Journal of Neuroscience Research

Journal of Neuroscience Research 90:1126–1135 (2012)

Neuropeptide Y and Peptide YY Have Distinct Roles in Adult Mouse Olfactory Neurogenesis

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Neuropeptide Y (NPY) and peptide YY (PYY) are differentially expressed throughout the olfactory neuroepithelium (ON), with NPY expression present in sustentacular cells, olfactory ensheathing cells, and olfactory receptor neurons and PYY expressed only in sustentacular cells. Examination of the anatomical morphology of the ON in NPY knockout ($NPY^{-/-}$) and PYY knockout ($PYY^{-/-}$) mice shows that there are significantly more neurons in $PYY^{-/-}$ mice and significantly fewer neurons in $NPY^{-/-}$ mice. Interestingly, the mature neurons of $NPY^{-/-}$ mice were undergoing apoptosis. The transcription factor Mash1, which is critical in the production of olfactory precursors, is also differentially expressed in $NPY^{-/-}$ and $PYY^{-/-}$ ON. It is upregulated in the neurons of $NPY^{-/-}$ mice and unchanged in $PYY^{-/-}$ mice. Furthermore, significantly fewer olfactory neurospheres are present in cultures prepared from $PYY^{-/-}$ mice in the first 2 weeks compared with $NPY^{-/-}$ and wild-type mice. Together these results suggest that, during olfactory neurogenesis, NPY acts as a trophic factor for the maturation and survival of olfactory receptor neurons, whereas PYY has an important role in the regulation of olfactory neuron differentiation.

Key words: neuropeptide Y; peptide YY; olfactory neuroepithelium; olfactory precursors

Olfactory neurogenesis occurs throughout adulthood and is possible because of the proliferation and differentiation of multipotent precursor cells adjacent to the basement membrane of the olfactory neuroepithelium (ON) (Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 1991). The inner basal layer of the ON is composed of two cell types, horizontal (HBC) and globose (GBC) basal cells (Graziadei and Graziadei, 1979). Precursor cells isolated from the ON possess the ability to differentiate into either mature olfactory receptor neurons (ORNs) or nonneuronal supporting cells (Huard et al., 1998).

The regenerative capacity of the ON has been studied extensively (Schwob, 2002). Less is known, however, about the genetic and molecular events and local environmental trophic influences that control olfactory stem cell activity. Both GBCs and HBCs have been identified as olfactory precursor cells that generate ORNs and nonneuronal cells (Leung et al., 2007; Iwai et al., 2008). Specifically, HBCs are capable of giving rise to ORNs and nonneuronal cells following injury and during normal neuronal turnover (Leung et al., 2007; Iwai et al., 2008), whereas GBCs are sufficient for ORN generation during normal neuronal turnover (Leung et al., 2007).

The development of the ON is under transcriptional control. One of the key regulators in the control of neuronal differentiation in the ON involves Mash1 (Guillemot et al., 1993; Cau et al., 1997, 2002). The mouse Mash1 gene encodes a transcription factor expressed in neural precursors. In the ON of mice with a null mutation of the gene, olfactory precursors are not produced, although nonneuronal supporting cells are present (Guillemot et al., 1993). Further analysis of the olfactory pathway in these mice reveals a generalized loss of ORNs (Guillemot et al., 1993).

The identification of factors that promote neurogenesis within the ON can provide clues to the general process of mammalian nervous system repair. Neuropeptide Y (NPY) is expressed at high levels in the olfactory system (Ubink and Hökfelt, 2000; Hansel et al., 2001; Montani et al., 2006). Previous studies suggest that NPY regulates neuroproliferation of olfactory, hippocampal, and subventricular zone precursor cells via the Y1 and Y2 receptors (Hansel et al., 2001; Howell et al., 2003, 2005; Doyle et al., 2008; Stanic et al., 2008). Mice with a targeted deletion of NPY (Erickson et al., 1996) con-

Contract grant sponsors: Mason Foundation, Rosemary Pryor Trust, Perpetual Trust, NHMRC of Australia; Contract grant number: 427606 (to H.H.).

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Received 25 September 2011; Revised 22 November 2011; Accepted 23 November 2011

Published online 22 February 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23008

tain only half as many dividing olfactory neuronal precursor cells and significantly fewer postmitotic ORNs compared with control animals (Hansel et al., 2001). Another member of this family of peptides is peptide YY (PYY), which shares extensive sequence homology with NPY (Renshaw and Batterham, 2005) and is also expressed in neurons, though to a lesser extent (Boey et al., 2007). More specifically, PYY is expressed in neurons of the paraventricular, arcuate, and supraoptic nuclei of the human hypothalamus (Morimoto et al., 2008).

We have used mouse models with both NPY and PYY knockouts to study the effect of their absence on olfactory neurogenesis. In vitro neuroproliferation studies were performed on olfactory neurospheres derived from wild-type (WT) and NPY and PYY knockout mice.

MATERIALS AND METHODS

Animals

Experiments were carried out on WT (C57/BL6 SvJ) mice bred and housed in the Biological Testing Facility at the Garvan Institute of Medical Research. The germline NPY ($NPY^{-/-}$), PYY ($PYY^{-/-}$), and NPYPYY ($NPYPYY^{-/-}$) knockout mice used in the experiments have been described previously (Boey et al., 2006; Karl et al., 2008) and were also C57/BL6 SvJ mice. Male and female mice of approximately 6 weeks of age were held in holding rooms with 12-hr light/dark cycles, were allowed access to food and water ad libitum, and were free of microbiological pathogens. All animal experiments were approved by the Garvan and St.Vincent's Animal Experimentation Ethics Committee and conducted in accordance with National Health and Medical Research Council of Australia guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

Immunochemistry

Adult WT (n = 6 mice), $NPY^{-/-}$ (n = 5 mice), $PYY^{-/-}$ (n = 5), and $NPYPYY^{-/-}$ (n = 3) mice aged approximately 6 weeks were given an overdose of intraperitoneal Euthal (80 mg/kg) and transcardially perfused with ice-cold normal saline, followed by 4% paraformaldehyde (PFA; ProSciTech, Thuringowa, Australia). Olfactory tissue was dissected and processed for paraffin embedding. Cross-sections of ON (6 μm) were collected onto electrostatic glass slides (Menzel-Gläser, Braunschweig, Germany). Sections were dewaxed in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated through a graded series of alcohols.

