2003, 2005). Moreover, the DG of mice lacking the Y1 receptor display lowers levels of cell proliferation and fewer immature neurons (Howell et al., 2005, 2007).

Here, we investigated whether exogenous NPY stimulates DG neuronal precursors proliferation in vivo. For this, we performed intracerebroventricular (ICV) injection of NPY in wild type (WT) mice combined to BrdU labeling and analyzed cellular proliferation and fate of newborn cells in the DG, respectively 48 h or 3 weeks after treatment. We also determined the phenotype of newly generated cells and the receptor subtype involved in this effect with specific Y1 and Y2 receptor agonists, antagonists, as well as Y1$^{-/-}$ mice.

Adult (4-months-old) female C57BL/6 and Y1 receptor knockout mice (Pedrazzini et al., 1998) were anesthetized with isoflurane, placed in a stereotaxic frame, and were randomly assigned to receive 1 µl of freshly prepared solution of either NPY (2.5 pmol, NaCl 0.9%) (n = 12), Y1 agonist ([Leu^{31},Pro^{34}]) (2.5 pmol, NaCl 0.9%) (n = 12), Y2 agonist (NPY$_{3–36}$) (2.5 pmol, NaCl 0.9%) (n = 12) (Bachem, USA) or saline solution (NaCl 0.9%) (n = 12) through a Hamilton syringe. Another set of animals (n = 12) received a mixed solution of NPY (2.5 pmol) and Y1 antagonist (BIBP3226) (10 pmol).

Injections were made in the posterior part of the lateral ventricle at the following coordinates relative to the bregma: anteroposterior: −0.3 mm; mediolateral:

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+1.0 mm; dorsoventral: −2.0 mm according to the stereotaxic atlas to study the effects of NPY on acute cell proliferation at 48 h, mice received a single injection of 5-bromo-2 deoxyuridine (BrdU) (Sigma, France) (50 mg/kg, 0.1 M NaOH, NaCl 0.9%, i.p.) 44 h after ICV injection of one of the above-indicated molecules and were killed 4 h later. To study long-term effects of NPY on cellular differentiation, animals received the first injection of BrdU 1 h after the ICV injection, then b.i.d. during the five following days and were killed 3 weeks after the ICV injection.

Tissue processing and immunohistochemistry were performed as described before (Decressac et al., 2009). Briefly, the brains were cut in five rostrocaudal series, and the free floating sections were processed for immunohistochemistry, by using the following antibodies, rat anti-BrdU antibody (1:100, AbCys, France), goat anti-DCX (1:1,000, Santa Cruz Biotechnology, USA), mouse anti-Nestin (1:500, Chemicon, USA), chicken anti-GFAP (1:2,000, Abcam, UK), rabbit anti-NeuN (1:1,000, Chemicon).

Quantification was performed on eight serial coronal sections (40-µm-thick) spaced 200-µm apart throughout the entire rostral/caudal extent of the hippocampus (from −1.4 to −3.0 mm from bregma). All cells that were BrdU+ and double-labeled with a specific neural marker were on a confocal laser scanning microscope (BX60, Olympus) and FV10 1.6 software (Olympus). For DAB labeling, sections were photographed (SMZ1500 microscope and NIS Elements F 2.20 software Nikon) and cell counting was performed using Image J software (NIH, USA).

Figures show group means ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) or factorial ANOVA with treatment, cell type, or genotype as between-subject factors. For all analyses, upon confirmation of significant main effects, differences among individual means were analyzed using the Newman–Keuls posthoc test. For all analyses, significance was accepted at $\alpha = 0.05$. For cell type normalization, the value of each cell type for each individual was divided by the mean of the saline value for each cell type.

Modulation of NPY function differentially affected cellular proliferation in the DG [$F_{5,30} = 56.832$, $P < 0.0001$] as analyzed 48 h after treatment (Fig. 1A). Compared to saline (57 ± 8, Fig. 1B), an ICV injection of NPY (2.5 pmol) significantly increased the number of BrdU+ cells in the DG (97 ± 11, Fig. 1C) ($P < 0.001$). This effect was specifically mediated by Y1 receptors since the application of Y1 but not Y2 receptor agonist increased the number of DG BrdU+ cells compared to saline (104 ± 13, $P < 0.001$ and 56 ± 11, $P = 0.82$, respectively). The effect of Y1 receptor agonist was actually as potent as the one of NPY ($P = 0.29$) unlike Y2 receptor agonist ($P < 0.001$). The specificity of the Y1 receptor involvement in the enhancing effect of NPY on DG cellular proliferation was further supported by the abolishment of NPY effect following the coadministration of the Y1 receptor antagonist ($P < 0.001$ vs. NPY). Indeed, as presented in Figure 1A, following this treatment, the number of BrdU+ cells in the DG did not differ from the saline group ($P = 0.2$). The critical role of Y1 recep-

