K⁺ Channel Kv3.4 Is Essential for Axon Growth by Limiting the Influx of Ca²⁺ into Growth Cones

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Membrane excitability in the axonal growth cones of embryonic neurons influences axon growth. Voltage-gated K⁺ (Kv) channels are key factors in controlling membrane excitability, but whether they regulate axon growth remains unclear. Here, we report that Kv3.4 is expressed in the axonal growth cones of embryonic spinal commissural neurons, motoneurons, dorsal root ganglion neurons, retinal ganglion cells, and callosal projection neurons during axon growth. Our *in vitro* (cultured dorsal spinal neurons of chick embryos) and *in vivo* (developing chick spinal commissural axons and rat callosal axons) findings demonstrate that knockdown of Kv3.4 by a specific shRNA impedes axon initiation, elongation, pathfinding, and fasciculation. In cultured dorsal spinal neurons, blockade of Kv3.4 by blood depressing substance II suppresses axon growth via an increase in the amplitude and frequency of Ca²⁺ influx through T-type and L-type Ca²⁺ channels. Electrophysiological results show that Kv3.4, the major Kv channel in the axonal growth cones of embryonic dorsal spinal neurons, is activated at more hyperpolarized potentials and inactivated more slowly than it is in postnatal and adult neurons. The opening of Kv3.4 channels effectively reduces growth cone membrane excitability, thereby limiting excessive Ca²⁺ influx at subthreshold potentials or during Ca²⁺-dependent action potentials. Furthermore, excessive Ca²⁺ influx induced by an optogenetic approach also inhibits axon growth. Our findings suggest that Kv3.4 reduces growth cone membrane excitability and maintains [Ca²⁺]_i at an optimal concentration for normal axon growth.

Key words: axon growth; Ca²⁺ influx; growth cone; membrane excitability; voltage-gated K⁺ channels

Significance Statement

Accumulating evidence supports the idea that impairments in axon growth contribute to many clinical disorders, such as autism spectrum disorders, corpus callosum agenesis, Joubert syndrome, Kallmann syndrome, and horizontal gaze palsy with progressive scoliosis. Membrane excitability in the growth cone, which is mainly controlled by voltage-gated Ca²⁺ (Cav) and K⁺ (Kv) channels, modulates axon growth. The role of Cav channels during axon growth is well understood, but it is unclear whether Kv channels control axon outgrowth by regulating Ca²⁺ influx. This report shows that Kv3.4, which is transiently expressed in the axonal growth cones of many types of embryonic neurons, acts to reduce excessive Ca²⁺ influx through Cav channels and thus permits normal axon outgrowth.

Introduction

Precise axon growth (including elongation, pathfinding, and fasciculation) is crucial for establishing initial neural circuits in embryos. The growth cone, located at the tip of a growing axon,

guides the growth of an axon toward its target by responding to extrinsic guidance cues (Lowery and Vactor, 2009). Interestingly, membrane excitability in the growth cone can also regulate axon elongation (Spitzer, 2006), axon turning in response to guidance cues *in vitro* (Ming et al., 2001), and the pathfinding of motor axons *in ovo* (Hanson and Landmesser, 2004). An optimal concentration of [Ca²⁺]_i in the growth cone is required for normal axon growth (Kater and Mills, 1991; Henley and Poo, 2004; Rosenberg and Spitzer, 2011). [Ca²⁺]_i is controlled by membrane excitability (Ming et al., 2001), but how membrane excitability in axonal growth cones is regulated remains elusive.

Kv channels are the principal regulators of membrane excitability (Hille, 2001). Nonspecific blocking of Kv channels by tetraethylammonium chloride or by 4-aminopyridine has been shown to inhibit the axon growth of *Xenopus* retinal ganglion cells (RGCs) and cause aberrant axon routing in *Xenopus* brain slice explants. Furthermore, Kv4.3 immunoreactivity (IR) has

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been detected in the growth cone of cultured RGCs dissociated from the *Xenopus* retina (McFarlane and Pollock, 2000). In a later study, Kv1.5-like IR, Kv3.4-like IR, and Kv4.2-like IR were observed in RGC growth cones (Pollock et al., 2002). Both reports suggest that Kv1.5, Kv3.4, Kv4.2, and Kv4.3 are expressed in axonal growth cones. By mapping eight voltage-gated K⁺ channels (Kv1.1, Kv1.2, Kv1.3, Kv3.1, Kv3.2, Kv3.3, Kv3.4, and Kv4.3) in nine axonal tracts (including the developing optic nerve and corpus callosum) of the embryonic rat brain, we have shown that Kv3.4 is the only Kv channel expressed in early growing axons (Huang et al., 2012). Together, these studies imply a role for Kv3.4 in axon growth.

Kv3.4, the only A-type K⁺ channel with a high activation threshold (≥ -10 mV), is activated during the rising phase of action potentials and then is rapidly inactivated when expressed in heterologous cells (Rudy et al., 1999). Kv3.4-mediated K⁺ currents have been detected in hippocampal dentate granule cells and DRG neurons, where they are generated to accelerate membrane repolarization during action potentials and thus shorten action potential duration (Riazanski et al., 2001; Ritter et al., 2012). However, whether Kv3.4 regulates axon growth in embryonic neurons is unknown.

In this report, we investigated the functions of Kv3.4 in developing chick spinal commissural axons and rat callosal axons, which are two commonly used animal models for studying axon growth *in vivo* (Pekarik et al., 2003; Chédotal, 2011). Cultured dorsal spinal neurons prepared from chick embryos were the main cell model for our *in vitro* studies. We found a dominant expression of Kv3.4 protein and currents in axonal growth cones. Knockdown of Kv3.4 expression results in axon growth errors, and blocking Kv3.4 channel activity also impedes axon growth. Our findings suggest that Kv3.4 reduces growth cone membrane excitability and thereby maintains the [Ca²⁺]_i at an optimal concentration for normal axon growth.

Materials and Methods

Animals. White leghorn chick embryos were provided by the Animal Health Research Institute in the Council of Agriculture (Taiwan). Fertilized eggs were incubated in a humid chamber at 38.5°C, and the developmental stages were assessed according to Hamburger and Hamilton (1951). Sprague Dawley pregnant female rats were provided by the Animal Center of National Yang-Ming University. National guidelines of animal care were followed, and all the experiments were approved by the Ethics Committee of National Yang-Ming University. Timed pregnancies were established by checking for vaginal smears on the morning following mating, and noon on the day sperm was detected was designated as the embryonic day (E) 0.5.

Plasmids. The pLKO.1 plasmid encoding rat Kv3.4shRNA or *Escherichia coli* LacZshRNA was driven by human U6 promoter and provided by the National RNAi Core Facility of Academia Sinica in Taiwan. The target sequence of Kv3.4shRNA was GTCGGAGAAGATCATCATCAA (nucleotides 102–122 of rat Kv3.4 coding sequence; NM_001122776.1). The corresponding sequence of chick Kv3.4 cDNA was CTCGGACAA-GATCACCATCAA (ENSEMBL: ENSGALT00000061883.2), with the three italicized nucleotides being the ones different from the rat's sequence. LacZshRNA was used as the control shRNA because its target sequence (GCGTTGGCAATTTAACCGCCA in *E. coli* lacZ gene; CP012633.1) was not homologous to any chick or rat sequences reported in GenBank. Rat Kv3.4 cDNA in a prokaryotic vector was a gift from Dr. Olaf Pongs (Schröter et al., 1991). To express a Kv3.4shRNA-resistant Kv3.4 (abbreviated as resKv3.4) in eukaryotic cells, rat Kv3.4 cDNA was subcloned into the pGW vector and four nucleotides (italicized) within the Kv3.4shRNA target sequence were mutated into GTCCGAAAAGAT-TATCATTAA. The other plasmids included rat Kv3.1b cDNA in pcDNA3 (Dr. Leonard Kaczmarek; Luneau et al., 1991), pCAG-en-

hanced yellow fluorescent protein (EYFP) (Drs. Tetsuichiro Saito and Jun-ichi Miyazaki; Saito and Nakatsuji, 2001), pCAGGS-GCaMP3 (Dr. Loren Looger; Tian et al., 2009), pCAGGS-channelrhodopsin-2 (ChR2)-Venus (Dr. Karel Svoboda; Addgene plasmid #15753) (Petreanu et al., 2007), and pGP-CMV-NES-jRCaMP1a (Dr. Douglas Kim; Addgene plasmid #61562; Dana et al., 2016). All plasmid DNAs were prepared using an endotoxin-free plasmid purification kit (QIAGEN).

Reagents. Reagents included Blood depressing substance II (BDSII), ω -conotoxin-GVIA, nifedipine, TTA-P2 (Alomone Labs), thapsigargin (Sigma), SKF-96535 (Tocris Bioscience), recombinant chicken netrin-1, ionomycin (Abcam), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine (DiI), fura-2 (Thermo Fisher Scientific), and all-trans-retinal (Sigma). Stock solutions were prepared by solubilizing reagents in H₂O, PBS, or DMSO according to the manufacturer's recommendations.

Cultures of dissociated neurons. Dorsal spinal neurons, motoneurons, and DRG neurons were isolated from the dorsal spinal cord, ventral spinal cord, and DRGs of HH21–HH23 chick embryos, respectively. RGCs and collosal projection neurons were isolated from the retina and cingulate/frontal cortices of E18.5 rat embryos, respectively. Fresh tissues were incubated with a solution containing 0.25% trypsin/0.038% EDTA (Thermo Fisher Scientific) in a 37°C water bath for 5 min. After trituration with a flamed glass pipette in Neurobasal medium containing 10% FBS, 2 mM L-glutamate, 100 units/ml penicillin, and 100 μ g/ml streptomycin (Thermo Fisher Scientific), single-neuron suspension was plated onto 12-mm-diameter coverslips coated with 50 μ g/ml poly-L-lysine and 10 μ g/ml laminin (Sigma). There were $\sim 1.5 \times 10^4$ dissociated neurons on each coverslip. Neurons were cultured in a humid chamber with 5% CO₂ at 37°C for various times before use.

