Role of Neuropeptide Y (NPY) in the differentiation of Trpm-5-positive olfactory microvillar cells

Kharen L Doyle[a,b*], Carla Cunha[c], Yvonne Hort[a], Ramon Tasan[d], Günther Sperk[d], John Shine[a,b] and Herbert Herzog[a,b]

[a]Garvan Institute of Medical Research, [b]UNSW Sydney, Australia, [c]i3S – Instituto de Investigação e Inovação em Saúde, Portugal, [d]Department of Pharmacology, Medical University of Innsbruck, Austria.

[*]Corresponding Author:
Dr Kharen Doyle, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, New South Wales, 2047, Australia.
Email: k.doyle@garvan.org.au

carla.cunha@ineb.up.pt
y.hort@garvan.org.au
ramon.tasan@i-med.ac.at
guenther.sperk@i-med.ac.at
j.shine@garvan.org.au
h.herzog@garvan.org.au

List of Abbreviations

BSA	bovine serum albumin
ChAT	Cholineacetyltransferase
MVC	microvillar cell
NPY	Neuropeptide Y
NPYGFP	Neuropeptide Y green fluorescent protein
OMP	olfactory marker protein
ON	olfactory neuroepithelium
ORN	olfactory receptor neuron
PFA	paraformaldehyde
PBS	phosphate buffered saline
Trpm5	transient receptor potential channel M5

Abstract

The mouse olfactory neuroepithelium (ON) is comprised of anatomically distinct populations of cells in separate regions; apical (sustentacular and microvillar), neuronal (olfactory sensory neurons) and basal (horizontal and globose basal cells). The existence of microvillar cells (MVCs) is well documented but their nature and function remains unclear. An important transcription factor for the differentiation of MVCs is Skn-1a, with loss of function of Skn-1a in mice resulting in a complete loss of Trpm-5 expressing MVCs, while olfactory sensory neuron differentiation is normal. Our previous research has shown that neuropeptide Y (NPY) is expressed in MVCs and is important in the neuroproliferation of olfactory precursors. This study showed that following X-ray irradiation of the snout of wildtype mice, which decreases the proliferation of basal precursor cells, the numbers of Trpm-5-positive MVCs is increased at 2 and 5 weeks post-irradiation compared to controls. Skn-1a expression in the ON following X-ray irradiation also increases at 2 weeks post-irradiation in a regionally specific manner matching the expression pattern of Trpm-5-positive MVCs. In parallel, NPYCre knock-in mice were used to examine the expression of Skn-1a following activation of NPY unilaterally in the ON (unilateral nasal irrigation of AAV-NPY-FLEX). These experiments demonstrated that Skn-1a is only expressed when NPY is activated in MVCs. Therefore the expression of NPY is necessary for the transcription factor-mediated differentiation of olfactory MVCs.

Keywords

Olfactory neuroepithelium, Neuropeptide Y, Microvillar cells, NPYcre mice, X-ray irradiation

1. Introduction

The mouse olfactory neuroepithelium (ON) is comprised of anatomically distinct populations of cells in separate regions: the apical layer (sustentacular and microvillar cells), the intermediate neuronal layer (olfactory sensory neurons) and the basal layer (horizontal and globose basal cells) (Graziadei and Graziadei, 1979). The intermediate layer contains neurons exhibiting a basal to apical maturation gradient such that mature olfactory receptor neurons (ORNs) are found closer to the apical layer (Calof and Chikaraishi, 1989). Multipotent stem cells isolated from the basal layer have the ability to proliferate and subsequently differentiate into olfactory receptor neurons or non-neuronal supporting cells (Leung et al., 2007; Huard et al., 1998; Schwob et al., 1994).

The existence of MVCs in the ON apical layer is well documented but their nature and function remain unclear. They have been proposed to represent a second class of ORNs with two distinct lines of evidence to support this. Firstly morphological analysis revealed a bipolar olfactory cell (Jourdan, 1975; Moran et al., 1982). Secondly, injection of horseradish peroxidase into the olfactory bulb leads to the retrograde labelling of olfactory MVC bodies (Rowley et al., 1989). However, there is also evidence to support their non-neuronal nature: following olfactory bulbectomy, olfactory MVCs do not degenerate (Carr et al., 1991).

Morphologically at least three different types of MVCs have been described (Hansen and Finger, 2008). Two of them express the monovalent cation channel transient receptor potential channel M5 (Trpm5). Trpm5 is expressed in type II taste cells, solitary chemosensory cells and thermosensitive cells (Matsumoto et al., 2011). Therefore, Trpm5-positive MVCs are considered to be chemo and/or thermosensitive (Yamaguchi et al., 2014). Non-neuronal Trpm5-positive MVCs also express choline acetyltransferase (ChAT) and can react to certain chemical or thermal stimuli and release acetylcholine to modulate activities of neighbouring supporting cells and ORNs via muscarinic acetylcholine receptors (Ogura et al., 2011).

Skn1a (also known as Pou2f3), a POU (Pit-Oct-Unc) transcription factor, is expressed in the Trpm5-expressing taste and solitary chemosensory cells. Furthermore, it is required for the generation of Trpm5-expressing MVCs in the mouse main ON because Skn1a knockout mice do not have any MVCs (Yamaguchi et al., 2014). Yamaguchi et al. (2014) also showed there was a small population of Skn1a-positive cells co-labelled with Mash1, a transcription factor necessary for the differentiation of ORNs in the ON (Cau et al., 2002; Cunha et al., 2012). Therefore, Skn1a was only transiently expressed in some ORN progenitors and Skn1a knockout mice still contain ORNs indicating its major role as a transcription factor for the differentiation of MVCs.

Olfactory MVCs which span the ON have also been shown to express neuropeptide Y (NPY) and to mediate the regulation of olfactory receptor neuronal apoptosis and regeneration by stimulus-induced release of NPY (Montani et al., 2006). NPY is important in the neuroproliferation of hippocampal, subventricular zone and olfactory precursors (Hansel et al., 2001; Howell et al., 2003, 2005; Doyle et al., 2008; Malva et al., 2012; Zaben and Gray, 2013). This raises the possibility that NPY-expressing olfactory MVCs link peripheral signals on the surface of the olfactory mucosa to assist in the proliferation and differentiation of olfactory precursor cells at the base of the ON. *Additionally in the olfactory neuropeithelium and bulb, NPY is expressed in olfactory ensheathing cells during prenatal development as well as in the adult (Ubink et al., 1994; Ubink and Hökfelt, 2000). During olfactory development another proposed and distinct role of NPY is the growth and/or guidance of ORN axons toward their target glomeruli in the olfactory bulb (Ubink and Hökfelt, 2000). During olfactory axonal regeneration, NPY expression in olfactory ensheathing cells is a source of growth- and neurite-promoting factors (Ubink et al., 1994). Less is known about the role of NPY in MVCs and for the purposes of this manuscript, the focus will be to study the role of NPY in MVCs.*

To study the role of NPY in the differentiation of Trpm-5-positive olfactory MVCs we have employed two independent mouse models. The first mouse model involved X-ray irradiation of the mouse snout, which decreases the proliferation of basal precursor cells and expression of transcriptional regulators. The subsequent expression levels and pattern

of localisation of NPY, Trpm-5 and Skn-1a in the ON was examined at various time points post-irradiation. The second model was NPYCre knock-in mice model which allows NPY to be specifically expressed only in NPY-expressing cells in an organ and age dependant manner. A nasal irrigation using AAV-NPY-FLEX vector was performed to express NPY unilaterally in the adult mouse ON to examine the expression of Skn-1a and Trpm-5 following age and site specific activation of NPY.

