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Connective Tissue Growth Factor Regulates Interneuron Survival and Information Processing in the Olfactory Bulb

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SUMMARY

Neurogenesis underlies plastic changes in defined neuronal circuits in the postnatal and adult brain. Here we identify connective tissue growth factor (CTGF) as a critical factor in the mouse olfactory bulb (OB) in determining the efficiency of incorporation of postnatally born inhibitory neurons, thus gating the output of glomeruli, the first relay station of olfactory processing in the brain. In the OB, CTGF expression was restricted to prenatally born external tufted cells. CTGF enhanced the proapoptotic activity of glial-derived TGF- β 2, decreasing the survival of periglomerular inhibitory neurons. Changes in CTGF expression levels in the OB led to modifications in local neuronal circuitry and olfactory behaviors. We show that the odorant-specific recruitment of distinct glomeruli resulted in enhanced local CTGF expression levels in the activated glomeruli. Collectively our data reveal a molecular mechanism controlling the survival of defined postnatally born neurons, thus adapting neuronal integration to the sensory experiences.

INTRODUCTION

In many mammals, including rodents, olfaction is a key sensory modality governing vital behavior such as food seeking, spatial orientation, and sexual and maternal behavior. Olfactory stimuli are conveyed by the olfactory epithelium to the olfactory bulb (OB), the first central relay station of the olfactory system that sends processed information to the cortex (Mombaerts, 2006; Shipley and Ennis, 1996). The two main excitatory neuronal cell types in the OB, namely mitral and tufted cells, are born embryonically (Hinds, 1968; Imamura et al., 2011). In sharp contrast,

the vast majority of OB interneurons are born postnatally. Interestingly, the generation of OB interneurons is not restricted to early postnatal stages but persists in the adult (Lledo et al., 2006). Thus, the postnatal OB continues to be invaded by thousands of new neurons daily. Postnatally generated OB interneurons originate in the subventricular zone (SVZ) of the lateral ventricles and migrate via the rostral migratory stream (RMS) to the OB. There, the majority matures into distinct interneuron subtypes, namely granule and periglomerular cells (Lledo et al., 2008).

The life-long addition of postnatally generated OB interneurons into pre-existing local circuits contributes to olfactory information processing and olfactory learning (Kageyama et al., 2012; Lazarini and Lledo, 2011). The survival of neuroblasts that are integrating into the pre-existing circuitry depends not only on bottom-up and top-down inputs to the OB (Yokoyama et al., 2011), but also on intrinsic cellular programs, including regulation of pro-apoptotic gene expression (Kim et al., 2007). Importantly, at the site of their destination, the survival of OB interneurons is activity-dependent (Petreanu and Alvarez-Buylla, 2002; Saghatelian et al., 2005), and can be modulated by factors provided by neighboring cells (Lin et al., 2010).

In microarray studies we identified connective tissue growth factor (CTGF) whose expression is high in the OB but not detectable in the SVZ, the site of origin for neuroblasts, and the migratory pathway (Khodosevich et al., 2007, 2009). CTGF is a small extracellular protein (38 kDa) encoded by an early response gene (Bradham et al., 1991). Both Ctgf mRNA and protein have a short half-life (Kroening et al., 2009). The various modes of CTGF action have been mapped to distinct functional domains. For instance, CTGF binds and modulates the activity of other growth factors, including IGF, TGF, and BMP (Abreu et al., 2002; Kim et al., 1997), and also interacts with cell receptors, e.g., integrins (Schober et al., 2002). CTGF was shown to have a pleiotropic action during pre- and postnatal development (Friedrichsen et al., 2005; Ivkovic et al., 2003; Stritt et al., 2009). In the adult, CTGF is expressed in some cell types of cardiovascular and reproductive tissues, and in the brain its expression is

restricted to layer VI of the cortex and the mitral cell and glomerular layers of the OB (Friedrichsen et al., 2005; Williams et al., 2007).

In contrast to the wealth of information regarding the involvement of CTGF in a number of pathogenic processes, e.g., fibrosis, wound healing, or cancer (de Winter et al., 2008; Shi-Wen et al., 2008), little is known so far about its function under physiological conditions in the postnatal and adult organism. The lack of studies is not surprising, given the scarce expression of CTGF postnatally and the perinatal lethality of *Ctgf* knockout mice (Ivkovic et al., 2003).

In this study we overcame the drawback of the global knockout by using virus-mediated overexpression and knock-down approaches *in vivo*, and demonstrated activity-dependent regulation of CTGF expression in prenatally born external tufted cells. Furthermore, we provided evidence that, in conjunction with glial-derived TGF- β 2, CTGF controls the survival of newly generated neurons, thus modifying local network activity and olfactory behavior.

RESULTS

CTGF Expression in the Postnatal Brain

To determine the regional expression pattern of CTGF in the postnatal brain, we performed *in situ* hybridization experiments on sagittal brain sections from 2-month-old wild-type mice using 38 nt oligoprobes complementary to *Ctgf* mRNA. As previously shown (Stritt et al., 2009; Williams et al., 2007), *Ctgf* mRNA was detected in layer VI of the cortex as well as in the mitral cell and glomerular layers of the main and accessory OB (Figure 1A). At the immunohistochemical level, cortical CTGF expression was confined to a thin layer just above the corpus callosum, most likely comprising layer VIb neurons, also known as layer VII or subplate neurons (Figure 1B). In the OB, CTGF immunolabeling was restricted to the glomerular layer (Figure 1C). In the somata of individual cells, CTGF expression was more intense in the vicinity of the major process (Figure 1D). CTGF was barely detectable in the mitral cell layer (Figure 1C).

Since the glomerular layer of the OB comprises different excitatory and inhibitory neuronal subtypes (Batista-Brito et al., 2008; Kiyokage et al., 2010), we analyzed the cell-type-specific expression of CTGF. CTGF-positive cells were colabeled exclusively by cholecystokinin (CCK) antibodies (Figure 1E), but not interneuron- (calretinin, calbindin, tyrosine hydroxylase, GAD) or glia (Olig2 and GFAP)-specific antibodies (see Figures S1A–S1D online, or data not shown, respectively). Since it was previously shown that in the OB CCK positivity can be detected by and large only in the external tufted cells (Liu and Shipley, 1994; Shipley and Ennis, 1996), it can be inferred from our colabeling experiments that CTGF expression is restricted to this cell type.

Next, we analyzed the time of birth for CTGF-positive cells by taking recourse to labeling with 5-bromo-2'-deoxyuridine (BrdU), a marker of dividing cells, and injected BrdU at several time points before and after birth of the animals. We found that CTGF-positive neurons were generated mainly around E16–18 (37% of CTGF-positive cells were labeled by BrdU at E16.5 and 42% at E17.5) (see Figure 1F for E17.5). The production of

CTGF-positive neurons was completed by the time of birth (none of the analyzed neurons generated at P0 ($n > 500$) and P7 ($n > 500$) coexpressed CTGF) (Figures S1E and S1F, respectively). This profile corresponds to the one reported for external tufted cells (Hinds, 1968).

Finally, to further confirm the glutamatergic nature of CTGF-positive cells, we injected adeno-associated virus (AAV) expressing tdTomato into the OB (to visualize cell processes) and analyzed 1 week later the expression of CTGF and of the vesicular glutamate transporter 1 (vGluT1), known to be expressed in tufted cells (Ohmomo et al., 2009). All virus-labeled CTGF-positive cells (26/26) coexpressed vGluT1 (Figure S1G). Together these data provide evidence that CTGF-positive cells in the glomerular layer are prenatally born excitatory external tufted cells.

The postnatal developmental expression profile revealed that CTGF began to be detectable around P3 and was expressed in the glomerular and mitral cell layers by P5 (Figure 1G). While CTGF expression in the mitral cell layer gradually decreased and was barely detectable by P12, expression in the glomerular layer remained stable throughout postnatal development and persisted in the adult (Figure 1G).

CTGF Regulates Neuronal Survival in the Glomerular Layer of the OB

Given the postnatal lethality of *Ctgf*^{-/-} mice around birth (Ivkovic et al., 2003), we used an alternative approach and knocked down CTGF expression in the OB (Figure 2A). We produced AAVs expressing tdTomato together with control shRNA or any of two shRNAs against CTGF and infected the entire OB of P3-old wild-type mice (Figure 2A1). Simultaneously, we injected a retrovirus expressing EGFP into the SVZ to label newborn neurons around P3, and analyzed the survival of labeled neurons in the OB at P31 and P59. Retroviruses infect only fast-dividing cells, the majority of which migrate from the SVZ into the OB within 7 days (Khodosevich et al., 2011). This approach allows the labeling of SVZ-generated neuroblasts that are born around the same time and will thus have approximately the same “age” when maturing in the OB. We confirmed efficient OB infection by AAVs 4 weeks postinjection (Figure 2B; note the high intensity of red fluorescence visible even in normal daylight). At the same time, we were able to track EGFP-positive infected cells that had migrated from the SVZ into the OB and had integrated into local circuits. CTGF expression knockdown was confirmed by western blot (Figure 2C) and immunohistochemistry in the OB (Figure 2D).

Injection with any of the two CTGF knockdown viruses led to an increase in the number of infected EGFP-positive cells located in the glomerular layer (Figures 2E and 2F). The effect of CTGF knockdown mediated by shRNA *Ctgf*-2 AAV was completely rescued when coinjecting another AAV expressing a modified CTGF that was resistant to shRNA *Ctgf*-2 (see Figure 2A2 for the rationale and Figure 2F for the results). Similar results were obtained when AAV infections were restricted to the glomerular layer (data not shown).

