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Semaphorin 3A induces Ca_v2.3 channel-dependent conversion of axons to dendrites

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Abstract

Polarized neurites, axons and dendrites, form the functional circuitry of the nervous system. Secreted guidance cues often convert the polarity of neuron migration and neurite outgrowth by regulating ion channels. Here, we show that secreted semaphorin 3A (Sema3A) converts the neurite identity of *Xenopus* spinal commissural interneurons (xSCINs) by activating Ca_v2.3 channels (Ca_v2.3). Sema3A treatment converted the identity of axons of cultured xSCINs to that of dendrites by recruiting functional Ca_v2.3. Inhibition of Sema3A signalling prevented both the expression of Ca_v2.3 and acquisition of the dendrite identity, and inhibition of Ca_v2.3 function resulted in multiple axon-like neurites of xSCINs in the spinal cord. Furthermore, Sema3A-triggered cGMP production and PKG activity induced, respectively, the expression of functional Ca_v2.3 and the dendrite identity. These results reveal a novel mechanism by which a guidance cue controls the identity of neurites during nervous system development.

Developing neurons become polarized to form axons and dendrites^{1–3}. How immature neurites of developing neurons acquire identities as axons/dendrites is unexplained. It was recently revealed that extracellular signaling molecules induce the axon identity^{4, 5}, but it remains unknown whether they also induce the dendrite identity, and if so, how.

In vivo, acquisition of an identity as either an axon or dendrite occurs at different phases of neuronal polarization; a developing neuron initially acquires an axon extending from the soma and subsequently either a single dendrite as in *C. elegans*⁶ or multiple dendrites as in most vertebrates^{1, 2}. Dendrites develop from undifferentiated neurites that emanate either

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AUTHOR CONTRIBUTIONS

M.N., T.K., M.J.vS. and K.H. designed, carried out experiments and analyzed the data; C-S.L. helped immunocytochemistry; N.Y. and Y.G. provided proteins; M.N., T.K., S-I.M. and S.I. carried out or supervised *in vivo* imaging analyses; M.N. and K.H. supervised the project and wrote the manuscript.

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directly from the soma, e.g., of cerebellar granule cells², or from the tip of the leading process, e.g., of cortical pyramidal neurons², or from the axonal initial segment (AIS), e.g., of xSCINs^{7, 8}. For example, after xSCIN axons cross the midline of the floor plate and bifurcate into ascending and descending axon branches on the opposite side of the lateral fascicle, multiple neurites extend toward the pial surface and ventrally into the marginal zone from the AIS at tailbud stages 32–34 (ref. 7). How these undifferentiated neurites acquire their dendrite identity is unknown. It is likely that specific, spatiotemporally-regulated extracellular signals activate intracellular signalling pathways to specify the dendrite identity.

Extracellular guidance factors can induce either attraction or repulsion of growth cones^{9, 10}, or reverse the direction of neuron migration¹¹ by causing changes in intracellular Ca^{2+} levels, suggesting that external factors trigger early signalling events that initiate cellular processes by regulating ion channels. *Sema3A*, a secreted molecule, functions in several neuronal processes, including the orientation and maturation of dendrites^{12–15}. *Sema3A* binds to its receptor, neuropilin-1 (Npn-1), in *Xenopus* spinal neuron growth cones and triggers local production of cGMP by soluble guanylate cyclase (sGC)¹⁶. Depending on their intracellular cGMP level ($[\text{cGMP}]_i$), growth cones are either repelled from or attracted toward *Sema3A*; low $[\text{cGMP}]_i$ induces repulsion¹⁶ through the activation of cyclic nucleotide-gated cation channels¹⁷ and high $[\text{cGMP}]_i$ induces attraction^{16, 18} through the activity of an unidentified channel(s). High level *Sema3A* expressed at the pial surface of the neocortex contributes to the attraction of dendrites¹². Interestingly, a relatively high level of *Sema3A* is expressed in the marginal zone of the *Xenopus* spinal cord at tailbud stages 30–32 (ref. 17), immediately before the emergence of putative dendrites from the AIS of spinal cord CINs (ref. 7). Here, we report that, both *in vitro* and *in vivo*, *Sema3A* induces the dendrite identity and simultaneously suppresses the axon identity of xSCINs by regulating cGMP signalling and the expression of functional $\text{Ca}_v2.3$ channels ($\text{Ca}_v2.3$).

