Ntf3 acts downstream of Sip1 in cortical postmitotic neurons to control progenitor cell fate through feedback signaling

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ABSTRACT
Cortical progenitors undergo progressive fate restriction, thereby sequentially producing the different layers of the neocortex. However, how these progenitors precisely change their fate remains highly debatable. We have previously shown the existence of cortical feedback mechanisms wherein postmitotic neurons signal back to the progenitors and promote a switch from neurogenesis to gliogenesis. We showed that Sip1 (Zeb2), a transcriptional repressor, controls this feedback signaling. A similar mechanism was also suggested to control neuronal cell type specification; however, the underlying mechanism was not identified. Here, we provide direct evidence that in the developing mouse neocortex, Ntf3, a Sip1 target neurotrophin, acts as a feedback signal between postmitotic neurons and progenitors, promoting both apical progenitor (AP) to basal progenitor (BP) and deep layer (DL) to upper layer (UL) cell fate switches. We show that specific overexpression of Ntf3 in neocortical progenitors promotes an overproduction of BP at the expense of AP. This shift is followed by a decrease in DL and an increase in UL neuronal production. Loss of Ntf3, by contrast, causes an increase in layer VI neurons but does not rescue the Sip1 mutant phenotype, implying that other parallel pathways also control the timing of progenitor cell fate switch.

KEY WORDS: Cell fate, Cortical development, Feedback signaling, Ntf3, Sip1, Zeb2

INTRODUCTION
Cortical progenitors sequentially give rise to the different layers of the cortex (Angevine and Sidman, 1961; Rakic, 1974). Neurons occupying the different layers show a high degree of heterogeneity with respect to their transcriptome, axonal targeting, dendritic complexity and physiology (Fishell and Hanashima, 2008). Understanding how such a heterogeneous population of neurons is born from a smaller, relatively homogeneous population of progenitors has always invited great attention.

Previous work has shown that cortical progenitors undergo progressive fate restriction (Desai and McConnell, 2000). This was proposed to be an intrinsic phenomenon (Tempel, 2001; Shen et al., 2006). However, it is also well established that environmental cues influence cell fate decisions (Dehay et al., 2001; Zarbalis et al., 2007; Lehtinen et al., 2011; Siegenthaler and Pleasure, 2011).

Previous work from our laboratory and others showed that cortical progenitors receive cues from postmitotic neurons through a feedback signal instructing a shift from neurogenesis to gliogenesis (Barnabe-Heider et al., 2005; Seuntjens et al., 2009). We showed that Sip1, a transcription repressor, is a master regulator of this feedback mechanism. Within postmitotic neurons it controls Fgf9 expression, which in turn regulates this shift. Furthermore, in Sip1 mutants the neocortex contains more UL and fewer DL neurons (Seuntjens et al., 2009). However, it is not clear whether feedback signaling also controls a shift from producing DL to UL neurons. It is also unknown whether such a shift is due to Sip1 intrinsic effects in postmitotic cells or Sip1 controlled cell extrinsic signals.

In this study, using in utero electroporation (IUE), we show that inactivating Sip1 in a mosaic manner in neocortical neurons could reproduce the Sip1 mutant phenotype. We show that Ntf3, a Sip1 target neurotrophin, is expressed in the developing cortex. We then overexpressed Ntf3 specifically in postmitotic neurons to determine whether it could influence progenitor fate. We show that Ntf3 can act as a feedback signal between postmitotic neurons and progenitors to control both AP to BP and DL to UL neuronal shift. This Ntf3-mediated feedback signaling eventually leads to a radial expansion of the cortex. Finally, we show that deleting Ntf3 leads to an increase in layer VI neurons. Thus, our results demonstrate that postmitotic neurons can indeed influence progenitor fate and that feedback signaling is an important means of regulating cell fate decisions.

