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Effect of intranasal administration of neurotrophic factors on regeneration of chemically degenerated olfactory epithelium in aging mice

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In the mammalian olfactory epithelium (OE), neurogenesis continues throughout the lifetime, by replacing olfactory receptor neurons (ORNs) lost by normal turnover in the postnatal period. However, this ability decreases with age and/or because of various toxic factors. To date, no effective treatment for olfactory dysfunction' especially because of aging, is available in clinical practice. Here, we examined the effects of intranasal administration of fibroblast growth factor-2 and insulin-like growth factor-1 in gelatin hydrogel on the degenerated OE of aging mice induced by methimazole administration. These topical treatments led to increases in the number of olfactory marker protein-positive cells, which identified mature ORNs, resulting in the increased thickness of OE. These results indicate that both fibroblast growth factor-2 and insulin-like growth factor-1 promote the proliferation of basal cells and differentiation of immature ORNs into mature ORNs in the degenerated OE of aging mice. These agents might be promising candidates for the treatment of degenerated OE of aging humans. *NeuroReport* **29:1400–1404 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

The mammalian olfactory epithelium (OE) located in the nasal cavity is a specialized sensory epithelium involved in odor perception. In the OE, neurogenesis continues throughout the lifetime, replacing olfactory receptor neurons (ORNs) lost by normal turnover in the postnatal period [1,2]. This unique ability of the OE, which most other neural systems except the dentate gyrus and the subventricular zone do not have [3,4], decreases with age and/or because of various toxic factors including viruses, smoking [5], and environmental factors. In clinical practice, topical or oral corticosteroids are used for the treatment of olfactory dysfunction. These have been shown to be efficacious in the reduction of mucosal swelling and improving access of chemical substance to the ORNs in rhinosinusitis, but not effective in olfactory dysfunction resulting from damaged OE because of other factors except rhinosinusitis.

Here, we focus on OE regeneration by attempting to develop a strategy to promote it in a chemically damaged aging mouse model.

The OE is a pseudostratified epithelium, consisting of three types of cells: supporting cells, ORNs, and basal cells, which are layered from the apical to basal. The ORNs are derived from stem cells located in the basal layer of OE, which are divided into two subclasses: globosal and horizontal basal cells. Continuous mitotic division of globose basal cells produces ORNs under normal conditions and that of horizontal basal cells produces all the cells in the OE in the event of extensive damage [6]. Continuous mitotic division of basal cells produces immature ORNs. Cell bodies of immature ORNs migrate apically with differentiation into mature ORNs. Many studies have reported that various neurotrophic factors regulate the proliferation of stem cells and differentiation into ORNs at distinct developmental stages [7]. Above all, fibroblast growth factor (FGF) superfamily members are important in the development of the OE as well as other neural tissues [8]. FGF2 is expressed in the OE in adult rodents [9] and promotes the proliferation of stem cells in vitro [9–12]. Another neurotrophic factor considered essential for the regeneration of OE is insulin-like growth factor-1 (IGF1). The OE expresses IGF1 and its receptors, and IGF1 stimulates proliferating precursors to differentiate into ORNs *in vitro* [13]. McCurdy *et al.* [14] report that neurotrophic factors are essential for regeneration and maintenance of the OE in adulthood. Actually, several studies have

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reported the effects of neurotrophic factors on the regeneration of OE in adult mice [5,14–16].

In our previous study, in aging mice, intranasal administration of FGF2 promoted the production of immature ORNs, but not mature ORNs [14]. In this study, we assessed the effectiveness of topical application of FGF2 or IGF1 on methimazole (MMI)-damaged OE. We examined the expression of mRNA of FGF2 and Igf1, and their receptors, Fgfr1 and Igf1r, during regeneration in the damaged OE [15], to confirm the time of administration of FGF2 or IGF1. In an attempt to administer FGF2 or IGF1 more effectively, we included these factors in a gelatin-based hydrogel, which has the advantage of the sustained release of drugs [17].

Materials and methods

Animal preparation and methimazole *injection*

Male Institute of Cancer Research mice (7 months old) were used in this study. All mice were housed in acrylic cages with wood chip bedding and unlimited access to food/water at a temperature of 22 ± 1°C with a 12 h light/dark cycle. All the procedures were approved by the Animal Care Ethics Committee of Kobe University Graduate School of Medicine (registration no. P120912, P090116). The mice were injected intraperitoneally with 100 mg/kg body weight of MMI. Saline-injected mice served as controls. In this study, the day of MMI injection was defined as day 0.

