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Inhibition of focal adhesion kinase increases adult olfactory stem cell self-renewal and neuroregeneration through ciliary neurotrophic factor

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Abstract

Constant neuroregeneration in adult olfactory epithelium maintains olfactory function by basal stem cell proliferation and differentiation to replace lost olfactory sensory neurons (OSNs). Understanding the mechanisms regulating this process could reveal potential therapeutic targets for stimulating adult olfactory neurogenesis under pathological conditions and aging. Ciliary neurotrophic factor (CNTF) in astrocytes promotes forebrain neurogenesis but its function in the olfactory system is unknown. Here, we show in mouse olfactory epithelium that CNTF is expressed in horizontal basal cells, olfactory ensheathing cells (OECs) and a small subpopulation of OSNs. CNTF receptor alpha was expressed in Mash1-positive globose basal cells (GBCs) and OECs. Thus, CNTF may affect GBCs in a paracrine manner. CNTF^{-/-} mice did not display altered GBC proliferation or olfactory function, suggesting that CNTF is not involved in basal olfactory renewal or that they developed compensatory mechanisms. Therefore, we tested the effect of increased CNTF in wild type mice. Intranasal instillation of a focal adhesion kinase (FAK) inhibitor, FAK14, upregulated CNTF expression. FAK14 also promoted GBC proliferation, neuronal differentiation and basal stem cell self-renewal but had no effective in CNTF^{-/-} mice, suggesting that FAK inhibition promotes olfactory neuroregeneration through CNTF, making them potential targets to treat sensorineural anosmia due to OSN loss.

Keywords

Olfactory stem cell; Basal cell proliferation; Neuronal differentiation; Olfactory function; Neuroregeneration

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Disclosures and Conflict of Interest
None for all authors.

Appendix A. Supplementary data
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2020.102061>.

1. Introduction

The sense of smell is maintained by a robust regeneration in the olfactory epithelium throughout life by continuously generating olfactory sensory neurons (OSNs) from basal stem cells. OSNs are directly exposed to the external environment and, therefore, are highly vulnerable to inspired toxins and chemicals (Jia et al., 2010, 2011) as well as viruses (Lee et al., 2014), including SARS-CoV-2 (Bilinska et al., 2020; Butowt and Bilinska, 2020). These injuries can lead to hyposmia or anosmia which is restored by stimulating regeneration in the olfactory epithelium (Jia et al., 2010, 2011; Schwob et al., 1995). However, the regenerative potential is diminished during aging, leading to anosmia in many of the elderly (Attems et al., 2015; Doty, 2018). No FDA approved treatments to increase adult olfactory stem cells to proliferate and differentiate are currently available but understanding the mechanisms should provide novel therapeutic targets. Basal stem cell proliferation and differentiation in the olfactory epithelium are tightly regulated by multiple mechanisms in basal stem cell niche, which is complex and not fully understood (Schwob, 2002; Schwob et al., 2017).

CNTF is almost exclusively expressed in the nervous system (Stockli et al., 1989). In the central nervous system, CNTF is produced by astrocytes (Yang et al., 2008; Stockli et al., 1991) and increases after injury (Kang et al., 2012; Park et al., 2000). Ischemic stroke upregulates CNTF expression which increases neurogenesis in the adult mouse subventricular zone of the brain (Stockli et al., 1989; Kang et al., 2012, 2013; Keasey et al., 2013). Exogenous CNTF enhances adult hippocampal neurogenesis, neuronal plasticity and spatial memory in mice (Blanchard et al., 2010; Emsley and Hagg, 2003). Thus, CNTF seems to act as an injury-induced paracrine signal to facilitate neural repair. In the olfactory epithelium, CNTF is present in the basal layer where olfactory stem cells, including horizontal (HBC) and globose (GBC) basal cells reside, and in a small subpopulation of OSNs (Buckland and Cunningham, 1999; Langenhan, 2006; Langenhan et al., 2005). However, the cell type(s) in the basal layer expressing CNTF has not been identified by co-localization. CNTF is also expressed in olfactory ensheathing cells (OECs) that wrap OSN axons and assist axonal growth (Stockli et al., 1991; Wewetzer et al., 2001; Asan et al., 2003). CNTF expression in basal stem cells has been reported to persist over three months during active repair following bulbectomy-induced olfactory epithelium injury (Buckland and Cunningham, 1998), suggesting CNTF could contribute to bulbectomy-induced regeneration. In the CNS, the specific CNTF receptor alpha (CNTFR α) is expressed by neurons (Hagg et al., 1992; Ip et al., 1991; LaVail et al., 1992; Sendtner et al., 1992) and astrocytes (Emsley and Hagg, 2003). In the olfactory epithelium, CNTFR α expression has not been documented. However, the other members of the hetero-trimer CNTF receptor, leukemia inhibitory factor receptor β (LIFR β) and gp130, are present in the olfactory epithelium (Bauer et al., 2003; Getchell et al., 2002) and LIFR β is expressed in GBCs and OECs (Nan et al., 2001).

We previously discovered that focal adhesion kinase (FAK), a non-receptor tyrosine kinase which mediates integrin signaling (Schaller, 2010), represses CNTF expression in astrogloma C6 cells (Keasey et al., 2013). Moreover, inhibition of FAK by systemic administration of FAK inhibitor, FAK14, enhances neurogenesis in the subventricular zone

of adult naïve mice through upregulation of CNTF (Jia et al., 2018). Here, we investigated whether this pathway also regulates adult regeneration in the mouse olfactory epithelium.

2. Materials and methods

2.1. Mice

A total of 158 mice were used. Our CNTF mouse line has been backcrossed to C57BL/6 eight times. Heterozygotes were bred to yield CNTF^{+/+}, CNTF^{+/-} and CNTF^{-/-} littermates. The CNTF null mice were produced by replacing the first exon of the CNTF gene with the bacterial lacZ gene, thus disrupting CNTF transcription while allowing expression of β -galactosidase (β -gal) protein which can be used as a reporter for “CNTF” expression (Yang et al., 2008; Asan et al., 2003). The CNTF^{-/-} mice have two copies and CNTF^{+/-} have one copy of the reporter gene, which can be used to identify cells expressing even low levels of CNTF (Yang et al., 2008; Kang et al., 2012; Langenhan et al., 2005; Jia et al., 2018). Both male and female CNTF littermate mice at 8–12 weeks old were used in the experiments. Adult male C57BL/6 mice (JAX 000664) at 8–10 weeks old were purchased from Jackson Laboratory and used in the experiments two weeks later. For Mash1-td-Tomato reporter mice, floxed td-Tomato mice (B6.Cg-*Gt(ROSA)26Sor^{tm6(CAG-tdTomato)Hze/J}*) were crossed with Mash1^{CreERT2} mice (*Asc1^{tm1.1(Cre/ERT2)Jejo/J}*) to obtain double heterozygous mice (Rodriguez-Gil et al., 2015). To induce td-Tomato expression in Mash1-positive cells, double heterozygous mice at 6–8 week old were injected with a single dose of 4-hydroxi-tamoxifen (intraperitoneal, i.p., 75 mg/kg) and the olfactory epithelium tissue was collected 24 h later. Mice were bred and maintained in the animal facility at East Tennessee State University. All animal work was approved by University Committee on Animal Care and complied with the NIH Guide on Care and Use of Animals.

2.2. Intranasal instillation and i.p. Injections

Anesthetized (4% isoflurane) C57BL/6, CNTF^{+/+} and CNTF^{-/-} mice aspirated a bolus of saline (50 μ l) or FAK14 (100 μ M, 50 μ l, #3414, Tocris) placed on the nares once a day for 3 days (Jia et al., 2013). In order to label proliferating cells, some mice received BrdU injections (i.p., 50 mg/kg) 4 h after saline or FAK14. To measure basal cell proliferation, the olfactory epithelium tissue was collected at 2 h after last BrdU injection. To measure neuronal differentiation and maturation, BrdU was pulse-chased for 9 days or 20 days. To measure basal levels of basal stem cell proliferation, CNTF^{+/+} and CNTF^{-/-} mice were injected i.p. with BrdU (50 mg/kg) once a day for 3 days. To measure CNTF-mediated basal cell proliferation, C57BL/6 mice aspirated saline or recombinant human CNTF (1 μ g/ml or 5 μ g/ml in 50 μ l) followed by BrdU injections (i.p., 144 mg/kg) at 18, 20 and 22 h (Jia et al., 2009) and tissue collection at 24 h.