2. Materials and methods

2.1. Animals

All experiments were carried out using adult (approximately 8 weeks) male wild-type C57BL/6J, NPYGFP and NPYCre mice and have been approved by the Garvan Institute of Medical Research/St Vincent's Hospital Animal Ethics Committee and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes (8^{th} edition, 2013). Animals were kept in a 12-hour light/dark cycle, with food and water *ad libitum* and handled for 1 week before experimentation in order to diminish their stress or anxiety.

2.2. X-ray irradiation

Adult wild type male C57BL/6J mice were anaesthetized with an intraperitoneal injection of 75 mg/Kg Ketamine (Maylab, Slacks Creek, Qld) plus 15 mg/Kg Xylazine (Troy Laboratories Pty Ltd, Glendenning, NSW) and placed in a circular Perspex pie chamber with 12 compartments. This was covered with a lead shield designed to protect the whole body except the nose snout and mice were irradiated at 8 Gγ/min as previously described (Cunha et al., 2012). Irradiated mice were assigned to one of 5 groups with survival times of 2 hours (n=3 mice), 24 hours (n=8 mice), 1 week (n=8 mice), 2 weeks (n=8 mice), and 5 weeks (n=8 mice).

2.3. Viral vector infusions

The NPYCre (NPYCre/NPYCre homozygote) knock-in mouse model, which represents a global NPY knockout model (Shi et al., 2013) allows NPY to be specifically expressed in NPY expressing cells only, since Cre-recombinase gene is under the control of the endogenous NPY promoter. Eight-week old mice were anaesthetized with an intraperitoneal injection of 75 mg/Kg Ketamine plus 15 mg/Kg Xylazine to allow for the unilateral nasal infusion of AAV-NPY-FLEX (n=6) or AAV-empty vector (n=4). 1µl of 1 X 10^9 Pfu/µl of virus was prepared and drawn up into a 1µl Hamilton syringe with flexible tubing attached to the needle and unilaterally inserted approximately 3mm into the nasal cavity. The solution was slowly infused into one nostril and the needle was left

in place for 10 minutes to allow diffusion of the virus. The mice were placed on heating mat to allow recovery and then returned to their home cage. 1-2 days post viral infusion, 1µl of Tamoxifen (Sigma, St. Louis, MO) (20µg/µl) was infused into the same nostril as the AAV-NPY-FLEX or AAV-empty vector. The mice were frequently weighed and checked for adverse effects such as increased nasal discharge. Following 14 days post infusion the mice were perfused as per protocol described below.

2.4. Perfusion, tissue collection and processing

Mice were anaesthetized with an intraperitoneal injection of 80 mg/kg Euthal and perfused with heparinized saline (10 IU/ml) followed by 4 % paraformaldehyde (PFA, Proscitech, Kirwan, Qld). Olfactory tissue was dissected and post-fixed in 4 % PFA overnight. The ON was decalcified for 7 days in 15%EDTA/4 % PFA and processed for paraffin embedding. Coronal serial sections of 6 µm were collected via a microtome (Leica, Heidelberg). Tissue sections were left at 60°C for 1-2 hours for melting of the paraffin, dewaxed in Histoclear solution (National Diagnostics, Atlanta, GA) and rehydrated through a graded series of alcohol.

2.5. Immunohistochemistry

Immunohistochemical analysis was performed using the following antibodies: Trpm-5, Skn1a, NPY, GFP, OMP and ChAT (see table 1 for suppliers). Non-specific staining was blocked in 10 % goat, rabbit and horse serum (Sigma) plus 1 % bovine serum albumin (Sigma) for 1 h. Sections of ON from WT control, X-ray irradiated, NPYGFP and NPYCre mice were incubated with the primary antibodies and incubated overnight at 4°C (for concentrations see table 1). Control sections were incubated with 1 % bovine serum albumin and processed in parallel. Endogenous peroxidase activity was quenched in 0.3% H_2O_2 (Ajax Finechem Pty Ltd, Taren Point, NSW) in PBS for 15 min. Sections were incubated with the respective biotinylated goat anti-rabbit, horse anti-mouse or rabbit anti-goat secondary antibodies (1:300, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Avidin-Horseradish Peroxidase complex was prepared

using the Vector ABC Elite kit (Vector Laboratories) according to the manufacturer's recommendations. Immunoreactivity was visualised using 3, 3'-diaminobenzidine (Dako, Carpinteria, CA) as the chromogen and slides mounted using Aquamount (BDH, VWR).

For double labelling immunofluorescence, the following antibody combinations were adopted: anti-ChAT/anti-Trpm5, anti-Skn1a/Trpm5 and NPY/Trpm5. Alexa Fluor® secondary anti-rabbit (488 and 594), anti-mouse (488) and anti-goat (488) (Molecular Probes, Abcam, Cambridge, UK) were used at a concentration of 1:50. Fluorescent slides were mounted using Fluoromount (Dako).

Table 1: The experimental details of the primary antibody used in immunohistochemistry. (BF=brightfield; IF=immunofluorescence)

Primary antibody	**Species**	**Concentration**	**Antigen retrieval**	**Supplier**
Trpm-5	goat rabbit	1:20 1:800 BF/1:700 IF	TE pH9.0/60^{o}C TE pH9.0/60^{o}C	Abcam
Skn-1a	rabbit	1:600 BF & IF	nil	Abcam
NPY	mouse	1:30 BF & IF	Citrate buffer	Sigma
GFP	rabbit	1:1000	TE pH9.0/60^{o}C	Invitrogen (Carlsbad, CA)
OMP	goat	1:1000	nil	Santa Cruz (Santa Cruz, CA)
ChAT	goat	1:30 BF & IF	TE pH9.0/60^{o}C	Millipore (Temecula, CA)

2.6. Imaging and Cellular Quantification

For all brightfield and fluorescence immunolocalisation analysis, sections were examined using an upright fluorescence microscope (Axiophot, Carl Zeiss, Oberkochen, Germany)

and photomicrographs were acquired using a 40X, 63X and 100X oil objectives on an AxioCam digital camera (Carl Zeiss MicroImaging GmbH).

Cellular quantification at the various time points following X-ray irradiation examined coronal sections randomly chosen from anterior to posterior regions. For each animal and marker, 5 different areas of the ON in the coronal section of the nose were quantified by counting the number of positively stained cells, corresponding to the middle of the nasal septum, one more superior and one more inferior area of both the left and right turbinates. Each area quantified corresponds to 300 μm^2 of the ON. The mean number of positive cells between areas was determined for each section and these values used to determine the mean number of positive cells between animals (n=3-5).