RESULTS

Sema3A converts the identity of axons to dendrites *in vitro*

Unlike cultured hippocampal neurons¹⁹, cultured xSCINs from stage 26 embryos^{16, 17} have neurites that are predominantly mono- (Fig. 1a–d, f–i) or bi-polar (Supplementary Fig. S1a, b), without substantial branches (Supplementary Fig. S2) or other distinguishing features of axons and dendrites. When cultured on poly-L-lysine for 16 hours, most neurons were unpolarized, immunonegative for both axonal markers tau-1 and GAP-43 and dendritic marker MAP2 (Supplementary Fig. S3a, b). When cultured on laminin for either three or six hours, most of these unpolarized neurites became tau-1-positive axons²⁰ (Fig. 1a, e). However, when these neurites were treated with *Sema3A* for three hours, the tau-1 immunoreactivity (IR) was markedly decreased (Fig. 1b, e) while MAP2-IR was significantly increased (Fig. 1d, e). Both changes were prevented by bath-applied SNP-1, the soluble Npn-1 ectodomain peptide that sequesters *Sema3A* (Fig. 1e)²¹, indicating the specificity of *Sema3A*. *Sema3A* promotes the outgrowth and branching of dendrites of cortical pyramidal neurons^{13, 14} and axotomy causes conversion of pre-formed axons of cultured hippocampal neurons to dendrites^{22, 23}. We did not observe significant morphological changes in the length or branching of xSCIN neurites after *Sema3A* treatment (Supplementary Fig. S2). Netrin-1, another secreted factor that effects axon initiation in *C. elegans*⁴, had no significant effect on the expression of tau-1- or MAP2-IR (Fig. 1e). We also monitored functional α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, which are present predominantly in dendrites^{22, 24}, by measuring holding current shifts induced by a gradient of AMPA. Significant AMPA currents were detected in almost all growth cones treated with *Sema3A*, but only in less than

30% of control growth cones (Supplementary Fig. S4). Thus, *Sema3A* likely initiates conversion of the identity of axons to dendrites before morphological changes occur.

To determine whether conversion of the neurite identity initiates within the growth cone, we applied a gradient of *Sema3A* to one growth cone of bipolar neurons (Supplementary Fig. S1a). The majority of those neurites that faced the source of the *Sema3A* became MAP2-positive dendrites but those that faced away from the source remained MAP2-negative (Supplementary Fig. S1b, c). Downstream signalling events induced by *Sema3A* in the growth cone, therefore, are likely sufficient to initiate conversion of the neurite identity.

Sema3A-induced conversion of neurite identity requires $\text{Ca}_v2.3$

Guidance cues induce growth cone membrane potential shifts; attractants cause depolarization and repellents cause hyperpolarization¹⁶. A relatively high concentration of *Sema3A* induced growth cone depolarization of ca. 15 mV from the resting potential of control growth cones, but not in growth cones pre-treated with SNP-1 (data not shown) or that overexpressed a mutant Npn-1 [NP-1 (0111)]^{16, 17} (Supplementary Fig. S5b, c). This 15 mV depolarization should be sufficient to cause Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Functional L-, N-, R ($\text{Ca}_v2.3$)- and T-type Ca^{2+} channels, but not P/Q types²⁵, have been detected in cultured embryonic *Xenopus* spinal neurons^{26–29}. We used a computed biophysical growth cone model¹⁷ to identify those Ca^{2+} channels that would be active preferentially during *Sema3A*-induced membrane depolarization. This model predicted that mainly $\text{Ca}_v2.3$ would conduct Ca^{2+} , that T-types would undergo voltage-dependent inactivation, and that activation of L-/N-types would require further depolarization (Supplementary Fig. S5d). We then examined the effect of $\text{Ca}_v2.3$ on the ability of *Sema3A* to induce the dendrite identity. A specific $\text{Ca}_v2.3$ inhibitor, SNX-482, in the bath, indeed, prevented conversion of the tau-1-positive axons to the MAP2-positive dendrites (Fig. 1h–j), but inhibitors of neither L-types (nimodipine) nor N-types (ω -conotoxin GVIA) prevented the conversion (Fig. 1j). Thus, *in vitro*, *Sema3A*-induced conversion of xSCIN axons to dendrites depends on $\text{Ca}_v2.3$ activity.

Sema3A recruits functional $\text{Ca}_v2.3$

To determine whether *Sema3A* causes activation of $\text{Ca}_v2.3$, we measured Ca^{2+} currents in the growth cone^{17, 29} in the presence of a gradient of *Sema3A*. High voltage-activated (HVA) Ca^{2+} currents were measured in the presence of bath-applied pimezide, a T-type channel blocker. We then isolated N-type and $\text{Ca}_v2.3$ currents by blocking residual T- and L-type currents with nimodipine²⁹. *Sema3A* caused an increase in total N-type and $\text{Ca}_v2.3$ currents (Fig. 2b, e) without affecting the magnitude of total HVA currents (Fig. 2a, e), indicating that *Sema3A* suppressed L-type currents. We further isolated the *Sema3A*-induced growth cone Ca^{2+} currents using Ni^{2+} ($\text{Ca}_v2.3$ and T-type channel blocker) and nimodipine and found that N-type currents were significantly decreased (Fig. 2c, e). In contrast, *Sema3A* caused a 4–5 fold increase in $\text{Ca}_v2.3$ currents isolated in the presence of nimodipine and ω -conotoxin GVIA (Fig. 2d, e) that were abolished by bath-applied SNX-482 (Fig. 2d, e). Therefore, *Sema3A* caused an increase in the active pools of $\text{Ca}_v2.3$ that conduct Ca^{2+} during the ca. 15 mV membrane depolarization (Supplementary Fig. S5d) and suppressed L-/N-type currents. Since *Sema3A* did not alter the activation/inactivation time constants of the $\text{Ca}_v2.3$ currents (Supplementary Table S1), it likely caused an increase in the number of $\text{Ca}_v2.3$ in the growth cone plasma membrane rather than modulating their gating properties (Supplementary Fig. S5e).