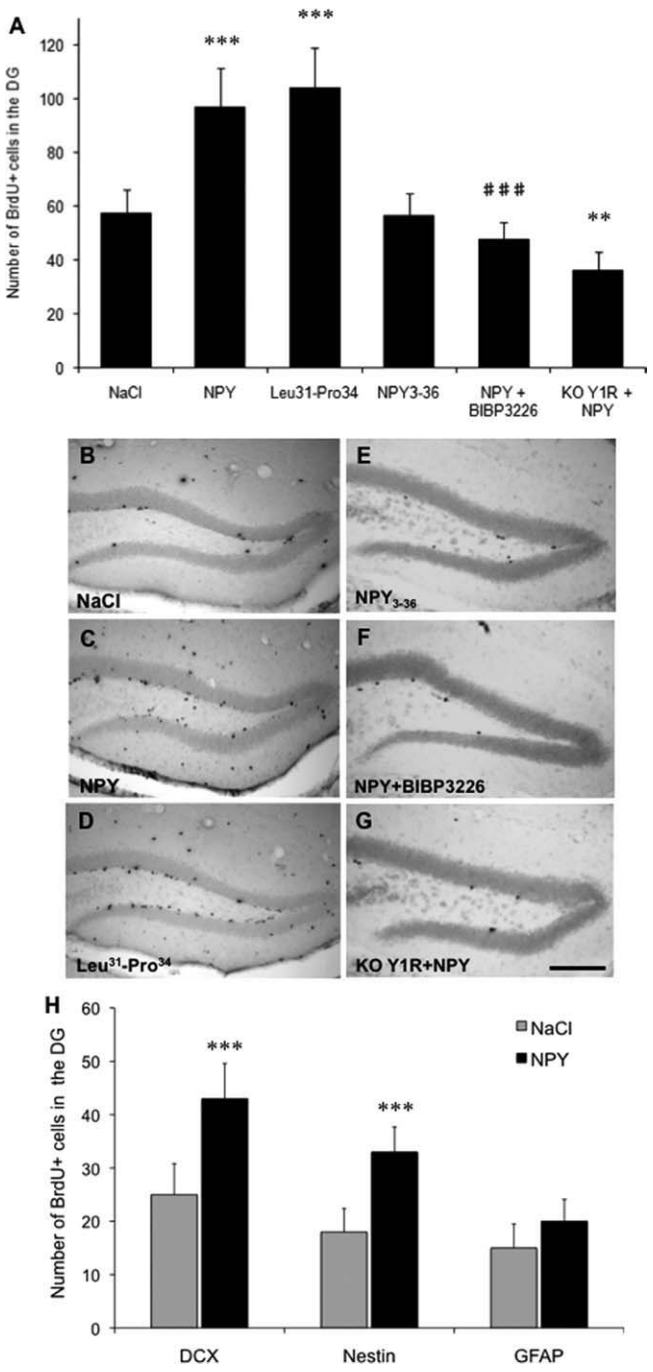

FIGURE 1. NPY stimulates cellular proliferation in the DG of the hippocampus via the Y1 receptor. Quantification of the number of BrdU+ cell in the DG 48 h after a single ICV injection (A). Immunolabeling of BrdU in WT mice injected with either NaCl (B), NPY (C), a Y1 agonist ([Leu^{31},Pro^{34}]) (D), a Y2 agonist (NPY_{3-36}) (E), or NPY + Y1 antagonist (BIBP3226) (F) and in $Y1^{-/-}$ mice injected with NPY (G). Scale bar: A–F: 200 µm. These data show that NPY upregulates cellular proliferation in the DG via the Y1 receptor. A quantification of BrdU+ cells double-labeled with DCX, Nestin, or GFAP in either saline or NPY-injected mice revealed that NPY specifically acts onto the amplifying progenitors and the neuroblasts (H). Data are expressed as mean ± SEM; ** $P < 0.01$, *** $P < 0.001$ compared to saline group; ### $P < 0.001$ compared to NPY group.

tor in the mediation of the NPY-associated proliferative effects in the DG was also confirmed in $Y1^{-/-}$ mice: the injection of NPY in these transgenic mice, which had significantly reduced DG cell proliferation (Howell et al., 2007), did not increase cellular proliferation (36 ± 5) as did NPY in WT mice ($P < 0.001$) (Fig. 1F). Interestingly, NPY ICV injection in $Y1^{-/-}$ mice even failed to bring cellular proliferation to the level of saline-injected WT mice ($P < 0.01$). Together, these results demonstrate that NPY promotes cellular proliferation in the DG of the hippocampus through the specific activation of Y1 receptor subtype (Fig. 1G).

