DCC functions as an accelerator of thalamocortical axonal growth downstream of spontaneous thalamic activity

Mar Castillo-Paterna, Verónica Moreno-Juan, Anton Filipchuk, Luis Rodríguez-Malmierca, Rafael Susín & Guillermina López-Bendito*

Abstract

Controlling the axon growth rate is fundamental when establishing brain connections. Using the thalamocortical system as a model, we previously showed that spontaneous calcium activity influences the growth rate of thalamocortical axons by regulating the transcription of *Robo1* through an NF- κ B-binding site in its promoter. *Robo1* acts as a brake on the growth of thalamocortical axons *in vivo*. Here, we have identified the Netrin-1 receptor DCC as an accelerator for thalamic axon growth. *Dcc* transcription is regulated by spontaneous calcium activity in thalamocortical neurons and activating DCC signaling restores normal axon growth in electrically silenced neurons. Moreover, we identified an AP-1-binding site in the *Dcc* promoter that is crucial for the activity-dependent regulation of this gene. In summary, we have identified the *Dcc* gene as a novel downstream target of spontaneous calcium activity involved in axon growth. Together with our previous data, we demonstrate a mechanism to control axon growth that relies on the activity-dependent regulation of two functionally opposed receptors, *Robo1* and DCC. These two proteins establish a tight and efficient means to regulate activity-guided axon growth in order to correctly establish neuronal connections during development.

Keywords axon growth; development; Netrin-1/Dcc signalling; spontaneous activity; thalamus

Subject Category Neuroscience

DOI 10.15252/embr.201439882 | Received 20 November 2014 | Revised 12 April 2015 | Accepted 13 April 2015 | Published online 6 May 2015

EMBO Reports (2015) 16: 851–862

Introduction

Axon growth is a key developmental process that is necessary for axons to establish appropriate connectivity in the central nervous system (CNS). Developing neurons extend their axons following stereotype pathways in which distinct axon guidance molecules and intermediate targets play a fundamental role [1–3]. Thus, the course of

axon growth is a tightly controlled process. Using the thalamocortical system as a model, we previously identified a mechanism that regulates the growth rate of developing axons [4]. We demonstrated that spontaneous Ca^{2+} activity modulates axonal growth through the transcriptional upregulation of the guidance receptor *Robo1*, which appears to function as a brake for thalamocortical axon (TCA) growth [4]. Therefore, modifications to *Robo1* transcription that are triggered by spontaneous activity mediate the developmental decrease in axon growth as TCAs approach the cortex. However, tight regulation of axon growth rate probably relies on different factors controlling speed in both senses, instead of just one specific factor acting as a brake. Thus, other activity-dependent factors might contribute to this process.

Several studies have demonstrated that spontaneous electrical activity plays a fundamental role in modulating early embryonic developmental events such as neurotransmitter specification and axon guidance [5, 6]. Moreover, spontaneous activity modulates the growth rate of developing thalamocortical and sensory axons of the spinal cord [4, 7, 8]. In fact, specific parameters of this activity, such as the frequency of Ca^{2+} spikes, drive precise changes in the expression of downstream target genes that are critical for the control of these developmental processes, including the axon growth program [4, 9]. Thus, spontaneous activity appears to emerge as a general mechanism to influence axonal extension of developing neurons through the regulation of gene expression.

The chemotropic guidance cue Netrin-1 mediates the attraction of migrating axons during CNS development through the receptor deleted in colorectal cancer (DCC) [10–12]. Activation of DCC by Netrin-1 is required for the outgrowth of cortical and commissural axons [13–16]. Indeed, the significance of this pathway during brain development was highlighted by the absence of spinal and cerebral commissures when Netrin-1 or DCC expression is abolished [17, 18]. Netrin-1 has also been shown to promote TCA outgrowth *in vitro*, and in *Netrin-1*^{−/−} mice, fewer TCAs reach the cerebral cortex [19]. Thus, Netrin-1 signaling through the DCC seems to be a good candidate to participate in the activity-dependent regulation of axon growth during development.

Here, we determined whether DCC might act together with *Robo1* to modulate the growth rate of TCAs downstream of

spontaneous activity. We found that spontaneous Ca^{2+} activity regulates DCC expression in an opposite fashion to that of Robo1. When thalamic neurons are silenced, DCC expression is significantly dampened at both the mRNA and protein levels. Through *in vitro* and *in vivo* loss- and gain-of-function experiments, we showed that DCC functions as an accelerator for TCA growth. Moreover, we showed that Ca^{2+} spikes modulate axonal growth through the transcriptional upregulation of *Dcc* acting via an AP-1 transcription factor-binding site in its promoter. In sum, our data identify a mechanism by which spontaneous activity can efficiently control axon growth through the transcriptional regulation of two functionally opposite genes, Robo1 and DCC.

Results

Silencing spontaneous activity decreases the amount of Dcc in growth cones

Developing axons might be guided along specific pathways to establish correct connections by controlling their growth rate. We recently demonstrated that by regulating the Robo1 gene, spontaneous Ca^{2+} activity is a key modulator of the growth rate of developing TCAs [4]. Robo1 acts as a brake for axonal growth, and its expression is upregulated in silenced thalamocortical neurons [4]. However, the efficient regulation of axon growth speed might not only rely on the function of a brake but also on that of an accelerator. Thus, we investigated whether other genes regulated by spontaneous thalamic activity also modulate the growth rate of TCAs.

To identify additional candidate genes involved in axon growth and that might be regulated by spontaneous activity, we overexpressed the inwardly rectifying potassium channel Kir2.1 (Kir) in thalamic neurons which will provoke hyperpolarization and dampening of neural activity [4, 20–24]. Dissociated thalamic cells were transfected with plasmids expressing Kir2.1 (Kir) and/or *Gfp*, and they were analyzed after 72 h in culture (Fig 1A). As expected, spontaneous somatic Ca^{2+} transients were silenced in Kir-expressing thalamic cells (Supplementary Fig S1A), and thus, we assessed whether the expression of putative downstream target genes might have changed in these thalamic silenced neurons. We identified *Dcc* as being one of the most strongly downregulated genes after silencing thalamic spontaneous activity. Silencing thalamic neuronal activity decreased *Dcc* expression 1.95-fold in Kir-expressing thalamic neurons (*Gfp*, 1.05 ± 0.28 , $n = 7$; *Kir*, 0.53 ± 0.33 , $n = 14$; Fig 1B), as measured by quantitative RT-PCR (qPCR). Moreover, DCC protein levels diminished accordingly in these neurons (Fig 1C and Supplementary Fig S1B). On the contrary, when we elevated the extracellular KCl concentration in the cultures to increase neuronal activity, the axons of thalamic neurons grew longer and accumulated more DCC protein (Supplementary Fig S1C–D). Moreover, and as embryonic development proceeded, the levels of expression of *Dcc* transcripts gradually diminished in thalamic neurons *in vivo* from 1.46 ± 0.34 at E12.5 ($n = 5$) to 0.57 ± 0.19 at E18.5 ($n = 4$) (Fig 1D). This decrease was also observed at the level of DCC protein, which declined from 100 ± 9.6 at E13.5 ($n = 9$) to 24.5 ± 4.5 at E15.5 ($n = 8$) (Fig 1E). Therefore, these results suggest the existence of a mechanistic link between the downregulation of DCC and the decrease in the growth rate previously described in these thalamic neurons [4].

