JOURNAL OF NEUROCHEMISTRY | 2008 | 105 | 641–652 doi: 10.1111/j.1471-4159.2007.05188.x

Y1 receptors are critical for the proliferation of adult mouse precursor cells in the olfactory neuroepithelium

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Abstract

While the regenerative capacity of the olfactory neuroepithelium has been well studied less is known about the molecular events controlling precursor cell activity. Neuropeptide Y (NPY) is expressed at high levels in the olfactory system, and NPY has been shown to play a role in neuroregeneration of the brain. In this study, we show that the numbers of olfactory neurospheres derived from NPY, NPY/peptide YY, and Y1 receptor knockout mice are decreased compared with wild type (WT) controls. Furthermore, flow cytometric analysis of isolated horizontal basal cells, globose basal cells, and glandular cells showed that only glandular cells derived from WT mice, but not from NPY and Y1 receptor knockout mice, formed secondary neurospheres suggesting a critical role for NPY signaling in this process. Interestingly, olfactory function tests revealed that olfaction in Y1 knockout mice is impaired compared with those of WT mice, probably because of the reduced number of olfactory neurons formed. Together these results indicate that NPY and the Y1 receptor are required for the normal proliferation of adult olfactory precursors and olfactory function.

Keywords: flow cytometry, neuropeptide Y, olfactory epithelium, stem cells.
J. Neurochem. (2008) **105**, 641–652.

The persistence and ability of the olfactory system to regenerate its neuroepithelium throughout adulthood by replacing damaged or dead neurons is unique in the mammalian nervous system. Such replacement and proliferation are possible as a result of the presence of precursor, multipotent cells in the olfactory neuroepithelium (ON). These precursor cells possess the ability to differentiate into either mature olfactory receptor neurons (ORNs) or non-neuronal supporting cells (Huard *et al.* 1998). The regenerative capacity of the ON, its reconstitution after injury and the re-innervation of the olfactory bulb have been studied extensively (Gage 2000; Schwob 2002). There is increasing knowledge regarding olfactory stem cell regulation and the transcriptional changes that occur during regeneration of the ON (Beites *et al.* 2005; Shetty *et al.* 2005; Nicolay *et al.* 2006). However, questions still remain about the genetic and molecular events and local environmental trophic influences that control and up-regulate olfactory stem cell activity. In fact, the precise identity of the olfactory stem cell is still unknown. The structure of the ON is well defined and contains distinct proliferating cell populations that give rise to transit amplifying progenitors that can differentiate into ORNs (Beites *et al.* 2005). Previous studies have shown that horizontal basal cells (HBCs) that reside on the basal lamina are proliferative and are able to differentiate into neurons and supporting cells (Mackay-Sim and Kittel 1991; Carter *et al.* 2004; Comte *et al.* 2004; Leung *et al.* 2007). Retroviral studies *in vivo* show that following injury globose basal cells (GBCs) are able to give rise to ORNs as well as supporting cells (Calof and Chikaraishi 1989; Schwartz Levey *et al.* 1991; Caggiano *et al.* 1994; Schwob *et al.* 1994; Chen *et al.* 2004). Furthermore, sustentacular cells express a neural stem

Received August 15, 2007; revised manuscript received November 4, 2007; accepted November 21, 2007.

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Abbreviations used: BrdU, bromodeoxyuridine; BSA, bovine serum albumin; GBC, globose basal cell; HBC, horizontal basal cell; ICAM-1, intercellular adhesion molecule-1; NPY, neuropeptide Y; ON, olfactory neuroepithelium; ORN, olfactory receptor neuron; PBS, phosphate-buffered saline; PYY, peptide YY; STFP, social transmission of food preference; WT, wild type.

cell marker making this cell type another possible candidate as an olfactory stem cell (Doyle *et al.* 2001; Merkle *et al.* 2004).

The identification of factors that promote neuroproliferation or regeneration within the ON can provide clues to the process of mammalian nervous system repair and treatment. Recent studies suggest that neuropeptide Y (NPY) regulates neuroproliferation of both olfactory and hippocampal precursor cells (Hansel *et al.* 2001; Howell *et al.* 2003, 2005). NPY is a 36-amino acid peptide that is widely expressed in the CNS and PNS during development and adulthood (Allen *et al.* 1983; Danger *et al.* 1990). Another member of this family of peptides is peptide YY (PYY) that 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). The actions of these peptides are mediated via a family of G protein coupled receptors designated as Y1, Y2, Y4, Y5, and Y6 with NPY and PYY exerting almost identical binding properties (Michel *et al.* 1998).

Neuropeptide Y is highly expressed in the ON primarily in sustentacular cells, where it is thought to stimulate proliferation of olfactory precursor cells (Hansel *et al.* 2001) and in microvillar cells where it is thought to have trophic influences in the regulation of olfactory receptor neuronal apoptosis and regeneration (Montani *et al.* 2006). NPY has also been demonstrated in the olfactory ensheathing cells of axon bundles suggesting its involvement in the guidance and/or growth of ORN axons toward their target glomeruli in the olfactory bulb (Ubink *et al.* 1994; Ubink and Hokfelt 2000). Mice with a targeted deletion of NPY (Erickson *et al.* 1996) contain only half as many dividing olfactory neuronal precursor cells and significantly fewer post-mitotic ORNs compared with controls (Hansel *et al.* 2001). Together with the demonstration that NPY acts via the Y1 receptor subtype on olfactory neuronal precursors or basal cells to activate a specific subgroup of mitogen-activated protein kinases during neuroproliferation (Hansel *et al.* 2001), these observations indicate that NPY may play a major role in the regulation of olfactory precursor cell proliferation in the adult.

The cellular mechanisms regulating olfactory precursor proliferation, differentiation of ORNs and their subsequent connection to mitral cells in the olfactory bulb are essential for normal olfactory function. Bilateral olfactory bulbectomy leads to anosmia and knockout mice where olfactory bulbs fail to develop, such as protein receptor 2 knockout mice, are anosmic (Matsumoto *et al.* 2006). In humans, olfactory bulb volume correlates with olfactory function (Rombaux *et al.* 2006). Bilateral olfactory bulbectomy causes long-term increases in the expression of the NPY gene in the piriform cortex and dentate gyrus suggesting that NPY plasticity in the olfactory/limbic system may contribute to the olfactory bulbectomy syndrome in rats (Holmes *et al.* 1998). In addition, NPY acting through the Y1 receptor regulates the 5-hydroxytriptamide system in mice, co-ordinately linking physiological survival mechanisms such as food intake with territorial aggressive behavior; (Karl *et al.* 2004) both of these behaviors are linked to the animal's ability to smell and detect pheromones.

The studies described in this manuscript were undertaken to gain an understanding of the role of NPY on the neuroproliferation of adult olfactory precursor cells. Anatomical analysis of the ON from wild type (WT), $NPY^{-/-}$, $NPY/PYY^{-/-}$, and $Y1^{-/-}$ mice using immunohistochemical markers was performed. Primary olfactory neurosphere cultures were also isolated from WT, $NPY^{-/-}$, $NPY/PYY^{-/-}$, and $Y1^{-/-}$ mice and examined *in vitro*. Isolation of HBCs, GBCs, and glandular cells from WT, $NPY^{-/-}$, and $Y1^{-/-}$ mice was performed using flow cytometry. Behavioral analysis of WT and $Y1^{-/-}$ mice was performed to examine olfactory function *in vivo*.

