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Neurobiology of Disease

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Neurobiology of Disease 26 (2007) 174–188

Neuropeptide Y is important for basal and seizure-induced precursor cell proliferation in the hippocampus

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Received 31 August 2006; revised 21 November 2006; accepted 14 December 2006
Available online 30 December 2006

We have shown that neuropeptide Y (NPY) regulates neurogenesis in the normal dentate gyrus (DG) via Y_1 receptors (Howell, O.W., Scharfman, H.E., Herzog, H., Sundstrom, L.E., Beck-Sickinger, A. and Gray, W.P. (2003) Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells. *J Neurochem*, 86, 646–659; Howell, O.W., Doyle, K., Goodman, J.H., Scharfman, H.E., Herzog, H., Pringle, A., Beck-Sickinger, A.G. and Gray, W.P. (2005) Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. *J Neurochem*, 93, 560–570). This regulation may be relevant to epilepsy, because seizures increase both NPY expression and precursor cell proliferation in the DG. Therefore, the effects of NPY on DG precursors were evaluated in normal conditions and after status epilepticus. In addition, potentially distinct NPY-responsive precursors were identified, and an analysis performed not only of the DG, but also the caudal subventricular zone (cSVZ) and subcallosal zone (SCZ) where seizures modulate glial precursors. We show a proliferative effect of NPY on multipotent nestin cells expressing the stem cell marker Lewis-X from both the DG and the cSVZ/SCZ *in vitro*. We confirm an effect on proliferation in the cSVZ/SCZ of Y_1 receptor$^{-/-}$ mice and demonstrate a significant reduction in basal and seizure-induced proliferation in the DG of NPY$^{-/-}$ mice.

Keywords: NPY; Neurogenesis; Progenitor cell; Stem cell; Epilepsy; Cell culture; Nestin; Subventricular zone; Subcallosal zone; Dentate gyrus

Introduction

Neurogenesis, the production of new neurons, is a restricted event in the postnatal and adult mammalian brain (Altman and Das, 1965; Kaplan and Hinds, 1977; Gross, 2000), confined to the subventricular zone (SVZ) around the anterior lateral ventricle and rostral migratory stream to the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994), the subgranular zone (SGZ) of the dentate gyrus (Altman and Das, 1965) and the cornu ammonis of the hippocampus (Rietze et al., 2000). There has been much interest in elucidating the role of adult neurogenesis in the dentate gyrus in the normal brain, where it appears to regulate hippocampal-dependent learning and memory (Shors et al., 2001; van Praag et al., 2002) as well as mood control (D'Sa and Duman, 2002; Santarelli et al., 2003).

Adult neurogenesis in the dentate gyrus may also contribute to the sequelae that follow seizures. Seizures increase neurogenesis in both the SGZ and rostral SVZ (Bengzon et al., 1997; Parent et al., 1997, 2002; Gray and Sundstrom, 1998; Scott et al., 1998) and gliogenesis in the caudal SVZ (cSVZ) (Parent et al., 2006). The mechanisms underlying seizure-induced neurogenesis/gliogenesis are incompletely understood, but clearly involve an increase in precursor cell proliferation. GFAP-expressing astrocytes appear to give rise to transiently amplifying C cells and proliferating neuroblasts (D cells) in the anterior SVZ (Doetsch et al., 1999), while radial glia-like astrocytes give rise to proliferating neuroblasts (D1 cells) either directly (Seri et al., 2001, 2004), or via a weakly nestin-positive amplifying cell population (Encinas et al., 2006), in the SGZ. Seizures increase the proliferation of neuroblasts in the rostral SVZ (Parent et al., 2002) and of both radial glia-like precursor cells (Huttmann et al., 2003) and doublecortin-positive cells in the SGZ of the dentate gyrus (Jessberger et al., 2005).

Dissecting the precise mechanisms underlying the effects of seizures on different precursor cell types has been hindered by the

Abbreviations: BrdU, bromodeoxyuridine; DG, dentate gyrus; GFAP, glial fibrillary acidic protein; HSVZ, hippocampal subventricular zones; SVZ, subventricular zone; SCZ, subcallosal zone; LeX, Lewis-X; PFA, paraformaldehyde; SGZ, subgranular zone.

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Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2006.12.014

lack of a unique marker for precursor cells in these neurogenic niches. The intermediate filament protein nestin is expressed in precursor cells during development, and in subpopulations of cells in both neurogenic and non-neurogenic regions of the postnatal brain (Wei et al., 2002). In vivo studies have shown that nestin expression can be detected immunohistochemically in stem cells of the cSVZ (Gates et al., 1995; Wei et al., 2002) and in GFAP-positive radial glia-like precursor cells in the dentate SGZ (Seri et al., 2004). Nestin expression is controversial in SGZ neuroblasts (D1 or Type 2 cells), which are the mitotic progeny of GFAP-positive radial glia-like precursor cells. Nestin expression has been inferred in these Type 2 cells by transient GFP expression in nestin reporter transgenic mice (Fukuda et al., 2003; Kronenberg et al., 2003) but has not been confirmed by direct immunohistochemical detection (Seri et al., 2004). However, a recent study using a nuclear-localized reporter of nestin gene expression, has identified a nestin-positive/β-tubulin-negative, transiently amplifying mitotic cell population, that arises from GFAP/nestin-expressing precursors in the SGZ, and which divides to give rise to β-tubulin-expressing immature neurons (Encinas et al., 2006).

Amidated neuropeptides, such as neuropeptide Y (NPY) (Hansel et al., 2001a) and pituitary adenylate cyclase-activating polypeptide (PACAP) are emerging as significant regulators of adult neural stem cells in the olfactory epithelium (Hansel et al., 2001b) and hippocampus (Howell et al., 2003, 2005; Mercer et al., 2004; Ohta et al., 2006). We have previously shown that an inhibitory peptide neurotransmitter, neuropeptide Y (NPY), released by GABAergic interneurons in the dentate gyrus and pyramidal layers of the hippocampus, is a potent proliferative factor acting through its Y_1 receptor, for neuroblasts and nestin-positive sphere-forming stem cells in cultures of the whole hippocampus from early postnatal rats (Howell et al., 2003). We have recently shown that the Y_1 receptor-mediated proliferative effect on neuroblasts (D1 or Type 2 cells) is confined to those neuroblasts isolated from the DG, but not from the hippocampus or adjacent subependymal regions (Howell et al., 2005). However, the nature and location of the nestin-positive NPY-responsive population remain unknown.

Since NPY is significantly up-regulated in the DG and hippocampus after status epilepticus (Marksteiner et al., 1989) and status epilepticus increases precursor proliferation in the DG leading to neurogenesis, and subventricular areas of the hippocampus leading to gliogenesis, we examined the effect of NPY on region-specific primitive nestin-positive precursor cells and sought to determine if NPY plays a role in seizure-induced precursor cell proliferation.

Materials and methods

Animals

Animals were maintained on a 12-h light:dark cycle and provided food and water ad libitum. Rats (Wistar) were obtained from (Harlan; UK). NPY Y_1 receptor$^{-/-}$ mice and their wild-type controls were obtained from Herbert Herzog, details of which have been previously published (Howell et al., 2003). Animal experimentation was approved by a University Bio-Ethics Committee and performed under UK Home Office license or NY State Department of Health Guidelines. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

NPY$^{-/-}$ mice and their wild-type controls were obtained from Richard Robinson (Columbia University, New York, NY, USA) and were originally from the colony established by Richard Palmiter. Experiments using NPY$^{-/-}$ mice or their wild-type controls were approved by the Columbia University Institutional animal care and use committee and met the guidelines of the National Institutes of Health.

Primary cell cultures

Wistar rat pups (8–10 days old) were decapitated, the hippocampus dissected, and 1mm transverse sections cut on a McIlwain tissue chopper and placed in 5 ml of GEYs solution (Life Technologies, Paisley, UK). Under a dissecting microscope and using a scalpel blade and tungsten needle, the DG (not including the septal tip of the DG that contacts the 3rd ventricle) and the remaining hippocampus were separated and removed and primary cell cultures were generated from each tissue as described in detail elsewhere (Howell et al., 2005). The hippocampal tissue consisted of a number of stem cell niches, including the caudal subventricular zone (cSVZ) (Parent et al., 2006) and hippocampal subventricular zone (Navarro-Quiroga et al., 2006) located under the posterior lateral ventricle, and the adjacent subcallosal zone (SCZ) (Seri et al., 2006) between the hippocampus and corpus callosum collectively referred to here as the hippocampal subventricular zones (HSVZ) for simplicity. Primary cell cultures of DG or HSVZ were grown in parallel on poly-L-lysine (Sigma)-coated sterile glass slides (for confocal microscopy), or onto poly-L-lysine-coated 24- or 96-well plates (for cell counting; Co-star, Fisher Scientific, USA).

Immunocytochemistry

Antibodies

Antibodies used in these experiments included: mouse IgG1 anti-nestin (1:1000; BD Biosciences, Oxford, UK), mouse anti-class III β-tubulin (1:1000; Babco, Richmond, CA, USA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:400, a gift from P. Steer, University of Southampton), sheep anti-Y_1 (1:200; Biogenesis, Poole, UK), mouse IgM anti-Lewis-X (1:200, BD Biosciences), rat anti-BrdU (1:200, Harlan Sera Labs, Loughborough, UK) and rabbit anti-Ki-67 (1:500; Novocastra Laboratories, UK). Secondary antibodies to mouse (IgG1, IgM or IgG (H+L)), rabbit, sheep or rat, conjugated to the appropriate fluorophore, were used at 1:200 dilution (Jackson Immuno research; Soham, Cambs, UK).

Immunocytochemistry protocol

Standard immunofluorescence staining was performed as previously described (Howell et al., 2003, 2005). Cell nuclei were counterstained with DAPI (1 μg/ml, Sigma), and imaged on an inverted Leica DM IRB microscope with a mercury bulb and filter cubes for visualizing DAPI, FITC, and rhodamine separately (Leica microsystems, Milton Keynes, UK) linked to Open Lab image capturing system (Version 2.1; Improvision, Lexington, MA, USA). For confocal imaging, cultures grown on glass coverslips were mounted in Moviol (Harco; Harlow, UK), sealed with clear nail-varnish and viewed with a Zeiss LSM 510 meta system (Carl Zeiss Ltd., Oberkochen, Germany) by sequential scanning of the appropriate channels and merged images generated with LSM software (LSM Version 5). All immunostaining procedures were conducted with secondary antibody-only controls,

which were devoid of primary antibody. There was no immunostaining that could be detected in the controls.

Cell counting and statistical analysis

Fluorescence cell counting was performed on an inverted Leica DM IRB microscope, as described previously (Howell et al., 2003, 2005), and cell numbers were reported as cells/mm^2 per well, based on an average of 2–4 determinations per well. Each separate experiment consisted of 4–12 wells per condition and was performed 2–4 times. One experiment consisted of tissue pooled from 2 animals. Data were plotted by Prism (GraphPad Software Inc., San Diego, CA, USA) and mean values were compared using Student's *t*-test or ANOVA, with appropriate post-testing, as detailed in the figure legends.

Investigating the response of dentate-derived cell cultures to NPY

Cells from primary cultures were plated and grown for 24, 48 or 72 h in the presence or absence of NPY (1 μM). Although we have published an effect of NPY on precursor cell proliferation at doses as low as 1 nM (Howell et al., 2003), our detailed analysis of proliferation was performed at 1 μM and so we used NPY at this concentration throughout this study. At each time point, the number of nestin-positive cells per mm^2 was calculated. To examine whether NPY had a direct proliferative effect, we grew DG cultures under control conditions (NB/B27 and glutamine) and then exposed one group at day 3 to 10 μM BrdU for 4 h and another group to both 10 μM BrdU and 1 μM NPY for 4 h, after which all cultures were immediately fixed. Cultures were immunostained for BrdU and nestin, and the proportions of nestin-positive cells incorporating BrdU of the total nestin+ population under NPY vs. control conditions were calculated. Confocal imaging of 3 DIV primary dentate cultures (1 μM NPY) was performed following a 4-h exposure to BrdU (10 μM) to detect BrdU/nestin-immunoreactive, cells. To determine the effect of NPY on nestin/Lewis-X (LeX)-positive cells, the total number of cells, the number of nestin-positive cells and the number of LeX-positive cells were calculated. Confocal imaging of 3 DIV primary dentate cultures (1 μM NPY) was performed following immunostaining for nestin and LeX expression.