2.7. Statistical analysis

All results were expressed as mean ± SEM plotted on a graph and analysed using GraphPad Prism software (version 6.0). Statistical significance was set at $p \leq 0.05$. A one-way repeated measures ANOVA was performed for comparison between all groups (control, 24 hour, 1, 2 and 5 week survival).

3. Results

3.1. Subcellular localisation of NPY, Trpm5 and ChAT in olfactory MVCs

MVCs have been described in the ON as bearing microvilli at their apex, with their cell body situated in the upper third of the ON and contain a thick basal process that projects towards the base of the neuroepithelium without penetrating the basal lamina. In the experiments performed in this study, the MVCs were shown to express NPY, Trpm5 and ChAT. As shown in Figure 1A, anti-NPY labelling in wildtype mice is shown to be in the cell soma of MVCs. The NPYGFP mice express GFP under the NPY promoter and in Figure 1B, GFP expression is evident in the apical region of the cell soma in MVCs. *In Figures 1A and B, NPY is also present in the olfactory ensheathing cells in the lamina propria (as indicated by asterisks).* Further immunolocalisation experiments using anti-Trpm5 and anti-ChAT antibodies have also been able to show expression of these factors

in the cytoplasm of MVCs situated apically within the ON (Fig. 1C and D). Some MVC processes are immunopositive for these factors that terminate just above the basal lamina. To ascertain whether NPY, Trpm5 and ChAT were labelling the same or different types of MVCs, double labelling immunohistochemistry was performed. Figure 1E shows that the MVCs are co-labelled with NPY and Trpm5 in the cell soma. The same is true for ChAT and Trpm5 double labelling immunofluorescence (Fig. 1F).

3.2. Mapping of regions within the ON expressing Trpm5 and Skn1a following X-ray irradiation of the wildtype mouse snout

Figure 2 shows representative diagrammatic images of positive and negative regions of the ON labelled with Trpm5 and Skn1a in control and irradiated mice. Various sections ranging from posterior to anterior were analysed in control mice and those mice with various survival times following irradiation (24 hours, 1, 2 and 5 weeks). At least three mice from each group were analysed. The results show that the presence of Trpm5 labelled cells appear in a symmetrical fashion on both sides of the septum and become more widespread 5 weeks following irradiation, particularly in dorsomedial and dorsolateral regions (Fig. 2 A and B). Analysis of Skn1a also shows a symmetrical pattern of localisation throughout the ON (Fig. 2C). 5 weeks post-irradiation Skn1a-positive cells become more widespread ventrally (Fig. 2D).

3.3. Quantification of Trpm5-, Skn1a- and NPY-positive MVCs following X-ray irradiation

Analysis of NPY-positive MVCs throughout the ON in control and post-irradiated ON, indicates there were no significant differences in the numbers of NPY-positive MVCs due to irradiation treatment of the mouse snout (Fig. 2E). However, analysis of Trpm5-positive MVCs shows that compared to control tissue, there were significantly increased numbers of Trpm5-positive MVCs at both 2 and 5 weeks post irradiation (Fig. 2F). Two weeks post-irradiation, the numbers of Skn1a-positive cells throughout the ON was significantly increased (Fig. 2G).

3.4. Pattern of NPY localisation in MVCs post-irradiation

Immunolocalisation of NPY in control wildtype mice and 2 hours, 24 hours, 1, 2 and 5 weeks post-irradiation is shown in Figures 3A-F. NPY localisation in MVCs is mostly confined to the cell soma with an occasional process being labelled for this neuropeptide. Two hours post-irradiation NPY labelling can be seen in a representative image (Fig. 3B) in MVC soma and processes that extends down to the basal region of the process situated in the basal lamina. This is also the case 24 hours, 1 and 2 weeks post-irradiation (Fig. 3C-E). By 5 weeks post-irradiation (as with the non-irradiated control tissue), only a subset of MVCs show NPY in the processes as well as in the cell soma (Fig. 3F). *The presence of NPY expression in olfactory ensheathing cells in the lamina propria is consistent at all time points post-irradiation and unaffected by X-ray irradiation (Fig. 3A-F).*

3.5. Unilateral nasal irrigation of NPYCre mice with AAV-NPY-FLEX vector

To examine MVCs with and without NPY expression in adult mice, NPYCre knock-in mice were employed and NPY was specifically re-introduced only in NPY MVCs within the ON while lacking everywhere else. In this model, the Cre-recombinase gene is under the control of the endogenous NPY promoter. A unilateral nasal irrigation with an AAV-NPY-FLEX vector guarantees that Cre-recombinase is only active in NPY expressing MVCs and that inversion of the inactive NPY gene in the AAV-NPY-FLEX vector to the active form only occurs in these NPY expressing cells. Successful activation of NPY expression in the ON through this method is shown in figure 4A, by the expression of the vector tag mCherry red fluorescence gene. No mCherry was expressed when the nasal cavity was irrigated with the empty vector (Fig. 4D and E). The septum is indicated with a dotted line and separates the right and left hand sides of the nasal cavity. Only when the vector is introduced unilaterally to the nasal cavity are mCherry-expressing MVCs *and olfactory ensheathing cells* present. When NPY is expressed unilaterally, there are differences in morphological characteristics throughout the ON. For example the thickness of the ON is less when no vector has been introduced (Fig. 4B-E). Therefore, we analysed the presence of mature ORNs throughout the ON using by OMP expression. The results show that OMP-positive mature ORNs are present following the introduction

of the viral vector but are absent without the presence of the vector (Fig. 4B and C) which would explain why there is a difference in the thickness of the ON. This occurred in all of the animals analysed (n= 6).

In figure 5 the NPYCre mice were used to examine the expression of Trpm5 and Skn1a upon NPY expression in the ON. Trpm5 expression is shown in the cytoplasm of the MVCs following infusion of the AAV-NPY-FLEX vector, whereas Skn1a expression can be seen in the nucleus of a subpopulation of cells throughout the ON, mostly in layer adjacent to the basal lamina. There are also some cells where Trpm5 and Skn1a are co-localised indicating sustained expression of the transcription factor Skn1a during differentiation of the Trpm5-positive MVCs. There was no expression of either Trpm5 or Skn1a in the no vector control side of the nasal cavity.

4. Discussion

Immunolocalisation experiments in this study have confirmed the expression of NPY, Trpm5 and ChAT in MVCs of the wildtype mouse ON (Montani et al., 2006; Ogura et al., 2011). In addition, GFP is expressed in the olfactory MVCs in NPYGFP mice. This expression is evident in the cell soma that is situated apically within the ON and some of whose processes extend down to the basal lamina. *NPY immunolocalistion is also present in olfactory ensheathing cells within the lamina propria of the ON. The functional implications of olfactory ensheathing glia are their association with elongating axons during development and regeneration and the presence of NPY in these cells suggests its role as a growth and survival factor (Ramon-Cueto and Avila, 1998). However, the role of NPY and the differentiation of olfactory MVCs is not as well examined and is the focus of these studies.*

The description of Trpm5-positive MVCs has been well documented (Hansen and Finger, 2008). Trpm5a and Trpm5b type MVCs have been documented in addition to non-Trpm5 type MVCs. Using electron microscopy, the point of difference of the MVCs is their morphological appearance as well as the appearance of the microvilli. However, what has been shown in common is that they are not ORNs. Our study analysed the

immunolocalisation of MVCs at the level of the light microscope. What is evident from our study is that NPY-, Trpm5- and ChAT-positive MVCs used in our analysis are the same population of MVCs. These antibodies have been co-localised in the microvillar cell soma. Co-localisation of Trpm5 and ChAT in MVCs as well as the spatial distribution pattern of these cells has also been described previously by Ogura et al., 2011 and Lin, 2008.

