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14-3-3 Proteins Regulate a Cell-Intrinsic Switch from Sonic Hedgehog-Mediated Commissural Axon Attraction to Repulsion after Midline Crossing

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SUMMARY

Axons must switch responsiveness to guidance cues during development for correct pathfinding. Sonic Hedgehog (Shh) attracts spinal cord commissural axons ventrally toward the floorplate. We show that after crossing the floorplate, commissural axons switch their response to Shh from attraction to repulsion, so that they are repelled anteriorly by a posterior-high/anterior-low Shh gradient along the longitudinal axis. This switch is recapitulated in vitro with dissociated commissural neurons as they age, indicating that the switch is intrinsic and time dependent. 14-3-3 protein inhibition converted Shh-mediated repulsion of aged dissociated neurons to attraction and prevented the correct anterior turn of postcrossing commissural axons in vivo, an effect mediated through PKA. Conversely, overexpression of 14-3-3 proteins was sufficient to drive the switch from Shh-mediated attraction to repulsion both in vitro and in vivo. Therefore, we identify a 14-3-3 protein-dependent mechanism for a cell-intrinsic temporal switch in the polarity of axon turning responses.

INTRODUCTION

Commissural axons are subject to numerous guidance cues as they navigate through the developing spinal cord. They are initially repelled from the roofplate by BMPs and attracted along the dorsoventral (DV) axis to the floorplate by Netrin-1 (Kennedy et al., 1994), Sonic Hedgehog (Shh) (Charron et al., 2003), and VEGF (Ruiz de Almodovar et al., 2011). After reaching and crossing the midline, commissural axons become sensitive to repellents secreted by the floorplate, such as Slits and Semaphorins (Long et al., 2004; Zou et al., 2000), which ensure that they exit and do not recross the floorplate. Following floorplate exit, axons turn anteriorly and migrate along the anteroposterior (AP) axis.

In mammals, this anterior turn has been attributed to Wnt4, which is expressed in an AP gradient at the mRNA level and attracts postcrossing axons anteriorly (Lyuksyutova et al., 2003). However, in chick, this anterior turn has been suggested to result from Shh repulsion, with a posterior-high/anterior-low gradient of Shh repelling postcrossing axons anteriorly along the AP axis (Bourikas et al., 2005). In contrast, Shh in mammals has been postulated to induce the responsiveness of commissural axons to Semaphorin repulsion during midline crossing (Parra and Zou, 2010). Consistent with this, disruptions of Shh signaling in rat open-book cultures affect midline crossing, with stalling, knotting, and looping of commissural axons at the floorplate. Intriguingly, disruption of Shh signaling in rat open-book cultures also generates postcrossing AP guidance phenotypes with a significant number of postcrossing axons turning posteriorly instead of anteriorly (Parra and Zou, 2010). This observation suggests that Shh may have an additional role in AP guidance of mammalian postcrossing commissural axons, independent of midline crossing. Furthermore, if the posterior-high/anterior-low gradient of Shh observed in chick is conserved in mammals, this would imply that commissural axons switch their response to Shh from attraction along the DV axis (precrossing) to repulsion along the AP axis (postcrossing), via an unknown mechanism. Although many factors have been shown to modify axon turning responses to guidance cues in vitro (e.g., Song et al., 1997, 1998), very few switches in the polarity of the turning response have been identified in vivo during

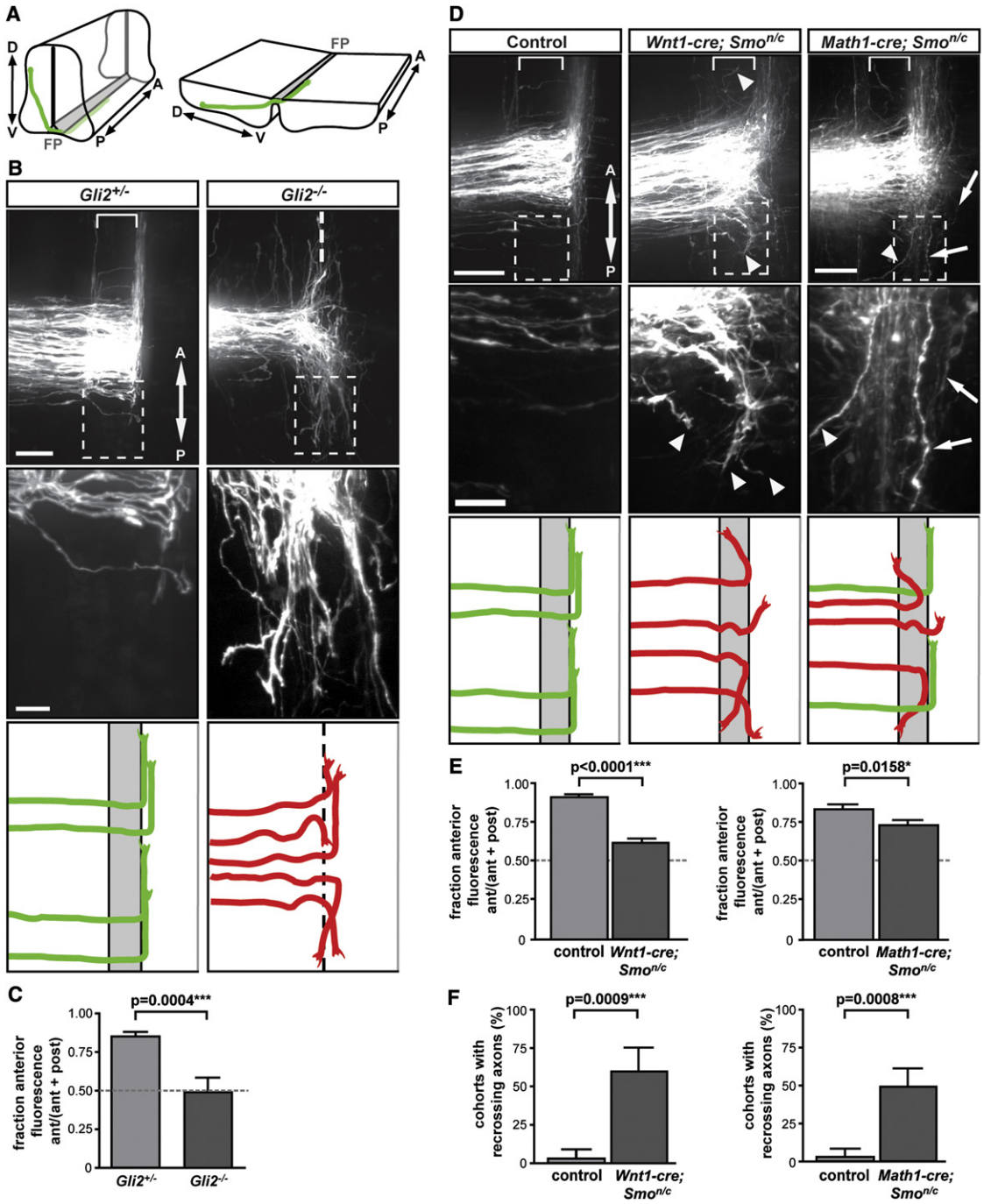

Figure 1. *Smo* Is Required for Proper Postcrossing Commissural Axon Guidance along the AP Axis

(A) Schematic of the stereotypical commissural axon trajectory in an intact spinal cord (left) and open-book preparation (right). Axons travel ventrally toward the floorplate (FP) (gray) where they cross the midline. After crossing, axons turn anteriorly and travel along the FP. D, dorsal; V, ventral; A, anterior; P, posterior.

(B) Top view shows DiI labeling of postcrossing commissural axons in open-book preparations of *Gli2*$^{+/-}$ and *Gli2*$^{-/-}$ E11.5 mice embryos. Middle view is a zoom of boxed region. Bottom view is a schematic of the axon guidance phenotype. Control *Gli2*$^{+/-}$ axons make a stereotypical anterior turn after exiting the FP (three embryos, 13 cohorts). *Gli2*$^{-/-}$ axons migrate ventrally to the midline but become severely disorganized at the midline and turn randomly along the AP axis (three embryos, 12 cohorts). Brackets indicate the floorplate and dashed lines the midline.

(C) Relative fluorescence (±SEM) of the anterior-directed axons versus total fluorescence of anterior- and posterior-directed axons (n = 11 cohorts per genotype).

(D) Top view shows DiI labeling of postcrossing commissural axons in open-book preparations of *Wnt1-Cre; Smo*$^{n/c}$ and *Math1-Cre; Smo*$^{n/c}$ E11.5 mice embryos. Middle view is a zoom of boxed region. Bottom view is a schematic of the axon guidance phenotype. Axons from control littermates turn anteriorly after exiting the FP and do not reenter the FP (seven embryos, 33 cohorts). Axons of *Wnt1-Cre; Smo*$^{n/c}$ cohorts have a similar number of axons turning posteriorly and anteriorly

development. The best-characterized example to date is the switch from attraction to repulsion to Netrin-1 in retinal ganglion cell axons, which depends on cAMP levels (Höpker et al., 1999; Shewan et al., 2002).