To study the role of the NPY Y_1 receptor, we prepared primary cell cultures as discussed above from DG that were grown in the presence of 1 μM NPY, or in the presence of 1 μM NPY and 1 μM of the Y_1 receptor antagonist BIBP3226 (Bachem Ltd., St. Helens, UK). Cells were also cultured with the specific Y_1 receptor agonist, [F7, P34]-NPY (1 μM). All cells were grown for 3 days, after which they were fixed. BrdU was added 4 h before fixation.

Sphere cultures

Non-clonal spheres

Non-clonal free-floating sphere cultures were established from DG tissue or hippocampal tissue (as described above in Primary cell cultures) by plating 100,000 viable cells/ml into untreated 6-well tissue culture plates (Co-star) under standard control growth conditions for 6 days. Comparison of control cultures and those grown in the presence of 1 μM NPY for 6 days was performed by quantification of neurosphere numbers by imaging a single 5× field per well from 18 dentate, and 24 hippocampal culture wells from two separate experiments. Only spheres with a diameter greater than 100 μm were counted and plotted as spheres per 5× field. Confocal imaging of spheres was performed on 6-day-old cultures by carefully aspirating the culture media and spheres to poly-L-lysine-coated 13-mm diameter glass slides placed within a 24-well plate (Co-star). Spheres were left for 3 h to allow adherence, before fixation and processing for nestin, GFAP and β-tubulin immunoreactivity.

To quantify cell spheres with regard to β-tubulin-positive cell content, 200 spheres from two wells of dentate-derived and hippocampus-derived cultures were scored. Eight groups of 25 spheres were scored for high, low or undetectable β-tubulin-positive cell numbers under a high power objective (40×) and epi-fluorescence. Data were expressed as mean±SE of total neurospheres and groups compared using the Student's *t*-test.

Clonal spheres

Tissue was processed as for non-clonal spheres except that viable cell suspensions were diluted to 20,000 cells/ml, using the trypan blue exclusion technique. Culturing at this density has been shown to result in the formation of clonal neurosphere colonies (Tropepe et al., 2000) rather than aggregated spheres. Cells were plated at a density of 10,000 cells/well onto uncoated 24-well plates. All wells were treated with antibiotic/antimycotic at a dilution of 1:100. Eight wells were used per condition. Spheres were grown in either 1 μM NPY or in medium containing 25 ng/ml of FGF2 and 20 ng/ml of EGF (Sigma). Spheres were left for 7 DIV before fixation and immunostaining or transfer to differentiating conditions as described below. To quantify the number and phenotype of cells within a single sphere, an average of 15 spheres for each condition were imaged, and cells counts were made and expressed as a proportion of DAPI+ cells/sphere.

Differentiating NPY cultured neurospheres

To determine the phenotype of progeny of dentate and hippocampal spheres cultured in NPY, we transferred free-floating cell spheres to NPY-deficient, differentiating conditions as described previously (Howell et al., 2003). Spheres were allowed to differentiate for 6 days before fixation, and subsequent processing for β-tubulin, GFAP or nestin immunostaining.

Proliferation in NPY $Y_1^{-/-}$ and $Y_1^{lox/lox}$ mice

Five 35-day-old male NPY $Y_1^{-/-}$ and five age- and generation-matched male $Y_1^{lox/lox}$ controls (Howell et al., 2005) were given 1 IP dose of BrdU (50 mg/kg) each day for 5 days. Animals were terminally anesthetized, perfused with saline and 4% PFA, the brains removed and post-fixed in PFA. Coronal sections containing hippocampus were cut at 40 μm on a Leica VT100M vibratome. Every 6th ($n=8$) systematically randomly sampled section was stained for BrdU. Immunopositive cells were counted in the left subgranular zone and cSVZ/SCZ using a Leitz Dialux22 microscope with a 63× oil objective and the investigator blind to the experimental group.

Seizures in NPY$^{-/-}$ and NPY$^{+/+}$ mice

To examine if NPY could be a modulator of proliferation in response to seizures, we examined SGZ cell proliferation following kainic acid-induced seizures in 3-month-old adult male NPY$^{-/-}$ mice and controls. Mice were maintained under constant environmental conditions, a 12-h:12-h light/dark cycle, and given mouse

chow and water ad libitum. The generation of the NPY$^{-/-}$ mice used has been previously described (Erickson et al., 1996; Baraban et al., 1997). Kainic acid (stock solution, 4 mg/ml) was dissolved in neutral-buffered saline. Seizure experiments began with a bolus injection of KA (20 mg/kg, i.p.) followed by a KA injection (10 mg/kg) every 1 h until either a full behavioral seizure was elicited or the specified dose was achieved. Control animals (both NPY$^{-/-}$ and NPY$^{+/+}$: $n=7$ in both groups) received equivalent IP injections of sterile vehicle.

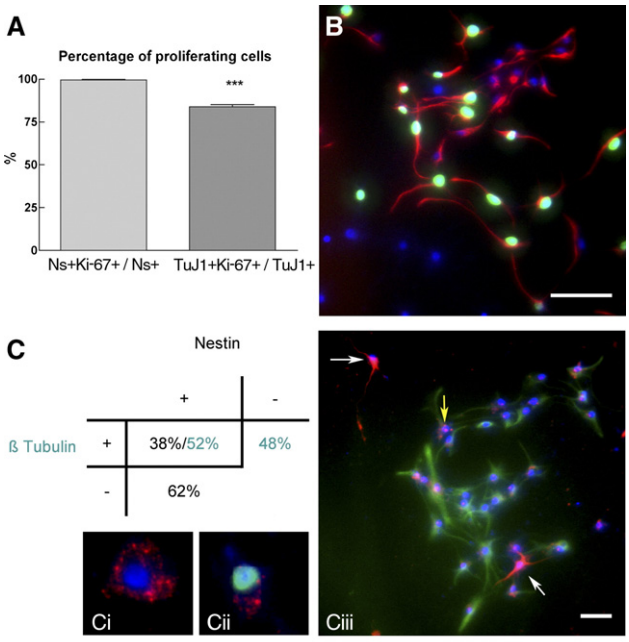
NPY$^{-/-}$ animals are more sensitive to seizure induction (Erickson et al., 1996) and required a "low dose" of kainic acid to display prominent seizures (intermittent tremors and high, intensive jumps), typically 20–30 mg/kg. Age-matched, wild-type animals were matched either to the dose of kainic acid " low dose" (seven animals; 4 received 20 mg/kg and 3 received 30 mg/kg kainic acid) or seizure severity (seven animals received kainic acid at 10 mg/kg/h IP until an equivalent level of seizures was attained) typically requiring 50–70 mg/kg of kainic acid. Cell proliferation was evaluated after 5 daily IP injections of 50 mg/kg of BrdU dissolved in saline, and filtered through a 0.22-μm Millipore membrane, beginning after the first kainate injection.

All animals were killed and perfused 2 h after the last IP BrdU injection for proliferation studies. After removal, the brains were post-fixed in 4% PFA overnight, serially sectioned at 40 μm on a Leica VT100M vibratome and stored in PFA until required. Sections were processed for BrdU immunohistochemistry as previously detailed (Howell et al., 2005). Every 6th systematically randomly sampled section per animal from the rostro-caudal extent of the hippocampus, was scored for nuclear BrdU staining using a 63× objective by an investigator blind to the animal's status. A stereological estimate of BrdU cells per hippocampal subgranular zone was calculated for the complete hippocampus. For a comparison of baseline cell proliferation in control wild-type and control NPY$^{-/-}$ animals, mean BrdU SGZ cell counts were compared using a Student's *t*-test. Statistical analysis comparing seizure-induced cell proliferation between control animals, 'low-dose" and "high-dose" kainic acid groups was performed using one-way ANOVA with Student–Newman–Keuls multiple comparison post-test (GraphPad Prism).

Results

Nestin-positive and Class III β-tubulin-positive cells are distinct but variably overlapping populations at 3 days, in whole hippocampal cultures

Whole hippocampus-derived cultures were examined at 3 days in culture. 100% of nestin cells were actively proliferating (Ki67-positive) compared to 80% of β-tubulin cells (Figs. 1A and B). Immunostaining for both nestin and β-tubulin revealed overlapping populations, with 38%±7.8% of nestin cells co-labeled for β-tubulin and 52%±6.4% of β-tubulin cells stained for nestin (Fig. 1C). Interestingly 86% of β-tubulin/nestin-positive cells had a punctate and largely cytoplasmic staining pattern of β-tubulin (Figs. 1Ci and Cii), consistent with early expression of β-tubulin in a nestin-positive proliferating neuronal precursor (Fig. 1Ciii). Exposing cultures to a 4-h pulse of BrdU to identify cells passing through the S-phase of the cell cycle revealed greater numbers of nestin than β-tubulin cells (Fig. 1D), and significantly greater proportions of BrdU+Ki67+nestin cells (24%±3%) than BrdU+/Ki67+/β-tubulin+ cells (6%±1.2%) ($p<0.01$; Student's *t*-test),



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	Cells / mm² ± SE	LI (Labelling Index) after 4h BrdU
Nestin	63 ± 3.8	24%±3%
Class III ß-tubulin	36 ± 1.9	6%±1%

Fig. 1. Nestin and β-tubulin-positive cells are partially overlapping populations in whole hippocampus-derived cultures. (A) The growth fraction (percent of cells staining for the cell proliferation marker ki67) of nestin-positive cells was significantly greater than β-tubulin-positive cells at 3 DIV ($p<0.001$; *t*-test). (B) Fluorescent micrograph of BrdU-positive (green), nestin-positive (red) cells counterstained with the nuclear stain DAPI. Note the largely bipolar morphology of nestin cells in the S-phase of the cell cycle. Scale bar=40 μm. (C) Nestin and β-tubulin cells at 3 DIV are distinct but overlapping populations, with 38% of nestin cells being β-tubulin-positive and 52% of β-tubulin-positive cells being nestin-positive. Panel Ci shows a high power view of a DAPI stained nucleus with punctate cytoplasmic β-tubulin staining. Panel Cii shows a similar view of a proliferating cell which is BrdU-positive (green) again showing punctate cytoplasmic β-tubulin staining. Panel Ciii shows nestin-positive cells (green) many of which have punctate cell body β-tubulin staining (yellow arrow). β-Tubulin-positive nestin-negative cells are indicated by white arrows. Scale bar=30 μm. (D) Table showing a greater number of nestin cells with a significantly higher labeling index (%cells labeled with BrdU and ki67 after a 4-h pulse of BrdU) than β-tubulin cells ($p<0.01$; *t*-test) demonstrating a greater proportion of cycling nestin cells in the S-phase consistent with a shorter cell cycle time. Data based on experiments with 4–12 wells per condition in 1–3 experiments.

consistent with a more rapidly cycling nestin-positive precursor population (i.e., greater labeling index) (Fig. 1D). From these data we can conclude that there are cycling populations of nestin+/TUJ1− cells, nestin+/Tuj1+ cells, and nestin−/TUJ1+ cells, of which the nestin+/TUJ1− population is the largest, and the nestin−/TUJ1+ population the smallest. These populations are similar to those described in the hilar tertiary matrix (Namba et al., 2005) and adult SGZ (Seri et al., 2004; Encinas et al., 2006) and in this study we focus on the nestin-positive cell population.

