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Research report

Regeneration of olfactory receptor neurons following chemical lesion: time course and enhancement with growth factor administration

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Abstract

Although it has been known for over 50 years that olfactory receptor neuron (ORN) neurogenesis and subsequent reinnervation of the olfactory bulb (OB) occurs following ORN injury, the precise intrinsic and extrinsic factors that regulate this dynamic process have not yet been fully identified. In the first of two experiments, we characterized the time course of anatomical recovery following zinc sulfate ($ZnSO_4$) lesion of ORNs in adult male Sprague–Dawley rats. $ZnSO_4$ produced a near complete deafferentation of OB within 3 days following intranasal administration. A time-dependent increase in ORN reinnervation of OB was observed following 10, 20, and 30 day recovery intervals. Given the evidence that bFGF, EGF, and TGF-α have mitogenic effects on ORNs in vitro, a second experiment examined the extent to which these growth factors (GFs) might enhance ORN regeneration and subsequent reinnervation of OB in vivo. Rats received intranasal infusions of $ZnSO_4$ on day 0, followed by subcutaneous injections of either bFGF (5, 10, or 50 μg/kg), EGF (5, 10, or 50 μg/kg), or TGF-α (5 or 10 μg/kg) on days 3–6. Horseradish peroxidase (HRP) histochemistry of OB following a 10-day recovery period revealed a dose-related enhancement in reinnervation of OB for each of the three growth factors examined, with the greatest enhancement produced by TGF-α. These data suggest that GFs may regulate ORN mitogenesis in vivo in a way similar to that which has been characterized in vitro.

Keywords: Regeneration; Neurogenesis; Olfactory system; Rat; Zinc sulfate; Growth factor

1. Introduction

The olfactory epithelium (OE) contains a population of mature olfactory receptor neurons (ORNs) that project directly to the olfactory bulb (OB) within the central nervous system (CNS). There is also a population of continuously-dividing neuronal progenitor cells within the OE, the globose basal cells. Although ORN neurogenesis occurs continuously in normal, undamaged OE, the rate of neurogenesis is greatly enhanced following physical or chemical damage to OE proper, the axons of ORNs, or the OB [7,13,25,33,34]. Anatomical, biochemical, and electrophysiological studies clearly indicate that progeny of globose basal cells differentiate into ORNs, send axons to the OB, extend a dendritic process through the mucosal lining, and ultimately display chemical and physiological characteristics of mature ORNs [2,6,21,24,28,36,37]. Together with the results of several behavioral studies suggesting that a recovery of odor-guided behavior accompanies ORN neurogenesis following chemical or surgical destruction [1,15,38,44], these data indicate that ORN regeneration results in intact and functionally meaningful olfactory input to the CNS.

The capacity of ORNs to regenerate and establish functional connections with the CNS following injury makes the olfactory system an attractive model not only for the study of neurogenesis per se, but also for the development of treatment strategies and the testing of bioactive compounds designed to promote the repair of damaged neural tissue. Among the many promising compounds in this regard are several peptide growth factors (GFs) that are known to play a critical role in the regulation of neurogenesis in the developing CNS. Persistent expression of some GFs and their receptors continues into adulthood within a number of regions including the hippocampus, subventricular zone, and the OE [4,11,27,35]. Recent data suggest that endogenous GFs may play a role in the regulation of neurogenesis within these cell populations in both the developing and adult nervous systems. For example, administration of basic fibroblast growth factor (bFGF) stimulates neuronal precursor proliferation in the hippocampus

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and subventricular zone in the rat pup [40]. Intraventricular infusion of bFGF also stimulates precursor proliferation within the subventricular zone of the adult rat [17]. Two other GFs, epidermal GF (EGF) and transforming GF-α (TGF-α), also appear to be involved in the regulation of proliferation and differentiation of many cell types, most notably those of epithelial origin (see Ref. [4] for a review). The receptor for both of these GFs continues to be expressed postnatally in several regions undergoing active neurogenesis into adulthood, including the subventricular zone, the dentate gyrus, and OE [16,27,35].

