K⁺ channel Kv3.1 associates with OSP/claudin-11 and regulates oligodendrocyte development

Seema Tiwari-Woodruff, Luis Beltran-Parrazal, Andrew Charles, Thomas Keck, Trung Vu, and Jeff Bronstein
UCLA Multiple Sclerosis Program, Department of Neurology, David Geffen School of Medicine at UCLA, Los Angeles, California
Submitted 12 October 2005; accepted in final form 12 April 2006

Tiwari-Woodruff, Seema, Luis Beltran-Parrazal, Andrew Charles, Thomas Keck, Trung Vu, and Jeff Bronstein. K⁺ channel Kv3.1 associates with OSP/claudin-11 and regulates oligodendrocyte development. Am J Physiol Cell Physiol 291: C687–C698, 2006. First published April 19, 2006; doi:10.1152/ajpcell.00510.2005.—K⁺ channels are differentially expressed throughout oligodendrocyte (Olg) development. Kv1 family voltage-sensitive K⁺ channels have been implicated in proliferation and migration of Olg progenitor cell (OPC) stage, and inward rectifier K⁺ channels (Kir)4.1 are required for OPC differentiation to myelin-forming Olg. In this report we have identified a Shaw family K⁺ channel, Kv3.1, that is involved in proliferation and migration of OPC and axon myelination. Application of anti-Kv3.1 antibody or knockout of Kv3.1 gene decreased the sustained K⁺ current component of OPC by 50% and 75%, respectively. In functional assays block of Kv3.1-specific currents or knock-out of Kv3.1 gene inhibited proliferation and migration of OPC. Adult Kv3.1 gene-knockout mice had decreased diameter of axons and decreased thickness of myelin in optic nerves compared with age-matched wild-type littermates. Additionally, Kv3.1 was identified as an associated protein of Olg-specific protein (OSP)/claudin-11 via yeast two-hybrid analysis, which was confirmed by coimmunoprecipitation and coimmunohistochemistry. In summary, the Kv3.1 K⁺ current accounts for a significant component of the total K⁺ current in cells of the Olg lineage and, in association with OSP/claudin-11, plays a significant role in OPC proliferation and migration and myelination of axons.

OLIGODENDROCYTES (Olg), which are responsible for central nervous system (CNS) axon myelination, arise from the proliferating neuroepithelial cells of the ventricular and subventricular zones in mammalian brain and the ventral midline and dorsal origin in the spinal cord (23, 37). During development, Olg lineage cells show stage-dependent K⁺ channel expression. Olg progenitor cells (OPC) express all six members of the delayed rectifier Shaker family K⁺ channels, voltage-sensitive K⁺ channels (Kv)1.1–Kv1.6, and show large voltage-dependent outward currents, some of which is due to Kv1 channels (1, 7, 13, 18, 30), which have also been suggested to have a key role in OPC cell cycle progression (7, 14, 28). Olg lineage cells also express Ca²⁺-sensitive and inwardly rectifying K⁺ (Kir) channels, and during differentiation, as the cells elaborate the myelin, the inward rectifiers are upregulated (2, 5, 8, 17, 25, 32, 34). Despite this rich population of K⁺ channels, a large component of sustained outward K⁺ current is still unaccounted for. Here we report that the bulk of OPC K⁺ current is due to Kv3.1 from the Shaw family of K⁺ channels, which are expressed early but persist in differentiated cells and play an important role in proper myelination.

Kv3.1 has been well characterized in different neuronal subtypes, including cerebellar granule cells, substantia nigra reticulata, thalamic nuclei, inferior colliculus, cochlear nuclei, and the superior olive medial nucleus of the trapezoid body, and nodes of Ranvier (10, 19, 20, 24, 25, 27, 41). The presence of Kv3.1 has not been investigated in OPC/Olg lineage cells. Our attention focused on this channel because it was identified by yeast two-hybrid analysis as an associated protein of Olg-specific protein (OSP)/claudin-11 (38, 39), a member of the claudin family of tight junction proteins and a probable autotoxin in the development of autoimmune demyelinating disease (6, 36). We hypothesized then that Kv3.1 is expressed in significant quantities in Olg cells and plays a role in their structure and function. In this report we present findings in support of our hypothesis, including 1) the presence of significant Kv3.1 message and protein in Olg lineage cells, 2) structural changes in myelin of Kv3.1-knockout (Kv3.1−/−) mice, 3) significant reduction of outward K⁺ currents by Kv3.1 antibody, and 4) significant decrease in outward K⁺ current in OPC of Kv3.1−/− mice. Additionally, the association of Kv3.1 with OSP/claudin-11 was further tested by coimmunoprecipitation, coimmunohistochemistry, and electrophysiology analysis, and the functional effects of this association were probed by proliferation and migration assays, functions previously shown to be affected by OSP/claudin-11 knockout and immunoblock (38, 39).

MATERIALS AND METHODS

Materials. Rabbit anti-Kv1.3, -Kv1.5, -2.1, -SK1, -SK2, -SK3, -Kir4.1, -Kv3.2, -Kv3.3, -Kv3.4, and -Kv3.1 antibodies (1:200 to 1:500), agitoxin-2 (Alomone Labs, Israel), anti-bromodeoxyuridine (BrdU) (1:20; Oncogene, San Diego, CA), rabbit anti-Kv1.2, -Kv3.1b, and -Kir4.1 antibodies (1:500); mouse anti-O4 antibody, GalC, mouse anti-Caspr antibody (1:250); and rat anti-myelin basic protein (MBP) (1:30) and rabbit anti-NG2 (1:100) antibodies were purchased from Chemicon (Temecula, CA). Rabbit anti-Kv3.1loop was previously kindly donated by Dr. X. Y. Huang (44); later we generated a similar antibody, using the same peptide sequence [rabbit anti-Kv3.1loop, against a 14-amino acid peptide (GAQPNDP-SASEHTH) from the external vestibule of the Kv3.1 channel protein; Sigma Genesis]. The peptide sequence has high homology to other members of the Kv3 family (Kv3.2, -3.3, and -3.4). Kv3.1loop anti-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
body recognized only Kv3.1a and Kv3.1b protein subunits in wild-type (WT) homogenates and none in Kv3.1a−/− homogenates. Rat anti-platelet-derived growth factor receptor (PDGF)-α and anti-BrdU antibodies were obtained from BD Biosciences-Pharmingen and Oncogene. The second antibody step was performed by labeling with antibodies conjugated to tetramethylrhodamine isothiocyanate, FITC, and Cy5 (Vector Labs and Chemicon). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Invitrogen (Carlsbad, CA).