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 ($NPY^{-/-}$), Y1 knockout mice ($Y1^{-/-}$), and double knockout mice ($NPY/PYY^{-/-}$) used in the experiments have been previously reported (Howell *et al.* 2003; Karl *et al.* 2004). Mice were held in holding rooms with 12 h light/dark cycles, 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), $Y1^{-/-}$ (n = 6 mice), $NPY^{-/-}$ (n = 4 mice), and $NPY/PYY^{-/-}$ (n = 4) mice aged ~6 weeks were given an overdose of intraperitoneal Euthal (80 mg/kg) and transcardially perfused with ice-cold normal saline followed by 4% *p*-formaldehyde (ProSciTech, Thuringowa, Qld, Australia). Olfactory tissue was dissected and processed for paraffin embedding. Cross-sections of ON and olfactory bulb (6 μm) were collected onto electrostatic glass slides (Menzel-Gläser, Braunschweig, Germany). Sections were dewaxed in Histoclear (National Diagnostics, Atlanta, GA, USA) and rehydrated through a graded series of alcohols.

Non-specific staining was blocked in 10% serum in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h. This was followed by incubation with the primary antibodies [anti-carnosine, rabbit, 1 : 100 (Chemicon, Temecula, CA, USA); anti-keratin, rabbit, 1 : 400 (Dako Corporation, Carpintera, CA, USA); anti-NPY, sheep, 1 : 200 (Auspep, Parkville, Vic., Australia); anti-GBC-1, mouse, 1 : 10 (kind gift of Professor James Schwob); anti-intercellular adhesion molecule-1 (ICAM)-1, mouse, 1 : 100 (BD Biosciences, North Ryde, NSW, Australia); anti-CD15, mouse, 1 : 100 (BD Biosciences); anti-Mash1, rabbit, 1 : 20 (Chemicon);

anti-neurogenin-1, rabbit, 1 : 100 (Chemicon)] for 1 h. Control sections were incubated with 1% normal serum of the same species as the primary antibody and processed in parallel. For immunoperoxidase studies, sections were washed in PBS submerged in 0.3% H_2O_2 for 15 min. Following washing, sections were incubated with biotinylated goat anti-rabbit, horse anti-mouse and donkey anti-sheep secondary antibodies (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1 : 300, washed in PBS, and coverslipped using aquamount (BDH, Poole, England). Quantification of Mash1-positive nuclei was calculated by counting randomized regions ($n = 6$), with an area of 70 μm^2, of the ON labeled with Mash1. Statistical analysis of the data were analyzed with Fisher's protected least significant difference ANOVA test using STATVIEW software (SAS, Cary, NC, USA). A value of $p < 0.05$ was considered significant. Images were captured using a Leica DC480 (Leica Camera AG, Solms, Germany) digital camera attached to a Zeiss Axiophot microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

Olfactory neurospheres in culture were characterized by immunocytochemistry with neuronal [anti-β III tubulin, mouse, 1 : 100 (Sigma, St Louis, MO, USA)] and non-neuronal (keratin) differentiation markers. After 10 days in culture, cells were rinsed in PBS and fixed in 2% *p*-formaldehyde (ProSciTech). Following fixation, cells were permeabilized in 0.1% Triton X-100, rinsed in PBS, blocked in 10% normal serum from the species in which the secondary antibody was made, and then incubated with primary antibodies. Unless otherwise stated all incubations were at 24°C in a humidified chamber. Following three washes in PBS, the cells were incubated with the secondary fluorescent antibodies for 30 min in the dark. Slides were mounted in Vectashield (Vector Laboratories) and images were captured using a Leica DC480 digital camera (Leica) attached to a Zeiss Axiophot microscope (Carl Zeiss).

Double-labeling immunocytochemistry was performed using the primary antibody combination of anti-keratin and anti-β III tubulin. Cells were permeabilized in 0.1% Triton X-100 for 10 min and subsequently blocked in 10% normal goat serum in BSA PBS for 1 h followed by incubation with primary antibodies for 1 h. This was followed by secondary antibody incubation for 30 min with goat anti-rabbit (Alexa Fluor® 594) (1 : 50) (Molecular Probes, Eugene, OR, USA) and goat anti-mouse (Alexa Fluor® 488) (1 : 50) (Molecular Probes). Slides were mounted in Vectashield (Vector Laboratories) and images were captured using a Leica DC480 (Leica) digital camera attached to a Zeiss Axiophot microscope (Carl Zeiss).

Primary adult olfactory precursor cells

To isolate olfactory precursor cells, olfactory turbinates were dissected from 10 to 15 adult WT, $Y1^{-/-}$, $NPY^{-/-}$, and NPY/$PYY^{-/-}$ mice aged ~6 weeks. Mice were anesthetized with CO_2, decapitated, and olfactory turbinates were removed and placed in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 9.6 mg/mL HEPES buffer (Sigma). Tissue was centrifuged at 700 *g* and supernatant was removed before placing minced tissue into Dulbecco's modified Eagle's medium containing 1% (w/v) BSA (Sigma Chemicals, St. Louis, MO, USA), 50 μg/mL Dnase (Sigma Chemicals), 1 mg/mL hyaluronidase (Sigma Chemicals), 1 mg/mL collagenase (Roche Diagnostics, GmbH Mannheim, Germany), and 5 mg/mL dispase (Roche Diagnostics) for 1 h at 37°C. Tissue suspension was triturated, filtered (150-μm wire mesh; Small Parts Inc., Miami Lakes, FL, USA), centrifuged at 700 *g*, and resuspended in Neurobasal medium (Invitrogen), containing 10% (w/v) dialyzed fetal calf serum (ThermoTrace, Melbourne, Vic., Australia), 1000 U/mL Penicillin G (Sigma Chemicals), 50 μg/mL gentamycin sulfate (Sigma Chemicals), 20 mmol/L glutamine (Invitrogen), and 2.5 mg/mL Amphostat B (ThermoElectron, Melbourne, Vic., Australia). Cells were filtered twice more through a 40-μm Nylon mesh filter (BD Falcon, Bedford, MA, USA) 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, NSW, 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), 20 ng/mL fibroblast growth factor-2 (Promega, Madison, WI, USA), and 20 ng/mL epidermal growth factor (Promega). Media was not changed for the duration of the experiments.

For the bromodeoxyuridine (BrdU) incorporation assay, the olfactory neurospheres derived from WT and $Y1^{-/-}$ mice ($NPY^{-/-}$ and NPY/$PYY^{-/-}$ neurospheres did not remain in culture long enough to be assayed for BrdU incorporation), were incubated with BrdU labeling reagent (Roche Diagnostics) that was added to the growth medium at a final concentration of 50 μmol/L. Cells were grown in Labtek four-well chamber slides (Nalge Nunc, Rochester, NY, USA) for 5 days at 37°C in 5% CO_2. The cells were fixed in 70% ethanol (in 50 mmol/L glycine buffer) for 20 min at −20°C, washed in PBS, and permeabilized with 0.3% Triton X-100 (Sigma Chemicals) for 15 min at 24°C. Cells were incubated with monoclonal anti-BrdU (1 : 10) (Chemicon) for 1 h at 24°C and detected using goat anti-mouse Alexa Fluor® 488 (1 : 50) (Molecular Probes) for 30 min at 24°C. Cells were washed in PBS and mounted in Vectashield (Vector Laboratories) mounting medium. Fluorescence was visualized using a laser scanning confocal microscope (Leica).

Quantification of olfactory neurospheres

Olfactory neurospheres were grown in two-well Labtek tissue culture chamber slides (Nalge Nunc). The numbers of neurospheres derived from WT ($n = 3$ experiments), $NPY^{-/-}$ ($n = 3$ experiments), $Y1^{-/-}$ ($n = 4$ experiments), and NPY/$PYY^{-/-}$ ($n = 2$ experiments) mice were counted at 1, 2, and 3 weeks of continuous culturing, without a change of media, and are expressed per well (2 cm^2). Statistical analysis of the data were analyzed with Fisher's protected least significant difference ANOVA test using STATVIEW software (SAS). A value of $p < 0.05$ was considered significant.

