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Original Article

Orexin-A increases the differentiation of human olfactory sensory neurons through orexin receptor type 1

Yin-Tzu Chen [a, 1], Tai-Horng Young [a, 1], Yu-Hsin Wang [a], Chih-Hsuan Huang [a], Yu-Yun Gao [a], Tsung-Wei Huang [b, c, *]

[a] Department of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei, Taiwan
[b] Department of Otolaryngology, Far Eastern Memorial Hospital, Taipei, Taiwan
[c] Department of Electrical Engineering, College of Electrical and Communication Engineering, Yuan Ze University, Taoyuan, Taiwan

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ABSTRACT

Introduction: Sensorineural olfactory dysfunction significantly impairs the life quality of patients but without effective treatments to date. Orexin is a neurotrophic factor activates neuronal network activity. However, it is still unknown whether orexin can promote differentiation in human olfactory sensory neurons (OSNs). This study seeks to explore the impact of orexin on the differentiation of human olfactory neuroepithelial cells (HONCs).

Methods: The primary olfactory epithelium cells were cultured with or without orexin-A. The neural maturation-related and functional proteins were analyzed through immunofluorescence staining and Western blot. The function of HONCs were evaluated through the synaptic vesicle recycling assay.

Results: The results showed that HONCs in the orexin-A group expressed higher levels of stage-specific markers, including achaete-scute homolog 1, βIII-tubulin, and olfactory marker protein. Additionally, more components of signaling transduction pathways compared to the control group. The orexin-A-mediated differentiation of OSN effect can be nullified with dual orexin receptor antagonist suvorexant and the selective orexin receptor type 1 antagonist SB674042, instead of selective orexin receptor type 2 antagonist TCS-OX2-29.

Conclusions: Orexin-A elevates the expression of protein markers in human olfactory neuronal progenitor cells to stimulate the differentiation of OSN and enhances the formation of components of the olfactory-specific signaling transduction pathway via orexin receptor type 1.

1. Introduction

Olfactory dysfunction impairs the ability to identify hazards in the surrounding environment and can lower quality of life by decreasing appetite and sexual ability [1]. A common cause of olfactory malfunction is the atrophy of the olfactory neuroepithelium (ON) with aging, along with upper respiratory viral infections and disruptions to neuron connections from head injuries [2,3]. Over the past 20 years, the occurrence of olfactory dysfunction has steadily increased, rising from 3 % to 20 %, and has increased significantly in recent years due to the coronavirus disease 2019 (COVID-19) pandemic [4]. Previous studies have shown that more than 80 % of COVID-19 patients experience hyposmia or anosmia, and more than 30 % of patients with COVID-19 have sequelae of anosmia after recovery [5]. Clinically, glucocorticoids are still the treatment option for sensorineural olfactory dysfunction, but their

Abbreviations: ADCY3, adenylate cyclase type 3; ANOVA, analysis of variance; ASCL1, achaete-scute homolog 1; BSA, bovine serum albumin; CK5, cytokeratin 5; COVID-19, coronavirus disease 2019; DAPI, 4′,6-diamidino-2-phenylindole; GAP43, growth associated protein 43; Golf, olfactory neuron specific-G protein; HBSS, Hanks' Balanced Salt Solution; HONCs, human olfactory neuroepithelial cells; OSNs, olfactory sensory neurons; OMP, olfactory marker protein; ON, olfactory neuroepithelium; OX1R, orexin receptor type 1; OX2R, orexin receptor type 2; PBS, phosphate-buffered saline; SD, standard deviation.

* Corresponding author. Department of Otolaryngology, Far Eastern Memorial Hospital, 21, Section 2, Nan-Ya South Road, Pan Chiao 220, Taipei, Taiwan.
E-mail address: huangtw@ntu.edu.tw (T.-W. Huang).

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[1] These authors contribute equally to the work.

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side effects limit their clinical application and there is controversy about the effectiveness of treatment [6,7]. Therefore, new treatment methods need to be developed for patients with sensorineural olfactory loss. Unlike other cranial neurons, the olfactory system of mammals can continuously replace olfactory sensory neurons (OSNs) throughout adulthood, as a result of normal turnover or injury [8]. However, this unique ability of the mammalian olfactory system deteriorates with age, in response to various toxic factors, including viruses, smoking, inflammation, drugs, and environmental factors, leading to olfactory dysfunction [9,10]. New treatment approaches involve creating new drugs to revive damaged olfactory neuroepithelium and transplanting or implanting progenitor cells into the affected area.

Most animals, including humans, rely on their sense of smell to find, choose and evaluate food [11]. Additionally, olfactory neural processing is closely tied to an organism's physiological and nutritional state. The olfactory system becomes more active and sensitive when an organism is hungry, but its activity decreases after the organism is satiated [12,13]. Orexin is a hunger-stimulating peptide that regulates feeding behavior [14]. In particular, when orexin elicits hunger responses, the olfactory system becomes more active and sensitive as orexin enhances the reactivity of the OSNs during times of starvation [15]. The peripheral olfactory system contains orexins and their receptors in proximity to the primary olfactory nerves. These can be found in the dendritic knobs and cilia of olfactory sensory neurons, as well as in the microvilli of supporting cells in the upper part of the olfactory epithelium [16]. In addition, orexin also participates in early postnatal brain development and maturation as a neurotrophic factor by directly or indirectly activating neuronal network activity [17]. Based on the unique properties related to olfaction, orexin may promote the differentiation and maturation of OSNs and can be adopted in treating human olfactory dysfunction. Therefore, this study aims to investigate the effect of orexin on the differentiation of human olfactory neuroepithelial cells (HONCs) *in vitro*.

2. Materials and methods

2.1. Specimen collection and cell preparation

This study was approved by Far Eastern Memorial Hospital Review Board (105104-F). All patients gave informed consent. In patients with chronic rhinitis, ON were collected from the superior turbinate in the nasal cavity near the roof during nasal surgery. For the explant culture of HONECs, biopsy specimens were washed with Hank's balanced salt solution, finely minced, and then treated with 0.125 % Trypsin/1 % EDTA solution for 30 min with gentle shaking. The tissue samples were collected through centrifugation and resuspended in IMDM culture medium (Iscove's Modified Dulbecco's Media; Invitrogen, USA) with the addition of 10 % fetal bovine serum and 1 % antibiotics [18]. Then, they were seeded in culture dishes that were coated with laminin-co-fibronectin and incubated for 28 days. The culture wells were divided into control and experimental groups, the antagonist was added into the medium at day 0, and the orexin was added after a week of expansion culture.

2.2. Orexin and antagonists preparation

Orexin-A (06012, Merck, Germany) and Orexin-B (scp0204, Merck) were diluted in the deionization and distilled water with 1 μg/mL. Suvorexant, an orexin receptor type 1 and type 2 antagonist was diluted in methanol at 1 μg/mL. The orexin receptor type 1 (OX1R) antagonist, SB-674042 (HY-10898, MedChem Express, NJ, USA), and orexin receptor type 2 (OX2R) antagonist, TCS-OX2-29 (HY-100452, MedChem Express) were diluted in the dimethyl sulfoxide, DMSO with 1 μg/mL and 10 μg/mL, respectively. All chemical stocks were packaged and conserved at −20 °C for further use.