Here, we show that Shh has a direct effect on the guidance of postcrossing commissural axons. Shh protein is expressed in a posterior-high/anterior-low gradient along the longitudinal axis of rodent spinal cord, and Shh signaling is required for correct AP guidance of postcrossing commissural axons in vivo. This suggests that after midline crossing, commissural axons switch their response to Shh from attraction (precrossing) to repulsion (postcrossing). Remarkably, we demonstrate that commissural neurons in vitro switch from Shh attraction to repulsion after an extended time in culture, indicating that there is an intrinsic, time-dependent switch in Shh responsiveness that mimics the different in vivo behavior of pre- and postcrossing axons. We found that 14-3-3 proteins, previously identified as important for the repulsive effects of MAG and NGF in postnatal neurons (Kent et al., 2010), are enriched in postcrossing commissural axons and also increase in a time-dependent manner in vitro. Inhibition of 14-3-3 function switches the response to Shh from repulsion to attraction in vitro and prevents the correct AP turning of postcrossing commissural axons in vivo. Conversely, premature overexpression of 14-3-3 proteins in vitro and in vivo drives the switch in Shh response from attraction to repulsion. 14-3-3 proteins switch the turning response to Shh by reducing PKA activity. Hence, we identify a 14-3-3 protein-dependent mechanism for a cell-intrinsic time-dependent switch in the polarity of axon turning responses. This allows commissural axons, which are first attracted ventrally toward the floorplate by Shh, to switch their response to Shh so that they become repelled by Shh after crossing the floorplate and migrate anteriorly along the longitudinal axis.

RESULTS

Guidance of Postcrossing Commissural Axons In Vivo along the AP Axis Requires Smoothened

To evaluate the role of the floorplate and floorplate-derived cues in the migration of postcrossing commissural axons, we analyzed *Gli2*$^{-/-}$ mouse embryos, which lack a floorplate. In these mutants, commissural axons still project to the midline in response to Netrin-1 in the ventral ventricular zone (Matise et al., 1999). We used DiI anterograde labeling of commissural axons of E11.5 embryos, shortly after commissural axons have begun to cross the floorplate, to visualize the trajectory of postcrossing commissural axons. After diffusion of the DiI, the neural tube was prepared in the open-book format for analysis of the commissural axon trajectories (Figure 1A). In control *Gli2*$^{+/-}$ embryos, labeled axons exhibited the stereotypic commissural axon trajectory: most axons migrated ventrally toward the midline, crossed the floorplate, and turned anteriorly (Figure 1B). In *Gli2*$^{-/-}$ neural tubes, axons still migrated ventrally to the midline but became severely disorganized at the midline. Although axons still switched from a DV to an AP axis of migration at the midline, their AP directionality appeared random (Figure 1B), consistent with previous studies by Matise et al. (1999). Approximately 50% of the total fluorescence of the axons was distributed anteriorly, indicating complete randomization of the AP guidance of axons (Figure 1C). Thus, whereas the floorplate is not required for axons to switch from a DV to an AP axis of migration, it is required for the axons to correctly turn anteriorly after midline crossing. This suggested that a floorplate-derived cue is important for correct anterior turning of postcrossing commissural axons.

One candidate floorplate-derived molecule that could act as a guidance cue along the longitudinal axis is Shh, which attracts precrossing commissural axons ventrally to the floorplate in mammals (Charron et al., 2003) and has been implicated in chick as a repellent for postcrossing commissural axons (Bourikas et al., 2005). To test the involvement of Shh signaling in AP guidance, we analyzed conditional knockout mice for *Smoothened* (*Smo*), a key positive regulator of the Shh signaling pathway (Charron et al., 2003; Yam et al., 2009). The use of conditional knockouts allowed for the cell-autonomous inactivation of *Smo* in neurons without neural tube repatterning (Charron et al., 2003). To generate commissural neurons null for *Smo*, we used *Wnt1-Cre* to express Cre recombinase in the dorsal spinal cord (Charron et al., 2003) in *Smo*$^{n/c}$ embryos, which have one null allele (*Smo*^{n}) and one floxed allele (*Smo*^{c}). DiI labeling of commissural axon trajectories showed that all of the controls (littermates lacking the *Cre* and/or *Smo*^{n} allele) displayed normal commissural axon projection patterns with postcrossing commissural axons turning anteriorly (Figure 1D, left). In *Wnt1-Cre; Smo*$^{n/c}$ embryos, in which all dorsal commissural neurons are null for *Smo*, cohorts showed severe axon guidance defects (Figure 1D, middle). Most had no clear anterior bias, and the ratio of anterior versus posterior axons was almost 50% (Figure 1E), indicating almost complete randomization of AP turning, similar to the *Gli2* mutants. In addition, about half of the cohorts had axons that recrossed the floorplate or failed to completely cross and turned back to the ipsilateral side (Figure 1F). These axons were present on both the anterior and posterior sides of the main bundle of axons crossing the floorplate (Figure 1D, arrowheads).

The *Wnt1-Cre* driver inactivates *Smo* in all dorsal interneurons, which include, but are not limited to, commissural neurons. To delete *Smo* specifically in commissural neurons, we used the *Math1-Cre* driver, which inactivates *Smo* only in the Math1^{+} subpopulation of commissural neurons. Because Math1 is

(17 of 21 cohorts, four embryos). Several axons stall or reenter and recross the FP (arrowheads). Axons of *Math1-Cre; Smo*$^{n/c}$ cohorts (n = 18 cohorts) also turn posteriorly (arrows) and/or recross the FP (arrowheads). Brackets indicate the floorplate and dashed lines the midline.

(E) Relative fluorescence (±SEM) of the anterior-directed axons versus total fluorescence of anterior and posterior axons.

(F) Quantification of recrossing axons as percentage of cohorts (±SEM) per embryo.

Unpaired t test was used for all statistical comparisons.

Scale bars, 50 μm (B and D, top) and 20 μm (B and D, middle).

See also Figure S1.

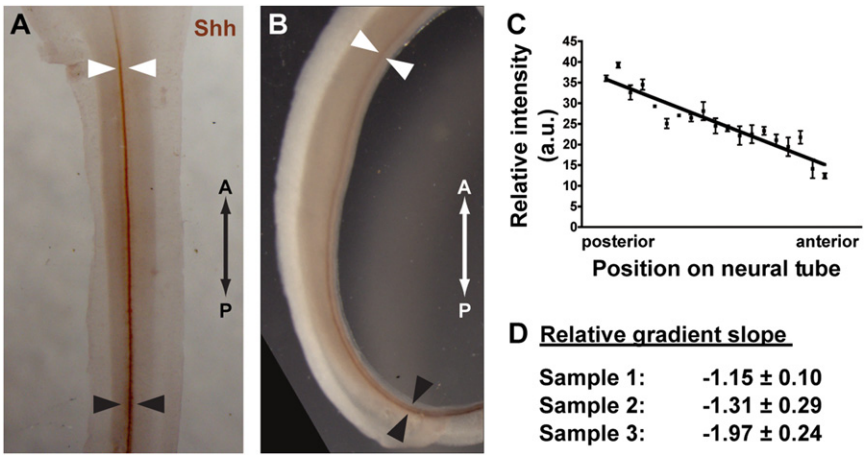

Figure 2. Shh Protein Accumulates in an AP Gradient in the Neural Tube

(A) Open-book view and (B) side view of whole-mount Shh immunostaining of a rat E13.5 neural tube. A gradient of Shh protein is present along the neural tube with high Shh levels posterior (black arrowheads) and low Shh levels anterior (white arrowheads).

(C) Plot of relative Shh staining intensity versus relative position along the neural tube (mean ± SD for each point). a.u., arbitrary unit.

(D) Each independent neural tube measured (n = 3) had a negative slope for the gradient.

expressed only in a subset of commissural neurons (see Figure S1 available online; Gowan et al., 2001), not all commissural neurons in *Math1-Cre;Smo$^{n/c}$* embryos are null for *Smo*. Thus, postcrossing commissural axons of *Math1-Cre;Smo$^{n/c}$* embryos were not as disorganized as those from *Wnt1-Cre;Smo$^{n/c}$* embryos, but they still displayed clear guidance defects along the AP axis (Figure 1D, right). The proportion of anteriorly directed axons was significantly lower than the control (Figure 1E), and many cohorts had axons that recrossed the floorplate (Figure 1F).

Together, these results show that Smo is required cell autonomously in commissural neurons for correct floorplate crossing and exit and for their postcrossing axons to project in the proper direction along the AP axis. That *Smo* mutant axons have defects in floorplate crossing is consistent with a role for Shh in inducing the response to Semaphorin, which is required for correct floorplate crossing and exit (Parra and Zou, 2010). However, the AP randomization we observed indicates that signaling through Smo also plays a role in guidance of postcrossing commissural axons along the longitudinal axis.

Shh Protein Is Present in a Longitudinal Gradient along the AP Axis of the Spinal Cord

We hypothesized that the failure of *Smo* mutant axons to turn correctly along the AP axis might be due to an inability of *Smo* mutant postcrossing axons to respond to Shh. To visualize the distribution of Shh protein in the neural tube, we performed anti-Shh staining on open-book preparations (Figures 2A and 2B). Shh protein was present in a posterior-high/anterior-low gradient. Plotting the staining intensity versus the relative position along the AP axis showed that the gradient was approximately linear (Figures 2C and 2D; correlation coefficient R^2 = 0.88). To our knowledge, this is the first demonstration at the protein level of a diffusible guidance cue accumulating in a gradient along the AP axis. The Shh protein gradient we observed is consistent with observations of a Shh mRNA gradient in the developing chick spinal cord (Bourikas et al., 2005) and demonstrates that this gradient is conserved in mammals. The presence of Shh in an AP gradient along the floorplate, together with our results showing that Smo is required cell autonomously for postcrossing commissural axons to turn anteriorly along the AP axis, supports a model where a Shh gradient directs AP guidance of postcrossing commissural axons in mammals.

