Neuropeptides 78 (2019) 101964

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

Neuropeptide Y increases differentiation of human olfactory receptor neurons through the Y1 receptor

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ARTICLE INFO

Keywords:
Neuropeptide Y
Y1 receptor
Anosmia
Olfactory receptor neurons
Olfactory mucosa

ABSTRACT

Olfactory dysfunction significantly impedes the life quality of patients. Neuropeptide Y (NPY) is not only a neurotrophic factor in the rodent olfactory system but also an orexigenic peptide that regulates feeding behavior. NPY increases the olfactory receptor neurons (ORNs) responsivity during starvation; however, whether NPY can promote differentiation of human ORNs remains unexplored. This study investigates the effect of NPY on the differentiation of human olfactory neuroepithelial cells *in vitro*. Human olfactory neuroepithelium explants were cultured on tissue culture polystyrene dishes for 21 days. Then, cells were cultured with or without NPY at the concentration of 0.5 ng/mℓ for 7 days. The effects of treatment were assessed by phase contrast microscopy, immunocytochemistry and western blot analysis. The further mechanism was evaluated with NPY Y1 receptor-selected antagonist BIBP3226. NPY-treated olfactory neuroepithelial cells exhibited thin bipolar shape, low circularity, low spread area, and long processes. The expression levels of Ascl1, βIII tubulin, GAP43 and OMP were significantly higher in NPY-treated cells than in controls ($p < 0.05$). NPY-treated olfactory neuroepithelial cells expressed more components of signal transduction apparatuses, G_{olf} and ADCY3, than those without NPY treatment. Western blot analysis also further confirmed these findings ($p < 0.05$). Additionally, the expression levels of Ascl1, βIII2 tubulin, GAP43, OMP, ADCY3, and Golf in BIBP3226 + NPY and controls were comparable ($p > 0.05$). NPY not only increases expressions of protein markers of human olfactory neuronal progenitor cells, but also promotes differentiation of ORN and enhances formation of components of olfactory-specific signal transduction pathway through Y1 receptors.

1. Introduction

Olfactory dysfunction impedes identification of nearby environmental hazards, and lowers quality of life by reducing appetite and sexual ability (Croy et al., 2014). The incidence of olfactory dysfunction has continuously risen from 3% to 20% in general population in the past two decades (Hoffman et al., 1998; Rombaux et al., 2016). Olfactory malfunction is commonly attributed to the atrophy of the olfactory neuroepithelium (ON) with increasing age, viral infection of upper respiratory tract, and disorder of neuron connections due to head trauma (Doty, 2009; Kern et al., 2000). The loss in olfaction may result from decreased number and degree of differentiation of olfactory receptor neurons (ORNs) (Jafek et al., 2002; Yamagishi et al., 1994). The ON, which consists of specialized olfactory glands, ORNs, basal cells, microvillous cells, and sustentacular cells, has the unique ability of continual neurogenesis in the peripheral neuron systems of adult mammals (Leung et al., 2007). Although many approaches, such as vitamin A, minerals, and various growth factors have achieved promising findings to treat olfactory dysfunction, they are still inadequate in clinical treatment (Jiang et al., 2015; Reden et al., 2012). Topical or systemic application of glucocorticoids is a common clinical treatment, but its therapeutic effect is controversial. Oral steroids can improve olfaction in sinonasal disease, but the reduced sense of smell tends to recur shortly after the treatment is stopped (Blomqvist et al., 2003). Consequently, alternative treatment modalities are required for patients with olfactory loss.

Most animals, including humans, rely on their sense of smell for food seeking, food choice and the appreciation of food palatability (Rolls, 2005). Moreover, olfactory neural processing is closely linked to the physiological and nutritional status of an organism. Activity and

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https://doi.org/10.1016/j.npep.2019.101964
Received 4 April 2019; Received in revised form 4 September 2019; Accepted 6 September 2019
Available online 09 September 2019