2.3. Tissue collection and histological analyses

For tissue collection, mice were anesthetized with Avertin (i.p., 400 mg/kg 2,2,2-tribromoethanol in 20 ml of 2% 2-methyl-2-butanol in saline, T48402, Sigma). For mRNA and protein analysis, fresh olfactory epithelium tissue from both sides was dissected, flash frozen in liquid nitrogen and stored at -80° C. For histology, mice were perfused with ice-

cold phosphate buffered saline followed by 4% paraformaldehyde. The lower jaw and skin were removed and then the tissue was post-fixed with 4% paraformaldehyde overnight, decalcified in EDTA (0.5 M, PH = 8) for 4–5 days and cryoprotected in 30% sucrose as previously described (Jia and Hegg, 2015).

Frozen coronal olfactory epithelium cryostat sections (20 μ m) at the level of the second palatal ridge in the mouse nasal cavity were collected on glass slides as we have described previously (Jia et al., 2013, 2009). Tissue sections were permeabilized with 0.3% triton X-100, blocked with 10% normal donkey serum and then incubated with mouse anti- β galactosidase (β -gal, 1:500, Z378A, RRID:AB_2313752), mouse anti-CNTF (1:200, MAB338, RRID:AB_2083064), goat anti-olfactory marker protein (OMP, 1:1000, #544–10001, RRID:AB_664696), mouse anti-GAP43 (1:1000, G9264, RRID:AB_477034), rabbit anti-CK5 (1:500, ab53121, RRID:AB_869889), rabbit anti-GFAP (1:1000, AB5804, RRID:AB_2109645), rabbit anti-brain lipid binding protein (BLBP, 1:1000, AB9558, RRID:AB_2314014) or mouse anti-CNTF α (1:500, #558891, RRID:AB_397144) antibody overnight at 4 °C. Immunoreactivity was detected by 488- or 594-conjugated donkey anti-mouse, rabbit or goat immunoglobulin (1:200, Molecular Probes). CNTF is secreted in a paracrine mode with a very short half-life (Dittrich et al., 1994). To increase detection of CNTF in the cells, a biotinylated secondary antibody and streptavidin were used for signal amplification. The nuclei were counterstained with DAPI. Detection of BrdU was described previously using rat anti-BrdU antibody (1:100, ab6362, RRID:AB_305426) (Jia et al., 2009). Immunoreactivity was visualized on a Leica TCS SP8 confocal laser scanning microscopy. The specificity of β -gal, CNTF and CNTF α immunostaining was shown by using purified IgG as primary antibody. The specificity of the CNTF antibody was further examined using CNTF $^{-/-}$ mice. The immunostaining specificity of p75, CK5, GFAP, OMP, and BLBP was checked by omitting the primary antibody or using purified isotype IgG as primary antibody. All of these controls showed no staining.

Quantification of BrdU-positive nuclei in the olfactory epithelium was performed as we have described previously (Jia et al., 2013). The numbers of BrdU-positive or BrdU-positive/CK5-positive nuclei or cells in the basal layer, middle layer or all layers of the ecto-turbinate 2 and endo-turbinate II (Fig. 3A) on three coronal sections in each mouse were counted by a researcher blinded to the treatments and genotypes and then normalized to the linear length of the ecto-turbinate 2 and endo-turbinate II. The integrated density of β -gal in olfactory sensory neurons or the basal layer of the ecto-turbinate 2 was analyzed on three sections in each mouse using the threshold method in Image J.

2.4. Protein and mRNA measurements

CNTF mRNA and protein in the olfactory epithelium were measured by RT-qPCR and western blot as described previously (Jia et al., 2019). Briefly, total RNA was isolated using RNeasy Mini kit (Qiagen) and reverse transcribed using MMLV-reverse transcriptase (Promega). The qPCR was performed using the following primers (Applied Biosystems), mouse GAPDH (4352932E), CNTF (mM00446373_ml) and Ki67 (mM01278608_ml). Data were analyzed using 2^{-Ct} method. The protein portion of olfactory epithelium tissue from RNA isolation was suspended by sonication in RIPA buffer supplemented with protease and

phosphatase inhibitors. The antibodies used in the western blot included mouse anti-CNTF (1:500, MAB338, RRID:AB_2083064), mouse anti-CNTFR α (1:1000, #558891, RRID:AB_397144), rabbit anti- β actin (1:2000, #4970, RRID:AB_2223172) and rabbit anti-GAPDH (1:2000, #5174, RRID:AB_10622025).

2.5. Olfactory function tests

Olfactory function was measured by buried food test and olfactory habituation/dishabituation test described previously (Jia and Hegg, 2015; Le Pichon et al., 2009).

2.5.1. Buried food test—The latency to find buried food measures the ability to detect novel odorants, while latency with presented food is used to control for locomotor ability (Jia and Hegg, 2015; Le Pichon et al., 2009). Two trials were included and mice were fasted 16–18 h prior to each trial. Mice were acclimated for 5 min in a cage filled only with fresh bedding twice, and then transferred to a cage with a piece of sugary cereal that was buried beneath the bedding (trial 1) or placed on the surface of bedding (trial 2) in a randomly selected location. The latency to uncovering and eating the cereal was measured for each trial. Trial 1 measures naïve olfactory-mediated finding, while trial 2 assesses locomotor ability. Only mice that could find the buried food within 5 min and eat it were chosen for data analysis.

2.5.2. Olfactory habituation/dishabituation test—This test measures odor discrimination without cognitive or learning-dependent training, nutritional deprivation and sensorimotor control (Yang and Crawley, 2009; Sundberg et al., 1982; Linster et al., 2002; Lee et al., 2011). The test includes the measurement of novel odorant discrimination or dishabituation in the first trial and odorant habituation by exposure to the same odorant. Generally, mice spend more time to investigate a novel odorant and less time on a previously investigated odorant. Mice were acclimated in the test cage with a clean dry cotton applicator inserted through the hole on the cage lid for 30 min to reduce novelty-induced exploratory activity. Distilled water (100 μ l), almond or peppermint extract (1:100, 100 μ l, McCormick & Co.) was applied to a cotton applicator inserted through the hole on the cage lid. We used a total nine trials, including three trials of distilled water followed by three trials of almond and three trials of peppermint. Each trial was run for 2 min with a 30 s delay before the next trial began. The investigation time by the mouse during the 2 min odorant presentation was recorded by a single observer blinded to genotypes. Investigation time differences between repeated exposures to the same odorant assess olfactory habituation, while differences between different odorants measure olfactory dishabituation or discrimination.

2.6. Statistical analyses

Data are presented as mean + SEM. Significance was set at $p < 0.05$ and determined by two-tailed t test (2 groups) or two-way ANOVA followed by Newman-Keuls post hoc tests (more than 2 groups). Behavioral data was analyzed by two-way repeated measures (RM) ANOVA followed by Newman Keuls post hoc test (GraphPad Prism 6.0). All analyses were done blinded to the genotype, sex, or treatment.