Specificity of Kv channel antibodies. All Kv channel antibodies used were raised from rabbits. Rabbit anti-Kv1.5 (Alomone Labs catalog #APC-004, RRID:AB_2040156) was raised against a peptide corresponding to amino acids 513–602 of mouse Kv1.5. This antibody recognized a band at 75 kDa (similar to the molecular weight of Kv1.5) in the protein lysate of Human embryonic kidney (HEK) 293 cells transfected with rat Kv1.5 cDNA (Li et al., 2015). Kv1.5-IR has been detected in the astrocytic processes within the granule cell layer and Bergmann glial fibers within the molecular layer of rodent cerebellum (Roy et al., 1996), and our immunostaining showed the same pattern. Rabbit anti-Kv3.1b (custom-made and affinity-purified by GeneTex; RRID:AB_2566819) was raised against the peptide (C)DQALTPDEGLPFTRTS corresponding to amino acids 523–537 of rat Kv3.1b, with an extra cysteine added to the N terminus. Western blot showed two bands at 70 and 110 kDa in HEK293 cells transfected with Kv3.1b cDNA (Bae et al., 2014), and our result was the same. Kv3.1b-IR has been observed in the rat neocortex, hippocampus, and globus pallidus (Weiser et al., 1995), and our immunostaining pattern was the same. The specificity of anti-Kv3.2 (Alomone Labs catalog #APC-011, RRID:AB_2040168), anti-Kv3.3 (Alomone Labs catalog #APC-102, RRID:AB_2040170), and anti-Kv3.4 (Alomone Labs catalog #APC-019, RRID:AB_2040172) has been described previously (Huang et al., 2012). Anti-Kv4.2 (RRID:AB_2372612) and anti-Kv4.3 (RRID:AB_845371) were custom-made and affinity purified by GeneTex, and the specificity of each antibody has been reported (Cheng et al., 2016).

Immunohistochemistry. Chick or rat embryos were perfused transcardially with a fixative (PBS containing 4% PFA and 0.25% glutaraldehyde) for 5 min and then postfixed in the same fixative for 30 min. Following dehydration in 0.1 M phosphate buffer (PB, 0.08 M K₂HPO₄, 0.02 M NaH₂PO₄, pH 7.4) containing 30% (w/v) sucrose for cryoprotection, all specimens were kept at -72°C before sectioning. Transverse sections of spinal cord were cut with a cryostat into 30 μ m and mounted directly onto gelatin-coated slides. After wash in low-salt Tris-buffered saline (LTBS; 25 mM Tris, 0.85% NaCl, pH 7.5) and then LTBS containing 0.1% Triton X-100 (LTBST), sections on slides were treated with 0.2% hydrogen peroxide in LTBS for 30 min. Nonspecific binding was blocked by 3% normal donkey serum and 2% BSA in LTBST for 1.5 h. Primary antibodies included rabbit anti-Kv1.5 (1:100), rabbit anti-Kv3.1b (1:100), rabbit anti-Kv3.2 (1:100), rabbit anti-Kv3.3 (1:100), rabbit anti-Kv3.4 (1:100), rabbit anti-Kv4.2 (1:250), and rabbit anti-Kv4.3 (1:1500). Sections were incubated overnight with primary antibody in LTBST containing 3% normal donkey serum. After wash in high-salt Tris-buffered saline (25

mm Tris, 1.7% NaCl, pH 7.5) containing 0.1% Triton X-100 for 30 min, sections were incubated with biotinylated secondary antibody (donkey anti-rabbit, donkey anti-mouse, or donkey anti-goat; 1:500; Jackson ImmunoResearch Laboratories) for 1.5 h, followed by reacting with avidin-biotin-peroxidase complex (Thermo Fisher Scientific) for another 1.5 h. Antigen was visualized by combining equal volumes of an ammonium nickel sulfate solution and a diaminobenzidine solution in the presence of 0.01% hydrogen peroxide. Images were acquired with DMX1200 digital camera connected to Olympus BX51 microscope.

Fluorescence immunostaining. Sections of spinal cord attached on coverslips were prepared as described above. Dissociated neurons attached on coverslips were fixed in PBS containing 4% PFA for 20 min and permeabilized with LTBST. Except that treatment with hydrogen peroxide was omitted, the procedure was similar as described in Immunohistochemistry. Primary antibodies included mouse anti-axonin-1 (1:100; Abcam catalog #ab93082, RRID:AB_10585126), rabbit anti-Kv3.1b (1:100), rabbit anti-Kv3.4 (1:100), mouse anti-Islet1/2 (1:200; DSHB catalog #39.4D5, RRID:AB_2314683), and mouse anti-TAG-1 (1:100; DSHB catalog #4D7/TAG1, RRID:AB_531775). Secondary antibodies were AlexaFluor-488-conjugated donkey anti-rabbit IgG and AlexaFluor-594-conjugated donkey anti-rabbit or anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories). To label F-actin, neurons were incubated with AlexaFluor-594-labeled phalloidin (1:200; Thermo Fisher Scientific) for 1 h. For Dil labeling, neurons were incubated with 25 μ g/ml Dil in HTBS for 30 min. After rinse with LTBST, sections on slides or cells on coverslips were mounted with DAPI-containing antifading medium (Fluoromount-G; Southern Biotechnology). Images were acquired using Olympus FV1000 confocal microscope with a 512×512 pixel resolution.

For Figure 3E, 60–61 EYFP⁺ neurons (pooled from three independent experiments) were imaged by the Olympus BX51 microscope after immunostaining with anti-Kv3.4, and Kv3.4 fluorescence intensity in the whole neuron (including the soma, axon, and growth cone) was measured by the ImageJ software. Kv3.4 fluorescence intensity in 60–61 EYFP[−] neurons was measured, too. The relative Kv3.4 protein level was obtained via dividing the Kv3.4 fluorescence intensity in EYFP⁺ neurons by that in EYFP[−] neurons.

In ovo electroporation. The procedure was similarly described (Pekarik et al., 2003). Plasmid DNAs encoding EYFP (0.2 μ g), LacZshRNA (1.8 μ g), Kv3.4shRNA (1.8 μ g), resKv3.4 (1.8 μ g), GCaMP3 (1 μ g), ChR2-Venus (1 μ g), jRCaMP1a (1 μ g), and rat Kv3.4 (1.8 μ g) were used. HH15–HH17 eggs were windowed after incubation for 1–2 d in a humidified oven at 38.5°C. Capillary glasses were made into needle-like micropipettes by a pipette puller (model P-97; Sutter Instruments) and polished by a micropipette grinder (Narishige). DNAs in 0.01% fast green solution were filled into micropipettes, injected into the lumen of neural tube, and unilaterally electroporated into one side of spinal cord, through a pair of L-shaped electrodes (3 mm in length) connected to the ECM 830 electroporator (BTX Harvard Apparatus). Three electrical pulses were applied at 25 V for 50 ms duration with 950 ms intervals. The window was sealed with adhesive tape, and eggs were further incubated before analysis.

Analysis of spinal commissural axon growth in ovo. Chick embryos with *in ovo* electroporation at HH15–HH17 were fixed at HH22–HH23. The spinal cord segment (~3 mm in length) with EYFP⁺ green fluorescence was isolated, opened at the midline, and mounted onto a slide in an open-book configuration. Using FV1000 confocal microscope with identical acquisition parameters, the open-book preparation was scanned and images were collected from at least 12–18 z slices (2 μ m interval) over a 22- to 34- μ m-thick z stack. To quantify the axon projection defects, one square region (310 μ m $\times$ 310 μ m with the floor plate in the middle as shown in Fig. 4B–E) was chosen from each embryo, and all EYFP⁺ axons within the region were assessed. The percentage of EYFP⁺ axons crossing the midline, stalling, misguided, or overshooting was obtained via dividing the number of defective EYFP⁺ axons by the total number of EYFP⁺ axons. For the width of commissural axon bundle, an average value was obtained after measuring the widest and narrowest parts. The number of open-book preparations in each group (equal to the number of embryo) was pooled from ≥ 3 independent experiments done on different days.

Western blotting. HEK293 cells were cultured with DMEM containing 10% FBS (Thermo Fisher Scientific), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Sigma), in a humid chamber with 5% CO₂ at 37°C. Plasmid DNAs encoding EYFP (0.4 μ g, as the control, see Fig. 4I, left), rat Kv3.4 cDNA (3.6 μ g), LacZshRNA (3.6 μ g), Kv3.4shRNA (3.6 μ g), and resKv3.4 (3.6 μ g) were used for transfection. The cells were incubated with a mixture of plasmid DNAs and Lipofectamine 2000 (Invitrogen) in serum-free medium overnight. After replacement with serum-containing medium, cells were cultured for another 24 h. To prepare crude cell lysate, ice-cold lysis buffer (20 mM Tris, pH 8, 137 mM NaCl, 1% NP-40, and 10% glycerol) containing 1.15 mM PMSF, 0.07 mM calpain inhibitor I, 0.06 mM calpain inhibitor II, and 1% protease inhibitor mixture (Sigma) was added to cells in dish. The cells were scraped from dish and lysed by two rounds of freeze and thaw. The supernatant was collected after centrifugation at 14,000 rpm for 30 min at 4°C, and protein concentration was determined by the BCA protein assay (Thermo Fisher Scientific).

Approximately 20 μ g total protein content of cell lysate was solubilized in a loading buffer (50 mM Tris, pH 6.8, 2% SDS, 0.2% bromophenol blue, and 10% glycerol). After denature by boiling for 5 min in the presence of 5% 2-mercaptoethanol, proteins were separated by 8% SDS-PAGE and electrophoretically transferred to a PVDF membrane (Millipore) using a semidry method (Bio-Rad). Following a brief rinse in HTBS containing 0.1% Tween 20 (HTBSW), the membrane was blocked for nonspecific binding with 5% nonfat milk in HTBSW for 1 h, washed in HTBSW 10 min for three times, and incubated in HTBSW containing 2% BSA and anti-Kv3.4 (1:200) at 4°C overnight. After wash, donkey anti-rabbit secondary antibody conjugated with HRP (1:5000; Jackson ImmunoResearch Laboratories) was applied to the membrane at 4°C for 1 h. In the presence of chemiluminescent reagent (Luminata Forte; Millipore), immunoreactive bands were visualized by exposing the membrane in a luminescence image analyzer (Fujifilm LAS4000). The membrane was then stripped and immunoblotted for anti-GADPH (1:10,000) as a control for protein loading.