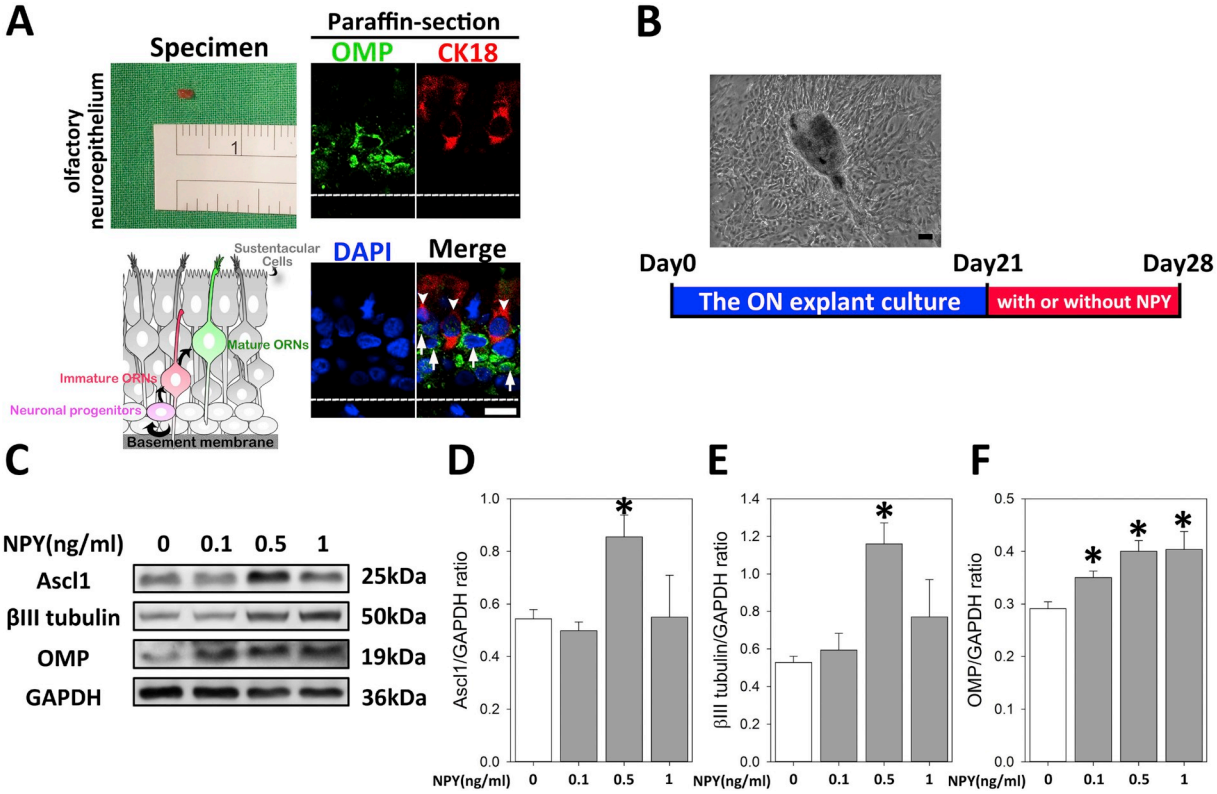

Fig. 1. Establishment of a primary HONCs culture. A tissue specimen of human olfactory neuroepithelium and the schematic diagram (A, left). Immunohistochemical staining of the ON fluorescently double labeled with OMP (green, arrow), CK18 (red, arrowhead) and DAPI (blue) representing mature ORNs, sustentacular cells and nuclei, respectively (A, right, scale bar = 20 μm). Dashed lines indicate basal membrane of the ON. Time course of the experimental design and the migrated cells from the ON explant (B, scale bar = 50 μm). The optimal concentration of NPY in altering the expression of different markers is determined using western blot (C) followed by densitometric analyses of Ascl1 (D), βIII Tubulin (E), and OMP (F). Asterisks denote $p < 0.05$, n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sensitivity in the olfactory system are increased under starvation, but reduced after satiation (Aime et al., 2007; Chaput and Holley, 1976; Prud'homme et al., 2009). Neuropeptide Y (NPY), a 36 amino-acid neuropeptide, is involved in various physiological and homeostatic processes in both the central and peripheral nervous systems (Sabban et al., 2016). Previous studies suggested that NPY regulates neuroproliferation of precursor cells in rodents in olfactory, hippocampal, and subventricular zone (Doyle et al., 2008; Hansel et al., 2001; Howell et al., 2005; Howell et al., 2003; Stanic et al., 2008). NPY is also an orexigenic peptide that controls feeding behavior (Beck, 2006). Particularly, the olfactory system becomes more active and sensitive when NPY elicits hunger responses, because NPY increases the ORNs responsivity during starvation (Negroni et al., 2012). In the ON, the microvillar cells are responsible for the production of NPY, involving the maintenance of adult ON by regulating the mitotic activity of its neuronal basal cells (Montani et al., 2006). Previous study also demonstrated that NPY operates mainly through Y1 receptors, presenting as heterogeneous forms in the ORNs of the ON (Montani et al., 2006). However, whether NPY can be adopted in treating human olfactory dysfunction remains unexplored. This study aims to investigate the effect of NPY on the differentiation of human olfactory neuroepithelial cells (HONCs) *in vitro*.

2. Materials and methods

2.1.1. Cell preparation and culture

HONCs were harvested from nasal superior turbinates near the roof of the nasal cavity in patients with chronic rhinitis during septomeatoplasty. This study was approved by an institutional review board of Far Eastern Memorial Hospital (105104-F). All patients gave informed consent. In the ON explant culture, biopsy specimens were washed with Hank's balanced salt solution; minced finely and then digested by 0.125% Trypsin/EDTA for 30 min at 37 °C. The pellets were gathered with centrifuge and resuspended in culture medium (Iscove's Modified Dulbecco's Media; Invitrogen, CA, USA) containing 10% fetal bovine serum and 1% antibiotics, and then seeded to six-wells tissue culture polystyrene (TCPS) dishes coated with laminin-co-fibronectin for 21 days. The media were then replaced with induction medium (DMEM/F12; Invitrogen) with and without NPY (0.5 ng/ml in H_2O, C932; Novoprotein, Shanghai, China) for one week. The cultures were randomly divided into two groups, namely control (medium only) and NPY. Subsequently, to investigate their mechanism, HONCs were incubated with NPY or NPY Y1 agonist [Leu^{31}, Pro^{34}]-NPY (0.5 ng/ml in H_2O, 1176, Tocris Bioscience, Bristol, UK) for one week following pretreatment with NPY Y1 receptor (NPY Y1R)-selected antagonist (BIBP3226) (50 μM in 20% acetic acid, E-3620, Bachem, CA) or induction medium with the same amount of acetic acid for 1 h.

