Brief Communications

Slit1b-Robo3 Signaling and N-Cadherin Regulate Apical Process Retraction in Developing Retinal Ganglion Cells

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When neurons exit the cell cycle after their terminal mitosis, they detach from the apical surface of the neuroepithelium. Despite the fact that this detachment is crucial for further neurogenesis and neuronal migration, the underlying mechanisms are still not understood.

Introduction

Upon neurogenesis in the vertebrate CNS, progenitors undergo divisions at the apical surface of the neuroepithelium, which in the final round of division gives rise to postmitotic neurons (Götz and Huttner, 2005). These newly generated neurons often first adopt a neuroepithelial morphology but then quickly detach their process from the apical surface and migrate toward the basal side of the neuroepithelium (Nadarajah et al., 2001; Miyata et al., 2004). Recent studies in the mouse cortex disagree on whether integrin function (Loulier et al., 2009) or adherens junctions (Cappello et al., 2006; Imai et al., 2006; Loulier et al., 2009) regulate retraction of apical processes. It is also not known whether extrinsic signals regulate this event.

In a study focused on axon initiation in transgenically labeled retinal ganglion cells (RGCs), Zolessi et al. (2006) made the unexpected observation that slit1b morphants showed inhibition of apical retraction. They used this finding to demonstrate that axonogenesis, which happened on schedule, did not depend on apical retraction. However, Zolessi et al. (2006) did not investigate the mechanism of retraction.

Slit proteins act as repulsive guidance ligands for axonal growth cones expressing Robo receptors and it has been reported that activated Robo or addition of purified Slit can inhibit N-cadherin-mediated cell adhesion in chick retinal cell cultures (Rhee et al., 2002, 2007). In Drosophila, Slit-Robo signaling negatively regulates cadherin-mediated adhesion on cardioblasts to enable proper lumen formation (Medioni et al., 2008; Santiago-Martinez et al., 2008). These results raised the possibility that a similar mechanism mediates the retraction of apical processes in the neuroepithelium. We found that Slit1B or Robo3 downregulation in transgenically labeled RGCs leads to inhibition of apical retraction. Expression of dominant-negative N-cadherin in RGCs results in the opposite phenotype—premature apical detachment. N-cadherin dominant-negative overexpression rescues apical retraction in slit1b morphants. These results are consistent with a model in which Slit/Robo signaling downregulates N-cadherin activity allowing apical retraction.

Materials and Methods

Animals. Wild-type and transgenic zebrafish were bred and kept at 26.5°C. Embryos obtained from natural mating were raised in 0.003% phenylthiourea at 28.5°C to prevent pigment formation. Transgenic lines Tg(ath5:gapGFP) and Tg(ath5:gapRFP) were from Zolessi et al. (2006).

Constructs and antibodies. Constructs used for embryo injection: ath5:gapGFP (Zolessi et al., 2006), hsp70:mCherryCAAX, and hsp70:ncadhΔEC-ΔEC. mCherry. pDONR-ncadhΔEC was subcloned from pEGFP-N1-ncadhΔEC, a gift from Dr. James Jontes (Ohio State University, Columbus, OH), using primers to add att sites at the 5’ and 3’ ends of the N-cadherin sequence. hsp70:mCherryCAAX and hsp70:ncadhΔEC-mCherry constructs were made using the Tol2kit as published (Kwan et al., 2007). Entry clones, except pDONR-ncadhΔEC, were gifts from Drs. Kate Lewis (Syracuse University, Syracuse, NY) and Chi-bin Chien (University of Utah, Salt Lake City, UT).

Primary antibodies used were diluted in blocking solution as follows: Zn5, 1:500 (Zebrafish International Resource Center); anti-N-cadherin antibody, 1:200 (ab12221; Abcam); anti-GFP, 1:200 (11814460001; Roche); and anti-GFP-FITC-conjugated, 1:500 (ab6662; Abcam). Sec-
ordinary antibodies used were as follows: donkey or goat anti-mouse IgG conjugated to Alexa 488, 594, or 647 fluorophores (Invitrogen), diluted to 1:1000 in blocking solution.