Recent research has focused on further characterizing the potential role of these GFs in regulating stimulatory and/or inhibitory control over basal cell proliferation within the OE. Specifically, bFGF appears to have a direct stimulatory effect on ORN precursor proliferation in vitro [8,18]. In addition, using an organotypic culture system that parallels the normal mitotic rate of OE, Farbman and Buchholz [11] have found that both EGF and TGF-α can upregulate the mitotic division of ORN progenitors. Given the profile of effects these GFs have on ORNs in vitro, and since all of these GFs are expressed in rat olfactory mucosa [11,12,30], the present experiments were designed to determine in vivo the normal rate of ORN regeneration following chemical lesions of the OE (Experiment 1), and to further examine the extent to which this anatomical recovery could be enhanced following administration of bFGF, EGF, and TGF-α (Experiment 2).

2. Experiment 1: olfactory receptor neuron regeneration following chemical lesion

Experimentally-induced damage to mammalian OE by physical or chemical means has been used extensively in the study of the anatomical, biochemical, physiological, and behavioral consequences of degeneration and regeneration of ORNs. Although a number of chemical substances have been found to effectively lesion ORNs [29,34,43], intranasal infusion of $ZnSO_4$ has been one of the most commonly used [1,2,14,22,41–43]. Even though several factors appear to influence the time course and extent of degeneration and subsequent regeneration of ORNs following intranasal infusions of $ZnSO_4$, substantial loss of the OE by coagulation necrosis has been consistently observed using this approach [2,14,20,32], suggesting that it can be a useful means of producing ORN cell death.

In order to maximize the probability of detecting GF-related enhancements of ORN regeneration in Experiment 2, the primary purpose of this first experiment was to identify a recovery interval at which an intermediate extent of OB reinnervation would be observed following intranasal infusions of $ZnSO_4$. The extent of ORN degeneration and subsequent regeneration and reinnervation of OB was assessed using the anatomical tracer horseradish peroxidase (HRP). Since this enzyme is readily taken up by ORNs and transported axoplasmically, quantification of the extent of HRP in the nerve layer and glomeruli of OB following intranasal application was used as an index of the extent of ORN regeneration and reinnervation of the OB. Experiment 1 therefore characterized the extent of OB differentiation and time course of reinnervation using an intranasal $ZnSO_4$ infusion protocol similar to that described by Mayer and Rosenblatt [22], followed by quantification of the extent of HRP transported to OB following recovery intervals of either 3, 10, 20, or 30 days.

2.1. Materials and methods

2.1.1. Subjects

Twenty male Sprague–Dawley rats (Harlan, IN) weighing 200–250 g served as subjects. All animals were housed individually in suspended wire mesh cages and maintained on a 12/12 h light/dark cycle, with lights on at 0700 h. All experimental procedures were conducted during the light cycle. Food and water were available ad libitum throughout the duration of the experiment. Using a between-subjects design, rats were randomly assigned to one of eight treatment groups that differed in intranasal treatment (saline or $ZnSO_4$) and recovery interval (3, 10, 20, 30 days). Two saline and three $ZnSO_4$ treated subjects were sacrificed at each recovery interval.

2.1.2. $ZnSO_4$ Lesion / HRP administration

On day 0, all rats were anesthetized with an intramuscular injection of an anesthetic mixture (ketamine, 50 mg/kg; xylazine, 2.61 mg/kg; acepromazine, 0.65 mg/kg) and given bilateral intranasal infusions of 0.05 ml of either 0.9% sterile saline or 2% $ZnSO_4$ (Sigma-Aldrich). A 1 cm^3 syringe with a blunted and angled needle (23 gauge; 20°) was used for all intranasal infusions. Experimental subjects were again anesthetized on days 3, 10, 20, or 30, depending upon treatment condition, and given intranasal infusions of 1% HRP (Type VIa, Sigma-Aldrich; a total of 0.3 ml per nostril).