Expression of $\text{Ca}_v2.3$ *in vivo* requires *Sema3A*

To examine the role of *Sema3A* in $\text{Ca}_v2.3$ function *in vivo*, we first tested the effect of down-regulating endogenous *Sema3A* by injecting an antisense-morpholino oligonucleotide

(*A*-MO) against *Xenopus* *Sema3A* (*xSema3A*) mRNA [*xSema3A-A*-MO; a sense-MO (*xSema3A-S*-MO) served as a control] into one dorsal blastomere of four-cell stage embryos along with GFP mRNA as an injection marker. We then immunostained whole-mounts³⁰ and imaged the immuno- and GFP fluorescence (Fig. 3a) to compare the MO-injected side of the spinal cord with the uninjected side in the same embryo (Fig. 3a) and also with uninjected control embryos. Immunostaining with a polyclonal antibody against human *Sema3A* (*hSema3A*) revealed a high level of bilateral expression of *xSema3A* in stage 30–32 spinal cords (Fig. 3b, b') that was reduced significantly by *xSema3A-A*-MO (Fig. 3c, c', f and Supplementary Movie S1), but not by *xSema3A-S*-MO (Fig. 3d, d', f), attesting to the specificity of the *A*-MO. The reduction of *xSema3A*-IR was reversed by the injection of *hSema3A* mRNA along with *xSema3A-A*-MO (Fig. 3e, e', f), confirming the specificity of the *Sema3A* antibody.

We then examined the effect of *Sema3A* down-regulation on the expression of *Ca_v2.3*. Immunostaining with polyclonal antibodies against human *Ca_v2.3* α_{1E} revealed a pattern of expression of *Xenopus* *Ca_v2.3* α_{1E} (*xCa_v2.3* α_{1E}) in the lateral marginal zones of the spinal cord (Fig. 3g, g') similar to that of *xSema3A* (Fig. 3b, b'). The specificity of the anti-*Ca_v2.3* α_{1E} antibodies was confirmed by their detection of over-expressed mouse *Ca_v2.3* α_{1E} (*mCa_v2.3* α_{1E}) when endogenous *xCa_v2.3* α_{1E} was down-regulated by a specific *A*-MO (*xCa_v2.3* α_{1E} -*A*-MO; Supplementary Fig. S6). The endogenous expression of *xCa_v2.3* α_{1E} became prominent at stage 32–34, following the prominent expression of *xSema3A* at stage 30–32. Strikingly, the expression of *xCa_v2.3* α_{1E} was reduced markedly by injection of *xSema3A-A*-MO [Fig. 3h, h', k and Supplementary Movie S2] but not the control *xSema3A-S*-MO (Fig. 3i, i', k). The effect of *xSema3A-A*-MO was specific since over-expression of *hSema3A* reversed the reduction of *xCa_v2.3* α_{1E} (Fig. 3j, j', k). These results demonstrate that *Sema3A* is required for the expression of *Ca_v2.3* in the *Xenopus* spinal cord *in vivo*.

Sema3A*-*Ca_v2.3* signalling is required for the dendrite identity *in vivo

We next confirmed that *Sema3A* signalling is required to specify the dendrite identity *in vivo* by analyzing MAP2-IR in whole-mount spinal cords. Dendrites of developing spinal neurons first became prominently MAP2 positive (Fig. 4a, b) at the stage 42 tadpole (Fig. 4a, left). MAP2-IR showed relatively simple dendritic arbors projecting ventrally (Fig. 4b, c, c'). Their morphology and the location of their soma suggest these neurons are highly likely to be either CINs or ascending interneurons³¹. We found that MAP2-IR in the *Sema3A-A*-MO injected (Fig. 4d, d', g and Supplementary Movie S3), but not the *xSema3A-S*-MO injected (Fig. 4e, e', g) side of spinal cords was significantly reduced compared to the uninjected side of the same spinal cords, and this effect was reversed by over-expression of *hSema3A* (Fig. 4f, f', g). Injection of *A*-MO against *xNpn-1* (Fig. 4g) but not the control *xNpn-1-S*-MO (Fig. 4g) also significantly reduced the MAP2-IR in the injected side of the spinal cord. Thus, *Sema3A* is required to specify the dendrite identity.