Table 1
NPY responsive nestin-positive cell populations exist within dentate and hippocampal micro-dissected cultures

	Dentate cultures		Hippocampal cultures	
	Control	1 μM NPY	Control	1 μM NPY
BrdU	225.4±7.6	312.5±6.9**	159.3±12.2	212.7±11.1**
Nestin	272.2±29.5	451.3±67.5*	125.3±9.9	183.2±19.6*
Nestin/BrdU	117.1±11.4	251.8±33.2**	74.7±7.4	131.7±14.6**

Cell cultures were established from micro-dissected dentate area tissue and remaining hippocampus including the caudal and hippocampal subventricular zones, plated at 300–500,000 viable cells per milliliter and grown for 2 days in control conditions prior to the application of 1 μM NPY to half the wells. 20 μM BrdU was added to all wells. NPY increased BrdU incorporating cells, nestin-positive cells and the number of nestin/BrdU double-positive cell numbers in both dentate and hippocampus derived cell cultures. Values are mean cells counts per $mm^2 \pm$SE of 8–16 determinations per well. Student's *t*-test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Both micro-dissected dentate gyrus and hippocampal subventricular zones cultures contain an NPY-responsive nestin-positive cell population

Micro-cultures were established from dentate gyrus (DG) (plating density 500×10^3 viable cells ml^{-1}) and hippocampal subventricular zones (HSVZ) tissue (plating density 300×10^3 viable cells ml^{-1}) to investigate the effect of NPY on cell proliferation and expression of the stem cell/progenitor cell marker, nestin. Cultures were grown for 2 days in standard control conditions in poly-L-Lysine-coated wells before the application to half the wells of either 1 μM NPY and 20 μM BrdU or 20 μM BrdU alone for 24 h prior to fixation. Table 1 shows that cultures from both regions yielded nestin-positive cells and that 24-h exposure to 1 μM NPY resulted in a significant increase in the total number of nestin-positive cells, BrdU-positive cells and nestin+BrdU co-localized cells.

A subpopulation of nestin-positive NPY-responsive cells exhibit the stem cell marker Lewis-X in both HSVZ-derived and dentate-derived cultures

Immunostaining for the stem cell marker Lewis-X (Le-X), a cell surface polysaccharide moiety expressed on stem cells (Capela and Temple, 2002) on DG-derived cultures grown in NPY conditions for 3 days and exposed to BrdU for 4 h before fixation showed many Le-X-positive cells in the S-phase of cell division confirming the Le-X-positive population as proliferative (Figs. 2A–C). Le-X, also known as CD15 or SSEA-1, is expressed on GFAP- and nestin-positive multipotent neural stem cells derived from the neurogenic niches of the adult forebrain (Imura et al., 2006), and its expression is controlled by Pax-6 (Shimoda et al., 2002) which is necessary for postnatal hippocampal neurogenesis (Maekawa et al., 2005). To further examine the phenotype of nestin-positive cells, both DG and HSVZ tissue cultures were plated at a viable cell density of 100×10^3 viable cells/ml, and grown in either 1 μM NPY or control conditions for 3 days. Immunostaining for Lewis-X (Le-X), revealed co-localization with nestin-expressing cells in both DG and HSVZ cultures (Fig. 2D). All Le-X-positive cells were nestin-positive in these and other experiments and an Le-X-positive/nestin-negative cell was never encountered (data not shown). Total cell counts assessed by nuclear DAPI staining, were again significantly increased in DG and HSVZ cultures following a 3-day growth in 1 μM NPY (Fig. 2E) compared to control conditions where the cell densities of DG and HSVZ cultures were almost identical (being 146.9±10.1 and 143.9±10.1 cells/mm^2, respectively). The number of nestin-positive cells was also significantly increased in DG and HSVZ cultures under NPY conditions compared to controls, and the number of nestin-positive cells in the HSVZ control cultures was significantly greater than in DG control cultures ($p<0.01$) (Fig. 2F). Immunostaining for the stem cell marker Lewis-X (Le-X) showed a significant increase in Le-X stained cells under NPY conditions in both DG and HSVZ cultures ($p<0.01$) (Fig. 2G). Again, Le-X-positive cell counts were greater in HSVZ cultures than in DG-derived cultures ($p<0.05$) (Fig. 2G).

NPY supports the generation of sphere-forming cultures from both dentate and hippocampus/caudal subventricular zone tissue

In a further examination of the growth characteristics of the nestin-positive cell populations, we sought to examine if NPY could support the generation of free-floating cell spheres from primary cell cultures of dissected dentate and hippocampal tissue (Fig. 3). Cells were cultured for 6 days in untreated tissue culture wells to encourage sphere formation. NPY supports the generation of a greater number of spheres from both DG and HSVZ tissue compared to control conditions (Fig. 3A). The numbers of cell spheres with a diameter greater than 100 μm were scored and HSVZ cultures yielded a greater number of these spheres than DG cultures under NPY conditions ($p<0.05$) (Fig. 3B).

To examine the phenotype of cells within these spheres, 6 DIV NPY cultured DG and HSVZ-derived cell spheres were transferred to poly-L-lysine-coated glass coverslips for 3 h to allow for attachment prior to fixation and processing for immunofluorescence. Separate wells from the same experiments were stained for nestin expression (precursor cell marker), β-tubulin (neuronal marker) or GFAP (astrocytic marker) expression, and all spheres were counterstained with the nuclear dye DAPI. DG-derived neurospheres contained nestin-positive (Fig. 3C) and β-tubulin-positive (Figs. 3D, E) cells. Spheres were largely negative for GFAP staining (Fig. 3F). β-Tubulin-positive cells were not detectable in all spheres, but were present as either a very low proportion of total cells (Fig. 3D); or were present at high levels (Fig. 3E). 200 DAPI stained neurospheres were scored for â-tubulin immunoreactivity in two wells of DG and two wells of HSVZ-derived neurospheres. The proportion of spheres with no-detectable β-tubulin-positive cells was very similar in DG-derived and HSVZ-derived cultures (16.0±3% and 15.4±3% respectively) (Fig. 3G). The proportion of spheres with a high β-tubulin-positive cell content was significantly greater in DG cultures compared to HcSVZ cultures (44.0±5.3% vs. 13.7±2.6%; $p<0.001$) (Fig. 3G).

To assess the phenotype of sphere progeny, cell spheres cultured for 6 DIV in NPY were transferred to NPY-deficient control media and allowed to differentiate for a further 6 days (Figs. 3H and I) (Howell et al., 2003). In the absence of the NPY growth stimulus, spheres from both DG and HSVZ cultures attached to the substratum and began to migrate away from the core cell mass. Immunostaining for β-tubulin and GFAP revealed that all spheres examined with confocal microscopy (100 spheres from each of the dentate and hippocampus groups) gave rise to both β-tubulin-positive cells and GFAP-positive cells (Figs. 3H and I). Interestingly, HSVZ-derived spheres appeared to generate a lower number

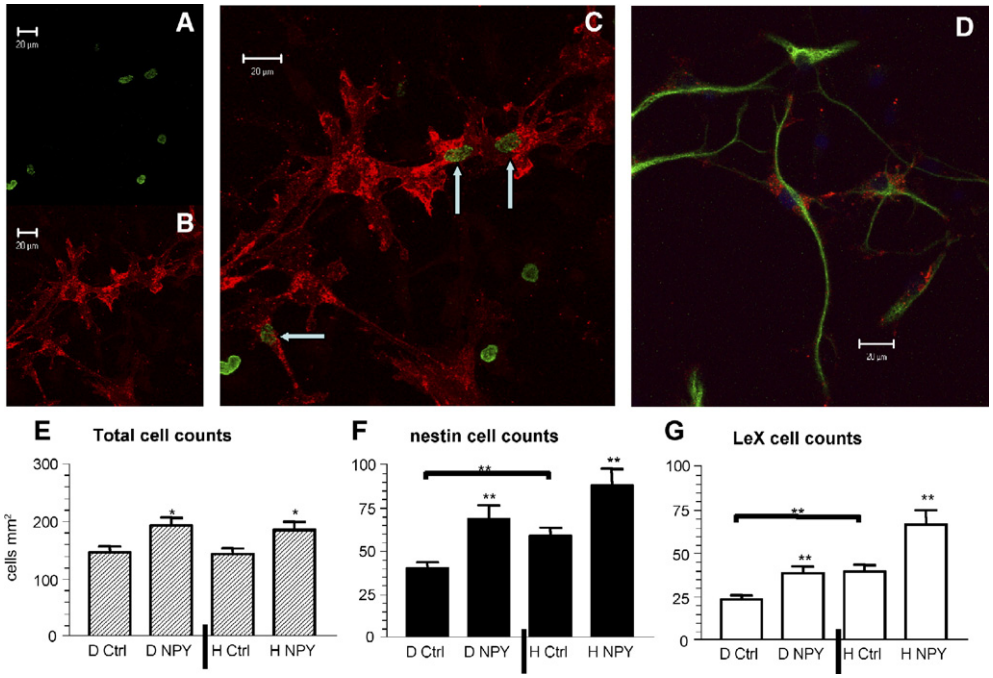


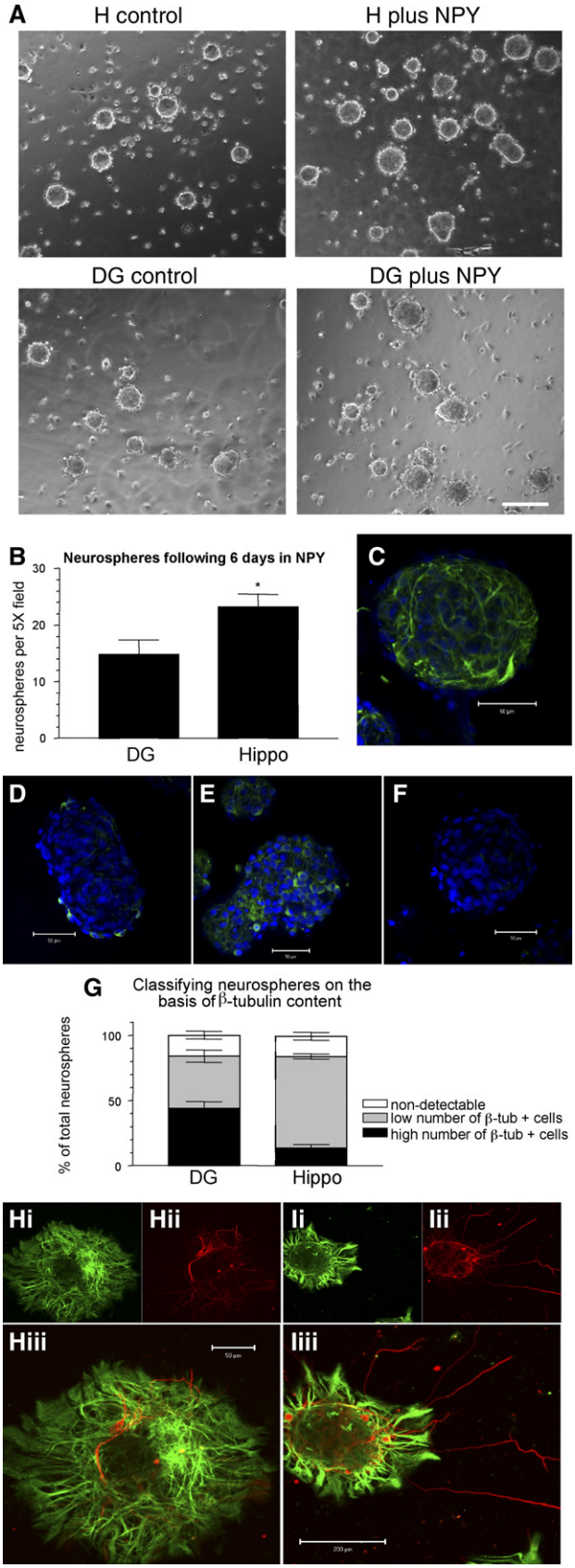
Fig. 2. NPY increases the number of progenitor cells immunopositive for nestin and LeX in dentate and hippocampal subventricular zones (HSVZ) cultures. (A–C) NPY supports the proliferation of Lewis-X-positive cells in culture. Cultures from dissected dentate tissue containing the neurogenic subgranular zone were cultured for 3 days in the presence of NPY and pulsed with 20 μM BrdU for 4 h before PFA fixation. Cultures probed with antibodies to BrdU (A) and the stem cell/progenitor marker LeX (B) revealed the presence of proliferating (BrdU-positive) LeX-positive cells (C), when grown in NPY. Images were captured by sequential scanning of the Cyanin2 and Cyanin3 channels and 3D projections generated. Scale bar, 20 μm. (D) Cells cultured from the dentate gyrus in 1 μM NPY for 3 DIV were immunopositive for nestin (green) and the progenitor/stem cell marker LeX (red). LeX stains cell perikarya and all LeX-positive cells expressed nestin. Images are 3D reconstructions from individual z-stacks captured by sequential scanning of the appropriate channels. Scale bar, 20 μm. (E–G) NPY increases the number of nestin-positive and Lewis-X-positive cells in dentate and HSVZ cultures. Separate cell cultures were established from dissected dentate and HSVZ and grown for 3 days under control conditions or conditions containing 1 μM NPY. (E) The total cell number (DAPI-positive cells) was similar between control dentate and control HSVZ cultures. Cell numbers increased following growth in NPY. (F) The absolute number of nestin-positive cells is increased in NPY growth conditions in dentate and HSVZ cultures. The number of nestin-positive cells in HSVZ cultures is significantly greater than for dentate cultures. (G) The absolute number of LeX-positive cells is increased in NPY growth conditions and the number of LeX-positive cells in HSVZ cultures is greater than for dentate cultures. Values are mean ± SE of 10–12 wells per condition from 2 separate experiments. Student's *t*-test: *, $p<0.05$; **; $p<0.01$; ***, $p<0.001$. D; dentate gyrus, H; hippocampal subventricular zones, Ctrl; Control, LeX; Lewis-X.

of β-tubulin-positive neurons in comparison to DG-derived spheres (Figs. 3H and I).