DNA microarray analysis

The procedures are as described previously [15]. Total cellular RNA was isolated from the damaged OE, at 12 h, on day 1, 3, 7, and 14 ($n = 10$ in each subgroup) after MMI. It was quantified using spectrophotometry and adjusted to equal concentrations. DNA microarray analysis was carried out using CodeLink Mouse Whole Genome Bioarrays (Applied Microarrays, Tempe, Arizona, USA) according to the manufacturer's protocol. The data were analyzed using Microarray Data Analysis tool version 3.2 supplied by the manufacturer (Filgen, Nagoya, Aichi, Japan).

Preparation of fibroblast growth factor-2-impregnated or insulin-like growth factor-1-impregnated gelatin hydrogels

Gelatin hydrogels were prepared just before administration by dripping 10 μl of a solution of saline or undiluted solution of 100 μg/ml human recombinant FGF2 (Kaken Pharmaceutical Co., Tokyo, Japan) or 10 mg/ml human recombinant IGF1 (Orphan Pacific, Tokyo, Japan) onto a 1 mm^2 sheet of freeze-dried gelatin hydrogel (Medgel Co., Tokyo, Japan).

Nasal instillation of topical agents

The mice were anesthetized by an intraperitoneal combined injection of midazolam, medetomidine chloride, and butorphanol (4, 0.3, and 5 mg/kg body weight). The saline ($n = 4$), or FGF2 ($n = 5$), or IGF1 ($n = 4$)-added gelatin hydrogel were applied on the right side of the nasal cavity on day 3, through a nostril, to the anterior portion of the lateral endoturbinate.

Tissue preparation

Mice were killed on day 18. Under deep anesthesia with isoflurane, mice were killed by cervical dislocation and then decapitated. Heads were fixed with 10% formaldehyde at room temperature for 24 h. The specimens were then decalcified in a decalcification solution (10% EDTA, pH 7.0) over 7 days, dehydrated through a graded alcohol and xylene series, and embedded in paraffin. The specimens were serially sectioned at 4 μm thickness.

Immunohistochemical staining

The sections were deparaffinized and rehydrated through a graded xylene and alcohol series. The sections were placed in 0.01 M of citric acid buffer solution and heated at 100°C in a microwave oven for 20 min for antigen retrieval. Endogeneous peroxidase was blocked with 3% hydrogen peroxide and nonspecific binding was blocked with 2.5% normal horse serum. The sections were incubated with the following anti-mouse polyclonal antibodies: GAP43 (1 : 500 dilution; MAB347; Chemicon International, Temecula, California, USA), which is expressed by immature ORNs, and olfactory marker protein (OMP) (1 : 900 dilution; 019-22291; Wako, Osaka, Japan), which is expressed by mature ORNs, respectively, for 60 min. Antibody binding was visualized using the ImmPRESS (Filgen, Nagoya, Aichi, Japan) HRP anti-mouse or goat IgG (Peroxidase) Polymer Detection Kit (MP-7402 or MP-7405; Vector Laboratories, Burlingame, California, USA), respectively. Diaminobenzidine was used for coloration and nuclei were counterstained with hematoxylin.

Quantitative analysis

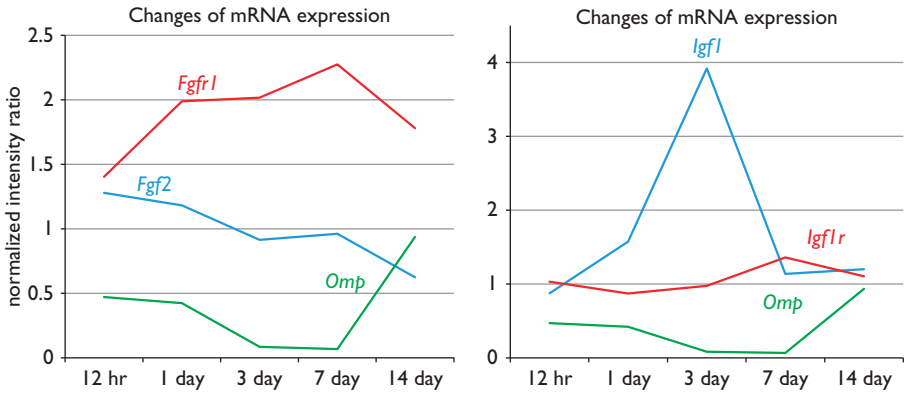
The OE on the nasal septum, in coronal sections through the olfactory bulbs, was observed at a magnification of × 200 under a Nikon microscope (Tokyo, Japan). The thickness of OE and the number of cells positive for GAP43 and OMP were examined. Three fields per each mouse were examined. To avoid variations between the regions of interest, the observation was restricted to the same areas of septal OE. The mean values of saline or FGF2-administered or IGF1-administered groups were compared statistically by one-way analysis of variance before being analyzed post-hoc by the Tukey–Kramer test. Differences were considered significant when the P value was less than 0.05.