Commissural Neurons Switch Their Response to Shh from Attraction to Repulsion over Time In Vitro

The directionality of the Shh gradient, decreasing anteriorly, implies that Shh acts as a repellent on postcrossing commissural axons. Although Shh has been proposed to function as a guidance cue for postcrossing commissural axons in the chick (Bourikas et al., 2005), Shh gradients have not been shown to directly repel commissural axons in any species. Explant-based assays in which an explant is cultured a short distance from a source of the guidance cue, such as those performed by Bourikas et al. (2005), cannot distinguish between biased outgrowth of axons and actual turning. To test whether Shh gradients can directly cause commissural axons to turn away, we used an in vitro assay for axon guidance based on the Dunn chamber (Yam et al., 2009). Commissural neurons were dissociated from the dorsalmost part of E13 rat spinal cords, an age where the dorsal spinal cord is populated mostly by neural precursors and young neurons that have not yet extended long neurites (Helms and Johnson, 1998). Hence, these cells have not been in proximity to the floorplate and are floorplate naive. The neurons were grown in culture and then exposed to a gradient of Shh in the Dunn chamber after a specified numbers of days in culture. With this assay, the turning of axons can be imaged and measured in response to a defined gradient of a chemical cue over a short time period. Because the response of commissural neurons to guidance cues such as Slit changes with the age of the neurons (Stein and Tessier-Lavigne, 2001), we assayed neurons cultured from 2 to 4 DIV (days in vitro). We found that for commissural neurons at 3–4 DIV, axons dramatically changed their direction of growth on exposure to a Shh gradient, turning away from higher concentrations of Shh (Figures 3A–3C). The Shh gradient rapidly stimulated the repulsion of axons with turning commencing within 1 hr of application of the gradient, indicating that the effect of Shh is direct. Quantification of the angle turned (Figure 3D) indicated that axons in a control gradient have no net turning (angle turned of $-0.82^\circ \pm 4.3^\circ$, mean ± SEM; Figures 3E and 3F). In a Shh gradient, however, axons from commissural

neurons at 3–4 DIV had a significant bias toward negative angles turned (−14.8° ± 5.0°; $p < 0.05$, one-way ANOVA; Figures 3E and 3F), indicating repulsion by Shh. The degree of repulsion by Shh was even more dramatic when those axons oriented toward increasing Shh concentrations, i.e., with initial angles between 0° and 90°, were considered. In this case, the mean angle turned was −23.9°. Shh appeared to only affect turning, not growth, of these axons because the Shh gradient did not significantly change the growth rate of the axons compared to the control ($p = 0.8287$) (Figure 3G). Furthermore, the net axon growth in a Shh gradient showed no correlation with the angle turned (Figure 3H).

As previously shown (Yam et al., 2009), commissural axons at 2 DIV were attracted up a Shh gradient, with a mean angle turned of 11.1° ± 4.6° (Figures 3E and 3F). This contrasts sharply with the repulsion by Shh that we observed at 3–4 DIV and suggests that the response of commissural neurons in vitro to Shh gradients changes over time. The length of the axon had no bearing on the degree of repulsion by Shh (Figure 3I), suggesting that the switch from attraction to repulsion by Shh is independent of axon length. This change in response to Shh over time is reminiscent of the change in response of commissural neurons in vivo to Shh gradients during development, with younger precrossing axons attracted to Shh along the DV axis and older postcrossing axons repelled by Shh along the AP axis. That isolated commissural neurons in culture maintain the ability to switch their response to Shh gradients suggests that the switch is cell intrinsic and temporally regulated.

Unlike the switch in commissural axon response to Shh, silencing of the Netrin-1 response at the floorplate is not cell intrinsic and depends on physical encounter with the floorplate (Shirasaki et al., 1998). Indeed, we found that commissural neurons do not change their response to Netrin-1 over time in vitro. Commissural axons were attracted to Netrin-1 both at 2 DIV (mean angle turned of 17.4° ± 4.0°) and 3–4 DIV (mean angle turned of 12.8° ± 3.6°) (Figure 3J). Thus, the cell-intrinsic switch in the polarity of the response to guidance cues regulates the response to Shh, but not to Netrin-1.

14-3-3 Protein Expression in Commissural Neurons Is Time Dependent

We next looked for endogenous proteins that are expressed in a time-dependent manner and that could mediate the switch in Shh response. 14-3-3 proteins were good candidates because they are major constituent proteins of growth cones and are important for the repulsive response to MAG and NGF in postnatal DRG neurons (Kent et al., 2010). 14-3-3 proteins are adaptor proteins that interact with phosphoserine/threonine motifs in their binding partners. They control the spatial and temporal activity of their binding partners through regulating their subcellular localization, conformation, or accessibility (Bridges and Moorhead, 2004).

We used immunostaining to examine the expression of five 14-3-3 isoforms in the developing mouse spinal cord at E10.5 and E11.5, when commissural axons are crossing the floorplate. To visualize commissural axon tracts, we stained for Tag1, a marker of precrossing commissural axons, and for L1, a marker of postcrossing commissural axons. As illustrated by Tag1 staining at E10.5 and E11.5, precrossing commissural axons have a stereotyped DV trajectory toward the floorplate (Figure 4A, arrows). For postcrossing commissural axons, L1 expression is present predominantly at the floorplate and ventral funiculus at E10.5, when the axons have just crossed the floorplate. At E11.5, L1 expression extends up the lateral funiculi and widens in the ventral funiculi, illustrating the progression of the postcrossing axons (Figure 4A, arrowheads).

The different 14-3-3 isoforms are all expressed in neural tissue (Figure 4A). Strikingly, both 14-3-3β and 14-3-3γ have an expression pattern in the neural tube that correlates with that of L1. Although 14-3-3β is expressed faintly in precrossing commissural axons, at E10.5, both 14-3-3β and 14-3-3γ are enriched at the floorplate and ventral funiculi, and at E11.5, their expression expands along the lateral funiculi and widens in the ventral funiculi. These changes in the distribution of 14-3-3β and 14-3-3γ mimic the changes in the pattern of L1 expression and indicate that 14-3-3β and 14-3-3γ are enriched in postcrossing commissural axons. 14-3-3τ is also present in postcrossing commissural axons, being present at the floorplate, ventral funiculi, and lateral funiculi. However, it is also expressed at significant levels in precrossing commissural axons, with staining along the DV axonal tracts. 14-3-3ε and 14-3-3ζ are also present in neural tissue but are expressed predominantly in cell bodies, rather than axonal processes. Hence, isoforms β and γ are those enriched in postcrossing commissural axons.

If 14-3-3 proteins are involved in the switch in Shh responsiveness, their expression should also change in vitro over time. We cultured dissociated commissural neurons for 2 or 3 DIV and analyzed the levels of 14-3-3 isoforms in cell lysates by western blotting. 14-3-3β, 14-3-3γ, and 14-3-3τ, all of which are expressed in postcrossing commissural axons, all have higher expression at 3 DIV compared to 2 DIV (Figures 4B and 4C). Of the two isoforms that are predominantly expressed in cell bodies, 14-3-3ε also had higher expression at 3 DIV compared to 2 DIV, but 14-3-3ζ did not. Given that 14-3-3β and 14-3-3γ are strongly expressed in postcrossing commissural axons compared to precrossing commissural axons, and that their expression increases in vitro over time in culture, they are good candidates for mediating the switch in Shh response from attraction to repulsion (Figure 4D).

14-3-3 proteins have been postulated to modulate growth cone turning by stabilizing the interaction between the regulatory and catalytic subunits of PKA, thereby reducing PKA activity (Kent et al., 2010). Therefore, an increase in 14-3-3 proteins should lead to a decrease in PKA activity. To assess the levels of active PKA, we used an antibody that recognizes the activated form of the catalytic subunit of PKA: phospho-PKA. Western blotting of lysates from dissociated commissural neurons showed that the levels of phospho-PKA at 3–4 DIV were about one-third lower than the levels at 2 DIV (Figure 4E). PKA phosphorylates the PP-1 inhibitory protein I-1 (phospho-I-1) in growth cones; thus, phospho-I-1 staining is another indicator of PKA activity (Han et al., 2007). Consistent with the decrease in phospho-PKA observed by western blotting, phospho-I-1 staining in commissural neuron growth cones was also significantly lower at 3 DIV compared to 2 DIV ($p = 0.0158$)