NPY has a proliferative effect on nestin-positive cells cultured from both the dentate gyrus (mediated via the NPY Y1 receptor) and from the HSVZ

To further examine whether the putative precursor cells of the dentate gyrus proliferate in response to the application of NPY, we generated cultures from micro-dissected DG and examined changes in the total number of cells, the number of nestin-positive cells with time, as well as the effect of a short pulse of NPY on the proportion of nestin-positive cells incorporating the S-phase marker of cell proliferation, BrdU (Fig. 4). Dissociated monolayer cell cultures grown in the presence of 1 μM NPY had significantly greater numbers of nestin-positive cells by 48 and 72 h after plating in comparison to cells cultured in control growth media (neurobasal A/B27 and L-glutamine) (Fig. 4A). The proportion of cells displaying a nestin-positive phenotype was also significantly different from control by 72 h in vitro (0.42 ± 0.03 and 0.52 ± 0.03 for control and NPY conditions at 72 h) ($p<0.05$).

In a separate set of experiments on DG-derived cultures we found no difference in the number of GFAP-positive cells or the proportion of GFAP-positive cells expressing BrdU after 4-h exposure at day 3 *in vitro* in response to NPY (data not shown). However, a 4-h exposure of BrdU and 1 μM NPY to day 3 cultures showed a direct proliferative effect of NPY on nestin-expressing cells by increasing the proportion of nestin cells incorporating BrdU (Fig. 4B). Confocal microscopy of these cells demonstrated the presence of BrdU-positive cells (green) (Fig. 4Ci), nestin-positive cells (blue) (Fig. 4Cii) and BrdU- and nestin-positive cells (Fig. 4Ciii). The NPY Y_1 receptor selective antagonist BIBP3226 blocked NPY-induced increases in the number of nestin and nestin/BrdU-positive cells in 3 DIV DG cell cultures (Figs. 4D and E). The Y_1 receptor selective agonist [F7, P34] NPY increased the total number of nestin-expressing cells, and the number of nestin/BrdU double-positive cells to the same levels as 1 μM NPY (Figs. 4D and E). Confocal imaging demonstrated co-localization of the Y_1 receptor upon nestin-positive cells with a non-neuronal morphology (Fig. 4F). In a separate set of experiments a 4-h pulse of BrdU and 1 μM NPY also significantly increased the proportion of BrdU incorporating nestin-positive cells cultured from the HSVZ (Fig. 4G).



NPY increases the number of clonal spheres but does not influence fate determination

The neurosphere assay has been shown to represent a population of cells demonstrating stem/progenitor cell characteristics (Seaberg and van der Kooy, 2002). Previous studies show that sphere colonies arise from the proliferation of single cells, when plated at a cell density of 20 cells/μl (Tropepe et al., 2000; Seaberg et al., 2005). This assay was used to investigate the phenotype and multipotency of neural precursor cells.

Both NPY and EGF/FGF2 increased the number of spheres after 7 days in culture (Figs. 5A and B).

There was a significant difference in the cellular phenotype of cells between NPY generated and EGF/FGF2 generated spheres

Fig. 3. NPY supports the generation of cell spheres from hippocampus and dentate cell isolates that give rise to new neurons and astrocytes. (A) Representative phase contrast images demonstrating the presence of free-floating cell spheres under control conditions from micro-cultures from the hippocampal subventricular zones (HSVZ) and dentate gyrus, respectively. Note the greater number of spheres resulting from the growth in the presence of NPY. Scale bar, 250 μm. H; hippocampal subventricular zones cultures, DG; dentate gyrus cultures. (B) A greater number of spheres can be cultured from HSVZ tissue in comparison to dentate tissue. Cultures grown for 6 days in 1 μM NPY were imaged by capturing one 5× field per well and scoring neurospheres with a diameter greater than 100 μM. Data are expressed as the number of spheres per field sampled and quoted as mean±SE of 18–24 wells of dentate and HSVZ cultures, from two separate experiments. Student's *t*-test; *, $p<0.05$. DG; dentate gyrus, Hippo; hippocampal subventricular zones. (C–F) Neurospheres cultured in 1 μM NPY for 6 DIV express nestin and β-tubulin, but are largely negative for GFAP. Cell nuclei are denoted by DAPI staining (blue) in all images. (C) All neurospheres investigated contained cells immunopositive for nestin (green). (D and E) Neurospheres were composed of variable numbers of β-tubulin-positive cells. (D) Example of a neurosphere with very little detectable β-tubulin staining. (E) Neurosphere containing many β-tubulin cells, both around the edge and at the center of the sphere. All spheres were largely negative for GFAP staining at 6 DIV (F). Confocal images were captured by merging individual z-stack images. Scale bars, 50 μm. (G) Neuronal β-tubulin content varies between neurospheres from dissected dentate and neurospheres from the HSVZ. 200 dentate and 200 HSVZ neurospheres were scored for β-tubulin-positive cells. Neurospheres were deemed to have 'low' neuron numbers if the only visible β-tubulin cells were located near the extreme edges of the sphere while 'high' β-tubulin cell numbers refer to positive cells both around the outer margins and within the center of the sphere. The number of spheres with a low-level of β-tubulin-positive cells was different between the two groups ($p<0.001$; Student's *t*-test). The number of spheres with a high β-tubulin-positive cell content was greater in dentate only cultures ($p<0.001$). Counting was performed under epi-fluorescence with an oil-immersion 40× objective. Values were expressed as mean±SE of eight groups of 25 spheres (200 in total). (H and I) NPY neurospheres from the dentate or HSVZ differentiate and give rise to neurons and astrocytes. Cultures from dissected dentate and HSVZ were grown as free-floating neurospheres in 1 μM NPY for 6 DIV. Spheres were then harvested, washed free of media and replated onto poly-L-lysine-coated glass slides in control media alone. Following a further 6 days spheres were processed for GFAP and β-tubulin immuno-fluorescence. (Hi–iii) Images of neurospheres derived from dentate cultures demonstrating the presence of both GFAP-positive astrocytes (green) and β-tubulin-positive neurons (red). (Ii–iii) Neurospheres from HSVZ cell cultures also contain GFAP and β-tubulin-positive cell populations. HSVZ-derived neurospheres contain a lower number of β-tubulin-positive neurons in comparison to dentate-derived neurospheres. Images were captured by sequential scanning of the appropriate channels under a 40× objective. Scale bars, 50 μm.