2.1.2. Morphological examination

Cell morphology was observed under an inverse phase contrast microscope (TS-100, Nikon, Tokyo, Japan) and scanning electron microscope (SEM) (S-4800; Hitachi, Tokyo, Japan). The procedure of sample preparation for SEM was referred to our previous studies (Huang et al., 2018). Briefly, the cultures were rinsed, and fixed with 2.5% glutaraldehyde for 1 h. The specimens were then dehydrated in a graded series of ethanol solutions, dried by critical point technique with liquid CO_2, and sputter coated with gold to a nominal thickness of 25 nm. Finally, the samples were examined under a SEM. The circularity ($4\pi \times \text{area/perimeter}^2$), the percentage of spread area (cell spread area per view) and process length were measured using ImageJ

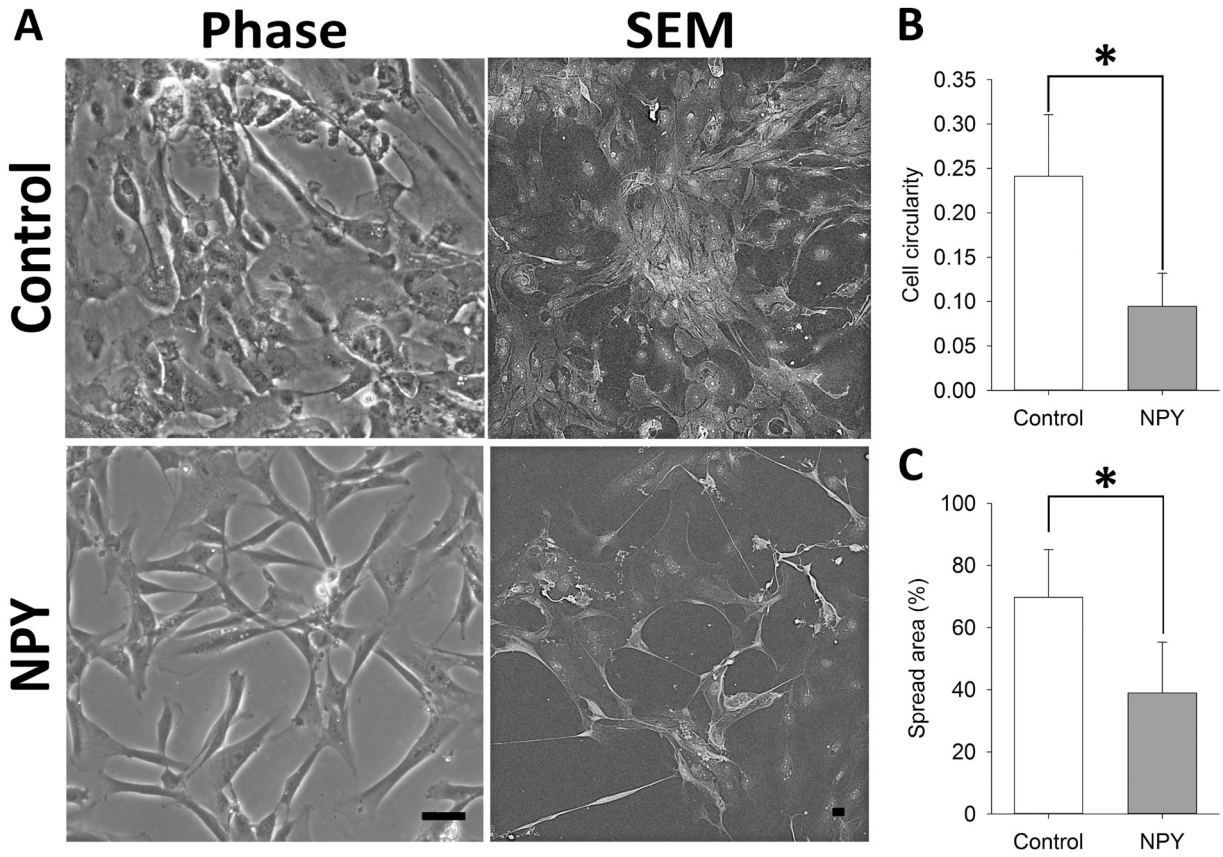

Fig. 2. Phase contrast and SEM images of HONCs cultured with or without NPY (0.5 ng/ml) (A). Cell circularity (B) and the percentage of HONC spread area (C). Data were calculated from five independent experiments with 20 randomly selected fields of view for each group (n = 5). The data Asterisks denote $p < 0.05$. Scale bar = 20 μm.

in at least 20 randomly selected fields of SEM for each group (Walters et al., 2017). Data were calculated from five independent experiments.