Animals. WT, OSP/claudin-11 heterozygous knockout (C57BL/6 × C3H; Ref. 15), and Kv3.1 heterozygous knockout (C57BL/6 from Dr. Bernardo Rudy, New York University, New York, NY; Refs. 16, 24) mouse breeding pairs were used to obtain postnatal day (P)0–P50 animals. Littermates were genotyped by using tail DNA as the template for amplification by PCR as shown previously (12, 15). Animals were housed under guidelines set by the National Institutes of Health and experiments were conducted in accordance with the UCLA Chancellor’s Animal Research Committee and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Perfusion. Mice were deeply anesthetized with isoflurane and perfused transcardially with ice-cold 0.9% saline followed by 4% paraformaldehyde. Brains with optic nerves (ON) attached were removed, postfixied overnight in 4% paraformaldehyde, and cryoprotected with 20% sucrose solution in PBS. Free-floating sections (25 µm thick) were cut coronally with a sliding microtome and collected serially in PBS.

Yeast two-hybrid screen, yeast two-hybrid bait, and library constructions. Three OSP/claudin-11 constructs were used as bait for yeast two-hybrid screen: the entire OSP/claudin-11 open reading frame (ORF), the first extracellular domain of OSP/claudin-11 between amino acids 35 and 84, and the COOH terminus of OSP/claudin-11 between amino acids 122 and 207. These cDNAs were cloned into the pGBT9 backbone (Matchmaker system; Clonetech Labs). GAL4 activation domain cDNA fusion libraries were constructed in modified pGAD GH vector (Clonetech), using mRNA from mouse brain. Yeast two-hybrid screening was performed according to Tiwari-Woodruff et al. (38).

Isolation and differentiation of O2A cells. OPC were isolated from P0–P2 mouse brains by immunopanning with anti-A2B5 (3) or with the shaking technique (22). For differentiation to mature Olg medium was supplemented with T3-T4-containing Sato medium, and cells were postfixed after 2 (O4+), 4 (GalC+), and 8 (MBP+) days to maximize stage-specific Olg.

RT-PCR. RNA was isolated from P0 and P25 A2B5+ OPC and MBP+ Olg, and first-strand cDNA was synthesized with random hexamer primers and Superscript II reverse transcriptase (Invitrogen). PCR primers were designed according to the mouse/rat Kv3.1 cDNA sequence: [Kv3.1a (accession no. NM008421): primer pairs to cover bp 1018–1650; Kv3.1b (accession no. M68880): primer pairs to cover bp 591–1042] mRNA was performed simultaneously.

Western blot analysis. Immunoprecipitation and Western blot analysis were performed as described previously (38, 39). OPC and mouse brain were homogenized in buffer A (mM: 150 NaCl, 1 EDTA, and 10 Tris-HCl and 1% Triton X-100) or radioimmunoprecipitation assay (RIPA) buffer (containing 0.1% SDS, 0.5% deoxycholate in addition to 1% Triton X-100) in the presence of protease and phosphatase inhibitors. Twenty micrograms of protein in sample loading buffer containing 10% β-mercaptoethanol and 5 mM DTT was subjected to SDS-PAGE and transferred to nitrocellulose. Specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibody were used and visualized with chemiluminescence substrate (Pierce, Rockford, IL). For immunoprecipitation 100 µg of homogenates was incubated with antibodies on protein A agarose beads.

Beads were washed and boiled in 40 µl of sample buffer (containing 2% SDS, 10% β-mercaptoethanol, and 10 mM DTT), subjected to SDS-PAGE, and transferred to nitrocellulose. Antibody concentration and immunoprecipitation efficiency (by Western blot) were confirmed for each antibody used. Images were scanned in Adobe Photoshop (Adobe Systems, San Jose, CA).

Proliferation and migration assays. To identify proliferating cells in vitro, primary OPC from WT, transgenic Kv3.1, and OSP/claudin-11 littersmates were isolated and counted and then labeled with BrdU for 24 h. Cell migration was quantified by counting the number of cells moving from agarose drops as described previously (38). Statistical significance was assessed with the Student’s paired t-test.

Histopathology and immunohistochemistry. Serial sections were mounted on slides and stained with hematoxylin and eosin. Consecutive sections were also examined by immunohistochemistry. Brain slices and Olg lineage cells were immunostained as previously described (38, 39). Gelatin-embedded optic nerves were similarly immunostained. IgG control experiments were performed for all primary antibodies, and no staining was observed under these conditions.

Briefly, sections and cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, immunostained with primary followed by secondary antibodies, washed and mounted on cover slides, dried, and coverslipped with Fluoromount G. For better penetration of antibodies (Kv3.1, OSP/claudin-11, and MBP staining) to label white matter structures, some spinal cord and brain sections were pretreated with 95% ethanol-5% acetic acid for 15 min, before proceeding through the permeabilization steps. Confocal imaging was performed with a Leica TCS-SP (Mannheim, Germany) microscope, and some images were also taken with a fluorescence microscope (BX51W1; Olympus, Tokyo, Japan) equipped with Plan Fluor objectives connected to a camera (DP70, Olympus). Digital images were collected and analyzed with Leica confocal and DP70 camera software. Images were assembled with Adobe Photoshop.