Flow cytometry

Olfactory neurospheres for flow cytometric analysis were grown in Labtek 25-cm^2 tissue culture flasks for 3–6 weeks. The cultures were triturated into single cell suspension and centrifuged (310 *g*) for 5 min and resuspended in PBS (Invitrogen) and centrifuged again (310 *g*, 5 min). The cells were resuspended in primary antibodies containing 10% fetal calf serum and incubated for 20 min at 4°C [anti-GBC-1 (1 : 50); anti-ICAM (1 : 25); anti-CD15 (1 : 20)]. Following centrifugation, the cells were resuspended in secondary antibody, R-PE (Jackson, West Grove, PA, USA) (1 : 50) for 20 min. Positive cells (580 nm) were collected using the FACS

Calibur (BD Biosciences). Gating parameters were set by side and forward scatter to eliminate debris, and dead and aggregated cells. The flow rate was ~1000 events per second for high purity and recovery. The cells that were collected from the flow cytometer were plated into Neurobasal medium (Invitrogen) containing B27 supplement into either eight-, four-, or two-well chamber slides to achieve a density of $\sim 1 \times 10^4$ cells/mL (Nalge Nunc). The numbers of cells retrieved from cell sorting experiments were expressed as a percentage of the total number of cells. One-way ANOVA analyses were performed to determine statistical significance (Prism, GraphPad Software, San Diego, CA, USA).

The olfactory neurospheres used in the single cell deposition experiments were triturated and sorted on a flow cytometer (FACS Calibur; BD Biosciences) into 96-well plates (Nalge Nunc) containing Neurobasal medium (Invitrogen) containing B27 supplement (Invitrogen), 20 ng/mL fibroblast growth factor-2 (Promega), and 20 ng/mL epidermal growth factor (Promega).

Determination of olfactory function in WT and Y1$^{-/-}$ mice

It is important to consider an individual's stress response as a confounding factor for olfactory function tests. Recently, we discovered circadian rhythm- and stress-dependent alterations in anxiety levels of Y1$^{-/-}$ mice (Karl *et al.* 2006). Thus, we handled our animals thoroughly before any testing was carried out to reduce the impact of stress on the mice's performance. Furthermore, we started with any behavioral paradigm 1 h after onset of the light phase, which is reportedly a period of the circadian rhythm, in which Y1 depleted mice do not show any gross abnormalities in anxiety-related behaviors.

Buried food olfactory test

This basic test measures the mouse's ability to find a buried piece of food using olfactory cues. To avoid problems associated with neophobia, mice are habituated to eat the new food by placing cookie pieces in the home cage overnight. The mice are then food-deprived for 24 h, with free access to water. The next day an identical piece of cookie is placed underneath the bedding material within the home cage. The latency to find the cookie and start eating is recorded using a stopwatch. A maximum cut-off time of 2 min is used.

Habituation/dishabituation olfactory test

Another basic olfactory test measures habituation to a repeatedly exposed odor, and subsequent dishabituation when the mouse is presented with a novel scent. Mice will generally thoroughly investigate a novel scent, but will spend less time investigating a repeatedly presented odor that is based on habituation processes. Odorants are prepared by diluting extracts (i.e. almond and peppermint) 1 : 100 in clean drinking water. Mice are placed individually into a clean cage. Cotton buds are dipped into plain water or odorant and are then placed through the wire cage lid and fixed with the tip at a height of 5 cm above the cage floor. The cumulative time spent sniffing the stimulus is measured over the following 3 min, before the next stimulus is presented. Stimuli are each presented 3 times consecutively (e.g. water only × 3, almond odor × 3, peppermint odor × 3). The behavior *sniffing* is defined as orienting the nose towards the stimulus within 2 cm of the cotton tip or in contact with the tip. Chewing the cotton tip or contacting it with an open mouth is not counted as sniffing.

Social transmission of food preference test

Social transmission of food preference (STFP) is a test of olfactory memory based on a simple olfactory conditioning process and the phenomenon that rodents are neophobic and will consequently avoid new foods and flavors. In this test, a mouse, which smells a novel flavor presented by a cage mate (i.e. odor in mouth, whiskers, or on snout/fur), will subsequently prefer food with the presented flavor over food with another novel flavor. All mice are first habituated to eat powdered lab chow from small glass/ceramic jars with holes in the lid to allow recording of feeding bouts and amount eaten, while minimizing digging and spillage. Afterwards, one mouse from each cage is randomly chosen as a demonstrator and is placed individually in a clean cage with free access to water, but not food. The other mice of the home cage (observers) are placed into a new clean cage (food and water *ad libitum*). After 24 h the demonstrator mouse is given access to a jar with flavored chow for 1 h. During this time the demonstrator has to eat at least 0.2 g of the flavored chow to be included in the study. This mouse is then placed into the cage with the observer cage mates. As the STFP test can be confounded by genotype-specific differences in social interaction, we addressed this issue by quantifying the motivation for social interaction (occurring between demonstrator and the observers): after the demonstrator is rejoined with the observers the experimenter scores the number of times the snout of the demonstrator mouse is sniffed by the observers for 30 min. The behavior *sniffing* is defined as an observer orienting towards the demonstrator's muzzle, within 2 cm of, or contacting the front or side of the muzzle. The cumulative time the demonstrator's muzzle is sniffed can also be recorded using a stopwatch. We also recorded the time and frequency of *anogenital sniffing* for the observer mice as a measure of social interaction. Following this demonstration period, observers are housed individually with free access to water, but not food, and tested 24 h later. Two jars, one containing flavored food eaten by the demonstrator and one containing a novel flavored food (e.g. cocoa and cinnamon or ginger and coriander), are placed in each observer's cage. At 1 min intervals, the experimenter records for 60 min the number of times the mouse is on top of each jar, and the number of eating bouts from each jar. The amount of food consumed is also measured by weighing the two food jars at the end of the 60 min test session.

The statistical analysis used for the behavioral testing was a one-way ANOVA design for most tasks and a two-way ANOVA (factor genotype and habituation) for the STFP (Statview).

Results

Expression of NPY in the olfactory neuroepithelium of WT, NPY$^{-/-}$, and NPY/PYY$^{-/-}$ mice *in vivo*

The ON of WT, NPY$^{-/-}$, and NPY/PYY$^{-/-}$mice was examined immunohistochemically using an anti-NPY antibody. However, as a result of the close homology between NPY and PYY (~70%) cross-reactivity with PYY cannot be excluded. In the ON of WT mice, NPY is expressed in the sustentacular cells, olfactory ensheathing cells and faintly in

the ORNs (Fig 1a). This staining is reduced in the NPY$^{-/-}$ mice (Fig 1b), and is completely ablated in the NPY/PYY−/− mice (Fig 1c).

Characterization of the olfactory neuroepithelium in WT, NPY$^{-/-}$, Y1$^{-/-}$, and NPY/PYY$^{-/-}$ mice *in vivo*

The ON of WT, NPY$^{-/-}$, Y1$^{-/-}$, and NPY/PYY$^{-/-}$ adult mice was examined using histochemical markers for ORNs and basal cells. An anti-carnosine antibody was used to label the ORNs and axon bundles in the olfactory neuroepithelial tissue. Figure 2(a, d, g, and j) clearly shows anti-carnosine-positive neurons throughout the epithelial layer of each animal with cytoplasmic staining of the ORNs and axon bundles in the WT and NPY$^{-/-}$ mice. However, in the Y1$^{-/-}$ and NPY/PYY$^{-/-}$ mice, the anti-carnosine antibody labels fewer neurons and is localized to the soma. In addition, there is no staining of the axon bundles in the Y1$^{-/-}$ mice. Furthermore, carnosine labeling of the olfactory bulb in WT and NPY$^{-/-}$ mice labels fibers throughout the bulb (Fig. 2c and i), but there was no carnosine-positive labeling of the nerve fibers