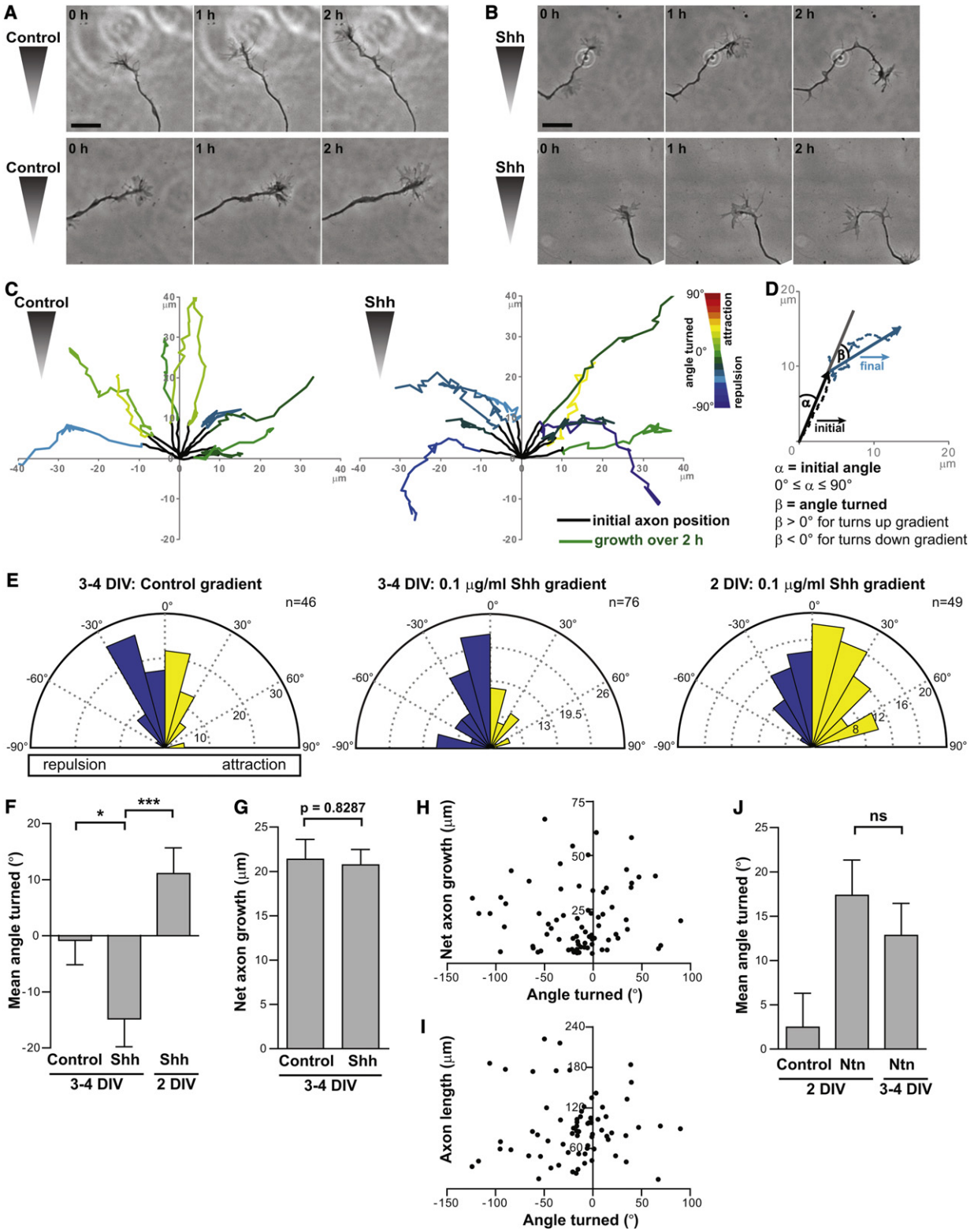

Figure 3. Commissural Axons Are Repelled by Shh at 3–4 DIV but Are Attracted by Shh at 2 DIV

(A) Commissural neurons were isolated from E13 rat embryos. Axons of dissociated commissural neurons do not change their direction of growth when exposed to a control gradient.

(B) When exposed to a 0.1 μg/ml Shh gradient at 3–4 DIV, axons are repelled by high concentrations of Shh and turn toward low concentrations of Shh.

(Figure 4F). Hence, the increase in 14-3-3 protein expression at 3 DIV correlated with a decrease in PKA activity.

Inhibition of 14-3-3 Proteins Converts Shh Repulsion to Attraction

We hypothesized that the increase in 14-3-3 protein levels may mediate the switch in Shh response from attraction to repulsion. To test this hypothesis, we inhibited 14-3-3 activity with R18 (PHCVPRDLSWLDLEANMCLP), a peptide antagonist that inhibits binding of all 14-3-3 isoforms to their Ser/Thr phosphorylated targets. In particular, R18 has been shown to inhibit the binding of 14-3-3γ to PKA (Kent et al., 2010). The control WLKL peptide (WLDL mutated to WLKL) does not bind to 14-3-3. Both the R18 peptide and WLKL control peptide were fused to YFP and to Tat to allow entry into cells (Dong et al., 2008).

Commissural axons, which are normally repelled by Shh at 3 DIV (Figures 3A–3F), continue to do so in the presence of the control Tat-WLKL-YFP, with a mean angle turned of −9.5° ± 3.8° (Figures 5A and 5B). Remarkably, in the presence of the inhibitory Tat-R18-YFP, 3 DIV commissural axons were attracted by a Shh gradient, with a mean angle turned of 8.1° ± 3.5° (Figures 5A and 5B). There was a dramatic shift in the distribution of the angles turned from mostly negative in the presence of WLKL, to mostly positive when 14-3-3 proteins were inhibited by R18 (Figure 5A). In contrast, R18 had no effect on net axon growth under the same conditions (Figure S2A).

To exclude the possibility of R18 having nonspecific effects, we also used shRNAmir targeted against 14-3-3β and 14-3-3γ, the two isoforms most prominently expressed in postcrossing commissural axons, to knock down 14-3-3 proteins in commissural neurons. Commissural neurons were transfected with plasmids encoding shRNAmir against 14-3-3β or 14-3-3γ. We were able to reduce 14-3-3β and 14-3-3γ protein levels to about 30% of control levels (Figures S2B and S2C). Commissural neurons expressing control scrambled shRNAmir were repelled by a Shh gradient (mean angle turned of −19.8° ± 6.1°) (Figures 5C and 5D). When either 14-3-3β or 14-3-3γ was knocked down, the response to a Shh gradient switched from repulsion to attraction (mean angle turned of 9.0° ± 5.1° and 14.2° ± 4.5°, respectively), similar to that observed with R18 inhibition of 14-3-3 function. Together, this demonstrates that 14-3-3 activity is important for conferring the repulsive response to Shh at 3 DIV.

14-3-3 Proteins Switch the Axon Turning Response to Shh through PKA Activity

Our previous work suggests that 14-3-3 proteins stabilize the PKA holoenzyme and, consequently, suppress its activation (Kent et al., 2010). Consistent with this, in commissural neurons where 14-3-3 protein levels have been knocked down, there was a small but consistent increase in phospho-PKA measured by western blotting of whole-cell lysates (Figure 5E).

To further delineate the relationship between 14-3-3 proteins and PKA, we tested whether 14-3-3 and PKA act functionally in the same pathway. R18 inhibition of 14-3-3 activity in 3 DIV commissural neurons switched the response to Shh from repulsion to attraction (Figures 5A and 5B). We hypothesized that this was due to an increase in PKA activity resulting from 14-3-3 inhibition, and we predicted that we could rescue the effect of 14-3-3 inhibition by modulating PKA activity. Indeed, addition of the PKA inhibitor KT-5720 to R18-treated commissural neurons reverted the response to Shh to repulsion (Figure 5B, mean angle turned of −17.1° ± 6.0°). The degree of repulsion in the presence of R18 and KT-5720 was comparable to the control WLKL treatment, suggesting that 14-3-3 proteins act mostly, if not entirely, through PKA to switch the turning response to Shh. To test whether changes in PKA activity alone are sufficient to modulate Shh-mediated axon guidance, we inhibited PKA activity in young 2 DIV dissociated commissural neurons with KT-5720 and found that this switched the response to Shh from attraction to repulsion (Figure 5F). Conversely, increasing PKA activity with 6-BNZ-cAMP in older 3 DIV dissociated commissural neurons switched the response to Shh from repulsion to attraction (Figure 5F). Thus, PKA downstream of 14-3-3 can modulate the turning response to Shh gradients.

14-3-3 Protein Inhibition In Vivo Perturbs AP Guidance of Postcrossing Commissural Axons

Our in vitro experiments implicate the increase in 14-3-3 protein levels in the switch from attraction to repulsion of commissural neurons by Shh. To test whether 14-3-3 proteins are important in vivo for the repulsion of postcrossing commissural axons anteriorly along the longitudinal axis, we treated embryonic rat open-book cultures with Tat-R18-YFP to inhibit 14-3-3 activity or the control Tat-WLKL-YFP. One day later, the cultures were fixed and the trajectories of postcrossing commissural neurons visualized with DiI anterograde labeling. Postcrossing axons in the presence of control WLKL exhibited a stereotyped commissural

(C) Trajectory plots of a sample of ten axons in a control (left) or 0.1 μg/ml Shh (right) gradient. The initial axon position is black and the axon growth over 2 hr colored according to the angle turned. Axons in the Shh gradient tend to turn down the gradient.
(D) Definition of the initial angle, α, the angle between the initial axon position and the gradient, and angle turned, β, the angle between the vectors representing the initial and final position of the axon.
(E) Rose histograms of the distribution of turned angles of commissural neurons at 3–4 DIV or 2 DIV in a 0.1 μg/ml Shh gradient. Responses of individual neurons were clustered in 15° bins, and the percentage of total neurons per bin is represented by the radius of each segment.
(F) The mean angle turned (±SEM) for axons in a control and 0.1 μg/ml Shh gradient for commissural neurons at 2 and 3–4 DIV. $p = 0.0007$, one-way ANOVA, Newman-Keuls posttest, $^{*}p < 0.05$, $^{***}p < 0.001$.
(G) Net axon growth (±SEM) of commissural neurons at 3–4 DIV in a 0.1 μg/ml Shh gradient compared to those in a control gradient ($p = 0.8287$, unpaired t test).
(H) Scatterplot of the net axon growth versus angle turned for axons at 3–4 DIV in a 0.1 μg/ml Shh gradient. The angle turned is independent of the net axon growth.
(I) Scatterplot of the axon length versus the angle turned for axons at 3–4 DIV in a 0.1 μg/ml Shh gradient. The angle turned is independent of the initial axon length.
(J) The mean angle turned (±SEM) for axons in a control and 0.2 μg/ml Netrin gradient for commissural neurons at 2 and 3–4 DIV. $p = 0.0199$, one-way ANOVA, Dunnett's posttest; ns, not significant.
Scale bars, 20 μm (A and B).