Previous reports have shown that MVCs are involved in the stimulus release of NPY for the generation of ORNs (Montani et al., 2006). NPY-mediated neurogenesis is likely to be caused by its neuroproliferative effect on multipotent olfactory stem cells (Doyle et al., 2008). Neuropeptides and neurotransmitters are also thought to be involved in the peripheral modulation of the sense of smell (Kanekar et al., 2009; Lucero, 2013).
As NPY continues to be expressed in fully differentiated olfactory MVCs it is thought to have additional roles in the ON. NPY signalling is a target for improving recovery following olfactotoxicant exposure (Jia & Hegg, 2014), however, bulbectomy does not evoke NPY release (Jia & Hegg, 2014) suggesting that NPY from MVCs is induced by peripheral modulation. This is supported by experiments performed by Kanekar et al. (2009) that show the release of NPY is induced from olfactory slices following the addition of ATP, a signal of cellular stress. The olfactory mucosa is innervated via sympathetic, parasympathetic, trigeminal and terminal nerves. It has been suggested that Trpm5/ChAT-expressing MVCs react to certain chemical or thermal stimuli and release acetylcholine from parasympathetic fibres to modulate activities of neighbouring supporting cells and ORNs (Lucero, 2013; Ogura et al., 2011).

Following X-ray irradiation of the ON, we have previously shown a reduction of proliferating multipotent olfactory stem cells coincident with a reduction in expression of transcription factors (Mash1 and Pax6) as well as a decrease in the sense of smell (Cunha et al., 2012). Therefore we used this model to examine the effect on olfactory NPY-, Trpm5- and Skn1a-positive MVCs. It is very well known that the expression of olfactory receptor genes throughout the ON is spatially regulated into zones (Ressler et al., 1993) such that responses to a single odorant may be confined to a single zone within the ON.

Following X-ray irradiation, the regions within the ON containing Trpm5-positive and Skn1a-positive MVCs changed according to survival times post irradiation. This indicates that external factors effect expression of Trpm5 and Skn1a in MVCs in a regional manner. Furthermore, X-ray irradiation also effects the protein expression within the MVC such that NPY becomes increasingly expressed in the basal region of the MVC as a response. The majority of MVCs respond to odours (Hegg et al., 2010; Ogura et al., 2011) however do not send axons to the olfactory bulb. MVCs are associated with the trigeminal fibres although direct evidence to show they project to olfactory bulbs is lacking. NPY-containing MVCs under the control of odorant or trigeminal nerve do play a role in providing trophic factors to the ON. The detection and discrimination of odours is affected by the differential presence of nasal mucous (Cunha et al., 2012) following X-ray irradiation with a decrease in the sense of smell occurring at the 24 hour-1 week time point (Cunha et al., 2012) returning to normal 2-5 weeks post-irradiation. It is possible that NPY-mediated cell signalling responds to external factors causing a reduced sense of smell and hence the numbers and regional expression of Skn1a- and Trpm5-positive cells 2-5 weeks post-irradiation.

Our analysis employing NPYCre knock-in mice shows that in order for the transcription factor Skn1a to be expressed along with subsequent differentiation of Trpm5-positive MVCs, it is essential for NPY to be present. Following unilateral irrigation of the nasal cavity with AAV-NPY-FLEX vector, NPY expressing cells are evident and additionally they also express Trpm5. There is also co-expression of Skn1a and Trpm5 in a subpopulation of differentiating MVCs within the ON when NPY is expressed. Previous work by Yamaguchi et al. (2014) clearly shows that in Skn1a knock-out mice, MVCs are not produced. Here we were able to show that expression of NPY is required for the expression of Skn1a and the subsequent differentiation of Trpm5-MVCs. Previous work by our laboratory and others has shown that NPY is involved in the neuroproliferation of Mash1-positive neuronal progenitor cells (Hansel et al., 2001; Doyle et al., 2008). Yamaguchi et al. (2014) showed there was only a small population of progenitors that co-expressed Skn1a and Mash1 indicating that Skn1a-positive progenitors predominantly differentiate into Trpm5-positive MVCs whereas as Mash1-positive progenitors

differentiate into ORNs. Therefore, NPY is involved in the differentiation of both ORNs and MVCs in addition to the normal functioning of MVCs to act as cells linking peripheral signals to the normal functioning of the olfactory system.

Conclusion

Using various scientific models, our study confirms that NPY-expressing MVCs have an important role in regulating neurogenesis of the ON. Post-irradiation there was not an increase in the overall numbers of NPY-positive olfactory MVCs in the X-ray irradiated mice. However, the expression within the MVCs was cytoplasmic in control animals and became more widespread throughout the processes reaching to the basal lamina post-irradiation indicating NPY signalling to basal cells following external or peripheral events at the apical level of the ON. This results in the expression of the transcription factor Skn1a that promotes the differentiation of Trpm5-positive MVCs. Confirmation of the necessity for the expression of NPY for MVC differentiation was shown in NPYCre knock-in mice where Skn1a was only expressed to give rise to Trpm5-positive MVCs when NPY was present. Therefore, NPY is essential for the differentiation of olfactory MVCs, which then relays neuroproliferative signals to basal precursor cells maintaining neurogenesis in the ON.

Acknowledgements

We acknowledge Tanya Wyatt for technical assistance in the embedding and sectioning of the paraffin embedded tissue blocks. This work was funded by philanthropic grants and donations from the Mason Foundation and Rosemary Pryor Trust. Carla Cunha acknowledges Fundação para a Ciência e a Technologia for Fellowship SFRH/BDP/87071/2012. The authors indicate no potential conflict of interests.

References

Calof, A.L., Chikaraishi, D.M., 1989. Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. Neuron 3, 115-127.

Carr, V.M., Farbman, A.I., Colletti, L.M., Morgan, J.I., 1991. Identification of a new non-neuronal cell type in rat olfactory epithelium. Neuroscience 165, 584-600.

Cau, E., Casarosa, S., Guillemot, F., 2002. Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development. 129, 1871-1880.

Cunha, C., Hort, Y., Shine, J., Doyle, K,L., 2012. Morphological and behavioural changes occur following the X-ray irradiation of the adult mouse olfactory neuroepithelium. BMC Neurosci. 13, 134-147.

Doyle, K.L., Karl, T., Hort, Y., Duffy, L., Shine, J., Herzog, H., 2008. Y1 receptors are critical for the proliferation of adult mouse precursor cells in the olfactory neuroepithelium. J Neurochem. 105, 641-652.