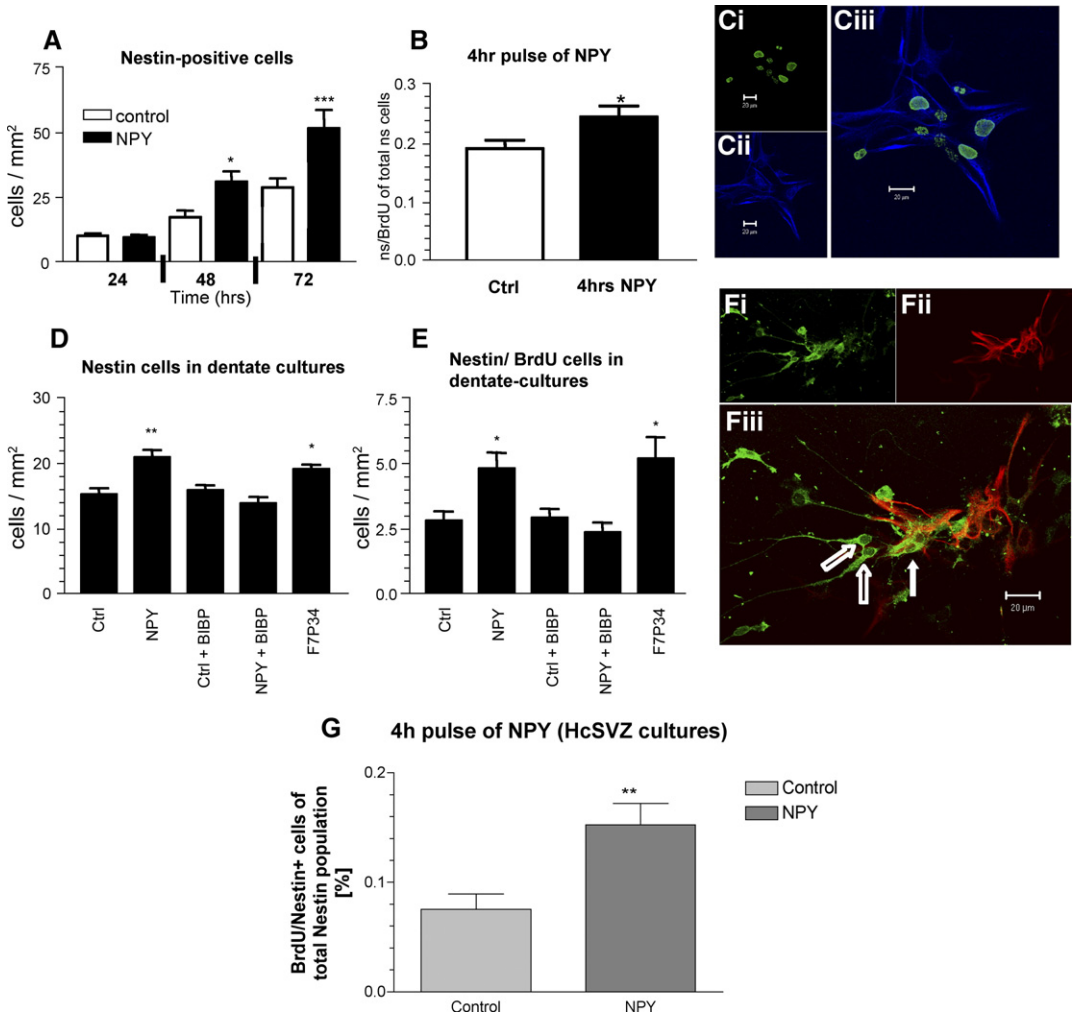


Fig. 4. NPY supports the proliferation of nestin-positive cells in cultures established from the dentate gyrus. (A) NPY increases the number of nestin-positive cells in dentate cultures in comparison to those grown under standard control conditions by 48 and 72 h. Values represent mean±SE based on a sample of 8 wells per condition from two separate experiments. One-way ANOVA with Dunnett's multiple comparison post-test (*, $p<0.05$; ***, $p<0.001$). (B) Dentate-derived cell cultures grown for 3 DIV in control conditions were exposed to BrdU and/or NPY for 4 h. The proportion of nestin cells incorporating BrdU over a 4-h period is increased with a 4-h exposure to NPY, indicating that NPY directly stimulates nestin-positive cells to divide. Values are mean±SE of 10–12 wells per condition from 3 separate experiments, analyzed using Student's *t*-test (*, $p<0.05$). (C) Primary cultures of micro-dissected dentate gyrus were cultured for 3 days in the presence of NPY and pulsed with 20 μM BrdU for 4 h before fixation. Cultures probed with antibodies to BrdU (green; Ci) and nestin (blue; Cii) revealed the presence of proliferating (BrdU-positive) nestin-positive cells (Ciii). Images were captured by sequential scanning of the appropriate channels and 3D projections generated. (D and E) Nestin-positive cell proliferation is mediated by the NPY Y_1 receptor. Dentate cell cultures were grown in control, NPY, control plus the Y_1 receptor antagonist BIBP3226, NPY and BIBP3226 or [F7, P34] NPY; a specific Y_1 receptor agonist for 3 DIV. NPY, BIBP3226 and [F7, P34] NPY were all used at 1 μM concentration. (D) NPY significantly increases the number of cells immunopositive for nestin and the number of cells (E) double-positive for nestin and BrdU (following a 4-h exposure on day 3 to 20 μM BrdU). Cells cultured in NPY plus the Y_1 receptor antagonist BIBP3226 demonstrate no change in nestin expression or BrdU incorporation (D and E). Conversely, the presence of the Y_1 receptor agonist [F7, P34] NPY elicits similar increases in nestin and nestin/BrdU cells counts to that seen for NPY alone (D and E). Experiments were performed on 16–18 wells per condition in 3 separate experiments. ANOVA with Dunnett's multiple comparison post-test (comparing control wells to all other conditions); *, $p<0.05$; **, $p<0.01$. (F) Confocal images showing Y_1 antibody staining (green; Fi) and nestin staining (red; Fii) in 3 DIV dentate cultures. A number of nestin-positive cells that co-label for Y_1 receptor staining can be seen (Fiii—solid arrows). Note that cells with a neuronal morphology on Y_1 antibody staining were negative for nestin (Fiii—open arrows). Scale bars, 20 μm. Ctrl; control media. (G) NPY is proliferative for nestin cells derived from hippocampal subventricular zones (HSVZ) cultures. HSVZ-derived cell cultures grown for 3 DIV in control conditions were exposed to BrdU and/or NPY for 4 h. The proportion of nestin cells incorporating BrdU over a 4-h period is increased, indicating that NPY directly stimulates nestin-positive cells to divide. Values are mean±SE of 12–16 wells per condition from 4 separate experiments, analyzed using Student's *t*-test (**, $p<0.01$).

with a significantly greater proportion of nestin+/GFAP+ co-localized cells in NPY spheres and nestin+/GFAP− cells in EGF/FGF2 spheres (Figs. 5C–F).

Spheres grown under all three conditions followed by differentiation in control medium, were immunopositive for both GFAP and TuJ1, demonstrating multipotentiality (Fig. 5G). Interestingly there was no difference between the proportions of GFAP+ cells and TuJ1+ cells under control or NPY conditions indicating a lack of fate determination by NPY, while there was a significant increase in the proportion of cells that were immuno-

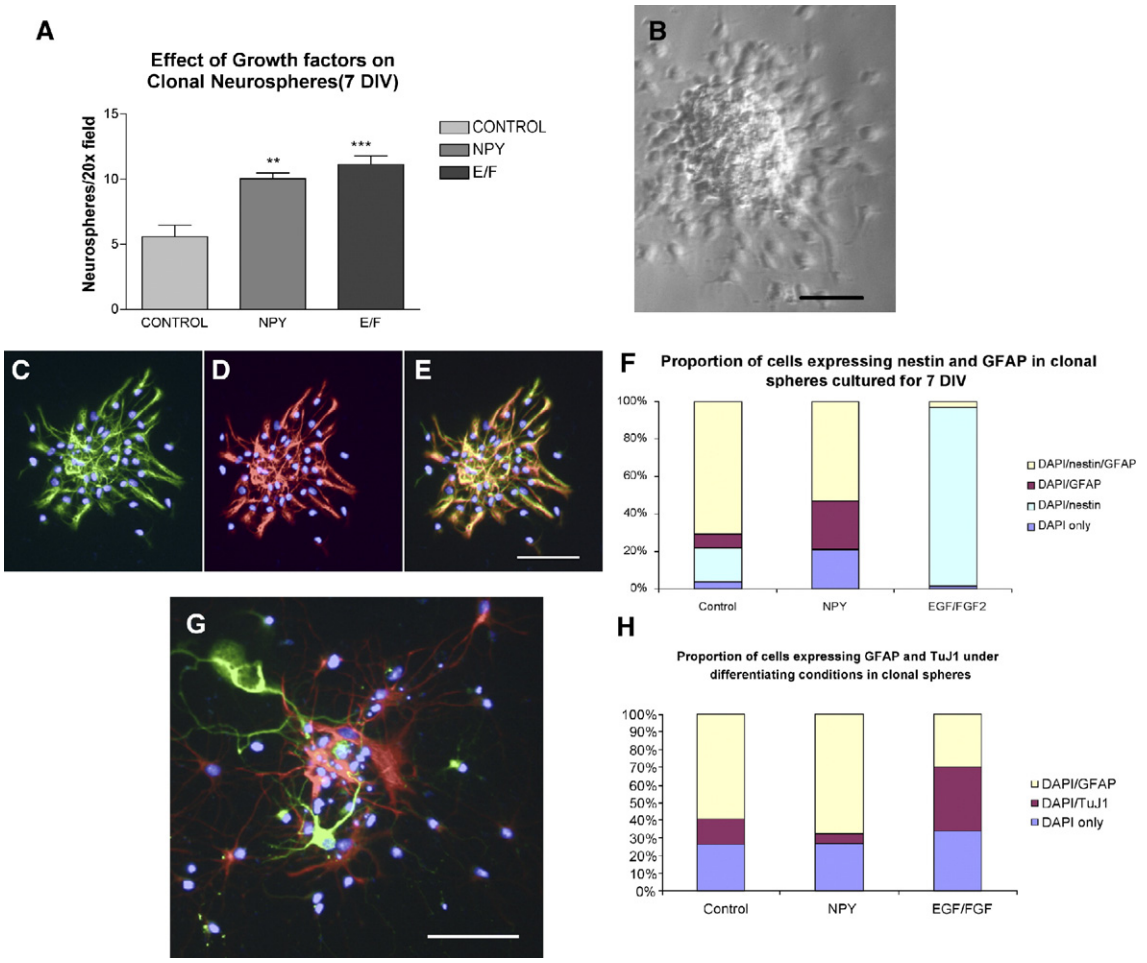


Fig. 5. Effects of NPY on clonal neurospheres. (A) NPY increases the number of clonal neurospheres derived from whole hippocampal cultures. Cells were plated at 20×10^3 cells/ml in non-adherent wells and grown for 7 days under control conditions or in the presence of 1 μM NPY or 20 ng/ml of EGF and FGF2 (E/F). Both NPY and combined EGF/FGF2 conditions had significantly greater number of spheres. Values are mean±SE of 8 wells per condition from two separate experiments, analyzed using ANOVA with Dunnett's post hoc test comparing all values to control (**, $p<0.01$; ***, $p<0.001$). (B) Brightfield image of a clonal neurosphere grown in EGF/FGF2 after adhering to a poly-L lysine-coated coverslip. Scale bar = 40 μm. (C–E) Images of a clonal neurosphere grown under NPY conditions for 7 days and stained for nestin (green, C) and GFAP (red, D) counterstained with DAPI (blue). Merged image (E) shows that most nestin-positive cells were also GFAP-positive under NPY growth conditions. Scale bar = 60 μm. (F) Quantification of the proportion of cells expressing nestin and GFAP based on counts from 15 spheres per condition in a single experiment, showing a significantly greater proportion of GFAP expression in nestin cells under control and NPY conditions, compared to EGF/FGF2 ($p<0.01$; ANOVA with Dunnett's post hoc comparing all to EGF/FGF2). (G) A neurosphere grown under NPY conditions and allowed to differentiate for 5 days showing the generation of TUJ1−positive neurons (green) and GFAP-positive astrocytes (red), counterstained with DAPI (blue). Scale bar = 60 μm. (H) Quantification of GFAP and TuJ1 staining based on counts from 15 spheres per condition from a single experiment, showing a significantly greater proportion of TuJ1-positive neurons generated from EGF/FGF2 spheres ($p<0.05$, *t*-test comparing EGF/FGF2 to either Control or NPY).

positive for TUJ1 under EGF/FGF2 conditions, consistent with a neurogenic effect (Fig. 5H).

NPY $Y_1^{-/-}$ animals show reduced BrdU incorporation in the caudal subventricular zone and subcallosal zone

Stereological estimation of SGZ BrdU cell labeling from these mice has been previously reported (Howell et al., 2005) showing an approximate 40% decrease in the number of BrdU-positive cells in comparison with age- and generation-matched $Y_1^{lox=lox}$ controls (6685±729 vs. 4176±217 cells per subgranular zone for control and $Y_1^{-/-}$ groups, respectively; one-way ANOVA with Student–Newman–Keuls multiple comparison post-test; $p<0.01$) (Figs. 6A and B). The number of BrdU-positive cells of the caudal SVZ lining the ventricular surface of the hippocampus and the adjacent subcallosal zone (SCZ) between the hippocampus and the corpus callosum, was significantly reduced in $Y_1^{-/-}$ animals in comparison to controls (1253±1304 vs. 8650±774 cells per cSVZ/SCZ for $Y_1^{lox=lox}$ and $Y_1^{-/-}$ groups respectively) (Figs. 6C and D). There was no difference in BrdU cell counts from the hilus or areas CA1–3, demonstrating no difference in BrdU bioavailability between the $Y_1^{-/-}$ mice and controls.

SGZ BrdU incorporation is reduced in $NPY^{-/-}$ mice under control conditions and after kainate-induced seizures

$NPY^{-/-}$ animals had a 41% reduction (1788±114.8 and 1062±125.9 cells) in BrdU-immunopositive cells within the subgranular

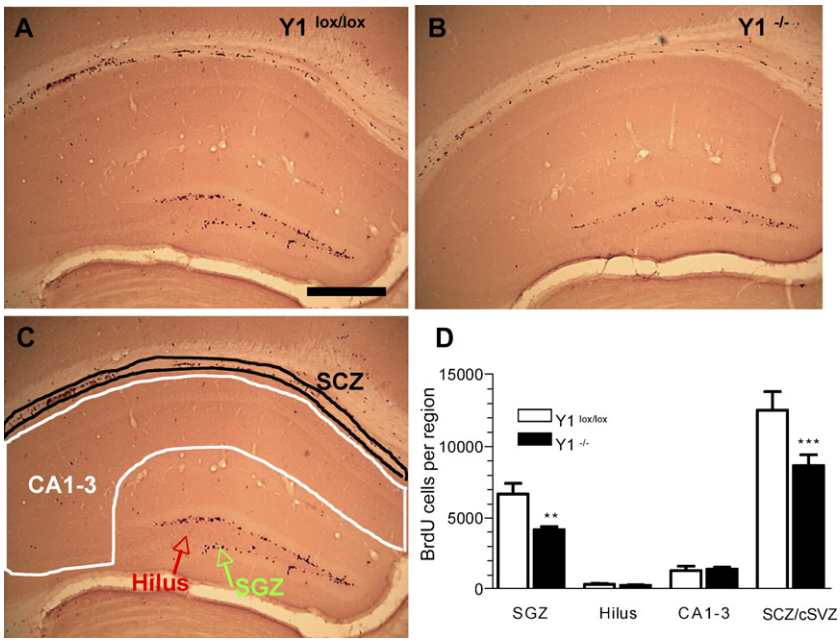


Fig. 6. Y_1 homozygous knockout animals have reduced subgranular zone (SGZ) and subcallosal (SCZ)/caudal subventricular zone (cSVZ) cell proliferation. Five $Y_1^{lox/lox}$ controls and five $Y_1^{-/-}$ 35-day-old mice were administered BrdU (50 mg/kg) intraperitoneally once per day for 5 days and sacrificed 5 h after the final injection. (A and B) Immunohistochemical detection of BrdU in representative sections clearly illustrates many cells that have incorporated the marker lying along the subgranular zone of the dentate and along the subcallosal zone (SCZ) between the hippocampus and corpus callosum. In $Y_1^{-/-}$ tissue there is a visible decrease in the number of BrdU-positive cells within the SGZ and SCZ. (C) BrdU cell counts were recorded from the dentate SGZ, dentate hilus, CA1–3 area of the hippocampus and the SCZ and cSVZ (not shown). (D) 8 Sections per animal serially sampled along the entire rostro-caudaul extent of the hippocampus were scored for BrdU-positive cell nuclei at high magnification. $Y_1^{-/-}$ animals had a significant reduction in the number of BrdU-positive cells counted in the dentate SGZ and SCZ/cSVZ (one-way ANOVA with Student–Newman–Keuls multiple comparison post-test; **, $p<0.01$; ***, $p<0.001$).

zone (SGZ) of the dentate gyrus in comparison to age-matched (eight wild-type animals, aged 9.0±0.4 weeks; seven NPY KO animals, aged 9.1±0.7 weeks) wild-type littermates of the same genetic strain (Figs. 7A–D). This is consistent with our previous report of a similar reduction in SGZ cell proliferation in $Y_1^{-/-}$ mice (Howell et al., 2005).