2.3. Morphological examination

Cell morphology was stained with CellMask™ Plasma Membrane Stains (C10046, Thermo Fisher Scientific) and 4′,6-diamidino-2-phenylindole (DAPI) (ab228549, Abcam, Cambridge, UK) to demonstrate the cell morphology after 28 days of culture. For better visualization of bipolar OSNs, cytosine-β-d-arabinofuranoside was used (10 μM) on day 21 in both groups to eliminate non-neuronal cells [19,20]. Circularity (calculated as $4\pi \times$ area/perimeter2), the percentage of cell spread area (cell spread area/cell numbers per view), and the cell dendrite/axon were determined using ImageJ software.

2.4. Immunofluorescence analyses

The biopsy specimens were fixed in 10 % formalin (HT501128, Merck) at 4 °C overnight, followed by decalcification, and subsequently embedded in paraffin. After heat-mediated antigen retrieval in pH 6 citrate buffer (TA00H01, BioTnA, KHH, ROC) and blocking nonspecific binding for 2 h at room temperature, sections were incubated with anti-olfactory marker protein (OMP) (sc-365818, 1:100; Santa Cruz Biotechnology, TX, USA), anti-cytokeratin5 (CK5) (ab64081, 1:1000; Abcam), and anti-β4 tubulin (ab82254, 1:200; Abcam) primary antibody diluted with PBS overnight at 4 °C diluted in the blocking solution. Sections were washed 3 times with PBS, and then incubated with anti-mouse IgG secondary antibody conjugated with AlexaFluor 488 (A-11029, Invitrogen; RRID:AB_2534088) and anti-rabbit IgG conjugated to AlexaFluor 546 (A-11035, Invitrogen; RRID: AB_2534093) for 1 h at room temperature. Sections were washed with PBS, and then mounted with Fluoroshield™ with DAPI (GTX30920, Genetex, CA, USA) for further observation. The cultured cells were fixed with 10 % formalin or 70 % ice methanol for 10 min at room temperature, and blocked in 2 % bovine serum albumin (BSA; A70370, Merck) or 10 % goat serum buffer (GTX73249, Genetex) at 4 °C overnight according to the use suggestions of antibodies. Continually, the cells were incubated with primary antibodies diluted in 2 % BSA, namely, anti-achaete-scute homolog 1 (ASCL1) (ab74065, 1:1000; Abcam), anti-growth associated protein 43 (GAP43) (ab75810, 1:500; Abcam), anti-βIII tubulin (ab7751, 1:1000; Abcam), anti-olfactory marker protein (OMP) (ab62144, 1:100; Abcam), anti-olfactory neuron specific-G protein (Golf) (ab74049, 1:1000; Abcam) and anti-adenylate cyclase type 3 (ADCY3) (ab125093, 1:1000; Abcam). Visualization of the primary antibodies was done with the species-specific secondary antibody conjugated to Alexa488 (Invitrogen, catalog A-11029; RRID:AB_2534088 or catalog A-11029; RRID: AB_2576217) for 1 h at room temperature. Additionally, the nucleus and cytoskeleton of HONCs were counterstained with DAPI, and Phalloidin-iFluor 555 reagent (ab176756, Abcam), respectively. Further, Images were taken from a fluorescence microscope (LSM510, Carl Zeiss, Jena, Germany).

2.5. Synaptic vesicle recycling assay

The cultured HONCs were incubated with 5 μg/mL FM1-43 dye (T3163, Thermo Fisher Scientific) in cold Hanks' Balanced Salt Solution (HBSS) containing 90 mM potassium ions for 1 min at 37 °C. The fluorescence intensity measured at this point was designated as “full staining.” After washing with HBSS, the cells were re-exposed to cold HBSS containing 90 mM potassium ions for 150 s

and then washed again. The fluorescence intensity measured at this point was designated as "destaining." The fluorescence intensity was detected at fluorescence excitation at 510 nm and emission detection at 626 nm through ELISA microplate reader (SpectraMax, Molecular Devices, CA, USA).

2.6. Western blot analysis

The migrating cells and explants were lifted from the plates simultaneously. Subsequently, the explants were filtered out through a mesh before undergoing digestion. Western blot samples were collected after 28 days of incubation, and both the experiment and control groups were conducted concurrently. The protein was collected from lysate and quantitated by bicinchoninic acid protein assay (Bio-Rad Laboratories, CA, USA). Denatured proteins were separated by 10 % and 12 % SDS-PAGE electrophoresis, and transferred to polyvinylidene fluoride membranes (Merck Millipore, MA, USA). The membranes were blocked in a 3 % albumin buffer for 2 h, immune-stained with the specific primary antibodies at 4 °C overnight, washed, and probed in corresponding HRP-conjugated secondary. The western blotting images were acquired with enhanced chemiluminescence (Merck Millipore) by UVP Bio-Spectrum 810, and analyzed by imageJ. The use of the primary antibodies, namely anti-ASCL1 (ab74065, Abcam), anti-βIII tubulin (ab7751, Abcam), anti-Golf (ab74049, Abcam) and anti-ADCY3 (ab125093, Abcam), anti-OMP (NB110-74751, Novus Biologicals, CO, USA), GAPDH (ab22555, Abcam), anti-Synapsin I (ab254349, Abcam) antibody, anti-PI3 kinase p110 alpha antibody (GTX100462, Genetex), anti-AKT1 (phospho S473) antibody (ab81283, Abcam), anti-mTOR antibody (GTX638220, Genetex).

2.7. Statistical analysis

The samples in each cell culture preparation are pooled from 2 to 3 subjects, and the "n" refers to the number of independent cell culture preparations. The results were presented as mean ± standard deviation (SD) using SigmaPlot 12.5 statistical software. The outcome of the normality testing was analyzed through the Shapiro-Wilk Normality Test and Equal Variance Test. The comparison between the two groups was performed using a Student's paired *t*-test, and differences among multiple groups were analyzed using a one-way analysis of variance (ANOVA), followed by the Tukey-Kramer test. The tests were two-tailed and statistical significance was defined as p value < 0.05 and marked with asterisks, compared to the control group.

3. Results

3.1. Morphological characterization of HONCs and dose-response analyses of orexin-A

To investigate the human ON, selected samples underwent histological analysis and were stained with OMP and CK5 (Fig. 1a, and b). OMP serves as a marker for mature OSN in adulthood, and CK5 marks the basal cells in ON [21]. In native tissues, OMP staining was observed in the cell body, dendrites, and axons, while CK5+ cells were located around the basal layer of the ON (Fig. 1b, and c). β4 tubulin staining ruled out the presence of respiratory epithelial cells. Fig. 1d showed the time course of the experimental design, where ON explants adhered to the substrate successfully, and HONCs migrated from the ON during culture.