In utero electroporation. The method was similar as described previously (Saito and Nakatsuji, 2001). A pregnant rat bearing E15.5 embryos was anesthetized by isoflurane inhalation, and its uterus was exposed through a 2 cm incision in the ventral peritoneum. Embryos within the uterus were carefully lifted through the incision and placed on a PBS-humidified gauze pad. The exposed uterus was humidified by frequent drops of warm PBS during surgery. Plasmids encoding EYFP (0.4 μ g), LacZshRNA (1.6 μ g), Kv3.4shRNA (1.6 μ g), and resKv3.4 (1.6 μ g) were used. Through a glass pipette connected to a syringe, a small volume of PBS containing endotoxin-free DNAs and fast green dye (0.01%, Sigma) was injected through the uterine wall into the lateral ventricle of each embryo brain. A pair of 5-mm-diameter platinum tweezer-style electrodes was placed in parallel with the midline of the embryo brain, and five pulses of 50 V with 100 ms duration were produced at 900 ms intervals by the ECM830 square wave electroporator (BTX Harvard Apparatus). After electroporation, the uterine horns were placed back to the abdominal cavity, warm PBS containing 10,000 units/ml penicillin and 10 mg/ml streptomycin (Sigma) was dropped into the abdominal cavity of the pregnant rat, and the abdomen wall and skin were sutured. Embryos were kept growing within the uterus of the pregnant rat.

To analyze callosal axon projection in E20.5 embryos, the pregnant rats with treatment were killed by intraperitoneal injection of sodium pentobarbital (100 mg/kg). Embryos were isolated for fixation, and coronal sections (30 μ m) of brain were examined. Only embryos with EYFP green fluorescence exclusively in the cingulate cortex and frontal cortex were included for analysis. Five square regions (320 μ m $\times$ 320 μ m in each region) along the corpus callosum (−2, −1, 0, 1, and 2; see Fig. 4J) were selected, and green fluorescence intensity in each region was quantified by the ImageJ version 1.46r software and normalized by the fluorescence intensity in region 2. The average of relative fluorescence intensity in each region was plotted along the corpus callosum (see Fig. 4N).

Time-lapse analysis of axon elongation. The dorsal spinal cord of HH21–HH23 chick embryos was dissociated, and neurons were plated onto a 10-mm-diameter glass within the center of a culture dish. After

15–20 h of culture, the dish was placed on the stage of an inverted microscope with time-lapse image function (Olympus IX81). Only one neuron, which had a single axon longer than twofold of the soma diameter, was analyzed in each dish. During 1 h observation, neurons in culture medium were maintained at 37°C and supplied with a mixture of 95% air and 5% CO₂ (Olympus MIU-IBC). Axons that retracted from the initial position during the first 0.5 h period (–0.5 to 0 h) were excluded. To analyze the effect of BDSII on axon growth, a small volume of medium containing vehicle or BDSII stock solution was added to bath at 0 h and incubated for 0.5 h (0–0.5 h). To analyze the effect of photostimulation on axon growth, ChR2⁺ or EYFP⁺ neurons were bath incubated with all-trans-retinal (1 μM) and then stimulated by blue light (473 nm, 1 s, 0.7 mW/mm², emitted from a mercury lamp through a 20× objective lens) at frequencies of 6, 12, 24, 60, or 120 h^{–1}. Images were taken every 5 min during –0.5 to 0.5 h. The length of axon was measured by the Analysis LS Research software (Olympus). The net axon growth was obtained by subtracting the length at –0.5 h from the length at 0 or 0.5 h, and the axon growth rate per hour during 0–0.5 h was obtained by multiplying the average length by 2. The total number of neurons in each group shown in Figure 5C and Figure 8E was pooled from four independent experiments done on different days.

Spinal cord explants. The procedure was similar as reported previously (Moore and Kennedy, 2008). In the open-book configuration of spinal cord prepared from HH21–HH23 chick embryos, the dorsal spinal cord was collected, trimmed to a size of ~500 × 500 μm², and transferred onto a 12-mm-diameter coverglass coated with poly-L-lysine (50 μg/ml) and laminin (10 μg/ml). Then, 4 μl of mixed gel (3:2:1 for collagen: Matrigel: medium) was added onto each explant. After gel polymerization, each explant in one well of a culture dish was covered with Neurobasal medium containing 10% FBS, 2 mM L-glutamate, 100 μg/ml streptomycin, 100 units/ml penicillin, and recombinant netrin-1 protein (R&D Systems; 200 ng/ml). BDSII was bath applied to explants at 30 h after plating, and explants were fixed at 15 h later. To visualize the axons projected from explants, explants were incubated with mouse anti-axonin-1 (1:100; Abcam catalog #ab93082, RRID:AB_10585126) overnight at room temperature. After rinse six times with LTBS containing 1% Triton X-100, explants were incubated with AlexaFluor-594-conjugated donkey anti-mouse IgG overnight at 4°C. Following rinse with LTBS containing 1% Triton X-100, explants on coverglasses were mounted directly onto slides with antifading medium, and images were taken by an Olympus BX51 fluorescence microscope. The areas with and without axonal spread in each explant were measured, similar as described previously (Cregg et al., 2010). The axonal area was obtained by subtracting the area without axonal spread in the center from the total area. The percentage of axon bundles in each explant was obtained by dividing the number of axon bundles with diameters >5 μm by the total number of axon bundles with all diameters. The total number of explants for each treatment was pooled from at least three independent experiments done on different days.

Calcium imaging analysis in GCaMP3⁺ neurons. The plasmid encoding GCaMP3 was electroporated into the right side spinal cord of HH15–HH17 chick embryos. After incubation, dorsal spinal cord was dissociated at HH21–HH23, and neurons were plated onto a 10-mm-diameter glass in the center of a culture dish. Following 15–20 h of culture, a dish with neurons was placed on the stage of an inverted microscope (Zeiss Axiovert 200, equipped with the bright field, phase contrast, and fluorescent optics) and covered with the HEPES buffer, which contained the following (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, 2.2 CaCl₂, 10 HEPES, and 5 glucose; pH was adjusted to 7.3 with HCl. Neurons were illuminated by a Xenon lamp equipped with an ultrahigh speed switcher (Lambda DG-4 Plus, Sutter Instruments), and the excitation wavelength 490 nm was selected by a computer-controlled rotating filter. The fluorescence emitted at 525 nm was detected by the image-intensifying Photometrics CoolSNAP HQ Digital Monochrome CCD camera system (Roper Scientific). Ca²⁺ images were taken every 2 s (150–500 ms exposure) for 10 min, and the baseline was obtained during 0–1 min. In addition to the HEPES buffer (vehicle), neurons were preincubated for 20 min in the Ca²⁺-free HEPES buffer supplemented with 2 mM EGTA or the HEPES buffer containing a channel blocker (ω-conotoxin-GVIA, nifedipine, TTA-P2,

thapsigargin, or SKF-96535). Then, a micropipette (with a tip opening of 2.5 μm in diameter) filled with the HEPES buffer containing BDSII (5 μM) or netrin-1 (5 μg/ml) was focally applied during 1–10 min, with a distance ~20 μm away from the growth cone. A positive pressure of 2.5 psi (2 Hz frequency, 10 ms duration) was applied to the micropipette by a pressure ejector (NPI; PDES-01D-LA-2) connected to a pulse generator (GW Instek; GFG-8200A).

To measure the relative level of [Ca²⁺]_i in the growth cone (not including the filopodia) after drug treatment, the fluorescence intensity (F) in each image was digitally quantified with the MetaFluor software (Universal Imaging). A value (ΔF/F = (F – F₀)/F₀) was obtained by subtracting the baseline fluorescence intensity (F₀, the fluorescence at 0–20 s within the 0–1 min period) from the fluorescence intensity (F) at a time point within the 1–10 min period and normalized to F₀. All fluorescence data were background corrected. The incidence of Ca²⁺ influx in the growth cone was counted using a threshold of 20% of (F – F₀)/F₀, and the frequency of Ca²⁺ transients per hour was obtained by multiplying the number of Ca²⁺ transients by 6. Appearance of Ca²⁺ transients was confirmed by frame-by-frame examination of the time-lapse movie.

Fura-2 calcium imaging. After 15–20 h of culture, dissociated dorsal spinal neurons were loaded with cell-permeable fura-2 AM (2 μM) in the serum-free medium for 30 min in a CO₂ incubator, washed by warm medium, and incubated in the HEPES buffer before analysis. A coverslip with neurons was placed on the stage of inverted microscope and covered with the HEPES buffer. Neurons were illuminated by a Xenon lamp equipped with an ultrahigh speed switcher, and the excitation wavelengths of fura-2 (340/380 nm) were selected by a computer-controlled rotating filter. The emission light at 510 nm was detected by the image-intensifying camera system. Images were taken every 2 s (150–500 ms exposure) for 10 min, including 1 min before and 9 min after focal application of BDSII to the growth cone. The minimum ratio (R_{min}, 340/380 ratio under Ca²⁺-free conditions) and the maximum ratio (R_{max}, 340/380 ratio under Ca²⁺-saturating conditions) were yielded in the growth cone of ionomycin (5 μM)-permeabilized neurons, in Ca²⁺-free (2 mM EGTA and 2 mM Mn²⁺) and saturating Ca²⁺ (10 mM Ca²⁺) media for 1 min, respectively. Data were analyzed by the MetaFluor software. [Ca²⁺]_i in the growth cone was estimated using the equation of Grynkiewicz et al. (1985): [Ca²⁺]_i = K_Dβ[(R – R_{min})/(R_{max} – R)], where K_D (for Ca²⁺ binding to fura-2 at 37°C) = 224 nM, and β = ratio of the baseline fluorescence (380 nm) under Ca²⁺-free and -bound conditions.

Electrophysiology. Dorsal spinal neurons, dissociated from the dorsal spinal cord of HH21–H23 chick embryos, were sparsely cultured for 12–18 h to avoid innervation between cells. Right before recording, neurons were covered with the HEPES buffer. Patch pipettes (7–12 MΩ) for outside-out patch and whole-cell recording were pulled from borosilicate glass capillaries (outer diameter, 1.5 mm; inner diameter, 0.86 mm; Harvard Apparatus), heat-polished, and filled with an internal solution containing the following (in mM): 120 K-gluconate, 24 KCl, 0.2 EGTA, and 10 HEPES (pH was adjusted to 7.3 with KOH). Pipette capacitance was compensated in the cell-attached configuration. Signals were amplified using the Multiclamp 700B amplifier (Molecular Devices) and low-pass filtered at 4 kHz (4-pole Bessel) with a sampling rate of 10 kHz using the Digidata 1440 interface (Molecular Devices). Data were acquired using the pClamp 10.2 software (Molecular Devices). Recordings were made at 22°C–24°C.