3. Results

3.1. CNTF is expressed in HBCs, OECs, and a small population of OSNs

CNTF expression in the olfactory epithelium was first detected using β -gal antibody in CNTF-LacZ reporter mice. Cells with high β -gal expression (Fig. 1A, arrows) resided mainly in the basal layer beneath the middle layer of OMP-positive OSNs (Lee et al., 2011). Some of β -gal-positive cells (arrowheads) were located in the lamina propria and close to OMP-positive olfactory nerve bundles containing the OSN axons (asterisks, Fig. 1A). The absence of staining with IgG confirmed the specificity of β -gal antibody (Fig. 1B). β -gal immunostaining in some cells in the basal layer was co-localized with the HBC marker, CK5 (Fig. 1C–D, arrows) (Iwai et al., 2008), which was confirmed by CNTF antibodies (Fig. 1E–F, arrows) in the olfactory epithelium of C57BL/6 mice. Some of the CK5-positive cells did not have CNTF-immunostaining (Fig. 1E, arrowheads), indicating that only a subset of HBCs expresses CNTF under physiological conditions. In the basal layer, some β -gal-positive or CNTF-positive (Fig. 1G–H, arrows) cells resided beneath CK5-positive HBCs (arrowheads) and were not co-localized with CK5 immunostaining, suggesting they are not HBCs and probably OECs. The specificity of CNTF immunostaining was validated by the absence of staining with purified IgG as primary antibody (Fig. 1I) and with CNTF antibody in CNTF^{-/-} mice (Fig. 1J). Further, β -gal-positive cells in the basal layer were also double labeled for an OEC marker, GFAP (Au et al., 2002; Kawaja et al., 2009), identifying them as OECs (Fig. 2A, arrow indicates cell body and arrowheads indicate cell process). These data indicate that CNTF is expressed in some HBCs and OECs in the basal layer of the olfactory epithelium. Mash1 is a pro-neural transcription factor expressed in a subpopulation of GBCs (Krolewski et al., 2012), which are located immediately above the HBCs. There was no co-localization of CNTF immunostaining with td-Tomato (tdT) expression in Mash1-tdT reporter mice (Fig. 2B). Consistent with previous report (Langenhan et al., 2005), CNTF measured by β -gal was also expressed in a small subpopulation of OSNs located in the middle layer and expressing OMP (Fig. 2C–D). In the lamina propria of CNTF-LacZ reporter mice, β -gal-positive cells surrounded OMP-positive OSN axons (Supplement Fig. 1A). Although β -gal expression was not co-localize with an OEC marker, BLBP (Supplement Fig. 1B) (Rodriguez-Gil and Greer, 2008), many of them expressed OEC marker, the NGF receptor p75 (Supplement Fig. 1C, D) (Franceschini and Barnett, 1996). CNTF and p75 staining also overlapped (Supplement Fig. 1E, F). Some of these cells also expressed GFAP (Supplement Fig. 1G, H), another OEC marker (Au et al., 2002; Kawaja et al., 2009). These data confirm by double-immunostaining previous reports that HBCs and OECs express CNTF.

3.2. CNTFR α is expressed in GBCs and OECs in the lamina propria

CNTFR α protein was detected in olfactory epithelium tissue of C57BL/6 mice, as shown by western blot (Fig. 3A). A prominent band between 50 and 75 kDa was detected, which is consistent with previous reports of 60 kDa of CNTFR α in various types of tissue (Lin et al., 2009; Tripathi and McTigue, 2008; Zvonic et al., 2003). A weaker band at 75 kDa was also seen in the blot, which is very similar to the CNTFR α in brain tissue and may be a differentially glycosylated form of CNTFR α (Zvonic et al., 2003). CNTFR α immunostaining was located mainly in the basal layer and lamina propria (Fig. 3B). In the

basal layer, CNTFR α immunostaining was surrounding td-Tomato (tdT)-positive nuclei in Mash1-tdT reporter mice (Fig. 3B–C), indicating CNTFR α is expressed in the cell membrane of Mash1-positive GBCs. In the lamina propria, CNTFR α immunostaining was among OMP-positive axon bundles and did not co-localized with OMP (Fig. 3D–E). CNTFR α expression was co-localized with p75 (Fig. 3F–G), indicating that OECs express CNTFR α . The lack of staining with IgG as primary antibody validated the specificity of CNTFR α staining (Fig. 3H). Collectively, these data suggest that CNTF produced by HBCs and OECs in the basal layer might affect GBC function.

3.3. Deletion of CNTF does not affect GBC proliferation and olfactory function

GBC proliferation is responsible for regeneration at physiological condition (Caggiano et al., 1994; Leung et al., 2007). To determine whether CNTF is involved, CNTF^{+/+} and CNTF^{-/-} mice received 3 daily of i.p. injections of BrdU (50 mg/kg) to label proliferating cells. Two hours after the last injection, BrdU-incorporated nuclei in the endoturbinates II and ectoturbinates 2 (Fig. 4A) were quantified. BrdU-positive nuclei were distributed in all layers of olfactory epithelium in both CNTF^{+/+} and CNTF^{-/-} mice (Fig. 4B), but most of the BrdU-positive nuclei were located in the basal layer (96% in CNTF^{+/+} and 98% in CNTF^{-/-} mice, data not shown). The few BrdU-positive nuclei in the middle and apical layer could be Bowman's gland ducts cells and sustentacular cells, respectively. The numbers of BrdU-positive nuclei in the basal layer, as an indicator of GBC proliferation, were comparable between CNTF^{+/+} and CNTF^{-/-} mice (Fig. 4C), suggesting that CNTF does not affect rapid GBC proliferation under normal conditions. To determine whether CNTF has a role in odorant detection, we tested CNTF^{+/+} and CNTF^{-/-} mice in the buried food test. CNTF^{-/-} mice exhibited comparable latencies with CNTF^{+/+} mice in both trials (Fig. 4D). Next, we used an olfactory habituation/dishabituation test that examines spontaneous odorant discrimination. CNTF^{+/+} and CNTF^{-/-} mice displayed similar habituation to water, almond and peppermint, and dishabituation from almond to peppermint (Fig. 4E). Collectively, both behavioral tests suggest that deletion of CNTF does not affect olfactory function.

3.4. Inhibition of FAK increases CNTF expression which promotes GBC proliferation

To determine whether FAK regulates CNTF and neurogenesis in the olfactory epithelium, saline or FAK inhibitor, FAK14 (100 μ M, 50 μ l), was intranasally instilled into both nares of C57BL/6 and CNTF-LacZ reporter mice once a day for 3 days. Two hours after the last aspiration, FAK14 significantly increased CNTF mRNA (Fig. 5A) and protein (Fig. 5B–C) by 28% and 65%, respectively, compared to saline control in C57BL/6 mice. Thus, FAK inhibition increases CNTF like it does in the subventricular zone (Keasey et al., 2013; Jia et al., 2018). FAK14 also increased the cell proliferation marker Ki67 mRNA expression (Fig. 5A) by 56% above saline control in C57BL/6 mice. To measure FAK14-induced increases of CNTF expression in different cell types, β -gal integrated density in OSNs or the basal layer including HBCs and OECs (as shown in 1C–K) were quantified in CNTF-LacZ reporter mice (Jia et al., 2018). FAK14 increased β -gal expression by more than two fold in the basal layer (Fig. 5D–E), while it did not alter expression in OSNs (Fig. 5D, F, 78 and 186 neurons were measured in saline and FAK14, respectively), suggesting FAK inhibition increases CNTF expression in HBCs and/or OECs, but not OSNs.

To count proliferating cells, C57BL/6, CNTF^{+/+} and CNTF^{-/-} received intranasal instillation of saline or FAK14 followed by BrdU injections (i.p., 50 mg/kg) 4 h later, for 3 days. Two hours after the last BrdU injection, BrdU-positive nuclei in the basal layer of olfactory epithelium were increased 109% and 73% in FAK14-treated groups compared to saline controls in C57BL/6 (Fig. 6A–B) and CNTF^{+/+} mice (Fig. 6C), respectively. In CNTF^{-/-} mice, FAK14 treatment did not affect the level of BrdU-positive basal nuclei (Fig. 6C). These data collectively indicate that intranasal instillation of FAK14 promotes basal cell proliferation through CNTF. The number of BrdU-positive basal nuclei in saline-treated CNTF^{+/+} vs. CNTF^{-/-} mice was the same (Fig. 6C) and comparable to the BrdU data in Fig. 4C. In order to estimate the percentage of BrdU-positive GBCs among the basal cells, double immunostaining of BrdU with OMP or CK5 was performed. BrdU-positive nuclei in the basal layer resided immediately above CK5-positive HBCs (Fig. 6D) and barely reached the middle OMP-positive OSN layer (Fig. 6E), suggesting they are GBCs. In saline- and FAK14-treated C57BL/6 mice, only 1.16% and 1.27% of total BrdU-positive basal nuclei were in the layer of CK5-positive HBCs or co-localized with CK5 (data not shown), suggesting that FAK inhibition induces GBC proliferation. To measure whether CNTF can directly alter GBC proliferation, saline or CNTF (1 or 5 µg/ml in 50 µl) was intranasally instilled into C57BL/6 mice and BrdU-labeled proliferating cells were quantified at 24 h. Compared to saline, CNTF at 5 µg/ml significantly enhanced the number of BrdU-positive nuclei by 36% (Fig. 6F).