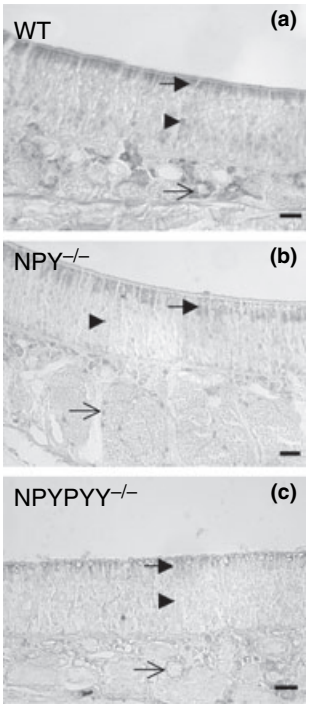

Fig. 1 Immunolocalization of NPY in WT, NPY$^{-/-}$, and NPY/PYY$^{-/-}$ mice. (a) Anti-NPY antibody staining in WT mice is localized to the cytoplasm of sustentacular cells (black arrow), faint labeling of the soma of ORNs (black arrowhead), and olfactory ensheathing glia (black line arrow). (b) NPY$^{-/-}$ mice have reduced levels of anti-NPY immunoreactivity compared with WT in sustentacular cells (black arrow), ORNs (black arrowhead), and olfactory ensheathing glia (black line arrow). (c) No staining of this antibody is observed in the sustentacular cells (black arrow), ORNs (black arrowhead), and olfactory ensheathing glia (black line arrow) of the ON of NPY/PYY$^{-/-}$ mice.

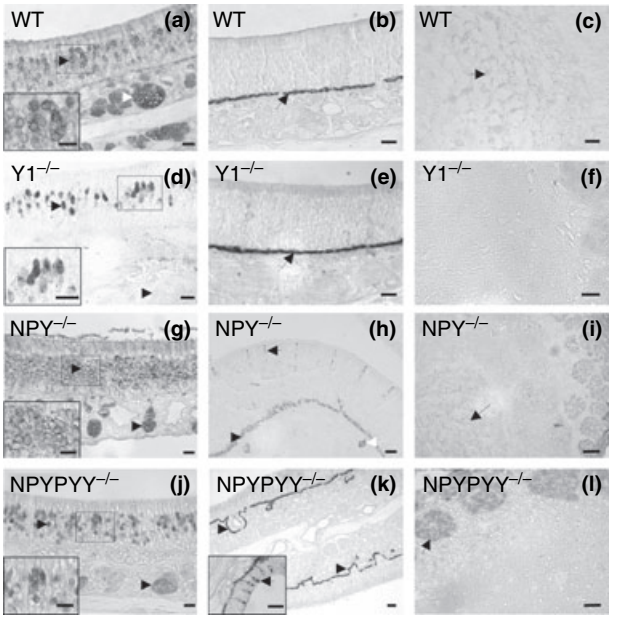

Fig. 2 Neuroanatomical analysis of WT, Y1$^{-/-}$, NPY$^{-/-}$, and NPY/PYY$^{-/-}$ olfactory neuroepithelium and olfactory bulb. (a) Carnosine labels the cytosol of ORNs throughout the neuroepithelial layer (black arrow) and axon bundles in the lamina propria (white arrow) of WT mice (inset shows ORNs at higher magnification). (b) Keratin-positive HBCs in WT sections are found in the basal layer of the neuroepithelium (black arrow). (c) Carnosine labeling of axonal fibers (black arrow) throughout the olfactory bulb of WT sections. (d) Carnosine immunoreactivity of ORNs in Y1$^{-/-}$ tissue is localized to both the nucleus and cytoplasm (black arrow) of ORNs and is absent from the axon bundles (black arrowhead) in the lamina propria (inset shows ORNs at higher magnification). (e) Keratin-positive HBCs (black arrow) in Y1$^{-/-}$ olfactory neuroepithelium appear in the basal layer. (f) Axonal fibers throughout the olfactory bulb do not label positively with carnosine in Y1$^{-/-}$ mice. (g) NPY$^{-/-}$ olfactory neuroepithelial section showing carnosine-positive cytoplasmic staining of ORNs (black arrow) and axon bundles (black arrowhead). The labeling of ORNs in the NPY$^{-/-}$ is very similar to that observed in the WT control (compare with a) (inset shows ORNs at higher magnification). (h) Keratin immunoreactivity of horizontal basal cells in NPY$^{-/-}$ olfactory neuroepithelium extends into cells beyond the basal layer (black arrows) where HBCs normally reside. Immunoreactivity of keratin in NPY$^{-/-}$ also labels the cytoplasm of cells within the sustentacular cell layer of the neuroepithelium (black arrowhead). (i) Neuronal fibers (black arrow) are carnosine-positive in the olfactory bulb of NPY$^{-/-}$ mice. (j) Anti-carnosine immunoreactivity is seen in the cell soma of ORNs (black arrow) and also in the axon bundles of NPY/PYY$^{-/-}$ mice (inset shows ORNs at higher magnification). (k) Keratin immunoreactivity in the horizontal basal cells of NPY/PYY$^{-/-}$ mice shows that the basal lamina is disrupted and is not straight (compare with b and e) (black arrowheads). Keratin immunoreactivity is also present in cells of the sustentacular cell layer (see inset, black arrow). (l) Axonal fibers throughout the olfactory bulb do not label positively with carnosine in NPY/PYY$^{-/-}$ mice; however, glomeruli are carnosine-positive (black arrow). Scale bars: (a–l) 20 μm.

throughout the olfactory bulb of $Y1^{-/-}$ mice (Fig. 2f). NPY/$PYY^{-/-}$ mice express anti-carnosine in the glomeruli but not in the nerve fibers of the olfactory bulb (Fig. 2l).

Examination of the HBCs situated in the basal layer of the ON was performed using an anti-keratin antibody (Fig. 2b, e, h, and k). Keratin was localized to the cytoplasm of HBCs in all of the mice examined. In $NPY^{-/-}$ mice, however, the HBCs did not form a continuous monolayer and cells above and below the basal layer were keratin-positive (Fig. 2h). This pattern of labeling was more pronounced in the NPY/$PYY^{-/-}$ mice (Fig. 2k). Furthermore, keratin-positive cells also appeared in the sustentacular cell layer of the $NPY^{-/-}$ and NPY/$PYY^{-/-}$ mice [Fig. 2h and k (inset)].

Mash1 immunohistochemistry was performed using olfactory neuroepithelial tissue from WT, $Y1^{-/-}$, $NPY^{-/-}$, and NPY/$PYY^{-/-}$ mice to examine the population of Mash1-positive transit amplifying cells. The nuclei of a subpopulation of sustentacular cells and olfactory ensheathing glial cells in the lamina propria were positively labeled in all the mice examined (Fig. 3a–d). In the WT control animals the nuclei of cells within the neuroepithelial layer that were Mash1-positive were confined to the basal region where transit amplifying cells and GBCs reside (Fig. 3a). In $Y1^{-/-}$ mice there appeared to be fewer Mash1-positive nuclei in the neuroepithelial layer, whereas in $NPY^{-/-}$ and NPY/$PYY^{-/-}$ there were Mash1-positive nuclei in the more apical regions of the neuroepithelium (Fig. 3b–d). The histogram in Fig. 3e shows the numbers of Mash1-positive nuclei reduced in $Y1^{-/-}$ mice and increased in $NPY^{-/-}$ and NPY/$PYY^{-/-}$ mice (WT vs. $Y1^{-/-}$, $p = 0.4$; WT vs. $NPY^{-/-}$, $p = 0.0002$; WT vs. NPY/$PYY^{-/-}$, $p = 0.0002$; $Y1^{-/-}$ vs. $NPY^{-/-}$, $p < 0.0001$; $Y1^{-/-}$ vs. NPY/$PYY^{-/-}$, $p < 0.0001$; and $NPY^{-/-}$ vs. NPY/$PYY^{-/-}$, $p = 0.8$). As a marker of immediate neuronal precursors, Neurogenin-1 was used to analyze the pattern of expression in tissue from WT, $Y1^{-/-}$, $NPY^{-/-}$, and NPY/$PYY^{-/-}$ mice. There was immunolocalization in the microvillar cells throughout the neuroepithelium with no differential patterns present in the mice studied (results not shown).