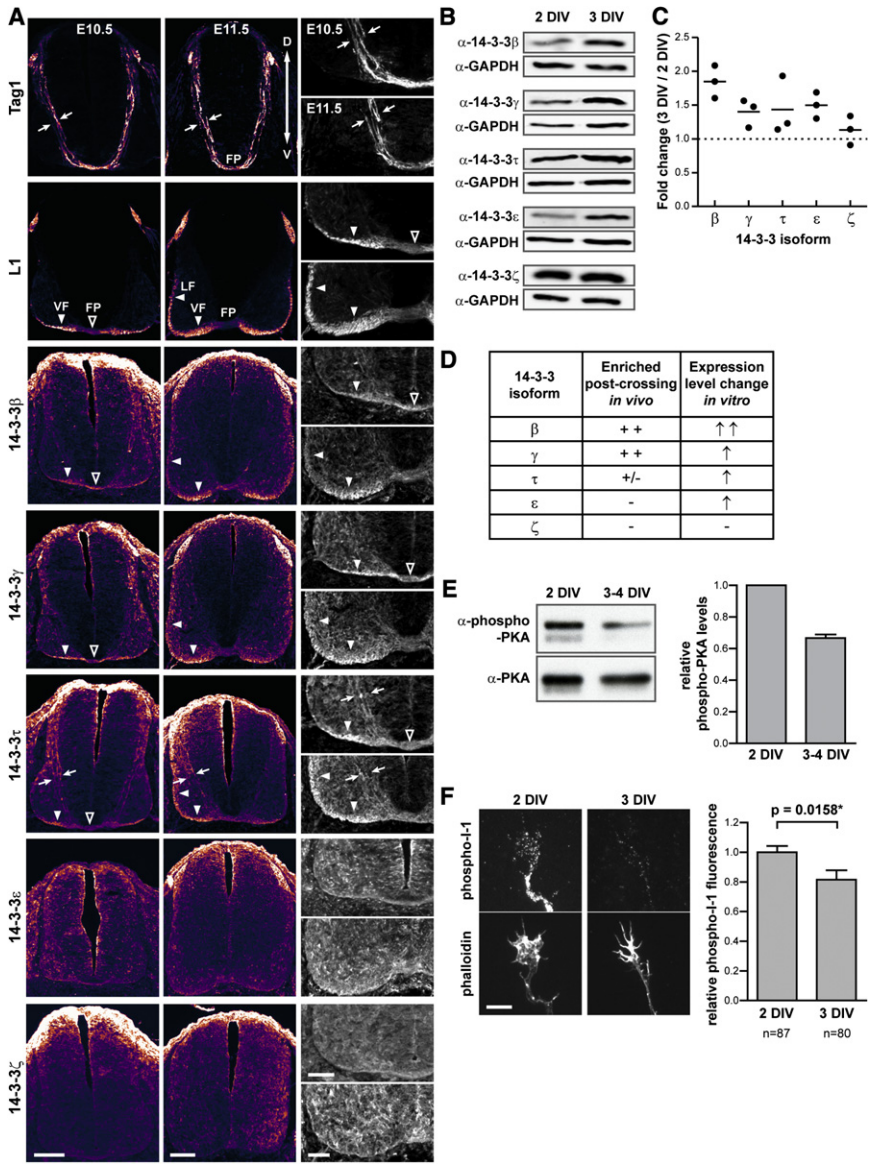

Figure 4. 14-3-3 Protein Expression and PKA Activity in Commissural Neurons Is Time Dependent

(A) Mouse E10.5 and 11.5 spinal cord cross-sections immunostained for Tag1, a marker of precrossing commissural axons, L1, a marker for postcrossing commissural axons, and five 14-3-3 isoforms. 14-3-3β and 14-3-3γ are enriched in postcrossing commissural axons. 14-3-3τ is present in pre- and postcrossing commissural axons. Arrows indicate precrossing commissural axon trajectories, filled arrowheads postcrossing commissural axon trajectories, and open arrowheads the floorplate. Left view is a pseudocolored heatmap of the immunostainings. Right view is a zoom of the ventral region of the spinal cord.

(B) Western blots for 14-3-3 isoforms from lysates of dissociated rat commissural neurons cultured for 2 or 3 DIV.

(C) 14-3-3 protein levels were normalized to GAPDH before calculating the ratio of expression at 3 to 2 DIV. Bar represents the mean of three independent experiments.

(D) Summary of the 14-3-3 isoform expression patterns. Both 14-3-3β and 14-3-3γ are enriched in postcrossing commissural neurons in vivo and increase in expression levels over time in vitro in dissociated commissural neuron culture.

(E) Phosphorylated PKA catalytic subunit and total PKA were detected by western blot of commissural neuron lysates at 2 and 3–4 DIV. Phospho-PKA levels were normalized to total PKA and quantified from two experiments. Graph represents mean ± SEM.

(F) Dissociated rat commissural neurons were cultured for 2 or 3 DIV, then fixed and immunostained for phospho-I-1, a target of phospho-PKA. Fluorescent phalloidin was used to label F-actin. The average phospho-I-1 fluorescence signal was measured for each growth cone and normalized to the mean growth cone fluorescence signal at 2 DIV (three independent experiments, ≥25 growth cones per condition). Graph represents mean ± SEM. The phospho-I-1 fluorescence intensity is significantly lower at 3 DIV compared to 2 DIV (unpaired t test).

Scale bars, 100 μm (A, left), 50 μm (A, right), and 10 μm (F).

VF, ventral funiculus; LF, lateral funiculus.

See also Figure S4.

axon trajectory, turning anteriorly after crossing the floorplate (Figure 6A). However, in the presence of R18, many axons failed to turn anteriorly upon floorplate exit. R18 increased the percentage of cohorts with randomization of AP guidance along the longitudinal axis ($p = 0.0041$), but there was no significant effect on the amount of stalling or recrossing axons (Figure 6B). When we quantified the degree of AP randomization by measuring the fraction of anterior fluorescence, there was a significant decrease in the amount of anterior axons in the presence of R18 (Figure 6C). The decrease was in between that observed for the *Wnt1-Cre;Smo*$^{n/c}$ and *Math1-Cre;Smo*$^{n/c}$ mice.

To inhibit 14-3-3 activity specifically in neurons, we turned to the developing chick embryo. Plasmids encoding R18 or a control WLRL peptide fused to EGFP (Kent et al., 2010) were injected into chick neural tubes and electroporated unilaterally. Two days later, the embryos were dissected and the commissural axon trajectories analyzed in the open-book format. Electroporated neurons were identified by EGFP expression. In neurons expressing control WLRL-EGFP, the vast majority (92%) of axons correctly made an anterior turn after crossing the floorplate, with very few axons making a posterior turn. In contrast, neurons expressing R18-EGFP had a different distribution of phenotypes, with significantly fewer axons (58%) making the correct anterior turn ($p < 0.001$) and significantly more axons (35%) turning posteriorly ($p < 0.001$) (Figure 6D). Notably, as with the Tat-R18-YFP-treated rat open-book cultures (Figures 6A and 6B), there was no significant difference in the percentage of axons stalled in the floorplate or after floorplate exit, indicating that 14-3-3 activity

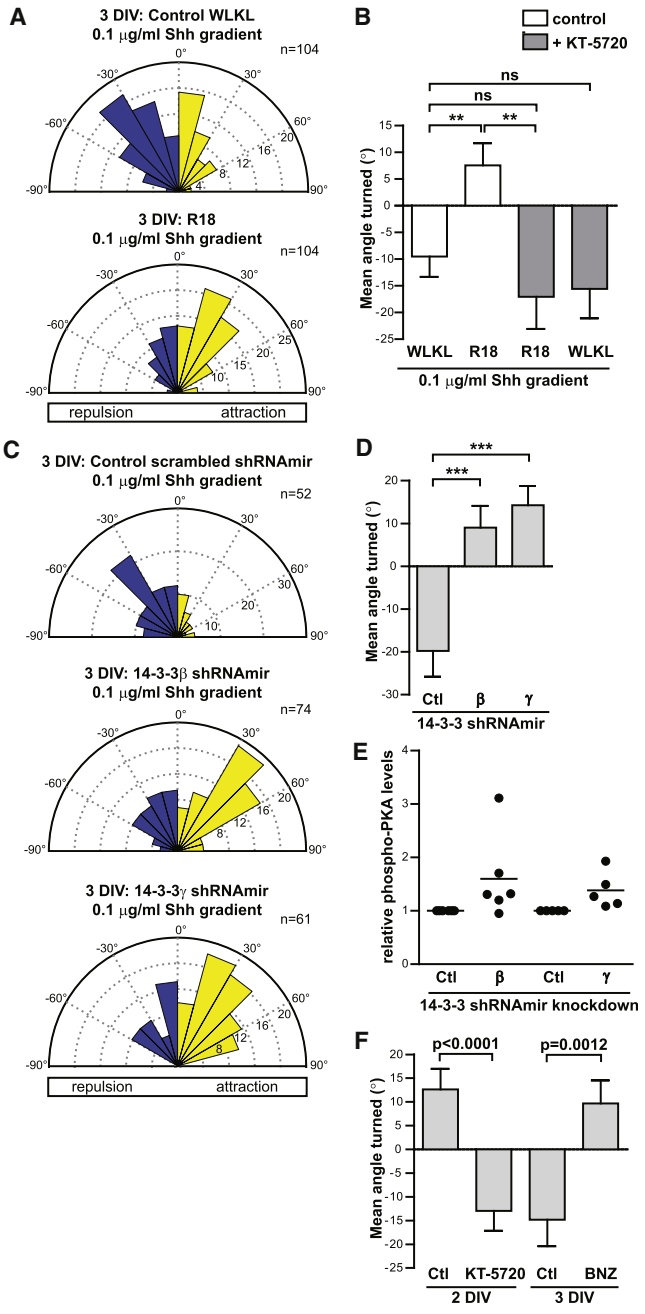

Figure 5. Inhibition of 14-3-3 Protein Function Converts Shh Repulsion to Attraction through PKA Activation

(A) Rose histograms of the angles turned of rat commissural neurons at 3 DIV in a 0.1 μg/ml Shh gradient. Neurons were treated with 100 ng/ml of either Tat-WLKL-YFP (control) or Tat-R18-YFP for 6 hr prior to placing the neurons in the Dunn chamber for the turning assay. Inhibition of 14-3-3 proteins with Tat-R18-YFP switches the response to Shh from repulsion to attraction.

(B) Mean angle turned (±SEM) in the presence or absence of the PKA inhibitor KT-5720 (200 nM) for neurons treated with either Tat-WLKL-YFP or Tat-R18-YFP; $p = 0.0001$, one-way ANOVA, Tukey's posttest. $^{**}p < 0.01$.