2.1.3. HRP histochemistry

Twenty-four hours following HRP infusion, rats were deeply anesthetized with sodium pentobarbitol (100 mg/kg) and transcardially perfused with 150 ml of 0.9% physiological saline followed by 350 ml of a fixative solution consisting of 1% paraformaldehyde/1.25% glutaraldehyde in 0.1 M PB, followed by 350 ml of ice cold 30% sucrose in 0.1 M PB. Brains were extracted, immersed in 30% buffered sucrose solution and stored at 4°C for at least 3 days prior to gelatin embedding. For gelatin embedding of OBs, brains were blocked at the optic chiasm and submerged individually into a 10% solution of Knox gelatin at 37°C for 1 h then stored at 4°C for 20 min. Brains were then submerged in 4% paraformaldehyde for 3 h at room temperature followed by 30% buffered sucrose at 4°C for at least 48 h prior to slicing. Coronal sections

through the OB were taken at 50 μm using a standard freezing microtome. Every third and fourth section throughout the extent of the OB was collected and stored in deionized water at 4°C. Detection of HRP in OB tissue was achieved using a peroxidase reaction with tetramethylbenzidine (TMB) as the chromogen (adapted from Ref. [23]). Free floating sections were first rinsed in distilled water and then placed for 20 min into a pre-incubation solution consisting of a TMB-nitroprusside solution in sodium acetate buffer and distilled water. Tissue was then reacted at room temperature on a shaker for 20 min by adding a diluted solution of H_2O_2 to the incubation solution. This reaction was stopped by rinsing all sections with ice cold 5% ammonium molybdate, followed by 0.1 M PB at room temperature. The reaction product was further stabilized using a cobalt-intensified diaminobenzidine peroxidase reaction, followed by multiple rinses in 0.1 M PB. All tissue was subsequently mounted onto gelatin-covered slides, coverslipped, and the area (mm^2) of HRP stain in OB was quantified by analysis of digitized images captured by a CCD camera interfaced with image acquisition hardware (Global Lab Image) and Sigma Scan software.

2.1.4. Histological analysis

Measurements were taken from coronal sections matched for their location in the anterior–posterior plane. Total area of HRP stain (mm^2) in nerve layer and glomeruli of OB was quantified using SigmaScan software and averaged across left and right hemispheres and across three adjacent coronal sections for each animal. All values were subsequently transformed to 'percent control' values by dividing the average HRP area determined for each subject by the mean HRP area for the respective saline-treated subjects in each condition.

3. Results and discussion

Fig. 1 illustrates the extent of HRP label in OB for saline and $ZnSO_4$ treated animals following either 3, 10, 20, or 30 days of recovery. Intranasal infusions of $ZnSO_4$ resulted in a 95% decrease in HRP area at the shortest recovery interval. Furthermore, the extent of HRP in OB was found to increase in a linear fashion as a function of the length of the recovery interval, with an approximate 70% recovery 30 days post-lesion. Two-way ANOVA revealed significant main effects of intranasal treatment [$F(1,12) = 259.8$, $p < 0.0001$] and recovery interval [$F(3,12) = 15.7$, $p < 0.0002$], as well as a significant interaction between the two [$F(3,12) = p < 0.0003$]. Subsequent post-hoc pairwise comparisons further revealed that although a substantial increase in area of HRP label was observed following longer recovery intervals, lesioned animals still differed markedly from saline treated subjects at the longest (30 days) recovery interval.