Additionally, to evaluate the optimal dose of orexin-A, HONCs were incubated with varying amounts of orexin-A from 0.1 ng/mL to 10 ng/mL after a week of expansion culture. A Western blot analysis showed that 0.1 and 1 ng/mL of orexin-A significantly increased the expression of ASCL1, βIII tubulin, and OMP (Fig. 2a, $p < 0.05$). A concentration of 10 ng/mL is ineffective for some of the proteins. Moreover, HONCs expressed the higher functional signal transduction markers, Golf, and ADCY3, with 1 ng/mL orexin-A (Fig. 2b, and c, $p < 0.05$). Therefore, 1 ng/mL orexin-A was adopted for experiments. After 28 days in vitro, plasma membrane stains revealed that most of the cells had a flattened, epithelial-like appearance in the control group (Fig. 3a). In contrast, cells treated with orexin displayed a thin, bipolar shape, with significantly lower

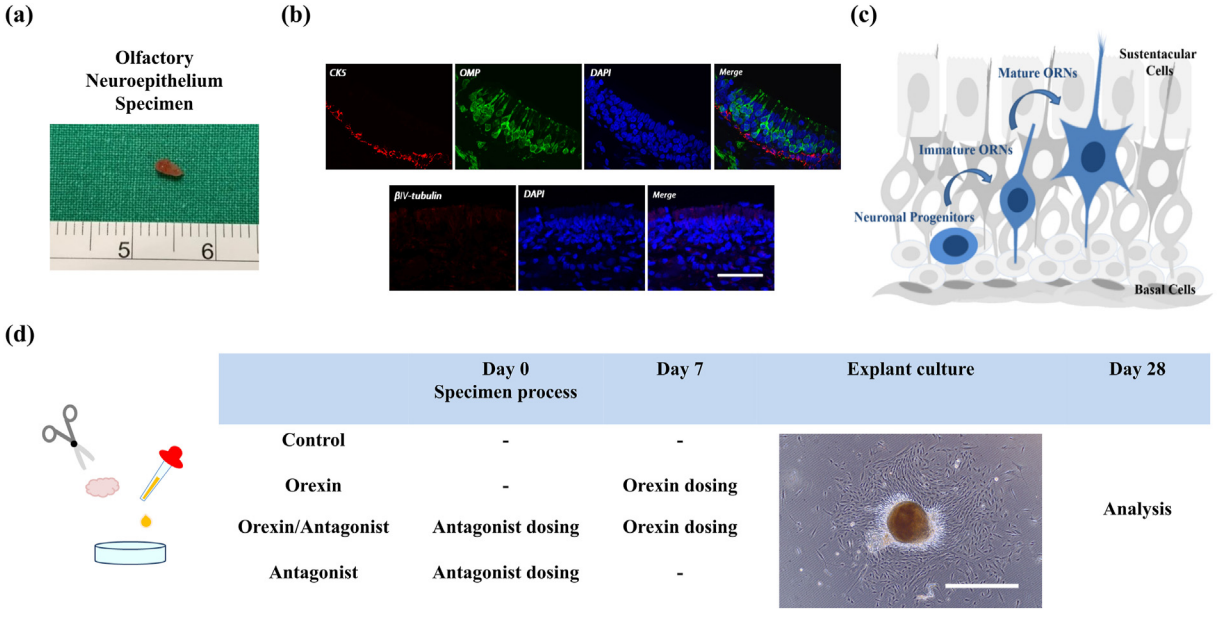

Fig. 1. Schematic diagram for primary HONCs culture establishment. (a) An excised specimen from human olfactory neuroepithelium. (b) Immunohistochemical stain with OMP (green), CK5 (red), and DAPI (blue) represents mature OSNs, basal cells, and nuclei, respectively (top). β4-tubulin (red) and DAPI (blue) immunohistochemical markers were to rule out respiratory epithelium (scale bar = 100 μm). (c) Schematic showing HONCs maturation process from progenitor cells to mature neuronal cells. (d) Time course of the experimental design with accompanying microscopy indicating cell migration from explants (scale bar = 1 mm).

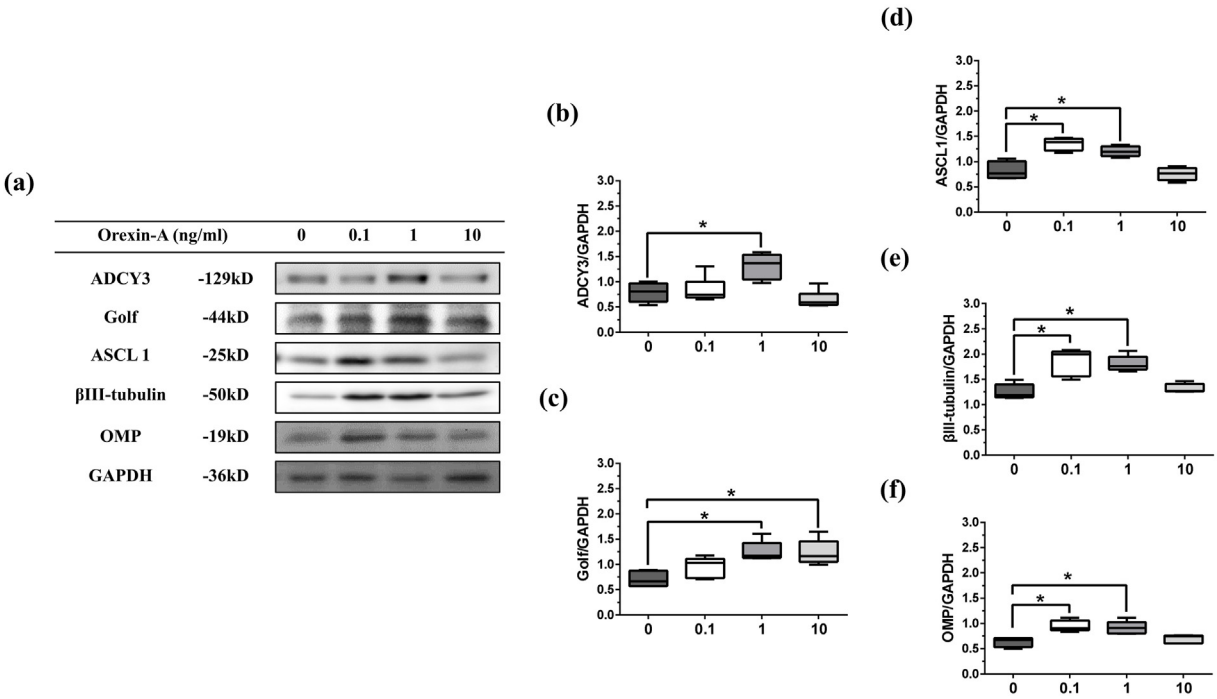

Fig. 2. Dose-response analyses of orexin-A. (a) Western blots of olfactory neuronal protein expression and olfactory signal transduction apparatus. (b–f) Western blot densitometric analysis. (Results are the mean ± SD. Asterisks indicate $p < 0.05$, compared to the control group, $n = 5$).