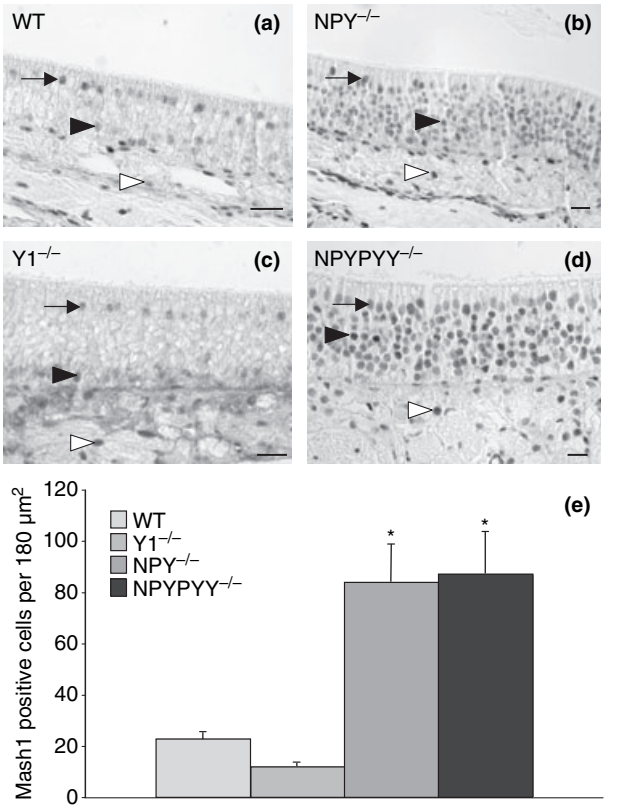

Fig. 3 Neuroanatomical analysis of WT, $Y1^{-/-}$, $NPY^{-/-}$, and NPY/$PYY^{-/-}$ ON using anti-Mash1. (a) Expression of Mash1 in WT mice shows localization in the nuclei of sustentacular cells (arrow), transit amplifying cells (arrowhead), and olfactory ensheathing cells (white arrowhead). (b) In $NPY^{-/-}$ mice Mash1 is localized to the nuclei of sustentacular cells (arrow), olfactory ensheathing cells (white arrowhead), and most nuclei throughout the neuronal layer of the ON (arrowhead). (c) Mash1 localization in $Y1^{-/-}$ mice is in sustentacular cells (arrow), transit amplifying cells (arrowhead), and olfactory ensheathing cells (white arrowhead). (d) Section from NPY/$PYY^{-/-}$ mice shows Mash1 immunolocalization in sustentacular cells (arrow), most nuclei throughout the ON (arrowhead), and olfactory ensheathing cells (white arrowhead). (e) Quantification of Mash1-positive nuclei throughout the neuroepithelial layer shows that compared with WT controls, $Y1^{-/-}$ mice have less positive nuclei and $NPY^{-/-}$ and NPY/$PYY^{-/-}$ have significantly higher numbers of positive nuclei (*$p = 0.0002$). The data are presented as mean percentage ± SEM. Scale bars: (a–d) 20 μm.

Quantification of olfactory neurospheres

Olfactory neurospheres in cultures derived from WT, NPY−/−, $Y1^{-/-}$, and NPY/$PYY^{-/-}$ mice were quantified over a period of three continuous weeks. Regardless of the genotype of the mice, all cultures produced neurospheres within 48 h of plating. These neurospheres were 50–100 μm with no differentiated cell types appearing during the initial stages of culturing. The graph in Fig. 4 shows that similar numbers of olfactory neurospheres formed within the first week *in vitro*, except for neurospheres from the NPY/$PYY^{-/-}$ mice that have reduced numbers. Between 1 and 2 weeks *in vitro*, all groups showed a moderate increase in the number of olfactory neurospheres, except for NPY/$PYY^{-/-}$ mice. However, while the number of olfactory neurospheres derived from WT mice increased between 2 and 3 weeks the number of neurospheres derived from $NPY^{-/-}$ (93% reduction compared with WT), $Y1^{-/-}$ (40% reduction compared with WT), and NPY/$PYY^{-/-}$ (100% reduction compared with WT) decreased (WT vs. $Y1^{-/-}$, not significant; WT vs. $NPY^{-/-}$, $p < 0.05$; $Y1^{-/-}$ vs. $NPY^{-/-}$, not significant; WT vs. NPY/$PYY^{-/-}$, $p < 0.0001$; $Y1^{-/-}$ vs. NPY/$PYY^{-/-}$, $p < 0.02$; and $NPY^{-/-}$ vs. NPY/$PYY^{-/-}$, $p < 0.01$).

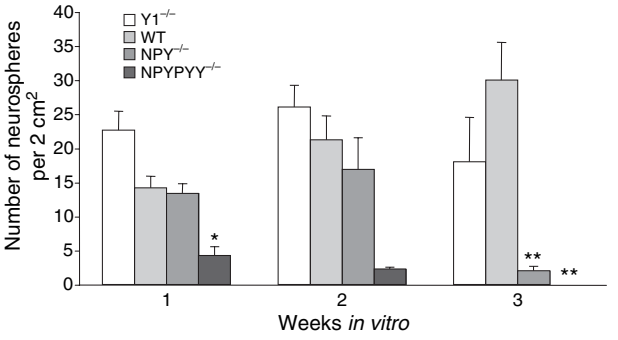

Fig. 4 Quantification of olfactory neurospheres derived from WT, NPY$^{-/-}$, NPY/PYY$^{-/-}$, and Y1$^{-/-}$ mice. Analysis of the number of neurospheres growing in culture for a period of 3 weeks shows that after 1 and 2 weeks *in vitro* the only statistically significant difference in the number of olfactory neurospheres that were growing in culture were the NPY/PYY$^{-/-}$ (*$p < 0.05$) compared with NPY$^{-/-}$, Y1$^{-/-}$, and WT controls. However, the number of olfactory neurospheres derived from the NPY$^{-/-}$ (**$p < 0.05$) and NPY/PYY$^{-/-}$ (**$p < 0.0001$) mice present at 3 weeks was significantly less compared with both the Y1$^{-/-}$ and WT controls. The number of olfactory neurospheres continued to increase in the WT controls but started to decrease in the Y1$^{-/-}$. The data are presented as mean ± SEM. (WT $n = 3$ experiments; Y1$^{-/-}$ $n = 4$ experiments; NPY$^{-/-}$ $n = 3$ experiments; NPY/PYY$^{-/-}$ $n = 2$ experiments).

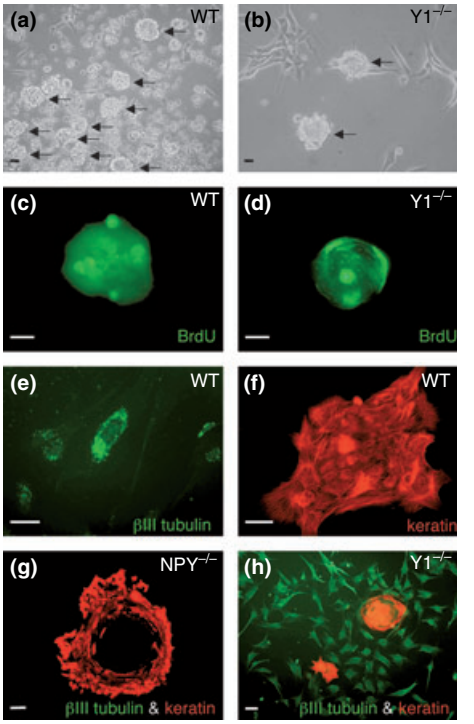

Fig. 5 *In vitro* characterization of olfactory neurospheres derived from wild type (WT), NPY$^{-/-}$, and Y1$^{-/-}$ mice. (a) Olfactory neurospheres derived from WT mice following 8 days *in vitro* (black arrows). These neurospheres are three-dimensional structures that are microscopically distinguishable from cellular aggregates that also form in culture. (b) Olfactory neurospheres derived from Y1$^{-/-}$ mice grown for 16 days *in vitro*. (c) A proliferating WT neurosphere showing BrdU-positive nuclei (green). (d) A proliferating Y1$^{-/-}$ neurosphere showing BrdU-positive nuclei (green). (e) β III tubulin-positive cytoplasmic labeling of neurons derived from a WT olfactory neurosphere culture. (f) Keratin-positive neuroepithelial cells that have differentiated from a neurosphere culture derived from WT mice. (g) Double labeling immunocytochemistry showing keratin-positive immunoreactive cells form around what appears to be the remnants of an olfactory neurosphere derived from NPY$^{-/-}$ mice. There were no β III tubulin-positive neurons. (h) Immunocytochemical analysis of olfactory neurospheres derived from Y1$^{-/-}$ double labeled with β III tubulin (green) and keratin (red) undergoing differentiation. Scale bars: (a–d, g, and h) 20 μm and (e and f) 10 μm.