DCC functions as an intrinsic accelerator for TCA

The aforementioned results prompted us to hypothesize that DCC plays a role in modulating TCA growth. To test this, we measured axonal outgrowth in dissociated thalamic neurons from wild-type and *Dcc*-deficient mice (Fig 2). In the absence of *Dcc*, thalamic axons grew significantly less compared to controls (*Dcc*^{+/+}, $100 \pm 1.16\%$, $n = 4$ brains; *Dcc*^{-/-}: $46.80 \pm 0.69\%$, $n = 6$ brains; Fig 2A,B), and thus, we predicted that increasing DCC levels in thalamic neurons would accelerate thalamic axon growth *in vitro*, overriding the effects of Kir-induced silencing of thalamic spontaneous activity. We augmented DCC levels by transfecting thalamic neurons with a full-length *Dcc* (*FL-Dcc*) at E13.5, and we quantified the length of thalamic axons. Overexpression of *Dcc* remarkably enhanced the extension of thalamic axons (*Gfp*, $100 \pm 4.84\%$, $n = 7$ independent cultures; *FL-Dcc*, $148.5 \pm 4.34\%$, $n = 7$ independent cultures; Fig 1C,D), further indicating that DCC acts as an intrinsic accelerator for thalamic axon progression.

Netrin-1, the best known ligand for DCC proteins, is expressed by thalamic neurons, and it is distributed along the entire TCA connectivity pathway *in vivo* [19, 25, 26]. As expected, we detected *Netrin-1* mRNA and protein expression in thalamic dissociated cultures at E13.5 (Supplementary Fig S2A). Thus, we first tested whether Netrin-1 fulfills a cell autonomous role in thalamocortical axon growth by culturing dissociated thalamocortical neurons from wild-type or *Netrin-1*^{-/-} mice. Like axons lacking DCC, axons without *Netrin-1* are significantly shorter than their wild-type controls (Supplementary Fig S2C,D). As Netrin-1 has been described as a survival factor in several brain structures, such as the spinal cord or the developing brainstem [27, 28], we assessed whether the effect on axon growth could somehow be related to increased thalamic neuronal death *in vitro*. Using release of lactate dehydrogenase activity (LDH) to detect cell death, we found no significant differences in *Netrin-1*^{+/+} and *Netrin-1*^{-/-} thalamic cultures (Supplementary Fig S2B). Then, we evaluated the extrinsic role of Netrin-1 in modulating thalamocortical axonal growth rate by placing thalamic explants from wild-type embryos onto telencephalic slices from *Netrin-1*^{-/-} mice or their wild-type littermates (Fig 3A). TCA extended much less when grown on *Netrin-1*^{-/-} slices than on the controls (*Netrin-1*^{+/+}, 1334 ± 126.9 , $n = 13$ explants; *Netrin-1*^{+/+}, 1630 ± 124.4 , $n = 20$ explants; *Netrin-1*^{-/-}, 665.7 ± 97.23 , $n = 15$ explants; Fig 3B,C). Altogether these results show that Netrin-1 functions to accelerate thalamocortical axonal extension in the ventral telencephalon.

Regulation of TCA extension *in vivo* via Netrin-1/DCC signaling

Our results *in vitro* and *ex vivo* showed that Netrin-1 and DCC play a role in the modulation of thalamic axonal extension. However, in order to determine whether Netrin-1/DCC signaling may also influence TCA extension *in vivo*, we performed dye-tracing studies in *Netrin-1*^{-/-} and *Dcc*^{-/-} mutant mice at E14.5. The lack of either *Netrin-1* or *Dcc* diminished TCA extension in the neocortex when compared with wild-type mice (*Netrin-1*^{+/+}, $n = 4$ brains; *Netrin-1*^{-/-}, $n = 5$ brains; *Dcc*^{+/+}, $n = 5$ brains; *Dcc*^{-/-}, $n = 8$ brains; Fig 3D). Thus, we expected that increasing DCC levels in thalamic neurons would promote TCA growth *in vivo*. We increased DCC levels through *in utero* electroporation of *FL-Dcc* into thalamic

Figure 1. Silencing thalamic activity downregulates DCC transcription.

A Embryonic dissociated thalamic neurons transfected with *Gfp* (green) and immunostained for Tuj1 (red). Nuclear counterstaining with DAPI is shown in blue. Scale bar, 100 μ m. Arrowheads indicate examples of Gfp-transfected cells labeled by Tuj1.

B Real-time PCR quantification of the transcripts for *Kir2.1* and *Dcc* in *Gfp*- and *Kir2.1*-transfected cells: *** $P < 0.001$, Mann–Whitney *U*-test. The data are presented as the means $\pm$ s.e.m.

C Immunocytochemistry for DCC in control and *Kir2.1*-expressing axons. Less DCC expression was found in growth cones transfected with *Kir2.1* compared to controls (arrowheads). Scale bar, 10 μ m.

D Semi-quantitative PCR for *Dcc* and *gapdh*, normalizing *Dcc* expression to that of *gapdh*: *** $P < 0.001$, one-way ANOVA with Tukey's *post hoc* analysis. ID, integrated density. The data are presented as the means $\pm$ s.e.m.

E Immunocytochemistry for DCC in E13.5 (GFP-positive; white arrowhead) and E15.5 (GFP-negative; blue arrowhead) isolated thalamic growth cones. Thalamic explants from E13.5 GFP mouse were cocultured together with explants from wild-type E15.5 embryos on polylysine/laminin coverslips. Scale bar, 10 μ m. Quantification of the data shown: *** $P < 0.001$, two-tailed Student's *t*-test. ID, integrated density. The data are presented as the means $\pm$ s.e.m.

neurons at E12.5, and we quantified the extension of TCA at E15.5 (Fig 3E). The overexpression of DCC notably increased TCA extension at both the rostral (*Gfp*, 1967 ± 143.8 , $n = 4$ brains; *FL-Dcc*, 3631 ± 315.6 , $n = 8$ brains) and intermediate (*Gfp*, 1653 ± 202.9 , $n = 4$ brains; *FL-Dcc*, 3561 ± 329.7 , $n = 8$ brains) telencephalic levels (Fig 3F,G). In sum, these results show that *Dcc* gain-of-function has the opposite effect to silencing thalamic spontaneous activity, supporting the role of DCC as an accelerator of TCA extension.