Results

Transitional patterns of fibroblast growth factor-2 or insulin-like growth factor-1 mRNA expression during the regeneration of olfactory epithelium

The intensity ratio of mRNA for FGF2, normalized to the control, was greater than 1 at 12 h after MMI injection

Fig. 1



Changes in mRNA expression of *Fgf2* and its receptor *Fgfr1*, and *Igf1* and its receptor *Igf1r* in the olfactory epithelium after the intraperitoneal injection of methimazole. Changes in the expression of *Omp* mRNA are shown in the graphs as references. Vertical axis: normalized intensity ratio of mRNA transcription in the olfactory epithelium of methimazole-injected mice divided by those of the control. FGF, fibroblast growth factor; IGF, insulin-like growth factor.

(Fig. 1), indicating that the expression began to increase sometime between 0 and 12 h. It was still higher than the control level at 1 day, indicating that the expression continued to increase after 12 h to 1 day. On day 3, the ratio was less than 1, indicating that mRNA expression decreased below its control level between days 1 and 3. The mRNA of Fgfr1 was higher than the control level at 12 h, 1, 3, 7, and 14 days. The intensity ratio of mRNA for IGF1, normalized to the control, was below 1 at 12 h, and greater than 1 at 1, 3, 7, and 14 days (Fig. 1), indicating that the expression began to increase sometime between 12 h and day 1, although it may have peaked either before or after day 3. The mRNA level of Igf1r was higher than the control level at day 7 and 14, indicating that the expression began to increase sometime between days 3 and 7. These results suggested that FGF2 and IGF1 were required for regeneration of the OE within the first 7 days after excessive injury. Previous studies reported that neurotrophic factors such as FGF2 [18,19], IGF1, and BDNF [20,21] showed a considerable decrease with age in rodents. Taken together, we hypothesized that the administration of FGF2 and IGF1 in gelatin hydrogels, at 3 days after MMI injection, would yield the maximum effect on the regeneration of OE.

Effects of fibroblast growth factor-2 or insulin-like growth factor-1 on the regeneration of degenerating olfactory epithelium in aging mice

No significant differences were found in the number of GAP43-positive cells among saline or FGF2-administered or IGF1-administered groups. In contrast, significant differences were found in the number of OMP-positive cells and wall thickness among these groups. The OE was significantly thicker in the FGF2-administered or IGF1-administered group than that in the control group (Figs 2 and 3). Between the FGF2-administered and IGF1-administered group, the thickness of OE was not significantly different.

The number of OMP-positive cells increased in the FGF2-administered and IGF1-administered group compared with that in the control group (Figs 2 and 3). Between the FGF2-administered and the IGF1-administered group, the number of OMP-positive cells was not significantly different.