**DNA, RNA, and morpholinos injections.** Plasmid DNA encoding the fluorescent proteins were injected into the cytoplasm of one-cell stage zebrafish embryos. ath5:gapGFP construct was injected together with translation blocking (5′-CCCTAACCTCAGTTACAATTTATA-3′); 0.2 ng of anti-slit1b, translation blocking (5′-GCTCGGTGTCGGCATCTCCAAAAG-3′) (Zolesi et al., 2006); 2 ng of anti-robo2, translation blocking (5′-GTAAAGGTTGTGTAAGGACCCCAT-3′); and a combination of 1 ng of anti-robo3var1, translation blocking (5′-CCCTAAAGGGCCTACAATCCACCTG-3′) and 1 ng of anti-robo3var2, translation blocking (5′-CTCTTATACGAAACGCACAGCCTC-3′) (Challa et al., 2005; Devine and Key, 2008).

**Blastomere transplantation.** Embryos were dechorionated with Pronase (30 mg/ml, Sigma). Blastomeres from transgenic donor embryos, Tg(ath5:gapRFP), injected with H2B-YFP mRNA, and morpholinos were transplanted into the animal poles of unlabeled host blastulas using a glass micropipette.

**Heat shock experiments.** Embryos injected with the heat-shock promoter-driven constructs were raised at 28.5°C until 20 or 24 h post-fertilization (hpf). The embryos were then incubated at room temperature for 30–60 min, transferred to a tube of prewarmed medium, and heat-shocked at 37°C for 1 h.

**RNA synthesis.** pCS2FA-transposase was a gift from Dr. Brian Link (Medical College of Wisconsin, Milwaukee, WI). The transposase mRNA was transcribed using the mMessage machine in vitro transcription kit (Ambion) from the SP6 promoter of pCS2FA-transposase, after linearization with NotI. The mRNA was subsequently purified using RNeasy RNA purification kit (Qiagen).

**In situ hybridization.** slit1b and robo3 antisense probes were generated by digesting pCR2.1-TOPO-slit1b-2C and pCR2.1-TOPO-robo3 with BamH1 and HindIII, respectively, then transcribing with T7 RNA polymerase. pCR2.1-TOPO-slit1b-2C and pCR2.1-TOPO-robo3 were gifts from Dr. Chi-bin Chien. Whole-mount in situ hybridization of slit1b mRNA was performed on wild-type embryos as previously described (Shimamura et al., 1994), hybridized embryos were subsequently sectioned for image acquisition. In situ hybridization for robo3 mRNA was performed on 20 μm cryosections as previously described (Butler et al., 2001).

**Statistical analysis.** The Mann–Whitney U test was used to compare the percentage of Ath5:gapGFP-expressing cells with un-retracted apical processes in WT and slit1b morphants per retina, using InStat software (GraphPad). Binomial test was used in all other experiments to assess statistical significance.

**Results**

slit1b and robo3 regulate apical process retraction of RGCs

To validate and extend the findings of Zolesi et al. (2006) on the role of Slit1b in apical retraction, embryos were injected with ath5:gapGFP with or without slit1b morpholino and fixed at 48 hpf. As Ath5:gapGFP is expressed by both the differentiated RGCs and their progenitors, Zn5, which labels the axon and soma of RGCs in zebrafish, was used as a definitive marker for the differentiated ganglion cells (Schmitt and Dowling, 1996). An Ath5:gapGFP+ RGC was judged to have an un-retracted apical process if its cell body was in the RGC layer and positive for Zn5 yet retained an apical process that extended outside of the ganglion cell layer (Fig. 1E, F, arrowheads). At 48 hpf, only few RGCs in control retinas had un-retracted apical processes, while a significant number of RGCs in the slit1b morphant retina retained un-retracted apical processes (Fig. 1). The reason that the RGC layer is thinner in slit1b morphants is probably that many RGC somas have trouble migrating basally when still attached apically (Zolesi et al., 2006). To check that this phenotype is not a result of a general developmental delay, we compared the relative timing of apical retraction to another RGC developmental event—axonogenesis using time-lapse analysis of Ath5:gapGFP-expressing RGCs.
mediated the Slit1b-regulated apical retraction. Among the four zebrafish robo genes, only robo2 and robo3 mRNA are detected in the developing retina (Lee et al., 2001). In zebrafish, two forms of robo3 exist: robo3v1 and robo3v2 (Challa et al., 2005). Therefore, to test whether Robo2 or 3 in RGCs is involved in apical process retraction, we injected Tg(ath5:gapRFP) embryos with control, robo2, or a combination of robo3v1 and robo3v2 morpholinos and H2B-YFP mRNA as a nuclear marker of donor cells. Blastomeres from injected embryos were then transplanted into uninjected wild-type hosts of the same stage. We found that Ath5:gapRFP-positive cells from control and robo2 morpholino-injected embryos retracted their apical processes normally, while Ath5:gapRFP-positive cells from robo3 morphants retained apical processes (Fig. 2 B, D, F). Thus, robo3 knockdown, but not robo2 knockdown, phenocopies slit1b morphants, suggesting that Robo3 is the receptor downstream of Slit1b during apical retraction.