Because the reduction of MAP2-IR caused by down-regulation of either *xSema3A* or *xNpn-1* could have caused defects in cell migration³², we examined the effects of down-regulation of *xCa_v2.3* α_{1E} on the dendrite identity. Consistent with the idea that *Ca_v2.3* is a *Sema3A* downstream effector required to specify the dendrite identity, MAP2-IR was significantly reduced by *xCa_v2.3* α_{1E} -*A*-MO, to an extent similar to that caused by *Sema3A-A*-MO (Fig. 5a, a', f and Supplementary Movie S4), but not by *xCa_v2.3* α_{1E} -*S*-MO (Fig. 5b, b', f). Injection of the *mCa_v2.3* α_{1E} mRNA together with *xCa_v2.3* α_{1E} -*A*-MO sufficiently reversed the reduction of MAP2-IR (Fig. 5c, c', f) resulting from injection of *xCa_v2.3* α_{1E} -*A*-MO alone. However, restoration of MAP2-IR by *mCa_v2.3* α_{1E} in the side of the spinal cord treated with *xCa_v2.3* α_{1E} -*A*-MO occurred only in the presence of endogenous *xSema3A* (Fig. 5c, c', f), not when *xSema3A* was down-regulated (Fig. 5e, e',

f), suggesting other parallel signal(s) are also required to specify the dendrite identity. We also tested the effects of pharmacological blockade of $\text{Ca}_v2.3$ on the acquisition of the dendrite identity by administering different doses of SNX-482 into the spinal cords of stage 30–32 tailbuds (Fig. 5g). Administration of 45 μM SNX-482 eliminated almost all dendrites (Fig. 5k, k', l) while lower doses caused significant reductions (Fig. 5i, i', j, j', l). The local administration of nimodipine (Fig. 5h, h', l) or ω -conotoxin GVIA (Fig. 5l) had no significant effect. These results support the idea that the *Sema3A* signal propagates through $\text{Ca}_v2.3$, whose activity is required for the acquisition of the dendrite identity.

$\text{Ca}_v2.3$ suppresses the axon identity *in vivo*

Since *Sema3A* induced the conversion of axons of cultured *xSCINs* to dendrites *in vitro* (Fig. 1), we tested whether down-regulation of $\text{Ca}_v2.3$ expression also affects the axon identity *in vivo*. Whole mounts of normal stage 42 tadpole spinal cords showed equal intensities of bilateral axon tracts along their longitudinal axis (Fig. 6a, b, b'). Injection of *xCa_v2.3* α_{1E} -A-MO (Fig. 6c, c', g), but not the control *xCa_v2.3* α_{1E} -S-MO (Fig. 6d, d', g), resulted in a significant increase of tau-1-IR in the injected side of the spinal cords (Fig. 6c', d'), as compared with control, uninjected animals (Fig. 6b, b', g). The increased tau-1-IR was particularly noticeable close to the ventral midline (Fig. 6c, c') where it is normally absent (Fig. 6b, b'), implying that the default neuronal polarization state in the absence of $\text{Ca}_v2.3$ is that axons. Furthermore, local administration of SNX-482 into the spinal cord caused a significant increase in tau-1-IR throughout the spinal cord (Fig. 6e, e', g). In contrast, administration of neither nimodipine (Fig. 6f, f', g) nor ω -conotoxin GVIA (Fig. 6g) had significant effect. Western blot analysis showed that the ratio of MAP2 to tau-1 in total protein extracted from the SNX-482-treated spinal cords was significantly lower than that from untreated, control, spinal cords (Supplementary Fig. S7), corroborating the immunohistochemical observations of spinal cords shown above. Thus, $\text{Ca}_v2.3$ activity is required not only for acquisition of the dendrite identity but also for suppression of the axon identity.

To exclude off-target effects of A-MO treatment, we examined the neuronal integrity of *xCa_v2.3* α_{1E} -A-MO-treated animals. We microinjected Texas Red conjugated with 70K-dextran into one dorsal blastomere of 64-cell stage embryos into which *xCa_v2.3* α_{1E} -A-MO had previously been injected at the four-cell stage (Fig. 6h). We then visualized individual spinal neurons in the MO-injected side of stage 42 tadpole spinal cords by their Texas Red. We imaged isolated, putative CINs whose soma were located ca. two thirds of the distance from the ventral midline and whose single axon and dendrites projected ventrally (Fig. 6i, k, control). Individual putative CINs in the side of spinal cords treated with *xCa_v2.3*-A-MO, indeed, showed multiple axon-like neurites that emanated from the proximal segment and many that projected longitudinally in the ipsilateral axon tract (Fig. 6j, l), but such axon-like neurites were only rarely observed in control animals (Fig. 6i, k). The ipsilateral projection (Fig. 6j, l) of these putative CIN axons may be a result of their reduced sensitivity to netrin-1 (ref. 33). These results clearly demonstrate that the activity of $\text{Ca}_v2.3$ suppresses the axon identity.

Cyclic GMP-dependent expression of $\text{Ca}_v2.3$ and PKG-dependent acquisition of the dendrite identity

We previously showed that *Sema3A* triggers the local production of cGMP by sGC (ref. 16). A recent study showed that cGMP-PKG signalling induces initiation of the dendrite identity in undifferentiated neurites of cultured hippocampal neurons³⁴. Interestingly, bath-application of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of sGC, prevented the induction of $\text{Ca}_v2.3$ currents by a gradient of *Sema3A* in *xSCIN* growth cones (Fig. 7a). Moreover, inhibition of sGC by local administration of ODQ abolished not only