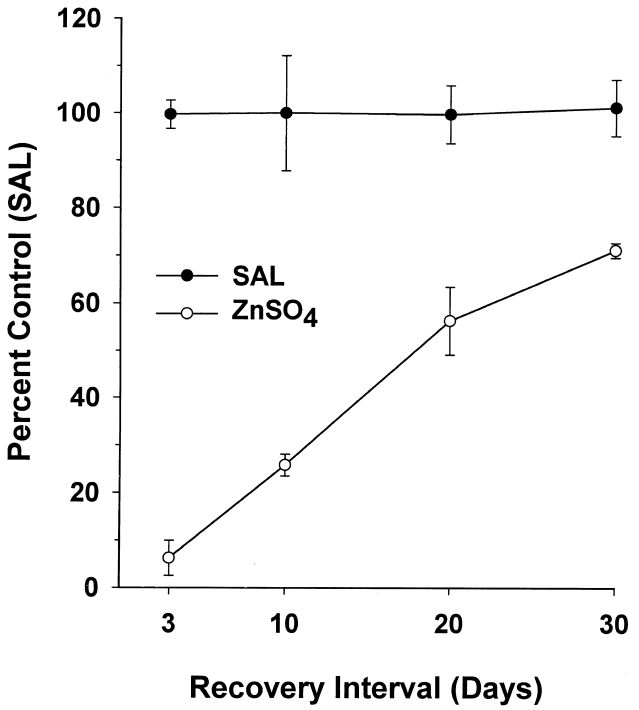

Fig. 1. Percent control area of HRP staining (mean ± S.E.M.) in OB following intranasal $ZnSO_4$ administration across recovery intervals of 3, 10, 20, and 30 days. A near complete loss of HRP staining was observed following a 3-day recovery interval, indicating substantial deafferentation of OB as a consequence of intranasal $ZnSO_4$ infusion. Longer recovery intervals were associated with greater HRP label in OB indicating a time-related anatomical recovery.

The results from the present experiment replicate and extend previous findings regarding ORN regeneration [2,14,20,24,42] and reinnervation of OB [2,14] following intranasal $ZnSO_4$ infusions. A substantial but, nevertheless, incomplete anatomical recovery (approximately 70% of saline-treated control) was observed following a 30-day recovery interval. Whether a more complete anatomical recovery would be observed following longer survival times using these lesion parameters remains to be determined. It should be noted that both concentration and volume of $ZnSO_4$ infusion are important factors that contribute to the time course and overall extent of anatomical, biochemical, and functional recovery [1,2,14,20,21, 26,38,42]. The relatively small volume and low concentration of $ZnSO_4$ used in the present study likely accounts for the more rapid rate of anatomical recovery compared to several prior reports [2,14]. Even though a very small number of $ZnSO_4$ lesioned subjects was utilized at each recovery interval, very little between-subject variability was observed. This finding is noteworthy since some investigators have reported that intranasal infusions of $ZnSO_4$ can produce lesions that vary dramatically in magnitude from animal to animal [20,21,38].

The data from the present experiment indicate that $ZnSO_4$ produced consistent and substantial deafferentation of OB followed by a time-dependent recovery of ORN

innervation of OB. As described earlier, the primary goal of this first experiment was to determine the rate of anatomical recovery following $ZnSO_4$ lesions of ORNs, and in so doing, determine a recovery interval at which a partial anatomical recovery was observed that could then be used to examine the effects of GFs on ORN regeneration. Given the pattern of findings in the present experiment, a 10 day post-lesion recovery interval (associated with approximately 25% anatomical recovery) was selected for Experiment 2.

4. Experiment 2: effects of growth factor administration on ORN regeneration following $ZnSO_4$ lesion

Several previous studies have demonstrated a prominent upregulation in the proliferative rate of dividing cells within the basal layer of OE following manipulations that result in loss of mature ORNs [5,7,24,25,31,39]. In addition, several GFs have been found to have mitogenic effects when added to the culture medium of ORNs maintained in vitro [8,11,12,18]. If endogenous GFs play a role in stimulating olfactory progenitor cell division and proliferation, then administration of GFs may further enhance the rate of ORN regeneration following lesions of mature ORNs. The purpose of this second experiment was to directly examine this possibility by assessing the effects of bFGF, EGF and TGF-α on ORN regeneration in vivo following $ZnSO_4$ lesion. As detailed above, we chose to study the effects of these GFs on the reinnervation of OB following an intermediate (10 days) recovery interval in order to ensure sensitivity to potential GF-related effects on ORN regeneration and reinnervation of the OB.

4.1. Materials and methods

4.1.1. Subjects

One hundred eight male Sprague–Dawley rats (Harlan, IN) weighing 185–235 g at the beginning of the experiment served as subjects. All housing and maintenance conditions were identical to those described in Experiment 1.