Since the SVZ produces granule and periglomerular cells, an increase in the number of periglomerular cells might result from one of two causes. (1) CTGF knockdown resulted in mistargeted

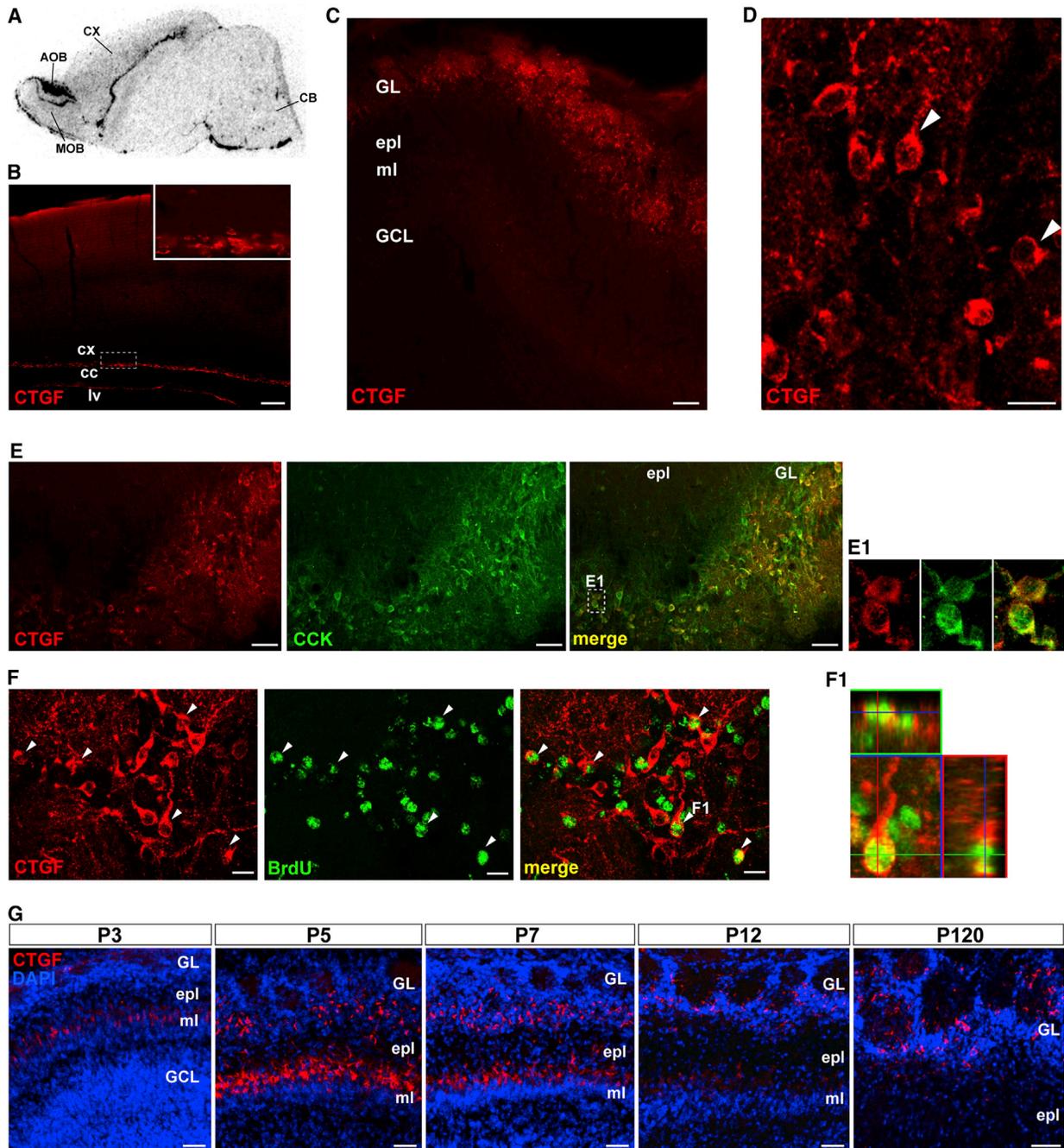


Figure 1. CTGF Is Expressed in a Cell-Type-Specific Fashion in the Mouse OB

(A) *Ctgf* mRNA is detectable by in situ hybridization in layer VI of the cortex and in the OB (lateral 0.8 mm).

(B and C) Corresponding protein expression in the two brain regions, i.e., cortical layer VI and OB. Most CTGF-labeled cells are located in the deeper part of the glomerular layer.

(D) In the somata of individual cells, CTGF expression is more intense in the vicinity of the major process (arrowheads).

(E) CTGF-positive cells coexpress CCK. Indicated cells are shown at higher magnification in (E1).

(F) Representative image from birth-dating experiments showing colabeling of CTGF-positive cells that are born at E17.5 with the cell-proliferation marker BrdU. Arrowheads point to double-labeled cells. Indicated cell is shown at higher magnification in (F1).

(G) Postnatal expression profile of CTGF in the OB. CTGF expression in the OB becomes detectable around P3 and reaches its maximum at P5. Notably, expression in mitral cells disappears at later stages, in contrast to CTGF expression in the glomerular layer, where it continues to be expressed in the adult.

Abbreviations are as follows: AOB, accessory OB; BrdU, 5-bromo-2'-deoxyuridine; CB, cerebellum; cc, corpus callosum; CCK, cholecystokinin; cx, cortex; epl, external plexiform layer; GCL, granule cell layer; GL, glomerular layer; lv, lateral ventricle; ml, mitral cell layer; MOB, main OB. Scale bars in (B) and (C), 100 μ m; (E) and (G), 50 μ m; (D) and (F), 20 μ m. See also Figure S1.

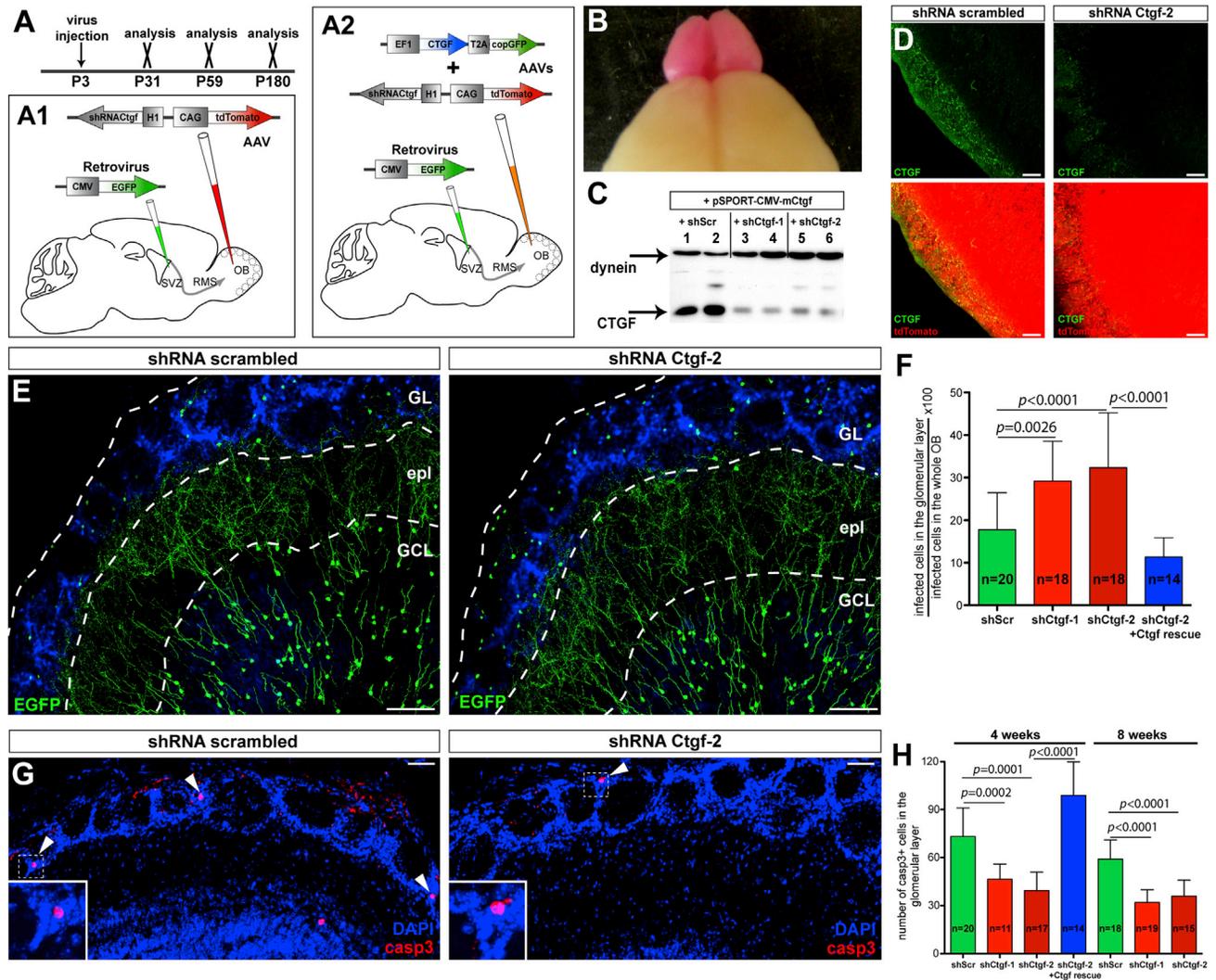


Figure 2. CTGF Regulates the Survival of Postnatally Generated Cells in the OB

(A) Overview of the experimental procedure. (A1) P3-old wild-type mice were injected into the OB by AAVs expressing tdTomato and control shRNA or one of two shRNAs against CTGF. The SVZ was co-injected with a retrovirus expressing EGFP to label newborn cells produced at the time of injection. Mice were analyzed 4 and 8 weeks postinjection. (A2) In rescue experiments, OBs of P3-old wild-type mice were injected by two AAVs: one expressing tdTomato and shRNA against CTGF and another expressing copGFP and shRNA-resistant CTGF.

(B) OB 4 weeks postinjection under normal daylight demonstrating high efficiency of infection.

(C) Confirmation of CTGF knockdown by western blot analysis in HEK cells after infection with CTGF shRNA-expressing viruses.

(D) Immunohistochemical evidence of CTGF knockdown in the OB after infection with CTGF shRNA-expressing viruses.

(E) CTGF knockdown results in an increase in the number of postnatally generated cells (visualized by EGFP) in the glomerular layer.

(F) Quantification showing the increase in glomerular layer cell number at 4 weeks after CTGF knockdown (bars in red) versus control (green bar) and “rescue” (blue bar). “n” indicates the number of analyzed OBs; one-way ANOVA, post hoc Tukey-Kramer (mean ± SD).

(G) CTGF knockdown decreases the number of activated caspase-3-positive cells in the glomerular layer.

(H) Quantitative evaluation demonstrating that CTGF knockdown decreases the number of apoptotic cells (bars in red) when compared to control (bars in green) or “rescue” (bar in blue) conditions 4 and 8 weeks postinjection. “n” indicates the number of analyzed OBs; one-way ANOVA, post hoc Tukey-Kramer (mean ± SD).