3.5. FAK inhibition promotes GBC proliferation and neurogenesis

In the subventricular zone and dentate gyrus, CNTF regulates progenitor proliferation without affecting normal neuronal differentiation (Kang et al., 2013; Blanchard et al., 2010). To determine whether FAK inhibition-induced CNTF affects neuronal differentiation, the FAK14 and BrdU treatments were followed (pulse-chase method) by 9 and 20 days before analyses. This extended period allows for basal stem cells to proliferate and differentiate into mature OSNs, a process that takes 10–20 days (Schwob et al., 1995; Jia et al., 2009; Liberia et al., 2019). To analyze the fate of FAK14-induced GBC proliferation, BrdU-positive nuclei in the total olfactory epithelium, middle OSN layer and basal stem cell layer were counted. Compared to saline controls, BrdU-positive nuclei in the total olfactory epithelium of C57BL/6 treated with FAK14 were increased 89% and 85% by 9 days (Fig. 7A–B) and 20 days (Fig. 7C–D), respectively. There was no change in BrdU incorporation in saline-treated mice between 9 and 20 days (9.3 ± 1.3 vs. 13.0 ± 3.2 , $N = 5$ mice/group, two-tailed t test). At 9 days post-treatment, BrdU-positive nuclei were found slightly above the basal layer and co-localized with immature OSNs expressing GAP43 (Fig. 7A). At 20 days post-treatment, most of BrdU-positive nuclei were present in the middle neuronal layer and co-localized with mature OSNs expressing OMP (Fig. 7C, arrowhead). A small population of BrdU-positive nuclei still remained in the basal layer (Fig. 7C, arrows), indicating GBC self-renewal or slowly dividing GBCs that serve as quiescent stem cells (Jang et al., 2014). Additionally, in both basal layer and middle OSN layer, FAK14 increased the numbers of BrdU-positive nuclei 20 days post-intranasal instillation by 2.6- and 2-fold of saline, respectively (Fig. 7E). Together, this suggests that FAK inhibition promotes GBC self-renewal and/or proliferation but does not interfere with subsequent normal neuronal differentiation or migration, consistent with the effects of increased CNTF in the SVZ (Kang

et al., 2013). Finally, intranasal instillation of FAK14 increased BrdU incorporation in the total olfactory epithelium, the basal layer and middle neuronal layer of CNTF^{+/+} mice at 20 days post-treatment but failed in CNTF^{-/-} mice (Fig. 7F–G), suggesting that FAK inhibition enhances GBC self-renewal and/or proliferation through CNTF.

4. Discussion

This study shows in adult mice that local treatment with a FAK inhibitor can increase synthesis of CNTF in the olfactory epithelium which promotes neurogenesis, while identifying the CNTF-expressing and responsive cell types by double-immunostaining. OECs are known to produce CNTF in vitro (Wewetzer et al., 2001) and in the rodent olfactory system (Stockli et al., 1991; Asan et al., 2003). Here, we showed co-localization with their markers p75 NGF receptor and GFAP. CNTF is reportedly expressed in basal cells that are morphologically similar to HBCs in rodent olfactory epithelium (Buckland and Cunningham, 1999). Here, we showed that CNTF was expressed in a subset of HBCs, identified by co-localization of their marker CK5. We do not know whether the CNTF-positive and CNTF-negative cells represent permanently different populations or are related to the physiological state of the HBCs. CNTF has no effect on HBC proliferation in vitro (Satoh and Yoshida, 1997), suggesting that HBCs are a resource of CNTF for other cells. Consistent with a previous study (Langenhan et al., 2005), CNTF was also expressed in a small subpopulation of OSNs. The expression of CNTFR α is mostly restricted to nerve tissues and is detected in neurons and astrocytes of the central nervous system (Emsley and Hagg, 2003; Hagg et al., 1992; Ip et al., 1991; LaVail et al., 1992; Sendtner et al., 1992). CNTF binding to its specific CNTFR α induces the formation of three-component receptor complex, including LIFR β and gp130, and promotes neurogenesis and axonal regeneration in the injured nervous system (Kang et al., 2013; Hagg et al., 1992; Askvig et al., 2012; Guthrie et al., 1997; Hagg and Varon, 1993). LIFR β and gp130 are present in GBCs and OECs in rodent olfactory epithelium (Bauer et al., 2003; Getchell et al., 2002; Nan et al., 2001). We identified for the first time that CNTFR α is expressed in Mash1-positive GBCs in mouse olfactory epithelium. Thus, CNTF produced by HBCs likely plays a paracrine role in promoting proliferation of neighboring GBCs. Consistent with previous in vitro studies (Wewetzer et al., 2001; Lipson et al., 2003), both CNTF and CNTFR α were detected in OECs of the lamina propria. In vitro, CNTF does not stimulate proliferation of OECs (Wewetzer et al., 2001), suggesting that their CNTF exerts paracrine effect on neighboring GBCs in vivo.

In the mature olfactory epithelium, basal stem cells proliferate to replace dying OSNs (Farbman, 1990; Graziadei and Graziadei, 1979). GBCs proliferate actively, with 1 division per day (Rodriguez-Gil et al., 2015; Gordon et al., 1995; Beites et al., 2005), while HBCs divide every 30–50 days (Carter et al., 2004). Thus, our proliferation labeling protocol mostly labeled GBCs. Lineage studies indicate that GBCs produce new OSNs (Schwob et al., 1995, 1994; Caggiano et al., 1994; Leung et al., 2007; Chen et al., 2004; Calof and Chikaraishi, 1989; Schwartz Levey et al., 1991). Under basal conditions, proliferation in the basal layer of the olfactory epithelium was not different between CNTF^{+/+} and CNTF^{-/-} mice, suggesting that CNTF does not affect neurogenesis in the naïve olfactory epithelium or that these mice developed compensatory mechanisms. LIF^{+/+} and LIF^{-/-} mice also have

similar levels of GBC proliferation (Bauer et al., 2003). However, after bulbectomy LIF is upregulated and promotes GBC proliferation through LIF (Bauer et al., 2003). Therefore, it is conceivable that pharmacologically increasing CNTF above baseline levels would also promote neurogenesis. In the subventricular zone, ischemic stroke injury upregulates CNTF expression which promotes neurogenesis (Yang et al., 2008; Kang et al., 2013). In the subventricular zone, integrin-FAK signaling represses CNTF expression and its inhibition upregulates CNTF expression and neurogenesis (Keasey et al., 2013; Jia et al., 2018). Here, FAK inhibition promoted GBC proliferation and olfactory neurogenesis. FAK is ubiquitously expressed in different cell types (Schaller, 2010). Our study suggests that FAK in HBCs or OECs regulates CNTF expression because of the increased β -gal CNTF-reporter expression in the basal layer of the olfactory epithelium, but not OSNs, in the middle layer of the olfactory epithelium after FAK14 treatment. These results also confirmed the qPCR measurements of CNTF expression. Chronic inflammation reduces olfactory neurogenesis and gene annotation enrichment analysis shows that focal adhesion signal is significantly upregulated in HBCs following chronic inflammation (Chen et al., 2019), perhaps reducing CNTF. It remains to be determined whether inhibition of focal adhesion signaling by FAK inhibitors may rescue neurogenesis through CNTF following chronic inflammation.

Neurogenesis in the olfactory epithelium is tightly regulated, including basal cell proliferation and neuronal differentiation. Intranasal instillation of FAK14 increased basal cell proliferation measured by BrdU-incorporation in the basal layer. FAK inhibition-induced BrdU-positive nuclei in the basal layer were not co-localized with HBC marker, CK5, indicating that FAK inhibition promotes GBC proliferation. FAK14 did not affect proliferation in CNTF^{-/-} mice, suggesting that FAK inhibition-induced GBC proliferation is mediated by CNTF. We used BrdU pulse-chase analysis to assess the locations of BrdU-positive nuclei in either the basal layer (indicative of GBC self-renewal) or neuronal layer (indicative of neuroregeneration). FAK14 significantly increased newly proliferating cells in both layers 20 days post-treatment but not in CNTF^{-/-} mice, suggesting that FAK inhibition promotes neuroregeneration and GBC self-renewal through CNTF. Thus, CNTF seems to act on GBC progenitors to promote neuroblast formation like it does on the C-cells in the subventricular zone (Kang et al., 2013), while also stimulating self-renewal, perhaps like LIF does (Bauer and Patterson, 2006). The dual roles of CNTF on GBCs in the olfactory epithelium could be due to a heterogeneous property of GBCs (Beites et al., 2005). GBCs expressing Mash1 are immediate precursor cells to OSNs (Gordon et al., 1995), while GBCs expressing p27^{Kip1}, a member of the Kip/Cip family of cyclin-dependent kinase inhibitors, are slowly dividing stem cells (Jang et al., 2014). The direct role of CNTF in promoting GBC proliferation is supported by the intranasal instillation of CNTF having a similar effect as the FAK inhibitor. Clearly, the inexpensive small molecule inhibitor has superior pharmacological properties than CNTF protein. FAK14 most likely penetrates the olfactory epithelium much easier than the CNTF protein. Growth factors such as IGF-1 can enter the brain after intranasal delivery (Lioutas et al., 2015; Nowrangi et al., 2019) but the mechanism of CNTF transport from the apical surface to the basal layer of the olfactory epithelium to reach the GBCs is not fully understood. Intranasal CNTF most likely was through the paracellular pathway between the cells, e.g., due to OSNs regeneration resulting

in decreased intercellular barrier function (Ruigrok and de Lange, 2015) and not through endocytosis of nerve endings or the sustentacular cells (Illum, 2002).