RESULTS AND DISCUSSION
Mosaic deletion of Sip1 in young cortical neurons phenocopies the Sip1 mutant

We have previously shown that deletion of Sip1 in postmitotic neurons has severe effects on cortical development (Miquelajaregui et al., 2007; Seuntjens et al., 2009). We had demonstrated that Sip1 deletion in young neocortical neurons changes the proportion of DL versus UL neurons. In order to test whether this effect is cell intrinsic or cell extrinsic, we decided to employ IUE. We introduced a plasmid encoding GFP-IRESCre in E12.5 Sip1flo/flo and Sip1flo/wt embryos. To verify if this relatively low efficiency deletion of Sip1 could replicate the phenotype of Sip1flo/flo:NexCre mutants, we stained for the layer V marker Ctip2 (Bcl11b). As in Sip1flo/flo:NexCre, the number of Ctip2+ cells decreased in Sip1flo/flo brains when compared with controls (Fig. 1A). Interestingly, within the electroporated region where the loss of Ctip2+ cells was observed, GFP+ cells did not show a preference for being either Ctip2 positive or negative [Fig. 1B; proportion of GFP+/Ctip2+ cells in Sip1flo/flo=0.21±0.06 (n=4) compared with 0.17±0.02 (n=3) in Sip1flo/flo, P>0.05]. This shows that Sip1 affects cell fate through cell-extrinsic mechanisms and also that IUE can be used to study these effects.

Ntf3 is upregulated in the Sip1 mutant cortex

We have previously shown that upon loss of Sip1, there is an ectopic upregulation of Ntf3 in the cortical plate (CP; Fig. 1C) and through ChIP assay that Sip1 directly binds to the Ntf3 promoter region.
(Seuntjens et al., 2009). In order to investigate the functionality of this binding, we performed a luciferase assay. The Ntf3 enhancer region (Fig. 1D) was inserted upstream of the gaussia-luciferase gene and transfected with or without Sip1 in COS7 cells. We observed a 1.9-fold decrease in transcriptional activity of the Ntf3 enhancer when Sip1 was co-transfected (Fig. 1D; n=3, P=0.0009). To check for Ntf3 expression dynamics in the developing cortex we used qRT-PCR, as the levels of Ntf3 expression were not quantifiable by in situ hybridization. We observed that the expression of Ntf3 at E13.5 was ~160-fold higher than at E12.5 (Fig. 1E; P=0.033). Furthermore, the expression doubled from E13.5 to E15.5 (Fig. 1E; P=0.027). This shows that the expression of Ntf3 correspondingly increases as the cortical plate grows. Additionally, the sudden increase in expression from E12.5 to E13.5 coincides with the appearance of the first neurons in the CP, thus indicating that the first postmitotic cells entering the cortical plate at E13.5 express Ntf3.

**Ntf3 promotes an increase in the BP population**

To study whether the upregulation of Ntf3 in the Sip1 mutant has any effect on the proportion of AP and BP, we overexpressed Ntf3 in the wild-type (WT) cortex. BPs are situated in the subventricular zone (SVZ) and are responsible for the vast expansion of UL neuronal production, though they have been shown to produce all layers of the cortex (Tabarykin et al., 2001; Haubensak et al., 2004; Englund et al., 2005; Gotz and Huttner, 2005; Arnold et al., 2008; Kowalczyk et al., 2009; Pinto et al., 2009; Vasistha et al., 2014).

In order to restrict the expression of Ntf3 to postmitotic neurons, we designed a plasmid system in which the Ntf3-IRES-EGFP cassette was preceded by a mCherry-polyA sequence flanked by

Fig. 1. Ntf3 is upregulated in the Sip1 mutant cortex. (A) In utero electroporation of Cre in Sip1fl/fl and Sip1fl/wt embryos at E12.5. Ctip2 decreases in the electroporated region of Sip1fl/fl embryos at E17.5, whereas no difference was observed in Sip1fl/wt embryos. Arrowheads indicate GFP+/Ctip2+; arrows indicate GFP+/Ctip2− cells. Images are composites of tiled images. (B) Proportion of GFP(Cre)+ cells also positive for Ctip2 in Sip1fl/fl=0.21±0.06 (n=4) compared with 0.17±0.02 (n=3) in Sip1fl/wt (P=0.05). (C) Ntf3 is upregulated in the Sip1 mutant cortex at E14.5, whereas no expression could be detected in wild type. Higher magnification images correspond to the boxed regions. (D) The Ntf3 enhancer region used. Luciferase assay shows that, upon co-transfection of Sip1, the transcription of the Ntf3 enhancer reduced by 1.9 fold (n=3, relative luminescence without Sip1=0.85±0.05 versus 0.44±0.03 with Sip1, P=0.0009). (E) qRT-PCR results showing the endogenous expression of Ntf3 (relative to Gapdh) during different stages of development. Expression at E13.5 (n=3) is ~160-fold higher compared with E12.5 (n=3, P=0.033). The expression at E15.5 (n=5) is ~2.2-fold higher compared with E13.5 (P=0.027). The expression at P0 (n=4) is ~74-fold higher compared with E13.5 (P=0.022).
loxP sites. Cre recombinase was driven by the postmitotic-specific NeuroD1 promoter, which switches expression from mCherry to Ntf3 and EGFP specifically in postmitotic neurons (Fig. 2A; supplementary material Fig. S1A,B). The location of the EGFP+ cells and the expression of Ntf3 verified by in situ hybridization confirmed exclusive postmitotic expression (Fig. 2A; supplementary material Fig. S2). In order to avoid variations that arise from comparing different mediolateral and