(C) Rose histograms of the angles turned for rat commissural neurons expressing shRNAmir targeted against 14-3-3β or 14-3-3γ at 3 DIV in a 0.1 μg/ml Shh gradient. Knockdown of 14-3-3β or 14-3-3γ switches the response to Shh from repulsion to attraction.

(D) Mean angle turned (±SEM). $p < 0.0001$, one-way ANOVA, Dunnett's posttest. $^{***}p < 0.001$.

(E) Relative phospho-PKA levels, normalized to actin levels, as assessed by western blotting of 3–4 DIV commissural neuron lysates expressing shRNAmir against 14-3-3β or 14-3-3γ. Bar represents the mean. Ctl, control.

(F) The mean angle turned (±SEM) for axons in a 0.1 μg/ml Shh gradient for commissural neurons at 2 and 3 DIV treated with either KT-5720 (200 nM) or 6-BNZ-cAMP (30 μM). Unpaired t test.

See also Figure S2.

is required for AP guidance, but not floorplate crossing and exit. This is different than what we observed in the *Wnt1-Cre;Smo$^{n/c}$* and *Math1-Cre;Smo$^{n/c}$* mice, which have defects in both AP guidance and floorplate crossing and exit. Together with our in vitro data demonstrating that 14-3-3 activity is required for the switch in Shh response from attraction to repulsion, this implicates 14-3-3 proteins in mediating the repulsive response to Shh in postcrossing commissural axons.

Overexpression of 14-3-3 Proteins Is Sufficient to Prematurely Switch the Response to Shh from Attraction to Repulsion

If 14-3-3 proteins are key mediators of the switch in the polarity of the turning response, overexpression of 14-3-3 proteins should be sufficient to prematurely activate the switching program in young neurons. We tested this in vitro by transducing dissociated commissural neurons with HSV (herpes simplex virus) expressing either 14-3-3β or 14-3-3γ, the two 14-3-3 isoforms that are enriched in postcrossing commissural axons. Control neurons transduced with GFP alone were attracted up a Shh gradient at 2 DIV (mean angle turned of 18.0° ± 5.1°) (Figures 7A and 7B). Expression of either 14-3-3β or 14-3-γ significantly changed the turning response of the neurons to a Shh gradient. Overexpression of 14-3-3β silenced the attractive response to Shh (mean angle turned of −2.4° ± 4.2°). Interestingly, overexpression of 14-3-3γ converted the attractive response to Shh to a repulsive response, with a large fraction of the population turning away from the Shh gradient (mean angle turned of −9.9° ± 4.9°) (Figures 7A and 7B). Therefore, overexpression of 14-3-3γ can prematurely induce commissural neurons at 2 DIV to become repelled by Shh gradients, a behavior that normally does not occur until 3 DIV when 14-3-3 levels are higher.

To test whether these results translate to differences in commissural axon behavior in vivo, we overexpressed 14-3-3β, 14-3-3γ, or 14-3-3ζ in the developing chick embryo. Plasmids encoding the different 14-3-3 isoforms, together with *Math1$_{promoter}$::GFP* to mark Math1^{+} commissural neurons, were injected into chick neural tubes and electroporated unilaterally. Two days later, the embryos were dissected and commissural axon trajectories analyzed in the open-book format. In control neurons, the axons of electroporated neurons migrated ventrally toward the floorplate (Figure 7D), and the vast majority (96%) turned anteriorly after crossing the floorplate (Figures 7C and 7D). In contrast, axons from commissural neurons overexpressing 14-3-3β or 14-3-3γ exhibit a striking phenotype. At various distances before reaching the floorplate, >21% of axons prematurely turn anteriorly. In addition, some also have a dorsal component to their trajectory, suggesting that they are also repelled from the floorplate (Figures 7C and 7D). This phenotype

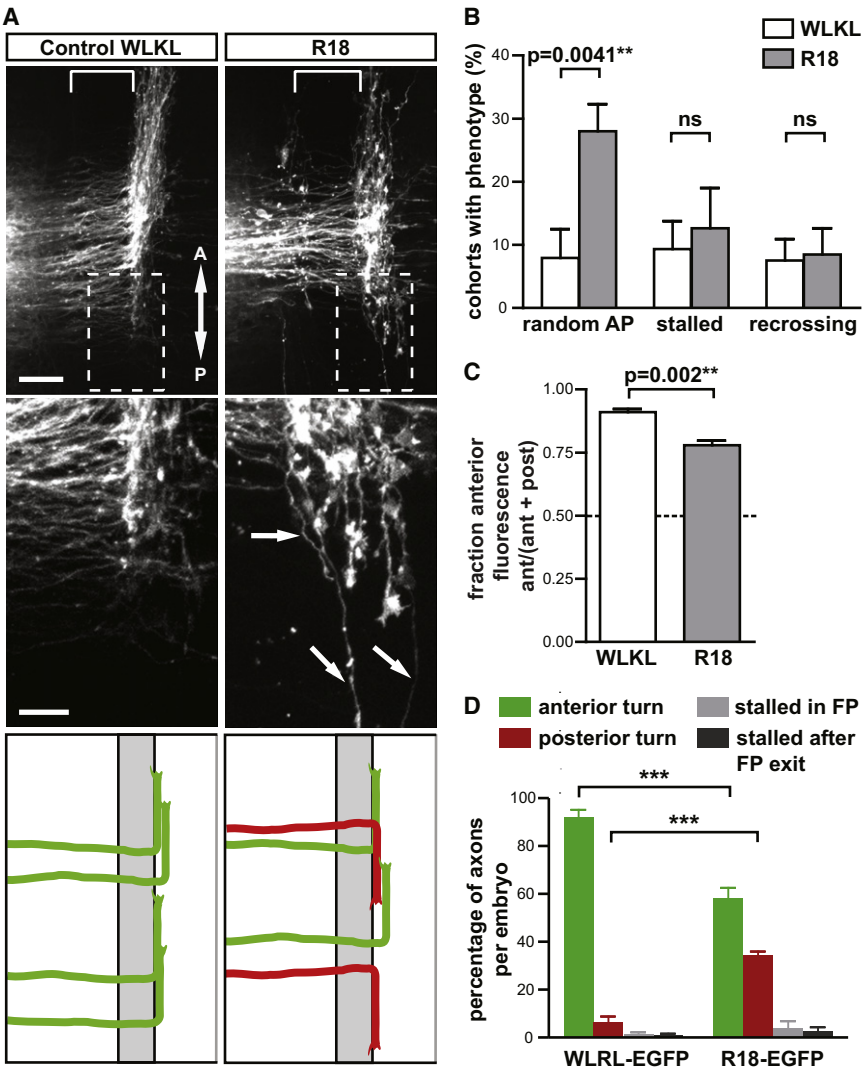

Figure 6. Inhibition of 14-3-3 Protein Function In Vivo Perturbs AP Guidance of Postcrossing Commissural Axons

(A) Top view shows DiI labeling of postcrossing commissural axons in rat open-book cultures treated with either 100 or 150 ng/ml Tat-WLKL-YFP or Tat-R18-YFP. Middle view is a zoom of boxed region. Bottom view is a schematic of the axon guidance phenotype. Control Tat-WLKL-YFP-treated axons make a stereotypical anterior turn after exiting the floorplate (12 open books, 64 cohorts). Tat-R18-YFP-treated axons show errors in turning after exiting the floorplate, with many axons turning posteriorly (arrows) (13 open books, 75 cohorts). Brackets indicate floorplate.

(B) Quantification of random AP turning, stalled axons in the floorplate, and recrossing axons, as percentage of cohorts (±SEM) per open book (unpaired t test).

(C) Relative fluorescence (±SEM) of the anterior (ant)-directed axons versus total fluorescence of anterior and posterior (post)-directed axons. In WLKL-treated axons (six open books, 37 cohorts), most of the fluorescence is on the anterior side of the label, whereas in R18-treated axons (seven open books, 43 cohorts), there is a significant decrease in the amount of anterior fluorescence ($p = 0.002$, unpaired t test).

(D) Chick neural tubes were electroporated with either WLRL-GFP or R18-GFP plasmids and allowed to develop for 2 days in vivo. The behavior of individual axons was quantified. Expression of R18-EGFP significantly decreased the number of axons that turn anteriorly and increased the number of axons that turn posteriorly after crossing the floorplate (n = 6 embryos per condition, minimum 199 axons per condition). Graph represents the percentage of axons per embryo (mean ± SEM); two-way ANOVA, ***$p < 0.001$.

Scale bars, 50 μm (A, top) and 20 μm (A, middle).

is consistent with overexpression of 14-3-3β or 14-3-3γ prematurely switching the response to Shh from attraction (toward the floorplate) to repulsion (movement away from the floorplate and anteriorly toward low concentrations of Shh). Overexpression of 14-3-3ζ, an isoform that does not increase in expression over time in vitro and is not enriched in postcrossing commissural axons in vivo, had no significant effect on the commissural axon trajectories, with most axons (93%) correctly turning anteriorly after crossing the floorplate (Figures 7C and 7D). Therefore, overexpression of 14-3-3β or 14-3-3γ is sufficient to switch the response of commissural axons to Shh gradients from attraction to repulsion, suggesting that they are key mediators of the switch in turning response to Shh.

DISCUSSION

Our data support a model where Shh acts as a bifunctional guidance cue, attracting commissural axons toward the floorplate and then repelling them anteriorly along the AP axis (Figure 8A). Furthermore, dissociated commissural neurons in vitro switch from being attracted to being repelled by Shh over time (Figure 8B). This recapitulates the change in response to Shh between pre- and postcrossing commissural axons in vivo, suggesting that the switch in response is intrinsic, cell autonomous, and time dependent. Our gain- and loss-of-function experiments in vitro and in vivo show that changes in 14-3-3 protein levels are sufficient to drive this switch in turning response to Shh: a reduction in 14-3-3 proteins switches the response from repulsion to attraction, and an increase in 14-3-3 proteins switches the response from attraction to repulsion. This is consistent with our observations that 14-3-3 levels increase over time in culture and that 14-3-3 proteins are enriched in postcrossing commissural axons. Furthermore, we demonstrate that 14-3-3 proteins mediate this switch through regulation of PKA activity.