2.1.3. Immunofluorescence analyses

Biopsy specimens were fixed using 10% paraformaldehyde at 4 °C overnight, decalcified with 10% EDTA (pH 7.4) for one week and embedded with paraffin. Sections of 4 μm were cut for characterization following deparaffinization and rehydration through xylenes and graded alcohol. Antigen retrieval was performed using citrate buffer (pH 6.0) at 95 °C for 15 min. For immunocytochemistry, the cultured cells were fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 (X100; Sigma-Aldrich, St. Louis, MO) for 7 min at room temperature. The targeted samples were subsequently blocked in 5% bovine serum albumin (BSA) for 10 min, and incubated with primary antibodies diluted in 5% BSA, namely anti-cytokeratin18 (CK18) (1:1000, ab82254, Abcam, Cambridge, UK), anti-achaete-scute homolog 1 (Ascl1) (1:1000, ab74065, Abcam), anti-growth associated protein 43 (GAP43) (1:1000, BD612262, CA), anti-βIII tubulin (1:1000, ab118627, Abcam), anti-olfactory marker protein (OMP) (1:100, NB110–74751, Novus, CO), anti-OMP (1:100, ab62144, Abcam), anti-olfactory neuron specific-G protein (G_{olf}) (1:100, GTX110520, GeneTex,CA, USA) and anti-adenylate cyclase type 3 (ADCY3) (1:100, ab125093, Abcam). These primary antibodies were visualized using species-specific secondary antibodies conjugated to AlexaFluor488 or AlexaFluor555 (Abcam) for 1.5 h at room temperature. All primary antibodies were known to react with human antigens and no non-specific immunoreactivity by examining with secondary antibody alone. Additionally, 4′,6-diamidino-2-phenylindole (DAPI) and rhodamine conjugated phalloidin were applied to counterstain nuclear and cytoskeletal markers, respectively. Images were further taken with a confocal microscope (LSM510, Carl Zeiss, Germany). After ensuring the images were not over contrasted, each marker between the control and the NPY-treated group was randomly acquired with the even expose time.

2.1.4. Western blot analysis

The protein concentration of each lysate was measured by protein assay (Bio-Rad, CA). Denatured proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes blocked in a CISblocking buffer (CIS-Biotechnology, Taichung city, Taiwan) were incubated with the specific primary antibodies at 4 °C overnight, washed, probed in horseradish peroxidase-conjugated secondary antibodies, and finally visualized by enhanced chemiluminescence (ECL; Millipore, Billerica, MA). The primary antibodies utilized in this investigation were anti-*Ascl1* (1:1000), anti-GAP43 (1:1000, BD612262, CA), anti-*βIII tubulin* (1:1000), anti-*OMP* (1:1000, ab98124, Abcam), anti-G_{olf} (1:1000), anti-*ADCY3* (1:1000) and *GAPDH* (1:5000, ab22555, Abcam). All antibodies were known to react with human antigens. The western blotting images were acquired by UVP BioSpectrum 810, and analyzed by Vision Works LS software (UVP, CA).

2.1.5. Statistical analysis

The results were observed from at least five independent experiments among 20 subjects, with the data expressed as mean ± standard deviation (SD). Comparative analysis of these two groups was conducted using a Student's paired *t*-test. Differences between multiple groups were compared using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. A p value < 0.05 indicated a significant difference.