Voltage-gated K⁺ currents were recorded from axonal growth cones. The outside-out patches were held in the voltage-clamp configuration at –70 mV. Outward K⁺ currents were evoked by voltage steps (–90 to 70 mV, 20 mV increments; 800 ms duration), while the leakage and capacitive currents were subtracted using a P/–4 procedure. The rise time was determined as the time interval between the points corresponding to 20% and 80% of the peak amplitude. The decay phase of the outward K⁺ current was fitted with the sum of two exponentials and a constant as follows:

$$f(t) = A_1 e^{-\frac{t}{\tau_1}} + A_2 e^{-\frac{t}{\tau_2}} + C$$

The decay time constant was defined as follows: $(A_1/A_1 + A_2)\tau_1 + (A_2/A_1 + A_2)\tau_2$. The conductance was calculated assuming a reversal potential of -88 mV and a linear current–voltage curve for open channels.

For action potential recordings, growth cones were whole-cell current-clamped at their resting membrane potentials. A current (50 pA, 200 ms duration) was injected into the growth cone to elicit an action potential. The rise time of the peak was determined as the time interval between the points corresponding to 20% and 80% of the peak amplitude. The decay time constant was obtained as described above.

Optical stimulation and calcium imaging. For Figure 8A–D, the right side spinal cord of HH15–HH17 chick embryo was electroporated with constructs encoding ChR2-Venus fusion protein (Petreanu et al., 2007) and jRCaMP1a (Dana et al., 2016) or jRCaMP1a alone. Dorsal spinal neurons were dissociated at HH21–HH23 and plated onto a culture dish with a 10-mm-diameter glass in the center. After 15–20 h of culture, a dish with neurons was placed on the stage of an inverted microscope (Olympus IX70) and covered with the HEPES buffer. All-trans-retinal ($1 \mu\text{M}$) was bath applied at 2 min before photostimulation. Under a $40\times$ oil objective (Olympus), an axon-bearing ChR2⁺/jRCaMP1a⁺ or ChR2⁻/jRCaMP1a⁺ neuron was stimulated with blue light (473 nm, 1 s, 0.7 mW/mm^2), which was delivered via a $200 \mu\text{m}$ multimode optical fiber (0.39 numerical aperture, Thorlabs) coupled with a diode-pumped solid-state laser (OEM Laser Systems). The distance between the tip of optic fiber and the recording neuron was $\sim 1 \text{ mm}$. jRCaMP1a⁺ neurons were illuminated by a Xenon lamp, the excitation wavelength 540 nm was selected by a monochromator (Polychrome V, Tillphotomics), and the fluorescence emitted at 610 nm was detected by a CCD camera (Micro-max YHS1300, Roper Scientific). Ca^{2+} images were taken every 2 s (100–300 ms exposure) for 10 min. Fluorescence intensity (F) in each image was digitally quantified with the MetaFluor software (Universal Imaging). $\Delta F/F$ ($F - F_0/F_0$) was calculated by subtracting the baseline fluorescence level (F_0 , the fluorescence at 0–20 s within the 0–1 min period) from the actual fluorescence response and normalized to F_0 . To estimate the total calcium influx within a specific time window (1–10 or 1–3.5 min), each value of $\Delta F/F$ (above the baseline 0%, taken every 2 s) was multiplied by 2 s, and then all of them were summed up as the area under the curve.

Statistics. All data are presented as mean $\pm$ SEM. Statistical tests were performed by SPSS 18.0 (IBM). Independent-samples *t* test was used to evaluate differences between samples in some experiments, and the indicated pairs were compared by Tukey's *post hoc* test after one-way analysis of variance (ANOVA) in some other experiments. Kolmogorov–Smirnov test was used in the analysis of cumulative distribution of axon length. Paired-samples *t* test was applied for the electrophysiological experiments.

Results

Kv3.4 in the axonal growth cones of spinal commissural neurons

During the development of the lumbosacral spinal cord in chick embryos, commissural neurons, with somata located in the dorsolateral spinal cord, project axons along the periphery of the dorsal spinal cord at Hamburger and Hamilton stage (HH) 19–HH20 (Hamburger and Hamilton, 1951). Commissural axons elongate ventromedially toward the floor plate at HH21, reach the floor plate and cross the ventral midline at HH23, and then turn anteriorly and move toward the brain by HH25 (Stoeckli and Landmesser, 1995). Using immunohistochemistry to map the spinal cord from HH17 to HH27, we detected Kv3.4-IR in commissural axons only during HH19–HH25, with the strongest intensity at HH23 (Fig. 1A–F). The temporal profile of the Kv3.4 transient expression coincides with the commissural axon growth period in chick embryos, and a similar phenomenon was observed in rat embryos during embryonic day (E) 12.5–E13.5 (Fig. 1S–U). By contrast, no Kv1.5-IR, Kv4.2-IR, or Kv4.3-IR could be detected in growing commissural axons (Fig. 1G–I). We also examined whether other Kv3 members are expressed in

growing commissural axons. Kv3.1b was found to be present in the commissural axons over the same developmental period as that of Kv3.4, but Kv3.2 and Kv3.3 were absent (Fig. 1J–L). Thus, after mapping seven Kv channels, we detected only Kv3.4 and Kv3.1b in growing spinal commissural axons.

The cell adhesion molecule axonin-1, also known as TAG-1 in mammals, is expressed in spinal commissural axons of chick embryos (Stoeckli and Landmesser, 1995). Fluorescence immunostaining revealed the presence of large Kv3.4⁺ puncta along axonin-1⁺ commissural axons, and these puncta looked like growth cones (Fig. 1M–M''). To verify this observation, we cultured neurons dissociated from the dorsal spinal cord of HH21–HH23 chick embryos because most commissural neurons project axons during this period of time (Stoeckli and Landmesser, 1995). An antibody against transcription factors Islet1 and Islet2 (Islet1/2) has been used to label dorsal interneurons 3 (dI3) and motoneurons (Helms and Johnson, 2003; Lu et al., 2015). Because most Kv3.4⁺ neurons ($87.8 \pm 1.1\%$, $n = 3$; 403 of 459 neurons) do not express Islet1/2, Kv3.4⁺/Islet1/2⁻ neurons are presumably dI1, dI2, and dI4–dI6 dorsal interneurons. dI1 and dI2 neurons form the spinal commissural neurons (Helms and Johnson, 2003). Furthermore, all axonin-1⁺ commissural neurons expressed Kv3.4 (Fig. 1N–N''), and Kv3.4-IR was enriched at the filopodia (densely filled with F-actin) of the growth cones (Fig. 1O–O''). By contrast, Kv3.1b was expressed strongly in the soma and axon shaft but weakly in the growth cone (Fig. 1P–P''). Enrichment of Kv3.4 in the growth cone was confirmed by normalizing the Kv3.4 immunofluorescence to that of DiI (a membrane marker) (Fig. 1Q–R). Thus, Kv3.4 is the major Kv3 channel expressed in the axonal growth cones of spinal commissural neurons.

Kv3.4 in the axonal growth cones of four other types of neurons

In the spinal cord of HH23 chick embryos, in addition to the commissural axons, we also detected Kv3.4-IR in the motoneuron axons and the bifurcation zone (Fig. 2A,B). The bifurcation zone is a distinct structure in the spinal cord where DRG sensory afferents bifurcate into the ascending and descending branches (Altman and Bayer, 2001). In rat embryos, Kv3.4 has been detected in the optic nerve (which comprises RGC axons) during E14.5–E19.5, the corpus callosum (which is made up of callosal axons) during E17.5 to postnatal day 1 (Huang et al., 2012), and the bifurcation zone during E13.5–E17.5 (Fig. 1T,U; E17.5 data not shown). Interestingly, the temporal profiles for Kv3.4 transient expression seem to coincide with the growing periods of these three types of axons (Huang et al., 2012; current study). Furthermore, it has been shown that anti-Islet1/2 can also label postmitotic motoneurons (Tsuchida et al., 1994), DRG neurons (Cui and Goldstein, 2000), and RGCs (Pimentel et al., 2000). Double immunostaining revealed Kv3.4-IR in the axonal growth cones of cultured motoneurons, DRG neurons, and RGCs (Fig. 2C–E). Using a TAG-1 antibody that has been used to mark the developing corpus callosum (Wolfer et al., 1994), we also found Kv3.4-IR in the axonal growth cones of cultured callosal projection neurons (Fig. 2F). Both our *in vivo* and *in vitro* results demonstrate that Kv3.4 is expressed in the axonal growth cones of motoneurons, DRG neurons, RGCs, and callosal projection neurons during axon growth.

Knockdown of Kv3.4 inhibits axon initiation and elongation

To test whether Kv3.4 is required for axon growth, we suppressed Kv3.4 protein expression using a construct that encoded a Kv3.4

Figure 1. Kv3.4 in the axonal growth cones of dorsal spinal commissural neurons. **A–F**, Transverse sections of the spinal cord of chick embryos were immunostained for Kv3.4. **A**, Absence of Kv3.4-IR in the dorsal spinal cord at HH17. Kv3.4-IR in precrossing commissural axons (**B–F**, arrowheads) is evident during HH19–HH25 but disappears at HH27. **D**, Arrows indicate postcrossing commissural axons projecting from the other side of spinal cord. FP, Floor plate. **G–L**, Transverse sections of the spinal cord at HH23 were immunostained as indicated. **G**, Absence of Kv1.5-IR. **H**, Kv4.2-IR in the somata and dendrites of motoneurons (MN). **I**, Kv4.3-IR in the bifurcation zone (BZ). In addition to the BZ, Kv3.1b-IR is strong in postcrossing commissural axons (**J**, arrow) but weak in precrossing commissural axons (**J**, arrowhead). **K**, Absence of Kv3.2-IR. **L**, Kv3.3 in motoneurons. **M–M''**, Double staining in transverse sections of the spinal cord at HH21 shows colocalization of Kv3.4 and axonin-1 in the growth cones (arrowheads) of commissural axons. **N–N''**, Colocalization of Kv3.4 and axonin-1 in cultured dorsal spinal neurons isolated from HH21–HH23 chick embryos. **O–O''**, Red fluorescence-tagged phalloidin colabeling reveals enrichment of Kv3.4 in the growth cone (**O–O''**) and Kv3.1b in the soma/axon shaft (**P–P''**) of (Figure legend continues.)