CNTF promotes progenitor C-cell proliferation in the subventricular zone through fibroblast growth factor-2 (FGF2) signaling (Kang et al., 2013). FGF2 and its mitogenic effect are seen in the mouse olfactory epithelium in response to purinergic receptor activation (Jia et al., 2011). It remains to be determined whether CNTF acts via FGF2 signaling. There were no differences in total numbers of BrdU-positive nuclei at 6 h compared to 9 days and 20 days post-treatment of FAK14. This suggests that the early effects of FAK inhibition on GBC proliferation are maintained and does not interfere with normal neuronal differentiation and migration. Increased CNTF does not interfere with normal neuronal differentiation in the subventricular zone either (Kang et al., 2013). Thus, no further cell death analysis was pursued in the present study. In the subventricular zone, FAK represses CNTF expression through JNK, whereas FAK or JNK inhibition upregulates CNTF expression and neurogenesis (Keasey et al., 2013; Jia et al., 2018). In the olfactory epithelium, activation of JNK signaling in OSNs is associated with injury and inflammation-induced cell death (Chen et al., 2017; Gangadhar et al., 2008; Victores et al., 2018). It remains to be determined whether intranasal instillation of FAK inhibitors may also be beneficial on neuroprotection via inhibition of JNK signaling under pathological conditions.

5. Conclusions

This study establishes that CNTF is expressed in HBCs and OECs and provide the first evidence that CNTFR α is expressed in GBCs of mouse olfactory epithelium, suggesting that CNTF may act as a paracrine signaling to modulate proliferation of neighboring GBCs. The finding that GBC proliferation is not affected in CNTF $^{-/-}$ mice but that increasing CNTF expression promotes olfactory neurogenesis raises the possibility that CNTF plays a role only under injury conditions. This study advances our understanding of olfactory stem cell lineage and cytokine signaling responsible for olfactory stem cell proliferation and self-renewal in the mouse olfactory epithelium. It points to opportunities to investigate whether FAK and CNTF signaling could be potential therapeutic targets for sensorineural anosmia due to loss of OSNs, such as in post-viral olfactory disorder, post-head trauma anosmia or presbyosmia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BrdU	5-bromo-2'-deoxyuridine
CK5	cytokeratin 5
CNTF	ciliary neurotrophic factor
CNTFRα	ciliary neurotrophic factor receptor alpha
FAK	focal adhesion kinase
FGF2	fibroblast growth factor-2
GAP43	growth associated protein 43
GBC	globose basal cell
HBC	horizontal basal cell
LIFRβ	leukemia inhibitory factor receptor beta
Mash1	mammalian achaete scute homolog-1
OEC	olfactory ensheathing cell
OMP	olfactory marker protein
OSN	olfactory sensory neuron

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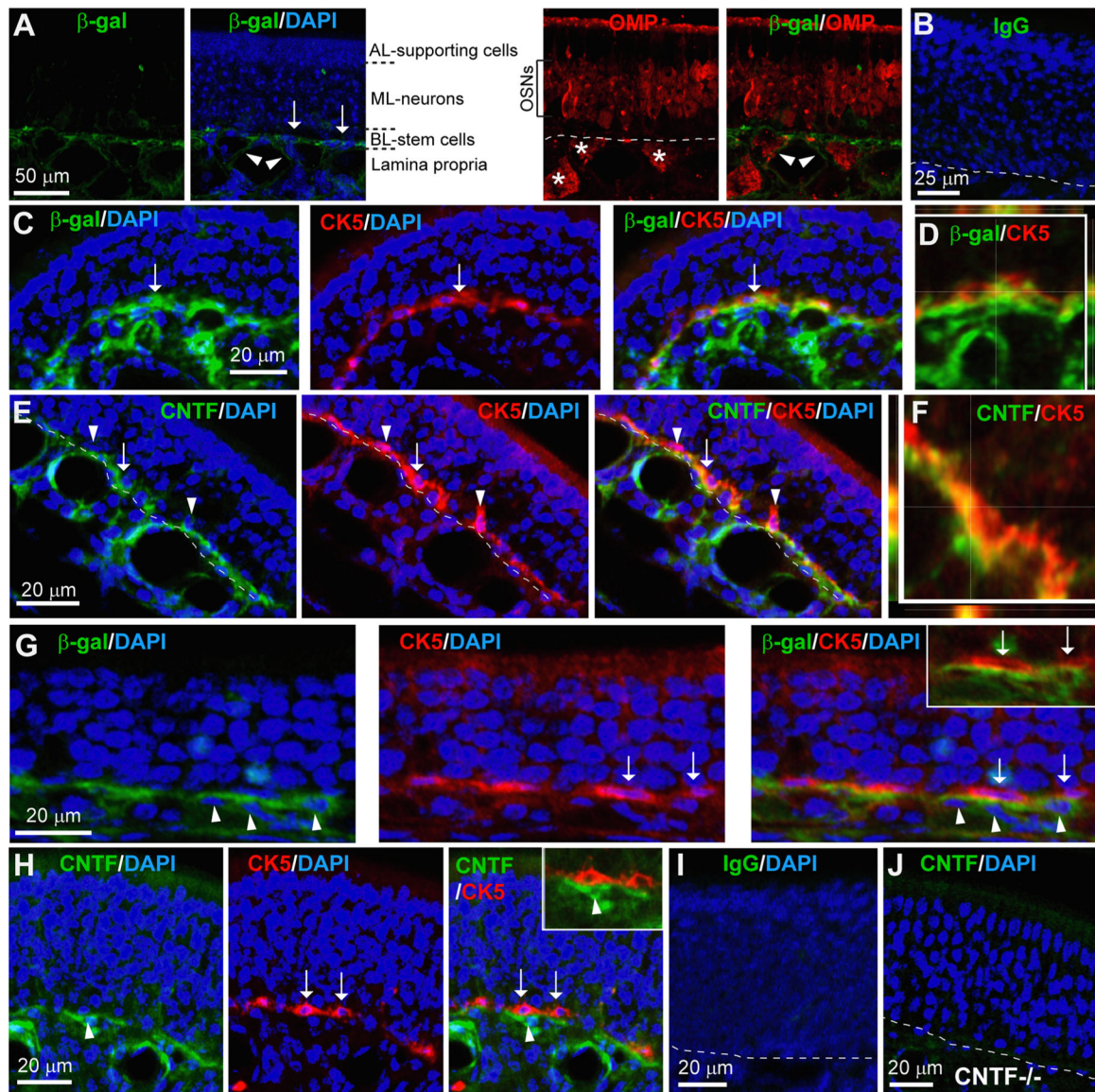


Fig. 1.