Characterization of primary olfactory neurosphere cultures derived from WT, NPY$^{-/-}$and Y1$^{-/-}$ mice

Neurospheres formed three-dimensional aggregates of cells that were derived from proliferation of a single olfactory precursor cell. Figure 5a illustrates olfactory neurospheres derived from WT mice after 8 days *in vitro* and Fig. 5b illustrates olfactory neurospheres derived from Y1$^{-/-}$ mice after 16 days *in vitro* (Fig. 5b). In fact, neurospheres were seen in all the cultures 24 h post-plating and increased in number (Fig. 4) and size over a 2-week period. Some neurospheres from WT and Y1$^{-/-}$ but not NPY $^{-/-}$ grew to be 200–300 μm in diameter over time. Neurospheres from both WT and Y1$^{-/-}$ cultures survived and increased in size for several months. Neurospheres from NPY$^{-/-}$ mice, however, did not survive for more than 3 weeks. More differentiated cell types appeared in the NPY$^{-/-}$ cultures following 3 weeks in culture (results not shown). Moreover, neurospheres derived from WT mice also gave rise to secondary neurospheres, whereas no secondary neurospheres were formed from neurospheres isolated from Y1$^{-/-}$ or NPY$^{-/-}$ mice (Table 1). Proliferation assays performed on the neurospheres resulted in BrdU-positive labeling of nuclei in WT and Y1$^{-/-}$ mice (Fig. 5c and d). However, neurospheres from NPY$^{-/-}$ and NPY/PYY$^{-/-}$ mice were not assayed for BrdU incorporation because of their lack of time in culture.

In order to more clearly characterize the type of cells formed in culture, immunocytochemistry was performed on the neurospheres. These studies demonstrated the differentiation of the olfactory precursor cells into neuronal and non-neuronal cell types. The cultures examined (WT, NPY$^{-/-}$, and Y1$^{-/-}$) showed differentiation into neurons (β III tubulin-positive) and neuroepithelial cells (keratin-positive). Neuroepithelial cells are differentiated cells *in vitro*, but are not HBCs that are keratin-positive *in vivo*. Figure 5e shows granular cytoplasmic labeling of β III tubulin-positive neurons derived from WT cultures. Keratin-positive neuroepithelial cells from WT mice are seen in Fig. 5f. NPY$^{-/-}$ cultures also differentiated with keratin-positive neuroepithelial cells surrounding the remnants of a neurosphere,

Table 1 Characteristics of olfactory neurospheres and putative olfactory stem cell populations derived from WT, $Y1^{-/-}$, and $NPY^{-/-}$ mice

Culture type/antibody markers for flow cytometry	WT		$Y1^{-/-}$		$NPY^{-/-}$	
	Survival	Secondary sphere	Survival	Secondary sphere	Survival	Secondary sphere
Neurospheres 'bulk' culture	+	+[a]	+	–	+(< 3 weeks)	–
CD15 +ve	+	+[b]	+	–	+	–
ICAM-1 +ve	+	–	–	–	–	–
GBC-1 +ve	+	–	+	–	–	–

(–) did not survive and did not form secondary neurospheres and (+) survived in culture and formed secondary neurospheres. [a]2% single cells formed secondary neurospheres; [b]0.02% CD15 +ve cells formed secondary neurospheres.

however, no β III tubulin labeled cells were identified (Fig. 5g). Figure 5h shows a culture of olfactory neurospheres derived from $Y1^{-/-}$ double labeled with keratin (red) and β III tubulin (green). The remnants of the neurosphere labeled positively for the neuroepithelial cell marker keratin. Many neurons were seen surrounding the neurosphere in this culture although the morphology of the neurons was quite different to those seen in the WT cultures (compare with Fig. 5e).

Flow cytometric analysis of olfactory neurospheres

The availability of extracellular markers to specific cell types within the ON has allowed the olfactory neurosphere cultures to be sorted into homogeneous populations of cells. In particular, ICAM-1 is an extracellular marker for HBCs, GBC-1 is an extracellular marker for GBCs, and CD15 is an extracellular marker for glandular cells of the Bowman's gland (Fig. 6a–c). These images are of WT control tissue. They are however, representative of $NPY^{-/-}$ and $Y1^{-/-}$ mice, as there was no evidence of differential patterns of localization amongst the different groups of animals. The olfactory neurosphere cultures from each group of mice were triturated and labeled with these markers and sorted on a flow cytometer to achieve homogeneous populations of specific cell types (Fig. 6d–f). Each of the homogeneous populations of cells formed single cell suspensions initially following flow cytometry. Table 1 shows which of the cells survived, proliferated, or were passaged. Interestingly, the ICAM-1-positive HBC population derived from $NPY^{-/-}$ and $Y1^{-/-}$ mice did not survive despite retrieving a similar percentage of cells compared with WT mice. Furthermore, the GBC-positive cells retrieved from the $NPY^{-/-}$ mice did not survive *in vitro*. The CD15-positive population of olfactory glandular cells derived from $NPY^{-/-}$, $Y1^{-/-}$, and WT mice was the only population to survive from each group and the CD15-positive glandular cells from the WT mice were the only population to form secondary neurospheres (Table 1).

The number of cells that were CD15-positive, ICAM-positive, or GBC-positive was calculated as a percentage of the total number of cells that were sorted. The histogram in Fig. 6g shows the average percentage of positive cells for each antibody within the WT, $Y1^{-/-}$, or $NPY^{-/-}$ groups. This graph shows that overall a higher percentage of CD15-, ICAM-, and GBC-positive cells were sorted from the $Y1^{-/-}$ mice compared with WT or $NPY^{-/-}$ mice. However, statistical analysis showed that this increased percentage failed significance.

Determination of olfactory function

To investigate if any of the alterations seen in the neurosphere cultures from the $Y1^{-/-}$ mice also translate in functional changes *in vivo* we performed some behavioral tests on olfactory-related parameters. We recorded a significantly increased frequency of *anogenital sniffing* for the $Y1^{-/-}$ observers in the STFP test (WT = 18.1 ± 2.8 vs. $Y1^{-/-}$ = 34.6 ± 3.1; $p < 0.01$) whereas the frequency of snout *sniffing* was identical for both genotypes (WT = 54.1 ± 4.9 vs. $Y1^{-/-}$ = 63.4 ± 4.9; $p = 0.2$) (Table 2). Furthermore, the habituation/dishabituation olfactory test revealed a strong trend ($p = 0.06$) for a genotype effect as Y1 depleted mice spent less time *sniffing* an almond-dipped stimulus when it was presented for the first time (Table 2). Two-way ANOVA also showed a trend for a significant genotype × habituation interaction for time *sniffing* the almond-dipped stimulus over the period of three trials: the Y1 knockout mice exhibited reduced habituation to this scent ($p = 0.06$).