Activity-dependent regulation of DCC in controlling TCA growth

The results obtained strongly suggest that DCC might act downstream of spontaneous activity to control axon growth in thalamic neurons. Hence, we hypothesized that augmenting DCC levels in silenced thalamic neurons should rescue the axon growth defect

observed after *Kir2.1* silencing. To test this, we quantified the extension of thalamic axons transfected with *Gfp*, *Kir*, *FL-Dcc* alone, or co-transfected with *Kir* and *FL-Dcc* (Fig 4A,B). Whereas silencing spontaneous activity significantly diminished thalamic axon growth (*Gfp*, 100 ± 4.8 , $n = 7$ independent cultures; *Kir*, 49 ± 2.3 , $n = 7$ independent cultures; Fig 4A,B), co-electroporation of *Kir* with *FL-Dcc* completely rescued the axon growth defect to the levels of *FL-Dcc* alone (*FL-Dcc* + *Kir*, 135 ± 6.4 , $n = 7$ independent cultures; *FL-Dcc*, 148 ± 4.3 , $n = 7$ independent cultures; Fig 4A,B). These results demonstrate that modifying DCC levels in silenced neurons can rescue the abnormal axonal extension of *Kir*-positive neurons *in vitro* (Fig 4C). To determine to what extent spontaneous thalamic activity influences TCA growth rate via DCC *in vivo*, we measured the TCA extension in the cortex following co-electroporation with *Kir* and *FL-Dcc* at E12.5 (Fig 4D). Remarkably, co-electroporation of both plasmids together rescued the delayed

Figure 2. Alterations to the axon growth rate by manipulating DCC expression.

- A Tuj1 immunostaining revealed that thalamic axon growth decreased in the absence of DCC (arrowheads). Scale bars: 100 μ m lower power panels; and 50 μ m, higher power panels.
- B Quantification of the data shown in A. *** P < 0.001, two-tailed Student's t -test. The data are presented as the means $\pm$ s.e.m.
- C Tuj1 staining revealed an increase in thalamic axon growth following *Dcc* overexpression with a full-length *Dcc* (*FL-Dcc*) construct (arrowheads). Scale bars: 100 μ m, lower power panels; and 50 μ m, higher power panels.
- D Quantification of the data in C. When DCC levels were elevated, axon growth increased significantly when compared to control conditions. *** P < 0.001, two-tailed Student's t -test. The data are presented as the means $\pm$ s.e.m.

axon progression found after *Kir* electroporation alone. TCA extended significantly further in the cortex, both at rostral (*Kir*, 1237 ± 75.6 , $n = 8$ brains; *Kir* + *FL-Dcc*, 3743 ± 208.3 , $n = 7$ brains; Fig 4E,F) and at intermediate levels (*Kir*, 1243 ± 69.7 , $n = 8$ brains; *Kir* + *FL-Dcc*, 3930 ± 172.5 , $n = 7$ brains; Fig 4E,F) compared to axons expressing *Kir* alone. These results indicate that the delayed axon growth triggered by the silencing of spontaneous thalamic activity can be rescued *in vivo* by activating *Dcc* signaling.

Spontaneous thalamic activity regulates *Robo1* and DCC levels in an opposite manner [4] (Fig 1). Silencing spontaneous activity increases *Robo1* mRNA and protein, while it decreases DCC protein and mRNA. Thus, we tested whether this dynamic regulation occurs in thalamic neurons after manipulating their rate of axonal extension *in vitro*. As such, we silenced thalamic neurons with

Kir to diminish their axon growth rate and measured the levels of DCC and *Robo1* proteins in their growth cones. Growth cones transfected with *Kir* had higher *Robo1* and lower DCC levels than *Gfp*-transfected axons (*Gfp*, 100 ± 16.5 Dcc and 100 ± 9.3 *Robo1*, $n = 9$; *Kir*, 40.7 ± 7.3 Dcc and 234.4 ± 9.3 *Robo1*, $n = 20$; Fig 4G, H). These results demonstrate that spontaneous activity influences axon growth rates by antagonistically modulating the expression of *Robo1* and DCC in TCA.

The activity-dependent DCC transcriptional regulation occurs through an AP-1-binding site

Calcium spontaneous activity could modulate DCC expression levels by regulating its transcription. In order to determine this,

Figure 3. DCC acts as an accelerator for thalamocortical axon growth *in vivo*.

- A Experimental paradigm used to analyze the involvement of Netrin-1 in thalamic growth.
- B Wild-type thalamic axons grow significantly less in *Netrin-1^{-/-}* slices than in control slices (arrowheads). Crystals of Dil (red)-labeled thalamocortical axons, and DAPI nuclear counterstaining is in blue. Scale bar, 300 μ m.
- C Quantification of the data shown in B. $**P < 0.01$ and $***P < 0.001$, Kruskal–Wallis test with Dunn's *post hoc* analysis. The data are presented as the means $\pm$ s.e.m.
- D Progression of TCA labeled by Dil in the E14.5 thalamus of *Dcc^{+/+}* and *Dcc^{-/-}* brains. The bars indicate the position of the pallial–subpallial boundary for reference, and the arrowheads indicate the delayed position of TCA in the mutants. Scale bar, 200 μ m.
- E Schematic representation of the experimental procedure used to test the effect of increasing the Dcc levels *in vivo*. A FL-Dcc plasmid was co-electroporated *in utero* with GFP into the thalamus at E12.5, and the brains were analyzed at E15.5, at the peak of TCA cortical extension.
- F GFP immunoreactive axons in coronal sections at intermediate cortical levels, with DAPI nuclear counterstaining shown in blue. Overexpression of Dcc led to a significant acceleration in the cortical extension of TCA compared to controls (arrowheads). Scale bar, 300 μ m.
- G Quantification of the data shown in F and data not shown. $**P < 0.01$, two-tailed Student's *t*-test. The data are presented as the means $\pm$ s.e.m.