Nonspecific staining was blocked in 10% serum in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hr. This was followed by incubation with the primary antibodies: anti-PYY, rabbit, 1:1,200 (Abcam, Cambridge, MA); anti-OMP, goat, 1:1,000 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Mash1, rabbit, 1:10 (Chemicon, Temecula CA); anti-caspase3, rabbit, 1:20 (Abcam); anti-βIII-tubulin, mouse, 1:500 (Promega, Madison, WI); and anti-keratin, goat, 1:400 (Abcam) for 1 hr at room temperature or overnight at 4°C. Control sections were incubated with 1% BSA and processed in parallel. For immunoperoxidase studies, sections were washed in PBS in 0.3% H_2O_2 for 15 min. After washing, sections were incubated with biotinylated goat anti-rabbit, horse anti-mouse and rabbit anti-goat secondary antibodies (Vector Laboratories, Burlingame, CA) at a concentration of 1:300 for 30 min at RT. Avidin-HRP complex was prepared using the Vector ABC Elite kit according to the manufacturer's recommendations. Immunoreactivity was visualized by using DAB as the chromogen and mounted using Aquamount (BDH, Poole, England). Images were captured with a Leica DC480 (Leica Camera AG, Solms, Germany) and Zeiss AxioCam (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) digital camera attached to a Zeiss Axiophot microscope.

Double-labelling immunochemistry was performed using the primary antibody combinations of anti-caspase3 and anti-OMP and anti-Mash1 and anti-βIII-tubulin and anti-Mash1 and anti-keratin. Cells were permeabilized in 0.1% Triton X-100 for 10 min and subsequently blocked in 1% BSA PBS for 1 hr, followed by incubation with primary antibodies for 1 hr. This was followed by secondary antibody incubation for 30 min with donkey anti-rabbit (Cy3; 1:200; Abacus ALS Pty Ltd.), donkey anti-goat (Alexa Fluor 488; 1:50; Molecular Probes, Eugene, OR), and goat anti-mouse (Alexa Fluor 488; 1:50; Molecular Probes). Slides were mounted in Dako fluorescent mounting media (Dako, Carpinteria, CA), and images were captured wth the Leica DC480 digital camera attached to a Zeiss Axiophot microscope.

Detection of Apoptosis Using a TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) measures fragmented DNA by incorporating fluorescein-12-dUTP at 3′-OH DNA ends, using the terminal deoxynucleotidyl transferase enzyme. The TUNEL assay was performed with the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Briefly, tissue was hydrated and permeabilized with proteinase K (20 μg/ml) digestion for 8 min at room temperature. No further PFA fixation was performed. Tissue was incubated for 1 hr at 37°C with terminal deoxynucleotidyl transferase (recombinant) and fluorescein-12-dUTP and reaction stopped by incubating slides in 2× SSC for 15 min. Cell nuclei counterstaining was performed with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA).

Quantification of ORNs, Mash1-Positive Cells, and Caspase3-Positive Cells in the Adult Mouse ON

ORNs stained with the anti-OMP antibody were quantified in WT (n = 4), $NPY^{-/-}$ (n = 5), and $PYY^{-/-}$ (n = 5) mice. Random fields of 120 μm^2 in nonadjacent sections were chosen, and immunopositive cells were counted (WT, n = 69; $NPY^{-/-}$, n = 82; $PYY^{-/-}$, n = 65). The numbers of neuronal, sustentacular, and horizontal basal cell bodies that expressed the transcription factor Mash1 were analyzed in WT (n = 3), $NPY^{-/-}$ (n = 4), and $PYY^{-/-}$ (n = 5) mice. Random fields of 180 μm^2 were chosen to quantify the total number of Mash1-positive neuronal nuclei (WT, n = 10; $NPY^{-/-}$, n = 30; $PYY^{-/-}$, n = 27), sustentacular cell nuclei (WT, n = 10; $NPY^{-/-}$, n = 30; $PYY^{-/-}$, n = 27), and

TABLE I. Sequences of Primers Used for RT-PCR of Transcription Factors in the Mouse ON Tissue

Gene		Sequence (5′ to 3′)	Temperature (°C)	Size (bp)
GAPDH	Forward	ACTTTGTCAAGCTCATTTCC	57	269
	Reverse	TGCAGCGAACTTTATTGATG		
Msi1	Forward	AAAGAGTGTCTGGTGATGCGGGAC	58	307
	Reverse	GCCTGTTGGTGGTTTTGTCG		
Mash1	Forward	GCCAACAAGAAGATGAGCAAGGTG	60	317
	Reverse	ATCTCCTGCCATCCTGCTTCCA		
Pax6	Forward	ATACCTACACCCCTCCGCACAT	56	268
	Reverse	CAGCCATTTCTCTTTCTTTCCTGA		
Wnt3a	Forward	TCACAACAATGAGGCTGGGC	59	304
	Reverse	TTCGGGGTTAGGTTCGCAGAAG		
Hes1	Forward	CACGCTCGGGTCTGTGCTGAGAGC	57	307
	Reverse	ATGCCAGCTGATATAATGGAG		

horizontal basal cell nuclei (WT, n = 10; $NPY^{-/-}$, n = 30; $PYY^{-/-}$, n = 27). The total number of sustentacular and horizontal basal cell nuclei was also calculated in sections immunolabelled with Mash1 and counterstained with hematoxylin in WT and knockout mice. Analysis of the number of caspase3-positive cells was performed in WT (n = 3), $NPY^{-/-}$ (n = 3), and $PYY^{-/-}$ (n = 3) mice. Statistical analysis of the data was performed with a one-way ANOVA and post hoc (Dunnet's) test in Graph Pad Prism 5.0 software. $P < 0.05$ was considered significant.

Primary Adult Olfactory Precursor Cells

To isolate olfactory precursor cells, olfactory turbinates were dissected from up to 30 adult WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice aged approximately 6 weeks. Mice were anesthetized with CO_2 and decapitated, and olfactory turbinates were removed and placed in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 9.6 mg/ml HEPES buffer (Sigma, St. Louis, MO). Tissue was centrifuged at 1,000*g*, and supernatant was removed before placing minced tissue into DMEM containing 1% (w/v) BSA (Sigma), 50 μg/ml DNase (Sigma), 1 mg/ml hyaluronidase (Sigma), 1 mg/ml collagenase (Roche Diagnostics, GmbH Mannheim, Germany), and 3 mg/ml dispase (Roche Diagnostics, Indianapolis, IN) for 1 hr at 37°C. Tissue suspension was triturated, filtered (150-μm wire mesh; Small Parts Inc., Miami Lakes, FL), centrifuged at 1,000*g*, and resuspended in Neurobasal medium (Invitrogen) containing 10% fetal calf serum (Invitrogen), 1,000 U/ml penicillin G (Sigma), 50 μg/ml gentamycin sulfate (Sigma), 20 mM glutamine (Invitrogen), and 2.5 mg/ml Amphostat B (ThermoElectron, Melbourne, Australia). Cells were filtered twice more through a 40-μm nylon mesh filter (BD Falcon, Bedford, MA) to remove olfactory neurons and ensheathing glia, and subsequently the olfactory precursor cells were collected on a 11-μm nylon mesh filter (Australian Filter Specialists, Huntingwood, Australia). Cultures were grown at 1×10^5 cells/ml at 37°C in 5% CO_2 in Neurobasal medium containing B27 supplement (instead of fetal calf serum; Invitrogen). Fibroblast growth factor-2 (20 ng/ml; Promega) and epidermal growth factor (20 ng/ml; Promega, Madison, WI) were added for neurosphere quantification experiments. Medium was not changed for the duration of the experiments.