We then sought to identify which cellular types were affected by a single ICV NPY injection. For this, we performed a quantification of either the nestin-expressing progenitors or the doublecortin-expressing neuroblasts that incorporated BrdU (Fig. 1H). Both the number of nestin+ and DCX+ cells were increased 48 h after an ICV injection of NPY (33 ± 6 for Nestin+/BrdU+ [$F_{1,10} = 32.22$, $P < 0.001$]; 43 ± 7 for DCX+/BrdU+ [$F_{1,10} = 40.72$, $P < 0.0001$]) compared to saline-injected mice (18 ± 4 for Nestin+/BrdU+; 25 ± 5 for DCX+/BrdU+).

As nestin is a protein expressed by both quiescent radial stem cells and actively proliferating progenitors, we also quantified the number of BrdU labeled cells coexpressing Glial Fibrillary Acidic Protein (GFAP). We found that there was no significant change in the number of BrdU+/GFAP+ cells in NPY-treated animals (20 ± 4) compared to control group (15 ± 5) [$F_{1,10} = 4.27$, $P = 0.066$]. This result indicates that NPY stimulates proliferation of both amplifying neural progenitors (nestin+/GFAP−) and neuroblasts (DCX+), while having no effect on quiescent neural progenitors and astrocytes (GFAP+). These results are consistent with previous reports showing that, in vitro, NPY promotes the proliferation of amplifying neural progenitors and neuroblasts (Howell et al., 2003, 2005).

Having established the enhancing effects of ICV NPY injections on in vivo cell proliferation in the DG, we then investigated the fate of these newly generated cells. For this, mice received daily injections of BrdU for five consecutive days following the ICV injection of either saline (Fig. 2A), NPY (Fig. 2B), Y1 (Fig. 2C), or Y2, receptor agonists (Fig. 2D) and were killed 21 days later to allow for cell differentiation to occur (Li et al., 2009). As observed 48 h after ICV injections, modulation of NPY function differentially altered the number of BrdU+ cells in the DG of the hippocampus 21 days after injection, [$F_{3,20} = 102.13$, $P < 0.0001$]. Indeed, compared to control mice (126 ± 14), NPY-induced an increase in the number of BrdU+ cells in the DG (239 ± 23, $P < 0.001$). This effect was partly dependent upon Y1 receptor activation, since a robust increase in BrdU+ cells in the DG was observed after Y1 receptor agonist injection as compared to saline ($P < 0.001$), though this effect was not as marked as with NPY ($P < 0.01$). Nevertheless, an injection of the Y2 receptor agonist failed to increase the number of newborn cells in the DG (141 ± 10) as compared to controls ($P = 0.15$); the number of BrdU+ cells after this treatment remaining significantly lower than after NPY injection ($P < 0.001$). The preferential implication of the Y1 receptor in this process was further supported by the absence of a significant change in the number of BrdU+ cells in the DG after administration of the Y1 receptor antagonist (113 ± 10) [$F_{1,10} = 3.27$, $P = 0.1$] and the reduced level of BrdU+ cells in $Y1^{-/-}$ mice treated with NPY at 3 weeks (77 ± 11) as compared to NPY-treated WT mice [$F_{1,10} = 281.61$, $P < 0.0001$] (Fig. 2I).

We then sought to determine whether a specific cell subpopulation was more affected by these manipulations of NPY function. For this, out of the overall BrdU+ cells quantified, we identified and quantified the number of cells expressing the quiescent neural progenitors and astrocytes marker (GFAP+ cells, Fig. 2E), progenitors marker (Nestin+ cells, Fig. 2F), the amplifying neuroblast marker doublecortin (DCX+ cells, Fig. 2G), and the mature neuron marker (NeuN+ cells, Fig. 2H).