Fig. 2. Ntf3 promotes an increase in BP and decrease in AP populations. (A) Plasmid system for restricting expression to postmitotic neurons. A representative image showing exclusive postmitotic expression of EGFP. (B) Overview image showing that upon Ntf3 overexpression, the Tbr2+ BP population is expanded. Images are composites of tiled images. (C,E) Electroporation of GFP does not lead to any change in the size or distribution of Tbr2+ (~0.013±0.021, n=5) and Ctip2+ cells. Overexpression of Ntf3 leads to an expansion of Tbr2+ cells (0.10±0.048 higher proportions of Tbr2+ cells, n=5, P=0.003). Ctip2+ cells are displaced, mostly as a secondary effect of this increase in Tbr2+ cells. The contralateral hemisphere serves as an internal control. (D,E) Pax6+ APs are also displaced and reduced in Ntf3 overexpression brains (the difference in the proportion of Pax6+ cells between the electroporated and non-electroporated hemispheres upon Ntf3 overexpression is 0.9±0.03 compared with 0.01±0.05 in controls; n=3, P=0.04). All electroporation was carried out at E12.5 and analyzed at E14.5. (F) Effect of radial expansion of the cortex on Ntf3 overexpression. White and orange bars indicate the thickness of the electroporated and non-electroporated hemisphere, respectively. Bars were placed parallel to the apical process of the cells. Arrows indicate the physical fold in the cortical plate. Images are composites of tiled images.
rostrocaudal levels, we present all analysis as the difference between the electroporated and non-electroporated hemispheres. We electroporated wild-type E12.5 embryos and analyzed the brains at E14.5. We observed that upon Ntf3 overexpression there was a vast expansion in the proportion of Tbr2+ BP and hence the size of the SVZ (Fig. 2B,C,E; the proportion of Tbr2+ cells was 0.10±0.048 more in the electroporated hemisphere compared with the contralateral hemisphere upon Ntf3 overexpression compared with −0.013±0.02 in EGFP electroporations; n=5, P=0.003). Interestingly, there was also a decrease in the proportion of Pax6+ AP upon Ntf3 overexpression (Fig. 2D,E; 0.09±0.03 decrease in the proportion of Pax6+ cells in the electroporated hemisphere upon Ntf3 overexpression compared with 0.01±0.05 in control plasmid electroporations; n=3, P=0.04). Interestingly, the expansion of Tbr2+ progenitors was not always restricted to the electroporated region (Fig. 2B,C). Furthermore, the number of GFP+ Ntf3-expressing cells required for causing the AP to BP switch was not high. However, as each neuron carries multiple copies of the plasmid, a crucial threshold for Ntf3 concentration might be attained with fewer cells.

Following the expansion in BPs, we also observed a radial expansion of the electroporated cortex compared with the contralateral hemisphere (Fig. 2F). A similar radial expansion of the cortex has been previously reported (Nonaka-Kinoshita et al., 2013; Stahl et al., 2013). Because in all these experiments Ntf3 expression was restricted to postmitotic neurons, the changes in progenitor populations are most likely to be the result of feedback signaling.

Ntf3 promotes UL neurogenesis at the expense of DL neurons

Next, we asked whether the Ntf3 initiated AP to BP shift could also lead to an alteration in proportions of different cell types. To test this, we overexpressed Ntf3 at E11.5 and analyzed the brains at E17.5. We observed a 0.077±0.027 decrease in the proportion of Tbr1+ layer VI neurons in the Ntf3 electroporated hemisphere compared with the contralateral hemisphere, whereas EGFP expression had no effect (0.002±0.008; Fig. 3A,D; n=3, P=0.028). Ntf3 electroporated hemisphere showed a 0.034±0.01 decrease in Ctip2+ (layer V) proportions compared with the contralateral hemisphere, whereas EGFP electroporated brains did not show any difference (−0.0005±0.01; Fig. 3B,D; n=5, P=0.003). We also observed a 0.05±0.03 increase in Brn2+ UL neuron proportions between electroporated and non-electroporated hemispheres in Ntf3 electroporated brains compared with EGFP electroporations (−0.0068±0.006; Fig. 3C,D; n=4, P=0.036). This shows that Ntf3 promotes a shift from DL to UL neurogenesis.
highlighting the presence of cortical feedback signals in layer specification.