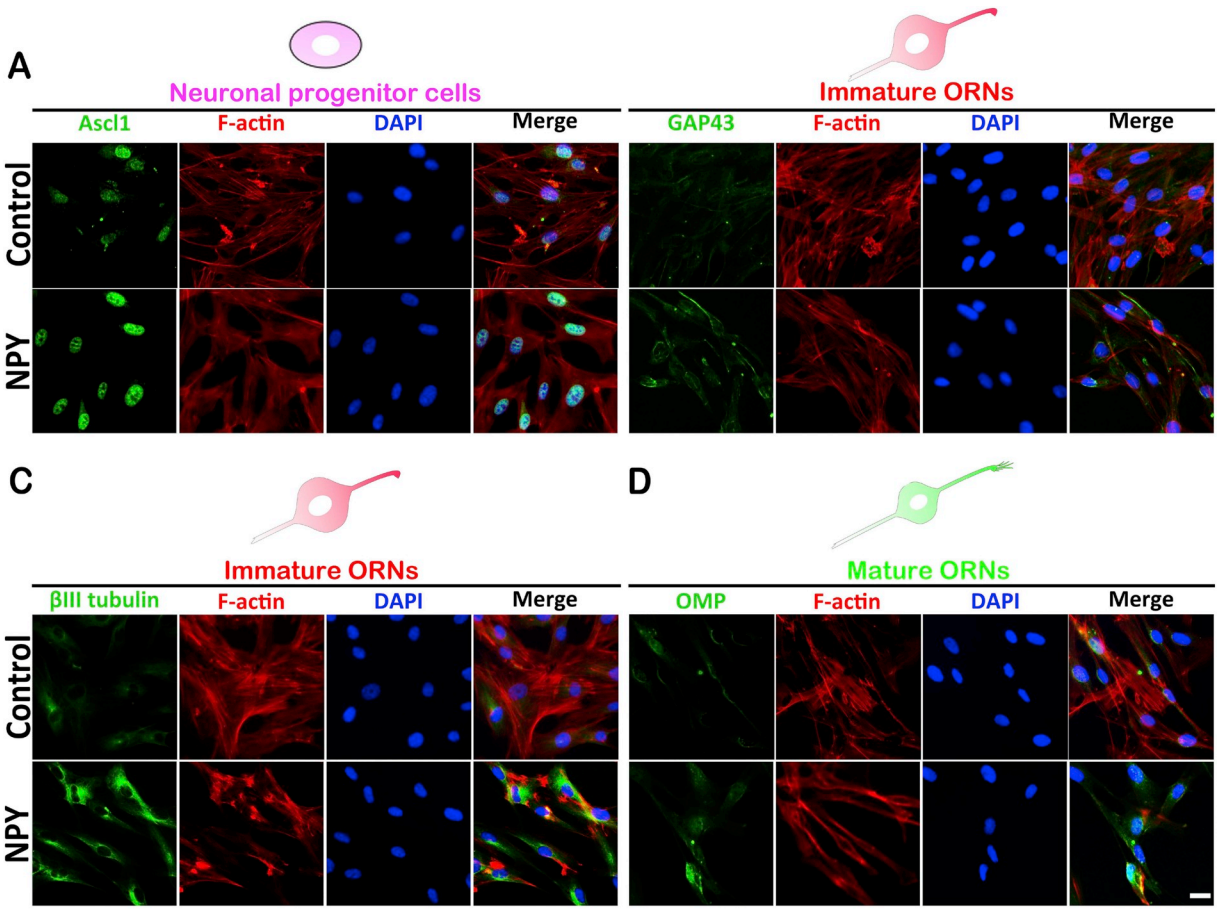

Fig. 3. Identification of neuronal progenitor cells, immature ORNs, and mature ORNs in cultures with or without NPY (0.5 ng/ml). Immunocytochemistry stained with markers (green) for Ascl1 (A), GAP43 (B), βIII Tubulin (C), and OMP (D). Cellular cytoskeletal shape (F-actin) and nuclei are labeled with rhodamine-phalloidin (red) and DAPI (blue), respectively. Scale Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1.1. Morphological characterization of HONCs and dose-response analyses of NPY

To characterize the human ON, a histological analysis was performed randomly on selected specimens, which were stained with OMP and CK18 (Fig. 1A). OMP is a representative marker of mature ORNs in adulthood, and CK18 is a marker of sustentacular cells (Holbrook et al., 2011). In native tissue, ORNs and sustentacular cells were identified in the ON (Fig. 1A). During the cultural period, the ON explant successfully adhered to the substrate and HONCs migrated from the ON. After three weeks *in vitro*, HONCs reached 70–80% confluence (Fig. 1B). Further, to determine the dose of NPY, HONCs were incubated with different concentrations of NPY ranging from 0.1 ng/mL to 1 ng/mL for 7 days after three weeks *in vitro*. A western blot analysis revealed that 0.5 ng/ml NPY significantly promoted expressions of Ascl1 and βIII tubulin (Fig. 1C & D–E, $p < 0.05$). Moreover, the expression of OMP significantly increased when the concentration of NPY was > 0.1 ng/ml (Fig. 1C–F, $p < 0.05$). Therefore, 0.5 ng/ml NPY was adopted for experiments. After 28 days *in vitro* culture, most of the cell morphology consisted of flattened and epithelial-like cells in controls (Fig. 2A). Simultaneously, the morphology in the NPY-treated cells exhibited not only thin bipolar shape with long processes measured in 83.4 ± 16.3 μm in contrast to 53.6 μm ± 15.3 μm in controls ($p < 0.05$), but also significantly lower circularity and spread area than controls ($p < 0.05$, Fig. 2).

3.1.2. The ORN differentiation of HONCs and expressions of signal transduction apparatus of ORNs

HONCs have been well characterized for stage-specific markers. In the HONCs development, a marker of neuronal progenitor cells, Ascl1, plays a crucial choice point at an early stage in ORN lineage to initiate a differentiation program (Kam et al., 2014). OMP is a prototypical form, and is expressed almost exclusively in mature ORNs, while βIII tubulin is a neuron-specific isoform of tubulin, and is widely accepted as a marker of immature ORNs (Kam et al., 2014; Iwai et al., 2008). GAP43 was also a widely used immature neuron marker (Neve et al., 1987). Immunofluorescence revealed that NPY-treated HONCs expressed higher stage-specific markers, namely Ascl1, βIII tubulin, GAP43, and OMP, than controls (Fig. 3). GAP43 and βIII tubulin were clearly present throughout the neuron, in the soma and neurites. Compared to controls, the expression of OMP was higher in NPY-treated cells (Fig. 3D). G_{olf} and ADCY3 are essential components of signal transduction pathway for odorant receptors and neurotransmitter response (Sakano, 2010). Results of immunostaining analysis showed that HONCs expressed G_{olf} and ADCY3 in their cytoplasm or cell membranes (Fig. 4). Further, western blot analysis confirmed that the expression levels of stage-specific markers and components of signal transduction pathway were higher in NPY-treated cells than in controls (Fig. 5D–J, $p < 0.05$). NPY-treated HONCs not only differentiated into ORNs, but also enhanced formation of components of signal transduction pathway.