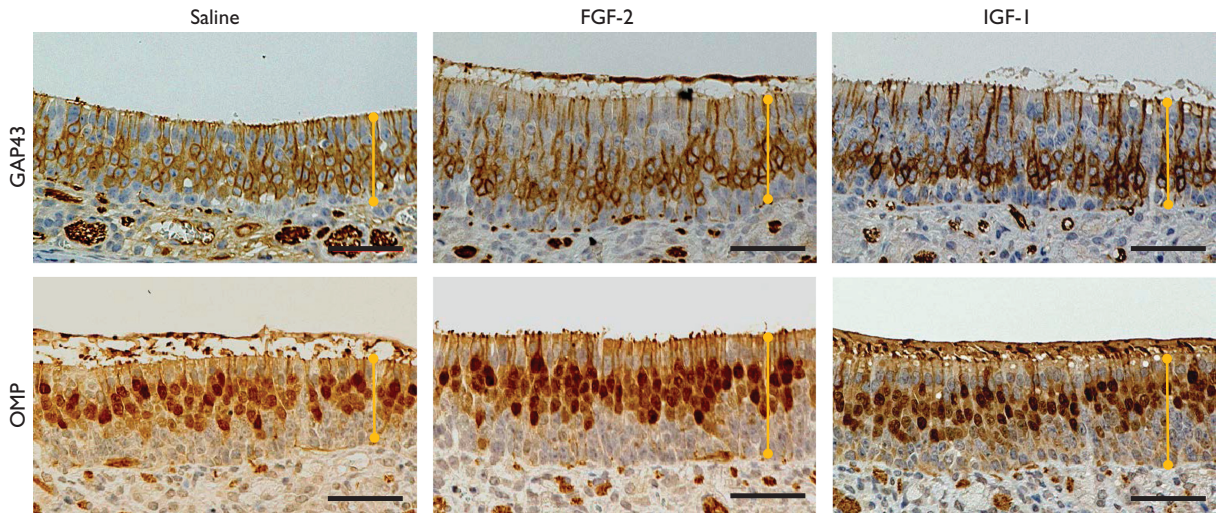
Effects of fibroblast growth factor-2 or insulin-like growth factor-1 on the regeneration of olfactory epithelium depending on locations in the nasal cavity

In this study, we placed gelatin hydrogels unilaterally (on the right side) to retain enough space for respiration. However, no significant differences were observed between the right and left sides of the nasal cavity in the thickness of OE, and the number of GAP43-positive or OMP-positive cells in each group (Fig. 4).

Discussion

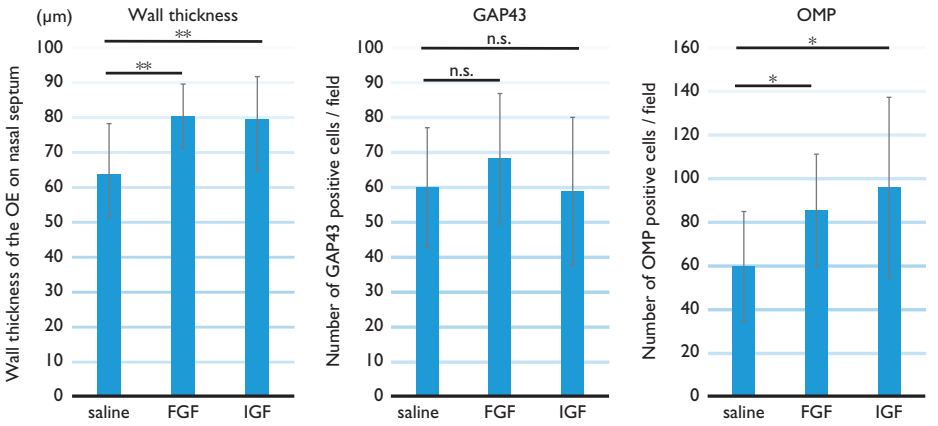
In this study, we demonstrated the effect of intranasal administration of FGF2 or IGF1 on OE regeneration in aging mice. In the FGF2-administered and IGF1-administered group, use of gelatin hydrogels led to an increase in the number of OMP-positive cells, resulting in an increased thickness of OE, in contrast with our previous report, in which intranasal administration of FGF2 did not lead to differentiation of mature ORNs [14]. The most plausible explanation for the different results in these two studies is the use of gelatin hydrogels. In our previous study, FGF2 was administered intranasally in PBS solution twice a day. In contrast, in the present study, the continuous effect of FGF2 was achieved owing to gelatin hydrogel placement, which led to the controlled release of growth factors, the half-life of which is very short in its free form [17]. Second, the condition of OE was significantly different in the two studies. The OE was under normal conditions in our previous study. In contrast, the OE was excessively damaged by MMI in the present study. Just after the injection of

Fig. 2



Immunostaining for GAP43 or olfactory marker protein (OMP)-positive cells in the olfactory epithelium 18 days after the methimazole injection in saline, FGF-2-administered, or IGF1-administered groups. Yellow bars represent the thickness of olfactory epithelium from the apical to basal. Scale bar, 50 mm. FGF, fibroblast growth factor; IGF, insulin-like growth factor.