Figure 2. Kv3.4 in the axonal growth cones of motoneurons, DRG neurons, RGCs, and callosal projection neurons. **A, B**, Transverse sections of the spinal cord (SC) of HH23 chick embryos were immunostained, showing Kv3.4-IR in the axonal bundle (arrowhead) of motoneurons (MN) (**A**) and the bifurcation zone (BZ) of DRG neuron afferents (**B**). **C, D**, Motoneurons and DRG neurons of HH21–HH23 chick embryos were dissociated, cultured for 20 h, and double immunostained for Kv3.4 and Isl1/2. **E, F**, RGCs and callosal projection neurons (CPNs) were dissociated from the retina and cingulate/frontal cortices of E18.5 rat embryo, respectively. After 16 h of culture, cells were double immunostained for Kv3.4 and Isl1/2 (**E'**) or TAG-1 (**F'**). Kv3.4-IR is evident in axonal growth cones (**C''–F''**, arrows). Scale bars: **A**, 38 μ m; **B**, 25 μ m; **C**, 16 μ m; **D**, 13 μ m; **E**, 16 μ m.

short hairpin RNA (Kv3.4shRNA), which was delivered into the dorsal spinal neurons of chick embryos by *in ovo* electroporation. After incubation, the dorsal spinal cord from the embryos was isolated, and the cells were dissociated for culture. The transfected neurons were identified by the expression of EYFP. A neurite shorter than twice the soma diameter was defined as a protrusion, whereas a neurite longer than twice the soma diameter was defined as an axon.

Among the control neurons (only expressing EYFP), ~32% had protrusions (Fig. 3F), ~22% had axons (Fig. 3G), and ~46% had no protrusions or axons. Kv3.4-IR was strong on the somatic surface of neurons without neurites (Fig. 3A, top), but it was more evident in the growth cone than the somatic surface in neurite-bearing neurons (Fig. 3A, bottom). LacZshRNA, which has no effect on eukaryotic cells, was used as a control for the shRNA. The characteristics of LacZshRNA⁺ neurons were similar to those of the control neurons (Fig. 3B, E–I). Through a decrease in the endogenous levels of Kv3.4 protein (Fig. 3C, E), Kv3.4shRNA treatment resulted in fewer neurons with protrusions or axons (Fig. 3F, G). In addition,

the Kv3.4shRNA⁺ neurons had shorter axons, as revealed by the average axon length and the cumulative distribution analysis of the axon length (Fig. 3H, I). Cotransfection with a construct encoding Kv3.4shRNA-resistant Kv3.4 (resKv3.4) rescued the defects caused by Kv3.4shRNA (Fig. 3D–I). Thus, Kv3.4 is required for axon initiation and elongation in cultured dorsal spinal neurons.

Knockdown of Kv3.4 impedes axon elongation, pathfinding, and fasciculation *in vivo*

To investigate the functions of Kv3.4 in spinal commissural axons, we delivered the Kv3.4shRNA construct into the spinal cord of chick embryos at HH15–HH17 and examined axon trajectory in an open-book configuration at HH22–HH23 (Fig. 4A). In the control or LacZshRNA⁺ embryos, the axons had crossed the midline, turned anteriorly, and compacted themselves into a bundle (known as the ventral funiculus) (Sakai and Kaprielian, 2012) adjacent to the floor plate (Fig. 4B, C). By contrast, in the Kv3.4shRNA⁺ embryos, fewer axons crossed the midline; more axons stalled or were misguided in the floor plate (Fig. 4D, F, G); and a dramatic increase in the width of the axon bundle was due to axon defasciculation (Fig. 4D, F, H). Coexpression of resKv3.4 rescued the defects in axon elongation, pathfinding, and fasciculation caused by Kv3.4shRNA (Fig. 4E, G, H).

Western blot analysis showed that Kv3.4shRNA reduced the expression of rat Kv3.4 cDNA after cotransfection into HEK293 cells (Fig. 4I), which confirms that the Kv3.4shRNA also worked in the rat. To elucidate the functions of Kv3.4 in the developing callosal axons of rat embryos, we delivered the Kv3.4shRNA construct by *in utero* electroporation to the right side of the neocortex

(Figure legend continued.) cultured dorsal spinal neurons. **Q–Q''**, Kv3.4 and Dil colabeling. White represents Kv3.4-abundant regions. Blue represents Kv3.4-sparse regions (**Q''**). **R**, Ratio of Kv3.4/Dil in the soma, axon shaft, or growth cone of each neuron was obtained by dividing the fluorescence intensity of Kv3.4 by that of Dil. Data are mean $\pm$ SEM ($n = 8$ neurons, pooled from three independent experiments done on different days). *** $p < 0.001$, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). **S–U**, Kv3.4-IR in spinal commissural axons (arrowheads) of rat embryos is evident during E12.5–E13.5 (**S, T**), but it disappears at E14.5 (**U**). Scale bar: (in **U**) **A**, 35 μ m; **B**, 35 μ m; **C**, 40 μ m; **D**, 50 μ m; **E**, 50 μ m; **F**, 60 μ m; **G–L**, 50 μ m; **M–M''**, 30 μ m; **N–N''**, 13 μ m; **O–O''**, 20 μ m; **P–P''**, 20 μ m; **Q–Q''**, 16 μ m; **S–U**, 100 μ m.

Figure 3. Knockdown of Kv3.4 inhibits neurite protrusion and axon elongation. **A–D**, The spinal cord of chick embryos at HH15–HH17 was electroporated with constructs encoding EYFP alone (control, **A**), LacZshRNA/EYFP (LacZshRNA, **B**), Kv3.4shRNA/EYFP (Kv3.4shRNA, **C**), or Kv3.4shRNA/resKv3.4[Kv3.4shRNA-resistant Kv3.4]/EYFP (Kv3.4shRNA + resKv3.4, **D**). The dorsal spinal cord was dissociated at HH21–HH23 and cultured for 20 h before immunolabeling Kv3.4 (red). **A–D**, Top, Neurons without neurites. Bottom, Axon-bearing neurons. Nontransfected neurons in each culture were used for comparison, and their nuclei were labeled by DAPI (blue). **A, B**, In the control or LacZshRNA⁺ neurons, Kv3.4-IR was strong in the somatic surfaces of neurons without neurites, but it became more evident in the growth cones of axon-bearing neurons. LacZshRNA did not suppress Kv3.4 expression. **C**, Kv3.4shRNA strongly reduced Kv3.4-IR in neurons without neurites (arrowhead) but had a weaker effect in axon-bearing neurons. **D**, Cotransfection of resKv3.4 rescued the knockdown effect caused by Kv3.4shRNA. **E**, After measuring the fluorescence intensity of neurons with or without neurites (30 neuron counts for each; total 60 counts), the relative Kv3.4 protein level was obtained by dividing the Kv3.4 fluorescent intensity of EYFP⁺ neurons by that of EYFP[−] neurons. **F**, The percentage of protrusion-bearing neurons. **G**, The percentage of axon-bearing neurons. **H**, The average of axon length. **I**, The cumulative distribution of axon length (Kolmogorov–Smirnov test) shows that the axons of Kv3.4shRNA-transfected neurons are shorter. Nontransfected neurons pooled from three independent experiments. Data are mean ± SEM. **p* < 0.05, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). ***p* < 0.01, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). ****p* < 0.001, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA).

at E15.5 (Fig. 4J), a developmental stage that occurs shortly after the neural progenitors of the cingulate and frontal cortices are generated in the ventricular zone (Fame et al., 2011). At E20.5, the callosal axons had extended to the contralateral frontal cortex in the LacZshRNA⁺ embryos (Fig. 4K,N), but they only reached the contralateral cingulate cortex in the Kv3.4shRNA⁺ embryos (Fig. 4L,N). Coexpression with resKv3.4 rescued the defect in axon elongation caused by Kv3.4shRNA (Fig. 4M,N). Both *in vivo* models indicate that axon growth is impeded by the decrease in Kv3.4 protein levels.

Blockade of Kv3.4 causes axon retraction and defasciculation
BDSII is a selective blocker of Kv3 channels (Diochot et al., 1998; Yeung et al., 2005). Kv3.4 is the only Kv3 channel present in axonal growth cones (Fig. 1). To test whether blockade of Kv3.4 is

able to inhibit axon growth, we measured the axon length of cultured dorsal spinal neurons using time-lapse microscopy before and after bath application of BDSII. Among the axons that grew during the control period of 0.5 h (from −0.5 to 0 h; average growth rate $21.0 \pm 3.6 \mu\text{m/h}$, *n* = 29), the BDSII incubation during the subsequent 0.5 h (from 0 to 0.5 h) caused axon retraction ($-21.6 \pm 6.6 \mu\text{m/h}$, *n* = 11), compared with that of the vehicle incubation ($16.8 \pm 7.8 \mu\text{m/h}$, *n* = 18) (Fig. 5A–D). Furthermore, the effect of Kv3.4 blockade on axon growth was investigated in the presence of netrin-1, an extracellular cue that guides spinal commissural axons to extend from the dorsal spinal cord to the floor plate (Serafini et al., 1996). In spinal cord explants, netrin-1 induced prominent axon elongation and fasciculation, but coapplication with BDSII resulted in shorter axons and defasciculated axons (Fig. 5E–H). Thus, blockade of Kv3.4 (Fig. 5)

Figure 4. Knockdown of Kv3.4 inhibits axon elongation, pathfinding, and fasciculation *in vivo*. **A–E**, The right side spinal cord of chick embryo at HH15–HH17 was electroporated with constructs encoding EYFP (control, **B**), EYFP/LacZshRNA (**C**), EYFP/Kv3.4shRNA (**D**), or EYFP/Kv3.4shRNA/Kv3.4shRNA-resistant Kv3.4 (resKv3.4) (**E**). Embryos were fixed at HH22–HH23, and their spinal cords in open-book configurations show the trajectories of EYFP⁺ commissural axons. A, Anterior; D, dorsal; P, posterior; V, ventral. **B–E**, Arrows indicate the bundle of commissural axons (ventral funiculus, VF). **D**, Arrowheads indicate stalling axons at the floor plate (FP). Asterisks indicate misguided axons. **F**, Summary of projection errors of spinal commissural axons. **G**, The percentage of EYFP⁺ axons with projection errors. **H**, The width of the ventral funiculus. **I**, Western blotting was performed using lysate of HEK-293 cells transfected with constructs encoding Kv3.4/LacZshRNA/EYFP, Kv3.4/Kv3.4shRNA/EYFP, or Kv3.4shRNA/resKv3.4/EYFP. The major protein band of Kv3.4 at position of 100 kDa was shown, and GAPDH was used as a loading control. **J–M**, In E15.5 rat brain, the ventricular zone (green) adjacent to the lateral ventricle (LV, blue) was electroporated with constructs encoding EYFP/LacZshRNA (**K**), EYFP/Kv3.4shRNA (**L**), or EYFP/Kv3.4shRNA/resKv3.4 (**M**). The positive electrode paddle was located on the left side of brain. Coronal sections of E20.5 rat brain were analyzed after embryos were grown *in utero*. EYFP⁺ callosal axons, which project from the cingulate cortex (CgC) and frontal cortex (FC), only reach the contralateral cingulate cortex in the Kv3.4shRNA-expressing brain. PC, Parietal cortex. **N**, Measurement of axon projection to the contralateral side. Relative intensity in each region (J, −2, −1, 0, 1, 2) is obtained by normalizing its fluorescence intensity with that in region 2. **G, H, N**, Numbers (Figure legend continues.)

has inhibitory effects on axon growth similar to those of knockdown of Kv3.4 (Figs. 3, 4).