CNTF is expressed in HBCs in adult mouse olfactory epithelium. A) CNTF was expressed in basal stem cells (arrows) located in the basal layer (BL) below OMP-positive OSNs in the middle layer (ML), as shown by β -gal immunostaining in CNTF-LacZ reporter mice at 10–12 weeks old. Some β -gal-positive cells (arrowheads) were detected in the lamina propria and were around OMP-positive OSN axons (asterisks). Dashed line = basement membrane. AL: apical layer where supporting cells are. B) No staining was present using purified isotype IgG as primary antibody, confirming β -gal immunostaining specificity. C) β -gal expression in the basal layer was co-localized with HBC marker, CK5. D) The co-localization of β -gal with CK5 is shown in a 0.67 μ m thick confocal image. E) CNTF immunoreactivity was detected in CK5-positive HBCs in the olfactory epithelium of C57BL/6 mice. Arrows indicate a CK5-positive HBC with CNTF immunostaining. Arrowheads indicate CK5-positive HBCs without CNTF. Dashed line = basement

membrane. F) A 0.67 μm thick confocal image showing the co-localization of CNTF with CK5 immunostaining. In the basal layer, some β -gal-positive G) or CNTF-positive H) cells, possibly OECs, resided beneath CK5-positive HBCs. Arrows indicate CK5-positive HBCs, while arrowheads indicate β -gal or CNTF-positive cells. Inserts show arrow-indicated areas with high magnification. CNTF immunostaining specificity was validated by using purified IgG as primary antibody I) and using CNTF^{-/-} mice J).

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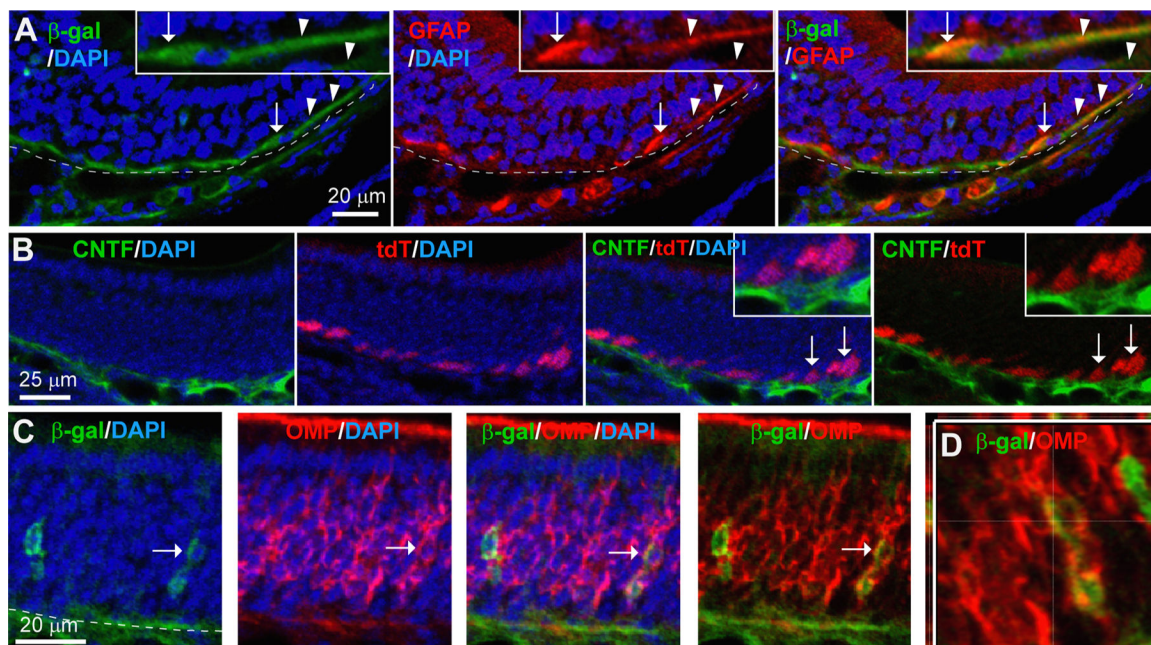


Fig. 2. CNTF is expressed in OECs in the basal layer and some OSNs in adult mouse olfactory epithelium. A) β -gal-positive cells in the basal layer expressed OEC marker, GFAP. Arrow points to cell body and arrowheads indicate cell process. Inserts show arrow-indicated areas with high magnification. B) CNTF was not expressed in GBCs, as shown CNTF immunostaining was not co-localized with nuclear td-Tomato (tdT) in Mash1-tdT reporter mice. Inserts are higher magnification of areas indicated by arrows. C) A small subpopulation of β -gal-positive cells were located in the middle layer of olfactory epithelium in CNTF^{+/-} mice and expressed OSN marker, OMP. Dashed line = basement membrane. D) A 0.67 μ m thick confocal image showing the co-localization of β -gal with OMP.

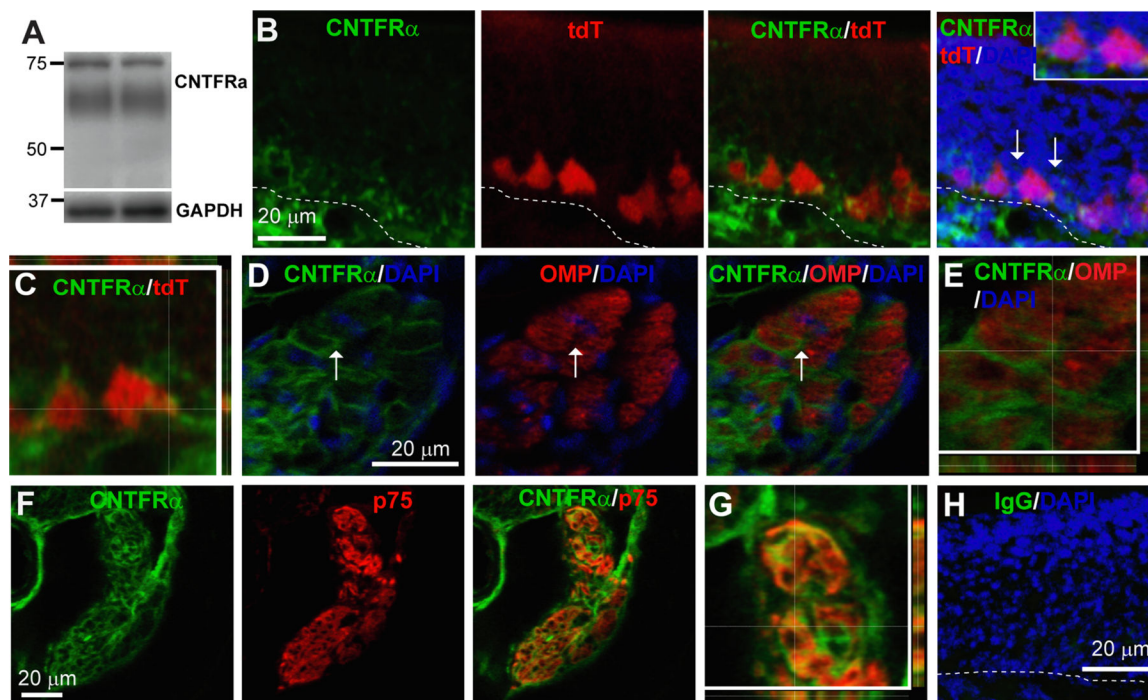


Fig. 3.

CNTFR α is expressed in GBCs and OECs in the lamina propria. A) CNTFR α protein was expressed in the olfactory epithelium tissue of C57BL/6 mice at 10–12 weeks old, as shown by the expected 60 kDa band in a western blot. The 75 kDa band may be glycosylated CNTFR α as seen in the brain. B) CNTFR α immunostaining was expressed in the cell membrane of GBCs identified by nuclear tdT in Mash1-tdT reporter mice. C) A 0.67 μ m confocal image showing the co-localization of CNTFR α with tdT-marked GBCs. D–E) In the lamina propria of the olfactory epithelium, CNTFR α was expressed among axon bundles of OSNs, but did not co-localized with OMP. F–G) CNTFR α immunostaining was co-localized with p75. H) No staining with purified IgG as primary antibody validated the specificity of CNTFR α immunostaining. Dashed line = basal basement membrane.