Graziadei, P.P., Graziadei, G.A., 1979. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol. 8, 1-18.

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

Hansen, A., Finger, T.E., 2008. Is Trpm5 a reliable marker for chemosensory cells? Multiple types of microvillous cells in the main olfactory epithelium of mice. BMC Neurosci. 9, 115-126.

Hegg, C.C., Jia, C., Chick, W.S., Restrepo, D., Hansen, A., 2010. Microvillous cells expressing IP3 receptor type 3 in the olfactory epithelium of mice. Eur. J. Neurosci. 32, 1632-1645.

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

Huard, J.M., Youngentob, S.L., Goldstein, B.J., Luskin, M.B., Schwob, J.E., 1998. Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. J. Comp. Neurol. 400, 469-486.

Jia, C., Hegg, C.C., 2014. Effect of IP3R3 and NPY on age-related declines in olfactory stem cell proliferation. Neurobiol. Aging 36, 1045-1056.

Jourdan, F., 1975. Ultrastructure of the olfactory epithelium of the rat: polymorphism of the receptors. CR Acad. Sci. Hebd. Seances. Acad. Sci. D. 280, 443-446.

Kanekar, S., Jia, C., Hegg, C.C., 2009. Purinergic receptor activation evokes neurotrophic factor NPY release from neonatal mouse epithelial slices. J. Neurosci. Res. 87, 1424-1434.

Leung, C.T., Coulombe, P.A., Reed, R.A., 2007. Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat. Neurosci. 10, 720-726.

Lin, W., Ezekwe Jr, E.A., Zhao, Z., Liman, E.R., Restrepo, D., 2008. TRPM-5-expressing microvillous cells in the main olfactory epithelium. BMC Neurosci. 9, 114-126.

Lucero, M., 2013. Peripheral modulation of smell: Fact or fiction? Seminars in Cell and Dev. Biol. 24, 58-70.

Malva, J.O., Xapelli, S., Baptista, S., Valero, J., Agasse, F., Ferreira, R., Silva, A.P., 2012. Multifaces of neuropeptide Y in the brain – neuroprotection, neurogenesis and neuroinflammation. Neuropeptides 46, 299-308.

Matsumoto, I., Ohmoto, M., Narukawa, M., Yoshihara, Y., Abe, K., 2011. Skn-1a (Pou2f3) specifies taste receptor cell lineage. Nat. Neurosci. 4, 685-687.

Montani, G., Tonelli, S., Elsaesser, R., Paysan, J., Tirindelli, R., 2006. Neuropeptide Y in the olfactory microvillar cells. Eur. J. Neurosci. 24, 20-24.

Moran, D.T., Rowley, J.C 3rd., Jafek, B.W., 1982, Electron microscopy of human olfactory epithelium reveals a new cell type: the microvillar cell. Brain Res. 253, 39-46.

Ogura, T., Szebenyi, S.A., Krosnowski, K., Sathyanesan, A., Jackson, J., Lin, W., 2011. Cholinergic microvillous cells in the mouse MOE and effect of acetylcholine on olfactory sensory neurons and supporting cells. J. Neurophysiol. 106, 1274-1287.

Ramón-Cueto, A., Avila, J., 1998. Olfactory ensheathing glia: Properties and function. Brain Res. Bull. 46, 175-187.

Rowley, J.C., Moran, D.T., Jafek, B.W., 1989. Peroxidase backfills suggest the mammalian olfactory epithelium contains a second morphologically distinct class of bipolar sensory neuron: the microvillar cell. Brain Res. 502, 387-400.

Schwob, J.E., Huard, J.M., Luskin, M.B., Youngentob, S.L., 1994. Retroviral lineage studies of the rat olfactory epithelium. Chem. Senses 19, 671-682.

Shi, Y.C., Lau, J., Lin, Z., Zhang, H., Zhai, L., Sperk, G., Heilbronn, R., Mietzsch, M., Weger, S., Huang, X.F., Enriquez, R.F., Baldock, P.A., Zhang, L., Sainsbury, A., Herzog, H., Lin, S., 2013. Arcuate NPY controls sympathetic output and BAT function via a relay of tyrosine hydroxylase. Cell Metab. 17, 236-248.

Ubink, R., Halasz, N., Zhang, X., Dagerlind, A., Hökfelt, T., 1994. Neuropeptide tyrosine is expressed in ensheathing cells around the olfactory nerves in the rat olfactory bulb. Neuroscience 60, 709-726.

Ubink, R., Hökfelt, T., 2000. Expression of Neuropeptide Y in olfactory ensheathing cells during prenatal development. J. Comp. Neurol. 423,13-25.

Yamaguchi, T., Yamashita, J., Ohmoto, M., Aoude, I., Ogura, T., Luo, W., Bachmanov, A.A., Lin, W., Matsumoto, I., Hirota, J., 2014. Skn1a/Pou2f3 is required for the generation of Trpm5-expressing microvillous cells in the mouse main olfactory epithelium. BMC Neurosci. 15, 13-22.

Zaben, M.J., Gray, W.P., 2013. Neuropeptides and hippocampal neurogenesis. Neuropeptides 47, 431-438.