4.1.2. Procedure

Intranasal infusion of $ZnSO_4$ was performed as described in Experiment 1. All GFs (human recombinant; Sigma-Aldrich) were prepared immediately prior to each experiment, diluted to the appropriate concentration using 0.1 M PBS as vehicle, and stored at 4°C. Doses of GF were based on prior studies demonstrating relatively high potency of each of these GFs both in vitro [8,11] and in vivo [40]. Since each of these polypeptides appears to maintain potent biological activity following peripheral administration in the rat [10,40], and to avoid physical damage to the OE with repeated daily intranasal GF application, the subcutaneous (s.c.) route of administration was selected over an intranasal approach. All animals received injections of vehicle or GF (see below) once per day on days 3–6.

Twelve animals died within 24 h following intranasal $ZnSO_4$ ($n = 10$) or HRP ($n = 2$) infusions due to respiratory complications. The remaining 96 subjects were randomly assigned to one of several treatment conditions; these conditions, the final number of subjects in each, and treatment regimens were as follows: (1) Saline/vehicle (S/V; $n = 18$) treated animals received intranasal administrations of 0.9% saline on day 0 followed by s.c. injections of vehicle (0.1 M PBS) on days 3–6 and served as a pure control condition. (2) $ZnSO_4$/vehicle (Z/V; $n = 23$) treated animals received intranasal infusions of $ZnSO_4$ on day 0 followed by s.c. injections of vehicle on days 3–6. (3) $ZnSO_4$/bFGF animals were given intranasal infusions of $ZnSO_4$ on day 0 followed by s.c. injections of bFGF on days 3–6 in concentrations of either 5 μg/kg (F5; $n = 6$), 10 μg/kg (F10; $n = 7$), or 50 μg/kg (F50; $n = 7$). (4) $ZnSO_4$/EGF treated subjects received intranasal infusions of $ZnSO_4$ on day 0 followed by s.c. injections of EGF on days 3–6 in concentrations of either 5 μg/kg (E5; $n = 8$), 10 μg/kg (E10; $n = 7$), or 50 μg/kg (E50; $n = 7$). (5) $ZnSO_4$/TGF-α treated subjects were given intranasal $ZnSO_4$ infusions on day 0 followed by s.c. injections of TGF-α on days 3–6 in concentrations of either 5 μg/kg (T5; $n = 6$) or 10 μg/kg (T10; $n = 7$).

Ten days following $ZnSO_4$ infusions, all rats received intranasal infusions of HRP and were sacrificed 24 h later. All tissue was processed for visualization and quantification of HRP in OB in a manner identical to that described in Experiment 1. Temporal constraints on processing the tissue from 108 subjects required that the experiment be run in 3 replications. In contrast to Experiment 1 in which data were transformed to 'percent saline' values, however, all data in Experiment 2 were transformed to 'percent Z/V' values by dividing the mean area of HRP for each subject by the mean area of HRP for Z/V treated controls within each replication. This conversion served to minimize variability between replications potentially due to differences in extent of $ZnSO_4$ lesion or in HRP visualization, and to focus on the extent to which GF administration resulted in an enhancement of ORN reinnervation of OB relative to lesioned animals not receiving GFs.

5. Results and discussion

Consistent with the findings of Experiment 1, mean area of HRP label in Z/V treated subjects following a 10-day recovery interval was dramatically reduced relative to saline treated controls. A *t*-test comparing the area of HRP label of $ZnSO_4$ treated animals with a 10-day recovery interval in Experiment 1 (mean = 25.8%, S.E.M. = 2.3) to that of Z/V treated animals in Experiment 2 (mean = 23.79%, S.E.M. = 2.61) indicated that the magnitude of