Discussion

The role of NPY acting via the Y1 receptor was examined in the ON, with a particular focus on putative olfactory precursors. NPY is expressed in sustentacular cells, olfactory ensheathing glia and faintly in ORNs. This was abolished in mice with no NPY or PYY, but faint labeling was present using an anti-NPY antibody in $NPY^{-/-}$ mice suggesting a slight cross-reactivity with PYY, which we speculate may also be involved in olfactory precursor function. This hypothesis was tested by studying the cells of the ON both *in vivo* and *in vitro* in WT, $NPY^{-/-}$, $Y1^{-/-}$, and $NPY/PYY^{-/-}$ mice. Unlike WT and $NPY^{-/-}$ mice, $Y1^{-/-}$ mice had no carnosine-positive labeling of axon bundles or olfactory bulb

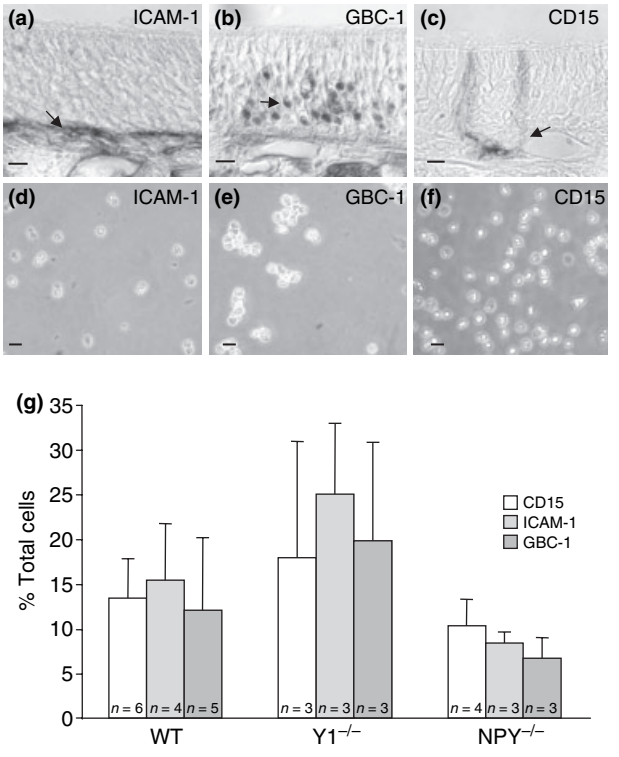

Fig. 6 Immunohistochemical and flow cytometric analysis of ICAM-1-, GBC-1-, and CD15-positive cells. (a) ICAM-1 antibody labels the HBCs of the olfactory neuroepithelium (black arrow). (b) GBC-1 antibody labels the GBCs of the olfactory neuroepithelium (black arrow). (c) CD15 labels Bowman's glands (black arrow). (d) ICAM-1-positive cells isolated using flow cytometry are small, flat cells appearing on their own or as doublets. (e) GBC-1-positive cells are round cells that form clusters and (f) CD15-positive cells are round cells mostly appearing as single cells in suspension. (g) Olfactory neurospheres from WT, $NPY^{-/-}$, and $Y1^{-/-}$ mice were labeled with CD15, ICAM-1, and GBC-1, and the number of positive cells was calculated as a percent of the total number of cells sorted. Overall, the percentage of CD15-, ICAM-1-, and GBC-1-positive cells from WT controls was between 10% and 15%. A slightly higher percentage (17–25%) of cells were retrieved from the $Y1^{-/-}$ mice. Compared with WT controls this was not statistically significant. The percentage of cells retrieved from the $NPY^{-/-}$ mice was consistently less than WT and $Y1^{-/-}$ mice; however, this did not reach statistical significance. The data are presented as mean percentage ± SEM. (*n* = number of experiments). Scale bars: (a–c) 10 μm and (d–f) 20 μm.

fibers. $NPY/PYY^{-/-}$ mice also had no carnosine-positive olfactory bulb fibers. Studies by Ubink and coworkers (Ubink *et al.* 1994; Ubink and Hökfelt 2000) have suggested that the expression of NPY in olfactory ensheathing cells might be involved in the guidance and/or growth of ORNs toward their target glomeruli in the olfactory bulb. Therefore, it is likely that in the $Y1^{-/-}$ mice a disruption of the signaling between the ON and the olfactory bulb occurs and is potentially responsible for the effects seen in the *in vivo* olfactory tests in these mice.

Keratin intermediate filaments are the specific markers for HBCs (Calof and Chikaraishi 1989). Interestingly, the keratin-positive HBCs in the $NPY^{-/-}$ and $NPY/PYY^{-/-}$ mice were not confined to the basal lamina of the ON, which is thought to be the region of the ON where the olfactory precursor cells reside (Mackay-Sim and Kittel 1991). The basal layer of the ON where keratin-positive HBCs reside has been likened to the stem cell niches of non-neuronal systems, which are defined by adhesion to a basal lamina or stroma where adhesion mechanisms regulate stem cell proliferation and inhibit differentiation (Zhu *et al.* 1999; Watt 2001). Perhaps in the $NPY^{-/-}$ and $NPY/PYY^{-/-}$ mice, there is a disruption of the laminin and/or integrins that are present in the stem cell niche of the ON. There is also keratin labeling of cells in the sustentacular cell layer of $NPY^{-/-}$ and $NPY/PYY^{-/-}$ mice that is not present in the WT or $Y1^{-/-}$ mice. A recent study by Montani *et al.* (2006) has suggested that these cells may be microvillar cells that express NPY and are likely to influence the signaling of olfactory neuronal apoptosis and neurogenesis. Mash1, a determination gene for olfactory receptor neurones (Cau *et al.* 2002) is expressed in sustentacular cells, olfactory ensheathing cells and transit amplifying cells that are situated in the basal region of the ON. In $NPY^{-/-}$ and $NPY/PYY^{-/-}$ mice, there is a statistically significant increase in the number of Mash1-positive nuclei in the ON. With an increase in Mash1 expression compared with WT and a decrease in neurosphere formation, it is unlikely that neurosphere forming cells reside within the Mash1-positive transit amplifying cell population. The differential pattern of keratin- and Mash1-positive cells in the $NPY^{-/-}$ and $NPY/PYY^{-/-}$mice could be an indication of developmental abnormalities because of the lack of these peptides.

In vitro, olfactory neurospheres were isolated from WT, $NPY^{-/-}$, $NPY/PYY^{-/-}$, and $Y1^{-/-}$ mice and the neurospheres isolated from WT control mice increased in size and number over a 3-week period. Neurospheres derived from $Y1^{-/-}$ mice also became larger over time but their numbers began to decrease between 2 and 3 weeks whereas the neurospheres derived from $NPY^{-/-}$ mice had decreased significantly by 3 weeks compared with WT and $Y1^{-/-}$ mice. The lack of NPY is exacerbated by the lack of PYY. These results indicate that in the absence of both NPY, PYY and their Y1 receptor, olfactory neurospheres are able to form *in vitro*, however over time are unable to maintain neuroproliferation. This would agree with work by Hansel *et al.* (2001) that showed NPY acting via its Y1 receptor stimulated olfactory neuronal precursor proliferation in the adult mammal, and neuronal precursor proliferation in the postnatal and adult dentate gyrus (Howell *et al.* 2005). The widespread expression of NPY throughout the nervous system and the discovery of precursor cells in various regions of the nervous system may indicate that NPY may have widespread actions on neurogenesis.