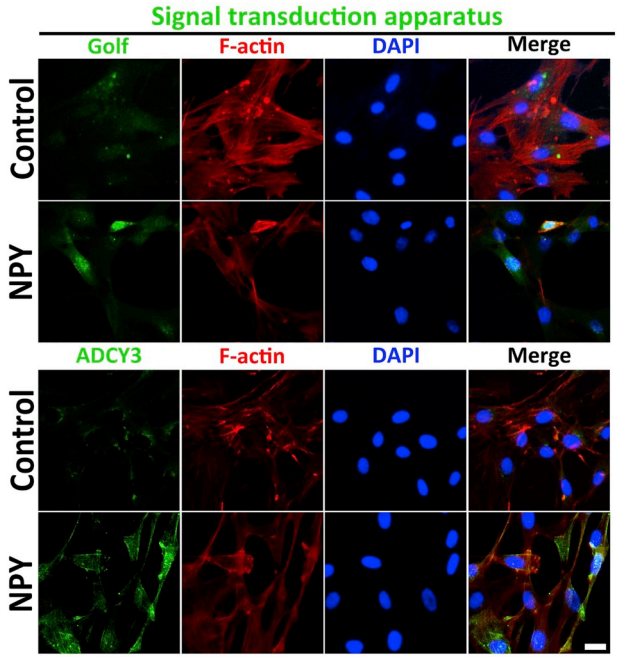

Fig. 4. Olfactory signal components in HONCs incubated with or without NPY (0.5 ng/ml). Immunocytochemistry stained with markers (green) for G_{olf} (A) and ADCY3 (B). Cellular cytoskeletal shape (F-actin) and nuclei are labeled with rhodamine-phalloidin (red) and DAPI (blue), respectively. Scale Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. The effect of NPY Y1R antagonist and NPY Y1 agonist [Leu^{31}, Pro^{34}]-NPY on the differentiation of HONCs

In order to verify the role of NPY in the ORN differentiation, the effect of NPY-mediated ORN differentiation was investigated with NPY Y1 agonist [Leu^{31}, Pro^{34}]-NPY and NPY Y1R antagonist (BIBP3226). First, immunofluorescence revealed that some of immature neurons expressed NPY Y1R (Fig. 5A). Further, to determine the dose of BIBP3226, HONCs were incubated with different concentrations of BIBP3226 ranging from 1 μM to 50 μM for 1 h. As shown in Fig. 5C, BIBP3226 did not influence the expressions of Ascl1, βIII tubulin, and OMP. Therefore, 50 μM BIBP3226 was adopted for further experiments. Western blot analyses revealed that the expression levels of Ascl1, βIII tubulin, GAP43, OMP, ADCY3, and G_{olf} were increased in NPY-treated and [Leu^{31}, Pro^{34}]-NPY-treated HONCs ($p < 0.05$, Fig. 5D–J). However, the expression levels of these stage-specific markers and components of signal transduction pathway decreased in BIBP3226 + NPY and BIBP322 + [Leu^{31}, Pro^{34}]-NPY groups and became non-significantly different from the control ($p > 0.05$). It indicated that the actions of NPY were mediated by the Y1 receptor (Fig. 5B).