we looked into the regulatory sequences of the *Dcc* gene and searched for conserved motifs associated with Ca^{2+} -sensitive transcription factors. Through sequence alignment, we revealed an evolutionarily conserved 1491-bp region upstream of the first exon of *Dcc* (Supplementary Fig S3), which we cloned upstream of the firefly luciferase gene. Silencing spontaneous activity with *Kir* transfection into dissociated thalamic cells at E13.5 (Fig 5A) produced a 43% decrease in *Dcc* reporter activity in these cells (*Gfp*, 100 ± 14.3 ; *Kir*, 56.3 ± 9.4 ; $n = 7$ experiments; Fig 5A). Thus, Ca^{2+} activity-responsive elements may exist in the *Dcc* promoter. We only found one nuclear factor-kappa B (NF- κ B) transcription factor-binding site and one activator protein 1 (AP-1) site, which sequence and position were conserved in the genomes

analyzed (Fig 5B and Supplementary Fig S3). Both of these transcription factors have been previously implicated in activity-dependent transcriptional regulation [29, 30]. The mutation of the NF- κ B site in the *Dcc* reporter induced a significant reduction in the basal transcription of *Dcc* (wild-type, 100 ± 14.3 ; NF- κ B, 65.5 ± 6.2 , $n = 7$ experiments; Fig 5C). This reduction was not observed when the AP-1 site was mutated (AP-1, 77.2 ± 6.3 , $n = 7$ experiments; Fig 5C). We next determined whether any of these sites might be involved in the activity-dependent regulation of *Dcc*. Interestingly, mutation of the NF- κ B site did not affect the decrease of *Dcc* transcription by *Kir* (wild-type + *Kir*, $56.3 \pm 9.4\%$; mutant NF- κ B + *Kir*, $66.3 \pm 5.1\%$, $n = 7$ experiments; Fig 5D). By contrast, mutation of the AP-1 site significantly reduced the effect

Figure 4.

of *Kir* overexpression on *Dcc* transcription (mutant AP-1 site + *Kir*, $87.2 \pm 10.6\%$, $n = 7$ experiments; Fig 5D). Thus, AP-1 appears to fulfill an important role in the activity-dependent transcription of *Dcc*.

Discussion

The mechanisms that control axon growth extension are steadily being elucidated. We recently identified spontaneous activity as an

Figure 4. DCC controls axon extension *in vivo* downstream of spontaneous activity.

- A GFP (green), Tuj1 (red), and DAPI (blue) staining in control (*Gfp*), *Kir*-transfected, and *Kir+FL-Dcc*-co-transfected thalamic cells. Increasing the levels of DCC in silenced thalamic neurons rescued the axon growth defect produced by *Kir* overexpression. The arrowheads indicate the length of the axons. Scale bar, 50 μ m.
- B Quantification of the data shown in A and data not shown. $**P < 0.01$ and $***P < 0.001$; Kruskal–Wallis test with Dunn's *post hoc* analysis. The data are presented as the means $\pm$ s.e.m.
- C Scheme representing the effect on axon growth after *Kir* transfection alone or co-transfection with *Kir* and *Kir+FL-Dcc*. Red lines represent the status of the spontaneous activity in the cell: no activity, straight line; or spontaneous activity, curved line.
- D Schematic representation of the experimental procedure used to test the effect of increasing the levels of Dcc in *Kir*-silenced TCA *in vivo*. A *Kir* plasmid was co-electroporated into the thalamus *in utero* at E12.5 with *Gfp* or with *Gfp* plus *FL-Dcc*, and the brains were analyzed at E15.5.
- E GFP immunoreactive axons in coronal sections at intermediate cortical levels, with DAPI nuclear counterstaining in blue. Overexpression of *Dcc* in *Kir*-silenced axons rescued the cortical extension of TCA silenced axons (arrowheads). Scale bar, 300 μ m.
- F Quantification of the data shown in E and data not shown. $*P < 0.05$, $***P < 0.001$, one-way ANOVA with Tukey's *post hoc* analysis. The data are presented as the means $\pm$ s.e.m.
- G Expression of DCC (red) and Robo1 (gray) protein levels in E13.5 thalamic growth cones electroporated with *Gfp* or *Kir2.1* plasmids. Scale bar, 5 μ m.
- H Quantification of the data shown in G: $**P < 0.01$, $***P < 0.001$; two-tailed Student's *t*-test. The data are presented as the means $\pm$ s.e.m.

intrinsic modulator of the rate of axon extension in the thalamocortical system [4]. An embryonic developmental switch in TCA growth rate from fast to slow occurs concomitantly with a decrease in the frequency of spontaneous Ca^{2+} activity in thalamic neurons. This reduction in Ca^{2+} activity is sufficient to slow down the cortical progression of TCA by regulating the transcription of the axon guidance receptor Robo1, which acts as a brake for thalamocortical axon extension [4]. Here, we found another key element that controls axon growth regulated by spontaneous activity. DCC, a receptor for Netrin-1, acts downstream of spontaneous activity to modulate the axon's growth rate but in an opposite manner to that

of Robo1, accelerating TCA growth. Silenced neurons augment their expression of Robo1 and decrease their DCC levels, leading to a reduction in axon extension. Moreover, the transcriptional regulation of DCC occurs through an AP-1-binding site in its promoter. In summary, we describe a mechanism to modulate axon growth whereby spontaneous calcium activity in developing neurons controls Robo1 (brake) and DCC (accelerator) transcription, exerting opposite functional effects on this process (Fig 6).

TCAs reduce their rate of axon extension when approaching cortical targets in order to more thoroughly explore potential target areas [4]. In this study, we found that altering DCC expression in thalamic

Figure 5. DCC expression is regulated at the transcriptional level through an AP-1-binding site.

- A Silencing spontaneous activity induced a 30.38% decrease in expression of the luciferase reporter. $*P < 0.05$, two-tailed Student's *t*-test. The data are presented as the means $\pm$ s.e.m.
- B Schematic representation of the wild-type and mutated forms of the 1491-bp cloned region of the *Dcc* promoter.
- C Significant reduction in the basal transcription of *Dcc* when NF- κ B but not the AP-1 site was mutated ($n \geq 3$ replicates). $*P < 0.05$, Mann–Whitney *U*-test. The data are presented as the means $\pm$ s.e.m.
- D Luciferase assays to evaluate the contribution of AP-1 and NF- κ B sites to the reduction in *Dcc* by *Kir*. Mutating only the AP-1 site significantly reduced the dampening of *Dcc* transcription after the silencing of spontaneous activity ($n \geq 3$ replicates). $*P < 0.05$, Mann–Whitney *U*-test. The data are presented as the means $\pm$ s.e.m.

Figure 6. Scheme representing the mechanism to regulate axon growth in developing TCA.