Blockade of Kv3.4 increases Ca^{2+} influx in axonal growth cones

To test whether Kv3.4 regulates Ca^{2+} influx in axonal growth cones, we examined the effect of Kv3.4 blockade on Ca^{2+} influx in cultured dorsal spinal neurons expressing GCaMP3, a genetically encoded Ca^{2+} indicator that emits green light following excitation. Ca^{2+} elevation and Ca^{2+} transients are two types of Ca^{2+} influx ($\Delta F/F > 20\%$) that are commonly detected in axonal growth cones. Ca^{2+} elevation is characterized by a sustained increase in $[\text{Ca}^{2+}]_i$ with a gradually elevated baseline (Hong et al., 2000), whereas Ca^{2+} transients represent rapid increases in $[\text{Ca}^{2+}]_i$ that quickly return to baseline (Spitzer, 2002). In vehicle-treated GCaMP3⁺ neurons, 12.5% (3 of 24) showed Ca^{2+} elevation, 12.5% (3 of 24) exhibited Ca^{2+} transients, and 75% (18 of 24) had neither (Fig. 6*A,D–F*). In BDSII-treated GCaMP3⁺ neurons, 48% (12 of 25) showed Ca^{2+} elevation, 32% (8 of 25) exhibited Ca^{2+} transients, and 20% (5 of 25) had neither (Fig. 6*B–F*). In addition to elevating the incidence of Ca^{2+} influx, BDSII also increased the frequency (from 8.0 ± 4.6 h to 19.5 ± 3.3 h) and amplitude of Ca^{2+} transients (from $42.8 \pm 10.3\%$ to $125.7 \pm 15.7\%$ of $\Delta F/F$) but did not induce changes in the duration (16.6 ± 5.6 ms vs 20.0 ± 1.9 ms) (Fig. 6*G–I*). To estimate the $[\text{Ca}^{2+}]_i$ concentration increased by BDSII, cultured dorsal spinal neurons were loaded with fura-2. The focal application of BDSII to growth cones elevated the $[\text{Ca}^{2+}]_i$ from a baseline of 23.0 ± 2.5 nM to a peak of 486.7 ± 103.9 nM, which was much higher than the peak amplitude (61.3 ± 8.8 nM) in the vehicle group (Fig. 6*J,K*). These data indicate the involvement of Kv3.4 in regulating Ca^{2+} influx.

Figure 5. Blockade of Kv3.4 causes axon retraction and defasciculation. *A–D*, Axons of cultured chick dorsal spinal neurons grew over the control period (from -0.5 to 0 h), followed by bath incubation of vehicle (*A*) or BDSII ($3 \mu\text{M}$, *B*) from 0 to 0.5 h. Representative images were captured at -0.5 , 0 , and 0.5 h using time-lapse microscopy. *A*, *B*, Arrowheads indicate growth cones. BDSII causes axon retraction. *E–H*, Chick dorsal spinal cord explants, with netrin-1 addition soon after plating and BDSII ($1.5 \mu\text{M}$) addition at 30 h after plating, were fixed at 45 h after plating. Axons in explants were visualized by immunolabeling for axonin-1 (red). *E*, *F*, Boxed areas are magnified in *E'* and *F'*, respectively. Quantification of the axonal area per explant (*G*) and the percentage of axon bundles with diameter $> 5 \mu\text{m}$ per explant (*H*). BDSII decreases netrin-1-induced axon growth and causes axon defasciculation. Numbers in parentheses indicate total number of neurons (*C*) or explants (*G*, *H*) analyzed. Data are mean $\pm$ SEM. $^{**}p < 0.01$, compared with vehicle (*D*) (independent-samples *t* test). $^{***}p < 0.001$, compared with netrin-1 (*G*, *H*) (independent-samples *t* test). Scale bars: *A*, *B*, $25 \mu\text{m}$; *E*, *F*, $200 \mu\text{m}$; *E'*, *F'*, $80 \mu\text{m}$.

Kv3.4 blockade-induced Ca^{2+} transients require T-type and L-type Ca^{2+} channels

In the growth cone, $[\text{Ca}^{2+}]_i$ is modulated by Ca^{2+} influx via surface Ca^{2+} -permeable channels or via Ca^{2+} release from intracellular Ca^{2+} stores (Gomez and Zheng, 2006). To identify the sources of Ca^{2+} responsible for the BDSII-induced Ca^{2+} transients, we depleted extracellular Ca^{2+} using EGTA or depleted intracellular Ca^{2+} stores using thapsigargin in GCaMP3⁺ dorsal spinal neurons. BDSII-induced Ca^{2+} transients were abolished by extracellular Ca^{2+} depletion but were unaffected by intracellular Ca^{2+} store depletion (Fig. 6*L,N*), which indicates that extracellular Ca^{2+} is required for BDSII-induced Ca^{2+} transients.

Voltage-gated Ca^{2+} channels (including T-type, L-type, and N-type) and transient receptor potential canonical (TRPC)

(Figure legend continued.) in parentheses indicate the total number of embryos analyzed. Data are mean $\pm$ SEM. $^{*}p < 0.05$, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). $^{***}p < 0.001$, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). $^{*}p < 0.05$, comparing "Kv3.4shRNA + resKv3.4" with Kv3.4 shRNA (Tukey's *post hoc* test after one-way ANOVA). $^{**}p < 0.01$, comparing "Kv3.4shRNA + resKv3.4" with Kv3.4 shRNA (Tukey's *post hoc* test after one-way ANOVA). $^{***}p < 0.001$, comparing "Kv3.4shRNA + resKv3.4" with Kv3.4 shRNA (Tukey's *post hoc* test after one-way ANOVA). Scale bars: *B–E*, $60 \mu\text{m}$; *K–M*, $670 \mu\text{m}$.

Figure 6. Blockade of Kv3.4 increases the influx of Ca^{2+} in axonal growth cones. **A–C**, During 10 min Ca^{2+} imaging analysis, the baseline was obtained during 0–1 min, and vehicle or BDSII (5 μM) was focally applied (arrows) to the growth cone of GCaMP3^{+} neurons during 1–10 min. Representative pseudocolor images show relative levels of $[\text{Ca}^{2+}]$, (purple to red, low to high) in growth cones, which are taken at 0, 3, 6, and 10 min, respectively. **D**, BDSII induces a sustained Ca^{2+} elevation in some GCaMP3^{+} neurons. **E**, BDSII induces Ca^{2+} transients in some other GCaMP3^{+} neurons. **F**, The incidence (% of neurons examined) of Ca^{2+} elevation and Ca^{2+} transients. **G–I**, The frequency (**G**, per hour) and amplitude (**H**, $\Delta\text{F}/\text{F}$, as shown in **E**) of Ca^{2+} transients are higher in BDSII-treated neurons than vehicle-treated neurons, but the duration (**I**, seconds) has no difference. **J**, In the representative growth cone of fura-2-loaded neurons, (Figure legend continues.)

channels are surface Ca^{2+} -permeable channels. Ca^{2+} currents through T-type and L-type Ca^{2+} channels produce Ca^{2+} transients (Gu and Spitzer, 1993; Tang et al., 2003), whereas Ca^{2+} currents through TRPC channels are involved in growth cone turning induced by netrin-1 (Wang and Poo, 2005). To identify which surface Ca^{2+} -permeable channels mentioned above contribute to BDSII-induced Ca^{2+} transients, we bath applied channel blockers to GCaMP3⁺ neurons. TTA-P2 (a T-type Ca^{2+} channel blocker, 3–10 μM) or nifedipine (a L-type Ca^{2+} channel blocker, 5–10 μM) abolished BDSII-induced Ca^{2+} transients, but ω -conotoxin-GVIA (a N-type Ca^{2+} channel blocker, 2–5 μM) or SKF-96535 (a TRPC channel blocker, 25 μM) had no effect (Fig. 6M,N). Thus, T-type and L-type Ca^{2+} channels are required for the Ca^{2+} transients induced by Kv3.4 blockade.

Blockade of Kv3.4 increases netrin-1-induced Ca^{2+} influx in axonal growth cones

Binding of netrin-1 depolarizes the plasma membrane by ~ 20 mV, which leads to Ca^{2+} influx into the growth cone (Hong et al., 2000; Wang and Poo, 2005). We found that focal application of netrin-1 to growth cones induced a small sustained Ca^{2+} elevation (5%–10% of $\Delta\text{F}/\text{F}$) in 53.3% (8 of 15) of GCaMP3⁺ neurons (Fig. 6O,Q). Preincubation with BDSII increased the amplitude of netrin-1-induced Ca^{2+} elevation (10%–20% of $\Delta\text{F}/\text{F}$) in 63.2% (12 of 19) of GCaMP3⁺ neurons (Fig. 6P,Q). This shows that blockade of Kv3.4 by BDSII is able to increase netrin-1-induced Ca^{2+} influx in the growth cone. Because the observation period only lasted for 10 min, whether netrin-1-induced axon guidance is also affected by Kv3.4 blockade requires further investigation.

Kv3.4 is the major Kv channel that reduces growth cone membrane excitability

To examine the electrophysiological properties associated with Kv3.4 in axonal growth cones, patch-clamp recordings were performed on cultured dorsal spinal neurons. The resting membrane potential (RMP) was -46.7 ± 1.3 mV (mean $\pm$ SEM; $n = 144$). After applying depolarizing pulses from a holding potential of -90 mV, we detected inactivating A-type K^+ currents (Fig. 7A) with a fast rise time (Fig. 7B), a slow decay time constant (Fig. 7C), and a high-voltage activation threshold (the slope factor was 23.9 mV, which reached a midpoint at 1.8 ± 6.6 mV; Fig. 7D). When the voltage was stepped from -90 to 70 mV, the rise time and the decay time constant were 0.9 ± 0.1 and 169.3 ± 35.5 ms, respectively (Fig. 7B,C). After focal application of BDSII, the peak amplitude of total outward K^+ current was reduced to ap-

proximately one-third of the control (Fig. 7E,F). The rise time and decay time constant of the BDSII-sensitive current were similar to those of the control (Fig. 7G,H). Because Kv3.4 is the only Kv3 channel in the growth cone (Fig. 1), the BDSII-sensitive K^+ current is mediated by Kv3.4. These results indicate that Kv3.4 is the major Kv channel in the axonal growth cones of embryonic dorsal spinal neurons.