Ca_v2.3 α_{1E} expression (Fig. 7b, b', h), but also dendrite formation (Fig. 7e, e', h), suggesting that cGMP signalling functions as an upstream regulator of the expression of Ca_v2.3 as well as acquisition of the dendrite identity. Surprisingly, a gradient of Rp-8-*p*CPT-cGMPS, a PKG-inhibiting cGMP analogue¹⁶ induced Ca_v2.3 currents (Fig. 7a) and pre-empted their induction by Semaphorin 3A (Fig. 7a). Moreover, Rp-8-*p*CPT-cGMPS-induced Ca_v2.3 currents were not affected by ODQ, concordant with the notion that cGMP is required to target functional Ca_v2.3 to the plasma membrane. Importantly, these Ca_v2.3 currents were eliminated by pretreatment with cycloheximide (CHX), a protein synthesis inhibitor (Fig. 7a), suggesting that cGMP, not PKG, mediates both the Semaphorin 3A-induced synthesis of Ca_v2.3 proteins and their targeting to the plasma membrane *in vitro*. Rp-8-*p*CPT-cGMPS also caused a significant increase in the expression of α_{1E} Ca_v2.3 (Fig. 7c, c', h), which extended beyond the marginal zones of the spinal cord. It is noteworthy that pre-treatment with CHX eliminated the Semaphorin 3A-induced expression of Ca_v2.3 both *in vitro* and *in vivo* (Supplementary Fig. S8), supporting the idea that cGMP regulates the expression of functional Ca_v2.3 at the translational level. In contrast, Rp-8-*p*CPT-cGMPS drastically reduced MAP2-IR (Fig. 7f, f', h) to an extent similar to that caused by treatment with either ODQ (Fig. 7e, e', h) or SNX-482 (Fig. 5i-l), demonstrating that PKG activity is required for the acquisition of the dendrite identity. Consistent with these observations, local administration of 8-Br-cGMP, a PKG-activating cGMP analogue, enhanced both the expression of α_{1E} Ca_v2.3 (Fig. 7d, d', h) and dendrite formation (Fig. 7g, g', h).

Finally, we down-regulated the expression of either α_{1E} Sema3A or α_{1E} Ca_v2.3 with specific A-MOs in one side of spinal cords and tested the effects of varying the levels of cGMP on the expression of α_{1E} Ca_v2.3 or acquisition of the dendrite identity. Despite the absence of Semaphorin 3A, local administration of either Rp-8-*p*CPT-cGMPS (Fig. 7j, j', o) or 8-Br-cGMP (Fig. 7k, k', o) induced the expression of α_{1E} Ca_v2.3, while local administration of ODQ caused their significant reduction (Fig. 7i, i', o), supporting the idea that cGMP induces the expression of α_{1E} Ca_v2.3. In contrast, ODQ (Fig. 7l, l', o) or Rp-8-*p*CPT-cGMPS (Fig. 7m, m', o) prevented the appearance of dendrites even in the presence of the α_{1E} Ca_v2.3. However, local administration of 8-Br-cGMP failed to rescue dendrites in the absence of α_{1E} Ca_v2.3 (Fig. 7n, n', o), indicating that Ca_v2.3 is required for the acquisition of the dendrite identity. Taken together, our results reveal that Semaphorin 3A induced cGMP-PKG signalling regulates the cGMP-dependent expression of functional α_{1E} Ca_v2.3 that together with PKG as a co-effector specify the dendrite identity while suppressing axon identity.

DISCUSSION

Extracellular factors signal to regulate the initiation and maintenance of the axon identity^{4, 5, 35} and dendrite growth and branching^{13–15}. However, the signalling events that regulate the acquisition of an identity as a dendrite in response to extracellular factors are unknown. We demonstrate *in vitro* and *in vivo* that a secreted guidance factor, Semaphorin 3A, induces the dendrite identity and concomitantly suppresses the axon identity by inducing the expression of functional Ca_v2.3. We further demonstrate that Semaphorin 3A-triggered cGMP production and PKG activity are required, respectively, for the expression of Ca_v2.3 and acquisition of the dendrite identity.

We found that the relatively high Semaphorin 3A concentration required to convert the axons of cultured xSCINs to dendrites caused growth cone depolarization and the recruitment of functional Ca_v2.3 to the plasma membrane. We also found that increased expression of Semaphorin 3A occurs in close spatiotemporal proximity to the expression of Ca_v2.3 in the spinal cords of tailbud stage animals, when putative dendrites emanate from pre-existing axons. We propose that a high Semaphorin 3A concentration is required to induce Ca_v2.3-mediated Ca²⁺ signalling in growth cones, which, in turn, causes the up-regulation of MAP2 required to

specify the dendrite identity while simultaneously down-regulating the unphosphorylated tau required to specify the axon identity (see Fig. 8). Local protein synthesis and trafficking can occur in cultured growth cones of *Xenopus* retinal ganglion cells (xRGCs) within 5 minutes of their exposure to Semaphorin 3A (ref. 36). Thus, Semaphorin 3A signalling may induce cGMP-dependent *de novo* synthesis of Ca_v2.3 and their targeting to the plasma membrane. Semaphorin 3A has been demonstrated to activate the ERK (extracellular-signal-regulated-kinase)-TOR (target-of-rapamycin) signalling pathway during repulsion/collapse of cultured xRGC growth cones^{37, 38}. CNrasGEF, a cyclic nucleotide-activated guanine exchanging factor that activates GTPase Ras, could couple Semaphorin 3A-induced production of cGMP to the ERK-TOR signalling pathway³⁹ to induce *de novo* synthesis of Ca_v2.3. A similar mechanism could account for the cGMP-mediated targeting of Ca_v2.3 to the plasma membrane, since Ras/Rap1 are known to induce vesicle transport that trafficks the ionotropic glutamate receptor⁴⁰, and Semaphorin 3A enhances axonal vesicle transport²¹.