4. Discussion

The ON occupies < 10% of the nasal cavity in humans, and it is thinner and more patchily scattered in adult human than in rodents from olfactory cleft to the middle turbinate (Nibu et al., 1999). However, the success rate of generating olfactory neurons rose from 16% to 80% over the last two decades, by isolating them from a cadaver or from biopsies during the nasal surgery (Borgmann-Winter et al., 2015; Winstead et al., 2005). Olfactory dysfunction may be due to decreased number and degree of differentiation of ORNs (Jafek et al., 2002; Yamagishi et al., 1994). Metaplasia of respiratory epithelium also occurs within the ON after severe damage to this neuroepithelium in endoscopic sinus surgery, resulting in loss in olfaction (Konstantinidis et al., 2010). Clinically, the new treatment strategies are developing new agents to regenerate the damaged ON or transplantation and engraftment of progenitor cells in the damaged ON (Goldstein et al., 1998; Li et al., 2018). The first step to apply this agent in clinical treatment is to evaluate its effect on the proliferation and differentiation of human ORNs *in vitro*. In this study, the ON was successfully obtained from patients and HONCs reach 70–80% confluence after three-week culture. The morphology in the NPY-treated cells significantly differs from controls. The development of new therapies for neurodegenerative disorders is focused on identifying molecules that affect the differentiation of neurons and neurites out growth. Newly formed neurons undergo a series of extensive morphological changes as they mature, involving neurites outgrowth. Therefore, these shape descriptors, cell circularity and process lengths, are initially direct ways to quantify cell morphology and reflect formation and maintenance of the nervous system (Harrill et al., 2010; Nichols et al., 2018; Yu et al., 2009). The reason to choose “7 days” for NPY treatment is that it usually takes 3 days to 8 days for HONCs to differentiate into ORNs (Chen et al., 2008). Our hypothesis is that NPY might regulate ORNs maturation; therefore, we choose the 7-day treatment to assess the role of NPY in HONCs. During neurogenesis progression, neuronal progenitor cells proliferate first, and then differentiate into ORNs or sustentacular cells through complex feedback mechanisms (Iwai et al., 2008; Hahn et al., 2005). Insufficient progenitor cells or the incorrect signaling may cause incomplete recovery of the olfactory function and the loss of olfactory neural homeostasis (Holcomb et al., 1996; Ogawa et al., 2014). Furthermore, the expressions of OMP and signal transduction apparatuses are important processes to define the restorative olfaction. Experimental results demonstrate that not only the expression levels of Ascl1 and OMP but also components of signal transduction are higher in the NPY than in controls, indicating that NPY can promote HONCs to differentiate into mature ORNs.

Recent studies have shown the presence of NPY in both the developing and the adult ON of rodents, and suggested its implication as a neuroproliferative factor (Doyle et al., 2008; Hansel et al., 2001; Jia and Hegg, 2010). NPY, evoking various physiological responses in mammals, is mainly expressed within a subset of developing embryonic neurons and neuronal progenitor cells, whereas in the postnatal ON it is mostly expressed in ORNs, sustentacular cells, microvillar cells, and olfactory ensheathing cells (Doyle et al., 2008; Hansel et al., 2001; Ubink et al., 1994). NPY is synthesized in the adult ON by microvillar cells, acting on neuronal progenitor cells to induce cell-cycle activation and division (Brogden et al., 2005). NPY not only plays a significant role in the regulation of feeding, but also acts as a trophic factor for the maturation and survival of ORNs during olfactory neurogenesis (Doyle et al., 2012). Mice with a targeted deletion of NPY had significantly reduced density of olfactory neurons by adulthood (Erickson et al., 1996; Hansel et al., 2001). Our experimental results reveal that NPY raises expression of neuronal progenitor cells marker, *Ascl1*, during *in vitro* culture, which is compatible with previous reports in rats (Hansel et al., 2001).

NPY can act through at least five receptors subtypes, including Y1, Y2, Y4, Y5 and Y6 (Michel et al., 1998). The Y6 receptor is not functional in humans. The Y1 and Y2 receptors are the most common NPY receptors in the mammalian brain, and all of these receptors are coupled to G-protein and influence response-specific second messenger cascades. NPY can signal through various intracellular pathways, including calcium and cyclic AMP (Balasubramaniam, 1997). The expressions of the NPY Y1R are located in brainstem nuclei and forebrain regions, including the cerebral cortex, the hippocampal formation, several amygdaloid, thalamic, and hypothalamic nuclei (Parker and Herzog, 1999). The NPY Y1R regulates or modulates food intake (Duhault et al., 2000), anxiety (Wahlestedt et al., 1993), and hormone secretions (Hastings et al., 2001). A previous report demonstrated that the ability of NPY to increase neuronal precursor proliferation is mediated by NPY Y1R; this effect is mediated downstream through a kinase cascade involving protein kinase C and ERK1/2 (Hansel et al.,