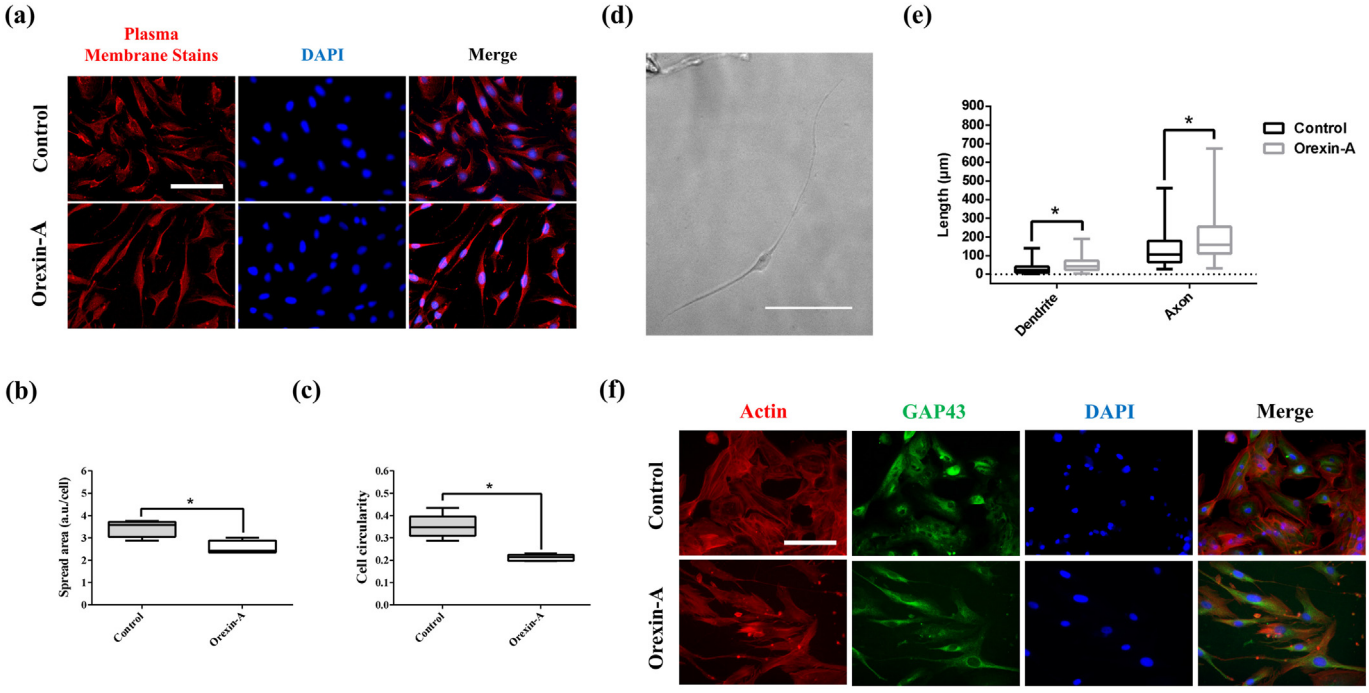

Fig. 3. Morphology observation and neural marker labeling. (a) Morphology observation of HONCs cultured with or without orexin-A (1 ng/mL) through fluorescence graphs. (b) The percentage of HONC spread area and (c) cell circularity of HONC (Asterisks indicate $p < 0.05$, scale bar = 100 μm). (d) Representative photo of bipolar OSNs (bar = 100 μm). (e) The axon and dendrite length of bipolar OSNs (Asterisks indicate $p < 0.05$). (f) Immunofluorescent staining with neuron marker, GAP43 (green). Scale bar = 100 μm.

circularity and reduced spread area compared to the controls ($p < 0.05$, Fig. 3b and c). Additionally, the lengths of OSN dendrites and axons were evaluated in both groups, and the average lengths were longer in the orexin-A treated group (Fig. 3d and e). The immunofluorescence revealed that HONCs expressed a neuron-specific marker, GAP43 (Fig. 3f).

3.2. The OSN differentiation of HONCs and the functional analysis of OSNs

HONCs have been thoroughly studied and are well-known for their specific stage markers. During the development of HONCs, the basal cell marker ASCL1 acts as a critical point at the early

stages of the OSN lineage to initiate the differentiation program (20). OMP is typically expressed in mature OSNs, while βIII-tubulin, the neuron-specific form of tubulin, is commonly used as a marker for immature OSNs [22,23]. Immunofluorescence revealed that HONCs in the orexin group expressed higher stage-specific markers, namely ASCL1, βIII-tubulin, and OMP, compared to controls (Fig. 4a, b, and c). βIII-tubulin was clearly present throughout the neurons, cell body, dendrites, and axons. Western blot analysis confirmed these findings (Fig. 2). Golf and ADCY3 play critical roles in the signal transduction pathways for odorant receptors and neurotransmitter response [24]. The results of the immunostaining analysis revealed the presence of Golf in cell bodies and ADCY3 in cell membranes, as shown in Fig. 5a and b. The Western blot analysis revealed elevated expression levels of Golf and ADCY3 in the orexin-A treated group compared to the control group ($p < 0.05$, Fig. 2). The treatment with orexin-A not only induced the differentiation of HONCs into OSNs but also increased the formation of signal transduction apparatuses. Furthermore, OSNs treated with orexin-A showed a significant increase in the protein expression of synapsin Ia and Ib as well as a higher percentage of difference in fluorescence intensity (dF %) compared to the control group. This suggests that the orexin-A treated group had a greater release of neurotransmitters, indicating an advanced level of OSN maturation ($p < 0.05$, Fig. 6d).

3.3. The effect of orexin receptor antagonists on the differentiation of HONCs

The role of orexin-A in OSN differentiation was examined by using orexin receptor antagonists to determine its effect. First, immunofluorescence revealed that the immature neurons expressed OX1R and OX2R (Fig. 7a). Further, to determine the dose of suvorexant, a dual orexin receptor antagonist [25], the HONCs were treated with various concentrations of suvorexant, ranging from 0.1 ng/mL to 10 ng/mL. As demonstrated in Fig. 7b, suvorexant did not influence the expressions of these stage-specific markers and was confirmed with the densitometry (Fig. 7c, d, and e). Therefore, the experiments were conducted using 1 ng/mL of the orexin receptor antagonist suvorexant. Analysis of Western blot showed that the levels of ASCL1, βIII tubulin, OMP, Golf, and ADCY3 were reduced in the group treated with orexin-A and suvorexant, and were no longer significantly different from the control group. (Fig. 8, $p > 0.05$). Further, to determine the receptor involved in the signaling pathway of orexin-A-mediated OSN differentiation, selective OX1R antagonist, SB674042 [26], and selective OX2R antagonist, TCS-OX2-29 [27], were adopted for experiments. As shown in Fig. 9, SB674042 abolished the action of orexin-A on promoting expression levels of ASCL1, βIII tubulin, OMP, Golf, and ADCY3 (Fig. 9a–f, $p > 0.05$ between orexin-A+SB674042 and