Abbreviations are as follows: casp3, activated caspase-3; epl, external plexiform layer; GCL, granule cell layer; GL, glomerular layer. Scale bars in (D) and (E), 100 μm; (G), 50 μm. See also Figure S2.

granule cells; i.e., granule cells migrate beyond their correct location into the glomerular layer. Although we cannot completely exclude this scenario, we believe that it is highly unlikely for the following reason. Since cell fate of neuroblasts is determined by the SVZ subarea where they are born (Merkle

et al., 2007), it would be expected that mistargeted “granule cell layer-fated” neuroblasts keep the morphology and marker expression of granule cells. This was not the case in CTGF knockdown mice. We did not detect cells with the typical granule cell-like morphology, i.e., long apical dendrite and short basal

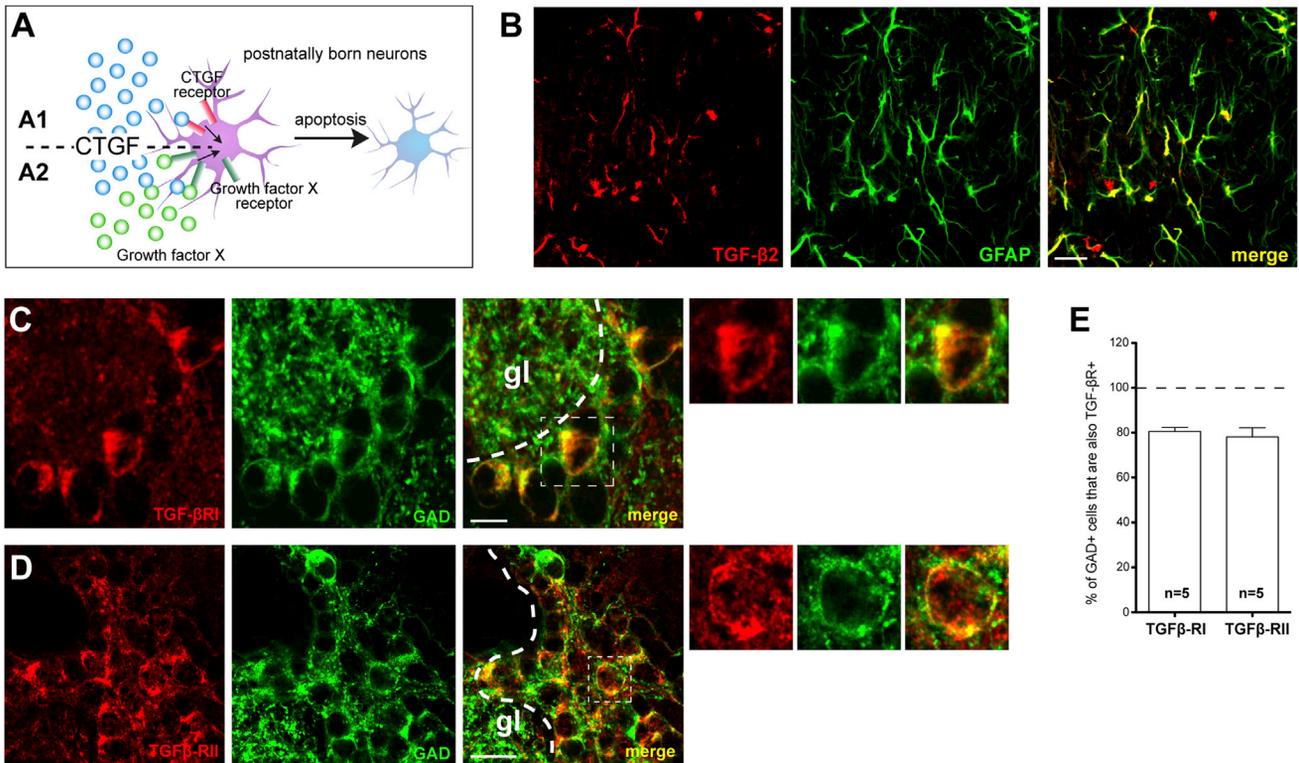


Figure 3. TGF- β 2 and Its Receptors Are Expressed in the Glomerular Layer and Act Downstream of CTGF In Vitro

(A) Two scenarios depicting possible mechanisms by which CTGF may regulate neuronal survival: (A1) CTGF binds to its cell surface receptor—this would require the receptor expression in maturing neuroblasts; (A2) CTGF interacts with another growth factor, modulating the activity of the latter—this would require the expression of the respective growth factor in the glomerular layer and the expression of the growth factor receptor in maturing neuroblasts. (B) In the glomerular layer, TGF- β 2 expression is restricted to GFAP-positive astrocytes. (C and D) TGF- β RI and TGF- β RII expression in the glomerular layer is restricted to GAD-positive interneurons. (E) Quantification of GAD-positive interneurons that coexpress TGF- β RI or TGF- β RII. “n” indicates the number of analyzed OBs. Abbreviations are as follows: gl, glomerulus. Scale bars in (B) and (D), 20 μ m; in (C), 10 μ m. See also Figure S3.

dendrites, in the glomerular layer (for typical morphology of periglomerular and granule cells, see Figures S2A and S2B, respectively). (2) Alternatively, the increase in periglomerular cells following CTGF knockdown resulted from altered apoptosis. Since approximately half of the newborn neurons undergo apoptotic cell death during the first few weeks after their arrival in the OB (Alonso et al., 2006; Mouret et al., 2008), we analyzed whether CTGF expression was linked to apoptosis. Indeed there was a significant decrease in the number of activated caspase-3-positive cells (apoptotic marker) in the glomerular layer following CTGF knockdown (Figures 2G and 2H), while in the granule cell layer there was no effect (Figure S2C). Furthermore, coinjection of AAV expressing shRNA-resistant CTGF mRNA did not only rescue the CTGF knockdown effect but even increased the number of apoptotic cells (Figure 2H). Reduction in the number of apoptotic cells following CTGF knockdown was still significant 8 weeks postinjection (Figure 2H). An increase in periglomerular neurons following CTGF knockdown was also reflected in the augmented number of calretinin (CR)-positive interneurons (Figures S2D and S2E) that constitute a subpopulation of postnatally generated interneurons residing in the glomerular layer (Batista-Brito et al., 2008).

Finally, to confirm that CTGF affects newborn interneurons only during critical period of their maturation when they are prone to cell death, around 10–25 days after birth (Mouret et al., 2008), but not when these neurons become mature, we extended our analysis of newborn cell survival after CTGF knockdown to 6 weeks postinjection and compared the data with those obtained at 4 weeks postinjection (Figure S2F). There were no differences in the number of survived periglomerular cells at 4 and 6 weeks postinjection (Figure S2F). Thus, mature periglomerular neurons were not responsive to the CTGF expression levels.

CTGF Regulates Neuronal Survival via TGF- β 2 Signaling

Subsequently, we investigated downstream signaling mediating CTGF-dependent neuronal survival. Since CTGF can bind to its own receptor, and can also interact with other growth factors, the following scenarios can be envisaged (see Figure 3A):

- (1) CTGF binds to its cell-surface receptor—if this were the case, the receptor should be expressed in maturing neuroblasts (Figure 3A1).
- (2) CTGF interacts with another growth factor X, modulating the activity of the latter—if this were the case, the growth

factor X should be expressed in the glomerular layer and the corresponding receptor in maturing neuroblasts (Figure 3A2).

To identify potential candidates that are expressed in the glomerular layer, we took recourse to published data and the Allen Mouse Brain In Situ Atlas (<http://www.brain-map.org>). The expression of several genes was analyzed in the glomerular layer by western blot analysis and immunohistochemistry. The first hypothesis could be refuted, since cell receptors mediating direct interaction with CTGF (i.e., TrkA, integrins α M β 2, α V β 3, α 6 β 1, and α 5 β 1) were not expressed in maturing neuroblasts (data not shown).

Pursuing the second hypothesis (Figure 3A2), we identified insulin-like growth factor 1 (IGF1) expression in a subpopulation of TH-positive interneurons and in CCK-positive external tufted cells. However, we could not detect IGF1 receptor expression in glomerular layer interneurons (data not shown).

Another potential candidate that might be involved in CTGF downstream signaling is the transforming growth factor β (TGF- β). CTGF was shown to interact via its N-terminal domain with both TGF- β 1 and TGF- β 2, enhancing their binding to TGF- β receptors and thus augmenting their activity (Abreu et al., 2002; Khankan et al., 2011). Furthermore, TGF- β signaling was demonstrated to activate apoptosis via a caspase-3-dependent pathway (Jang et al., 2002). We did not detect TGF- β 1 expression in the glomerular layer of the OB by western blot or immunohistochemistry (data not shown). In contrast, TGF- β 2 and its receptors TGF- β RI and TGF- β RII were all expressed in the glomerular layer (Figure S3A). Interestingly, TGF- β 2 was expressed exclusively in GFAP-positive astrocytes in the glomerular layer (Figure 3B). TGF- β Rs, on the other hand, were found in a subpopulation of GAD-positive interneurons of the glomerular layer (Figures 3C–3E). Furthermore, TGF- β RI-expressing cells colocalized 100% with TGF- β RII-expressing cells (Figure S3B). Thus, at least at the expression level, the TGF- β signaling components fulfill the requirements to mediate CTGF-dependent responses in the newly born glomerular layer neurons: CTGF and TGF- β 2 are expressed in the glomerular layer, whereas TGF- β RI and TGF- β RII can be detected in newly born neurons (see scheme in Figure 3A2).

To further substantiate the hypothesis of CTGF-TGF- β 2 coupling, we quantified activated caspase-3-positive cells in the glomerular layer of organotypic cultures obtained from coronal OB sections of 1-month-old wild-type mice that were cultured for 16 hr (Figure S3C). Addition of neutralizing anti-CTGF antibody decreased apoptosis in the glomerular layer, whereas recombinant CTGF increased it (Figure S3D). Anti-TGF- β 2, but not anti-TGF- β 1, neutralizing antibody abolished the effect of recombinant CTGF. Furthermore, both recombinant TGF- β RI-Fc and TGF- β RII-Fc (fusions of TGF- β Rs with the Fc immunoglobulin domain that bind to TGF- β and block its activity) abolished the CTGF effect. Likewise, neutralizing antibody to TGF- β 2, but not to TGF- β 1, reduced glomerular layer apoptosis, and recombinant TGF- β 2 enhanced it. When CTGF and TGF- β 2 were both added to the medium, there was a dramatic increase in the number of apoptotic cells in the glomerular layer. Blocking TGF- β signaling by TGF- β RI-Fc or TGF- β RII-Fc did not decrease

apoptosis in the granule cell layer (Figure S3E). The intracellular apoptotic effect of TGF- β is mediated by SMAD proteins (Shi and Massagué, 2003). SMAD3 inhibitor completely abrogated the enhanced apoptosis resulting from treatment with recombinant CTGF or with CTGF+TGF- β 2 (Figure S3D). Thus, CTGF potentiates TGF- β 2 activity in the glomerular layer and regulates apoptosis of newly generated cells via the TGF- β R-SMAD pathway.