Electrophysiology. Current recordings were obtained with the whole cell configuration of the patch-clamp technique as previously described (28, 33). OPC were perfused with (mM) 150 NaCl, 5.4 KCl, 1.5 CaCl2, 1.5 MgSO4, 10 glucose, and 10 HEPES with 0.5–1 µM tetrodotoxin (pH 7.3). K+ channel blockers 4-aminopyridine (4-AP) and tetraethylammonium chloride (TEA) and Kv3.1 channel antibodies were directly added to the bath solution. Patch-clamp pipettes were filled with internal solution containing (mM) 130 KCl, 2 MgCl2, 1.0 CaCl2, 10 EGTA, and 10 HEPES, pH 7.3. Delayed rectifier currents were routinely investigated by applying a series of voltage steps ranging from −80 to +80 mV for 500 ms from a holding potential (V0) of either −80 or −40 mV. Cell sealing and breakthrough into whole cell mode was performed in current-clamp conditions permitting an accurate determination of cell resting membrane potential. To determine the voltage dependence of steady-state activation, currents were elicited by 50-ms voltage pulses (−80 to +80 mV, 10-mV increments) from a V0 of −80 mV. After the steady-state peak outward currents (Ik) were converted into conductance (Gk = I(V0)/E0, where E0 is Nernst potential of K+ at 21°C, i.e., −80 mV), conductance (G) at various membrane potentials was normalized to maximal conductance (Gmax). Gmax was plotted vs. V0. Curves were fitted to Boltzmann functions to determine the voltage of half-maximal activation (V1/2). In some cases, the peak outward currents were directly plotted vs. V0. Patch-clamp recordings were performed with an Axopatch 200A amplifier; data were stored and analyzed with Axon Instruments (Foster City, CA) pCLAMP software and Origin 6.0 (Microcal Software) on a Pentium PC. All data were collected at 10 kHz and analog filtered at 5 kHz.

Data analysis and statistics. Data are reported as means ± SE. Statistical significance was assessed with a two-sided t-test for paired or unpaired samples at the significance level (P). SEs of fitted parameters were obtained by analyzing data of individual experiments separately. Results of electrophysiology were statistically evaluated.
with the Origin 6.0 software package. Boltzmann fits were performed with the same software.

**Assaying process length and complexity.** Differences in length and complexity of processes between Kv3.1+/− and littermate Olg were determined by using a modification of the Scholl method (29). A circular grid was constructed on a plastic transparency that was superimposed on a ×40 image of cells printed on paper. Twenty-five MBP-stained Olg from four different litters of Kv3.1+/− and control mice were photographed at ×40. The nucleus of the cell was positioned beneath the center of the grid, and each section was assigned a score equal to the diameter of the circle crossed by the longest process in the section. An approximate diameter score was obtained by averaging these numbers.

**Quantification of light and electron microscopy.** For light microscopy, semithin sections of ON and brain stem were cut at 2 μm, toluidine blue counterstained, and photographed. Three regions from each section were randomly chosen for quantification. Axon diameter was measured by tracing the perimeter of 50 neighboring axons in several samples for a total of 200 axons from Kv3.1+/− (n = 5) and Kv3.1+/-+ (n = 4) age-matched P50 littermates, and pooled data across individual animals within each group were used to calculate means ± SE. For electron microscopy, age-matched littermates were perfused with saline followed by fixative (2% glutaraldehyde + 2% paraformaldehyde). Serial ultrathin sections from fixed ON embedded in Epon oriented to visualize myelinated axons were stained with uranyl acetate–lead citrate, and representative areas were photographed. Montages (4–6) consisting of either ×4,800 or ×14,000 were obtained from select sections. To measure myelin thickness we overlaid a square grid on the photographs and measured the myelin width every time a vertical grid encountered myelin. We avoided measuring myelin areas that were loosely compacted or frayed. For most axons two encounters were measured. The ratio of axon diameter to total fiber diameter (g ratio) was measured from both Kv3.1+/− and Kv3.1+/-+ P25 and P50 ON and brain stem by dividing the circumference of an axon without myelin by the circumference of the same axon including myelin.

**RESULTS**

**Kv3.1 mRNA and protein are expressed in cells of Olg lineage.** Kv3.1 gene transcription can result in alternative-spliced products that differ only in their COOH-terminal sequence, with the last 18 amino acid sequences in Kv3.1a and Kv3.1b, bound three proteins of 96, 75, and 60 kDa in OPC and Olg (Fig. 1B, right). The 75-kDa and 60-kDa bands could be glycosylated and nonglycosylated Kv3.1, as the bands disappear when Kv3.1+/− homogenates are used. Even though the peptide (GAQPNDPSSAE)TH from the external vestibule of the Kv3.1 channel protein used to make Kv3.1loop antibody is highly homologous to Kv3.2 and to a lesser amount to Kv3.3 and Kv3.4, the antibody is specific to Kv3.1a and Kv3.1b, as no significant immunostaining is observed when Kv3.1+/− tissue is used. The specific bands were also not observed when appropriate antigenic peptides were used to preadsorb the antibodies.

The presence of Kv3.1 in OPC and Olg in culture was also detected immunohistochemically, with both Kv3.1b-specific and Kv3.1loop antibodies giving robust staining. Figure 1C, left, shows cultured Olg labeled with Kv3.1loop antibody. Figure 1C, middle, shows the same field labeled with MBP staining, a marker for late differentiating Olg. The merged images (Fig. 1C, right) show that a Kv3.1+ Olg was also MBP+. The inset in Fig. 1C shows an A2B5 immunopanned OPC in culture labeled with Kv3.1b antibody. No staining was observed when peptide-adsorbed antibodies were used or when OPC and Olg from Kv3.1+/− mice were used. OPC also immunolabeled moderately with antibodies to Kv1.3, Kv2.1, SK3-type Ca2+-activated K+ channels, and Kv3 subtypes Kv3.2 and Kv3.3 and intensely with antibodies to Kv1.5, SK2-type Ca2+-activated K+ channels, Kv4.1, and Kv3.4 (Fig. 1D). No immunostaining difference was observed between WT and Kv3.1+/− OPC and Olg for these Kv3+ channels.