An important question is why Slit1b and Robo3 do not cause neuroepithelial cells to detach before their final division. RGCs of the embryonic zebrafish retina go through their final S-phase between 30 and 40 hpf (Hu and Easter, 1999) and finish mitosis a few hours later (Poggi et al., 2005). Using in situ hybridization at a variety of time points between 24 and 40 hpf, we first detected a clear signal for both slit1b and robo3 mRNA in scattered cells at ~36–38 hpf (Fig. 2 H, I). Thus, the timing of expression of this receptor–ligand pair may provide part of the answer to this question.

Expression of dominant-negative N-cadherin leads to premature apical process retraction

Immunohistochemistry reveals that N-cadherin is expressed strongly in the developing zebrafish retina (Liu et al., 2001), consistent with a possible role in apical retraction. As N-cadherin is expressed in the apical processes of Ath5:gapGFP-positive RGC progenitors (Fig. 3 A), we investigated whether it is involved in apical process attachment using a dominant-negative construct lacking the extracellular N-cadherin domain (Niemann et al., 1999; Jontes et al., 2004). To bypass the severe defects that result from disrupting N-cadherin in early development (Pujic and Malicki, 2001), we controlled the expression of the dominant-negative protein by a heat-shock-inducible promoter. Expression was induced by a 1 h heat-shock at 37°C at 20 hpf in Tg(ath5:gapGFP) embryos either injected with hsp70:ncadh1ΔEC-mCherry or a control construct (hsp70:tmCherryCAAX). The injected embryos were fixed at 36 hpf. As most RGCs have just exited cell cycle at 36

starting at 35 hpf. We found that only 31.6 ± 10.6% of normal RGCs sent out axons before retracting their apical processes (n = 19), but in slit1b morphants, although axonogenesis was not premature, 68.4 ± 9.1% of RGCs extended an axon while retaining an apical process (n = 26). This significant difference (p < 0.01) indicates that apical retraction is inhibited compared with axonogenesis by the knocking down Slit1b.

Robo proteins are receptors for Slit ligands. Therefore, we wondered whether newborn RGCs express Robo, which could...
hp, we speculated that most of the newly born RGCs would still retain apical processes (Hu and Easter, 1999). As expected, the majority of the Ath5:gapGFP + cells expressing hsp70:mCherryCAAX had unretracted processes attached to the apical surface. However, Ath5:gapGFP + cells expressing the dominant-negative N-cadherin protein had fewer un-retracted apical process (Fig. 3D). Thus, N-cadherin function appears to be essential for the attachment of newborn RGC apical processes.

Dominant-negative N-cadherin rescues the retraction phenotype in slit1b morphants

As we found that blocking Slit1b/Robo3 signaling inhibits apical retraction, whereas interfering with N-cadherin function leads to precocious retraction in developing RGCs. One possible explanation for these opposing phenotypes is that Slit/Robo signaling normally downregulates N-cadherin-mediated apical adhesion. If this was the case, the dominant-negative N-cadherin phenotype should be epistatic to the Slit1b phenotype. To test this notion, embryos were coinjected with control morpholino and hsp70mCherryCAAX or hsp70:nCadhΔEC-mCherry, or with slit1b morpholino and hsp70mCherryCAAX or hsp70:nCadhΔEC-mCherry. Embryos were then heat-shocked at 24 hpf and fixed at 48 hpf. Knockdown of Slit1b was confirmed to inhibit apical retraction, as shown by a significantly higher proportion of Hsp70: mCherry-expressing RGCs with un-retracted apical processes in slit1b morphants compared with those in the control morphants (Fig. 4A–F). The Hsp70:nCadhΔEC-mCherry-expressing RGCs, however, retracted their apical processes efficiently in these morphants (Fig. 4G), showing that blocking N-cadherin function in the morphant cells rescues apical retraction. These results suggest a model in which Slit-Robo signaling in newborn RGCs downregulates N-cadherin-mediated attachment at the apically located adherens junction, allowing apical retraction upon cell cycle exit.