To obtain in vivo evidence that CTGF acts via the TGF- β pathway, we knocked down TGF- β RI expression in postnatally generated neuroblasts (Figure 4A). For these knockdown experiments, we employed microRNAs (miRNAs) rather than shRNAs, since they enable the use of RNA-polymerase II-specific promoters (e.g., synapsin promoter). P3-old wild-type mice were injected into the SVZ with retroviruses expressing EmGFP (Emerald GFP) and control miRNA or any of two miRNAs against TGF- β RI and were analyzed 4 weeks postinjection (Figure 4A1). The synapsin promoter assured the onset of miRNA expression only during neuroblast maturation in the OB, thus leading to restricted EmGFP fluorescence in the postnatally born OB interneurons. TGF- β RI expression knockdown was confirmed by western blot (Figure 4A3). Knockdown of TGF- β RI in maturing neurons of the OB increased the number of infected cells in the glomerular layer, mimicking the effect of CTGF knockdown in the OB (Figures 4B and 4C). Together these results demonstrated that the effects observed in vitro in organotypic cultures could be replicated in vivo.

To show that CTGF activity is TGF- β dependent in vivo, P3-old wild-type mice were injected into the SVZ with retroviruses expressing EmGFP and control miRNA or any of two miRNAs against TGF- β RI. Simultaneously, we injected AAV to knock down CTGF in the OB (Figure 4A2). If CTGF acted via a different receptor than TGF- β RI, then TGF- β RI-knockdown cells should continue to be responsive to changed CTGF levels in the glomerular layer. However, CTGF knockdown did not affect the survival of TGF- β RI-knockdown neurons (Figure 4C), demonstrating that CTGF regulates neuronal survival via TGF- β signaling.

To substantiate our finding that TGF- β Rs act downstream of CTGF, we employed P5-old *Tgf β 2* fl/fl mice that were injected into the SVZ with two retroviruses: one expressing tdTomato and another expressing Cre recombinase together with EGFP (Figure 4D, D1). This ensures that *Tgf β 2* knockout cells in the OB are EGFP positive, whereas control cells are tdTomato positive. To increase CTGF-dependent apoptosis in maturing neuroblasts, OBs were simultaneously injected with AAV that expressed CTGF and copGFP (since EGFP and copGFP are unrelated proteins, they can be distinguished by immunohistochemistry). As yet another control, we used the same injection protocol in wild-type mice. Mice were analyzed 7, 14, 28, and 40 days postinjection. Up to 14 days postinjection, the ratio between EGFP (= knockout) and tdTomato (= control) cells was around 1 for both granule cell and glomerular layers of the *Tgf β 2* fl/fl and wild-type mice (Figure 4F). However, at 28 days postinjection and later, the EGFP:tdTomato cell ratio increased in the glomerular layer of *Tgf β 2* fl/fl mice in comparison to wild-type mice (Figures 4E and 4F), indicating that *Tgf β 2* knockout led to enhanced cell survival. Furthermore, this effect was observed only in the glomerular layer, but not in the granule

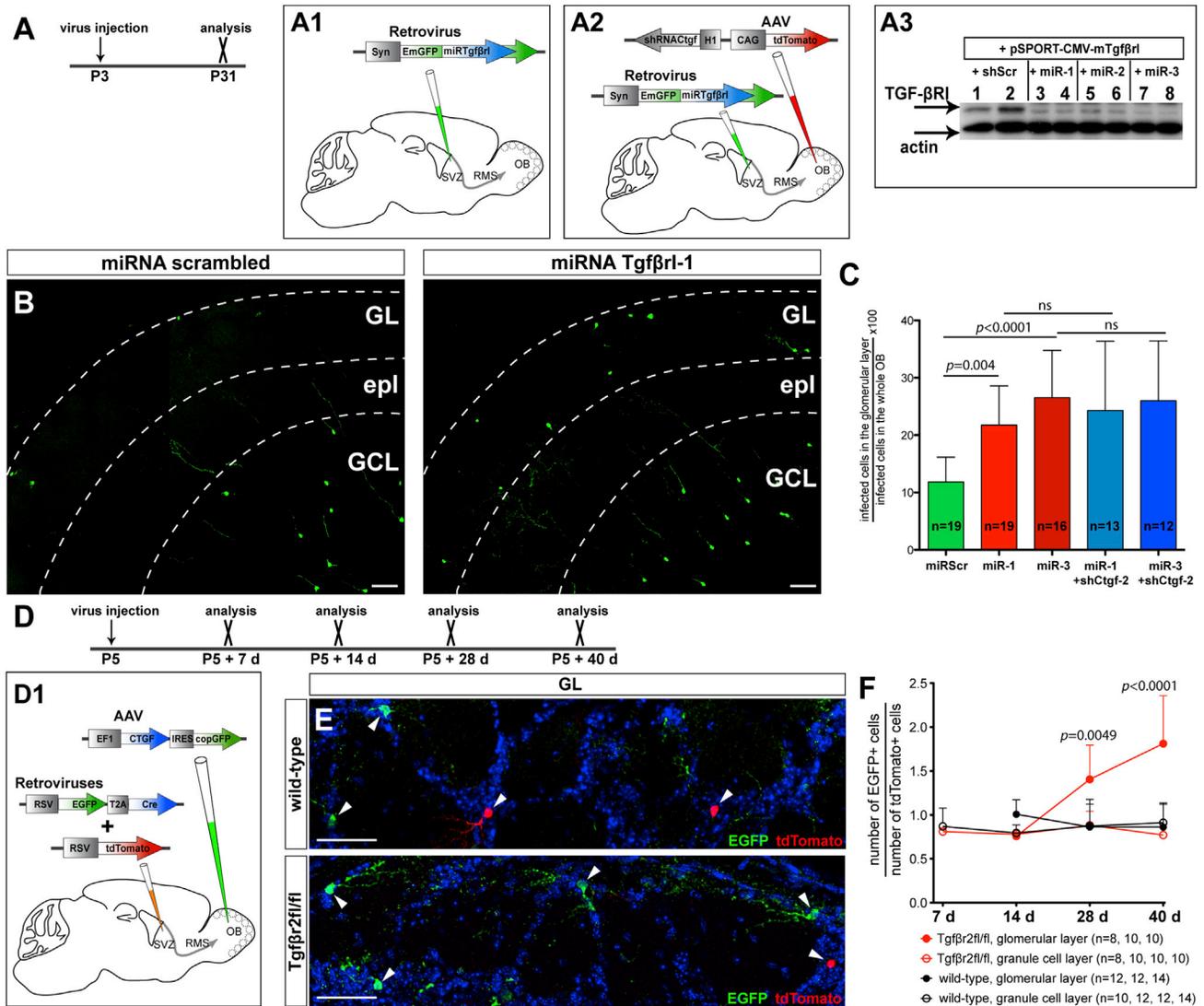


Figure 4. CTGF Regulates Neuronal Survival via TGF-β Signaling In Vivo

(A) Overview of the experimental procedure testing that TGF-βRI acts downstream of CTGF in vivo. (A1) To knock down TGF-βRI in postnatally generated neurons, P3-old wild-type mice were injected into the SVZ with a retrovirus expressing EmGFP and control miRNA or one of two miRNAs against TGF-βRI under the synapsin promoter. Mice were analyzed 4 weeks postinjection. If TGF-βRI acts downstream of CTGF, knockdown of TGF-βRI in postnatally generated neurons should mimic the phenotype obtained subsequent to CTGF knockdown in the OB. (A2) P3-old wild-type mice were injected into the SVZ with the same retroviruses as in (A1). However, this time OBs were coinjected with AAV expressing tdTomato and shRNA against CTGF. If TGF-βRI acts downstream of CTGF, TGF-βRI knockdown cells should not be responsive to CTGF knockdown. (A3) Confirmation of TGF-βRI knockdown by western blot analysis.

(B) Knockdown of TGF-βRI in maturing neurons in the OB resulted in an increase in the number of postnatally generated cells in the glomerular layer.

(C) Quantification showing the increase in glomerular layer cell number at 4 weeks after TGF-βRI knockdown (bars in red) versus control (green bar). Furthermore, TGF-βRI knockdown neuroblasts were not responsive to CTGF knockdown in the OB (blue bars). "n" indicates the number of analyzed OBs; one-way ANOVA, post hoc Tukey-Kramer (mean ± SD).

(D) Overview of the experimental procedure testing that TGF-βRII acts downstream of CTGF in vivo. (D1) To knock out TGF-βRII in postnatally generated neurons, *Tgfβ2fl/fl* P5-old mice were injected into the SVZ with two retroviruses: one expressing tdTomato and another coexpressing Cre recombinase together with EGFP. OBs were coinjected with an AAV expressing copGFP and CTGF. Mice were analyzed 7, 14, 28, and 40 days postinjection. If TGF-βRII indeed acts downstream of CTGF, *Tgfβ2* knockout neuroblasts should exhibit enhanced survival.

(E) Knockout of *Tgfβ2* in postnatally born neuroblasts (green cells) resulted in an increase in neuroblast survival in the glomerular layer in comparison to control neuroblasts (red cells).

(F) Quantification showing the increase in neuroblast survival following *Tgfβ2* knockout in the glomerular layer, but not in the granule cell layer. Injections into wild-type mice had no effect. "n" indicates the number of analyzed OBs; one-way ANOVA, post hoc Dunnett's (mean ± SD).

Abbreviations are as follows: epl, external plexiform layer; GCL, granule cell layer; GL, glomerular layer. Scale bars are 50 μm. See also Figure S4.

cell layer (Figure 4F, Figure S4A). Notably, enhanced cell survival following *Tgf β 2* knockout was also observed when CTGF-dependent apoptosis was not boosted by CTGF overexpression (Figure S4B). Together, these experiments imply TGF- β RII as the downstream effector of CTGF in postnatally generated periglomerular cells.