Kainic acid (KA) significantly increased the number of BrdU incorporating cells in wild-type dentate in comparison to control animals (Figs. 7A, 8A and B). Interestingly, the difference between low-dose KA (20–30 mg/kg, no noticeable seizures) and high-dose KA (50–70 mg/kg, resulting in severe seizures) was not statistically significant. Wild-type or $NPY^{-/-}$ animals treated with an equivalent dose of KA (20 mg/kg) demonstrated large increases in the numbers of BrdU-labeled cells, within the SGZ of the dentate granule cell layer, the hippocampal parenchyma, the cSVZ lining the ventricular surface of the hippocampus, and the subcallosal zone (SCZ) between the hippocampus and overlying corpus callosum (Figs. 8A–D).

The application of low-dose KA to $NPY^{-/-}$ mice that resulted in severe seizures, significantly increased the number of BrdU incorporating cells within the SGZ in comparison to control $NPY^{-/-}$ animals. However, $NPY^{-/-}$ animals that experienced severe seizures, had a level of hippocampal cell proliferation well below that of KA dose-matched or seizure-matched wild-type controls (Figs. 7A and 8), consistent with a role for NPY in seizure-induced SGZ cell proliferation. As control $NPY^{-/-}$ animals had a lower level of cell proliferation in comparison to wild types, the reduced number of BrdU cells following seizure-induction may reflect this lower starting point. Indeed, wild-type BrdU counts increase from 1788±114.8 cells/subgranular zone to 7800±890 cells/subgranular zone for high-dose kainic acid animals, an increase of 4.4-fold. $NPY^{-/-}$ control BrdU counts increase from 1062±126 cells/subgranular zone to 4991±272 cells/subgranular zone following KA, an increase of 4.7-fold.

At the time of increased cell proliferation, wild-type animals demonstrated an increase in NPY immunoreactivity. Tissue sections from wild-type controls and low-dose KA animals (20 mg/kg) were probed with an antibody to NPY (Figs. 7B and C). $NPY^{-/-}$ homozygous knockout sections were negative for staining (Fig. 7D). NPY-specific staining could be seen in cells of the dentate hilus in both wild-type control and KA-treated animals. KA increased the intensity of NPY staining in many cell processes lying within the hilus and also upon processes located between the hilus and granule cell layer, adjacent to the proliferative niche of the subgranular zone.

Discussion

Nestin-positive cells in the dentate gyrus and hippocampus

The rodent dentate gyrus is partly formed in the embryo, when progenitor cells migrate within the hippocampal migratory stream to the developing dentate gyrus around E18 to establish the tertiary germinal matrix in the hilus (Pleasure et al., 2000), which continues to directly generate granule cell neurons until P15–20, by which time it has receded to form the adult neurogenic subgranular zone (SGZ). Recently, a second transient (P0–P14) germinal matrix near the fimbria, called the hippocampal subventricular zone has been

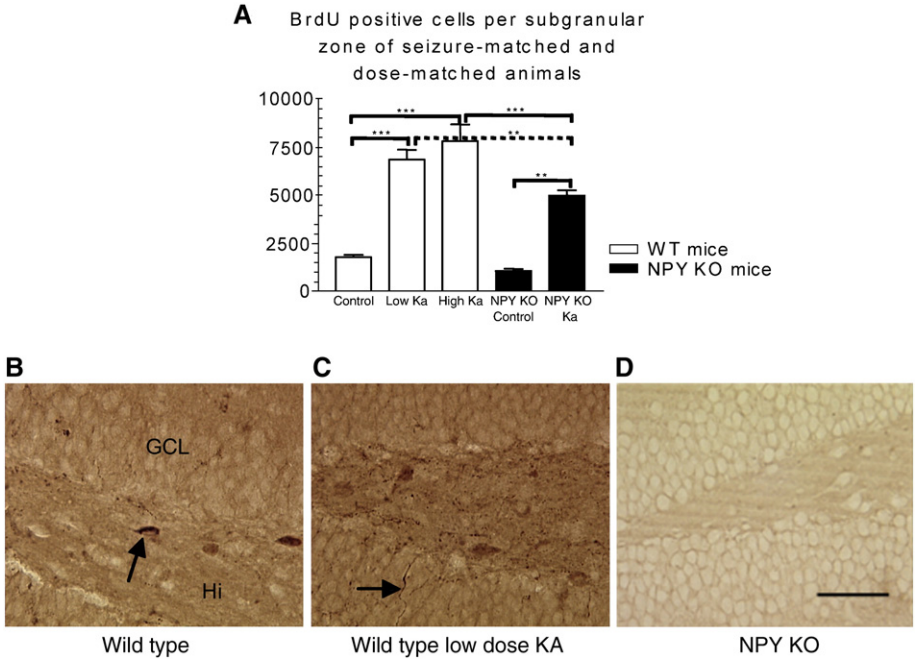


Fig. 7. Cell proliferation in NPY$^{-/-}$ animals is increased following seizures. NPY$^{-/-}$ animals are more sensitive to seizures and required a 'low dose' of kainic acid (20–30 mg/kg) to display prominent seizures (intermittent tremors and high, intensive jumps). Wild-type animals were matched according to the dose of kainic acid (KA) (seven animals; 4 × 20 mg/kg and three × 30 mg/kg KA) or seizure severity (seven animals were administered KA until equivalent seizures were attained; 50–70 mg/kg of KA). (A) Cell proliferation is reduced in NPY$^{-/-}$ animals in comparison to wild-type animals matched for dose of KA or for seizure severity. Following KA, the number of BrdU incorporating cells is significantly increased in wild-type and NPY$^{-/-}$ animals in comparison to respective untreated controls. The number of BrdU-positive cells does not increase to the same level as wild-type kainate dosed animals, though the relative scale of increase is similar. Eight systematically random sampled sections per animal from the rostro-caudal extent of the hippocampus were scored for BrdU-positive cell nuclei at high magnification. A stereological estimate of the total number of BrdU cells per hippocampal subgranular zone is reported. ANOVA with Student–Newman–Keuls multiple comparison post-test (**, $p<0.01$; ***, $p<0.001$). (B) Tissue sections from wild-type controls and low-dose KA animals (C) were probed with an NPY specific anti-serum. NPY-specific staining could be seen upon interneurons of the dentate hilus in control and treated animals (arrow in panel B). KA increased the intensity of NPY staining in many cell processes lying within the hilus and also upon processes located between the hilus and granule cell layer (arrow in panel C). NPY$^{-/-}$ sections were negative for staining (D). Scale bar = 100 μm.

described, which also contributes to the hilar germinal matrix and dentate gyrus neurogenesis (Navarro-Quiroga et al., 2006) as well as to forebrain gliogenesis. Therefore, in hippocampal sections from P8–10 pups, from which our cultures were generated, multipotent progenitor cells destined for the dentate gyrus are located predominantly in dentate but also in hippocampal cultures, while multipotent progenitors destined for the corpus callosum, fimbria and forebrain cortex reside in the hippocampal cultures alone.

The principal phenotype of proliferative hilar progenitors between P5 and P12 is astrocytic, typically expressing S100β protein and the intermediate filaments nestin and GFAP, as well as the astrocyte-specific glutamate transporter (GLAST) (Namba et al., 2005). A substantial proportion (66.8%) of these proliferating cells differentiate into proliferating neuroblasts and immature neurons in the hilus, which migrate into the granule cell layer. The role of, and changes to, glial progenitor cells in the transition between the postnatal and adult SGZ remains largely unknown, although glial precursors do appear to give rise to radial glia like cells in the adult SGZ (Namba et al., 2005). This is important as distinct transitional states have been found between the embryonic, postnatal and adult SVZ (Tramontin et al., 2003; Peretto et al., 2005).

We found evidence of multipotent precursors in cultures derived from the whole hippocampus containing both germinal zones, in agreement with the *in vivo* findings of Namba et al. (2005) and Navarro-Quiroga et al. (2006). The majority of cycling cells were S100β (data not shown) and nestin-positive, and when cultured under clonal sphere-generating conditions (Tropepe et al., 2000), were multipotent, because they gave rise to both β-tubulin-positive cells with a neuronal morphology, and GFAP-positive astrocytes. The nestin-positive cells in our selective hippocampal and dentate cultures had additional features of multipotent precursor cells, in that they stained for the stem cell marker Lewis-X.

As well as a population of proliferating neuroblasts (β-tubulin-positive, nestin-negative and Ki67-positive), we also describe a proliferative subpopulation of nestin cells that showed punctate β-tubulin immunostaining, consistent with a transitional stage between more primitive nestin-positive cells and cycling neuroblasts, as previously identified within the hilar tertiary matrix of the dentate (Namba et al., 2005). β-Tubulin immunostaining is one of the earliest markers for neuronal differentiation (Lee et al., 1990). Our results also show functional differences between cycling nestin and Class III β-tubulin-positive cells, with the nestin-positive population cycling more rapidly than the neuroblast population, because their labeling index was significantly greater after a 4-h BrdU pulse.