Quantification of Olfactory Neurospheres

Olfactory neurospheres were grown on two-well Labtek tissue culture chamber slides (Nalge Nunc, Rochester, NY). The numbers of neurospheres derived from WT (n = 3 experiments), $NPY^{-/-}$ (n = 3 experiments), and $PYY^{-/-}$ (n = 3 experiments) mice were counted at 1, 2, and 3 weeks of continuous culturing and are expressed per well (2 cm^2). Statistical analysis of the data was performed with Fisher's PLSD ANOVA test in Statview software (SAS, Cary, NC). $P < 0.05$ was considered significant. After 2–3 weeks in vitro, the differentiation of ONs was assessed, and the percentage of Mash1-positive cells double labelled with βIII-tubulin and keratin was calculated in $NPY^{-/-}$ and $PYY^{-/-}$ cultures.

Reverse Transcription-PCR

RNA extractions were carried out on the ONs of adult mice using Trizol reagent (Sigma) according to the manufacturer's instructions. RNA was quantified by spectrophotometry, and cDNA was synthesized from 1 μg of total RNA with random hexamers by using the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Reverse transcription-PCR was then performed with TaqDNA polymerase (Amplitaq Gold DNA polymerase; Applied Biosystems, Foster City, CA) using 2 μl cDNA per 25 μl total volume. PCRs were performed for 35 cycles with denaturing at 94°C and extension at 72°C. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to control for variations between samples. The specific primers and annealing temperatures used along with the resultant product sizes obtained are as detailed in Table I.

RESULTS

PYY Expression in the ON

Sections of mouse ON from WT, $NPY^{-/-}$, $PYY^{-/-}$, and $NPYPYY^{-/-}$ knockout mice were collected to analyze the expression of PYY. Because of sequence similarities and cross-reactivity with NPY,

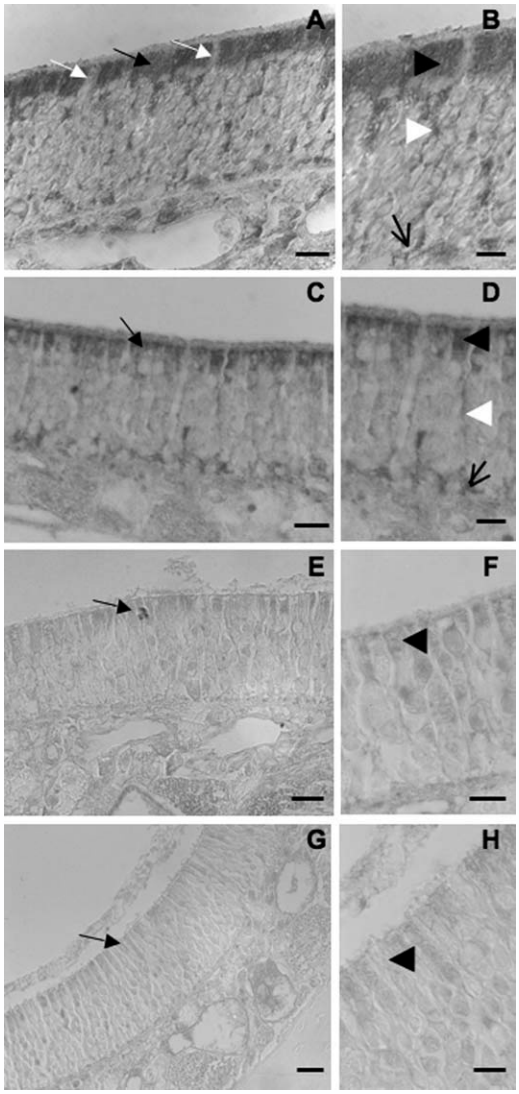

Fig. 1. Photomicrographs of PYY expression in the ON of WT, $NPY^{-/-}$, $PYY^{-/-}$, and $NPYPYY^{-/-}$ mice. **A:** PYY is expressed in the cell soma of sustentacular cells in the ON of WT mice (black arrow) but not microvillar cells (white arrows). **B:** High-magnification image showing PYY in the cytoplasm of sustentacular cells (black arrowhead), sustentacular cell process (white arrowhead), and sustentacular endfeet (open arrow). **C:** PYY is expressed in the cell soma of sustentacular cells in $NPY^{-/-}$ mice (black arrow). **D:** In the higher magnification image, PYY is expressed by the sustentacular cell soma (black arrowhead), process (white arrowhead), and endfeet (open arrow) in $NPY^{-/-}$ mice. **E,F:** PYY expression is decreased in the sustentacular cells of $PYY^{-/-}$ mice (black arrow and arrowhead) and is absent from sustentacular processes and endfeet (D). **G,H:** PYY expression is not present in the cell soma of sustentacular cells of $NPYPYY^{-/-}$ mice (black arrow), and the absence of PYY is more clearly seen at higher magnification (black arrowhead). Scale bars = 20 μm in A,C,E,G; 10 μm in B,D,F,H.

$NPYPYY^{-/-}$ mice were used to verify the specificity of the antibody for PYY. Using an anti-PYY polyclonal antibody, immunohistochemical analysis showed staining of the cell soma, processes, and endfeet of sustentacular cells in WT and $NPY^{-/-}$ mice (Fig. 1A–D). Microvillar cells were not labelled positively with anti-PYY. Importantly, the antibody staining strongly decreases in $PYY^{-/-}$ mice (Fig. 1E,F) and was not evident in $NPYPYY^{-/-}$ mice (Fig. 1G,H), confirming the presence of specific PYY-expressing sustentacular cells in WT and $NPY^{-/-}$ tissue.