Next, we sought direct proof that astrocytes are the source of TGF- β 2 that mediates CTGF signaling in vivo (Figure S4C). Wild-type P3-old mice were injected into the SVZ with retrovirus expressing tdTomato to label newborn neurons around P3 (Figure S4C1). Simultaneously, OBs were injected with a mix of two AAVs: one AAV overexpressing CTGF (to increase CTGF-dependent apoptosis) and another AAV to selectively knockdown TGF- β 2 expression in astrocytes. To this end, we used an AAV expressing EmGFP and control miRNA or any of two miRNAs against TGF- β 2 under the control of the astrocyte-specific promoter GFAP (Figure S4C1). In addition, we pseudotyped AAV particles with rh43 nonhuman AAV serotype that was shown to ensure glial cell tropism (Lawlor et al., 2009). The resulting AAV indeed guaranteed astrocyte-specific infection in the OB (98% of total infected cells were astrocytes; Figure S4D). TGF- β 2 expression knockdown was confirmed by western blot (Figure S4C2). Using this approach, we showed that TGF- β 2 knockdown resulted in a significant increase of tdTomato-positive cells in the glomerular layer (Figures S4E and S4F). TGF- β 2 knockdown rescued CTGF-induced neuronal apoptosis, thus identifying astrocytes as the source that provides TGF- β 2.

CTGF Regulates Local Circuit Activity in the Glomerular Layer

To evaluate the potential impact of CTGF-induced changes on olfactory information processing, we first tested whether an increase in the number of neurons in the glomerular layer changed local circuit activity in the OB. P3-old wild-type mice were injected into the OB by AAVs expressing tdTomato together with control shRNA or any of the two shRNAs against CTGF (Figure 5A). Simultaneously, a retrovirus expressing EGFP was injected into the SVZ. Around 4–5 weeks postinjection, we tested electrophysiological parameters of cells belonging to the two cell populations: EGFP-labeled neurons representing periglomerular neurons produced postnatally around P3, and tdTomato-labeled neurons born before P3 (only interneurons in the glomerular layer of the OB were recorded, identified by visual and electrophysiological characteristics). The frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from control periglomerular cells (0.37 and 0.27 Hz, for Figures 5B and 5C, respectively) was similar to what was published before (0.36 Hz) (Grubb et al., 2008). However, after CTGF knockdown, EGFP-labeled interneurons exhibited an increase in the sIPSC frequency, indicating an increase in the network inhibition on periglomerular cells, while the amplitude of sIPSCs was not affected (Figure 5B, Figure S5A). Frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) were not changed (Figure 5B, S5A). The same effect was observed for tdTomato-labeled interneurons (the bulk of this population is born prenatally) (Figure 5C, Figure S5B). As a consequence, there was a considerable decrease in the sEPSC/sIPSC ratio for both cell populations.

The glomerular layer contains at least five interneuronal subtypes (Parrish-Aungst et al., 2007) that are born at different prenatal/postnatal ages (Batista-Brito et al., 2008). The decrease in excitation/inhibition ratio appeared to affect all cell types. The electrophysiological results demonstrate that CTGF expression levels have a profound role in the regulation of local circuit activity by shifting the excitation/inhibition ratio in periglomerular interneurons.

We then tested if the increase in periglomerular cell number affects inhibition of the two main excitatory neuron types of the OB, i.e., mitral cells and external tufted cells. P3-old wild-type mice were injected into the OB with AAVs expressing tdTomato together with control shRNA or any of the two shRNAs against CTGF (Figure 5D, D1), and were analyzed around 30 or 45 days postinjection. At 30 days postinjection there was no difference in sIPSC frequency between CTGF knockdown and control mitral cells. However, at 45 days postinjection, mitral cells in CTGF knockdown mice exhibited significantly increased sIPSC frequency (Figure 5E). In contrast, CTGF knockdown did not increase significantly sIPSC frequency of external tufted cells that were recorded 45 days postinjection (Figure S5F). Neither mitral nor external tufted cell sIPSC amplitudes were modified by CTGF knockdown (Figures S5C and S5D for mitral cells and Figure S5G for external tufted cells). Mitral cells receive inhibition via periglomerular cells onto the most distal parts of their apical dendrites in the glomeruli, whereas granule cells impinge on mitral cell lateral dendrites considerably more proximally in the external plexiform layer (Liu and Shipley, 1994; Shipley and Ennis, 1996). Since dendritic filtering slows the kinetics of recorded synaptic inputs, we investigated if the increase in the electrotonically more distal inhibition of mitral cell dendrites provided by periglomerular cells leads to a slowing of sIPSC decay kinetics. There was indeed an increase in the sIPSC τ_{decay} in mitral cells of CTGF knockdown animals compared to that in control animals around 45 days postinjection (Figures 5F and 5G).

Activation of dopamine and GABA_B receptors on olfactory nerve reduces the probability of glutamate release (Aroniadou-Anderjaska et al., 2000; Kageyama et al., 2012). We tested if the periglomerular cell number increase affects the release probability by analyzing paired-pulse ratios of EPSCs evoked by two subsequent stimuli delivered on olfactory nerve. Paired-pulse ratios of EPSCs recorded in mitral and external tufted cells were around 0.7 for both control and CTGF knockdown conditions (Figures S5E and S5H) and were in accordance with published data (Aroniadou-Anderjaska et al., 2000; Grubb et al., 2008). Thus, unaltered paired-pulse ratios indicate that presynaptic properties of olfactory nerve input to the glomeruli were not affected by the genetic manipulation.

CTGF Expression Regulates Mouse Olfactory Behavior

Odorant detection, discrimination, and memory (Figure 6A) were tested in control and CTGF knockdown wild-type mice (Figure 6A1) 2 months postinjection (n control = 6, n shCtGF-2 = 11) using an olfactometer. Following the protocol shown in Figure 6A, we investigated olfactory sensitivity by determining the detection threshold for two odorants, namely pyridazine and 1-decanol, using the descending method of limits in two-odorant rewarded discrimination tasks (rewarded odorant, stimulus [S+]; solvent,

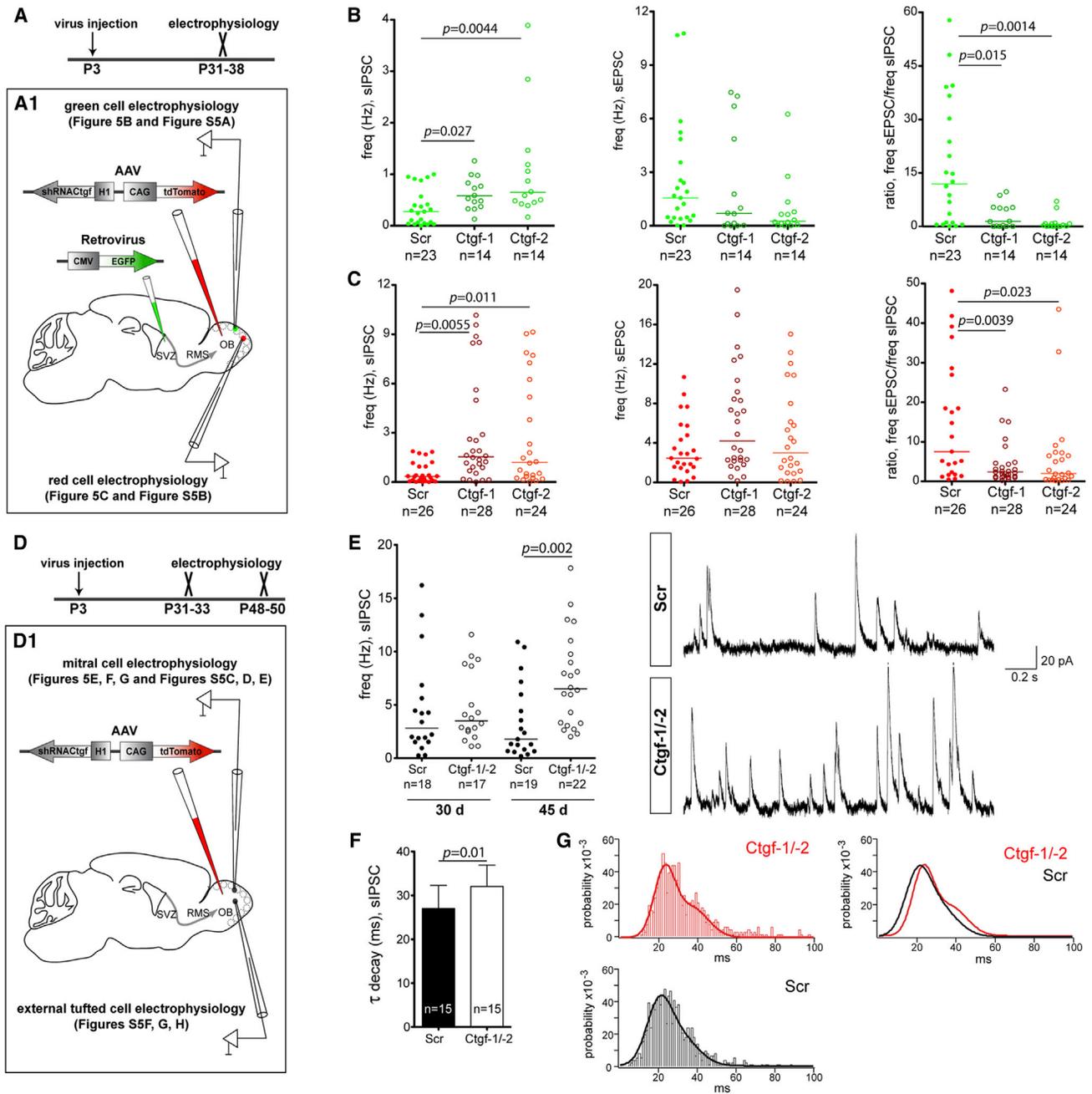


Figure 5. CTGF Expression Levels Control the Excitation/Inhibition Balance in OB Glomeruli

(A) Electrophysiological analysis of periglomerular cells. (A1) P3-old wild-type mice were injected into the OB with AAVs expressing tdTomato and control shRNA or one of two shRNAs against CTGF. The SVZ was coinjected with a retrovirus expressing EGFP to label newborn cells produced at the time of injection.