Shh Has Multiple Roles in Commissural Axon Guidance

Shh secreted from the floorplate has multiple roles in nervous system development, from cell fate specification to axon

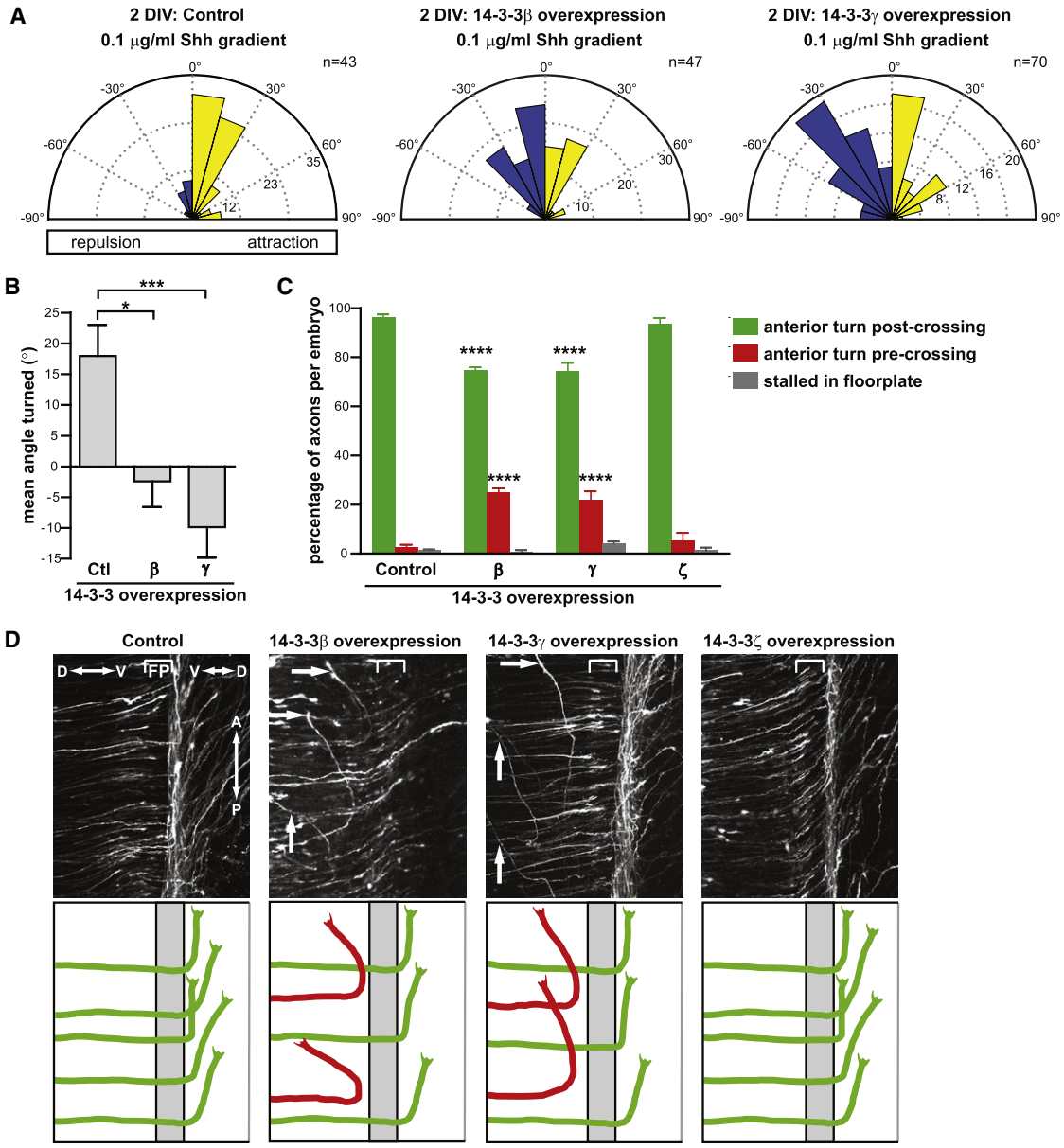

Figure 7. Overexpression of 14-3-3 Proteins Is Sufficient to Prematurely Switch the Response to Shh from Attraction to Repulsion

(A) Rose histograms of the angles turned for rat commissural neurons transduced with HSV expressing either 14-3-3β or 14-3-3γ, in a 0.1 μg/ml Shh gradient at 2 DIV. Overexpression of 14-3-3β silences attraction to Shh, whereas overexpression of 14-3-3γ switches the response to Shh from attraction to repulsion.

(B) Mean angle turned (±SEM). $p = 0.0004$, one-way ANOVA, Dunnett's posttest. *$p < 0.05$, ***$p < 0.001$.

(C and D) Chick neural tubes were electroporated with plasmids encoding various 14-3-3 isoforms together with *Math1*$_{promoter}$*::GFP* to mark electroporated Math1+ commissural neurons and allowed to develop for 2 days in vivo. Neural tubes were dissected and analyzed in the open-book format (D). Control and 14-3-3ζ-overexpressing axons make a stereotypical anterior turn after crossing the floorplate, whereas some axons overexpressing 14-3-3β or 14-3-3γ turn anteriorly before reaching the floorplate (arrows). (C) The behavior of individual axons was quantified. Overexpression of either 14-3-3β or 14-3-3γ significantly increased the number of axons that prematurely turn anteriorly before reaching the floorplate ($n \geq 4$ embryos per condition, minimum 101 axons per condition). Graph represents the percentage of axons per embryo (mean ± SEM); two-way ANOVA, ****$p < 0.0001$. Brackets indicate floorplate.

guidance. The DV gradient of Shh, together with BMPs from the roofplate, initially specifies the identity of various neuron types in the spinal cord. Subsequently, the DV Shh gradient, together with Netrin-1 and VEGF, is reused to guide precrossing commissural axons to the floorplate. Upon reaching the floorplate, Shh induces the response to Semaphorins, which allows for the correct exit of commissural axons from the floorplate (Parra and Zou, 2010). We now show that Shh also has a direct effect on postcrossing commissural axons in mammals, guiding them along the AP axis, consistent with evidence from chick (Bourikas

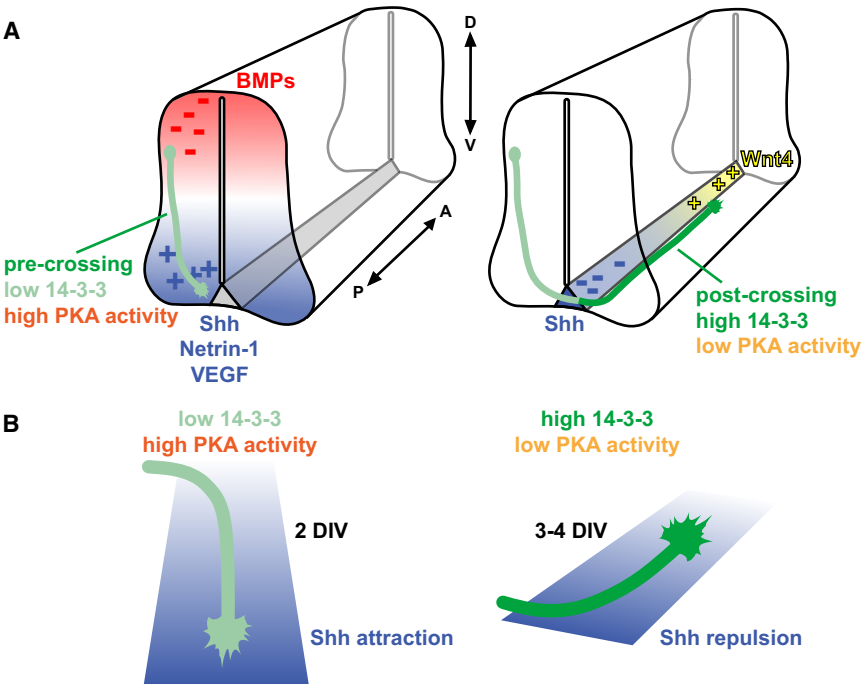

Figure 8. Differential Pre- and Postcrossing Commissural Axon Responses to Shh Are Regulated by a Cell-Intrinsic 14-3-3 Protein-Dependent Switch

(A) (left) During precrossing commissural axon guidance, commissural axons are attracted by Shh. The dorsal-high repellent gradient of BMPs (red) and the ventral-high attractant gradient of Netrin-1, Shh and VEGF (blue) act together to guide precrossing commissural axons ventrally toward the floorplate. (right) After crossing the floorplate, axon behavior switches and postcrossing commissural axons become repelled by Shh. Axons are guided anteriorly by the posterior-high repellent Shh gradient (blue) and an anterior-high attractant Wnt4 gradient (yellow). This switch in the polarity of the Shh response depends on 14-3-3 levels, which are low in precrossing commissural axons and high in postcrossing commissural axons. 14-3-3 proteins regulate the guidance response to Shh through modulating PKA activity. (B) The switch in Shh response is recapitulated in vitro with dissociated commissural neurons. At 2 DIV, commissural neurons are attracted toward high concentrations of Shh. At 3–4 DIV, commissural neurons are repelled by high concentrations of Shh. This switch also correlates with an increase in 14-3-3 protein levels and decrease in PKA activity.
See also Figure S3.

et al., 2005). Thus, Shh and Wnt4 both contribute to the correct AP guidance of commissural neurons.