Olg lineage cells also label with anti-Kv3.1 in vivo. Some perfusion-fixed P4–P12 brain sections were labeled with hematoxylin and eosin stain for orientation, and selected sections were coimmunostained with Kv3.1loop antibody and two Olg lineage markers, anti-PDGFR-α and anti-rip. Anti-PDGFR-α labels precursor cells committed to Olg lineage, and anti-rip specifically labels rip, a protein of unknown function, found in the cytoplasm of premyelinating and myelinating Olg. OPC in the corpus callosum (box in hematoxylin and eosin-stained section in Fig. 2A) of P4–P12 brains were Kv3.1loop+/Kv3.1b- and PDGFR-α- and Kv3.1- and rip-immunopositive (Fig. 2Bi–iii). Anti-chondroitin sulfate proteoglycan NG2, which identifies OPC in adult brain, also colabeled with Kv3.1b antibody (results not shown). P15 optic nerve Olg showed robust immunostaining with anti-Kv3.1loop (Fig. 2Bi, inset) and Kv3.1b data (not shown). It is important to note that although most of the positively indicated OPC and Olg cells were also positive for Kv3.1, Olg and OPC markers did not label many of the Kv3.1+ cells in vivo. These cells are likely neurons, but we did not analyze this directly.

**Myelin thickness and average axon diameter are reduced in optic nerve of Kv3.1+/− mice.** Anti-MBP immunostaining (Fig. 2, C and D) and electron microscopy analysis (Fig. 2, E and F) revealed five consequences of Kv3.1 knockout for Olg morphology and axon myelination. 1) Olg showed a significant (P < 0.05) decrease in diameter, 54 ± 8 μm (n = 25, 4 different litters) for Kv3.1+/− compared with 79 ± 4 μm for Kv3.1+/+ littermates (n = 25). 2) There was reduced Olg membrane branching complexity (Fig. 2C) [MBP immunostained 8–12 Olg per well per animal, and 6 different pairs of Kv3.1+/+ and Kv3.1+/− littermates were compared]. 3) As
shown in the white matter tracts near the corpus callosum (Fig. 2D), there was discontinuous and punctate MBP$^{+}$ staining in Kv3.1$^{-/-}$ compared with the even staining observed with Kv3.1$^{+/+}$ littermates [corpus callosum areas from 5 Kv3.1$^{-/-}$ and Kv3.1$^{+/+}$ littermate brains were compared]. 4) There is a substantial reduction in the number of large-diameter axons, also shown for the cross section of the ON and brain stem in Fig. 2Eii−iii. 5) The thickness of the myelin layer is reduced, as is shown for the ON in the electron microscopy sections of Fig. 2Eii. Axon diameter was measured by tracing the perimeter of 50 neighboring axons in several samples for a total of 200 axons from Kv3.1$^{-/-}$ (n = 5) and Kv3.1$^{+/+}$ (n = 4) P50 litters. Mean axon diameter was reduced in Kv3.1$^{-/-}$ (0.78 ± 0.04 μm compared with controls, 0.93 ± 0.14 μm; Fig. 2Eiv). ON myelin sheath thickness was 0.069 ± 0.04 μm in Kv3.1$^{-/-}$ compared with 0.089 ± 0.03 μm in Kv3.1$^{+/+}$ litters (Fig. 2Evi). These differences were significant (P < 0.05) across multiple sections of age-matched animals (n = 4).

The thickness of the myelin sheath is related to the axon diameter, and the g ratio, defined as the ratio of the inner fiber diameter to the outer diameter, presents the precise relation between the axon diameter and the myelin sheath thickness. To determine whether there was a variation in the amount of myelin around myelinated axons in the CNS of Kv3.1$^{-/-}$ mice, we calculated the ratio of the axon diameter to the total fiber diameter (g ratio). Axon regions that were without compact myelin were not included in this analysis. The calculated g ratio was not significantly different between Kv3.1$^{-/-}$ and Kv3.1$^{+/+}$ animals in either ON (Fig. 2Ev) or brain stem. To analyze whether there were any major changes in myelin protein of Kv3.1$^{-/-}$ animals we analyzed PLP and OSP protein levels of age-matched Kv3.1$^{-/-}$ and Kv3.1$^{+/+}$ PS0 brain by Western blot but saw no significant difference by densitometry of the gel bands (data not shown).

Kv3.1 channel is major component Ik in mouse OPC. From previous characterizations in neurons, Kv3.1-dependent currents should 1) not be inactivated at modestly depolarized potentials, 2) be sensitive to low concentrations of TEA (relative to Kv1 currents, which are sensitive to high TEA), and 3) be sensitive to anti-Kv3.1loop antibody. In addition, outward currents should be substantially reduced in Kv3.1$^{-/-}$. These predictions were tested in cultured A2B5$^{+}$ OPC.

The resting potential of OPC was −35 ± 6 mV (n = 62). K$^{+}$ currents activated from a V_h near the resting membrane potential (−40 mV) were slowly activating and noninactivating and had a V_{1/2} of +10−6.5 mV, similar to the range that has been described for Kv3.1 channels (21). K$^{+}$ currents activated from a more hyperpolarized potential of −80 mV were a combination of rapidly activating, fast inactivating A currents (I_A) and slowly activating, noninactivating currents (Fig. 3C).