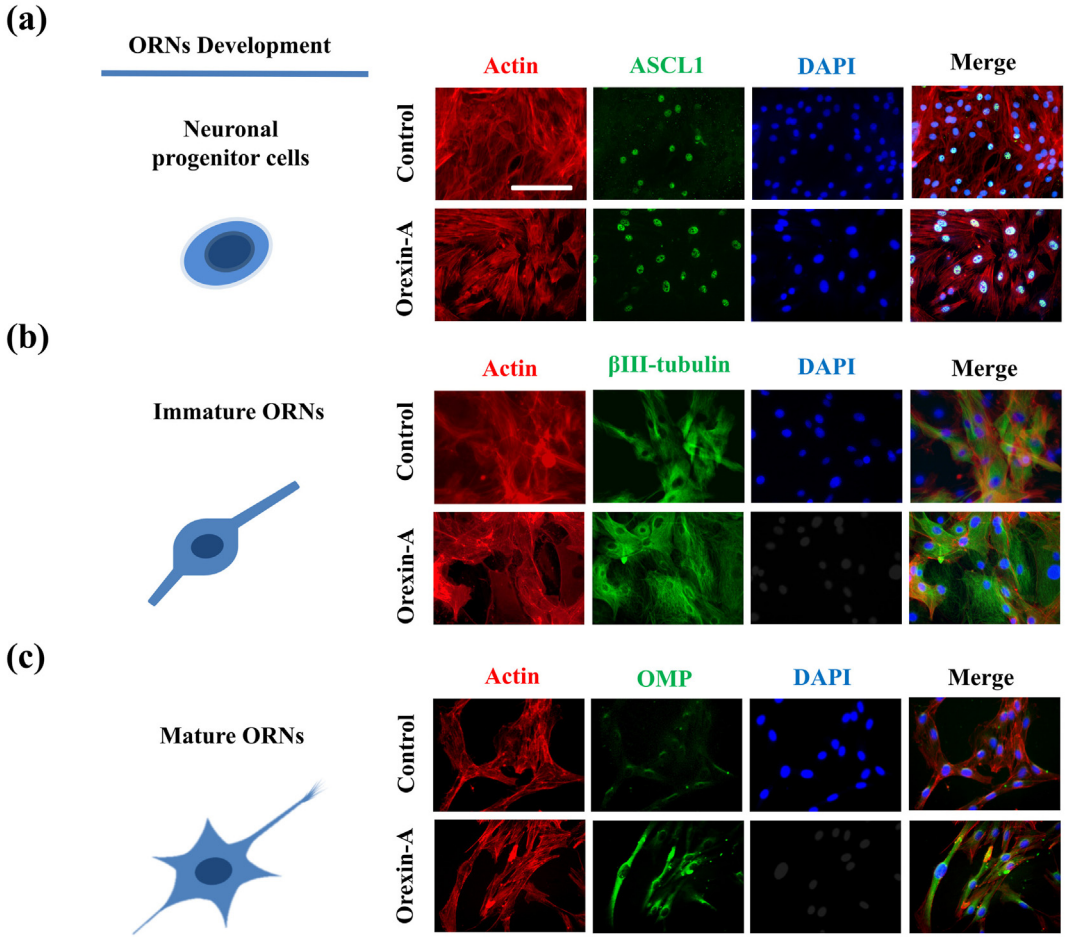

Fig. 4. Immunofluorescence of cultures with or without orexin-A (1 ng/mL). The cells stained with protein marker (green), (a) ASCL1, (b) βIII tubulin, and (c) OMP to label neuronal progenitor cells, immature OSNs, and mature OSNs, respectively.

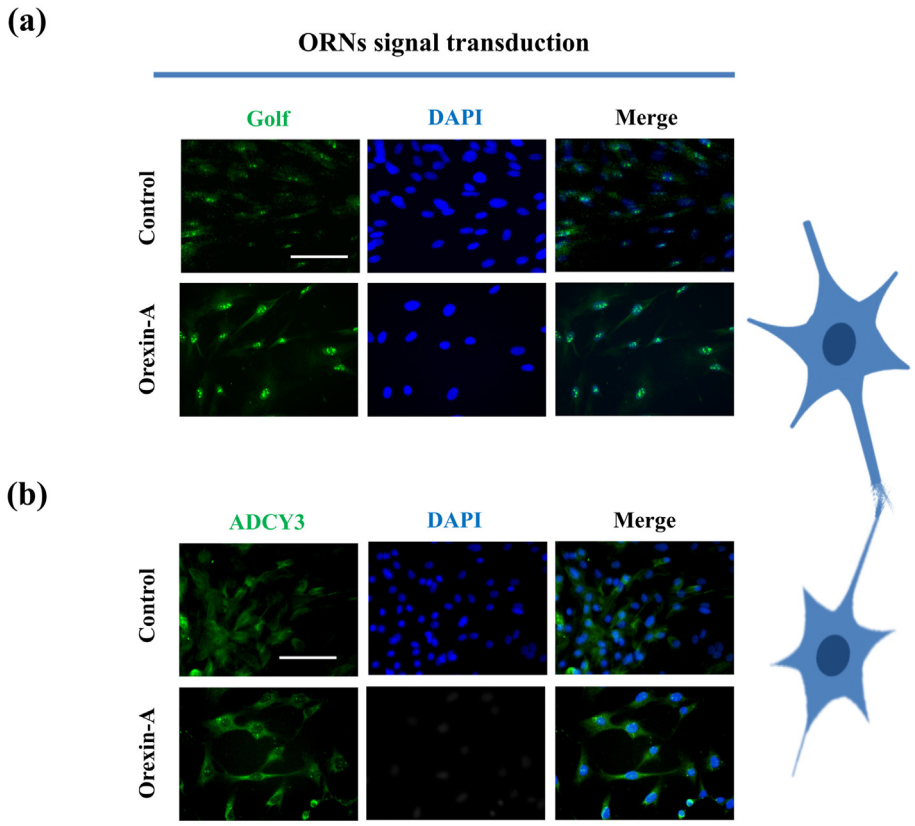

Fig. 5. Immunofluorescence of signal transduction protein for cultures with or without orexin-A (1 ng/mL). The cells stained with protein marker (green), (a) ADCY3 and (b) Golf protein markers are used to identify olfactory signal transduction. Cellular cytoskeletal shape (F-actin) and nuclei are visualized with staining using phalloidin-ifluor 555 (in red) and DAPI (in blue), respectively (scale bar = 100 μm).

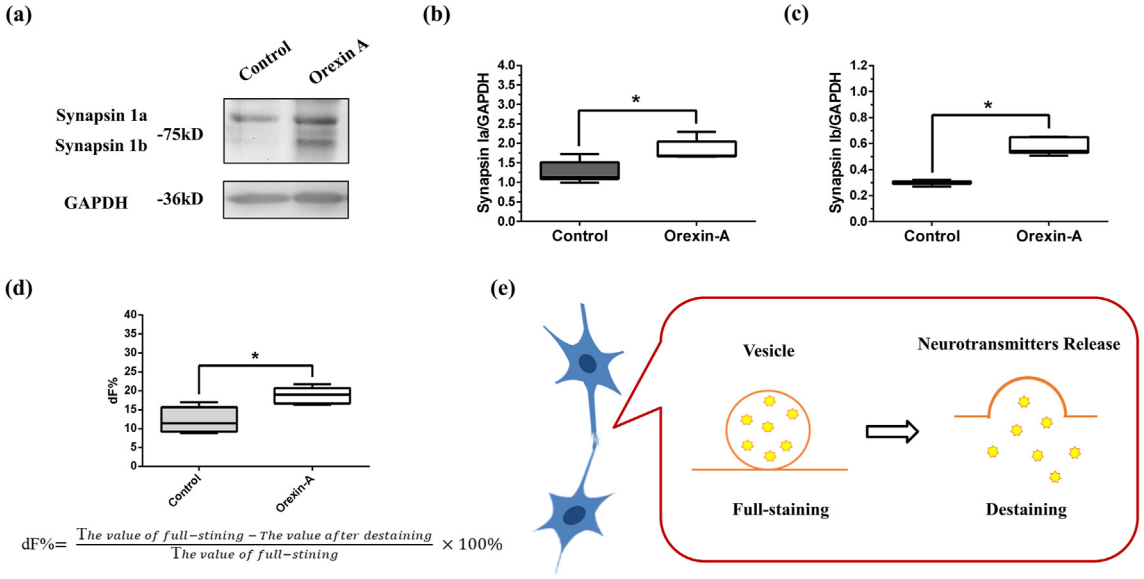

Fig. 6. The functional evaluation for cultures with or without orexin-A (1 ng/mL). (a) Western blots of olfactory functional protein expression in different groups followed by densitometric analyses of (b) synapsin Ia and (c) synapsin Ib. (d) The variation in fluorescence intensity during vesicle fusion and neurotransmitter release. (e) Schematic illustration of the vesicle fusion and neurotransmitter release.

controls). However, the expression levels of these stage-specific markers were not significantly different between the orexin-A and orexin-A+TCS-OX2-29 groups (Fig. 9 g-l, $p > 0.05$). It indicated that the actions of orexin-A were mediated by the OX1R. The downstream mechanisms of orexin-A in neural differentiation and maturation were evaluated. As shown in Fig. 10, the group