(B and C) Green (B) and red (C) fluorescent cells were electrophysiologically analyzed 4 weeks postinjection. Increased survival of inhibitory neurons following CTGF knockdown resulted in an increase of sIPSCs. The decreased EPSC/IPSC ratio reflects a change in the excitatory/inhibitory balance that is shifted toward increased inhibition in local circuits. "n" indicates the number of analyzed cells; Kruskal-Wallis, post hoc Steel-Dwass (raw data and median).

(D) Electrophysiological analysis of mitral and external tufted cells. (D1) P3-old wild-type mice were injected into the OB with AAVs expressing tdTomato and control shRNA or one of two shRNAs against CTGF.

(E) Increased survival of periglomerular interneurons resulted in an increase of sIPSCs in mitral cells around 45 days postinjection. Representative traces are shown on the right. "n" indicates the number of analyzed cells; Mann-Whitney (raw data and median).

(F) Increase in interneuron survival also increased the time constant of decay (τ_{decay}) for mitral cell sIPSCs. "n" indicates the number of analyzed cells; Student's t test (mean \pm SD).

(G) sIPSC τ_{decay} probability histograms were skewed toward bigger values. The overlay on the right illustrates that there was an increase of sIPSCs with slow τ_{decay} (30–60 ms) in mitral cells of CTGF knockdown mice when compared to control mice. See also Figure S5.

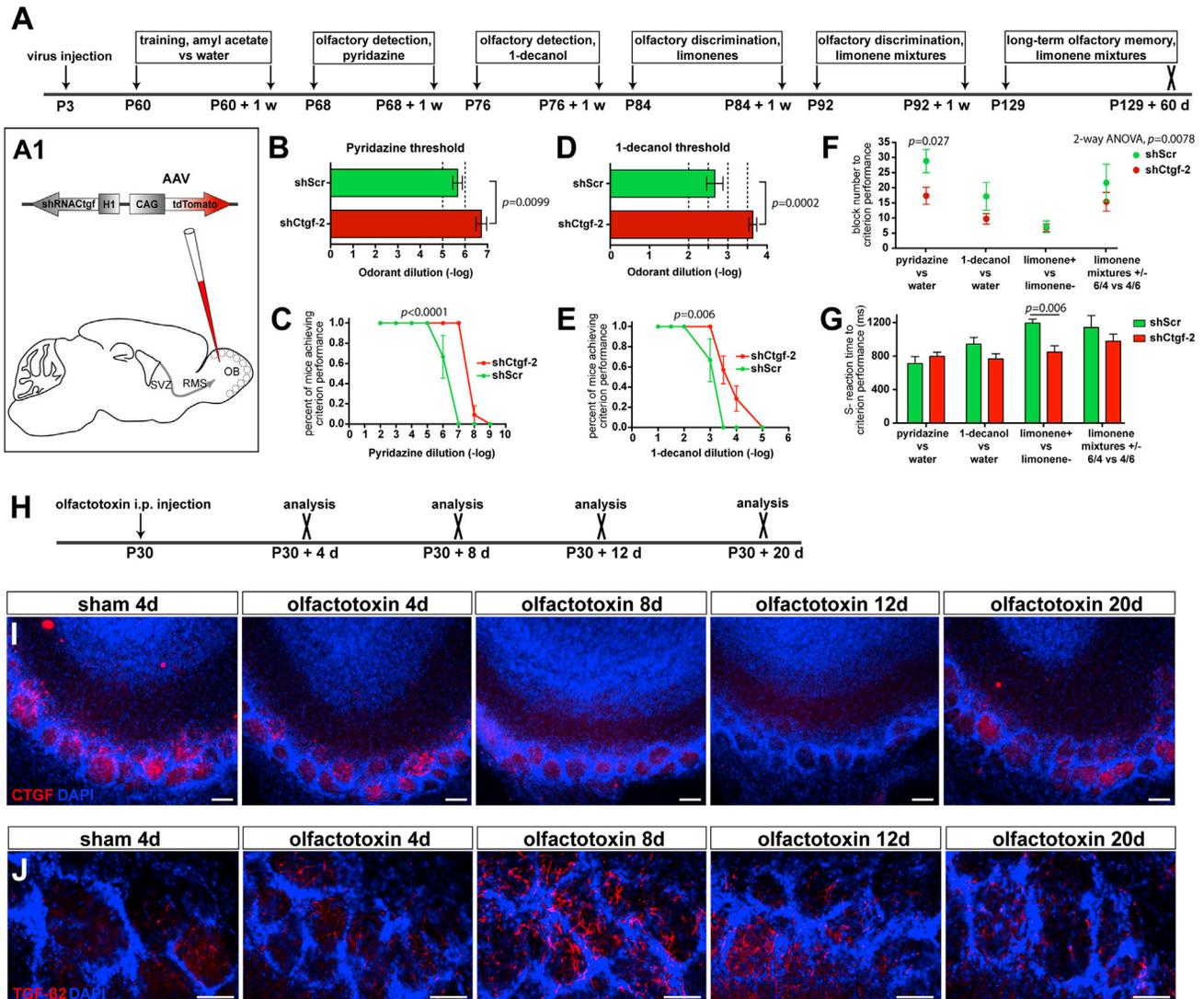


Figure 6. CTGF Knockdown Enhances Olfactory Discrimination, and CTGF Expression Is Dependent upon Olfactory Experience

(A) Overview of the experimental protocol. OBs of P3-old wild-type mice were infected by AAVs expressing tdTomato and control shRNA or shRNA Ctgf-2 (A1). Two-month-old mice were analyzed on olfactometers (n control = 6, n shCtgf-2 = 11).

(B and D) CTGF knockdown resulted in a decrease of the detection threshold for pyridazine and 1-decanol, respectively, Student's t test (mean \pm SEM).

(C and E) CTGF knockdown mice reached criterion performance ($\geq 90\%$ correct responses in the block) at 10–100 lower odorant concentrations than controls, two-way ANOVA (mean \pm SEM).

(F) CTGF knockdown mice needed fewer blocks of learning to discriminate between odorants, two-way ANOVA, post-hoc Bonferroni (mean \pm SEM).

(G) For some odorants, CTGF knockdown mice had a decreased reaction time for the negative (S-) odorant, Student's t test (mean \pm SEM).

(H) Overview of the experimental protocol for olfactory ablation. P30-old wild-type mice were i.p. injected with the olfactotoxin dichlobenil that impairs olfactory sensory neuronal input into the OB, and CTGF expression was analyzed at 4, 8, 12, and 20 days postinjection.

(I) CTGF expression was dramatically decreased already 4 days postinjection. Twelve days postinjection, CTGF expression was close to background. CTGF expression was restored to some extent 20 days postinjection, correlating with partial reinnervation of olfactory sensory input as previously reported (Alonso et al., 2008).

(J) TGF- β 2 expression was already increased 4 days postinjection. Eight days postinjection, TGF- β 2 expression level was highest and gradually decreased thereafter.

Scale bars, 50 μ m. See also Figure S6.

nonrewarded [S-]). Mice were given two sessions (eight blocks each) per day with one decimal dilution of the odorant per session. CTGF knockdown resulted in a decrease of the detection threshold for both odorants (Figures 6B and 6D, respectively)

and in shifting criterion performance (i.e., $\geq 90\%$ correct responses per block) to lower odorant concentration (Figures 6C and 6E, respectively). The same paradigm was used for olfactory discrimination between limonene pair (+ and - enantiomers) and

their binary mixtures. Overall, CTGF knockdown mice needed fewer blocks of trials to reach criterion performance (Figure 6F) and spent less time at negative (S⁻) odorant identification when discriminating between limonene enantiomers (Figure 6G). Analysis of long-term memory did not show a difference between CTGF knockdown and controls (data not shown). Thus, CTGF knockdown mice performed better in odorant detection and olfactory discrimination than did controls, but their olfactory memory remained unchanged.

CTGF Expression Is Dependent on Olfactory Activity

Finally, we investigated whether CTGF expression is sensitive to the degree of olfactory experience. To this end, we injected P30-old wild-type mice i.p. with the olfactotoxin dichlobenil, thus ablating sensory neurons of the main olfactory epithelium targeting the OB (Alonso et al., 2008), and analyzed CTGF expression 4, 8, 12, and 20 days postinjection (Figure 6H). Ablation of olfactory receptor neurons was confirmed by impairment in food search of olfactotoxin-injected mice (Figures S6A and S6B) and by decreased expression of the olfactory marker protein (OMP) (Figures S6C and S6D). Suppression of the sensory input led to a significant decrease in CTGF expression already 4 days postinjection, and at 12 days postinjection CTGF expression was barely detectable (Figure 6I and Figure S6E for quantification). CTGF expression was partially restored 20 days postinjection, correlating with partial reinnervation of olfactory sensory input as reported (Alonso et al., 2008). Decreased CTGF expression levels following olfactotoxin treatment did not result from a demise of external tufted cells (i.e., CTGF-expressing cells), as was evidenced by an unchanged number of CCK-positive cells (data not shown). Conversely, TGF- β 2 expression was strongly elevated during the period of olfactory input impairment and reached a maximum 8 days postinjection (Figure 6J).

Ctgf is an immediate-early gene with a short half-life (Kroening et al., 2009) and is thus suited to test whether environmental changes at a fast timescale alter CTGF expression levels. The first indication for the activity-dependent regulation of CTGF expression derives from its coexpression with *c-fos*, another activity-dependent immediate-early gene (Figure S6F). Since suppression of olfactory input resulted in a reduction of CTGF expression, we hypothesized that olfactory enrichment augments CTGF expression. Mice were presented daily with three distinct odors for three weeks (63 distinct odorants in total) (Figure S6G), and CTGF expression levels were evaluated thereafter in 200 glomeruli (Figures S6H and S6I). Glomeruli were subdivided according to their intensity of CTGF expression into four categories: very high (>10 units), high (5–10 units), intermediate (1–5 units), and low (0–1 units) (for details, see the Supplemental Experimental Procedures). There was no difference in CTGF expression levels between controls and mice subjected to an odor-enriched environment (Figure S6J). This is not too surprising, as one would expect to see a potential augmentation of the short-lived CTGF only in few glomeruli that were activated by the last few odorants. We also measured whether the enrichment paradigm resulted in changes of neuronal survival in the glomerular layer (see BrdU treatment rationale in Figure S6G) and found no difference in BrdU-positive cell number between control and odor-enriched mice (Figure S6K).