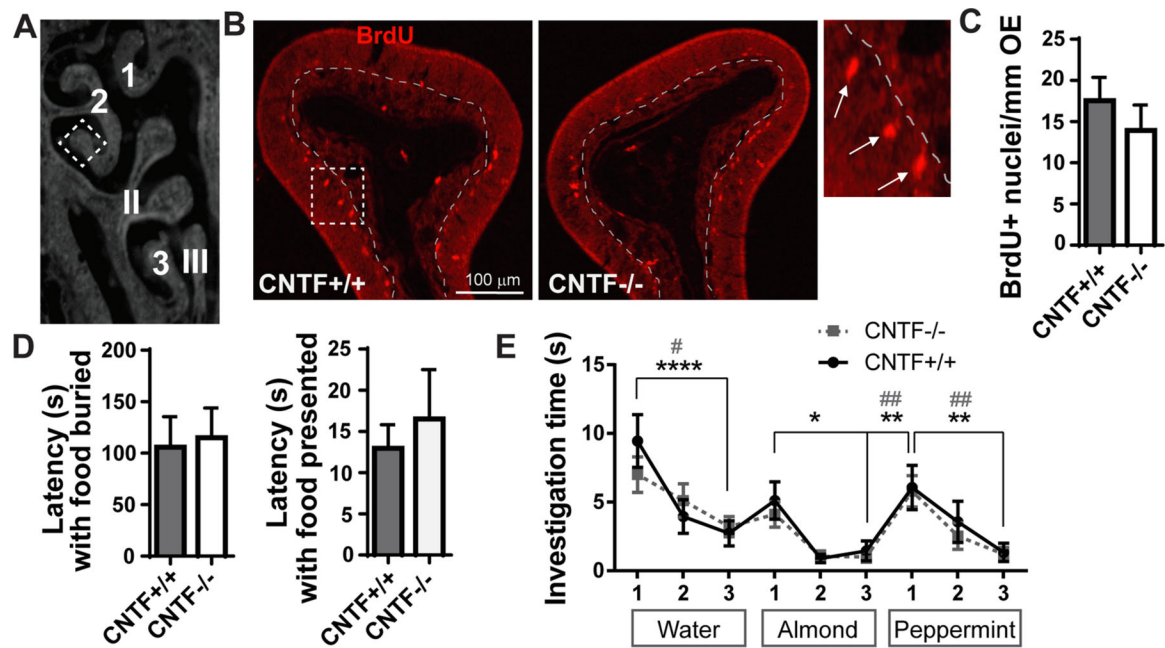


Fig. 4.

CNTF^{-/-} mice have normal basal cell proliferation and olfactory function. A) A schematic image of a coronal section of the left mouse olfactory epithelium (modified from (Jia et al., 2011)). The endoturbinates II–III and ectoturbinates 1–3 are numbered. Dashed square marks the area corresponding to images shown for BrdU incorporation in subsequent figures. B) Representative images of BrdU-positive nuclei in the ectoturbinate 2 of olfactory epithelium in CNTF^{+/+} and CNTF^{-/-} mice. A higher magnification image of the area in the dashed rectangle is shown in the right. Arrows indicate BrdU-positive nuclei in the basal layer, where counts were performed. C) The levels of BrdU-positive nuclei in the basal layer of the olfactory epithelium were comparable between CNTF^{+/+} and CNTF^{-/-} mice (N = 5 mice at 10–12 weeks old/group, $p = 0.42$, two-tailed t test). D) The latencies to find a buried cereal in a buried food test were not different between CNTF^{+/+} and CNTF^{-/-} mice. The latency to find a visible cereal was used as a locomotor control. N = 18,19 mice at 8–12 weeks old, $p = 0.83$ and 0.60 (two-tailed t test). E) CNTF^{+/+} and CNTF^{-/-} mice had similar habituation and dishabituation profiles in an olfactory discrimination test. Differences between trial 1 vs. 3 in water, almond and peppermint indicate olfactory habituation, while differences between trial 3 in water vs trial 1 in almond and trial 3 in almond vs. trial 1 in peppermint show olfactory discrimination. N = 18,19 mice at 8–12 weeks old, *, **, **** $p < 0.05$, 0.01 , 0.0001 in CNTF^{+/+} mice, #, ## $p < 0.05$, 0.01 in CNTF^{-/-} mice (two-way RM ANOVA followed by Newman-Keuls multiple comparisons test).

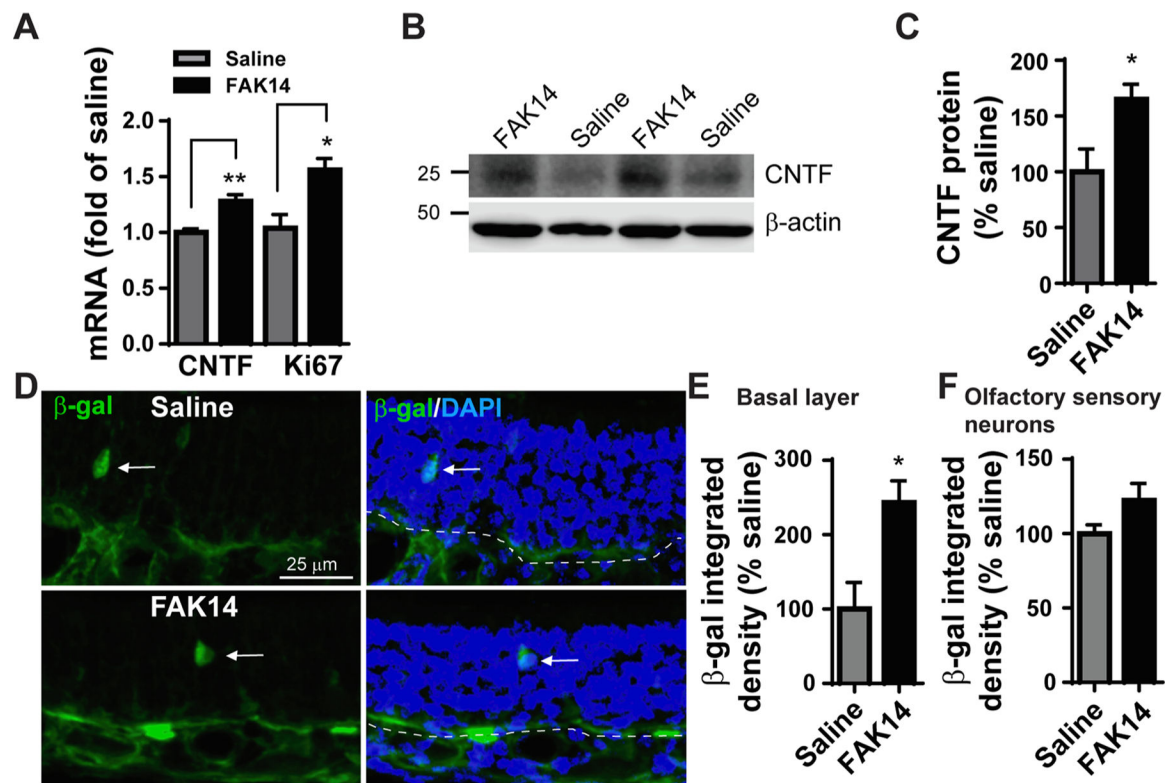


Fig. 5. Local inhibition of FAK increases CNTF expression and cell proliferation in the olfactory epithelium. Adult C57BL/6 or CNTF^{+/-} mice at 10–12 weeks old received intranasal instillation of saline or FAK inhibitor, FAK14, for 3 days. The olfactory epithelium tissue was collected 2 h after the last instillation. Compared to saline control, FAK14 increased CNTF mRNA A) and protein B-C) expression. FAK14 also increased mRNA expression of the cell proliferation marker Ki67 A), suggesting that it upregulates cell proliferation in the olfactory epithelium. N = 5 mice/group. ** p = 0.003, * p = 0.01 for CNTF and Ki67 mRNA and p = 0.03 for CNTF protein (two-tailed *t* test). In CNTF^{+/-} mice, FAK14 increased β -gal integrated density in the basal layer of the olfactory epithelium D–E), but did not alter it OSNs D, F), suggesting FAK14 enhances CNTF expression in HBCs and OECs, but not ONSs. Arrow indicates β -gal positive OSNs. Dashed line = basement membrane, N = 3 and 4 mice/group. * p = 0.03 (two-tailed *t* test).