The identity of neurites as either axons or dendrites depends on the relative signalling of cAMP-PKA and cGMP-PKG; greater cGMP-PKG signalling causes undifferentiated neurites of hippocampal neurons to become dendrites³⁴. PKG antagonizes acquisition of the axon identity induced by PKA-dependent phosphorylation of LKB1 (serine/threonine kinase 11)^{35, 41} and glycogen synthase kinase-3 β (GSK3 β)⁴². Semaphorin 3A-induced PKG activity¹² may gate Ca²⁺ entry through Ca_v2.3 by inducing membrane depolarization¹⁶ and simultaneously opposing the PKA activity required for the axon identity. The details of the mechanism that underlies the activation of Ca_v2.3 required for the acquisition of the dendrite identity is unknown, but may require an unidentified factor(s) (Factor "X", Fig. 8). This hypothesized factor is unlikely any of the known signalling molecules that promote the axon identity because disruption of the expression of these molecules does not cause the conversion of axons to dendrites. Semaphorin 3A causes the phosphorylation of collapsin-response-mediator-protein-2 (CRMP2) through the action of cyclin-dependent-kinase-5 and GSK3 β (ref. 43) that is known to suppress the axon identity⁴⁴. Whether Ca_v2.3 functions by modulating the phosphorylation states of either tau-1 (ref. 45) or CRMP2 (ref. 44) requires further study.

Although the localization of Ca_v2.3 predominantly to dendrites⁴⁶ and spine heads⁴⁷ has been demonstrated, their function *in vivo* has not been elucidated previously. Our study demonstrates the importance of the spatiotemporal and dynamic expression patterns of both guidance factors and ion channels in establishing neurite identity during nervous system development. Ca_v2.3-induced acquisition of the dendrite identity and the simultaneous suppression of the axon identity may also contribute to the Semaphorin 3A-induced failure of axons to regenerate after nerve injury⁴⁸.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Sema3A converts the identity of axons to dendrites by $Ca_v2.3$ activity. (**a–d, f–i**) Bright field (left) and immunofluorescence (right) images of *x*SCINs cultured on a laminin substrate for six hours under various experimental conditions. Insets, magnified fluorescent images of the same sections in either top or bottom. Untreated (Control; **a, c**), treated with Sema3A ($2 \text{ U}\cdot\text{ml}^{-1}$; **b, d, f, g**), or with Sema3A and SNX-482 ($1 \mu\text{M}$, +SNX-482; **h, i**), immunostained with tau-1 (**a, b, f, h**) or MAP2 (**c, d, g, i**). Scale bars, $10 \mu\text{m}$. (**e, j**) Summaries of the percentage of tau-1-positive growth cones and MAP2-positive neurites that emerged from the soma. Neurons were treated with Sema3A and SNP-1 (20 nM , +SNP-1; **e**), with netrin-1 ($5 \mu\text{g}\cdot\text{ml}^{-1}$; **e**), with Sema3A and SNX-482 (**j**), with Sema3A and nimodipine ($20 \mu\text{M}$, +nimo; **j**), or with Sema3A and ω -conotoxin GVIA ($1 \mu\text{M}$, +CgTX; **j**). The dark grey bar = tau-1 positive growth cones cultured on a laminin substrate for 3hrs (**e**).

Data are mean $\pm$ s.e.m. (n) = number of cultures (ca. 210 to 450 neurons with neurites of ca. 100–250 μ m long) examined. Significant differences from Control (**e**) and Sema3A (**j**) are indicated (**p < 0.01), respectively.

Figure 2.

Sema3A increases growth cone $\text{Ca}_v2.3$ currents. (**a–d**) Representative traces of Ca^{2+} currents (left) and summaries of the current (I)/voltage (V) relationship (right) in the presence of 500 nM pimoizide (PMZ, total HVA currents; **a**), 20 μM nimodipine (nimo, N- & R-type ($\text{Ca}_v2.3$) currents; **b**), 20 μM nimo + 300 μM Ni^{2+} (N-type currents; **c**), 20 μM nimo + 1 μM ω -conotoxin GVIA (CgTX, $\text{Ca}_v2.3$ -type currents; **d**). (**e**) Average peak Ca^{2+} currents shown in **a–d**. $\text{Ca}_v2.3$ currents were enhanced from 56 ± 4 pA to 246 ± 19 pA. Data are mean $\pm$ s.e.m. (n) = number of growth cones examined for each experimental condition.

Significant differences from the control (culture medium) are indicated (* $p < 0.05$; ** $p < 0.01$).