Figure Legends

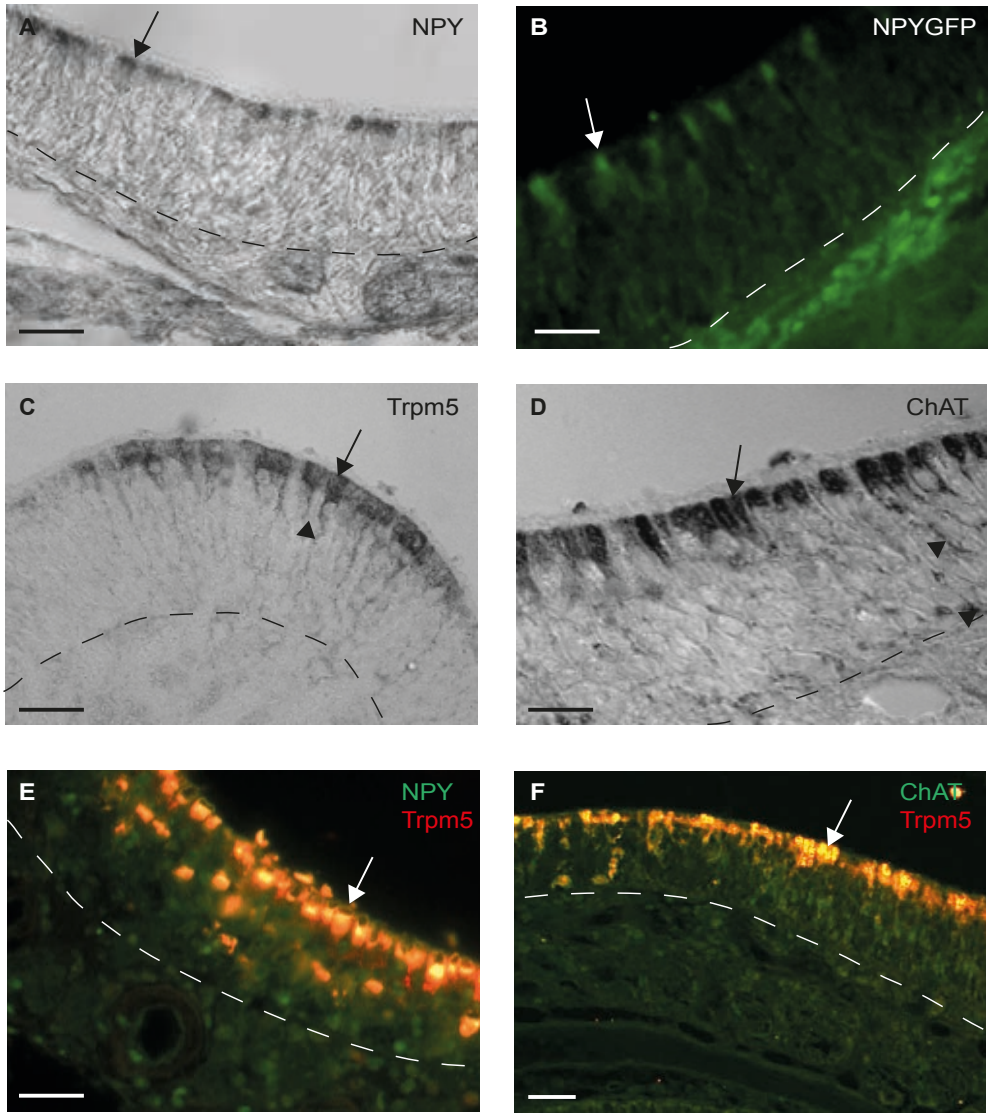
Fig. 1. Cellular localisation pattern of NPY, NPYGFP, Trpm5 and ChAT in olfactory microvillar cells (MVC). **A,** Coronal section of olfactory neuroepithelium (ON) prepared from an adult wild type mouse. NPY is localised in the cell soma of the microvillar cells situated apically in the ON (arrow) and *in olfactory ensheathing cells in the lamina propria (*);* **B,** Coronal section of the ON taken from an NPYGFP mouse. GFP-positive fluorescence appears in the cell soma (particularly the apex) of olfactory MVCs (white arrow) and *olfactory ensheathing cells in the lamina propria (*);* **C,** Trpm5-positive immunoreactivity appears in the cell soma of the MVCs with some MVC processes evident (arrowhead); **D,** Immunolocalisation of ChAT can be seen in the cytoplasm of MVCs (black arrow) as well as a process terminating just above the basal lamina (black arrowheads); **E,** Co-localisation of Trpm5 and NPY in olfactory microvillar cells (yellow) and **F,** Co-localisation of Trpm5 and ChAT in olfactory MVCs (yellow). Dotted lines indicate basal lamina/ON border. Scalebar=20μm

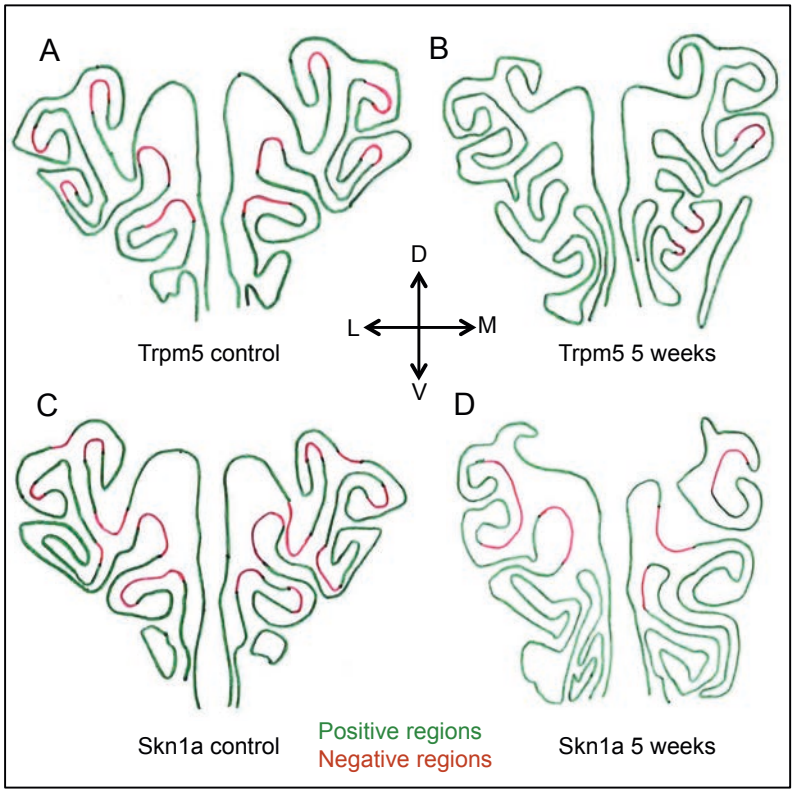
Fig. 2. Mapping and quantification of olfactory neuroepithelial regions before and after X-ray irradiation of the mouse snout. **A,** Non-irradiated mouse control showing areas containing Trpm5-positive MVCs shown in green; **B,** 5 weeks post-irradiation, Trpm5-positive MVCs are more widespread throughout the ON. Negative regions only appear medially; **C,** Non-irradiated mouse control showing areas containing Skn1a-positive cells (green); **D,** 5 weeks post-irradiation, Skn1a-positive cells are more widespread ventrally; **E,** There was no statistically significant difference in the number of NPY-positive MVCs following X-ray irradiation; **F,** At both 2 and 5 weeks post-irradiation there were statistically greater numbers of Trpm5-positive MVCs compared to controls and **G,** At 2 weeks post-irradiation there were statistically greater numbers of Skn1a-positive cells compared to controls. Green; immunopositive cells, Red; immunonegative cells. D; distal, M; medial, V; ventral, L; lateral. n.s. not significant

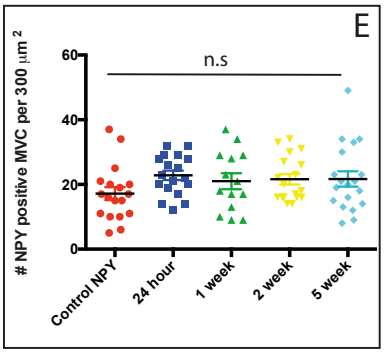
Fig. 3. Photomicrographs showing pattern of anti-NPY localisation in the ON of mice pre- and post-irradiation. **A,** Control mice (non-irradiated) express NPY in the apically situated cell soma (black arrow) and **B-F,** 2 hours, 24 hours, 1, 2 and 5 weeks post-irradiation, NPY expression extends further to the processes (white arrows) and end-feet of the MVC (white arrowhead) terminating at the basal lamina. Scalebar=10μm A; control, B; 2 hours, C; 24 hours, D; 1 week, E; 2 weeks, F; 5 weeks. *NPY-positive olfactory ensheathing cells (*).*