Spontaneous Ca^{2+} activity is regulated during development in thalamic neurons. The frequency of this activity drops from high to low when TCA slows down their progression through the cortex [4]. This switch encodes activity-dependent changes in expression of the downstream target genes, DCC and Robo1, which exert antagonistic activities on axon growth. The activity-dependent regulation of these two genes occurs through specific transcription factor-binding sites in their respective promoters.

neurons changes the rate of axon growth, revealing a novel role for DCC receptors as an accelerator of TCA extension. Our results show a significant decrease in thalamic axon growth in the *Netrin-1*^{-/-} and *Dcc*^{-/-} mice, both *in vitro* and *in vivo*. The reduced growth of wild-type axons in *Netrin-1*^{-/-} slices *ex vivo* further supports a Netrin-1-dependent function of DCC in controlling TCA growth. Indeed, previous studies have shown similar defects regarding axon outgrowth, and Netrin-1 has been shown to promote the outgrowth of commissural axons and attract their growth cones toward the ventral midline in the spinal cord, hindbrain, and telencephalon [11, 18, 31]. Moreover, Netrin-1 is expressed in the ventral telencephalon (vTel), and it also attracts and promotes axonal outgrowth of early cortical axons toward subcortical structures [2], as well as stimulating thalamic axon outgrowth [19]. In line with our results, the pathway of thalamic axons through the vTel is disorganized in the *Netrin-1*-deficient mouse and fewer axons reach the cortex [19]. However, as these axon guidance defects have been analyzed in a full *Netrin-1*-deficient mouse, it is possible that these errors might be indirect and due to the abnormal development of ventral telencephalic structures that are important for TCA development. Nevertheless, subsequent analyses of the *Netrin-1* mutant brains revealed topographical defects that included the invasion of more caudal territories by thalamic axons originating from the rostral thalamus [26]. In fact, Netrin-1 seems to exert a bifunctional effect on TCAs, attracting rostral thalamic axons and acting as a chemorepulsive cue for caudal thalamic axons [26]. Accordingly, DCC is expressed in a gradient in the thalamus [25, 26]. However, when Netrin-1 was tested in isolation, no attractive response was triggered, and only when Slit1 and Netrin-1 were presented together did

an attractive activity emerge that neither of the cues possesses alone [32].

Several studies have demonstrated that spontaneous activity plays a crucial role in early stages of neuronal development modulating processes such as axon pathfinding and targeting [4, 33–35]. In embryonic thalamic neurons, the frequency of Ca^{2+} transients diminishes as development proceeds (Fig 6; [4]). Moreover, we demonstrated that this change in spontaneous activity has a specific function in the axonal growth program [4]. The rapid TCA growth, which occurs at the ventral telencephalon, is correlated with a high frequency of Ca^{2+} transients, while the dampening of TCA cortical extension requires a reduction of spontaneous Ca^{2+} transients at late embryonic stages. When spontaneous thalamic activity was blocked, the speed of TCA extension was decreased both *in vitro* and *in vivo* [4].

Genetic reduction of spontaneous activity by *Kir2.1* overexpression diminishes DCC expression at the level of the mRNA and protein. This decrease is opposite to the one found when spontaneous thalamic activity is blocked [4], which suggests that the endogenous downregulation of DCC expression in thalamic neurons is regulated by activity-dependent mechanisms. This idea was further confirmed when the delayed TCA cortical extension was rescued by *in vivo* overexpression of a full-length DCC receptor with *Kir*. Thus, as demonstrated for Robo1 [4], electrical activity influences the axon growth speed by modulating the expression of at least two receptors, Robo1 and DCC, that function in an antagonistic manner in regulating this process.

It is well accepted nowadays that during brain development, spontaneous Ca^{2+} activity modulates early neural processes by the regulation of the expression of specific genes [36, 37]. We previously found that the activity-dependent regulation of *Robo1* relies on the binding of the NF- κ B transcription factor to a specific site of its promoter. When this site was mutated, the regulation of *Robo1* transcription by activity was dampened. Here, spontaneous activity appears to activate *Dcc* transcription through an AP-1-binding site and mutating this AP-1-binding site severely dampened the activity-dependent regulation of *Dcc*, suggesting that this site might be crucial for the involvement of DCC in growth (Fig 6).

The AP-1 transcription factor is comprised of a variety of dimers containing members of the *Fos* and *Jun* protein families [38]. In fact, *c-Jun* is a major component of the AP-1 transcription factor complex [39], and AP-1 activity is in part mediated, by the phosphorylation of *c-Jun* by the Jun-N-terminal kinases (JNKs). In function of the residues phosphorylated, *c-Jun* can activate or repress target gene transcription, playing a role in processes such as neurotransmitter specification or axonal regeneration [37, 40]. For example, regulation of the *tlx3* transcription factor, the activity of which is crucial for neurotransmitter specification in embryonic spinal cord neurons, occurs in an activity-dependent manner through a cAMP response element (CRE) motif in its promoter [37]. This CRE variant binds phosphorylated *c-Jun*, which in turn represses *tlx3* transcription [37]. Thus, Ca^{2+} levels can modulate both NF- κ B and *c-Jun* (AP-1) activity [37, 41], and an increase in this activity triggered by the switch in Ca^{2+} frequency occurring in late thalamic neurons [4] could have opposing effects on the transcription of target genes, such as the increase in *Robo1* and the decrease of *Dcc* transcription, reducing TCA growth. However, the mechanism by which AP-1 transcriptional activity is regulated in thalamic neurons during early and late phases of TCA growth remains to be elucidated.

In summary, the data presented here extend our knowledge on the factors and mechanisms implicated in axon growth and brain wiring that could be also considered in the context of axonal regeneration or repair.

Materials and Methods

Mouse strains

Wild-type mice maintained on a CD1 background were used to analyze gene expression and for the tissue culture experiments. *Netrin-1* heterozygous mice [42] were also maintained on a CD1 genetic background and crossed to produce homozygous embryos. *Dcc* heterozygous mice [17] were maintained on a 129SV/SvPasCrl genetic background and crossed to produce homozygous embryos. *Netrin-1* mutant mice are severe hypomorphs, and they were generated in a gene trap screen where the lacZ gene was inserted into the *Netrin-1* gene [18, 42]. *Dcc* knockout mice were generated by the targeted insertion of the neomycin resistance cassette into exon 3 by homologous recombination, resulting in a complete functional deletion [17]. In all cases, the day of vaginal plug was considered as embryonic day (E) 0.5. All the animals were maintained in accordance with Spanish and EU (Directive 2010/63/EU) regulations.

Primary thalamic cell culture, transfection, and pharmacological studies

To establish primary thalamic neuron cultures, pregnant mice were sacrificed and their embryos were recovered. The thalamus was dissected out of the brain, collected in Krebs solution, trypsinized, and then dissociated with a fire-polished Pasteur pipette, with 200,000–300,000 cells/well finally plated in a 24-well seeded in plating medium (sodium pyruvate 1 mM, Glutamax 1×, 45% glucose solution 50 mM, penicillin/streptomycin 100 U/ml, 10% FBS, and 86% MEM). Primary thalamic cells were cultured in maintenance medium (Glutamax 1×, 45% glucose solution 50 mM, penicillin/streptomycin 100 U/ml, 2% B27 and 95% neurobasal) at 37 °C with 5% CO₂, carefully replacing the medium every 2 days. The cells were transfected using Amaxa™ Basic Nucleofector™ Kit (VPI-1003). For pharmacological studies, KCl (2.5 mM) was added to the culture medium at the time of plating. For the detection of cell death by release of lactate dehydrogenase activity (LDH), thalamic cells (20,000 cells) were dissociated using the LDH Cytotoxicity Assay Kit (P40104; Innoprot), a colorimetric method to measure LDH activity using a reaction cocktails containing lactate, NAD⁺, diaphorase, and INT. LDH catalyzes the reduction of NAD⁺ to NADH in the presence of L-lactate, while the formation of NADH can be measured in a coupled reaction in which the tetrazolium salt INT is reduced to a red formazan product. The amount of the highly colored and soluble formazan can be measured at 490 nm spectrophotometrically.