Nestin-positive cells from the DG respond to NPY

Nestin-positive cells in the hilar tertiary germinal matrix are multipotent (Namba et al., 2005), and this matrix generates the adult

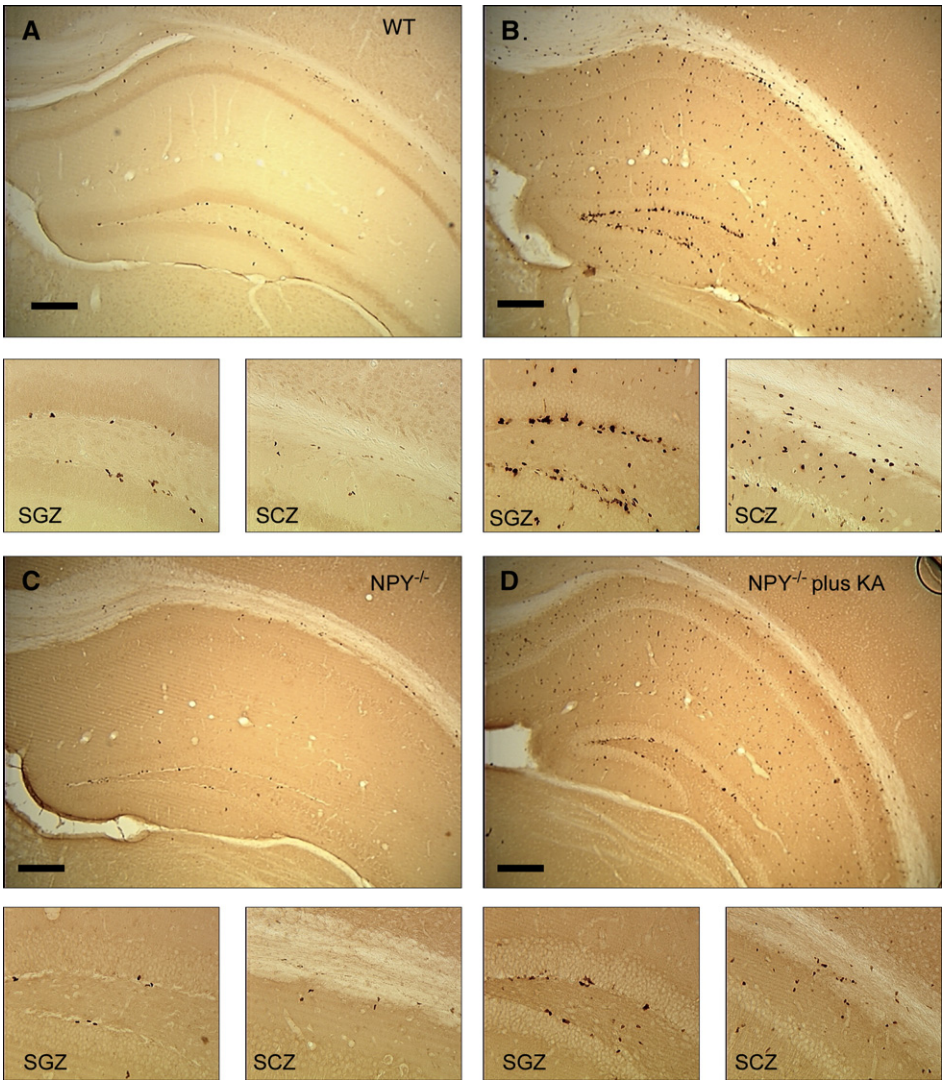


Fig. 8. Seizures increase cell proliferation. Representative images of wild-type (WT) (A and B) and NPY knockout ($NPY^{-/-}$) animals stained for BrdU immunoreactivity. WT (B) or $NPY^{-/-}$ animals (D) treated with 20 mg/kg kainic acid (KA) display an increase in proliferating cell numbers in all regions of the hippocampus, in comparison to saline-treated WT (A) or $NPY^{-/-}$ controls (C). Cell proliferation is particularly increased within the subgranular zone (SGZ) of the dentate, and within the subcallosal zone (SCZ) between the hippocampus and overlying corpus callosum (magnified images of the respective low power panels above). Note that WT animals have a greater number of BrdU-positive cells in control conditions and following KA. Scale bar = 100 μm.

SGZ, which contains GFAP-positive/nestin-positive precursor cells (Seri et al., 2001). We extend our previous work, showing here that the postnatal dentate also contains an NPY-responsive sphere-forming nestin-positive population, as well as the previously reported β-tubulin-responsive population (Howell et al., 2005). Our NPY-responsive/nestin population was GFAP-negative. While initial reports found conflicting evidence for the existence of an amplifying nestin-positive population in the adult SGZ (Filippov et al., 2003; Seri et al., 2004; Battista et al., 2006), a recent study has found that 50% of cycling cells in the adult SGZ are nestin-positive but GFAP and β-tubulin-negative (Encinas et al., 2006). Our results are therefore consistent with NPY affecting both intermediate nestin-positive precursors and committed neuroblasts, with no evidence of an effect on more primitive GFAP-positive precursors.

We have previously reported a proliferative effect of NPY on β-tubulin-positive cells cultured from the postnatal DG (Howell et al., 2005) via the Y_1 receptor, raising the possibility that the nestin-expressing and β-tubulin-expressing NPY-responsive cells in the postnatal dentate gyrus are the same population. This is unlikely as NPY-responsive β-tubulin-positive cells rapidly become postmitotic in culture (Howell et al., 2003), and postnatal dentate-derived neuroblasts are not sphere-forming *in vitro* (Seaberg and van der Kooy, 2002), in contrast to our dentate-derived nestin-positive cells. Our finding of significantly reduced precursor proliferation in the SGZ of adult NPY $Y_1^{-/-}$ mice (Howell et al., 2005) could be due to a reduced responsiveness in nestin- as well as β-tubulin-positive cells, but this will need to be clarified by further experiments.

Nestin cells in the hippocampus and hippocampal subventricular zone

NPY clearly had a proliferative effect on nestin-positive cells cultured from the hippocampus and HSVZ. As we have recently

shown that neuroblasts (β-tubulin-positive cells) derived from HSVZ cultures do not respond to NPY *in vitro* (Howell et al., 2005), the NPY-responsive nestin-positive cells from HSVZ cultures cannot be neuroblasts. In keeping with nestin-positive HSVZ cells being precursor cells, there was a significant overlap in the Lewis-X-positive and nestin-positive populations, as all Lewis-X-positive cells were nestin-positive and most, but not all, nestin-positive cells stained for Lewis-X. Lewis-X is a more stringent marker of neural progenitor cells (Capela and Temple, 2002), which is presumably why the number of LeX cells was always fewer than the number of nestin cells. It is interesting that LeX-positive cells are also found in the adult SVZ where the majority are GFAP-negative (Capela and Temple, 2002), consistent with transiently amplifying type C-cells (Doetsch et al., 1999). As we have shown that LeX-positive cells co-localize with nestin (Fig. 2A), and that neurospheres generated from the HSVZ are nestin-positive with very low levels of GFAP expression (Fig. 3F), then it is likely that the nestin-responsive population from HSVZ cultures are probably transiently amplifying type C cells (LeX+/GFAP−).

The developmental relationship between the early postnatal hippocampal subventricular zones and both the adult caudal SVZ lining the ventricular surface of the hippocampus and the adjacent SCZ between the hippocampus and corpus callosum, is unclear. However, it is intriguing to find an NPY-responsive nestin population in cultures of the postnatal HSVZ and reduced cell proliferation in the cSVZ and SCZ in adult NPY $Y_1^{-/-}$ mice. The SCZ is an important stem cell niche for producing myelinating oligodendrocytes to the corpus callosum and forebrain in the adult brain (Menn et al., 2006; Seri et al., 2006) and contains prominent NPY Y_1 receptor staining (Kopp et al., 2002).

NPY and seizure-induced proliferation in the hippocampus

As the majority of studies support the concept that increased neuronal activity increases DG neurogenesis, it is not surprising that seizures also increase neurogenesis (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998). The time course of proliferation in most studies shows an increase above baseline 2–3 days after status epilepticus (SE) (Parent et al., 1997; Nakagawa et al., 2000) although one study has shown an earlier increase (Radley and Jacobs, 2003). The mechanism of the increased cell proliferation is largely unknown. FGF-2 partially mediates progenitor proliferation after seizures (Yoshimura et al., 2001), seizure-induced neurogenesis is modulated by neurotrophins (Hagihara et al., 2005) and the cysteine protease cystatin C, which is expressed on astrocytes and microglia after SE, may have a role in baseline and seizure-induced neurogenesis (Pirttila et al., 2005).

There is also evidence that neurotransmitters are involved as 5HT1A (Radley and Jacobs, 2003; Zucchini et al., 2005) and galanin Type 2 receptors (Mazarati et al., 2004) appear important for seizure-induced precursor proliferation. NPY increases the proliferation of SGZ neuroblasts (Howell et al., 2005), its hippocampal expression is increased after limbic seizures (Marksteiner et al., 1989) and its expression in the granule cell layer precedes the time course of increased neurogenesis after kainate-induced SE (El Bahh et al., 2001), suggesting a possible role for NPY in the mediation of seizure-induced neurogenesis (Scharfman and Gray, 2006).

Here we show that NPY is necessary for baseline and seizure-induced precursor proliferation in the SGZ as proliferation was significantly reduced in $NPY^{-/-}$ mice under both conditions. Seizure-induced proliferation in $NPY^{-/-}$ animals was significantly reduced compared to both kainate-dose matched and seizure-severity-matched controls. There was no significant difference between the levels of proliferation when wild-type animals were given low or high doses of kainate, suggesting that the level of limbic seizure activity was similar in each. The greater intensity of hilar and granule cell layer NPY immunostaining was consistent with a seizure-induced increase in NPY expression as previously reported (Marksteiner et al., 1989) and we found no immunostaining for NPY in $NPY^{-/-}$ tissue. Interestingly, although the absolute levels of proliferation were lower in the $NPY^{-/-}$ animals in control conditions and after kainate, the relative increase after kainate was the same in the wild-type and $NPY^{-/-}$ groups (4.4-fold and 4.7-fold respectively), implying that SGZ proliferation under control conditions and after seizures is not mediated entirely via NPY, and that at least in the constitutive knock out, other mechanisms were able to induce a seizure-induced response but were unable to fully compensate for loss of the NPY-mediated effect.

The caudal SVZ and SCZ contain constitutively active oligodendrocyte precursors, a proportion of which arise from SVZ Type B stem cells, which generate postnatal oligodendrocytes that contribute to the myelination of white matter tracts in the corpus callosum and fimbria fornix (Menn et al., 2006). The cSVZ and SCZ also contain a prominent band of cellular Y_1 receptor staining (Kopp et al., 2002) and the proliferation of these stem/precursor cells in the cSVZ is increased after demyelination injury (Menn et al., 2006) and seizures (Parent et al., 2006). We confirmed an effect of NPY on proliferation in the adult cSVZ and SCZ under control conditions in NPY $Y_1^{-/-}$ mice. We did not quantify BrdU labeling in the cSVZ and SCZ after SE, but there was a clear reduction in $NPY^{-/-}$ mice under control conditions consistent with our findings in $Y_1^{-/-}$ mice, a clear increase after SE as reported by Parent et al. (2006) and (Figs. 8A and B) but an attenuated increase was again seen in $NPY^{-/-}$ mice after SE (Figs. 8C and D). Our results raise the intriguing possibility that some of the NPY-responsive β-tubulin-negative population in our postnatal HSVZ cultures are oligodendrocytes precursors, and that this activity continues into the adult cSVZ and SCZ, consistent with the reduced proliferation we observed in $Y_1^{-/-}$ animals, such that NPY modulates cSVZ and SCZ oligodendrogliogenesis as well as SGZ neurogenesis.

The significance of our results lies in the identification of NPY, and therefore NPY-releasing interneurons, as important modulators of the proliferation of precursor cells in both the SGZ and cSVZ under normal conditions and after seizures. Given that hilar and hippocampal interneuron loss is a prominent and early feature of mesial temporal lobe epilepsy, which is associated with cognitive decline, then targeting this population for neuroprotection, or pharmacological replacement, may ameliorate or reverse cognitive deficits by preventing or restoring the impaired neuropeptide drive on adult precursor cells.

Acknowledgments

The authors gratefully acknowledge the technical help of Dr. Matt Cuttle with confocal microscopy. This work was funded by the MRC (Grant No. G0300356), the University of

Southampton Grant No. D04 and the Wessex Medical Trust *Hope* to W.P.G.

References

Altman, J., Das, G.D., 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J. Comp. Neurol. 124, 319–335.

Baraban, S.C., Hollopeter, G., Erickson, J.C., Schwartzkroin, P.A., Palmiter, R.D., 1997. Knock-out mice reveal a critical antiepileptic role for neuropeptide Y. J. Neurosci. 17, 8927–8936.

Battista, D., Ferrari, C.C., Gage, F.H., Pitossi, F.J., 2006. Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus. Eur. J. Neurosci. 23, 83–93.

Bengzon, J., Kokaia, Z., Elmer, E., Nanobashvili, A., Kokaia, M., Lindvall, O., 1997. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. Proc. Natl. Acad. Sci. U. S. A. 94, 10432–10437.

Capela, A., Temple, S., 2002. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. Neuron 35, 865–875.

D'Sa, C., Duman, R.S., 2002. Antidepressants and neuroplasticity. Bipolar Disord. 4, 183–194.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97, 703–716.

El Bahh, B., Auvergne, R., Lere, C., Brana, C., Le Gal La Salle, G., Rougier, A., 2001. Decreased epileptic susceptibility correlates with neuropeptide Y overexpression in a model of tolerance to excitotoxicity. Brain Res. 894, 209–217.