Focal application of BDSII to the growth cone caused membrane depolarization from -49.6 ± 2.1 to -37.6 ± 6.1 mV (mean $\pm$ SEM; Fig. 7I), which indicates that Kv3.4 can reduce growth cone membrane excitability at subthreshold potentials. To test whether Kv3.4 can repolarize the growth cone membrane after Ca^{2+} -dependent depolarization (such as Ca^{2+} transients and Ca^{2+} elevation), we injected current into the growth cone to elicit an action potential. Under physiological conditions, the Ca^{2+} -dependent action potential exhibited a peak, which was then followed by a sustained plateau (a characteristic of L-type Ca^{2+} channel activity) (Fig. 7J), similar to the netrin-1-induced action potential recorded from the growth cone of embryonic *Xenopus* spinal neurons (Wang and Poo, 2005). Blockade of Kv3.4 by BDSII prolonged the duration of the peak (Fig. 7K), especially the decay time constant (Fig. 7M), but did not change the rise time or the amplitude (Fig. 7L,N). Because of a slower repolarization of the peak caused by BDSII, the amplitude of the plateau was also increased (Fig. 7O). Blockade of Kv3.4 increases the duration and amplitude of the Ca^{2+} -dependent action potential, which suggests that Kv3.4 can quickly repolarize the membrane potential and thereby limit Ca^{2+} influx into growth cones during Ca^{2+} -dependent action potentials.

Excessive Ca^{2+} influx inhibits axon elongation

Because blockade of Kv3.4 not only inhibited axon elongation (Fig. 5) but also increased Ca^{2+} influx (Fig. 6), we asked whether an increase in Ca^{2+} influx could inhibit axon elongation in axon-bearing neurons. An optogenetic approach was used. By expressing a fusion protein of ChR2 and Venus (a fluorescent protein that emits yellow light following excitation), ChR2⁺ neurons were identified with 510 nm light illumination.

To test whether photostimulation (473 nm, 1 s duration) can stimulate ChR2 and evoke Ca^{2+} influx into ChR2⁺ neurons, we cotransfected neurons with constructs encoding ChR2 and jRCaMP1a, a genetically encoded Ca^{2+} indicator that emits red light following excitation (Dana et al., 2016). In the growth cones of cultured dorsal spinal neurons during a 9 min (1–10 min) photostimulation at a frequency of 24 h^{-1} , there was a sustained Ca^{2+} influx (10–15% of $\Delta\text{F}/\text{F}$) in ChR2⁺ neurons (Fig. 8A), and this phenomenon was absent in ChR2[−] neurons (Fig. 8A'). When the frequency was reduced to 6 h^{-1} , only a brief Ca^{2+} influx (5%–10% of $\Delta\text{F}/\text{F}$) was observed in ChR2⁺ neurons (Fig. 8A''). Furthermore, there was a 2.5-fold enhancement of Ca^{2+} influx over the 9 min period of photostimulation when the frequency was increased from 6 to 24 h^{-1} (Fig. 8C). We also compared the effect of a single photostimulation between ChR2⁺ and ChR2[−] neurons. During 2.5 min (1–3.5 min) after the first photostimulation, Ca^{2+} influx was prominent in the ChR2⁺ neurons but not in the ChR2[−] neurons (Fig. 8D). By contrast, in the soma, the photostimulation paradigms did not evoke Ca^{2+} influx in ChR2⁺ or ChR2[−] neurons (Fig. 8B–B',C,D). These results indicate that a higher frequency of photostimulation can evoke more Ca^{2+} influx into the axonal growth cones of ChR2⁺ neurons.

To elucidate the relationship between Ca^{2+} influx and axon elongation, we permitted axons to elongate for 0.5 h (-0.5 to 0 h) and then photostimulated the ChR2⁺ neurons during the subse-

←

(Figure legend continued.) the ratio of 340/380 (pseudocolor) is low during 0–1 min, but it is increased after focal application (arrow) of BDSII (5 μM) during 1–10 min. **K**, Compared with vehicle, BDSII increases the peak amplitude of $[\text{Ca}^{2+}]_i$ transients in axonal growth cones. **L**, Representative patterns of BDSII-induced Ca^{2+} transients after depleting extracellular Ca^{2+} (Ca^{2+} -free) or depleting intracellular Ca^{2+} using thapsigargin (2 μM) in bath medium. **M**, Representative patterns of BDSII-induced Ca^{2+} transients after bath application of SKF-96535 (25 μM), TTA-P2 (3 μM), nifedipine (5 μM), or ω -conotoxin (2 μM). **N**, BDSII-induced Ca^{2+} transients are abolished by extracellular Ca^{2+} depletion, TTA-P2, or nifedipine. **O**, **P**, In bath medium preincubated with vehicle or BDSII (3 μM), the baseline is obtained during 0–1 min, and netrin-1 (arrows) is focally applied to the growth cone of GCaMP3⁺ neurons during 1–10 min. Representative pseudocolor images are taken at 0, 2, 5, and 10 min, respectively. **Q**, Netrin-1-induced Ca^{2+} elevation is increased by BDSII preincubation. Number in parentheses indicates the total number of neurons analyzed. Data are mean $\pm$ SEM. * $p < 0.05$, compared with vehicle (independent-samples t test). *** $p < 0.001$, compared with vehicle (independent-samples t test). Scale bar, 10 μm .

Figure 7. Kv3.4-mediated K^+ currents reduce membrane excitability in the axonal growth cones. **A**, An outside-out patch, isolated from growth cones of cultured dorsal spinal neurons, was held at -70 mV and hyperpolarized to -90 mV for 1.5 s before test pulses ranging from -70 to 70 mV (800 ms, 20 mV increments). Representative total outward K^+ currents are shown. **B**, **C**, Plot of 20%–80% rise time (**B**) or decay time constant (**C**) versus test pulse voltage. Number in parentheses indicates the total number of neurons analyzed. **D**, Normalized peak conductance ($G/G_{\max}$)–voltage relationship ($n = 3$). The solid curve is given by Boltzmann function raised to the fourth power: $[1/(1 + \exp[-(V - V_h)/k])]^4$, where V is the membrane potential, V_h is the potential at which the Boltzmann function is 0.5, and k is the slope factor. V_h is -35.4 mV, and the slope factor is 23.9 mV. This function reaches a midpoint at a value of 1.8 ± 6.6 mV. **E**, BDSII-sensitive K^+ current (red) is obtained by subtracting the sustained component (green) from the total current (control, black). **F**, Summary of the effect of $0.5 \mu\text{M}$ BDSII on peak current amplitude ($n = 6$). **G**, **H**, Summary of 20%–80% rise time and decay time constant between the control and BDSII-sensitive currents ($n = 4$). **I**, **J**, Whole-cell current-clamp recording was performed in growth cones of cultured dorsal spinal neurons. Membrane potential (V_m) was measured before and after focal application of BDSII ($n = 4$). **I**, Representative action potentials were elicited with 200 ms current pulse of 50 pA in the growth cone before (black trace) and after BDSII (red trace) (**J**). Peak is defined from the initial rising phase (blue arrow) to the first valley (**J**, black or red arrow). **K–O**, Properties of action potential before and after BDSII ($n = 6$). Data are mean $\pm$ SEM. n.s., no significant difference. * $p < 0.05$, compared with control (paired-samples t test). *** $p < 0.001$, compared with control (paired-samples t test).

quent 0.5 h (0 – 0.5 h). Photostimulation resulted in axon retraction at frequencies of 120, 60, or 24 h^{-1} , but not at frequencies of 12 or 6 h^{-1} (Fig. 8E). Different from the results for ChR2 $^+$ neurons, axon elongation in EYFP $^+$ neurons was not affected by photostimulation at any frequencies (Fig. 8E). Thus, axon elongation is inhibited by excessive Ca^{2+} influx, which is similar to the effects of Kv3.4 blockade.

Discussion

This study demonstrates that Kv3.4 is required for normal axon growth by controlling membrane excitability in the growth cones of neurons. The proposed mechanism is illustrated in Figure 9.

Roles of Kv3.4 in axon elongation, pathfinding, and fasciculation

Knockdown of Kv3.4 leads to fewer axon-bearing neurons and shorter axons, which can be rescued by reexpressing Kv3.4 (Figs. 3, 4), and blockade of Kv3.4 also results in shorter axons (Fig. 5). These findings demonstrate that Kv3.4 plays an essential role in axon elongation. Although we have observed axon stalling and misguidance after the protein level of Kv3.4 in spinal commissural axons is reduced (Fig. 4), further investigations are required to confirm the role of Kv3.4 in axon pathfinding.

Axon defasciculation occurs not only in the spinal commissural axons of living embryos in response to Kv3.4 knockdown

Figure 8. Excessive Ca^{2+} influx in the growth cone inhibits axon elongation. Constructs encoding ChR2 and jRCaMP1a or EYFP were electroporated into the spinal cord of HH15–HH17 chick embryos. Dorsal spinal neurons were dissociated at HH22–HH23 and cultured for 15–20 h. **A–B'**, The average of $\Delta F/F$ in the growth cone (red) or soma (green) under photostimulation (arrow) during 10 min calcium imaging analysis. The baseline was obtained during 0–1 min. Error bar (gray) indicates SEM. **C, D**, Estimate of Ca^{2+} influx by measuring the area under the curve (AUC) $>0\%$ of $\Delta F/F$ during 1–10 min (**C**) or 1–3.5 min (the effect of a single photostimulation, **D**). **E**, Photostimulation at frequencies of 120, 60, 24, 12, or 6 h^{-1} were applied to cultured neurons expressing only ChR2 or EYFP during 0–0.5 h. Neurite length was measured at -0.5 , 0, and 0.5 h, respectively. When the photostimulation frequencies were at 120, 60, or 24 h^{-1} , the axons of ChR2⁺ neurons were retracted, but the axonal growth rate of EYFP⁺ neurons was not affected. Numbers in parentheses indicate the total number of axon-bearing neurons analyzed. Data are mean $\pm$ SEM. **C, D**, $*p < 0.05$, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). **E**, $**p < 0.01$, comparison of the indicated pairs (Tukey's *post hoc* test after one-way ANOVA). **E**, $**p < 0.01$, compared with EYFP (independent-samples *t* test).