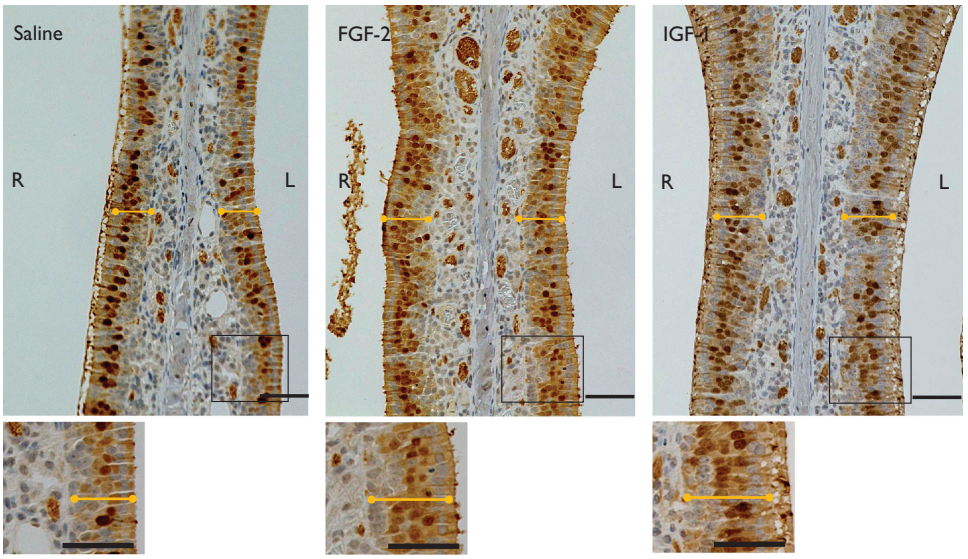
Fig. 3



Comparison of the wall thickness, number of cells positive for GAP43, or olfactory marker protein (OMP) in the olfactory epithelium (OE) 18 days after the methimazole injection among saline, FGF-2-administered, and IGF1-administered groups. Data were counted at 3 separate sites Fig. 2, and mean values of 3 groups were compared. *$P < 0.05$; **$P < 0.01$; NS. Results represent mean ± SD. $n = 4$ (saline, IGF-1), $n = 5$ (FGF-2), analysis of variance. FGF, fibroblast growth factor; IGF, insulin-like growth factor.

MMI, the whole OE degenerated and only basal cells were left. Three days after the injection of MMI, the OE was still thin; thus, the FGF2-administered might easily have reached the basal cells compared with the normal condition. In addition, processes of regeneration of ORNs between normal and excessively damaged conditions are obviously different [6]. The proliferation and differentiation of stem and progenitor cells in OE are stimulated and maintained by several secreted factors, and regulated by the feedback inhibitory signal expressed by ORNs under normal conditions [7]. In this study, the microarray data showed a higher level of FGF2 and IGF1 than the control in the OE after MMI damage, and regeneration of OE is promoted more after MMI damage than under the normal condition. This suggests that intranasal administration of FGF2 or IGF1 increases this regenerative capacity of progenitor cells. Interestingly, it was suggested that a part of the hydrogel in the nasal cavity may migrate to the opposite side through choanae, and that as found in this study, unilateral administration of gelatin hydrogel with FGF2 or IGF1 was effective on both sides of the OE. Taken together, our results suggest that a single intranasal administration of neurotrophic factors using gelatin hydrogel is effective for the treatment of degenerating OE in aged mice.

Fig. 4



Antiolfactory marker protein (OMP) immunohistochemical staining of coronal sections in the nasal septum on 18 days after the methimazole injection in saline, fibroblast growth factor-2 (FGF-2)-administered, or insulin-like growth factor-1 (IGF-1)-administered groups. Enlarged view of each olfactory epithelium as outlined by the black quadrangle is shown at the lower left of each figure. R, right nasal cavity; L, left nasal cavity. Yellow bars represent the thickness of olfactory epithelium from the apical to basal. Scale bar, 50 mm.