An ANOVA revealed that these different cell types were differentially affected by either NPY, Y1, and Y2 injections as compared to saline [significant effect of cell type: $F_{3,80} = 152.29$, $P < 0.0001$, treatment: $F_{3,80} = 127.81$, $P < 0.0001$, and significant cell type × treatment interaction: $F_{9,80} = 17.02$, $P < 0.0001$], thereby revealing that at least a specific cell type was differentially altered by NPY manipulations.

Considering that the levels of the different cell type populations already differed under saline conditions, we performed a normalization of the values whereby each cell type level after treatment was expressed as a percentage of the saline level so that the magnitude of each cell-type's number deviation from the saline level after treatment can be quantitatively addressed relative to each other within the same statistical model. The polar plot in Figure 2J illustrates the results of this analysis that are similar to those described above [significant effect of cell type: $F_{3,80} = 32.90$, $P < 0.0001$, treatment: $F_{3,80} = 125.37$, $P < 0.0001$, and significant cell type × treatment interaction: $F_{9,80} = 11.52$, $P < 0.0001$].

Posthoc analysis revealed that the general effect of the ANOVA was actually attributable to a preferential increase of the DCX+ and NeuN+ cells levels after both NPY (251 ± 16, $P < 0.001$) and Y1 agonist injections (280 ± 14, $P < 0.001$) as compared to saline. Indeed, even though Nestin+ cells levels were increased both after NPY (160 ± 8, $P < 0.01$) and Y1 agonist injections (190 ± 8, $P < 0.001$) as compared to saline, this effect was of smaller magnitude than the one observed for DCX+ and NeuN+ cells (NPY: $P < 0.001$ and $P < 0.001$, respectively; Y1 agonist: $P < 0.001$ and $P < 0.001$, respectively). DCX+ and NeuN+ cell types were equally enriched by NPY or Y1 receptor agonist injection; their levels did not differ at any time (ps > 0.06). This effect was specific to the Y1 receptor stimulation since we did not observe any significant modification in the level of Nestin+ (119 ± 6), DCX+ (102 ± 6), or NeuN+ (116 ± 13) cells after a single ICV injection of the Y2 receptor agonist as compared to saline. Additionally, no effect of treatment was observed for the GFAP+ cells, as their levels did not differ from those measured after saline injection.

Having identified the nature of the cell type that was responsive to NPY treatment through the activation of the Y1 recep-