Calcium imaging

Cell cultures transfected with the *Kir2.1* and/or *Gfp* plasmids were incubated for 45 min in 400 µl of maintenance medium (37°C) with Rhod-2 AM cell permanent calcium dye (1 mM; Molecular probes) prepared in DMSO + 20% w/v pluronic acid (2 µl of per

coverslip). Coverslips with loaded cultures were washed with maintenance medium, and they were placed in a recording chamber of an upright Leica DM LFSA stage, perfused with warmed (32°C) artificial cerebrospinal fluid (aCSF: in mM, 119 NaCl, 5 KCl, 1.3 MgSO₄ 7H₂O, 2.4 CaCl₂, 1 NaH₂PO₄, 26 Na₂HCO₃, 11 glucose) bubbled with 95% O₂/5% CO₂. Time-lapse recording of Ca²⁺ dynamics was obtained through a water immersion objective (L x10/0.30 Leica) after exciting the cultures at 552 nm with a mercury arc lamp. We acquired images with a digital CCD camera (Hamamatsu ORCA-R2 C10600-10B) at a time resolution of 250 ms. Calcium traces were analyzed with a custom routine developed in Matlab [43], and the proportion of the active cells among the *Gfp*- and *Gfp* + *Kir2.1*-transfected cells was calculated. Calcium transients were identified using asymmetric least squares baseline and Schmitt trigger analysis. We used 5% of the baseline noise as the high threshold and 2% as low threshold. Cells having at least one transient above this threshold were considered as active.

Western blots

Neuron cultures were lysed in NP-40 lysis buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 2 mM EDTA and 10% glycerol and 1% Nonidet P40 (NP-40)) supplemented with protease and phosphatase inhibitor cocktails (Roche). The protein lysates were clarified by centrifugation at 10,000× g for 10 min before the protein extracts were resolved by 8%–12% SDS-PAGE and transferred to a nitrocellulose membrane (Protran; Whatman). The membranes were then blocked for 30 min at room temperature with 5% nonfat powdered milk in Tris-buffered saline-Tween-20 (TBS-T: 50 mM Tris-HCl, 150 mM NaCl [pH 7.4], 0.05% Tween-20), and they were probed overnight at 4°C with the following primary antibodies: goat anti-DCC (diluted 1/500; R&D Systems) or rabbit anti-actin (diluted 1/3,000; Sigma). The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma), and antibody binding was visualized by chemiluminescence (Immobilon Western; Millipore) in a Luminescent Image Analyzer LAS-1000 Plus (Fujifilm).

In utero electroporation

In utero electroporation technique was performed as previously described [4]. Briefly, pregnant females from stage E12.5 were anesthetized with isoflurane and the embryos exposed through laparotomies. The *Kir-IRES-Gfp* (originally from Venkatesh Murthy) and *FL-Dcc* (a generous gift from Prof. Marc Tessier-Lavigne) DNA constructs were used at a concentration of 1.5 µg/µl and co-electroporated with a plasmid encoding *Gfp* at 0.9 µg/µl. Embryos were allowed to develop until E15.5 when TCA extension was analyzed.

Quantitative and semi-quantitative PCR

Total RNA from electroporated thalamic explants cultured for 60 h was extracted using the RNeasy Kit (Qiagen). This RNA was treated with RNase-free DNaseI for 30 min at 37°C prior to reverse-transcribing it into cDNA for 1 h at 42°C using SuperScript II Reverse Transcriptase and Oligo(dT)_{12–18} primers (Invitrogen). Quantitative RT-PCR (qPCR) was carried out on an Applied Biosystems 7300

real-time PCR unit using the Power SYBR Green PCR Master Mix (Applied Biosystems), 5 µl of cDNA, and the appropriate primers. Each independent sample was assayed in triplicate, and gene expression was normalized to that of GAPDH. The following primers were used for semi-quantitative PCR: Kir2.1³⁴⁶ (forward TTTGGGAACGGAAGAGTAAAGTC and reverse TTTTGGCTGTG TGTTTTGG); Kir2.1¹³⁴ (forward TTTTGGCTGTGTGTTTGG and reverse GGTGTCTGGGTCTCAATGG); DCC (forward GAATGAG GCATGTGCTCAGA and reverse AGAAGGCTCCAAAGGAGAGG); and GAPDH (forward AAAATGGTGAAGGTCGGTGT and reverse CTCACCCCATTTGATGTTAG). For qPCR, the following set of primers were used: GAPDH (forward CGGTGCTGAGTATGTCGTGG AGT and reverse CCAAGAAGAACTCGGAAAGC); DCC (CTGTCTG TGGACCGAGGTTT and GGTGGTCTCTCACTACAGA); and Kir2.1 (forward GTGTCCGAGGTCAACAGCTT and reverse GGTGTCTGG GTCTCAATGG).

Axon tracing, *in situ* hybridization, and immunohistochemistry

For axon tracing, embryonic brains or cultured slices were fixed in 4% PFA overnight or for 30 min, respectively. Small DiI crystals (1,1'-diiododecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes) were inserted into the thalamus and allowed to diffuse for 1–3 weeks at 37°C before obtaining vibratome sections (100 µm) of the brain. For immunohistochemistry, cultured slices/explants and mouse embryos were fixed in 4% PFA for 30 min and 2–3 h, respectively, and they were stained with the following antibodies: mouse anti-βIII Tubulin (1/1,000; Covance); rabbit anti-GFP (1/1,000; Invitrogen); goat anti-Robo1 (1/100; R&D systems); chicken anti-Netrin-1 (1/100; Abcam); and goat anti-DCC (1/100; R&D Systems). The following secondary antibodies (Molecular Probes) were used to visualize the binding of the primary antibodies: goat anti-mouse Alexa-546 (1/500); goat anti-chicken Alexa-488 (1/500); donkey anti-rabbit Alexa-488 (1/500); goat anti-rabbit Alexa 594 (1/500); donkey anti-goat Alexa-546 (1/500); and goat anti-rabbit Alexa-594 (1/400). DAPI (VWR) or Fast Red (Sigma) was used to counterstain the nuclei. For *in situ* hybridization, a probe for Netrin-1 (kindly provided by Prof. Tessier-Lavigne, Rockefeller University) was used. Briefly, coverslips were washed with PBT, post-fixed with PFA 4% 15 min, pre-hybridized 1 h at 65°C, and hybridized over night at 65°C with Netrin-1 probe at 6 µl/ml of hybridization solution, putting a total volume of 600 µl per well. Next day, coverslips were washed with different solution before incubating them with blocking solution 1 h at room temperature. After that, they were incubated in anti-DIG-Ab (antibody anti-digoxigenin) at a final concentration of 1:2,000 over night at 4°C. Then, they were washed first with TBS-T and later with NTMT before incubating in reaction mix (NTMT + 4.5 µl/ml NBT + 3.5 µl/ml BCIP) until the staining was observed. Finally, coverslips were washed with PBS, post-fixed with PFA 4% 20 min at RT, and mounted with Mowiol.