Neuronal and Nonneuronal Quantification of WT, $NPY^{-/-}$, and $PYY^{-/-}$ Mice

ON sections from WT (n = 4), $NPY^{-/-}$ (n = 5), and $PYY^{-/-}$ (n = 5) mice were collected for neuronal morphology and quantification analysis. The antibody marker for mature ORNs, olfactory marker protein (OMP), was used for analysis. OMP expression was seen in the cell soma of ORNs as well as the axon bundles in each of the animals examined (Fig. 2A–F). The immunohistochemical analysis of anti-OMP in WT mice shows avid immunoreactivity of the ORN cell bodies that span the ON (Fig. 2A,B). The cellular morphology and size of the ORNs remains unchanged when comparing WT with $NPY^{-/-}$ and $PYY^{-/-}$ mice. This is best seen in the higher magnification photomicrographs (Fig. 2B,D,F). The scatterplot in Figure 2G shows significantly greater numbers of OMP-positive ORNs are present in $PYY^{-/-}$ ($P < 0.05$) mice compared with WT controls. However, there are significantly fewer OMP-positive ORNs in $NPY^{-/-}$ ($P < 0.05$) mice compared with WT controls (Fig. 2G). There was a wide range in the number of OMP-positive cells in each of the random fields. This is due to the changing thickness of the neuronal layer of the ON across the section. There was no significant difference in the total numbers of sustentacular cells (WT 22.8 ± 1.1, $NPY^{-/-}$ 23.6 ± 0.9, $PYY^{-/-}$ 22 ± 1.45) or HBCs (WT 15.8 ± 0.65, $NPY^{-/-}$ 18.4 ± 0.75, $PYY^{-/-}$ 16.4 ± 2) when comparing WT controls with $NPY^{-/-}$ and $PYY^{-/-}$ mice.

Mash1 Expression and Quantification Comparing WT, $NPY^{-/-}$, and $PYY^{-/-}$ Mice

The expression of Mash1 was studied in ON sections from WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice. Mash1 was expressed in the cell soma of neurons, sustentacular cells, horizontal basal cells of the mouse ON, and olfactory ensheathing cells in the lamina propria (Fig. 3A,B). The expression pattern shown in Figure 3A is a WT control section. The nuclei of most, but not all, sustentacular cells show Mash1 immunoreactivity. This is also the case for ORNs and horizontal basal cells. The expression pattern was the same for $NPY^{-/-}$ mice. However, there appeared to be a greater number of Mash1-positive cells in the $NPY^{-/-}$ mice. Subsequently, quantification was performed to analyze the number of Mash1-positive neuronal, sustentacular, and horizontal basal cells in WT control and knockout mice. The histogram in Figure 3C shows a significantly greater number of Mash1-positive neuronal nuclei in $NPY^{-/-}$ ($P < 0.05$) mice. There was no significant difference

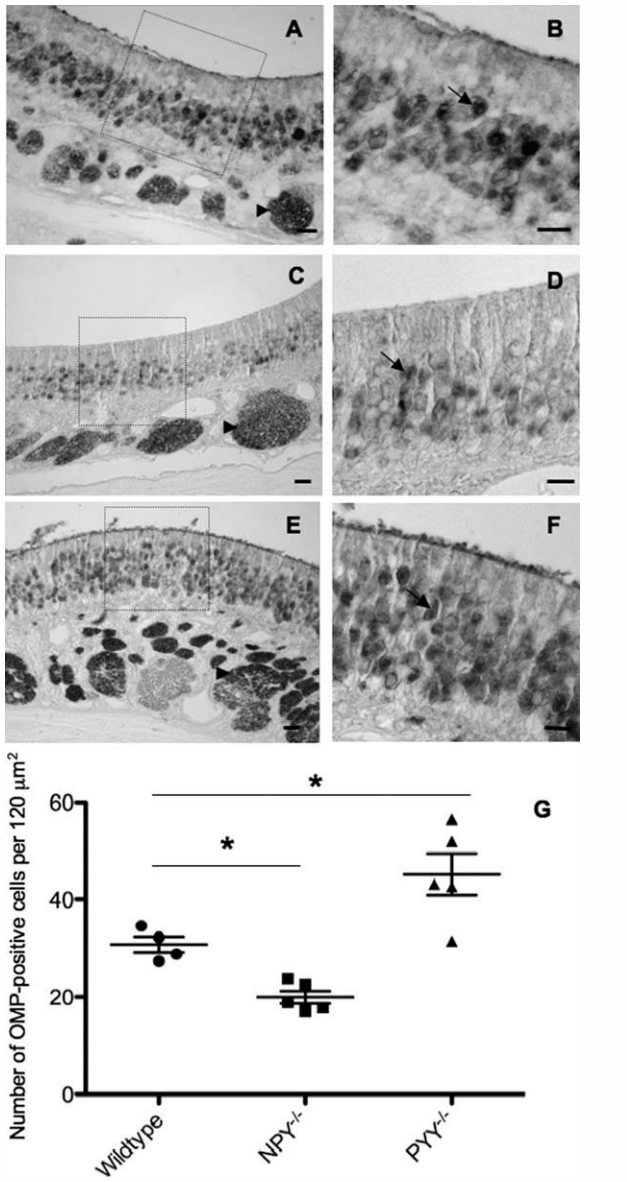

Fig. 2. Morphological analyses and quantification of ORNs in WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice. **A:** OMP immunoreactivity is present in the cell soma of ORNs and axon bundles (arrowhead) in WT mice. **B:** Higher magnification photomicrograph shows avid immunoreactivity of OMP in the cell soma of ORNs throughout the ON (arrow). **C:** The pattern of immunoreactivity of OMP in $NPY^{-/-}$ mice is the same as for wild-type mice, with no notable alterations in expression patterns or cell size. **D:** This can be seen more clearly in a higher magnification photomicrograph. **E,F:** In $PYY^{-/-}$ mice, OMP expression is in the cell soma of ORNs (arrow) and axon bundles (arrowhead). **G:** OMP-positive neurons were quantified per animal from WT (n = 4 mice), $NPY^{-/-}$ (n = 5 mice), and $PYY^{-/-}$ (n = 5 mice). The results show that there are significantly more OMP-positive neurons in $PYY^{-/-}$ mice and significantly fewer OMP-positive neurons in $NPY^{-/-}$ mice compared with WT controls. $^*P < 0.05$ was considered significant. Area within dashed lines (A,C,E) is 120 μm^2. Scale bars = 20 μm in A,C,E; 10 μm in B,D,F.