Discussion

In this report, we define a putative molecular pathway underlying the retraction of apical processes in newborn RGCs. We first show that knocking down Slit1b inhibits apical process retraction, corroborating previous data (Zolesi et al., 2006). We identified Robo3 as the likely receptor for Slit1b in this process. As work in other systems has shown that Slit/Robo signaling is capable of modulating cadherin function (Rhee et al., 2002; Medioni et al., 2008; Santiago-Martinez et al., 2008), we next investigated whether N-cadherin, which is known to be part of the adherens junctions at the apical complex in neuroepithelial cells, is also involved in the process of RGC apical retraction. We found that the expression of a dominant-negative N-cadherin in RGCs leads to premature detachment of their apical processes and rescues the apical detachment phenotype in slit1b morphants. Our results are thus consistent with the possibility that N-cadherin is downregulated by Slit-Robo signaling, leading to the loss of apical adhesion. To establish further details of this molecular pathway, it will be necessary to investigate the links between Slit/Robo signaling and N-cadherin. Epithelial/mesenchyme transition has been found to be mediated by Rab5-dependent endocytosis of E-cadherin (Fujita et al., 2002; Palacios et al., 2005; Emery and Knebelich, 2006), and E-cadherin internalization can be induced by soluble factors (Kamei et al., 1999; Ulrich et al., 2005; Bryant et al., 2007). Preliminary experiments
with endocytosis inhibitors and the expression of dominant-negative Rab5, however, failed to interfere with apical retraction of RGCs in our system (data not shown), suggesting that other effectors might be at play. Likely candidates include those discovered in cell culture systems by Rhee et al. (2002, 2007), who found that Slit activation of Robo leads to the phosphorylation of β-catenin for N-cadherin and reduces N-cadherin-mediated adhesion by severing its link to the cytoskeleton.

Transient disruption of integrin functions by injection of blocking antibody in mouse ventricular zone leads to detachment of apical processes from the ventricular surface (Loulier et al., 2009); this suggests that integrin signaling could also play a role in the apical retraction of neuroepithelial cells in the developing mouse cortex. Our results, though more consistent with a role for adherens junctions in apical attachment as suggested by Cappello et al. (2006) and Imai et al. (2006), do not rule out the possibility that both cadherins and integrins are involved in the attachments of apical processes, and that neither of them alone is sufficient. Given the expression of Slits, Robos, and Cadherin in different developing tissues (Bitzur et al., 1994; Challa et al., 2001; Lee et al., 2001; Hutson et al., 2003), we propose that Slit/Robo signaling and cadherin downregulation may be a common mechanism of apical processes retraction.

Precocious detachment affects the proliferation potential (Cappello et al., 2006; Imai et al., 2006), and failure to retract would inhibit the temporally orchestrated migration to distant sites, such as the cerebral cortex. Obviously a key issue for future research is what regulates the timing of this signaling. One attractive possibility is that Robo3 is only expressed when retinal cells have completed their final mitosis. Our in situ results on the timing and localized expression of robo3 mRNA in subsets of retinal cells are consistent with this idea. Interestingly, Slit1b expression is also turned on just at this stage, so, even if retinal cells prematurely express Robo3, they cannot detach until Slit1b is expressed and vice versa. We hope this work will stimulate further research into these relatively unexplored questions of developmental cell biology.

References


Figure 4. Expression of dominant-negative N-cadherin rescues apical retraction in slit1b morphant RGCs. A–H, Confocal images from control morpholino-injected (CoMO) embryos expressing Hsp70:mCherryCAAX (A, B) or Hsp70/NcadhΔEC-mCherry (C, D); confocal images from slit1b morphants expressing Hsp70:mCherryCAAX (E, F) or Hsp70/NcadhΔEC-mCherry (G, H) at 48 hpf. Zn5 (pseudocolored in green) labels the ganglion cells. Boxed regions in A, C, E, and G are enlarged in B, D, F, and H, respectively. Arrowheads in F indicate the un-retracted apical process of an Hsp70:mCherryCAAX-expressing RGC (defined as cells that are Zn5+) in a slit1b morphant; arrowheads in B, F, and H indicate mCherry-positive RGCs with retracted apical processes. Scale bars, 20 μm. I, Percentage of mCherry-positive RGCs with un-retracted apical processes at 48 hpf (n = 44 Hsp70:mCherryCAAX-expressing cells from control morpholino-injected embryos; n = 61 Hsp70/NcadhΔEC-mCherry-expressing cells from control morpholino-injected embryos; n = 38 Hsp70:mCherryCAAX-expressing cells from slit1b morphants; n = 41 Hsp70/NcadhΔEC-mCherry-expressing cells from slit1b morphants). Error bars indicate SEs (**p < 0.01; ***p < 0.001).