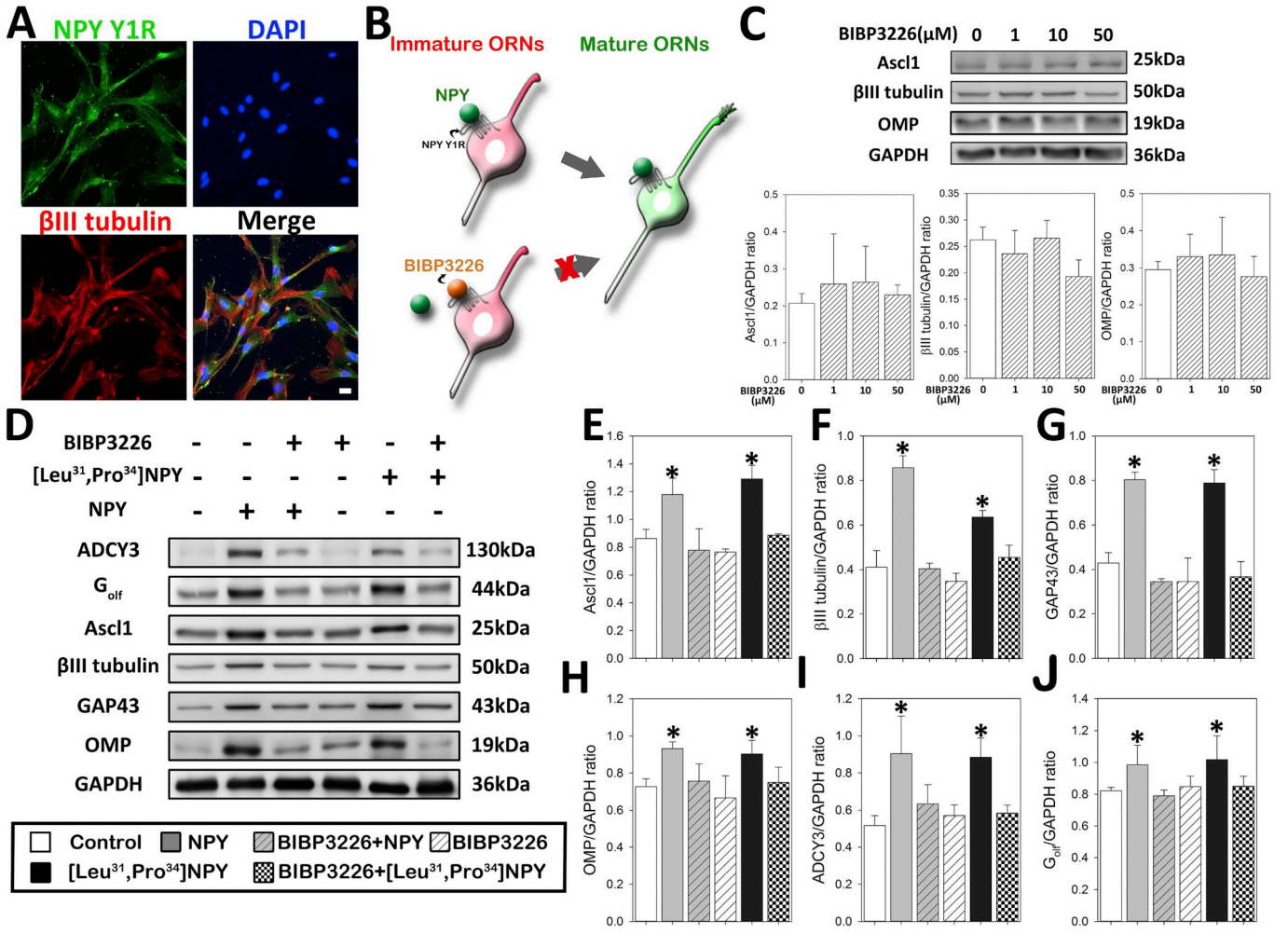

Fig. 5. The effect of NPY Y1 receptor antagonist (BIBP3226) and NPY Y1 receptor agonist ([Leu^{31}, Pro^{34}]-NPY) on differentiation of the ORN. A: Immunofluorescence images of immature ORNs are co-stained with βIII Tubulin (red) and NPY Y1R (green). B: Schematic showing proposed mechanism of NPY-mediated differentiation of ORNs. NPY-induced ORN differentiation is mediated by the NPY Y1R, which is blocked by BIBP3226. C: Western blot analyses for dose response of BIBP3226 on HONCs. The expression of different markers is determined using western blot (D) followed by densitometric analyses of Ascl1 (E), GAP43 (F), βIII Tubulin, (G), OMP (H), ADCY3 (I) and G_{olf} (J). Scale Bar = 20 μm. Asterisks denote $p < 0.05$, compared to controls, n = 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2001). In the adult rat ON, the NPY Y1Rs are expressed in neuronal progenitor cells and ORNs (Hansel et al., 2001; Montani et al., 2006). In this work, the pretreatment with BIBP3226 in NPY Y1R-positive HONCs incubated with NPY abolishes NPY-induced ORN differentiation. Additionally, NPY Y1 agonist [Leu^{31}, Pro^{34}]-NPY increases the differentiation of ORNs; whereas a pretreatment with BIBP3226 also eliminates the [Leu^{31}, Pro^{34}]-NPY-induced ORN differentiation. It indicates that the actions of NPY are mediated by the NPY Y1R.

In the study, NPY not only promotes differentiation of ORN, but also enhances formation of components of olfactory-specific signal transduction pathway in culture of HONCs. To our knowledge, this is the first report to demonstrate that exogenous NPY promotes differentiation and maturation of human ORNs through the NPY Y1R *in vitro*. However, whether NPY can be applied to facilitate regeneration of ON in clinical application needs further investigation.

5. Conclusion

The experimental results demonstrate that NPY not only increases expressions of protein markers of human olfactory neuronal progenitor cells, but also promotes differentiation of ORNs and enhances formation of components of olfactory-specific signal transduction pathway of HONCs through the NPY Y1R.

Financial support

Ministry of Science and Technology of Taiwan and Far Eastern Memorial Hospital (MOST-108-2314-B-418-009-MY3 and FEMH-2018-C-024).

Declaration of Competing Interest

None.

Acknowledgement

The authors would like to thank the Ministry of Science and Technology of Taiwan and Far Eastern Memorial Hospital for financially supporting this research under Contract No. MOST-108-2314-B-418-009-MY3 and FEMH-2018-C-024.

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