Recent work points to the existence of lineage-specific progenitors (Franco et al., 2012). One hypothesis for the switch from DL to UL neuron production mediated by Ntf3 would be the preferential effect of Ntf3 on UL specific progenitors (Franco and Muller, 2013), promoting their premature differentiation.

**Deletion of Ntf3 increases the proportion of layer VI neurons but the Ntf3-Sip1 compound mutant does not rescue the Sip1 mutant phenotype**

We have previously reported that in the absence of Sip1, cortical progenitors precociously produce UL neurons at the expense of DL neurons (Seuntjens et al., 2009). To investigate whether this phenotype can be reverted on Ntf3 deletion, we generated Sip1-Ntf3 double knockout (DKO). We did not find any significant difference in the proportions of Satb2+ (layer II-V), Ctip2+ (layer V), Bm2+ (layer II-IV) and Sox5+ (layer VI) cells between Sip1 single and Sip1-Ntf3 DKO (Fig. 4A-D, all \( P \geq 0.05 \)). Furthermore, we did not find any difference between wild type and Ntf3\(^{-/-}\) with respect to the proportion of Satb2, Bm2 and Ctip2+ populations (Fig. 4A,B,D). Interestingly, we noticed an increase in the proportion of Sox5+ layer VI population in Ntf3\(^{-/-}\) (0.365±0.029 in Ntf3\(^{-/-}\) compared with 0.293±0.023 in wild type; \( n=3, P=0.029 \)).

The increase in the proportion of Sox5+ layer VI neurons in Ntf3\(^{-/-}\) indicates that Ntf3 is involved in controlling important aspects of the cell fate switch. It is also noteworthy that the maximum effect of both Ntf3 overexpression and deletion was on layer VI cells. This also correlates with the onset of Ntf3 expression.

**Fig. 4. Deletion of Ntf3 from the Sip1 mutant cortex does not rescue the Sip1 mutant phenotype.** (A) Satb2 and Ctip2 immunohistochemistry marking UL and layer V cells in wild type, Ntf3\(^{-/-}\); Sip1\(^{fl/fl}\);NexCre and Sip1-Ntf3 DKO. The decrease in Ctip2 cells in Sip1 mutants is not restored in Sip1-Ntf3 DKO. Similarly, the increase in Satb2+ cells is also not restored. (B) Bm2, another UL marker, and (C) Sox5, a layer VI marker, also confirm that the Sip1-Ntf3 DKO does not rescue the Sip1 mutant phenotype. (D) Proportion of Sox5+ cells is higher in Ntf3\(^{-/-}\) compared with wild type \( \left[ n=3 \text{ for wild type, Sip1 and Ntf3\(^{-/-}\);} n=2 \text{ for Sip1-Ntf3 DKO (all } P>0.05 \right] \). The proportion of Sox5+ cells in Ntf3\(^{-/-}\) = 0.36±0.029 compared with 0.29±0.02 in wild type, *\( P=0.029 \). (E) Model depicting two possible modes of Ntf3-mediated signaling. Receptors located either along the radial glial process or the cell body can sense Ntf3 released by postmitotic neurons. This leads to an increased production of BPs from APs. Finally, the feedback signal initiated by Ntf3 leads to a shift from DL to UL neuronal production.
However, as the deletion of Ntf3 from the Sip1 mutant cortex does not rescue the phenotype, it is probable that multiple pathways exist for controlling cortical feedback signaling. Another possibility is that the receptors for Ntf3 (Trks) are also activated by alternate pathways in the developing cortex (Lee et al., 2002). For example, recent work has shown that EGFR can transactivate both TrkB and TrkC (Ernfors et al., 1994; Puehringer et al., 2013). Interestingly, Bdnf and Ntf3 have opposing functions in laminar fate specification (Fukumitsu et al., 2006). Whereas Ntf3 promotes UL neurogenesis, Bdnf has been shown to promote DL neurogenesis.