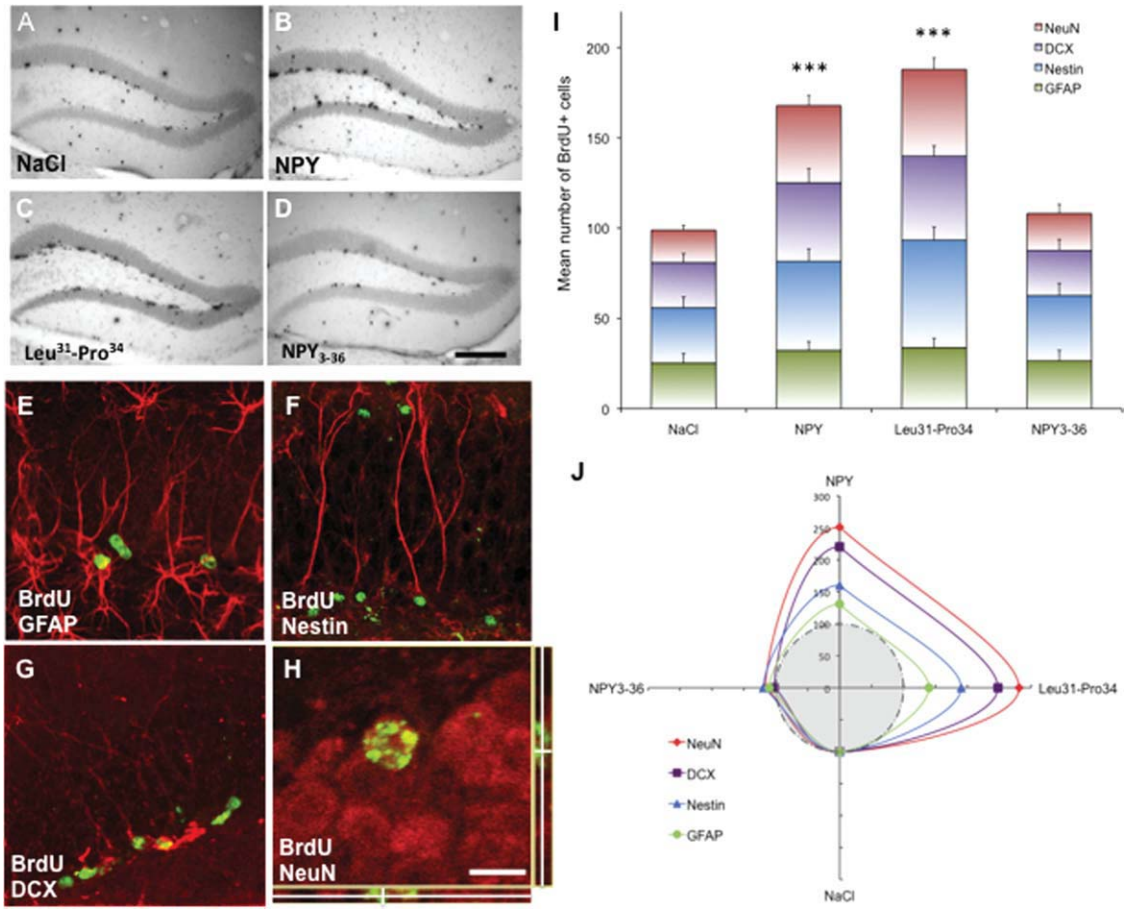

FIGURE 2. NPY promotes neuronal differentiation in the DG. Cells in the DG were labeled for BrdU following an ICV injection of either saline (A), NPY (B), a Y1 agonist ([Leu^{31},Pro^{34}]) (C), or a Y2 agonist (NPY$_{3–36}$) (D). Immunolabelings illustrating the differentiation of BrdU+ cells (green) in the granular zone of the DG into astrocytes (GFAP) (E), neural precursors (Nestin) (F), neuroblasts (DCX) (G), or mature neurons (NeuN) (H) (in red). Scale bar: A–D: 200 µm; F, H: 50 µm; G: 60 µm; H: 20 µm. Quantification of the number of BrdU+ cells that coexpressed DCX, NeuN, GFAP, or Nestin 3 weeks following treatments (I) shows that NPY or Y1 agonist significantly increase the number of newborn cells within the DG. Data are expressed as mean ± SEM, ***$P < 0.001$. Polar plot of the normalized values for each cell type relative to saline controls illustrating the differential enrichment in these different cell types observed 3 weeks after treatments. The gray circle overshadows the area referring to an absence of enrichment in the number of quantified cells 3 weeks after a single injection of either saline, NPY, Y1 agonist ([Leu^{31},Pro^{34}]), or Y2 agonist (NPY$_{3–36}$). Distance from this circle thereby illustrates the magnitude of enrichment in the different cell types in function of the treatment applied (J).

tor, we were poised to determine whether these cells actually expressed the Y1 receptor. We thus performed double immunostaining on sections of the adult DG and found that both neural precursors (nestin+) cells (Figs. 3A–C) and immature neurons (DCX+) (Figs. 3D–F) expressed the Y1 receptors. This result, similar to that previously described in vitro by Howell et al. (2003), is in agreement with the specific NPY-induced effect on cell proliferation that we reported 48 h and 3 weeks after a single ICV injection.

In this study, we have shown that a single ICV injection of a dose of NPY as low as 2.5 pmol promotes cellular proliferation in the DG of adult WT mice as early as 48 h after treatment. This effect is preferentially mediated by the Y1 receptor subtype as demonstrated by both pharmacological tools, that is, specific receptor agonists and antagonists, and genetic tools, that is, Y1$^{-/-}$ mice. Although further research is needed to specifically address the implication of the other NPY receptors, we demonstrated here that the sole stimulation of the Y1 receptor is sufficient to mimic the neurobiological effects of NPY injections. This result is consistent with a study we recently reported in which a similar dose of NPY had a proliferative effect on SVZ neural precursors (Decressac et al., 2009). Moreover, the NPY-induced neurobiological effects we observed after an ICV injection of a 2.5 pmol dose resonate well with previous studies that showed both behavioral and biological effects of NPY injections in the range of pico to micromolar (Kalra et al., 1997).