Coculture experiments

Organotypic slices of embryonic mice were prepared and cultured for 48 h as described previously [1]. Wild-type thalamic explants were transplanted onto wild-type or *Netrin*^{-/-} host coronal slices. Normalized fluorescence was measured using ImageJ.

Quantification of axon growth

ImageJ software was used for image processing and to analyze axon length *in vitro* and *in vivo*. To quantify axon growth in dissociated thalamic cells, we measured the length of the axons of 100–300 neurons per well using the NeuronJ plug-in. To quantify the ventral telencephalic extension of TCAs in the coculture slices and the cortical extension of TCAs labeled *in utero*, the 5-longest axons per slice were measured. For the *in utero* electroporation, only brains with a similar degree of electroporation in the thalamus (Th) were analyzed. Slices (60 µm) were obtained and images of the cortex were sampled at two different rostro-caudal levels (rostral and intermediate: 3 sections for each level and brain). A horizontal line was drawn from the edge of the pallial–subpallial boundary at the ventricle of each slice. The cortical area delimited by this line was then linearized using the Straighten plug-in of the ImageJ software. Within this area, the distance covered by labeled axons was measured and normalized to the total length of the region defined.

Luciferase assays

Dissociated thalamic cells plated in 24-well plates were co-transfected with a luciferase reporter plasmid as previously described [4]. A Dual Luciferase Reporter Assay Kit (Promega) was used to measure luciferase activity after 60 h of culturing. Each sample was assayed in triplicate, and luminescence was measured on a Sirius luminometer (Berthold DS). We determined the exact reporter activity by calculating the ratio of the pGL3-based luciferase reporter activity to that of the pRL-TK luciferase.

Bioinformatic analysis on the *Dcc* promoter

We performed a bioinformatic search within the mouse *Dcc* gene using the Ensembl Genome Browser (<http://www.ensembl.org/index.html>), and as described before [4]. Briefly, a 1491-bp fragment of the mouse *Dcc* gene was amplified from mouse brain genomic DNA using the following primers: Promoter *Dcc* F (5'-CATCATACG GATCGGCACAGA) and Promoter *Dcc* R (5'-CCTAGGCTTCTCTC TCCTCTTC), and further cloned into the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and, subsequently, into the pGL3-Basic Vector (Promega). We used the bioinformatic tools, TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and MATCH (<http://www.bioinfo.de/isb/gcb01/poster/goessling.html>) to search for putative transcription factor-binding sites within the identified *Dcc* promoter sequence. The ECR Browser (<http://ecrbrowser.dcode.org/> [44]) was used to identify conserved regions in the *Dcc* promoter applying the default settings (100 bp, 70% identity). We used site-directed mutagenesis GTGACTTCCC to AACAGAATTC (NF-κB site) and TTGAGTCAT to TTGGTGCAC (AP-1 site).

Statistical analyses

All data are shown as mean ± standard error of the mean (s.e.m.). Kolmogorov–Smirnov normality test was performed to evaluate Gaussian distribution of the samples and proper sample size. Depending on the results, a parametric or nonparametric test was applied for statistical analysis using the Prism software package (version 5). Difference was considered statistically significant when

$P < 0.05$. Assessment of data was performed blinded by different investigators in independent experiments. The selection of the wild-type embryos for this study was unbiased in terms of sex, size, and weight. Experiments were considered as independent when done from distinct litters.

Supplementary information for this article is available online: <http://embor.embopress.org>

Acknowledgements

We are grateful to Prof. Marc Tessier-Lavigne (Rockefeller University, New York) for the Netrin-1 and Dcc transgenic mouse lines, and Andras Nagy (Samuel Lunenfeld Research Institute, Toronto) for the EGFP mice. We are also thankful to members of G. López-Bendito's laboratory for stimulating discussions and comments. M.C-P was supported by a JAE-Doc position from the CSIC. This work was supported by grants from the Spanish MINECO BFU2012-34298 to G.L-B and an ERC Grant ERC-2009-StG_20081210. G. L-B is an EMBO YIP Investigator.