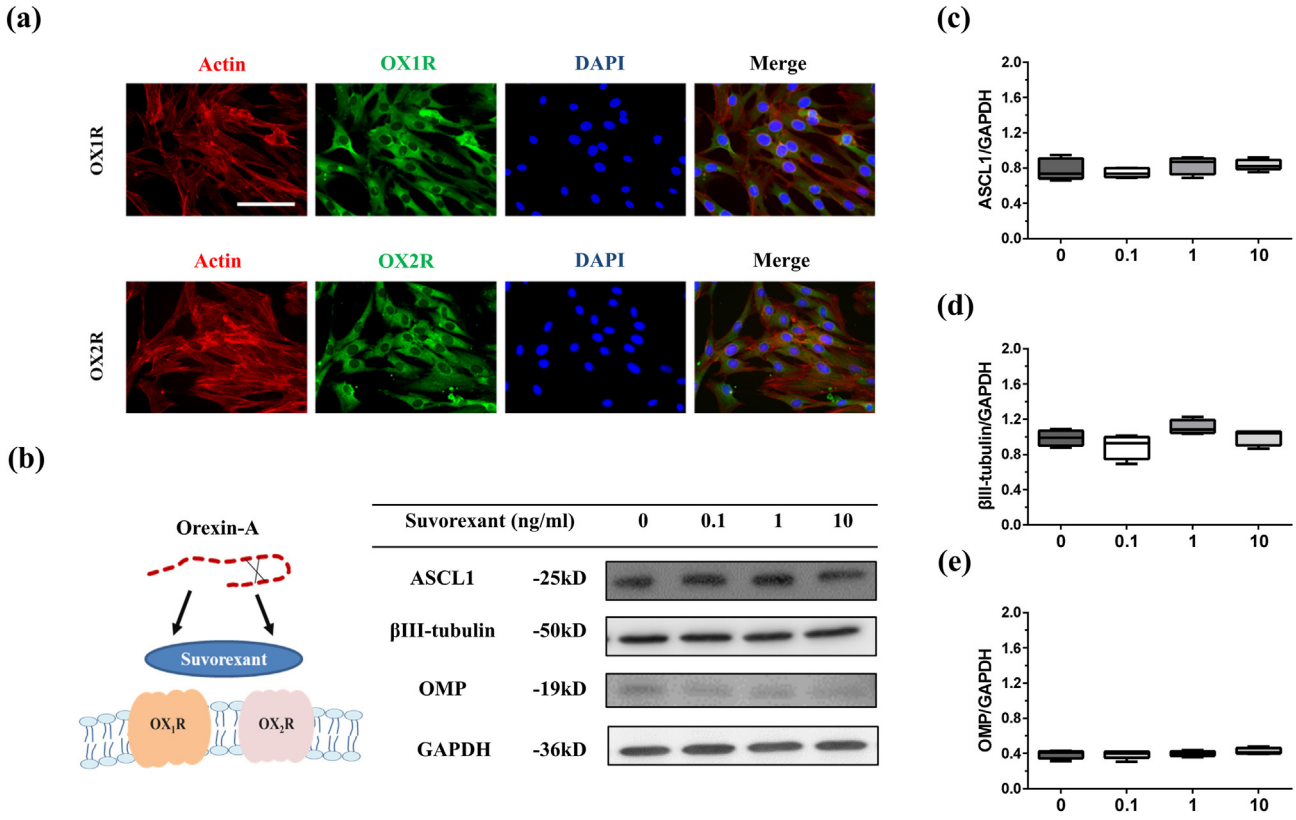

Fig. 7. Immunofluorescence of orexin receptors and dose-response analyses of orexin-A. (a) Immunofluorescence images of OSNs stained with OX1R and OX2R (green). (b) Schematic showing the proposed mechanism of orexin-mediated differentiation of OSNs and dose-response analyses of suvorexant. Western blot densitometric analysis of (c) ASCL1, (d) βIII Tubulin, and (e) OMP, respectively. ($p > 0.05$, compared to controls, $n = 5$).

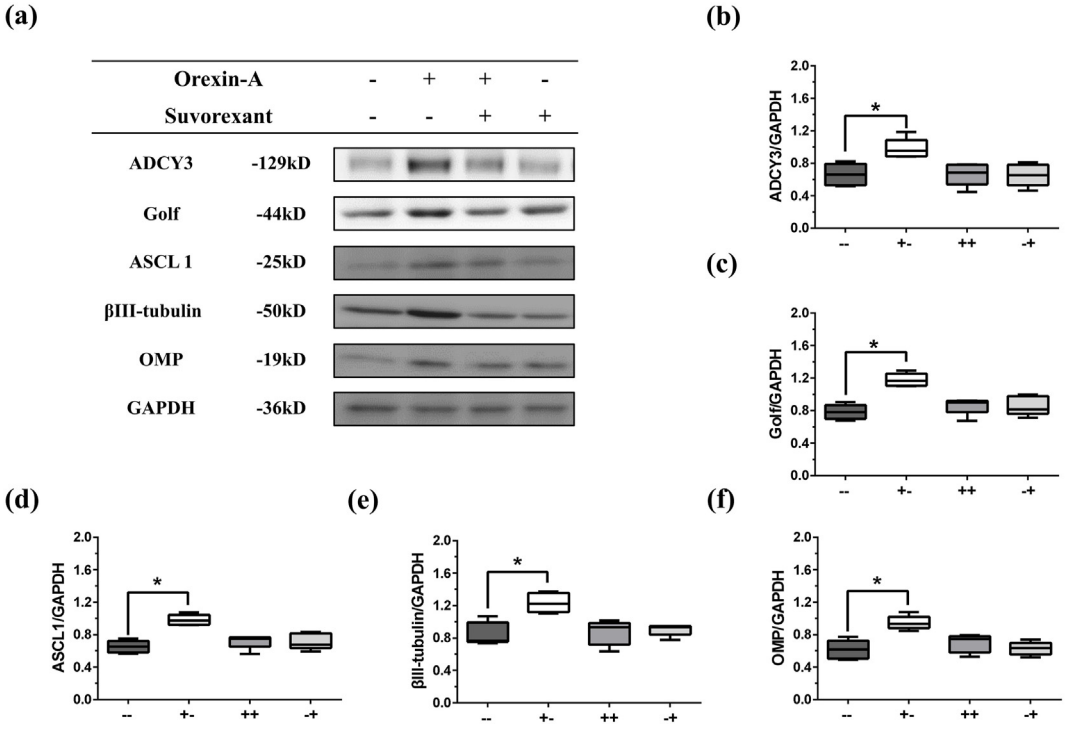

Fig. 8. The effect of orexin-A and orexin receptor antagonist (suvorexant) on the differentiation of OSN. (a) Western blots of olfactory neuron protein and olfactory signal protein expression in different groups, followed by densitometric analyses of (b) ADCY3, (c) Golf, (d) ASCL1, (e) βIII tubulin, and (f) OMP (Asterisks indicate $p < 0.05$, compared to controls, $n = 5–6$).

