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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-β2, 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; Saghatelyan 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 knockdown 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-β2, 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 Vb 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 processes (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 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).
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 μm; (E) and (G), 50 μm; (D) and (F), 20 μm. See also Figure S1.
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 dendrites.

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 coinjected 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.
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-β2 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

![Figure 3. TGF-β2 and Its Receptors Are Expressed in the Glomerular Layer and Act Downstream of CTGF In Vitro](image-url)
factor X should be expressed in the glomerular layer and
the corresponding receptor in maturing neuroblasts (Fig-
ure 3A2).

To identify potential candidates that are expressed in the
glomerular layer, we took recourse to published data and the
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 αM[i]2, αv[i]3,
α6[i]1, and α5[i]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-β1 and TGF-β2, 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-depen-
donent pathway (Jang et al., 2002). We did not detect TGF-β1
expression in the glomerular layer of the OB by western blot or
immunohistochemistry (data not shown). In contrast, TGF-β2
and its receptors TGF-βRI and TGF-βRII were all expressed in the
glomerular layer (Figure S3A). Interestingly, TGF-β2 was expressed exclusively in GFAP-positive astrocytes in the
glomerular layer (Figure 3B). TGF-βRs, on the other hand, were
found in a subpopulation of GAD-positive interneurons of the
glomerular layer (Figures 3C–3E). Furthermore, TGF-βRI-express-
ing cells colocalized 100% with TGF-βRII-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-β2 are expressed in the glomerular
layer, whereas TGF-βRI and TGF-βRII can be detected in newly
born neurons (see scheme in Figure 3A2).

To further substantiate the hypothesis of CTGF-TGF-β2
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-β2, but not anti-TGF-β1, neutralizing antibody abolished
the effect of recombinant CTGF. Furthermore, both recombinant
TGF-βRI-Fc and TGF-βRII-Fc (fusions of TGF-βRs with the Fc
immunoglobulin domain that bind to TGF-β and block its activity)
bogged the CTGF effect. Likewise, neutralizing antibody to
TGF-β2, but not to TGF-β1, reduced glomerular layer apoptosis,
and recombinant TGF-β2 enhanced it. When CTGF and TGF-β2
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-βRI-Fc or TGF-βRII-Fc did not decrease
apoptosis in the granule cell layer (Figure S3E). The intracellular
apoptotic effect of TGF-β1 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-β2 (Figure S3D). Thus, CTGF potenti-
ates TGF-β2 activity in the glomerular layer and regulates
apoptosis of newly generated cells via the TGF-βRI-SMAD
pathway.

To obtain in vivo evidence that CTGF acts via the TGF-β
pathway, we knocked down TGF-βRI expression in postnatally
generated neuroblasts (Figure 4A). For these knockdown exper-
iments, 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-βRI 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 inter-
neurons. TGF-βRI expression knockdown was confirmed by
western blot (Figure 4A3). Knockdown of TGF-βRI in mature
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 demon-
strated 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 ex-
pressing EmGFP and control miRNA or any of two miRNAs
against TGF-βRI. Simultaneously, we injected AAV to knock
down CTGF in the OB (Figure 4A2). If CTGF acted via a different
receptor than TGF-βRI, then TGF-βRI-knockdown cells should
continue to be responsive to changed TGF levels in the glomer-
ular layer. However, CTGF knockdown did not affect the survival
of TGF-βRI-knockdown neurons (Figure 4C), demonstrating that
CTGF regulates neuronal survival via TGF-β signaling.

To substantiate our finding that TGF-βRs act downstream
of CTGF, we employed P5-old Tgfαr2 fl/fl mice that were injected
into the SVZ with two retroviruses: one expressing tdTomato
and another expressing Cre recombinase together with EGF (Fig-
ure 4D, D1). This ensures that Tgfαr2 knockout cells in the
OB are EGFP positive, whereas control cells are tdTomato posi-
tive. 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 immuno-
histochemistry). 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
Tgfαr2 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 Tgfαr2 fl/fl mice in comparison to
wild-type mice (Figures 4E and 4F), indicating that Tgfαr2
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, Tgfbr2 fl/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, Tgfbr2 knockout neuroblasts should exhibit enhanced survival.

(E) Knockout of Tgfbr2 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 Tgfbr2 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 Tgfjir2 knockout was also observed when CTGF-dependent apoptosis was not boosted by CTGF overexpression (Figure S4B). Together, these experiments imply TGF-βII as the downstream effector of CTGF in postnatally generated periglomerular cells.

Next, we sought direct proof that astrocytes are the source of TGF-j2 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-j2 expression in astrocytes. To this end, we used an AAV expressing EmGFP and control miRNA or any of two miRNAs against TGF-j2 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-j2 expression knockdown was confirmed by western blot (Figure S4C2). Using this approach, we showed that TGF-j2 knockdown resulted in a significant increase of tdTomato-positive cells in the glomerular layer (Figures S4E and S4F). TGF-j2 knockdown rescued CTGF-induced neuronal apoptosis, thus identifying astrocytes as the source that provides TGF-j2.

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 (Parish-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 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 GABAA 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 paired tasks (rewarded odorant, stimulus [S+]; solvent, solvent).
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) 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; Kruskall-Wallis, post hoc Steel-Dwass (raw data and median).

(B) 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 ($\tau_{\text{decay}}$) for mitral cell sIPSCs. “n” indicates the number of analyzed cells; Student’s t test (mean ± SD).

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

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**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 ± SEM).

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

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

(G) For some odorants, CTGF knockdown mice had a decreased reaction time for the negative (S−) odorant, Student’s t test (mean ± 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-β2 expression was already increased 4 days postinjection. Eight days postinjection, TGF-β2 expression level was highest and gradually decreased thereafter.

Scale bars, 50 μm. See also Figure S6.

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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., ≥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
CTGF Regulates Interneuron Survival

DISCUSSION

In this study we identified CTGF as a modulator of postnatal/adult OB circuitry. Based on our results, we propose that
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-β2, whose activity it potentiates, promoting SMAD-dependent apoptosis of newborn neurons in the glomerular layer via TGF-βR.s. CTGF expression is enhanced by olfactory stimulation, thus leading to an activity-dependent potentiation of TGF-β2 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.
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-β 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; Saghatelyan 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 (Johnston 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 synapin or GFAP promoter, we used the BLOCK-IT PolII system (Invitrogen, Germany) and subcloned miRNA cassettes to viral vectors containing the synapin 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 × 10^6 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, Dunnnett’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.

**Lyric 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 μm 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–no 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**

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