Encinas, J.M., Vaahtokari, A., Enikolopov, G., 2006. Fluoxetine targets early progenitor cells in the adult brain. Proc. Natl. Acad. Sci. U. S. A. 103, 8233–8238.

Erickson, J.C., Clegg, K.E., Palmiter, R.D., 1996. Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. Nature 381, 415–421.

Filippov, V., Kronenberg, G., Pivneva, T., Reuter, K., Steiner, B., Wang, L.P., Yamaguchi, M., Kettenmann, H., Kempermann, G., 2003. Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Mol. Cell. Neurosci. 23, 373–382.

Fukuda, S., Kato, F., Tozuka, Y., Yamaguchi, M., Miyamoto, Y., Hisatsune, T., 2003. Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J. Neurosci. 23, 9357–9366.

Gates, M.A., Thomas, L.B., Howard, E.M., Laywell, E.D., Sajin, B., Faissner, A., Gotz, B., Silver, J., Steindler, D.A., 1995. Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres. J. Comp. Neurol. 361, 249–266.

Gray, W.P., Sundstrom, L.E., 1998. Kainic acid increases the proliferation of granule cell progenitors in the dentate gyrus of the adult rat. Brain Res. 790, 52–59.

Gross, C.G., 2000. Neurogenesis in the adult brain: death of a dogma. Nat. Rev., Neurosci. 1, 67–73.

Hagihara, H., Hara, M., Tsunekawa, K., Nakagawa, Y., Sawada, M., Nakano, K., 2005. Tonic–clonic seizures induce division of neuronal progenitor cells with concomitant changes in expression of neurotrophic factors in the brain of pilocarpine-treated mice. Brain Res. Mol. Brain Res. 139, 258–266.

Hansel, D.E., Eipper, B.A., Ronnett, G.V., 2001a. Neuropeptide Y functions as a neuroproliferative factor. Nature 410, 940–944.

Hansel, D.E., Eipper, B.A., Ronnett, G.V., 2001b. Regulation of olfactory neurogenesis by amidated neuropeptides. J. Neurosci. Res. 66, 1–7.

Howell, O.W., Scharfman, H.E., Herzog, H., Sundstrom, L.E., Beck-Sickinger, A., Gray, W.P., 2003. Neuropeptide Y is neuroproliferative for post-natal hippocampal precursor cells. J. Neurochem. 86, 646–659.

Howell, O.W., Doyle, K., Goodman, J.H., Scharfman, H.E., Herzog, H., Pringle, A., Beck-Sickinger, A.G., Gray, W.P., 2005. Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. J. Neurochem. 93, 560–570.

Huttmann, K., Sadgrove, M., Wallraff, A., Hinterkeuser, S., Kirchhoff, F., Steinhauser, C., Gray, W.P., 2003. Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis. Eur. J. Neurosci. 18, 2769–2778.

Imura, T., Nakano, I., Kornblum, H.I., Sofroniew, M.V., 2006. Phenotypic and functional heterogeneity of GFAP-expressing cells in vitro: differential expression of LeX/CD15 by GFAP-expressing multipotent neural stem cells and non-neurogenic astrocytes. Glia 53, 277–293.

Jessberger, S., Romer, B., Babu, H., Kempermann, G., 2005. Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. Exp. Neurol. 196, 342–351.

Kaplan, M.S., Hinds, J.W., 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197, 1092–1094.

Kopp, J., Xu, Z.Q., Zhang, X., Pedrazzini, T., Herzog, H., Kresse, A., Wong, H., Walsh, J.H., Hokfelt, T., 2002. Expression of the neuropeptide Y Y_1 receptor in the CNS of rat and of wild-type and Y_1 receptor knock-out mice. Focus on immunohistochemical localization(1). Neuroscience 111, 443–532.

Kronenberg, G., Reuter, K., Steiner, B., Brandt, M.D., Jessberger, S., Yamaguchi, M., Kempermann, G., 2003. Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. J. Comp. Neurol. 467, 455–463.

Lee, M.K., Rebhun, L.I., Frankfurter, A., 1990. Posttranslational modification of class III beta-tubulin. Proc. Natl. Acad. Sci. U. S. A. 87, 7195–7199.

Lois, C., Alvarez-Buylla, A., 1994. Long-distance neuronal migration in the adult mammalian brain. Science 264, 1145–1148.

Luskin, M.B., 1993. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11, 173–189.

Maekawa, M., Takashima, N., Arai, Y., Nomura, T., Inokuchi, K., Yuasa, S., Osumi, N., 2005. Pax6 is required for production and maintenance of progenitor cells in postnatal hippocampal neurogenesis. Genes Cells 10, 1001–1014.

Marksteiner, J., Sperk, G., Maas, D., 1989. Differential increases in brain levels of neuropeptide Y and vasoactive intestinal polypeptide after kainic acid-induced seizures in the rat. Naunyn-Schmiedeberg's Arch. Pharmacol. 339, 173–177.

Mazarati, A., Lu, X., Kilk, K., Langel, U., Wasterlain, C., Bartfai, T., 2004. Galanin Type 2 receptors regulate neuronal survival, susceptibility to seizures and seizure-induced neurogenesis in the dentate gyrus. Eur. J. Neurosci. 19, 3235–3244.

Menn, B., Garcia-Verdugo, J.M., Yaschine, C., Gonzalez-Perez, O., Rowitch, D., Alvarez-Buylla, A., 2006. Origin of oligodendrocytes in the subventricular zone of the adult brain. J. Neurosci. 26, 7907–7918.

Mercer, A., Ronnholm, H., Holmberg, J., Lundh, H., Heidrich, J., Zachrisson, O., Ossoinak, A., Frisen, J., Patrone, C., 2004. PACAP promotes neural stem cell proliferation in adult mouse brain. J. Neurosci. Res. 76, 205–215.

Nakagawa, E., Aimi, Y., Yasuhara, O., Tooyama, I., Shimada, M., McGeer, P.L., Kimura, H., 2000. Enhancement of progenitor cell division in the dentate gyrus triggered by initial limbic seizures in rat models of epilepsy. Epilepsia 41, 10–18.

Namba, T., Mochizuki, H., Onodera, M., Mizuno, Y., Namiki, H., Seki, T., 2005. The fate of neural progenitor cells expressing astrocytic and radial glial markers in the postnatal rat dentate gyrus. Eur. J. Neurosci. 22, 1928–1941.

Navarro-Quiroga, I., Hernandez-Valdes, M., Lin, S.L., Naegele, J.R., 2006. Postnatal cellular contributions of the hippocampus subventricular zone to the dentate gyrus, corpus callosum, fimbria, and cerebral cortex. J. Comp. Neurol. 497, 833–845.

Ohta, S., Gregg, C., Weiss, S., 2006. Pituitary adenylate cyclase-activating

polypeptide regulates forebrain neural stem cells and neurogenesis in vitro and in vivo. J. Neurosci. Res. 84, 1177–1186.
Parent, J.M., Yu, T.W., Leibowitz, R.T., Geschwind, D.H., Sloviter, R.S., Lowenstein, D.H., 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J. Neurosci. 17, 3727–3738.
Parent, J.M., Valentin, V.V., Lowenstein, D.H., 2002. Prolonged seizures increase proliferating neuroblasts in the adult rat subventricular zone-olfactory bulb pathway. J. Neurosci. 22, 3174–3188.
Parent, J.M., von dem Bussche, N., Lowenstein, D.H., 2006. Prolonged seizures recruit caudal subventricular zone glial progenitors into the injured hippocampus. Hippocampus 16, 321–328.
Peretto, P., Giachino, C., Aimar, P., Fasolo, A., Bonfanti, L., 2005. Chain formation and glial tube assembly in the shift from neonatal to adult subventricular zone of the rodent forebrain. J. Comp. Neurol. 487, 407–427.
Pirttila, T.J., Lukasiuk, K., Hakansson, K., Grubb, A., Abrahamson, M., Pitkanen, A., 2005. Cystatin C modulates neurodegeneration and neurogenesis following status epilepticus in mouse. Neurobiol. Dis. 20, 241–253.
Pleasure, S.J., Collins, A.E., Lowenstein, D.H., 2000. Unique expression patterns of cell fate molecules delineate sequential stages of dentate gyrus development. J. Neurosci. 20, 6095–6105.
Radley, J.J., Jacobs, B.L., 2003. Pilocarpine-induced status epilepticus increases cell proliferation in the dentate gyrus of adult rats via a 5-HT1A receptor-dependent mechanism. Brain Res. 966, 1–12.
Rietze, R., Poulin, P., Weiss, S., 2000. Mitotically active cells that generate neurons and astrocytes are present in multiple regions of the adult mouse hippocampus. J. Comp. Neurol. 424, 397–408.
Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301, 805–809.
Scharfman, H.E., Gray, W.P., 2006. Plasticity of neuropeptide Y in the dentate gyrus after seizures, and its relevance to seizure-induced neurogenesis. Exs 193–211.
Scott, B.W., Wang, S., Burnham, W.M., De Boni, U., Wojtowicz, J.M., 1998. Kindling-induced neurogenesis in the dentate gyrus of the rat. Neurosci. Lett. 248, 73–76.
Seaberg, R.M., van der Kooy, D., 2002. Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. J. Neurosci. 22, 1784–1793.
Seaberg, R.M., Smukler, S.R., van der Kooy, D., 2005. Intrinsic differences distinguish transiently neurogenic progenitors from neural stem cells in the early postnatal brain. Dev. Biol. 278, 71–85.
Seri, B., Garcia-Verdugo, J.M., McEwen, B.S., Alvarez-Buylla, A., 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J. Neurosci. 21, 7153–7160.
Seri, B., Garcia-Verdugo, J.M., Collado-Morente, L., McEwen, B.S., Alvarez-Buylla, A., 2004. Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J. Comp. Neurol. 478, 359–378.
Seri, B., Herrera, D.G., Gritti, A., Ferron, S., Collado, L., Vescovi, A., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 2006. Composition and organization of the SCZ: a large germinal layer containing neural stem cells in the adult mammalian brain. Cereb. Cortex 16 (Suppl. 1), i103–i111.
Shimoda, Y., Tajima, Y., Osanai, T., Katsume, A., Kohara, M., Kudo, T., Narimatsu, H., Takashima, N., Ishii, Y., Nakamura, S., Osumi, N., Sanai, Y., 2002. Pax6 controls the expression of Lewis X epitope in the embryonic forebrain by regulating alpha 1,3-fucosyltransferase IX expression. J. Biol. Chem. 277, 2033–2039.
Shors, T.J., Miesegaes, G., Beylin, A., Zhao, M., Rydel, T., Gould, E., 2001. Neurogenesis in the adult is involved in the formation of trace memories. Nature 410, 372–376.
Tramontin, A.D., Garcia-Verdugo, J.M., Lim, D.A., Alvarez-Buylla, A., 2003. Postnatal development of radial glia and the ventricular zone (VZ): a continuum of the neural stem cell compartment. Cereb. Cortex 13, 580–587.
Tropepe, V., Coles, B.L., Chiasson, B.J., Horsford, D.J., Elia, A.J., McInnes, R.R., van der Kooy, D., 2000. Retinal stem cells in the adult mammalian eye. Science 287, 2032–2036.
van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., Gage, F. H., 2002. Functional neurogenesis in the adult hippocampus. Nature 415, 1030–1034.
Wei, L.C., Shi, M., Chen, L.W., Cao, R., Zhang, P., Chan, Y.S., 2002. Nestin-containing cells express glial fibrillary acidic protein in the proliferative regions of central nervous system of postnatal developing and adult mice. Brain Res. Dev. Brain Res. 139, 9–17.
Yoshimura, S., Takagi, Y., Harada, J., Teramoto, T., Thomas, S.S., Waeber, C., Bakowska, J.C., Breakefield, X.O., Moskowitz, M.A., 2001. FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. Proc. Natl. Acad. Sci. U. S. A. 98, 5874–5879.
Zucchini, S., Barbieri, M., Simonato, M., 2005. Alterations in seizure susceptibility and in seizure-induced plasticity after pharmacologic and genetic manipulation of the fibroblast growth factor-2 system. Epilepsia 46 (Suppl. 5), 52–58.