To investigate the possible activity dependence of CTGF expression in specific glomeruli, we employed transgenic mice, namely MOR23-IRES-tauGFP, in which only two glomeruli per OB are EGFP labeled and hence can be surveyed over time (Vassalli et al., 2002). MOR23 receptors (and thus MOR23-GFP glomeruli) are activated by the odorant lylal (Grosmaître et al., 2006). In the first set of experiments, we presented lylal or acetophenone (unrelated to lylal odorant) to P60-old transgenic mice for 10 min or 8 hr and analyzed CTGF expression 3 hr post-exposure or immediately thereafter, respectively (Figure 7A). Lylal exposure increased CTGF expression levels in EGFP-labeled glomeruli in comparison to acetophenone (Figures 7B and 7C). We also investigated whether adjacent glomeruli might be affected, keeping in mind though that glomeruli detecting odorants with similar chemical functional groups might cluster together (Mori et al., 2006). Despite this caveat, differences in CTGF levels evoked by the two odorants were not significant (Figures S7A and S7B). Overall, our results indicate that olfactory activity indeed enhances CTGF expression precisely in specific odor-activated glomeruli. Thus, CTGF expression levels undergo rapid modifications in response to changes in olfactory activity.

Most previous studies employed “broad-spectrum” modifications of sensory input induced either by olfactory enrichment or sensory deprivation in order to study survival of postnatally generated OB neurons in the whole circuitry. Here, we aimed at studying activity-dependent modulation of neuronal survival in distinct glomeruli. To this end, we employed MOR23-IRES-tauGFP transgenic mice and labeled postnatally generated cells by adding BrdU in the drinking water from P20 to P27 (Figure S7C). Three weeks later, mice were exposed to lylal or acetophenone for different time periods—1 min, 10 min, 1 hr, 8 hr, and 24 hr—and analyzed 7 days postexposure. Exposure to lylal for 1 min had no effect on neuronal survival in MOR23 glomeruli, but all other treatments from 10 min onward decreased neuronal survival by 20% (Figures S7D and S7E). Thus, stimulation of olfactory activity by a distinct odorant decreases neuronal survival in the odorant-specific glomeruli.

Finally, we analyzed whether this decrease of neuronal survival is mediated by CTGF. We performed a similar experiment as the one above, in MOR23-IRES-tauGFP transgenic mice that were injected into the OBs by control or CTGF knockdown AAVs (Figure 7D, D1). CTGF knockdown mice exhibited higher cell survival in the glomerular layer in comparison to control mice, again confirming our data that CTGF stimulates neuronal apoptosis (Figures 7E and 7F). As expected, lylal reduced neuronal survival across MOR23 glomeruli in control AAV-injected mice (Figure 7F). However, CTGF knockdown completely abolished lylal-dependent reduction of neuronal survival (Figure 7F). These experiments demonstrate that modifications in CTGF expression levels in response to olfactory activity adjust the survival of postnatally born neurons in an odorant-specific fashion in the odorant-responsive glomeruli.

DISCUSSION

In this study we identified CTGF as a modulator of postnatal/adult OB circuitry. Based on our results, we propose that

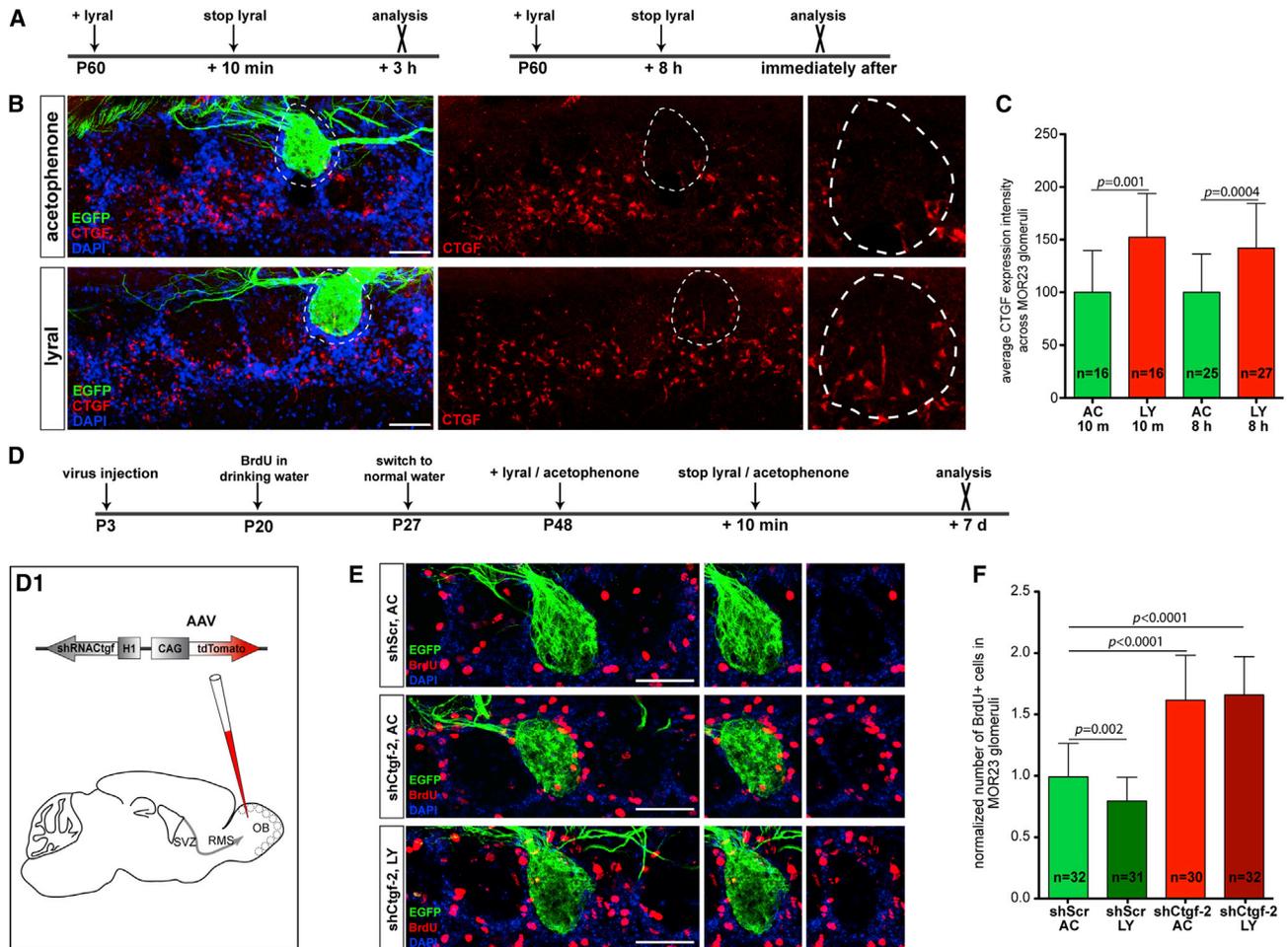


Figure 7. Activity-Dependent Expression of CTGF Regulates Survival of Interneurons in the Glomerular Layer

(A) Overview of the experimental protocol. P60-old MOR23-IRES-tauGFP mice were exposed to lyral for 10 min or 8 hr, and CTGF expression levels were measured.

(B) CTGF expression was increased after activation of MOR23 glomeruli, indicating that olfactory sensory activity enhanced CTGF expression.

(C) Quantitative evaluation demonstrating that lyral enhances CTGF expression in MOR23 glomeruli after 10 min and 8 hr exposure. "n" indicates the number of analyzed glomeruli; Student's t test (mean \pm SD).

(D) Overview of the experimental protocol. P3-old MOR23-IRES-tauGFP mice were injected into the OB with AAVs expressing tdTomato and control shRNA or shRNA against CTGF. Three weeks postinjection mice were given BrdU in the drinking water for 1 week. Mice were given normal water for 3 weeks more and exposed to lyral or acetophenone for 10 min. Mice were analyzed 1 week following odorant exposure.

(E) CTGF knockdown increased the number of BrdU-positive cells in MOR23 glomeruli in comparison to control mice, and this increase in survival was not reduced following lyral exposure.

(F) Quantification showing that CTGF knockdown increased neuronal survival in the glomerular layer (green bars, controls; red bars, CTGF knockdown). Furthermore, whereas in controls lyral exposure reduced cell survival, in CTGF knockdown mice lyral did not affect cell survival. "n" indicates the number of analyzed glomeruli; Student's t test (mean \pm SD).

Abbreviations are as follows: AC, acetophenone; LY, lyral. Scale bars, 50 μ m. See also Figure S7.

CTGF derived from prenatally born excitatory neurons regulates the survival of postnatally born inhibitory neurons in the glomerular layer in the following fashion (Figure 8): CTGF is expressed in external tufted cells that control the output of the glomerular layer, the first relay station of olfactory information processing (De Saint Jan et al., 2009; Hayar et al., 2004). CTGF acts via glial-derived TGF- β 2, whose activity it potentiates, promoting SMAD-dependent apoptosis of newborn neurons in the glomerular layer via TGF- β Rs. CTGF expression is enhanced by

olfactory stimulation, thus leading to an activity-dependent potentiation of TGF- β 2 signaling. At the functional level, changes in inhibitory neuron number modify the excitation/inhibition balance in stimulated glomeruli and OB output cells (i.e., mitral cells), thus affecting olfactory behavior. It is of note that the regulation of CTGF-mediated cell survival occurs in a region- and cell-type-specific manner. Thus, within the OB, only the survival of periglomerular cells, but not granule cells, is subject to CTGF regulation.