Conclusion

Intranasal administration of FGF2 or IGF1 using gelatin hydrogel is effective in stimulating OE regeneration after chemical damage in aged mice. FGF2 and IGF1 are already used in clinical pathology, and intranasal administration can be performed by an otorhinolaryngologist painlessly at an outpatient clinic. Theoretically, few side effects are expected in comparison with systemic administration. Our study provides a promising alternative therapeutic approach to targeting olfactory dysfunction following OE damage in the elderly. More detailed investigations should be performed for clinical application.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

References

1 Farbman AI. Olfactory neurogenesis: genetic or environmental controls. *Trends Neurosci* 1990; **13**:362–365.
2 Schwob JE. Neural regeneration and the peripheral olfactory system. *Anat Rec* 2002; **269**:33–49.
3 Seki T, Sawamoto K, Parent J, Alvarz-Buylla A. Neurogenesis in the adult brain I. *Neurobiology*. Tokyo: Springer; 2011. pp. 1–413.
4 Dalton P. Olfaction and anosmia in rhinosinusitis. *Curr Allergy Asthma Rep* 2004; **4**:230–236.
5 Nota J, Takahashi H, Hakuba N, Hato N, Gyo K. Treatment of neural anosmia by topical application of basic fibroblast growth factor-gelatin hydrogel in the nasal cavity: an experimental study in mice. *JAMA Otolaryngol Head Neck Surg* 2013; **139**:396–400.
6 Bakari A, Usman M. Olfactory function and dysfunction. *Arch Int Surg* 2012; **2**:2–6.
7 Leung CT, Coulombe PA, Reed RR. Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nat Neurosci* 2007; **10**:720–726.
8 Beites CL, Kawauchi S, Crocker CE, Calof AL. Identification and molecular regulation of neural stem cells in the olfactory epithelium. *Exp Cell Res* 2005; **306**:309–316.
9 Ford-Perriss M, Abud H, Murphy M. Fibroblast growth factors in the developing central nervous system. *Clin Exp Pharmacol Physiol* 2001; **28**:493–503.
10 Kawauchi S, Beites CL, Crocker CE, Wu HH, Bonnin A, Murray R, Calof AL. Molecular signals regulating proliferation of stem and progenitor cells in mouse olfactory epithelium. *Dev Neurosci* 2004; **26**:166–180.
11 DeHamer Mmguevara JL, Hannon K, Olwin BB, Calof AL. Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors. *Neuron* 1994; **13**:1083–1097.
12 Calof AL, Mumm JS, Rim PC, Shou J. The neuronal stem cell of the olfactory epithelium. *J Neurobiol* 1998; **36**:190–205.
13 Gokoffski KK, Kawauchi S, Wu HH, Santos R, Hollenbeck PLW, Lander AD, Calof AL. Feedback regulation of neurogenesis in the mammalian olfactory epithelium: new insights from genetics and systems biology. In: Menini A, editor. *The neurobiology of olfaction [chapter 10]*. Boca Raton: CRC Press; 2010.
14 McCurdy RD, Feron F, McGrath JJ, Mackay-Sim A. Regulation of adult olfactory neurogenesis by insulin-like growth factor-1. *Eur J Neurosci* 2005; **22**:1581–1588.
15 Nishikawa T, Doi K, Ochi N, Katsunuma S, Nibu K. Effect of intranasal administration of basic fibroblast growth factor on olfactory epithelium. *Neuroreport* 2009; **20**:764–769.
16 Uranagase A, Katsunuma S, Doi K, Nibu KI. BDNF expression in olfactory bulb and epithelium during regeneration of olfactory epithelium. *Neurosci Lett* 2012; **516**:45–49.
17 Ueha R, Ueha S, Sakamoto T, Kanaya K, Suzukawa K, Nishijima H, *et al*. Cigarette smoke delays regeneration of the olfactory epithelium in mice. *Neurotox Res* 2016; **30**:213–224.
18 Tabata Y. Significance of release technology in tissue engineering. *Drug Discov Today* 2005; **10**:1639–1646.
19 Shetty AK, Hattiangady B, Shetty GA. Stem/progenitor cell proliferation factors FGF2, IGF1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes. *Glia* 2005; **51**:173–186.
20 Mudo G, Bonomo A, Di Liberto V, Frinchi M, Fuxe K, Belluardo N. The FGF2/FGFRs neurotrophic system promotes neurogenesis in the adult brain. *J Neural Transm (Vienna)* 2009; **116**:995–1005.
21 Lai M, Hibberd CJ, Gluckman PD, Seckl JR. Reduced expression of insulin-like growth factor 1 messenger RNA in the hippocampus of aged rats. *Neurosci Lett* 2000; **288**:66–70.