Author contributions

GL-B conceived the idea. MC-P and GL-B designed the study. MC-P performed the *in vitro* and *ex vivo* experiments and the semi-qPCR, qPCR, luciferase, and LDH assay. VM-J and GL-B performed the *in utero* electroporation experiments. AF performed the calcium recordings in dissociated thalamic cells. RS and LM processed the *in utero* electroporation material and performed qPCR, *in situ* hybridization, and Western blot experiments. MC-P and GL-B conducted the data analysis and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- López-Bendito G, Cautinat A, Sánchez JA, Bielle F, Flames N, Garratt AN, Talmage DA, Role LW, Charnay P, Marín O *et al* (2006) Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. *Cell* 125: 127–142
- Metin C, Deleglise D, Serafini T, Kennedy TE, Tessier-Lavigne M (1997) A role for netrin-1 in the guidance of cortical efferents. *Development* 124: 5063–5074
- Garel S, Rubenstein JLR (2004) Intermediate targets in formation of topographic projections: inputs from the thalamocortical system. *Trends Neurosci* 27: 533–539
- Mire E, Mezzera C, Leyva-Díaz E, Paternain AV, Squarzonni P, Bluy L, Castillo-Paterna M, López MJ, Peregrín S, Tessier-Lavigne M *et al* (2012) Spontaneous activity regulates Robo1 transcription to mediate a switch in thalamocortical axon growth. *Nat Neurosci* 15: 1134–1143
- Ming G, Henley J, Tessier-Lavigne M, Song H, Poo M (2001) Electrical activity modulates growth cone guidance by diffusible factors. *Neuron* 29: 441–452
- Spitzer NC (2012) Activity-dependent neurotransmitter respecification. *Nat Rev Neurosci* 13: 94–106
- Fields R, Neale E, Nelson P (1990) Effects of patterned electrical activity on neurite outgrowth from mouse sensory neurons. *J Neurosci* 10: 2950–2964
- Cohan C, Kater S (1986) Suppression of neurite elongation and growth cone motility by electrical activity. *Science* 232: 1638–1640
- Borodinsky L, Root C, Cronin J, Sann S, Gu X, Spitzer N (2004) Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. *Nature* 429: 523–530
- Keino-Masu K, Masu M, Hinck L, Leonardo ED, Chan SS, Culotti JG, Tessier-Lavigne M (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* 87: 175–185
- Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 78: 425–435
- Richards LJ, Koester SE, Tuttle R, O'Leary DD (1997) Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. *J Neurosci* 17: 2445–2458
- Bouchard JF, Horn KE, Stroh T, Kennedy TE (2008) Depolarization recruits DCC to the plasma membrane of embryonic cortical neurons and enhances axon extension in response to netrin-1. *J Neurochem* 107: 398–417
- Liu G, Beggs H, Jürgensen C, Park HT, Tang H, Gorski J, Jones KR, Reichardt LF, Wu J, Rao Y (2004) Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. *Nat Neurosci* 7: 1222–1232
- DeGeer J, Boudeau J, Schmidt S, Bedford F, Lamarche-Vane N, Debant A (2013) Tyrosine phosphorylation of the Rho guanine nucleotide exchange factor Trio regulates netrin-1/DCC-mediated cortical axon outgrowth. *Mol Cell Biol* 33: 739–751
- Bouchard JF, Moore SW, Tritsch NX, Roux PP, Shekarabi M, Barker PA, Kennedy TE (2004) Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: a novel mechanism regulating commissural axon extension. *J Neurosci* 24: 3040–3050
- Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H *et al* (1997) Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* 386: 796–804
- Serafini T, Colamarino S, Leonardo E, Wang H, Beddington R, Skarnes W, Tessier-Lavigne M (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87: 1001–1014
- Braisted J, Catalano S, Stimac R, Kennedy T, Tessier-Lavigne M, Shatz C, O'Leary D (2000) Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. *J Neurosci* 20: 5792–5801
- Burrone J, O'Byrne M, Murthy VN (2002) Multiple forms of synaptic plasticity triggered by selective suppression of activity in individual neurons. *Nature* 420: 414–418
- Demarque M, Spitzer NC (2010) Activity-dependent expression of Lmx1b regulates specification of serotonergic neurons modulating swimming behavior. *Neuron* 67: 321–334
- Hartman KN, Pal SK, Burrone J, Murthy VN (2006) Activity-dependent regulation of inhibitory synaptic transmission in hippocampal neurons. *Nat Neurosci* 9: 642–649
- Wang C-L, Zhang L, Zhou Y, Zhou J, Yang X-J, Duan S-M, Xiong Z-Q, Ding Y-Q (2007) Activity-dependent development of callosal projections in the somatosensory cortex. *J Neurosci* 27: 11334–11342
- Mizuno H, Hirano T, Tagawa Y (2007) Evidence for activity-dependent cortical wiring: formation of interhemispheric connections in neonatal mouse visual cortex requires projection neuron activity. *J Neurosci* 27: 6760–6770
- Bonnin A, Torii M, Wang L, Rakic P, Levitt P (2007) Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nat Neurosci* 10: 588–597

26. Powell AW, Sassa T, Wu Y, Tessier-Lavigne M, Polleux F (2008) Topography of thalamic projections requires attractive and repulsive functions of Netrin-1 in the ventral telencephalon. *PLoS Biol* 6: e116
27. Furne C, Rama N, Corset V, Chedotal A, Mehlen P (2008) Netrin-1 is a survival factor during commissural neuron navigation. *Proc Natl Acad Sci USA* 105: 14465–14470
28. Llambi F, Causeret F, Bloch-Gallego E, Mehlen P (2001) Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. *EMBO J* 20: 2715–2722
29. Sugiyama C, Nakamichi N, Ogura M, Honda E, Maeda S, Taniura H, Yoneda Y (2007) Activator protein-1 responsive to the group II metabotropic glutamate receptor subtype in association with intracellular calcium in cultured rat cortical neurons. *Neurochem Int* 51: 467–475
30. Xiang G, Pan L, Xing W, Zhang L, Huang L, Yu J, Zhang R, Wu J, Cheng J, Zhou Y (2007) Identification of activity-dependent gene expression profiles reveals specific subsets of genes induced by different routes of Ca^{2+} entry in cultured rat cortical neurons. *J Cell Physiol* 212: 126–136
31. Shirasaki R, Tamada A, Katsumata R, Murakami F (1995) Guidance of cerebellofugal axons in the rat embryo: directed growth toward the floor plate and subsequent elongation along the longitudinal axis. *Neuron* 14: 961–972
32. Bielle F, Marcos-Mondéjar P, Leyva-Díaz E, Lokmane L, Mire E, Mailhes C, Keita M, García N, Tessier-Lavigne M, Garel S et al (2011) Emergent growth cone responses to combinations of slit1 and netrin 1 in thalamocortical axon topography. *Curr Biol* 21: 1748–1755
33. Butler A, Dantzer J, Shah R, Callaway E (2001) Development of visual cortical axons: layer-specific effects of extrinsic influences and activity blockade. *J Comp Neurol* 430: 321–331
34. Hanson MG, Landmesser LT (2004) Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron* 43: 687–701
35. Uesaka N, Hayano Y, Yamada A, Yamamoto N (2007) Interplay between laminar specificity and activity-dependent mechanisms of thalamocortical axon branching. *J Neurosci* 27: 5215–5223
36. Spitzer N (2006) Electrical activity in early neuronal development. *Nature* 444: 707–712
37. Marek KW, Kurtz LM, Spitzer NC (2010) cJun integrates calcium activity and *tlx3* expression to regulate neurotransmitter specification. *Nat Neurosci* 13: 944–950
38. Jochum W, Passegue E, Wagner EF (2001) AP-1 in mouse development and tumorigenesis. *Oncogene* 20: 2401–2412
39. Mechta-Grigoriou F, Gerald D, Yaniv M (2001) The mammalian Jun proteins: redundancy and specificity. *Oncogene* 20: 2378–2389
40. Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, Hristova M, Nateri AS, Makwana M, Riera-Sans L, Wolfer DP et al (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron* 43: 57–67
41. Riquelme D, Alvarez A, Leal N, Adasme T, Espinoza I, Valdés JA, Troncoso N, Hartel S, Hidalgo J, Hidalgo C et al (2011) High-frequency field stimulation of primary neurons enhances ryanodine receptor-mediated Ca^{2+} release and generates hydrogen peroxide, which jointly stimulate NF- κ B activity. *Antioxid Redox Signal* 14: 1245–1259
42. Skarnes WC, Moss JE, Hurtley SM, Beddington RS (1995) Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc Natl Acad Sci USA* 92: 6592–6596
43. Carron R, Filipchuk A, Nardou R, Singh A, Michel FJ, Humphries MD, Hammond C (2014) Early hypersynchrony in juvenile PINK1(-)/(-) motor cortex is rescued by antidromic stimulation. *Front Syst Neurosci* 8: 95
44. Ovcharenko I, Nobrega MA, Loots GG, Stubbs L (2004) ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. *Nucleic Acids Res* 32: W280–W286