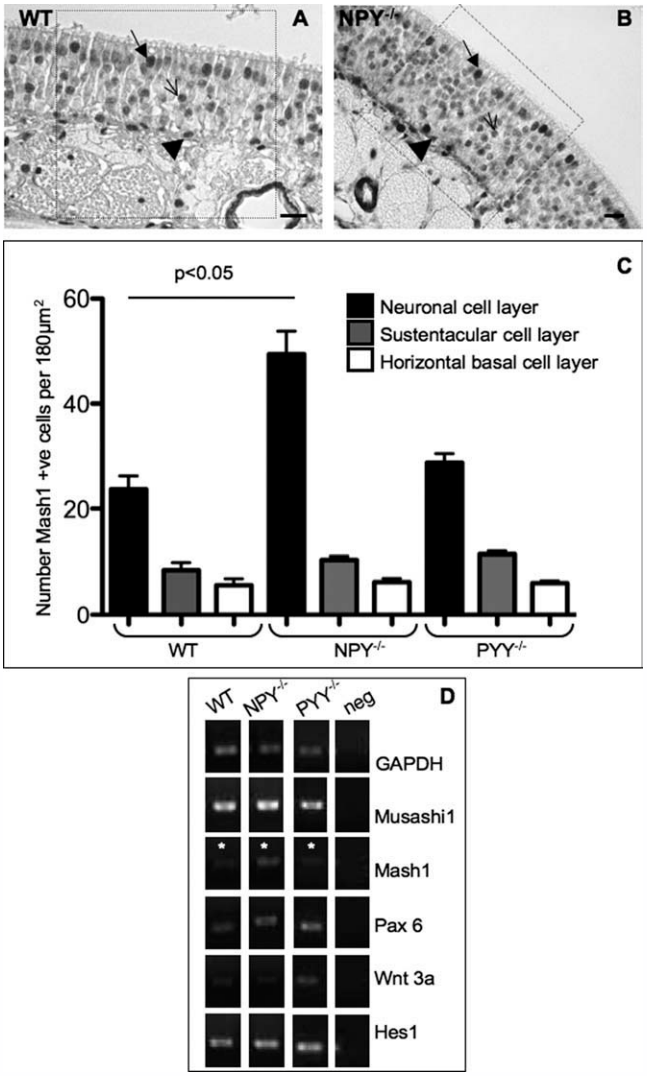

Fig. 3. Morphological analyses and quantification of Mash1-positive nuclei in horizontal basal cells, sustentacular cells and neurons of WT, $NPY^{-/-}$ and $PYY^{-/-}$ mice. **A:** Mash1 is present in the nuclei of horizontal basal cells (arrowhead), neurons (open arrow) and sustentacular cells (solid arrow) of WT mice. **B:** The same expression pattern is evident in $NPY^{-/-}$ mice compared with WT control. **C:** The results in the histogram show that there were significantly more Mash1-positive neuronal nuclei in $NPY^{-/-}$ compared with WT controls. There was no significant difference of Mash1-positive HBCs or sustentacular cells in $NPY^{-/-}$ and $PYY^{-/-}$ mice compared with WT controls. **D:** RT-PCR analysis of Musashi1, Mash1, Pax6, Wnt3a, and Hes1 shows expression in the ON of WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice. Mash1 expression is faint in all tissues from these mice, but in the ON Mash1 is more highly expressed in $NPY^{-/-}$ mice (asterisks). $P < 0.05$ was considered significant. Area within dashed lines is 180 μm^2. Scale bars = 20 μm.

when comparing the Mash1-positive sustentacular cells or HBCs from WT and knockout mice. To analyze the broader regulation of transcription during adult neurogenesis, RT-PCR of Musashi1, Mash1, Pax6, Wnt3a, and Hes1 was performed on the ON from adult WT,

$NPY^{-/-}$, and $PYY^{-/-}$ tissue. The results showed that Musashi1, Pax6, Hes1, and Wnt3a were all expressed in the ON. Interestingly, Mash1 expression is greater in $NPY^{-/-}$ compared with WT and $PYY^{-/-}$ mice (Fig. 3D).

Analysis of Apoptosis in the ON of Adult WT, $NPY^{-/-}$, and $PYY^{-/-}$ Mice

Apoptosis was detected in the ON of adult mice by using an anti-caspase3 antibody and a TUNEL assay. Figure 4 shows the pattern of caspase3 immunoreactivity and TUNEL-positive cells in WT and knockout mice. A differential pattern of caspase3-positive immunoreactivity in the cell soma of cells within the ON was seen when comparing WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice. Immunolabelling for caspase3 was evident in cells adjacent to the basement membrane (Fig. 4A) and cells in the globose basal cell layer (Fig. 4A) in WT mice. $PYY^{-/-}$ mice also showed caspase3-positive cells in the cells adjacent to the basal lamina and the globose basal cell layer of the ON (Fig. 4C). Caspase3 labelling of $NPY^{-/-}$ ON extended to include the neuronal layer (open arrow; Fig. 4E), and some OMP-positive neurons are shown to colocalize with caspase3 (open arrow, Fig. 4G). The scatterplot in Figure 4H indicates that there is a significantly greater number of caspase3-positive cells in $NPY^{-/-}$ compared with $PYY^{-/-}$ mice. A similar pattern of TUNEL-positive cells was evident in the WT and knockout mice (Fig. 4B,D,F). In WT mice, the TUNEL-positive nuclei appeared in the basal layer of the ON (Fig, 4). This was also the case in the $PYY^{-/-}$ mice (Fig, 4); however, there were also some TUNEL-positive nuclei positioned apically above the sustentacular cell layer (Fig. 4D). In $NPY^{-/-}$ mice, the TUNEL-positive nuclei were positioned within the neuronal layer of the ON (Fig, 4F) as well as the basal layer (white arrow, Fig. 4F).