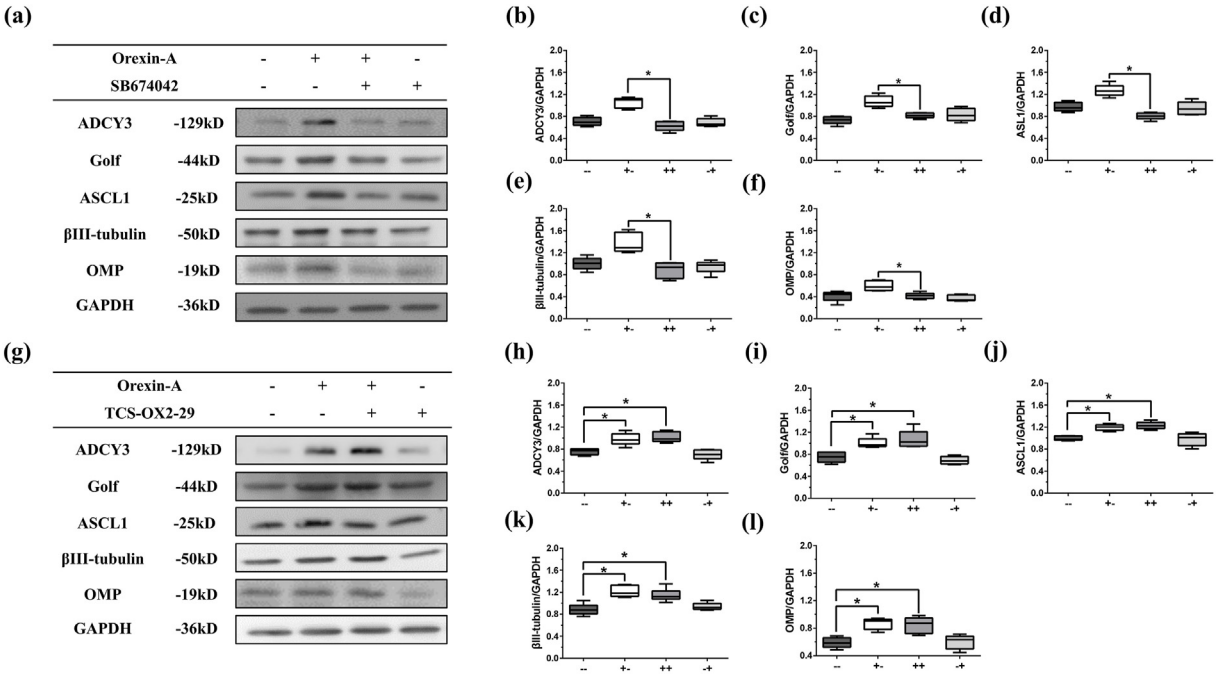

Fig. 9. The effect of OX1R antagonists (SB674042) and OX2R antagonists (TCS-OX2-29) on the differentiation of OSN. The expression of various markers was assessed through western blots (a and g) and subsequently analyzed densitometrically for ADCY3, Golf, ASCL1, βIII tubulin, and OMP (b-f and h-l). (Asterisks indicate $p < 0.05$, compared to controls, $n = 5–6$).

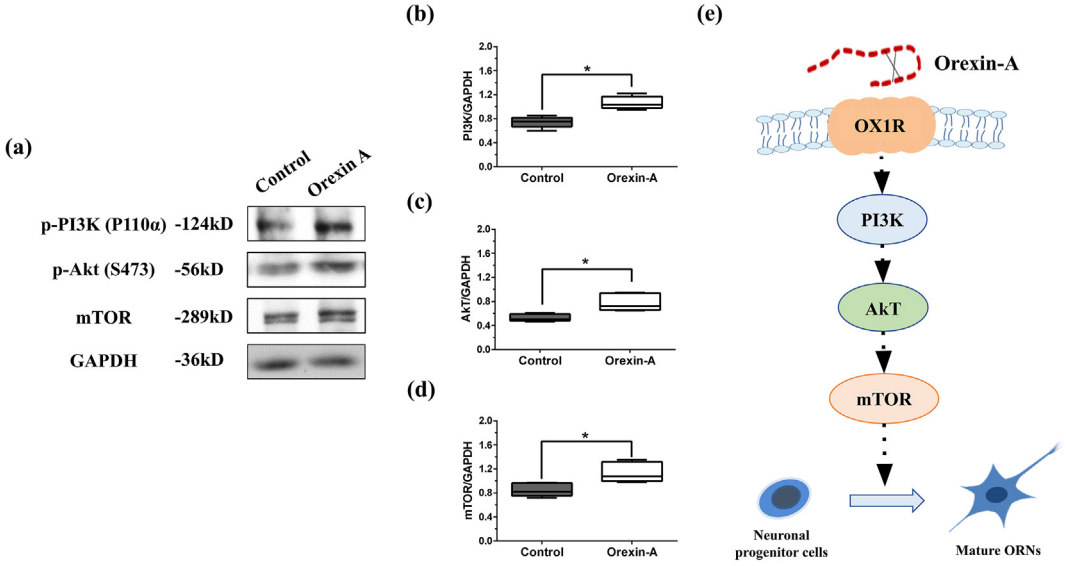

Fig. 10. The effect of orexin-A on the PI3K/Akt/mTOR pathway. (a and b) Western blot of protein level of PI3K/Akt/mTOR pathway, and subsequently densitometric analysis of (b) p-PI3K, (c) p-AkT, and (d) mTOR. (Asterisks indicate $p < 0.05$, compared to controls, $n = 5$). (e) Schematic diagram of the Orexin-A-mediated PI3K/Akt/mTOR pathway promoting HONCs differentiation and maturation.

treated with orexin-A exhibited increased expression levels of p-PI3K, p-Akt, and mTOR protein compared to the control group. Therefore, the PI3K/Akt/mTOR pathway is related to orexin-mediated differentiation and maturation of HONCs.

Further, the orexin family of peptides, orexin-B, was also evaluated. As shown in Fig. 11, neither orexin-B nor suvorexant influenced the expressions of ASCL1, βIII tubulin, and OMP compared with the controls.

4. Discussion

In humans, the olfactory neuroepithelium only takes up less than 10 % of the nasal cavity. Compared to rodents, it is thinner and more irregularly distributed in humans, extending from the olfactory cleft to the middle turbinate [28]. However, in the past twenty years, the success rate of isolating olfactory neurons through biopsies during nasal surgery or from a cadaver has risen from 16 % to