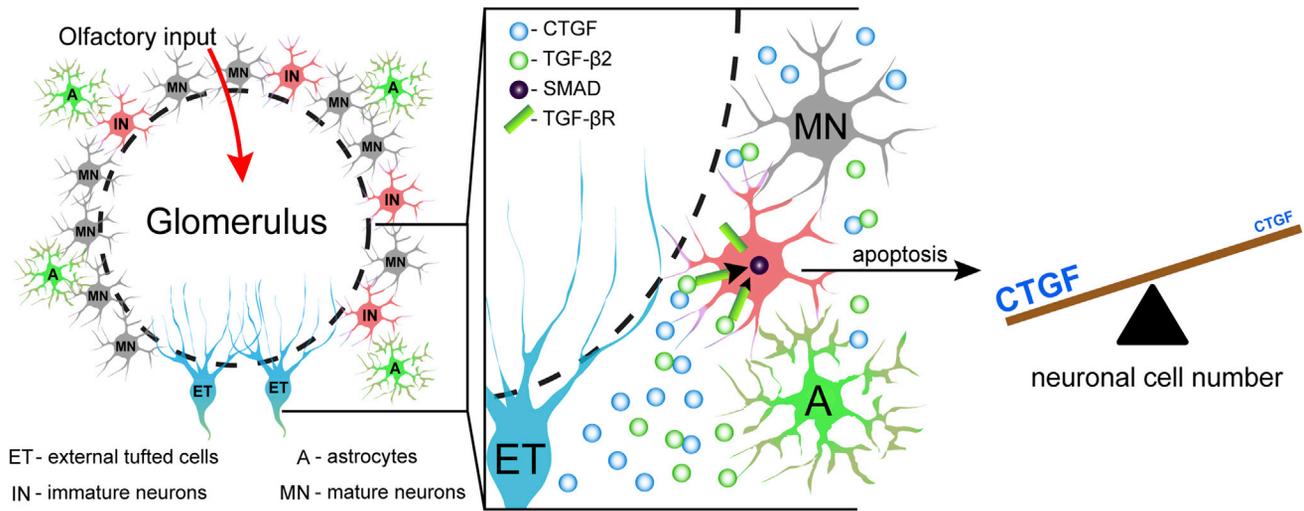


Figure 8. Proposed Mechanism by which CTGF Expression Levels Regulate the Survival of Periglomerular Interneurons

External tufted cells secrete CTGF that regulates the survival of newly integrating neurons in the glomerular layer by interacting and potentiating the proapoptotic activity of glial-derived TGF-β2 via SMAD-dependent cell death. CTGF expression is enhanced by olfactory stimulation.

Regulation of CTGF expression by olfactory stimuli changes apoptosis in the glomerular layer and thus adjusts the number of surviving inhibitory neurons according to olfactory cues in the environment. Since each odor often activates only few glomeruli, this adjustment of inhibitory drive provides a mechanism for glomerulus-specific plasticity. Thus, activation of distinct glomeruli increases CTGF expression, thereby reducing the number of interneurons in these glomeruli, whereas inactive glomeruli exhibit lower CTGF levels and hence more periglomerular interneurons. At the behavioral level the increased number of interneurons very likely lowers the threshold for odorant detection and enhances olfactory discrimination. The CTGF-dependent behavioral phenotype that we describe here is in accordance with results reported in a recent study, in which the authors demonstrated that an increase in mitral cell inhibition enhances odor discrimination, whereas a decrease in mitral cell inhibition interferes with odor discrimination (Abraham et al., 2010). The mechanism by which enhanced inhibition may result in better performance is most likely due to the fact that augmented inhibition causes better synchronization of mitral cell activity (Giridhar et al., 2011; Schoppa, 2006), thereby enhancing the recruitment of downstream cortical targets (Giridhar et al., 2011). Increased inhibition was shown to synchronize the activity not only of mitral cells in mice (Giridhar et al., 2011; Schoppa, 2006) but also of antennal lobe projection neurons in locusts (functional analogs of mitral cells) (MacLeod and Laurent, 1996). These studies and our own are compatible with the following scenarios, according to which CTGF levels modulate the olfactory detection threshold: (1) low CTGF levels augment the number of periglomerular interneurons, leading to an increase in odorant sensitivity; i.e., low abundant odorants can be detected (Kraemer and Apfelbach, 2004); (2) high CTGF levels reduce the number of periglomerular interneurons, leading to odorant habituation; i.e., reaction to strong odorants is decreased (Buonviso and Chaput, 2000; Dalton and Wysocki,

1996). Since changes in odorant sensitivity and habituation are long lasting, CTGF levels are ideally suited to link olfactory input and behavioral output.

Our data indicate that 10 min of odorant stimulation already significantly increases CTGF expression and decreases neuronal survival by 20% across odorant-stimulated glomeruli. Furthermore, it seems that the CTGF effect on cell survival is prone to “desensitization,” since longer exposure to an odorant (up to 24 hr) does not have a stronger effect than a short 10 min exposure. It goes without saying that in addition to CTGF there are other activity-dependent extracellular signals modulating periglomerular cell apoptosis. For instance, the availability of TGF-β2 per se might dictate as to how much CTGF is required to trigger cell apoptosis. Each of these signals very likely exhibits different kinetics of cell survival/death regulation. Little is known so far on how time of odorant exposure, odorant intensity, level of background noise in the environment, etc. control CTGF and other regulatory factors that participate in cell survival/death decision.

Numerous studies have investigated how modifications in olfactory sensory activity affect the survival of postnatally generated OB interneurons. Most of these studies focused on adult-born granule cells (e.g., Alonso et al., 2008; Petreanu and Alvarez-Buylla, 2002; Saghatelian et al., 2005), and only few also investigated periglomerular cells (Bovetti et al., 2009; Rey et al., 2012). In all these studies, the modification of sensory input was “extreme,” consisting either of a nonphysiological enrichment or complete ablation of olfactory receptor neuron activity. It is of note that a general olfactory enrichment did not affect periglomerular cell survival in our hands, while the selective stimulation of defined glomeruli (by lyral) decreased periglomerular cell survival in the respective glomeruli, clearly showing that these experimental regimes differentially affect outcome.

The restricted expression of CTGF in external tufted cells regulates the glomerular output on a long timescale (hours/days), adding therefore further temporal dimensions to the

well-described short timescale (millisecond range) regulation. External tufted cells exert a control of local synaptic processing in a glomerulus at several levels. Thus, the axons of external tufted cells connect intrabulbar isofunctional odor columns (Liu and Shipley, 1994), whereas intraglomerular connections between external tufted cells and periglomerular cells as well as short axon cells amplify the sensory input and synchronize glomerular output (De Saint Jan et al., 2009; Hayar et al., 2004). Low threshold activation and the propensity of spontaneous rhythmic burst firing designate external tufted cells to be excitatory pacemaker-like cells that drive glomerular output activity. External tufted cells exert this control at a fast timescale via chemical and electrical synapses. In contrast, we demonstrate here a mechanism by which external tufted cells regulate glomerular output at a much longer timescale.

CTGF responsiveness is reminiscent of two other immediate-early genes, *c-fos* and *Egr1* (also known as Zif268 protein). Expression of *c-fos* and *Egr1* in the glomerular layer already significantly increases 45 min after odor exposure (Johnson et al., 1995). This regulation contrasts with the activity-dependent regulation of tyrosine hydroxylase (TH) in periglomerular neurons that decreases significantly only after several days following sensory deprivation (Baker et al., 1993), again highlighting the diversity of temporal regulations that take place in glomeruli.

In summary, we here identified CTGF as a proapoptotic factor whose activity-dependent increase of expression eliminated newborn neurons in a locally restricted manner. Our experiments showed that even a small increase in the number of surviving cells dramatically changed olfactory behavior. Survival/death choice is regulated by external stimuli, and the number of surviving cells is “adapted” according to the animal’s local environment. Since olfaction is the most important sensory mode for many mammals, “olfactory competition” for food, mating, and predator versus prey relationship plays a decisive role during the life of an animal. Hence tight regulation of newly added neurons is a crucial mechanism enabling an adaptive response to environmental changes.

EXPERIMENTAL PROCEDURES

Materials and Reagents

All antibodies and chemicals are listed in the [Supplemental Experimental Procedures](#).

Animals

All animal procedures were performed according to the regulations of Heidelberg University/German Cancer Research Center or Pasteur Institute Animal Care Committees.

Plasmid Cloning

To obtain miRNA cassettes expressed under the synapsin or GFAP promoter, we used the BLOCK-iT PolII system (Invitrogen, Germany) and subcloned miRNA cassettes to viral vectors containing the synapsin or GFAP promoter, respectively. shRNA constructs were cloned as previously described (Khodosevich et al., 2009). For details of cloning, see the [Supplemental Experimental Procedures](#).

Analysis of mi/shRNA Silencing Efficiency

The efficiency of mi/shRNA silencing was tested as previously described (Khodosevich et al., 2009). For details, see the [Supplemental Experimental Procedures](#).

Production of Recombinant Viruses

Recombinant retroviruses and AAVs were produced as previously described (Khodosevich et al., 2009, 2012).

Injection of Recombinant Viruses into the Mouse Brain

The titer of the injected virus had been adjusted such as to be equal for all experiments— 4×10^8 units/ml for AAVs and 10^8 units/ml for retroviruses. For double SVZ/OB injections, mice were first injected into the OB using glass capillary and immediately after into the SVZ by a Hamilton syringe (Hamilton, Switzerland). Injection procedure is described in the [Supplemental Experimental Procedures](#).

Data Analysis and Statistics

Data were analyzed using GraphPad Prism (GraphPad Software, USA) and R software. Normally distributed data were analyzed by t test, ANOVA, Tukey, or Tukey-Kramer tests; non-normally distributed data were analyzed by Mann-Whitney, Kruskal-Wallis, Dunn’s, Dunnett’s, or Steel-Dwass tests. See the [Supplemental Experimental Procedures](#) for details of analysis procedures and statistics.

Olfactory Enrichment

Animals were exposed to odor-enriched environment for 3 weeks as described previously (Alonso et al., 2008). For details, see the [Supplemental Experimental Procedures](#).

Chemical Ablation of Olfactory Epithelium

Mice were given one i.p. injection of dichlobenil (2,6-dichlobenzonitrile, 100 mg/kg body weight) or DMSO and analyzed 4, 8, 12, and 20 days postinjection.

Lyrical Stimulation

MOR23-IRES-tauGFP mice were presented with tissue soaked in 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxaldehyde (= lyral) for different time periods. For details, see the [Supplemental Experimental Procedures](#).

Electrophysiology

Whole-cell recordings were performed at room temperature using sagittal OB slices of 250 μ m thickness from P31- to P50-old mouse brains. For details, see the [Supplemental Experimental Procedures](#).

Olfactory Behavior

Mice were partially water deprived for 1 week and then trained using an operant conditioning go-out procedure in computer-controlled olfactometers. In this paradigm, mice were trained to respond to the presence of a positive stimulus odorant (S+) by licking the water delivery tube and to refrain from responding to the presence of negative stimulus odorant (S−). Odorant detection threshold, odorant discrimination, and long-term olfactory memory were analyzed. See the [Supplemental Experimental Procedures](#) for details.

Additional experiments are described in the [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2013.07.011>.

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