Table 2 Summary of results of olfactory function tests

	WT (n = 10 mice)	Y1−/− (n = 8 mice)
Buried food olfactory test		
Latency to dig for hidden cookie [s]	272.8 ± 45.7	412.9 ± 79.2
Latency to eat cookie [s]	375.0 ± 72.3	468.5 ± 70.8
Habituation/dishabituation olfactory test		
Time spent sniffing at stimulus almond 1st [s]	9.1 ± 1.0	6.0 ± 1.0 (p = 0.06)
Time spent sniffing at stimulus almond 2nd [s]	3.1 ± 0.4	2.9 ± 0.5
Time spent sniffing at stimulus almond 3rd [s]	2.5 ± 0.2	2.2 ± 0.4
STFP Test		
Total time spent on anogenital sniffing [s]	18.1 ± 2.8	34.6 ± 3.1 (p < 0.01)
Percentage time the observer mouse spent with cued food [%]	72.6 ± 7.3	71.9 ± 10.2

Previous studies have identified GBCs (Goldstein and Schwob 1996) and HBCs (Carter *et al.* 2004) as putative olfactory precursors. During both normal developmental and regeneration, studies indicate the presence of transit amplifying progenitors (Calof *et al.* 2002) and immediate neuronal precursors (Calof and Chikaraishi 1989) within the basal compartment of the ON. In fact, a recent study by Leung *et al.* (2007) showed that both HBCs and GBCs act as olfactory precursors in which distinct cell populations mediate normal neuronal turnover and neuronal replacement upon traumatic injury. Flow cytometric analysis isolated GBCs and HBCs. Furthermore, anti-CD15, a marker expressed on pluripotent stem cells, adult stem cells, and bone marrow stem cells (Capela and Temple 2002, 2006) was used to isolate CD15-positive glandular cells. Interestingly, the percentage of GBC-, ICAM-, and CD15-positive cells isolated from the neurosphere 'bulk' cultures differed between WT control, $Y1^{-/-}$ and $NPY^{-/-}$ mice. The cells isolated from the $NPY^{-/-}$ mice had a lower percentage of these cells compared with WT and $Y1^{-/-}$ mice, however this did not reach statistical significance.

The subsequent culturing of ICAM-, GBC-, and CD15-positive cells isolated from WT, $Y1^{-/-}$, and $NPY^{-/-}$ mice did result in some characteristic differences. The ICAM-positive HBCs did not survive in culture when isolated from $Y1^{-/-}$ and $NPY^{-/-}$ mice. The WT HBCs did survive but did not form secondary neurospheres. Previous work by Carter *et al.* (2004) isolated HBCs using ICAM-1 and cultured them on a mixed collagen substrate to display an *in vitro* progenitor phenotype. They performed colony-forming assays and showed that 2–3% of cells expanded to form small and large clusters, as well as to form differentiated morphologies. Our experiments did not use a matrix for cell growth and that may have been the reason for finding differences in the *in vitro* characteristics of isolated HBCs. However, the culturing conditions used in our experiments were identical to the culturing conditions of our neurosphere 'bulk' cultures that led to the formation and survival of olfactory neurospheres.

Isolated GBCs survived when isolated from WT and Y1−/− mice, but not from $NPY^{-/-}$ mice. No secondary neurospheres were formed from isolated GBCs. Data from previous studies reported that GBCs were able to differentiate into olfactory neurons or supporting cells (Caggiano *et al.* 1994; Hahn *et al.* 2005) as well as showing the presence of neuronal precursor cells in a subpopulation of GBCs (DeHamer *et al.* 1994; Mumm *et al.* 1996; Calof *et al.* 1998). However, there is also evidence to show that GBCs are committed neuronal precursors (Hsu *et al.* 2001; McCurdy *et al.* 2005) and as such would not possess stem cell-like characteristics such as the formation of neurospheres.

The glandular cells isolated from the neurosphere 'bulk' cultures using anti-CD15 survived in culture when isolated from WT, $Y1^{-/-}$, and $NPY^{-/-}$ mice. In fact, of all the cells isolated by flow cytometry it was only CD15-positive glandular cells isolated from WT mice that formed secondary neurospheres. *In vivo* studies showed HBCs and GBCs are able to give rise to neurons and non-neuronal cells of the ON, not glandular cells (Leung *et al.* 2007). However, it is not known if a common progenitor exists for the neuronal and non-neuronal lineage of the ON and there is a suggestion that cells of the Bowman's gland may play a progenitor role in the establishment of the sustentacular cell population (Davis and Reed 1996). It is possible also that the microenvironment *in vitro* changes the capacity of CD15 cells to form neurospheres just as the microenvironment *in vivo* allows HBCs and GBCs to both behave as olfactory precursors (Leung *et al.* 2007).

In a battery of olfaction-related paradigms all animals of both genotypes (WT and $Y1^{-/-}$) exhibited the general ability to detect different odors/flavors and to distinguish between novel and familiar flavors. However, $Y1^{-/-}$ mice did show a reduced interest in investigating common smells such as almond compared with WT mice. Furthermore, the $Y1^{-/-}$ mice spent more time on *anogenital sniffing* – a behavior, which can be interpreted as a form of 'social exploration' of newly or re-introduced cage mates (Luo *et al.* 2003). A moderate impairment of olfactory abilities in $Y1^{-/-}$ mice

might be responsible for this phenotype. The attraction of investigating a novel flavor in the Habituation/Dishabituation Olfactory test was that it would not be as long-lasting for a mouse with a reduced sense of smell as for one with a more sensitive olfactory system. On the other hand, detecting a flavor less intensively compared with WT-like mice might result in reduced habituation to this particular flavor. The increase in time spent in *anogenital sniffing* in $Y1^{-/-}$ mice might highlight the importance of pheromone detection for rodents. Pheromones would help the observer mouse to determine sex and social status of the re-introduced demonstrator in the STFP test (Luo *et al.* 2003) and therefore it is important for the observer mouse to get this information. An impaired ability to detect smells and in particular pheromones might result in the increased time $Y1^{-/-}$ mice spent on this behavior. However, the general motivation to socially interact with a cage mate was not affected by Y1 depletion as shown by similar levels of snout sniffing. Although the differences detected between the WT and $Y1^{-/-}$ mice were not pronounced, the observed behavioral phenotype of $Y1^{-/-}$ mice could be based on a reduced sense of smell in these animals, which corresponds to the animals reduced ability for olfactory neurogenesis and olfactory precursor cell proliferation as well as the axonal connection between the ORNs and the mitral cells in the bulb.

Reduced olfactory function is supported by previous data that demonstrated a phenotype of increased aggression in $Y1^{-/-}$ mice, a behavior that strongly relies on smell in rodents (Karl *et al.* 2004).

In conclusion, we examined olfactory neuroepithelial cellular morphology *in vivo* in genetically modified mice that lacked NPY, PYY, and the Y1 receptor. These studies revealed differential patterns of staining in axonal fibers in the ON and olfactory bulb of $Y1^{-/-}$ mice, and in the basal lamina and apical supporting cells of $NPY^{-/-}$ and $NPY/PYY^{-/-}$ mice. The ability of the ON to generate new ORNs and for these neurons to subsequently make the correct connections and signals to the bulb are essential for olfactory function which has been shown in this study to be impaired in $Y1^{-/-}$ mice. In order to generate neurons, there is a population of olfactory multipotent precursor cells that are able to proliferate and differentiate via largely unknown mechanisms. For the first time, we have shown that the isolation of olfactory cells capable of forming neurospheres is compromised in $NPY^{-/-}$ mice and that is further exacerbated in $NPY/PYY^{-/-}$ mice. This indicates that although NPY is important for the normal proliferation of olfactory precursors, with PYY also likely to be involved. The major Y-receptor mediating this effect is likely to be the Y1, as $Y1^{-/-}$ mice also reveal abnormalities in olfactory precursor function. This indicates an important role for this neuropeptide family and its receptors in olfactory neurogenesis. In addition, we have shown that isolated glandular cells from the ON might be a putative olfactory precursor cell.

Acknowledgments

This work was funded by grants from the St Vincent's Clinic Foundation, Fairfax Foundation, and JO and JR Wicking Trust. The authors would also like to thank Ms Tanya Wyatt for her technical assistance in the cutting of paraffin embedded sections and Professor James Schwob for the kind donation of the GBC-1 antibody. The authors indicate no potential conflicts of interest.

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