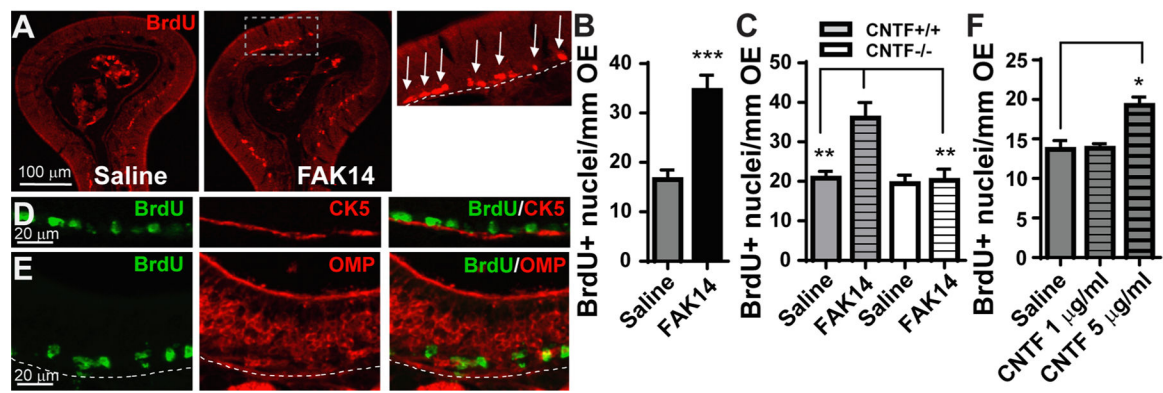


Fig. 6.

FAK inhibition promotes GBC proliferation through CNTF. Mice at 10–12 weeks old received intranasal instillation of saline or FAK14 followed by BrdU injection 4 h later every 24 h for 3 days. A) Representative images of BrdU-positive nuclei in the ectoturbinate 2 of the olfactory epithelium in saline- and FAK14-instilled C57BL/6 mice. The area in the dashed rectangle is shown at higher magnification in the right and arrows indicate BrdU-positive nuclei in the basal layer. B) Quantification of BrdU-positive nuclei in the basal layer showed that FAK14 increased basal cell proliferation by two fold of saline control in C57BL/6 mice (N = 6 mice/group, *** p = 0.0006, two-tailed *t* test). C) FAK14 also increased BrdU incorporation in the basal layer in CNTF^{+/+} but not CNTF^{-/-} mice (N = 3,3,3,5 mice, ** p < 0.01, two-way ANOVA followed by Newman Keuls multiple comparisons test). D) At 2 h post-instillation, BrdU-positive nuclei in the basal layer resided immediately above HBC and were barely co-localized with CK5, suggesting these BrdU-positive nuclei are proliferating GBCs. E) At 2 h post-instillation, BrdU-positive cells in the basal layer did not express OSN marker, OMP. F) Intranasal instillation of CNTF at 5 μ g/ml, but not at 1 μ g/ml, increased the number of BrdU-labeled proliferating cells in the olfactory epithelium of C57BL/6 mice at 24 h (N = 4,3,3 mice, * p < 0.05, one-way ANOVA followed by Bonferroni multiple comparisons test).

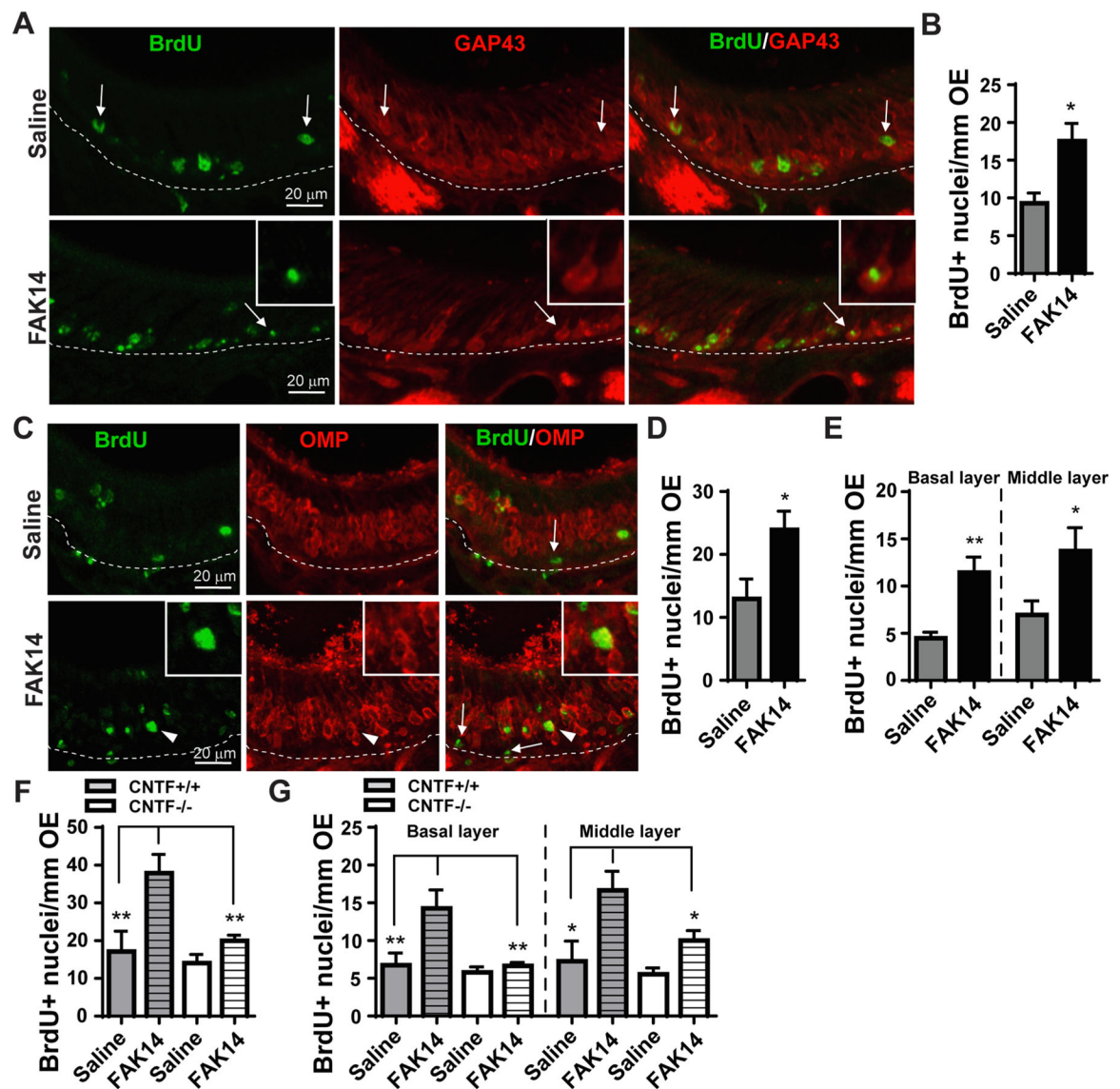


Fig. 7.

FAK inhibition promotes neurogenesis and GBC self-renewal through CNTF. Mice at 10–12 weeks old received intranasal instillation of saline or FAK14 followed by BrdU injection 4 h later every 24 h for 3 days. BrdU-positive nuclei in the total olfactory epithelium, basal layer and middle neuronal layer were quantified 9 days or 20 days after the last BrdU injection. A) At 9 days, BrdU-positive nuclei were present above the basement membrane (dashed line) and co-localized with the immature OSN marker, GAP43 (arrows), indicating neuronal differentiation. Inserts show a BrdU-positive nucleus in a GAP43-positive OSN. B) FAK14 increased the number of BrdU-positive nuclei in the total olfactory epithelium of C57BL/6 mice at 9 days ($N = 5$ mice/group, $* p = 0.016$, two-tailed t test). C) At 20 days, BrdU-positive nuclei were present in the middle layer and co-localized with mature OSN marker OMP (arrowhead), indicating neuronal maturation. In addition, a small population of BrdU-positive nuclei (arrows) remained in the basal layer, indicating slowly dividing GBCs or GBC self-renewal. Inserts show a BrdU-positive nucleus in an OMP-positive OSN. D)

FAK14 increased the number of BrdU-positive nuclei in the total olfactory epithelium of C57BL/6 mice at 20 days (N = 5 mice/group, * p = 0.036, two-tailed *t* test). E) FAK14 increased the number of BrdU-positive nuclei in the basal and middle layer of the olfactory epithelium at 20 days (N = 5 mice/group, * p = 0.028, ** p = 0.004, two-tailed *t* test). F,G) FAK14 also increased the numbers of BrdU-positive nuclei in the total olfactory epithelium, basal layer and middle layer of CNTF^{+/+}, but not CNTF^{-/-} mice (N = 3,3,3,4 mice, * p < 0.05, ** p < 0.01, two-way ANOVA followed by Newman Keuls multiple comparisons test).