Kv3.1 channels are relatively sensitive to TEA (IC$_{50}$ = 150−200 μM) compared with Kv1 channels (IC$_{50}$ = 2−50 μM). From V_h = −40 mV outward currents were decreased by 19 ± 5% by 100 μM TEA and 78 ± 4% by 1 mM TEA (Fig. 4, A and B). From V_h = −80 mV outward currents were decreased by only 5 ± 2% by 100 μM TEA and by only 50 ± 4% by 1 mM TEA (Fig. 3, C and D).

Kv3.1loop antibody (250 nM), directed to an epitope in the external vestibule of Kv3.1 channel, blocked ~79% of the Kv3.1 currents from Kv3.1-transfected HEK cells but not other related (Kv1.2) K$^{+}$ channels (44). In OPC Kv3.1loop antibody blocked 48 ± 6.4% of the outward K$^{+}$ conductance within 7–10 min of application (n = 6; Fig. 3, E and F). Additional use of 1 mM TEA reduced outward current to 25% of control (Fig. 3, E and F). G/G$_{max}$ vs. voltage curves show nearly 50% block with Kv3.1loop antibody present for 10 min and little effect on the voltage sensitivity. The antibody specific to splice variant Kv3.1b is directed to the cytoplasmic COOH termini of Kv3.1b and was used as a control. It did not show inhibition of the currents (data not shown).

Outward K$^{+}$ currents of OPC are significantly reduced in the absence of Kv3.1 protein. Kv3.1 current was also investigated by comparing the physiological properties of Kv3.1$^{-/-}$/OPC to Kv3.1$^{+/+}$ littersmate. The resting membrane potential of Kv3.1$^{-/-}$ OPC was −35 ± 3 mV (n = 12), not different from controls; however, outward K$^{+}$ currents from either V_h = −40 or −80 mV were dramatically reduced in Kv3.1$^{-/-}$ (Fig. 3G), primarily as a result of decrease in the sustained component. Kv3.1$^{-/-}$ OPC showed decreased current throughout the range of activating potentials (Fig. 3H), with the largest difference for large depolarizations. From V_h = −40 mV, a step to +60 mV produced 1.596 ± 284 pA (means ± SE; n = 42) in controls compared with only 147 ± 58 pA (n = 9) in Kv3.1$^{-/-}$.

Much of the voltage-activated OPC currents from V_h = −80 mV is fast I_A, which is inactivated when voltage is held at −40 mV. This current can be estimated by subtracting outward K$^{+}$ currents obtained from V_h = −40 mV from those obtained at −80 mV. If this is done for Kv3.1$^{+/+}$ and Kv3.1$^{-/-}$ the “subtracted” I_A components are very similar in amplitude and shape, suggesting that when Kv3.1 gene is knocked out the K$^{+}$ channels that underlie the I_A current are expressed normally and are unaffected. Consistent with this finding, V_{1/2} of activation in Kv3.1$^{-/-}$ obtained from V_h = −40 mV and −80 mV showed a significant difference (−5.1 ± 4 and −20 ± 2.5 mV, respectively) compared with Kv3.1$^{+/+}$ (8.6 ± 4 and 20 ± 6 mV) (P < 0.01). Thus the remainder of K$^{+}$ currents in...
K⁺ CHANNEL Kv3.1 REGULATES OLIGODENDROCYTE FUNCTION
K+ CHANNEL Kv3.1 REGulates OLIGODENDROCYTE FUNCTION

Fig. 2. Olg lineage cells express Kv3.1 in vivo. A: P12 brain section near the hippocampus (hp) and corpus callosum (cc), hematoxylin and eosin stained, showing the region immunolabeled in solid box in B. Bar, 0.5 mm. B: i: OPC labeled with platelet-derived growth factor receptor (PDGFR-α) antibody (green) also colabeled with anti-Kv3.1loop (red), shown more clearly for the few cells (boxed area in i) with individual staining (ii). Bar, 10 μm. Inset, optic nerve OPC immunolabeled with anti-Kv3.1loop (red; magnification ×40). iii: Same region of the brain labeled with anti-rip (green) and anti-Kv3.1loop (red) showing separate staining and merged images. iv: Similar region of the brain immunostained with PDGFR-α (green) and Kv3.1b (red). Merged image shows co-staining in 2 of 3 cells. C: Olg in differentiating medium are immunolabeled with MBP (×40 magnification). Kv3.1+/+ Olg in differentiating medium immunolabeled with MBP shows extensive myelin sheaths compared with Kv3.1−/− Olg. D: Kv3.1−/− MBP-immunostained white matter tracts near the outer borders of corpus callosum (×63 magnification) show bunched and discontinuous staining. E: i: 70-nm optic nerve cross sections were photographed with electron microscope at ×4,800 magnification. ii: Optic nerve cross sections photographed at ×19,000. Insets, examples showing the thinner myelin sheath in Kv3.1−/− (left) and Kv3.1+/− (right). Only the 20-mV increment steps are shown, for clarity. Calibration bars are for all 4 sets of data. E: steady-state current-voltage values for WT (○; n = 42) and Kv3.1−/− (●; n = 9) OPC for Vh = −40 mV. F: normalized conductance-voltage plots for the OPC in H.