Characterization of Neurosphere Proliferation in WT, $NPY^{-/-}$, and $PYY^{-/-}$ Mice

To gain further insight into the process of neuroproliferation, olfactory neurosphere cultures were prepared from WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice. The number of olfactory neurospheres was quantified at 1, 2, and 3 weeks in culture. The neurospheres derived from WT mice increased in number over 3 weeks, whereas the neurospheres derived from $NPY^{-/-}$ ($P < 0.05$) and $PYY^{-/-}$ ($P < 0.0001$) mice decreased significantly over this time. Significantly fewer neurospheres were present in culture at week 1 from $PYY^{-/-}$ mice (Fig. 5A). After 2–3 weeks in culture, olfactory neurosphere cultures derived from $NPY^{-/-}$ and $PYY^{-/-}$ mice were analyzed via double-labelling immunocytochemistry. Mash1 (red) is expressed in the nuclei of the cells. In Figure 5B, 21% of the Mash1-expressing cells (red) also expressed βIII-tubulin (green), indicating differentiation into immature ORNs in $NPY^{-/-}$ cultures. Figure 5C shows that, in $PYY^{-/-}$ cultures, 34% of the Mash1-positive cells also express βIII-tubulin. The cultures derived from $NPY^{-/-}$ and $PYY^{-/-}$ mice were also double labelled with Mash1 and keratin, which labels supporting cells derived from olfactory neurospheres. In $NPY^{-/-}$ cultures, 2.7% of Mash1-positive cells were keratin-positive (Fig. 5D). Figure 5E shows that, in $PYY^{-/-}$ cultures, 100% of Mash1-positive cells were also keratin positive. This analysis was performed on cell cultures that were fixed after 2–3 weeks in vitro. The lack of neurospheres and amount of differentiation correspond to the histogram showing a reduction in neurosphere proliferation at this time.

DISCUSSION

NPY and PYY are the two major peptides of the NPY family. NPY has previously been reported to have neurogenic effects, whereas the actions of PYY have been described mostly in neuroendocrine processes. We describe here for the first time the actions of both NPY and PYY in olfactory neurogenesis. NPY is expressed in ensheathing cells prior to the maturation of ORNs or the formation of glomeruli and is therefore thought to be involved in guidance and growth of sensory axons to their target glomeruli (Ubink and Hökfelt, 2000). NPY acting via the Y1 and Y2 receptors helps regulate the proliferation of precursor cells in the subventricular zone and rostral migratory stream as well as influencing the differentiation of olfactory bulb interneurons (Stanic et al., 2008). It has also been shown to be involved in olfactory neurogenesis, particularly at the level of the olfactory precursor cells. Its function in the neuroproliferation of olfactory precursors was shown to occur via the Y1 receptor (Doyle et al., 2008). It is expressed in the mouse ON in the cell soma of sustentacular cells (Hansel et al., 2001; Doyle et al., 2008), neurons (Doyle et al., 2008), olfactory ensheathing glia (Ubink and Hökfelt, 2000), and microvillar cells (Montani et al., 2006). PYY is also expressed in the cell soma, processes, and endfeet of sustentacular cells but not microvillar cells as shown here. Both sustentacular cells and microvillar cells span the ON from the apical layer to the basal lamina. Therefore, a likely neurogenic function of both NPY and PYY is to link signals from the surface of the ON that promote proliferation and maintenance of HBCs situated along the basal lamina to promote neurogenesis subsequently.

The in vivo role of NPY and PYY was studied via cellular quantification and morphology in WT and knockout mice. We showed that there are significantly fewer mature olfactory neurons (OMP-positive) in the ON of $NPY^{-/-}$ mice compared with WT controls, whereas there were significantly greater numbers of mature olfactory neurons (OMP-positive) in $PYY^{-/-}$ mice. The numbers of other olfactory cell types (sustentacular cells and horizontal basal cells) were unchanged when comparing WT and knockout mice. These effects were not due to an upregulation of NPY or PYY in the knockout mice, NPY and PYY expression in $PYY^{-/-}$