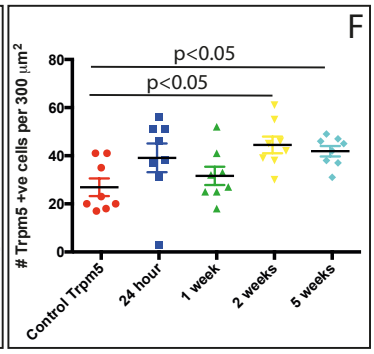
Fig. 4. Photomicrographs of the ON in NPYCre knock-in mice. **A,** mCherry expression in the ON following unilateral introduction of AAV-NPY-FLEX vector. mCherry is expressed in apically situated cell bodies of NPY expressing cells the the NPY knock-in mice (arrows) *as well as olfactory ensheathing cells (*).* The ipsilateral side (no vector) has no expression of mCherry and hence no NPY expressing cells; **B,** OMP is expressed in the ON following unilateral irrigation of AAV-NPY-FLEX vector; **C,** A higher magnification photomicrograph of the area indicated in Bi; **D,** Brightfield photomicrograph of no vector control section of ON. Thickness of either side of the septum is equivalent (see double sided arrows) and **E,** No vector control is negative for mCherry-expressing cells. Scalebar=30μm

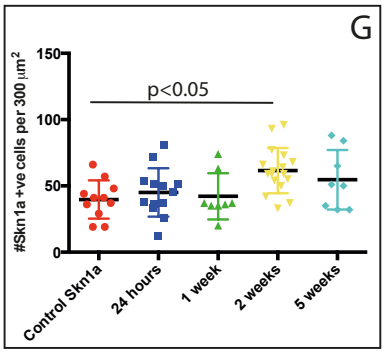
Fig. 5. Photomicrograph showing immunolocalisation of Trpm5 and Skn1a in NPYCre knock-in mice following unilateral infusion of AAV-NPY-FLEX vector. The septum that separates the right and left sides of the nasal cavity is indicated by a white dotted line. Unilateral exposure to the vector results in the expression of Trpm5 in olfactory MVCs (green) (example indicated by white arrow) and Skn1a in the nucleus of mostly basal cells (red) (example indicated by white arrowhead). Cells indicated with a white asterisk are co-labelled with Trpm5 and Skn1a. Scalebar=20μm