Fig. 3. OPC outward K+ currents are sensitive to tetraethylammonium chloride (TEA), anti-Kv3.1 antibody, and Kv3.1−/−. A: OPC outward currents when the cell was depolarized to −40 mV from holding potential (Vh) of −40 mV before (○) and after addition of 100 μM (●) and 1 mM (■) TEA. Calibration bars are for A, C, and E. B: currents from a Vh of −40 mV for 28 OPC were converted to conductance (G), and G/Vmmax was plotted against voltage before (○) and after TEA (●, 100 μM; ■, 1 mM). Error bars are SE. C: currents from the same cell from a Vh of −80 mV before (○) and after 100 μM (●) and 1 mM (■) TEA. D: as in B from a Vh of −80 mV. E: OPC K+ currents evoked from Vh = −40 mV to 60 mV before (○) and after addition of anti-Kv3.1loop antibody (250 nM) and after TEA (●). Only the 20-mV increment steps are shown. Calibration bars are for all 4 sets of data. F: conductance-voltage curves of the same experiment. G: outward OPC currents were generated by voltage steps in 10-mV increments from −80 mV to 80 mV from Vh = −40 mV (top) or −80 mV (bottom) in Kv3.1+/+ littermates (left) and Kv3.1−/− (right). The 20-mV increment steps are shown, for clarity. Calibration bars are for all 4 sets of data. H: steady-state current-voltage values for WT (○; n = 42) and Kv3.1−/− (●; n = 9) OPC for Vh = −40 mV.
CHANNEL Kv3.1 REGULATES OLIGODENDROCYTE FUNCTION

A

\[
\begin{align*}
96 \text{kDa} & \quad 70 \text{kDa} & \quad 55 \text{kDa} & \quad 23 \text{kDa} \\
\text{OSP/IP} & \quad \text{Kv3.1b} & \quad \text{Kv3.1a} & \quad \text{IgG} \\
\text{Kv3.1b IP} & \quad \text{OSP/claudin-11} & \quad \text{OSP/ Claudin-11} \\
\text{GFAP IP} & \quad \text{OSP/IP} & \quad \text{Kv3.1 IP} \\
\text{Kv3.1b IP} & \quad \text{OSP/IP} & \quad \text{Kv3.1 IP} \\
\text{Kv3.1a IP} & \quad \text{OSP/IP} & \quad \text{Kv3.1 IP} \\
\end{align*}
\]

B

\[
\begin{align*}
96 \text{kDa} & \quad 60 \text{kDa} & \quad 55 \text{kDa} & \quad 23 \text{kDa} \\
\text{OSP/IP} & \quad \text{Kv3.1b} & \quad \text{Kv3.1a} & \quad \text{IgG} \\
\text{Kv3.1b IP} & \quad \text{OSP/claudin-11} & \quad \text{OSP/ Claudin-11} \\
\text{GFAP IP} & \quad \text{OSP/IP} & \quad \text{Kv3.1 IP} \\
\text{Kv3.1b IP} & \quad \text{OSP/IP} & \quad \text{Kv3.1 IP} \\
\text{Kv3.1a IP} & \quad \text{OSP/IP} & \quad \text{Kv3.1 IP} \\
\end{align*}
\]

C

\[
\begin{align*}
\text{Kv3.1b} & \quad \text{OSP/claudin-11} & \quad \text{merged} \\
\text{OSP/claudin-11} & \quad \text{merged} \\
\text{caspr} & \quad \text{OSP} & \quad \text{Kv3.1} \\
\text{merge} & \quad \text{merge} & \quad \text{merge} \\
\text{Kv3.1} & \quad \text{OSP/claudin-11} & \quad \text{merge} \\
\end{align*}
\]

D

\[
\begin{align*}
\text{OSP/claudin-11} & \quad \text{OSP/claudin-11} & \quad \text{merge} \\
\text{OSP} & \quad \text{Kv3.1} & \quad \text{merge} \\
\text{Kv3.1} & \quad \text{OSP/claudin-11} & \quad \text{merge} \\
\end{align*}
\]

E

\[
\begin{align*}
\text{OSP/claudin-11} (-/-) & \quad \text{OSP/claudin-11} (+/+). \\
-80 \text{mV} & \quad 500 \text{pA} \\
-40 \text{mV} & \quad \\
\end{align*}
\]

F

\[
\begin{align*}
\text{OSP/claudin-11} (+/-) & \quad \text{OSP/claudin-11} (-/-) \\
\text{Kv3.1} +/+ & \quad \text{Kv3.1} -/- \\
\end{align*}
\]
KV3.1→/− OPC could be due to remaining KV1.3, KV1.5, and IA-producing K⁺ channels.

OSP/claudin-11 and KV3.1 form a protein complex in Olg progenitors. A mouse brain cDNA GAL4 activation domain library was screened with OSP/claudin-11 GAL4 binding domain baits in pGBT vector (38). Only transfection with OSP/claudin-11 C-portion bait (amino acids 122 and 207) resulted in positive clones. The two clones were called OSP/claudin-11-associated protein (OAP)-1 and OAP-2. OAP-1, found to be a novel member of the tetraspanin superfamily, forms a complex with OSP/claudin-11 and β₁-integrin (38). OAP-2 contained nucleotide sequence 372–1506 of the ORF, corresponding to amino acids 124–502 (COOH termini) of the voltage-sensitive K⁺ channel of the Shaw family, KV3.1. The region of KV3.1 associated with OSP/claudin-11 is common to both splice variants of the channel.

Coimmunoprecipitation experiments were done to confirm the association of KV3.1 with OSP/claudin-11. OPC homogenates were immunoprecipitated under relatively stringent conditions (1% Triton X-100; Ref. 9) with anti-OSP/claudin-11 or anti-KV3.1b specific antibodies, with anti-glia fibrillary acidic protein (GFAP) and anti-KV1.3, proteins not expected to associate with either KV3.1 or OSP/claudin-11, used as controls. Western blots were probed with anti-KV3.1b antibody (Fig. 4A, left) or anti-OSP/claudin-11 (Fig. 4A, right). There was reciprocal immunoprecipitation of OSP/claudin-11 with anti-KV3.1 and KV3.1 with anti-OSP/claudin-11. Neither KV3.1b nor OSP/claudin-11 was precipitated when the primary antibody was omitted or when primary antibodies to unrelated proteins, GFAP or KV1.3, were used.