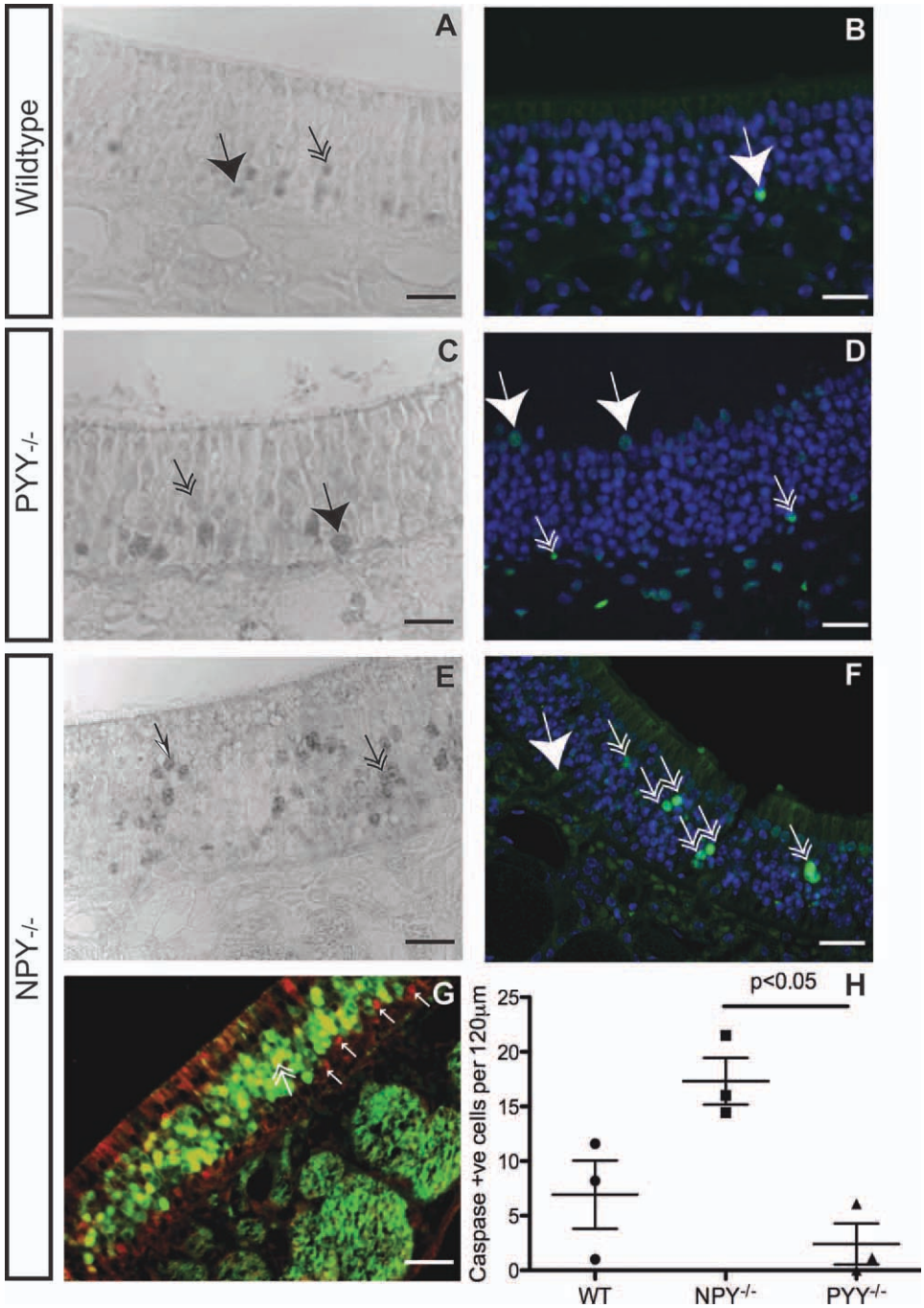

Fig. 4. Analysis of apoptosis in the mouse ON of WT, $NPY^{-/-}$, and $PYY^{-/-}$ mice. **A:** Caspase3 immunoreactivity is present in the nuclei of olfactory neuronal progenitors (black solid arrow) and immature olfactory neurons (black open arrow) in WT mice. **B:** TUNEL-positive nuclei are present in cells adjacent to the basal membrane (white arrow) **C:** The nuclei of immature neurons (black open arrow) and olfactory neuronal progenitors (black solid arrow) are caspase3-positive in $PYY^{-/-}$ mice. **D:** TUNEL-positive nuclei are present adjacent to the basal membrane (white open arrows) and apically above the sustentacular cell layer (white solid arrows). **E:** Nuclei of immature (open arrow) and mature neurons (black/white solid arrow) in $NPY^{-/-}$ are caspase3-positive. **F:** TUNEL-positive nuclei are present adjacent to the basal membrane (white solid arrow) as well as throughout the neuronal layer of the ON (white open arrows). **G:** Double labelling immunohistochemistry of caspase3 (red) and OMP (green) in $NPY^{-/-}$ mice showing caspase3-positive nuclei in immature neurons (white solid arrows) and some colocalisation of OMP-positive cells with caspase3 (yellow; white open arrow). **H:** Scatterplot of caspase3-positive cells shows that there are significantly greater numbers in $NPY^{-/-}$ compared with $PYY^{-/-}$ mice. $P < 0.05$ was considered significant. Scale bars = 20 µm.