The multiple roles of Shh at the floorplate are reflected in the phenotypes observed when Shh function or signaling is disrupted. In both chick and mouse, perturbation of Shh function results in defects in floorplate crossing and exit and defects in turning along the AP axis (Figure 1; Bourikas et al., 2005; Parra and Zou, 2010). Thus, it is reasonable to propose that these phenotypes reflect the dual role of Shh at the floorplate for crossing commissural axons, both to (1) induce Semaphorin repulsion of commissural axons at the floorplate for correct floorplate exit, and (2) guide postcrossing commissural axons anteriorly along the longitudinal axis. Intriguingly, inhibition of 14-3-3 function in rat and chick affected only AP guidance of postcrossing commissural axons, but not floorplate crossing and exit (Figure 6). Likewise, overexpression of 14-3-3 proteins had no effect on floorplate crossing and exit (Figure 7). This implies that 14-3-3 proteins are specifically involved in AP guidance of postcrossing commissural axons by Shh, but not in the induction of Semaphorin repulsion by Shh. This highlights that these two functions of Shh at the floorplate are distinguishable and act through different mechanisms.

Our genetic experiments selectively inactivate Smo in commissural neurons (Figure 1), convincingly demonstrating a cell-autonomous requirement for Smo in the AP guidance of postcrossing commissural axons. This is consistent with independent experiments showing that downregulation of Smo in rat open-book cultures leads to AP guidance defects (Parra and Zou, 2010). Both our results and those of Parra and Zou (2010) contrast with those obtained in chick with in ovo RNAi, which suggest that the guidance of postcrossing axons by Shh is independent of Smo (Bourikas et al., 2005). Additionally, in chick, the receptor mediating the effect of Shh on postcrossing axons has been proposed to be Hhip1 (Bourikas et al., 2005). We failed to find a role for Hhip1 in the AP guidance of commissural axons through genetic analysis in the mouse, with postcrossing commissural axons turning anteriorly in *Hhip1*$^{-/-}$ mice (Figures S3A–S3D). Although it is possible that Shh-mediated AP axon guidance in the chick and mammals uses different molecular mechanisms, this would be somewhat surprising given that all of the other guidance effects described so far for Shh are Smo dependent (Charron et al., 2003; Fabre et al., 2010; Sánchez-Camacho and Bovolenta, 2008; Yam et al., 2009).

A Novel Role for 14-3-3 Proteins in Switching the Polarity of the Turning Response to Shh

The ability of axons to change responsiveness to guidance cues is critical as axons navigate through complex environments. We show that the switch in Shh response from attraction to repulsion depends on 14-3-3 proteins, which are highly expressed in nervous tissue. In *Drosophila* motor neurons, correct axon pathfinding requires 14-3-3ε, which antagonizes Semaphorin-1a/PlexinA-mediated axon repulsion and allows axons to become more responsive to integrin-mediated adhesion (Yang and Terman, 2012). In postnatal rat DRG neurons, 14-3-3 proteins are important for conferring repulsive responses to NGF, and antagonism of 14-3-3 proteins converts this NGF-mediated repulsion to attraction (Kent et al., 2010), a process that could be harnessed to promote neuronal repair after injury. We now demonstrate a role for 14-3-3 proteins in a developmental switch in response to a guidance cue.

14-3-3 proteins function as homodimers and heterodimers to control the spatial and temporal activity of substrate proteins (Bridges and Moorhead, 2004). One way that 14-3-3 proteins modulate growth cone turning is by inhibiting PKA activity, through binding and stabilizing the PKA holoenzyme (Kent et al., 2010). Consistent with this, the increase in 14-3-3 levels in 3–4 DIV commissural neurons was accompanied by a decrease in active PKA levels, and PKA inhibition could rescue the effect of 14-3-3 inhibition on commissural axon turning.

According to our model, 14-3-3 levels regulate the global state of the neuron, changing the way the growth cone responds to Shh gradients. Our experiments showed that modulating 14-3-3 protein levels are sufficient to change the polarity of the turning response of commissural axons to Shh gradients. Thus, we hypothesize that 14-3-3 proteins regulate the turning response to Shh downstream of Shh reception, and we do not expect that Shh signaling itself regulates 14-3-3 levels. Consistent with this, neither treatment of commissural neurons with Shh nor a Smo antagonist affected 14-3-3 protein levels (Figure S3E).

Molecular Mechanisms Involved in Modulating Responses to Guidance Cues

In vitro, changes in the relative levels of other intracellular molecules, such as cyclic nucleotides and Ca^{2+}, can switch responses to guidance cues (e.g., Song et al., 1997, 1998; Wen et al., 2004). In vivo, cAMP is involved in the switch from Netrin-mediated attraction to repulsion of retinal ganglion cell axons (Shewan et al., 2002). However, we have not detected any differences in cAMP levels between commissural neuron growth cones at 2 and 4 DIV (Figure S4).

Alternatively, differential guidance responses can also result from differential expression patterns of receptors, as has been demonstrated for Sema3E, Netrin, and Slit (Chauvet et al., 2007; Chen et al., 2008; Hong et al., 1999; Shewan et al., 2002). For example, in the forebrain, neurons expressing PlexinD1 are repelled by Sema3E, whereas neurons expressing PlexinD1 and Neuropilin1 are attracted (Chauvet et al., 2007). In *Xenopus* axons in vitro, expression of Unc5 converts Netrin-mediated, DCC-dependent attraction to repulsion (Hong et al., 1999). Axon turning responses can also be modified by extrinsic signals such as extracellular matrix components or other guidance cues. In retinal ganglion cell axons, laminin can switch Netrin attraction to repulsion (Höpker et al., 1999). At the floorplate, activation of Robo by Slit silences the attractive effect of Netrin-1 on commissural axons (Stein and Tessier-Lavigne, 2001) whereas Shh and NrCAM trigger a gain of response to class 3 Semaphorins (Nawabi et al., 2010; Parra and Zou, 2010).

However, in our case, the switch in response to Shh is intrinsic and occurs in the absence of extrinsic cues. The switch we observed can be recapitulated in vitro with dissociated commissural neuron cultures in the absence of Shh and other cell types. This temporal switch from attraction to repulsion is reminiscent of the switch in responsiveness to Netrin-1 that has been observed in retinal explant cultures (Shewan et al., 2002). In the retinal explant cultures, it is possible that extrinsic factors may be involved because there is contact with neighboring cells and different cell types present in the explants. Because our commissural neuron cultures are almost pure (90%–98%; Yam et al., 2009) and cultured at very low density, it is unlikely that the switch we observe is triggered by other cell types. Furthermore, we isolate commissural neurons from the dorsal fifth of the spinal cord of E13 rats, an age where the dorsal spinal cord is populated with commissural neurons whose axons have not yet reached the floorplate. Hence, the neurons we use for our in vitro experiments are floorplate naive. Thus, our work reinforces the idea proposed by Holt and colleagues (Shewan et al., 2002) that time-dependent switches can regulate responses to axon guidance cues during development and in addition illustrates that these time-dependent switches can be intrinsic.

A cell-intrinsic switch that changes the response of neurons to midline cues adds another level of regulation to the switching of cellular responses at the midline. Crossing the floorplate is not required for commissural axons to become sensitive to the longitudinal gradients of Wnt4 and Shh because commissural axons in *Robo3* mutants make the correct anterior turn without crossing the floorplate (Chen et al., 2008). Thus, an intrinsic temporal switch may be involved in sensitizing the axons to these longitudinal gradients. Extrinsic factors in the spinal cord would add an additional level of regulation to modulate and fine-tune the guidance program. Extrinsic spatial and intrinsic temporal regulation might act together to switch commissural axon trajectory from DV to AP at the floorplate, ensuring high fidelity in axon turning at this intermediate target.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for further details on the experiments. All animal work was performed in accordance with the Canadian Council on Animal Care Guidelines and approved by the IRCM Animal Care Committee.

DiI Axon Tracing

Embryos were fixed in 4% paraformaldehyde (PFA) in PBS. Neural tubes were dissected from the fixed embryos, pinned open, and small 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) crystals were inserted to the medial neural tube dorsal of the motor column to label five to nine individual cohorts per embryo (Farmer et al., 2008). The DiI was allowed to diffuse for 1 or 2 days, and the neural tubes were then mounted open book and imaged.

Dissociated Commissural Neuron Culture

Dissociated commissural neuron cultures were prepared from the dorsal fifth of E13 rat neural tubes as previously described (Langlois et al., 2010; Yam et al., 2009). Neurons were assessed at 2 DIV (50–55 hr after plating) and 3–4 DIV (76–102 hr after plating).

Dunn Chamber Axon Guidance Assay

The Dunn chamber axon guidance assay, imaging, and analysis were performed as previously detailed (Yam et al., 2009). Gradients were generated with 0.1 μg/ml recombinant human Shh (C24II; R&D Systems), 0.2 μg/ml recombinant Netrin (a gift from T.E. Kennedy), or buffer containing BSA (the vehicle for Shh) as the control in the outer well.

Rat Open-Book Cultures

Open-book preparations of rat E13 spinal cords were isolated and cultured as previously described by Lyuksyutova et al. (2003). After 1 hr in culture, Tat-YFP-R18 or the control Tat-YFP- WLKL was added to the culture media to a final concentration of either 100 or 150 ng/ml and cultured for 24 hr.

Open-book explants were fixed at room temperature with 4% PFA, washed with PBS, and labeled with DiI.

Chick Electroporation

Chick spinal cord electroporation was performed at HH st. 18/19 as described by Luria et al. (2008). A total of 5–10 μg/μl solution of plasmid DNA was injected into the lumbar neural tube. The embryos were electroporated using platinum/iridium electrodes (FHC) with an ECM 830 Electro Square Porator (BTX; Harvard Apparatus; 30V, 5 pulses, 50 ms, at 1 s interval). Shells were sealed with Parafilm and incubated at 38°C until harvesting at HH st. 28/29.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2012.09.017.

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