Many other factors have been shown to have mitogenic actions within neurogenic regions in the adult brain (Abrous et al., 2005; Ming and Song, 2005). However, some of those

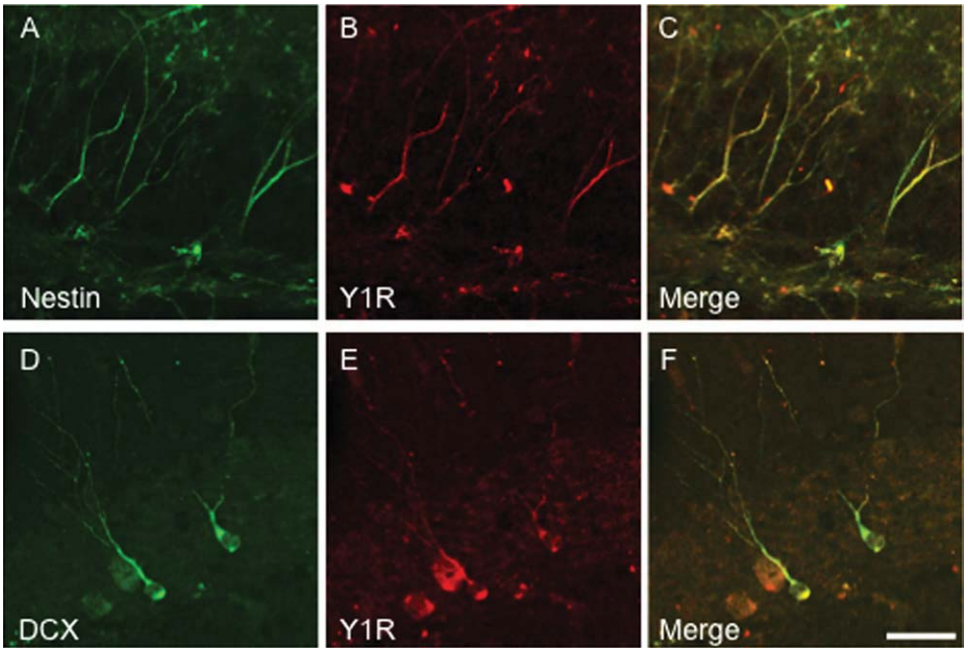

FIGURE 3. Expression of Y1 receptor in the DG of the hippocampus. A–F: Double staining of cells expressing the Y1 receptor (red) in the DG of the hippocampus showing that they are either Nestin+ amplifying progenitors (A) or DCX+ neuroblasts (D) (green). Scale bar: 50 μm.

that induce proliferation in the SVZ are often unable to stimulate neurogenesis in the DG (i.e., fibroblast growth factor and epidermal growth factor) (Kuhn et al., 1997). Here, we demonstrate that, in physiological conditions, NPY has the potency to stimulate cellular proliferation and neuronal differentiation at a level equal or even greater than, that observed with other trophic/growth factors such as ciliary neurotrophic factor, vascular endothelial growth factor, and transforming growth factor (Jin et al., 2002; Emsley and Hagg, 2003).

Not only have we shown that NPY promotes cell proliferation in the DG of mice, but we have also established that a single ICV injection of NPY actually promotes a preferential differentiation of newly generated cells toward a neuronal lineage (immature and mature neurons). This preferential enrichment of the DCX+ and NeuN+ cells is attributable at least to the stimulation of the Y1 receptor, as revealed by the effect of the ICV injection of the Y1 receptor agonist which, unlike the injection of the Y2 receptor agonist, had effects very similar to those of the injection of NPY.

The demonstration that the NPY-induced neuroproliferative effect was mediated by the Y1 receptor raised the question of the cellular location of this receptor. The Y1 receptor has previously been shown to be highly expressed in the DG of the hippocampus (Kopp et al., 2002; Shaw et al., 2003) but, to our knowledge, the cell type expressing this receptor in vivo was not determined. In the present study, we report that the Y1 receptor is expressed by both Nestin+ progenitors and DCX+ neuronal precursors, but not by GFAP positive cells. These data are in agreement with those of Howell et al. (2003), who previously showed in hippocampal cultures in vitro, that Y1 receptor staining colocalized with either nestin and class III β-tubulin positive cells. Together, these data show that the expression of the Y1 receptor is essential for the DG cell to be responsive to a NPY-induced proliferative signal.

Altogether, these data suggest that progenitors cells that express the Y1 receptor are reactive to the enhancing effects of NPY in physiological conditions, thereby potentially providing NPY with an important functional role in hippocampal-related behavioral functions. In this line, NPY has been shown to be involved in behavioral functions that depend upon the integrity of the hippocampus (Redrobe et al., 1999; Eaton et al., 2007).

However, the question as to whether NPY could exert similar functions in pathophysiological conditions where neurogenesis is impaired remains to be established.

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