Figure 3.

Sema3A is required for Ca_v2.3 expression in *Xenopus* spinal cords. **(a)** Schema depicting experimental paradigms. Injection of morpholino oligonucleotides (MOs) or mRNAs encoding either a green fluorescence protein (GFP) marker or *hSema3A* into one dorsal blastomere of a 4-cell stage embryo (left) to treat one side of the spinal cord while the contralateral side served as an internal control. Whole mount preparations of stage 32 (for Sema3A) or stage 34 (for Ca_v2.3) tailbud spinal tissues (centre) were immunostained with antibodies: anti-Sema3A to detect xSema3A and anti-Ca_v2.3 α_{1E} to detect xCa_v2.3 α_{1E} . Spinal cord schema is overlaid with an immunofluorescence image showing the expression of xSema3A in the non-injected side (left), but not in the injected side (right). **(b–e, g–j)**

Immunofluorescence images of *Xenopus* spinal cords immunostained for *xSema3A* (**b–e**) or *xCav2.3* α_{1E} (**g–j**), without MO injection (Control; **b, g**) and with injection of antisense (*A*-MO; **c, e, h, j**) or sense (*S*-MO; **d, i**) MO specific for either *xSema3A* or *xCav2.3* α_{1E} . Signals in red (**b'–e', g'–j'**) indicate the localization of *xSema3A* (**b'–e'**) and *xCav2.3* α_{1E} (**g'–j'**) IRs, which are overlaid with signals from the GFP co-injection marker (+GFP, green; **c'–e', h'–j'**). Scale bar, 20 μ m. (**f, k**) Summaries of normalized *xSema3A* (**b–e**) and *xCav2.3* α_{1E} (**g–j**) IRs. Injected sides of spinal cords were normalized to the contralateral, non-injected sides. For non-injected controls, the right sides of spinal cords were normalized against the left sides. Data are mean $\pm$ s.e.m. (n) = number of spinal cords examined. Significant differences from control are indicated (**p < 0.01).

Figure 4.

Sema3A signalling is required for acquisition of the dendrite identity of putative CINs *in vivo*. **(a)** A whole-mount preparation of a *Xenopus* stage 42 tadpole (left) and spinal cord schema overlaid with immunofluorescence images of MAP2-positive dendrites (right). **(b)** An immunofluorescence image of a lateral view of MAP2-positive dendrites (control, left) and schematic diagram of CIN neurite trajectories (right). MAP2-IR reveals dendrites projecting ventrally, suggesting that the majority of MAP2-positive dendrites are those of CINs. **(c–f)** Immunofluorescence images of spinal cords without MO-injection (Control; **c**, **c'**), or injected with antisense (A-MO; **d**, **d'**) or sense (S-MO; **e**, **e'**) MO specific to

xSema3A or *xSema3A-A-MO* together with *hSema3A* mRNA (**f**, **f'**). Traces in black (**c'**–**f'**) indicate the MAP2-positive dendrite trajectories and are overlaid with signals from the GFP marker (green). (**g**) Summaries of normalized MAP2-positive volumes of samples treated with either *xSema3A*- or *xNpn-1*-MOs. Injected sides of spinal cords were normalized against the contralateral, non-injected sides. For the non-injected controls, the right sides of spinal cords were normalized against the left sides. Data are mean $\pm$ s.e.m. (n) = number of spinal cords examined. Significant differences from the control are indicated (** $p < 0.01$). Scale bars, 20 μ m.

Figure 5.

$\text{Ca}_V2.3$ function is required for the dendrite identity of xSCINs *in vivo*. (**a–e, h–k**) Immunofluorescence images of spinal cords injected with antisense (A-MO; **a, a'**) or sense (S-MO; **b, b'**) MO specific to $\text{xCa}_V2.3 \alpha_{1E}$, or $\text{xCa}_V2.3 \alpha_{1E}$ -A-MO or $\text{xCa}_V2.3 \alpha_{1E}$ -S-MO together with $\text{mCa}_V2.3 \alpha_{1E}$ mRNA (**c, c', d, d'**) or with xSema3A -A-MO together with $\text{mCa}_V2.3 \alpha_{1E}$ mRNA (**e, e'**), or with local administration of either nimodipine (nimo; **h, h'**) or the indicated concentrations of SNX-482 (**i, i', j, j', k, k'**). Traces in black (**a'–e', h'–k'**) indicate the MAP2-positive neurite trajectories and are overlaid with the signals from the GFP marker (green). (**f, i**) Summaries of normalized MAP2-positive volumes of samples treated with $\text{xCa}_V2.3 \alpha_{1E}$ -MOs, xSema3A -A-MO, or the local administration of Ca^{2+} channel blockers. For animals treated with MOs, injected sides of spinal cords were normalized against the contralateral, non-injected sides. For animals treated with Ca^{2+} channel blockers, the MAP2-positive volumes of the bilateral sides of the spinal cord were normalized against the average MAP2-positive volumes in control animals. (**g**) Administration of Ca^{2+} channel blockers (FITC as an injection marker) into the hindbrain and spinal cords of stage 30–32 tailbuds. Data are mean $\pm$ s.e.m. (n) = number of spinal cords examined. Significant differences from control are indicated (* $p < 0.05$; ** $p < 0.01$). Scale bars, 20 μm .