The presence of proteins such as Ntf3 in the CP that act as feedback signals influencing progenitors located within the VZ and SVZ raises intriguing issues about how these signals are transmitted. Signals originating from the CP could either bind to cognate receptors along the radial glial process or diffuse to the VZ and bind to receptors on the cell body. Although our results do not answer this question, we believe that using such a system in combination with optical methods such as FRET could address this issue (Fig. 4E).

Using Ntf3 as a reporter of developmental events may allow the use of such a system in conjunction with optical methods that are currently under development. Our results demonstrate that, although Ntf3 is expressed in the mouse CP, it is likely to be active in a subpopulation of cells. Further studies are needed to determine whether Ntf3 expression represents a specific population of LT-HPCs or whether it is a marker of a larger population of cells. Nevertheless, the identification of Ntf3 as a marker of the CP may provide a useful tool for studying the development of the neocortex.

## Materials and Methods

### Mouse lines

All mouse experiments were carried out in compliance with German law and according to the guidelines approved by the Bezirksregierung Braunschweig or Landesamt für Gesundheit und Soziales Berlin. Both male and female mouse embryos were used. Sip1 conditional mutants carried a loxP-flanked exon 7 crossed to either Ncx1 or Emx1-Cre (Gorski et al., 2002; Higashi et al., 2002; Goebbels et al., 2006). The Ntf3 mutant was kindly provided by Dr Michael Sendtner (University of Würzburg, Germany).

### Tissue processing and antibodies

Tissue was processed as described previously (Seuntjens et al., 2009). The following primary antibodies were used: GFP (Abcam, chicken, 1:1000, ab13970/Rockland, goat, 1:500, 600-101-215), Ctip2 (Abcam, rat, 1:250, ab18465), Tbr2 (Abcam, rabbit, 1:150, ab23345), Pax6 (Millipore, rabbit, 1:300, AB2237), Satb2 (self-generated, rabbit, 1:1000), Bmi2 (Santa-Cruz, goat, 1:200, sc-6029), Sox5 (Santa-Cruz, goat, sc-17329) and Tbr1 (Abcam, rabbit, 1:200, ab31940). Dylight/Alexa-coupled secondary antibodies (Jackson ImmunoResearch) were used at 1:500. Hoechst (Sigma, 1:500) or rabbit, 1:200, ab31940). Dylight/Alexa-coupled secondary antibodies (Jackson ImmunoResearch) were used at 1:500. Hoechst (Sigma, 1:500) or rabbit, 1:200, ab31940).

### In utero electroporation (IUE)

IUE was performed as described previously (Saito, 2006).

### Luciferase assay

The following primers were used for amplifying the Ntf3 promoter region: 5′-AGGAAATTCCTGGCCTCCTTTCGCCCCGCAGCTT-3′ and 5′-TGATGTCCACCGTGCAATGAGGAAGGGTGGA-3′.

The amplicon was cloned into pMCS-Gaussia luciferase (Thermo Scientific). CMV-driven alkaline phosphatase served as the transfection control. COS7 cells were transfected using Lipofectamine 2000 (Invitrogen).

### Quantitative RT-PCR

Total RNA was extracted from E12.5, E13.5, E15.5 and P0 cortices using the RNeasy plus mini kit (Qiagen). FS cDNA was prepared using Maxima reverse transcriptase (Thermo Scientific) using a mixture of oligoD and random primers. qRT-PCR was performed using Fast SYBR Green master mix (Applied Biosystems) on an Applied Biosystems 7500 RT-PCR system. Primers were designed across an exon-exon boundary. A negative cDNA preparation (containing no reverse transcriptase) was used to verify the primer pairs. The following primers were used: Ntf3_fwd, 5′-AAGGGATCGGGAGGATGAC-3′; Ntf3_Rws, 5′-GCCCGAACCCACCATCACATCAATCAAAAAACCAAAAAACTGACCATT-3′; GAPDH_fwd, 5′-TACGGCCAAATCGGTTGACA-3′; GAPDH_Rws, 5′-GAGATGTTTCTCCGTGTCG-3′.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

S.P. and V.T. conceived the project. S.P., S.S. and A.N. performed experiments. S.P. and V.T. analyzed data. S.P. wrote the manuscript. S.S. and V.T. edited the manuscript.

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## Supplementary material

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