Immunoprecipitation was also done in a more stringent RIPA buffer, containing 0.5% deoxycholate, 0.1% SDS, and 1% Triton X-100 (Fig. 4B). Homogenates were immunoprecipitated with KV3.1b-specific, KV3.1loop, and OSP/claudin-11 antibodies, and Western blots were probed with either anti-KV3.1b (left) or anti-OSP/claudin-11 (right). The IgG band is visible near 56 kDa. The anti-KV3.1b-labeled band was 96 kDa; the OSP/claudin-11 band was 23 kDa; and the KV3.1a band was 60 kDa. The protein between 75 kDa. See text for details.

Steady-state current-voltage values for WT (data not shown). In adult brain, KV3.1b and KV3.1loop immunostaining was also found in neurons and in most white matter tracts, where it partially colocalized with OSP/claudin-11 (Fig. 4D, i and iv). OSP/claudin-11 colabeled with the paranodal protein Caspr, whereas some KV3.1loop and KV3.1b (not shown) immunostaining was observed in the nodes, between Caspr-immunostained paranodes, consistent with previous studies (Fig. 4D, ii and iii). KV3.1 and OSP/claudin-11 did not appear to colocalize in either the paranode or the nodal region. Magnified images in the corpus callosum and ON (not shown) indicated specific regions that had colabeling of the two proteins (Fig. 4Div, merged image). We have not been able to clearly define the colocalization of these proteins to the myelin tight junctions.

To understand the functional significance of KV3.1 and OSP/claudin-11 interaction in OPCs, we analyzed K⁺ currents in the absence of OSP/claudin-11. Resting membrane potential of OSP/claudin-11→/− OPC were −37 ± 3 mV (n = 14) and were similar to OSP/claudin-11+/− OPC. Outward K⁺ cur-

Fig. 4. KV3.1b associates with OSP/claudin-11. A: primary OPC homogenates (80–100 μg) were immunoprecipitated (IP) (43) with anti-OSP/claudin-11, -KV3.1b, -glial fibrillary acidic protein (GFAP), or -KV1.3 antibodies, and Western blots were probed with either anti-KV3.1b (left) or anti-OSP/claudin-11 (right). The IgG band is visible near 56 kDa. The anti-KV3.1b-labeled band was −96 kDa, and OSP/claudin-11 was at 23 kDa. B: IP of OPC homogenates performed with more stringent radioimmunoprecipitation assay (RIPA) buffer conditions. Top: probe with anti-KV3.1loop antibody. Bottom: probe with anti-OSP/claudin-11 antibody. The anti-KV3.1b-labeled band was 96 kDa; the OSP/claudin-11 band was 23 kDa; and the KV3.1a band was 60 kDa. The protein between KV3.1a and KV3.1b is −75 kDa. See text for details. C: i: primary cultures of GalC⁺ Olg and A2B5⁺ OPC (inset) were immunolabeled with anti-KV3.1b (green) followed by anti-OSP/claudin-11 (red). Merged images show regions of colocalization in yellow. ii: Live A2B5⁺ OPC were immunolabeled with anti-KV3.1loop and OSP/claudin-11 and then fixed. Colocalization is observed more on the membrane surface borders. iii: MBP⁺ Olg immunostained with KV3.1loop and OSP/claudin-11 showing maximal colocalization in the cell body and minimal in the processes. D: i: region of P45 brain including corpus callosum and corpus callosum and cortex immunostained with KV3.1loop (green) and OSP/claudin-11 (red) in merged confocal image. Only KV3.1 immunostaining is in neuronal cells (stars), whereas in some parts of the white matter it colabels with OSP/claudin-11, which is exclusively present in the white matter. A higher magnification of the area marked in white square is shown in iv (arrows denote colabeling of OSP/claudin-11 and KV3.1loop). ii: P25 optic nerve immunostained with anti-Caspr antibody labels the paranode regions and OSP/claudin-11 labels most (arrowheads), but not all (stars) paranodes. iii: Anti-KV3.1loop immunolabels a few nodes (arrowhead between Caspr’ paranodes; stars denote no KV3.1 immunostaining). Inset in iv shows anti-KV3.1b in P45 corpus callosum white matter. E: OPC K⁺ currents were generated by voltage steps in 20-mV increments from −80 mV to 80 mV from Vh = −40 mV (top) or −80 mV (bottom) in OSP/claudin-11+/− (left) and OSP/claudin-11−/− (right) littermates. Steady-state current-voltage values for WT (+, n = 42) and normalized conductance-voltage plots are shown. F: Pre-Olg from OSP/claudin-11+/− and OSP/claudin-11−/− cultured for 2 days in differentiating medium were immunostained with anti-KV3.1loop antibody (green). Anti-A2B5 immunopanned KV3.1−/− and KV3.1+/+ littermate OPC were cultured for 4 days and immunostained with anti-OSP/claudin-11 antibody (red). No significant difference in either KV3.1 or OSP/claudin-1 localization was observed.
The Kv3.1 channel is involved in OPC proliferation and migration. Proliferation assays were performed in OPC cultured for 24 h in the presence of mitogenic factors (PDGF and bFGF; Fig. 5A). Kv3.1 channel blockers TEA and 4-AP inhibited OPC proliferation in a concentration-dependent manner, and to an extent the inhibition was comparable to anti-Kv3.1loop antibody addition. Boiled anti-Kv3.1loop antibody had no effect. Anti-Kv1.3 antibody that targets an extracellular domain of Kv1.3 inhibited proliferation slightly. Anti-β1-integrin and anti-OSP/claudin-11 caused significant reduction in proliferation (38), and Kv3.1loop antibody did not block proliferation of Kv3.1+/− OPC (Fig. 5B). Kv3.1+/− OPC had a small (20%) but significant reduction in proliferation compared with Kv3.1+/+ OPCs (Fig. 5A, right).