Figure 6.

$\text{CaV}2.3$ activity suppresses the acquisition of the axon identity of xSCINs *in vivo*. (a) A schematic diagram of the spinal cord was overlaid with an image of the immunofluorescence of tau-1-positive axons. (b–g) Immunofluorescence images (b–f) and summaries (g) of tau-1-IR of *Xenopus* spinal cords without MO injection (Control; b, b') and with injection of antisense (A-MO; c, c') or sense (S-MO; d, d') MO specific to $\text{xCaV}2.3 \alpha_{1E}$, or with local administration of SNX-482 (e, e') or nimodipine (nimo; f, f'). Signals in orange (b'–f') indicate the localization of tau-1-IR and are overlaid with the signals from the GFP marker (green; c', d'). The injected side of the spinal cord was normalized against the contralateral, uninjected side. For the uninjected controls, the right sides were normalized against the left sides. For local administration of Ca^{2+} channel antagonists, tau-1-IR of the bilateral sides of the spinal cord was normalized against the average tau-1-IR in control animals. Data are mean $\pm$ s.e.m. (n) = number of spinal cords examined. Significant differences from control are indicated (* $p < 0.05$). (h) Visualization of individual xSCINs and their neurite projections after the $\text{xCaV}2.3 \alpha_{1E}$ MO treatment. After the microinjection of MO together with Alexa-488 conjugated with 10K-dextran as an injection marker into one dorsal blastomere of four-cell stage embryos, Texas Red conjugated with 70K dextran was injected into one dorsal blastomere of a 64-cell stage. In this preparation, approximately one

sixteenth of the spinal neurons in one side of the spinal cord are visualized. (**i–l**) Ventrolateral views of a *Xenopus* spinal cord showing CIN axons crossing the midline (white arrows) in non-treated (**i, k**) and treated with $\alpha\text{Ca}_v2.3\text{-AMO}$ (**j, l**). In animals treated with $\alpha\text{Ca}_v2.3\text{-AMO}$, multiple axon-like neurites extending from the initial segment and migrating longitudinally are observed (**j, l**, yellow arrows).

Figure 7. Sema3A-induced cGMP causes recruitment and expression of $\text{Ca}_v2.3$ and PKG is required as a co-activator for acquisition of the dendrite identity. **(a)** Representative traces of Ca^{2+} currents (left), summary of the current (I) / voltage (V) relationship (centre) of $\text{Ca}_v2.3$ currents and cumulative distribution of peak $\text{Ca}_v2.3$ currents (right) monitored in cultured *xSCIN* growth cones. Either ODQ (1 μM), Rp-8-pCPT-cGMPS (2.5 μM) or cycloheximide (CHX, 25 μM) was applied in the bath 30 min before experiments were performed. Sema3A (2,000 U·ml $^{-1}$ in micropipettes) was applied to the growth cone as a gradient. **(b-g, i-n)** Immunofluorescence images of *Xenopus* spinal cords immunostained for $\text{xCa}_v2.3 \alpha_{1E}$ (b-d,

i–k) or MAP2 (**e–g, l–n**), without MO injection (**b–g**) or with injection of *A*-MO specific for either *xSema3A* (**i–k**) or *xCav2.3* α_{1E} (**l–n**). Signals in red (**b'–d', i'–k'**) and traces in black (**e'–g', l'–n'**) indicate the location of the *xCav2.3* α_{1E} and MAP2 IRs, respectively, which are overlaid with signals from the GFP injection marker (green, **i'–k', l'–n'**). ODQ (1 mM), Rp-8-*p*CPT-cGMPS (0.25 mM) and 8-Br-cGMP (1 mM) were injected into the hindbrain and spinal cord of stage 30–32 tailbud animals (see Fig. 5g). Scale bar, 20 μ m. (**h, o**) Summaries of normalized *xCav2.3* α_{1E} IR (**b–d, i–k**) and MAP2-positive volumes (**e–g, l–n**). *xCav2.3* α_{1E} IR and MAP2-positive volumes in bilateral sides of the spinal cord treated with ODQ or cGMP analogues were normalized against the average IR and positive volumes in control animals (**h**). Injected sides of spinal cords were normalized to the average IRs and positive volumes in control animals (**o**). Data are mean $\pm$ s.e.m. (n) = number of growth cones and spinal cords examined. Significant differences from control are indicated (* $p < 0.05$; ** $p < 0.01$).

Figure 8.

Model depicting Sema3A-induced acquisition of the dendrite identity. Sema3A induces the cGMP-mediated expression of functional $\text{Ca}_v2.3$ and PKG-dependent depolarization. Thus, PKG gates Ca^{2+} signaling through $\text{Ca}_v2.3$ channels to promote the acquisition of the dendrite identity and suppresses the acquisition of the axon identity. Unidentified signaling molecule(s) "X" likely increases MAP2 and decreases unphosphorylated tau proteins.