(Fig. 4), but also in the axons projecting from spinal cord explants in response to Kv3.4 blockade (Fig. 5). Axon fasciculation is promoted by axon–axon interactions via cell adhesion molecules, such as L1 and other surrounding repulsive guidance molecules

(Jaworski and Tessier-Lavigne, 2012). Abnormal electrical stimulation, which reduces L1 expression in cultured mouse DRG neurons, is known to lead to axon defasciculation (Itoh et al., 1995). We speculate that abnormal electrical activity, resulting

Figure 9. A schematic summary of Kv3.4 function in the growth cone. **A**, In normal growing axons, the growth cone membrane is depolarized by spontaneous electrical activity (1) or after the binding of an attractive guidance cue (such as netrin-1) to its receptor (such as DCC) (2). Membrane depolarization allows Ca²⁺ influx through T-type and L-type Cav channels sequentially. Then, Kv3.4 channels are activated and Kv3.4-mediated A-type K⁺ outward currents reduce membrane excitability. **B**, After Kv3.4 knockdown by Kv3.4shRNA or Kv3.4 blockade by BDSII, excessive extracellular Ca²⁺ ions enter the growth cone, which leads to axon growth inhibition. BDSII-induced Ca²⁺ influx does not require the release of intracellular Ca²⁺ from the ER (endoplasmic reticulum). **C**, The membrane potential of growth cones can be depolarized by spontaneous electrical activity or by the binding of attractive guidance cues. Slight membrane depolarization induces sustained Ca²⁺ elevation, and the opening of Kv3.4 channels quickly reduces membrane excitability, which can inhibit the generation of action potentials. Substantial membrane depolarization evokes Ca²⁺-dependent action potentials to generate Ca²⁺ transients, and the activation of Kv3.4 channels repolarizes the membrane to reduce the amplitudes of action potentials, resulting in Ca²⁺ transients with smaller amplitudes. Thus, by controlling growth cone membrane excitability, Kv3.4 acts to maintain [Ca²⁺]_i at an optimal concentration for normal axon growth. AP, Action potential; Cav, voltage-gated calcium channel; DCC, deleted in colorectal cancer; IP₃R, inositol 1,4,5-triphosphate receptor.

from knockdown or blockade of Kv3.4, may inhibit the expression of cell adhesion and/or repulsive guidance molecules that are required for axon fasciculation.

Kv3.4 knockdown causes defects in axon pathfinding and fasciculation in spinal commissural axons but not in callosal axons (Fig. 4). There are two possible explanations for this discrepancy. First, these two sets of axons use different guidance cues for axon pathfinding and fasciculation. Second, the spinal commissural axons of chick embryos express Kv3.4, but not Kv3.3 (Fig. 1), whereas the callosal axons of rat embryos express both Kv3.3 and Kv3.4 (Huang et al., 2012). However, Kv3.3 also appears in the growth cone of pluripotent stem cells derived from human fibroblasts (Zhang et al., 2016), and the electrophysiological properties of Kv3.3 are similar to those of Kv3.4 (Rudy and McBain, 2001). Based on the above, it is possible that the presence of Kv3.3 allows axon pathfinding and fasciculation to occur in callosal axons after Kv3.4 knockdown.

Kv3.4 reduces the frequency and amplitude of Ca²⁺ transients during axon growth

Heightened Ca²⁺ transients inhibit axon elongation because the resultant [Ca²⁺]_i exceeds the optimal concentration of [Ca²⁺]_i for normal axon elongation (Kater and Mills, 1991; Gomez and Spitzer, 1999; Tang et al., 2003). The growth cones of chick dorsal spinal neurons exhibit Ca²⁺ transients at a frequency of 8 h⁻¹ (Fig. 6), similar to the frequencies of 1–10 h⁻¹ detected in *Xenopus* spinal neurons (Spitzer, 2002). Blockade of Kv3.4 increases the frequency (from 8 to 20 h⁻¹) and peak amplitude (from ~61.3 to ~486.7 nM) of Ca²⁺ transients (Fig. 6), which are

known changes that inhibit axon outgrowth (Spitzer, 2002; Tang et al., 2003) (Fig. 5). Our findings suggest that Kv3.4 acts as a suppressor to keep Ca²⁺ transients at a low frequency and amplitude, thereby maintaining [Ca²⁺]_i at an optimal concentration for normal axon outgrowth.

Several recent studies have suggested that Ca²⁺ transients in the growth cone may not be required for axon growth. For example, complete blockade of Ca²⁺ transients by overexpressing the K⁺ channel Kir2.1 or knock-out of the synaptic protein Munc18–1 does not affect axon elongation and pathfinding (Benjumeda et al., 2013; Law et al., 2016). Based on our findings, we hypothesize that the frequency of Ca²⁺ transients below the optimal range does not affect axon growth but that the frequency above the optimal range disrupts axon growth.

Kv3.4 repolarizes the membrane to limit Ca²⁺ influx through T-type and L-type Ca²⁺ channels

T-type and L-type Ca²⁺ channels in *Xenopus* spinal neurons are activated at membrane potentials positive to –60 and –30 mV, respectively (Gu and Spitzer, 1993). Activation of T-type Ca²⁺ channels causes a small elevation in [Ca²⁺]_i, which in turn activates L-type Ca²⁺ channels to produce much larger Ca²⁺ currents (Gu and Spitzer, 1993). By contrast, N-type Ca²⁺ channels do not contribute to Ca²⁺ currents in the axonal growth cones of embryonic cortical neurons (Tang et al., 2003). Consistently, our findings show that the Kv3.4 blockade-induced Ca²⁺ influx in axonal growth cones is mediated by T-type and L-type Ca²⁺ channels but not by N-type Ca²⁺ channels (Fig. 6). Moreover, blockade of Kv3.4 in axonal growth cones not only causes the

membrane to become more depolarized but also increases the amplitude and duration of the action potentials (Fig. 7). Based on these findings, we propose the following hypothesis. When the membrane is slightly depolarized, Kv3.4 channels act to repolarize the membrane to prevent Ca^{2+} influx through T-type Ca^{2+} channels; when the membrane is sufficiently depolarized to evoke a Ca^{2+} -dependent action potential, Kv3.4 channels act to repolarize the membrane to reduce Ca^{2+} influx through both T-type and L-type Ca^{2+} channels.

An increase in $[\text{Ca}^{2+}]_i$ in the growth cone can activate numerous target proteins, such as kinases and phosphatases, which leads to cytoskeleton reorganization, membrane trafficking (endocytosis and exocytosis), and adhesion dynamics (Henley and Poo, 2004; Gomez and Zheng, 2006). For example, Ca^{2+} influx inhibits neurite extension via the Ca^{2+} -dependent phosphatase calcineurin in *Xenopus* spinal neurons because activated calcineurin shifts cytoskeletal-regulating proteins to a dephosphorylated state, and this destabilizes actin filaments resulting in inhibition of neurite elongation (Lautermilch and Spitzer, 2000). Our findings show that Kv3.4 limits Ca^{2+} influx, which in turn maintains normal axon growth (Figs. 5, 6, 8); this is presumably mediated via Ca^{2+} -dependent effector proteins in the growth cone.

Kv3.4 is activated at more hyperpolarized potentials and inactivated more slowly in the growth cone

Kv3.4 currents have been detected in the following four types of neurons: the cell bodies of adult rat DRG neurons (RMP = -56.9 mV, the midpoint activation potential ($V_{1/2}$) = 16.8 mV) (Ritter et al., 2015), the somata of 7-day-old rat DRG neurons (RMP = -61 mV, $V_{1/2}$ = 21.6 mV) (Ritter et al., 2012), the axosomatic compartment of 4- to 10-day-old rat hippocampal dentate granule cells (RMP = -55 mV, $V_{1/2}$ = 3.3 mV) (Riazanski et al., 2001), and the growth cone of dorsal spinal neurons of chick embryos (RMP = -46.7 mV, $V_{1/2}$ = 1.8 mV; Fig. 7). If we compare the RMPs across these neurons, the RMP is more depolarized in embryonic neurons than in postnatal and adult neurons. Comparing the $V_{1/2}$ values of the Kv3.4 currents in these neurons, the $V_{1/2}$ values are more negative in embryonic neurons than in postnatal and adult neurons. Based on these two membrane properties (RMP and $V_{1/2}$), Kv3.4 channels in the growth cones of embryonic neurons are likely activated at more hyperpolarized potentials close to the resting potential. Furthermore, Kv3.4 blockade induces a more depolarized RMP (from -49.6 to -37.6 mV; Fig. 7) and thereby increases the incidence of Ca^{2+} influx (Fig. 6). Our findings suggest that some Kv3.4 channels are activated at subthreshold potentials to dampen slight membrane depolarization evoked by Ca^{2+} influx into the growth cone.

Consistent with the fast activation characteristic of A-type Kv channels, the rise time of the Kv3.4 channel in the growth cones of dorsal spinal neurons of chick embryos (20%–80% rise time ~ 1 ms; Fig. 7) is similar to that found in the somata of 7-day-old rat DRG neurons (~ 1 – 2 ms) (Ritter et al., 2012). However, the decay time constant of the Kv3.4 current is much slower in the former (~ 169 ms; Fig. 7) than in the latter (10–20 ms) (Ritter et al., 2012). The slower decay of the Kv3.4 current is likely due to channel modulation by cytoplasmic factors such as protein kinase C, which prolongs the decay time by phosphorylating the inactivation gate of the Kv3.4 channel (Covarrubias et al., 1994). A slower decay of the Kv3.4 channel can prolong its action to reduce membrane excitability in the growth cone.

In conclusion, this study demonstrates a novel role for Kv3.4 in maintaining normal axon growth by keeping $[\text{Ca}^{2+}]_i$ at an

optimal concentration in the growth cone. An increasing number of human genetic disorders resulting from aberrant axon growth have been identified, such as autism spectrum disorders (MacFadden and Minshew, 2013), corpus callosum agenesis, Joubert syndrome, Kallmann syndrome, and horizontal gaze palsy with progressive scoliosis (Engle, 2010). Further research is required to determine whether mutations in the Kv3.4 gene are associated with these neurodevelopmental disorders.

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