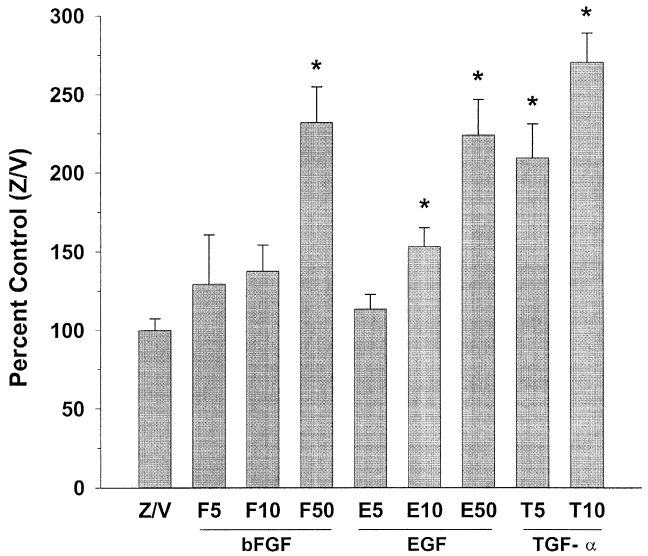

Fig. 2. Dose-related effects of bFGF (5, 10, 50 μg/kg), EGF (5, 10, 50 μg/kg), and TGF-α (5 and 10 μg/kg) on area of HRP label in OB (mean ± S.E.M.) relative to Z/V-treated controls following a 10-day recovery interval (* $p < 0.05$, Dunnett's post-hoc analysis vs. Z/V controls).

OB deafferentation did not differ between the groups [$t(24) = 0.28$, *ns*], suggesting both that the extent of OB deafferentation was similar in the two studies and that the administration of vehicle alone in Experiment 2 had no observable effect on ORN regeneration.

The dose-related effects of each GF on OB reinnervation are illustrated in Fig. 2. In order to directly examine the effect of GF administration on ORN regeneration, a one-way ANOVA was performed on percent control (Z/V) area of HRP label in OB which revealed a significant treatment effect [$F(8,69) = 15.7$, $p < 0.0001$]. Subsequent post-hoc analysis using Dunnett's method further revealed that each of these GFs produced dose-related enhancements in the area of HRP staining in OB compared to Z/V treated controls. Although EGF doses of 10 and 50 μg/kg produced a significant increase in HRP area compared to Z/V treated controls, only the highest dose of bFGF (50 μg/kg) was found to increase HRP area. In contrast, even the lowest dose of TGF-α (5 μg/kg) produced effects similar to those found following high doses of EGF and bFGF. The effects of the highest concentrations of each GF are illustrated photomicrographically in Fig. 3.

The findings from the present experiment indicate that intranasal $ZnSO_4$ infusions produced reliable and consistent damage to ORNs, and further suggest that the regeneration of ORNs and subsequent reinnervation of the OB can be enhanced by s.c. administration of growth factors. The finding that the high dose of bFGF produced an enhancement in ORN regeneration is consistent with prior work