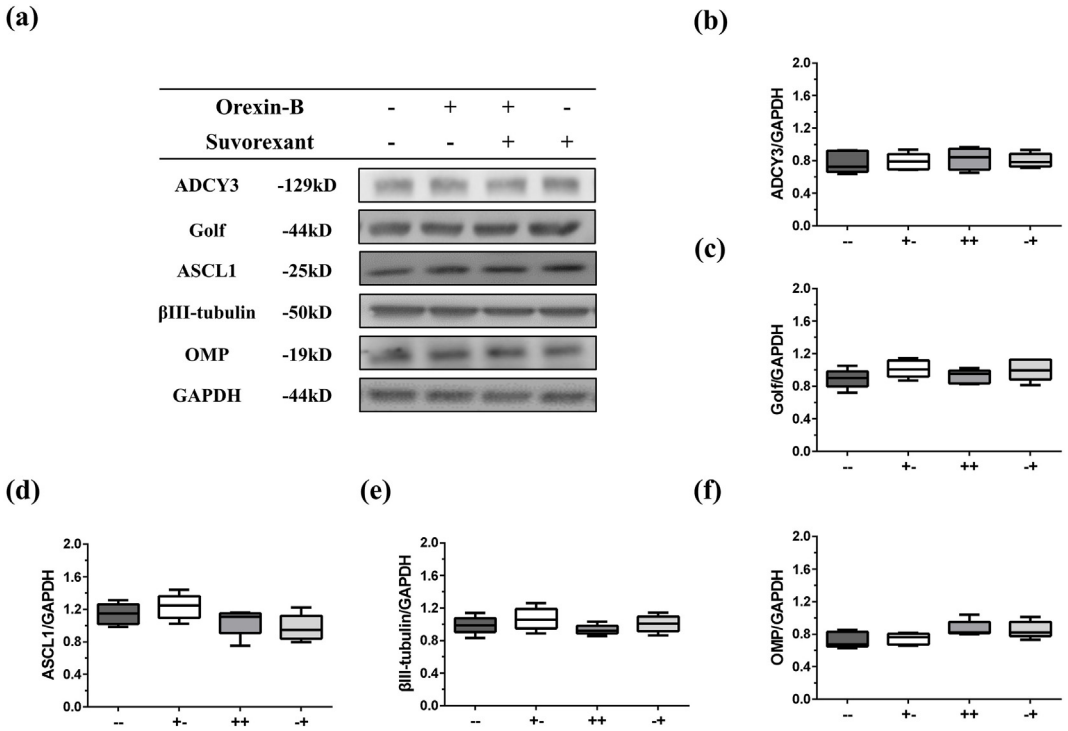

Fig. 11. The effect of orexin-B and suvorexant on the differentiation of OSN. (a) Western blots of olfactory neuron protein and olfactory signal protein expression in different groups, followed by densitometric analyses of (b) ADCY3, (c) Golf, (d) ASCL1, (e) βIII tubulin, and (f) OMP. There are no significant differences between groups (n = 5).

80 % [29]. In this study, the olfactory neuroepithelium was successfully obtained from patients, and HONCs migrated from the explant after culture. The morphological changes in cells treated with orexin-A are significantly distinct from the control group. Research for developing new treatments for neurodegenerative diseases focuses on locating molecules that influence the growth and differentiation of neurons and their projections. As they mature, newly formed neurons experience extensive morphological changes, including the growth of neurites. Thus, the initial evaluation of cell morphology can be directly performed through the use of shape descriptors and cell circularity, offering insights into the formation and maintenance of the nervous system [30,31]. The spread area of cells in epithelial cell culture is generally larger compared to that of neurons in culture. This is because epithelial cells are adherent and flat, and they grow in a monolayer on the culture substrate [32,33] In contrast, neurons have a more complex three-dimensional morphology, with dendrites and axons extending and branching in multiple directions [34]. OSNs, which are specialized bipolar neurons, are responsible for odor detection (Fig. 3d). OSNs have a dendrite that extends into the olfactory epithelium and a single, thin axon that projects into the olfactory bulb [34,35]. Additionally, the circularity of cells in epithelial cell cultures is typically higher when compared to neurons in culture. This difference arises because epithelial cells often have a polygonal or cuboidal shape, characterized by well-defined boundaries and a relatively uniform morphology [36]. Furthermore, GAP43, a neural-specific protein, is initially expressed in proliferating neuroblasts and plays a crucial role in neuronal maturation [37]. It is present in both immature and mature neurons [38,39]. Therefore, the expression of GAP43 in both groups confirms that the cultured cells indeed originate from the neuronal lineage, rather than having a different origin (Fig. 3f). During the early phase of regeneration, the first cells to proliferate are the basal cells, which then differentiate into OSNs or sustentacular cells through intricate feedback mechanisms [22]. A shortage of basal cells or incorrect signaling can result in incomplete restoration of olfactory function and disruption of olfactory neural homeostasis [40,41]. The new OSNs must have OMP and functional signal transduction apparatus to support the restoration of the sense of smell. Since the culture system is an explant culture, there are various cells in different stages of differentiation in the culture. The experiments show that orexin-A not only boosts the expression of protein markers in human olfactory basal cells but also fosters the differentiation of OSN and improves the formation of the HONCs olfactory apparatus and the functional protein (Figs. 5 and 6a, b and c). In addition to the increased protein expression, a functional analysis, specifically a synaptic vesicle recycling assay, was conducted. Synaptic vesicles play a critical role in neural communication by storing and releasing neurotransmitters in response to an action potential [42]. Mature OSNs can effectively respond to action potentials, leading to the fusion of the vesicles to the presynaptic membrane and the release of neurotransmitters from synaptic vesicles into the synaptic cleft [43]. These results indicate that orexin-A can promote HONCs to differentiate into mature OSNs.

Orexin-A and orexin-B are cleaved from a common precursor protein, prepro-orexin, and have 46 % identity. Orexins interact with two G protein-coupled receptors, OX1R and OX2R. Orexin-A has 100 times greater affinity for OX1R compared to orexin-B, which prefers binding to OX2R [44,45]. Orexin neurons have widespread projections throughout the brain and spinal cord, reaching areas involved in odor processing such as the olfactory bulbs, the locus coeruleus, and the raphe nuclei [16,46]. Orexin plays a crucial role in regulating olfaction and feeding and in promoting neurogenesis in the dentate gyrus of the hippocampus as a trophic factor [47]. In this work, the pretreatment with suvorexant, a dual orexin receptor antagonist, in HONCs incubated with orexin-A abolishes orexin-induced OSN differentiation. Additionally, the selective OX1R antagonist, SB674042, also eliminates orexin-

induced OSN differentiation, but the selective OX2R antagonist, TCS-OX2-29, does not. It indicates that the actions of orexin-A are mediated by the OX1R. Furthermore, orexin-A is a 33-amino acid peptide with identical sequences in humans, rats, mice, and bovine, while orexin-B is a 28-amino acid peptide that differs by two amino acids between the human, rat, and mouse sequences [45]. Experimental results demonstrate that orexin-B does not have the same effect as orexin-A.

The exact mechanisms underlying orexin's olfactory neuroregenerative effects are not yet fully understood. However, it is believed that orexin may act through various signaling pathways and interact with other growth factors and molecules involved in neuronal survival and regeneration. Previous studies indicate that orexins play a critical role in physiological and metabolic processes via the PI3K/Akt/mTOR pathway [48–50]. Consistent with this, our work observed an upregulation of protein expression in the PI3K-Akt-mTOR pathway, which is essential for promoting neuronal survival and growth [52,53]. Additionally, orexin has been shown to modulate the expression and release of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 [51–53]. These factors are known to promote neuron survival, growth, and regeneration. In addition to promoting neuronal health, orexin has been found to regulate inflammatory responses and reduce oxidative stress, which can contribute to neuronal damage and hinder regeneration [54]. By modulating these processes, orexin may create a more favorable environment for neuron regeneration.

To the best of our knowledge, this is the first study to show that orexin-A can enhance the differentiation and growth of human OSNs in vitro through its interaction with the OX1R. The study's limitation lies in the fact that, despite discovering that orexin-A encourages the differentiation of OSNs and enhances the development of elements linked to olfactory signal transmission within HONC culture, it's crucial to emphasize that research on the connection between orexin and the regeneration of olfactory neurons is still in its initial phases. Further investigations are required to clarify the exhaustive mechanisms and establish an in vivo model for potential therapeutic uses of orexin in promoting olfactory neuron regeneration under various circumstances.

5. Conclusion

The results of the experiments indicate that orexin-A not only boosts the expression of human olfactory basal cells but also enhances the differentiation of OSN and the development of the olfactory apparatus in HONCs via interaction with the OX1R.

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