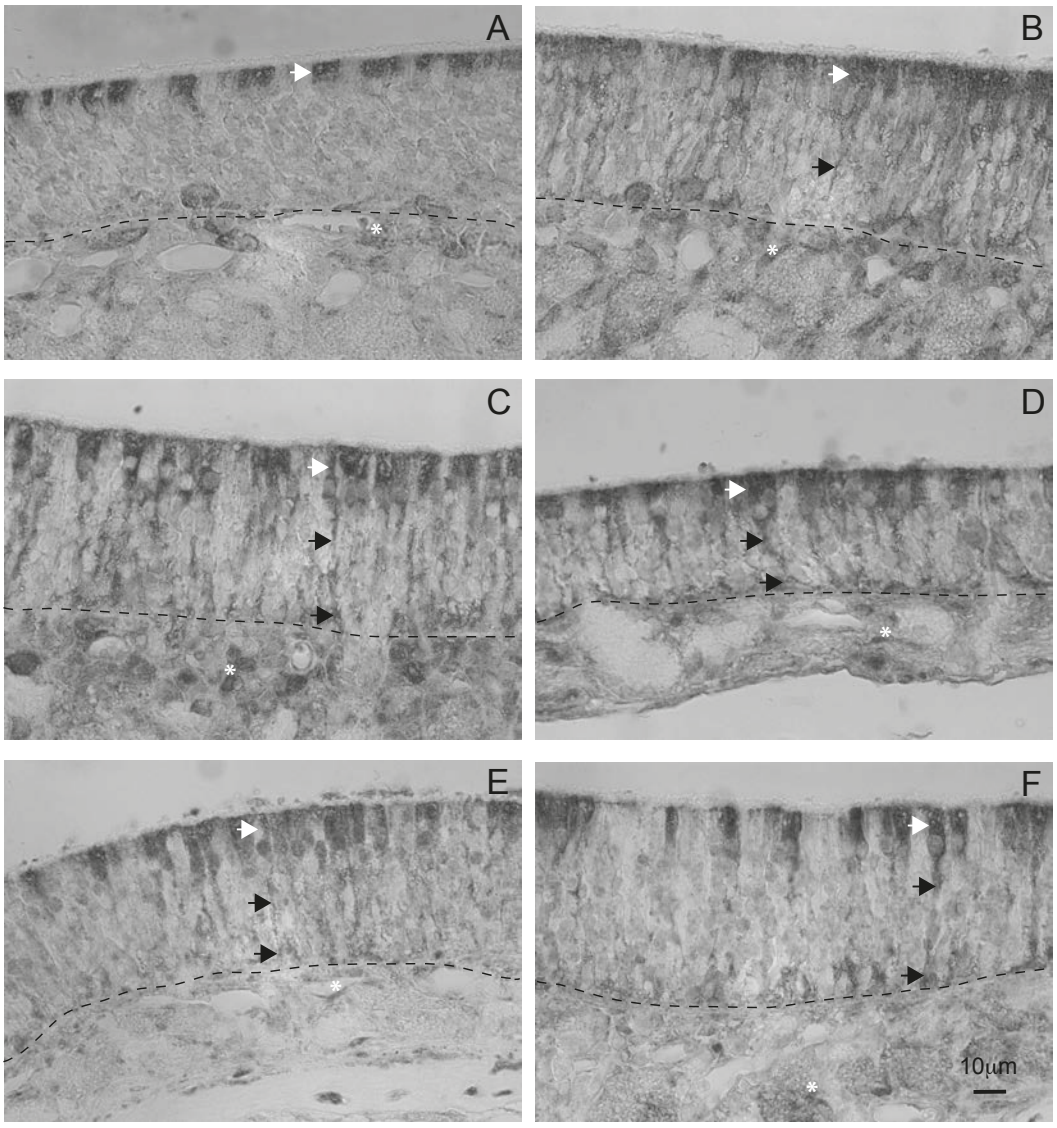

A
NPY
B
NPYGFP
C
Trpm5
D
ChAT
E
NPY
Trpm5
F
ChAT
Trpm5

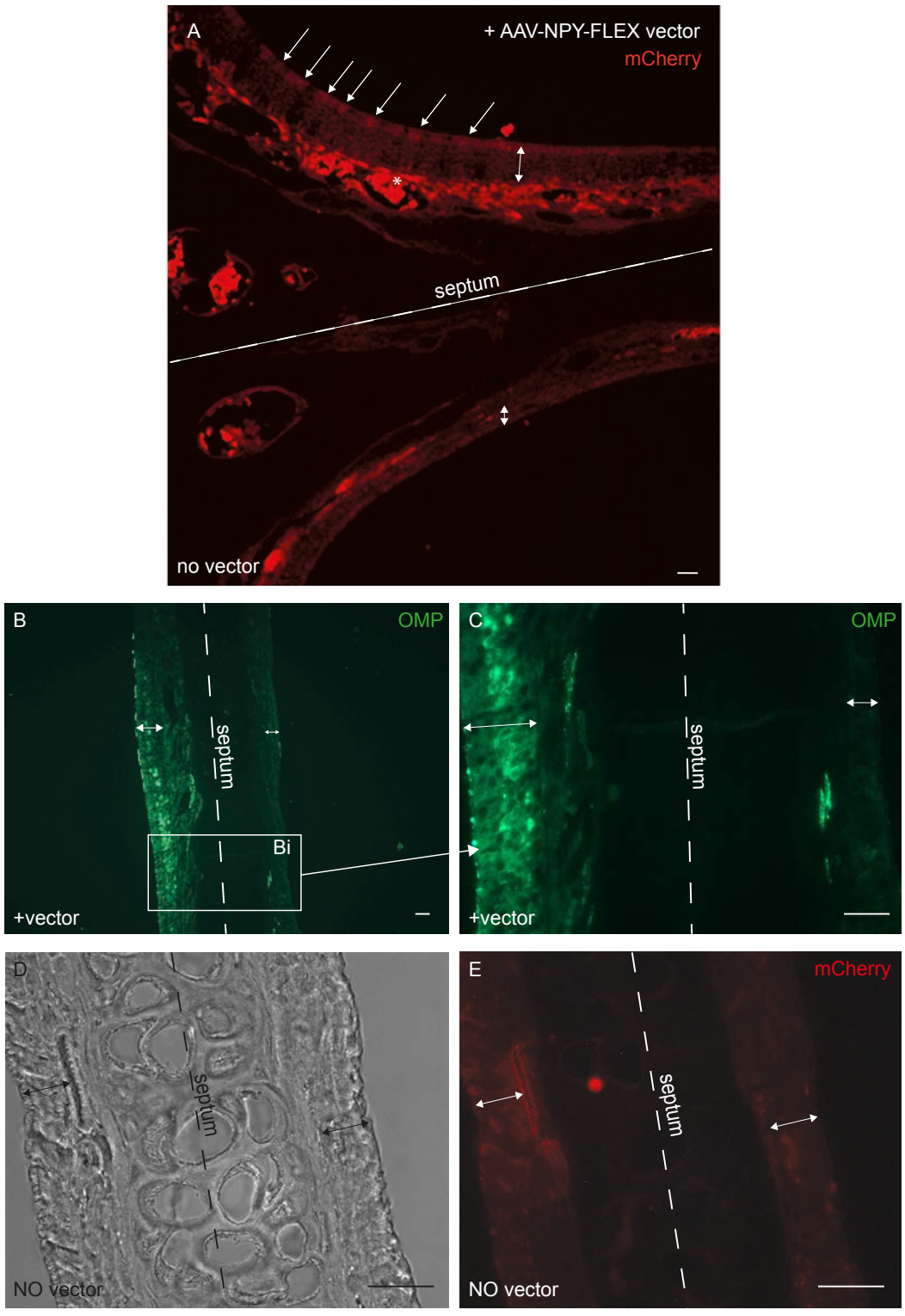

A
B
C
D
D
L
M
V
Trpm5 control
Trpm5 5 weeks
Skn1a control
Skn1a 5 weeks
Positive regions
Negative regions

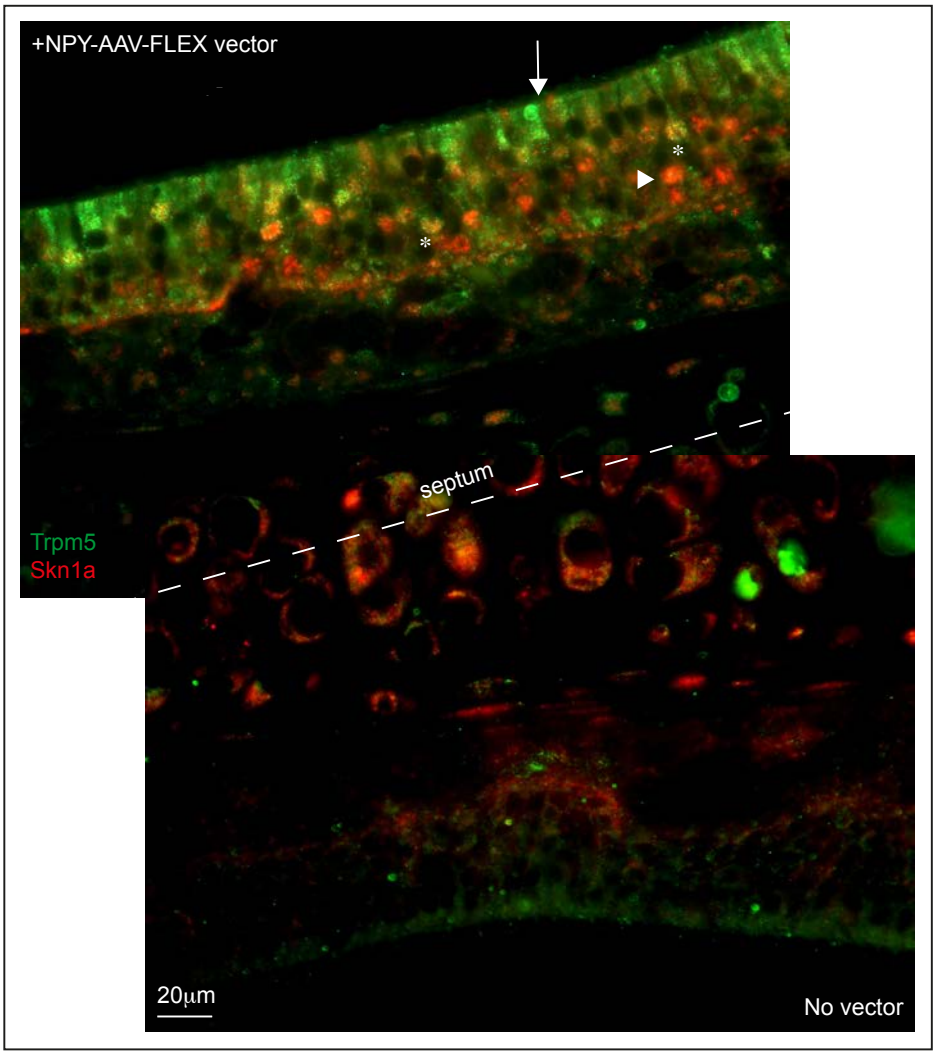

E
NPY positive MVC per 300 μm 2
n.s
Control NPY
24 hour
1 week
2 week
5 week


F
Trpm5 +ve cells per 300 μm2
p<0.05
p<0.05
Control Trpm5
24 hour
1 week
2 weeks
5 weeks


G
#Skn1a +ve cells per 300 μm2
p<0.05
Control Skn1a
24 hours
1 week
2 weeks
5 weeks


A
B
C
D
E
F
10μm


A
+ AAV-NPY-FLEX vector
mCherry
septum
no vector
B
OMP
septum
Bi
+vector
C
OMP
septum
+vector
D
septum
NO vector
E
mCherry
septum
NO vector


+NPY-AAV-FLEX vector
septum
Trpm5
Skn1a
20μm
No vector