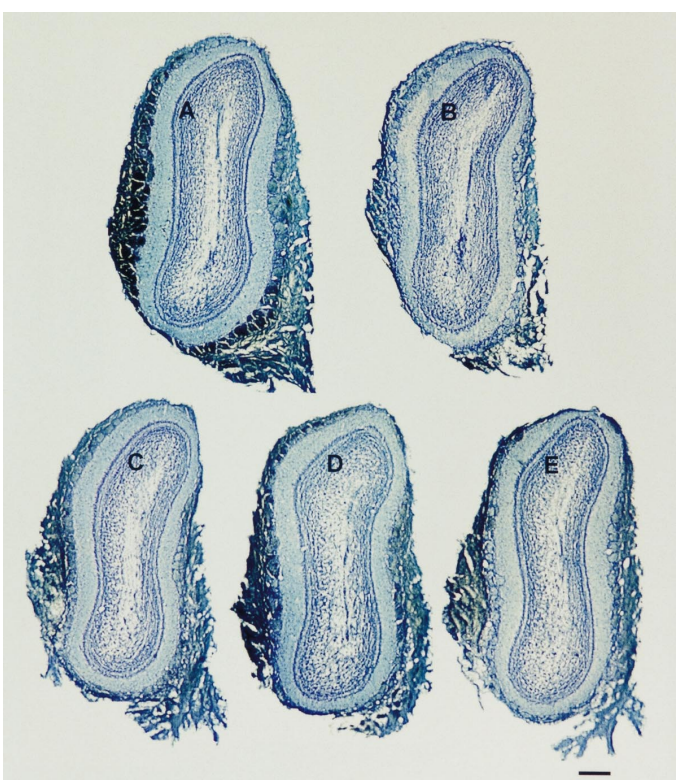

Fig. 3. Photomicrographs of representative coronal sections through the OB for (A) Saline/Vehicle, (B) $ZnSO_4$/Vehicle, (C) $ZnSO_4$/bFGF (50 μg/kg), (D) $ZnSO_4$/EGF (50 μg/kg) and (E) $ZnSO_4$/TGF-α (10 μg/kg) treated subjects. Histochemical analysis revealed the presence of significantly larger area of HRP reaction product in the olfactory nerve layer and glomeruli in GF-treated subjects compared to Z/V treated controls. Sections were counterstained with thionin following quantification for the purpose of this illustration. Scale bar is equal to 0.5 mm.

demonstrating its ability to regulate production of ORNs in vitro [8,18]. While the precise mechanism(s) by which exogenous bFGF acts to enhance the rate of ORN regeneration has not been fully characterized, several lines of evidence suggest that these effects may indeed be due to a direct effect of GFs on the proliferation of globose basal cells in the OE. Specifically, neuronal cells within the OE have been found to express the tyrosine kinase FGF receptors FGFR1 and FGFR2, to which bFGF has been shown to bind with high affinity [9,19]. Moreover, data suggest that bFGF may act to enhance neuronal proliferation by maintaining globose basal cells at a stage in which they can functionally amplify the number of progeny destined to become ORNs [8,12]. Since endogenous bFGF in OE has been found to be predominantly localized to nuclear regions of sustentacular and neuronal cells, it has been proposed that damage to OE may result in an increase in local bFGF signal that can serve to direct regeneration of OE [12]. For instance, injured or dying cells may release bFGF, and thus the effects observed in the present study may reflect an enhancement of this putative endogenous signal following lesion.

Both EGF and TGF-α were also found to produce a significant enhancement of ORN regeneration following $ZnSO_4$ lesions of OE. These findings are generally consistent with a host of prior studies demonstrating the mitogenic activity of these GFs on a variety of cell types (reviewed in Ref. [3]). Although both EGF and TGF-α were found to produce dose-related enhancements in ORN regeneration in the present study, TGF-α was found to be substantially more potent. These findings are consistent with prior work demonstrating that TGF-α is a more potent OE mitogen compared to EGF in vitro [11], and that TGF-α is a more potent activator of the EGF receptor in vivo [10]. It is important to note that since these GFs have mitogenic effects on supporting as well as neuronal cells within the OE [11], it remains unclear whether the effects of these GFs on ORN regeneration in the present experiment reflect *direct* effects on proliferation of globose basal cells, *indirect* effects on supporting cell replacement, or both. An enhanced rate of supporting cell replacement following damage as a consequence of this $ZnSO_4$ lesion approach may ultimately contribute to an enhanced rate of OE reconstitution, and therefore an enhanced rate of ORN recovery. Furthermore, it is conceivable that an increase in HRP area in the OB following GF administration may merely reflect enhanced uptake of HRP and/or alterations in the transport kinetics of ORNs. Although the precise mechanisms whereby GF administration may produce such effects remain unclear, studies examining this possibility are currently in progress.

6. General discussion

Intranasal infusions of $ZnSO_4$ resulted in a substantial but transient loss of ORNs as indicated by an initial reduction in HRP area in the OB followed by a time-related recovery. It was further found that daily s.c. injections of bFGF, EGF and TGF-α during recovery enhanced the area of HRP in the OB, evidence suggesting that these GFs can increase the normal rate of ORN regeneration in vivo. It will be important to determine if the GF-related enhancements of ORN regeneration persist beyond this relatively short (10 days) survival time. Furthermore, whether this enhanced anatomical recovery following GF-administration translates into an enhanced functional recovery remains to be determined.

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