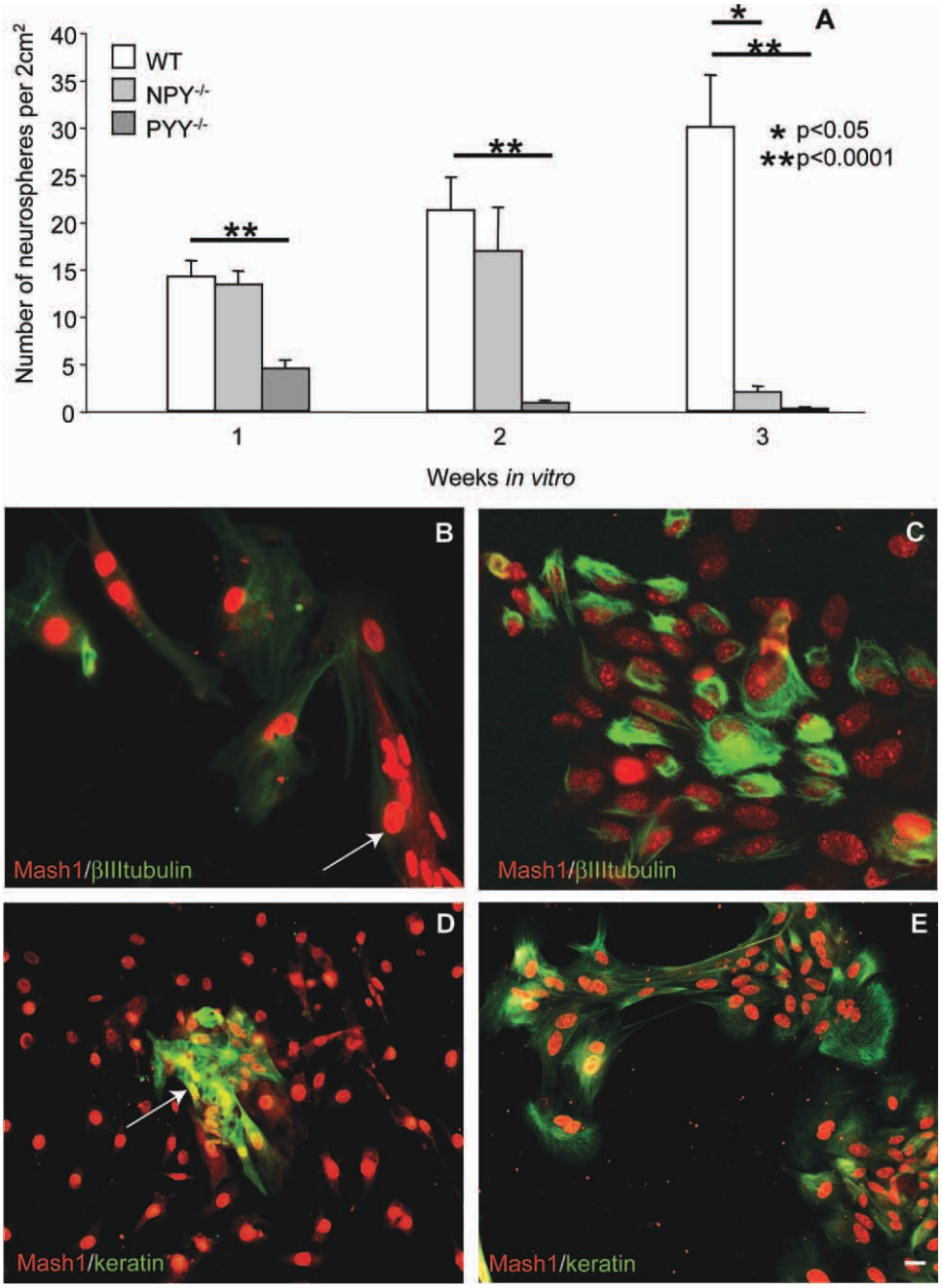

Fig. 5. Quantification of the number of neurospheres grown in vitro from WT, NPY$^{-/-}$, and PYY$^{-/-}$ mice. **A:** Histogram showing the number of neurospheres calculated at 1, 2, and 3 weeks in culture. The neurospheres derived from WT mice continue to increase in number over 3 weeks. The number of neurospheres from NPY$^{-/-}$ mice was equivalent to that in WT controls at week 1 but then decreased and was significantly reduced at week 3. PYY$^{-/-}$ mice produced significantly fewer neurospheres at week 1 in vitro, which also decreased in number up to week 3. $P < 0.05$ was considered significant. **B:** Cultures derived from NPY$^{-/-}$ mice showing Mash1-positive nuclei (red) and βIII-tubulin-positive cytoplasm in a subpopulation of cells (green). A cluster of Mash1-positive and βIII-tubulin-negative cells is also shown (arrow). **C:** Cultures derived from PYY$^{-/-}$ mice showing Mash1-positive nuclei (red) and βIII-tubulin-positive cytoplasm (green). **D:** Primary neurosphere cultures prepared from NPY$^{-/-}$ mice and grown for 17 days in vitro were labelled with anti-Mash1 (red) and antikeratin (green). A subpopulation of Mash1-positive cells was also keratin positive (arrow). **E:** PYY$^{-/-}$ cultures were stained following 3 weeks in vitro, and the cells were double labelled with Mash1 (red) and keratin (green). Scale bar = 10 μm in E (applies to D,E); 20 μm for B,C.

and $NPY^{-/-}$ mice, respectively, were not increased on RT-PCR analysis (results not shown). A previous study by Hansel et al. (2001) was able to show fewer olfactory receptor neurons in the ON of $NPY^{-/-}$ mice because of a reduction in olfactory precursor proliferation. This is also shown in the experiments presented here. In vitro experiments show there are fewer olfactory neurospheres and hence less precursor cell proliferation after 3 weeks in culture in $NPY^{-/-}$ mice. It is interesting that, in the first 22 weeks in vitro, neurospheres are formed and proliferate when derived from $NPY^{-/-}$ mice, but the number of neurospheres rapidly declines by week 3. In a previous study (Doyle et al., 2008), more differentiated cells types (neurons and nonneuronal cells) appeared in the $NPY^{-/-}$ cultures following 3 weeks in culture. Moreover, WT neurospheres gave rise to secondary neurospheres, whereas secondary neurospheres were not formed from $NPY^{-/-}$ mice (Doyle et al., 2008). There were also significantly fewer olfactory neurospheres in $PYY^{-/-}$ mice at 1, 2, and 3 weeks in culture. This is also the case in $NPYPYY^{-/-}$ mice (Doyle et al., 2008). It appears that, in the absence of PYY, neurospheres are less likely to proliferate and more likely to form differentiated cells (neurons and nonneuronal cells). Immunocytochemical analysis reveals that Mash1-positive cells in $PYY^{-/-}$ cultures are more likely to be βIII-tubulin-positive neurons and keratin-positive nonneuronal cells compared with $NPY^{-/-}$. We propose that, although precursor proliferation plays an important role in olfactory neurogenesis, there is another mechanism involving PYY in the growth and differentiation of ORNs in vivo and in vitro.

From the evidence shown here it becomes clear that NPY plays a role in the survival of ORNs. We performed analysis of cell death to elucidate the role of apoptosis on the differential regulation of NPY and PYY in the ON by using caspase3 and TUNEL analysis. Caspase3 is an effector caspase in neuronal apoptosis during development (Kuida et al., 1996). In the ON, caspase3 has been analyzed postbulbectomy and is expressed in the neuronal cell body after deafferentation (Cowan et al., 2001). In the animals studied (WT, $NPY^{-/-}$, $PYY^{-/-}$ mice), caspase3-positive cells are present in the globose basal cell layer of the ON. However, in $NPY^{-/-}$ mice, apoptotic cell bodies extend apically and were also present in the neuronal cell layer of the ON as shown by caspase3 immunoreactivity and the TUNEL assay. Therefore, the involvement of NPY, but not PYY, in olfactory neurogenesis is to prevent apoptosis of ORNs.

We investigated whether the differential roles of NPY and PYY in olfactory neurogenesis were due to transcription factor control. It is clear from the work of others that Mash1 (Guillemot et al., 1993), Ngn1 (Cau et al., 1997), Hes1 (Cau et al., 2002), Lhx2 (Hiroto and Mombaerts, 2004), Pax6 (Davis and Reed, 1996), and p63 (Packard et al., 2011) are essential for the genesis of olfactory neurons and supporting cells. Other transcription factors that are involved in neurogenesis are Msi1 (Okano et al., 2005; Watanabe et al., 2007) and Wnt3a (Otero et al., 2004). Msi1 and Wnt3a are expressed in the soma of ORNs in the adult mouse ON (data not shown) and may therefore be involved in the olfactory neurogenic process. Examination of transcription factors by RT-PCR showed they were present in the adult ON. Notably, there were differential levels of expression of Mash1 in the adult mouse ON in $NPY^{-/-}$ and $PYY^{-/-}$ mice, with an increase in Mash1 expression in $NPY^{-/-}$ compared with WT control. This agrees with the data showing a significantly greater number of Mash1-positive cells in the neuronal layer of the ON in $NPY^{-/-}$ mice compared with WT. This was not the case in the $PYY^{-/-}$ mice. It is possible that Mash1 continues to be expressed in the neuronal layer of the ON in $NPY^{-/-}$ mice as a consequence of the olfactory neurons having not reached maturity.

NPY (Doyle et al., 2008)- and PYY-expressing sustentacular cells also express Mash1 in the nucleus. One possible role for these peptides in sustentacular cells is to link environmental signals on the epithelial surface to the basement membrane of the ON, where olfactory precursors reside. The developmental origin of the sustentacular cell is still not fully understood. Sustentacular cells appear to have glial cell characteristics, but there is still the question of whether they share a common lineage with ORNs. It is possible that during embryogenesis (Beites et al., 2005) or following injury (Goldstein and Schwob, 1996; Chen et al., 2004) olfactory progenitors are bipotential and can differentiate into neurons or glia, but this may become restricted in adulthood. Therefore, during adult olfactory neurogenesis, which was studied here, the Mash1-positive sustentacular cells in $NPY^{-/-}$ and $PYY^{-/-}$ mice are unlikely to be playing a role in the neurogenic process.

This study shows that, in mice with no PYY, there are more olfactory neurons in vivo as well as more differentiation rather than neuroproliferation of olfactory precursors in vitro. This suggests that PYY plays a role in the regulation of olfactory neurogenesis, but its mechanism is different from the role of NPY during olfactory neurogenesis, which is to have trophic effects on the survival and maturation of olfactory neurons in the adult mouse ON.

ACKNOWLEDGMENTS

The authors thank Ms. Tanya Wyatt for her technical assistance in the cutting of wax-embedded sections. The authors indicate no potential conflicts of interest.

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