In vitro OPC migration, which depends on fibronectin substrate (Fig. 5B), was inhibited in the presence of Kv3.1 channel blockers TEA and 4-AP and anti-Kv3.1loop antibody. Antibody to the COOH terminus of Kv3.1b (anti-Kv3.1b) and anti-GFAP antibody, both added as controls, did not affect migration. Kv3.1+/− OPC showed a significant reduction in migration compared with Kv3.1+/+ (Fig. 5D).

DISCUSSION

Kv3.1 channel expression is widespread in the nervous system, with Kv3.1b mRNA far more abundant than the Kv3.1a transcript in adult brain (26, 42) but with both transcripts strongly expressed in neuronal subpopulations of the olfactory bulb, neocortex, hippocampus, basal nuclei, thalamus, brain stem, and cerebellum. It is therefore surprising that the knockout mouse has a mild phenotype (16). We were able to determine that a structural effect of Kv3.1 knockout is a reduction in myelin thickness and a decrease in the number of large-diameter axons (shown for ON in Fig. 2E). The expected functional consequence of this would be reduced conduction velocity and slowed response times, a nonlethal but suboptimal
situation for the organism, and could explain the motor skill deficit in these mice (16). The cause of these structural changes could reside in the neurons; because the g ratio is unchanged, the decrease in axonal width could be intrinsic to Kv3.1−/− neurons. However, because Olg and OPC also express Kv3.1, the principal finding of this report, the pathology could reside partially in the Olg.

In this report we present evidence that 1) Kv3.1 transcript and protein for both alternative spliced species are found in Olg and OPC (an earlier study using RNase protection assay to identify Kv3.1 mRNA in C6 glia cell line and mixed population of ON cultures was unsuccessful (26)); 2) ~50% of the voltage-dependent outward K+ current in OPC is due to Kv3.1; 3) Kv3.1 forms a complex with a major, Olg-specific protein, OSP/claudin-11; 4) specific block of Kv3.1 or Kv3.1 knockout inhibits proliferation and migration of Olg and OPC; and 5) Kv3.1 knockout affects normal myelination.

Kv3.1 may have functions not shared with other K+ channels, possibly through its association with OSP/claudin-11 and the other proteins shown to be members of the complex, OAP-1 and B1-integrin (38). The complex is important ultimately as a mediator of cell-cell contact and must have a role in myelination. The existence of a K+ channel as part of the complex raises the possibility that voltage-dependent local increases in extracellular K+ may affect tight junction formation. Also, having a voltage-dependent protein as a partner in the complex may confer voltage sensitivity to it, which may then allow voltage changes to influence tight junction formation. Aside from specialized functions, activation of the channel must also affect repolarization after depolarization stimuli, as would any of the voltage-dependent K+ channels. A direct way in which this could influence Olg is by affecting stimulus-dependent intracellular changes in Ca2+, an important second messenger that controls or modifies every cell behavior and function (4, 31). Restricting K+ conductance with anti-Kv3.1 or Kv3.1 knockout should increase stimulus-dependent Ca2+ increases in the cytoplasm of Olg and OPC, and we are presently examining this prediction.

The slowing of OPC proliferation and migration, shown here with Kv3.1 block or Kv3.1−/−, might be involved in the Kv3.1−/− dependent thinning of the ON myelin sheath; however, there is likely to be slowing of other Olg behaviors as well, for example, transitions in developmental stages and after differentiation in the rate of myelination once it has begun. If there is a critical period for myelination and Olg are slow to respond, the window of opportunity may close before the myelin sheath can obtain normal thickness. If this were the case it would imply that there is an independent mechanism for myelination termination, and this has important implications not only for developmental myelination but also for remyelination after injury and disease.

An interesting point made by the proliferation/migration experiments is that acute block of Kv3.1 by anti-Kv3.1 is more effective than Kv3.1 knockout. This suggests that Kv3.1−/− Olg have a mechanism of compensation, perhaps the upregulation of an alternative K+ channel. Another member of the Kv3 subtype, Kv3.3, is widely coexpressed with Kv3.1 in distinct neuronal populations in the CNS. Mice lacking both Kv3.1 and Kv3.3 K+ channel alleles display severe motor deficits (11, 12) compared with either single mutation. Immunohistochemical analysis indicates that other voltage-dependent K+ channels including Kv3.3 are probably not upregulated in the Kv3.1−/− Olg, but a Ca2+-dependent K+ channel would be a good candidate for compensation, especially if the principal effect of Kv3.1−/− is loss of feedback control over stimulus-dependent Ca2+ increases. Upregulation of Ca2+-dependent K+ channels would act as a substitute negative-feedback mechanism. If compensation does occur, and the mechanism of it is common to other cells, it would help explain the relatively mild phenotypic effects of Kv3.1 knockout.

Here we have demonstrated that Kv3.1 associates with OSP/claudin-11, contributes significantly to OPC K+ currents, and has a role in OPC development and axon myelination. Because distinct changes in the membrane K+ channel phenotype of OPC occur during lineage progression (3, 35, 40) and Kv3.1 along with Kv1 type channels and Kir4.1 has a major role in the regulation of OPC development and axon myelination, a complete understanding of the mechanism by which Kv3.1 and related proteins exert their influence on OPC development would be beneficial in developing therapies for demyelinating diseases.

ACKNOWLEDGMENTS

We thank Michael Woodruff for helpful discussion and comments on this manuscript. We acknowledge the Carol Moss Spivak Cell Imaging Facility at UCLA.

GRANTS

This work was supported by grants from the NMSS (RG355A1, S. Tiwari-Woodruff), the National Institute of Neurological Disorders and Stroke (NS-01596, J. Bronstein), the Department of Veterans Affairs, and the SW PA-DRECC (J. Bronstein).

REFERENCES

K+ CHANNEL Kv3.1 REGULATES OLIGODENDROCYTE FUNCTION


