Characterizing the conductance underlying depolarization-induced slow current in cerebellar Purkinje cells

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brief strong depolarization of cerebellar Purkinje cells, produced either through voltage-clamp commands or burst-activation of excitatory glutamatergic climbing fiber synapses, gives rise to a biphasic inward current. The early component of this inward current is mediated, at least in part, by a Ca-dependent Cl conductance triggered by Ca influx through voltage-sensitive Ca channels (Llano et al. 1991; Shin et al. 2008). We have described an unusually slow component of this inward current (time to peak: 2.0–3.0 s; 90–10% decay time: ~2.8 s), which we have named DISC (for depolarization-induced slow current; Shin et al. 2008; Crepel et al. 2011).

DISC is triggered by Ca influx through voltage-gated channels, being blocked by either a cocktail of Ca channel blockers or external Cd ions, and is strongly attenuated by internal application of either an inhibitor of vesicular neurotransmitter transporters (bafilomycin A) or vesicular membrane fusion (botulinum toxin D; Shin et al. 2008), suggesting autocrine action of a neurotransmitter released following Ca influx.

Here, we have sought to identify the current or currents underlying DISC. Our attention turned to the TRP superfamily of ion channels and in particular to transient receptor potential cation channel, subfamily M, members 4 and 5 (TRPM4 and TRPM5), which are the only members that are directly gated by internal Ca concentration. TRPM4 is a nonselective cation channel that conducts Na and K but not Ca (Launay et al. 2002). It is activated by internal Ca (Launay et al. 2004), and its sensitivity to Ca is strongly modulated by phosphatidylinositol-4,5-bisphosphate (Nilius et al. 2006) and protein kinase C (PKC; Earley et al. 2007). TRPM4 is expressed in a wide variety of tissues including heart, lung, skeletal muscle, intestine, prostate, kidney, and liver (Launay et al. 2002; Fonfria et al. 2006).

TRPM5 is ~45% homologous to TRPM4 and shares many of the same channel properties (Hofmann et al. 2003; Ullrich et al. 2005). Like TRPM4, it is activated by internal Ca and fluxes Na and K but not Ca. TRPM5 is expressed in taste cells, where it appears to be necessary for bitter, sweet, and umami taste transduction (Zhang et al. 2003; Damak et al. 2006). TRPM4 and TRPM5 have been reported to be expressed sparsely in brain (Launay et al. 2002; Fonfria et al. 2006; Mrjer et al. 2011). The electrophysiological profiles of TRPM4 and TRPM5 are provocative, having similarity to the nonspecific cation conductance (I\textsubscript{CAN}) of neurons and muscle cells (Guinamard et al. 2011). However, little is known about their electrophysiological function in neurons.

METHODS
Slice preparation. Cerebellar slices were prepared from juvenile postnatal day (P)16 to P22 C57BL/6 mice using standard techniques, in accordance with a protocol approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine. Sagittal slices of the cerebellar vermis (250 µm thick) were cut with a vibrating slicer (Leica VT 1000S) using a sapphire blade in ice-cold N-methyl-D-glucamine (NMDG)-based cutting solution. This solution...
contained the following (in mM): 135 NMDG, 1 KCl, 1.5 MgCl₂, 0.5 CaCl₂, 1.2 KH₂PO₄, 24.2 choline bicarbonate, and 13 glucose, bubbled with 95% O₂-5% CO₂ to yield pH 7.4. Slices were maintained thereafter in artificial cerebrospinal fluid containing the following (in mM): 124 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 20 glucose at room temperature. They were then placed in a submerged chamber that was perfused at 2 mL/min with artificial cerebrospinal fluid, also at room temperature and bubbled with 95% O₂-5% CO₂ to yield pH 7.4. GABAzine (5 µM) was added to the recording solution to block GABAergic receptors. Slices were visualized in a submerged chamber that was perfused at 2 mL/min with artificial cerebrospinal fluid containing the following (in mM): 135 NMDG, 1 KCl, 1.5 MgCl₂, 0.5 CaCl₂, 1.2 KH₂PO₄, 24.2 choline bicarbonate, and 13 glucose, bubbled with 95% O₂-5% CO₂ to yield pH 7.4. Slices were maintained thereafter in artificial cerebrospinal fluid containing the following (in mM): 135 CsCl, 2.5 NaCl, 1.5 MgCl₂, 0.5 CaCl₂, 1 NaH₂PO₄, 26.2 NaHCO₃, and 20 glucose at room temperature. To minimize phototoxicity, 0.1 mM Trolox-C was added to artificial cerebrospinal fluid from Purkinje cells in the cerebellum using conventional techniques.

Electrophysiology. Whole cell patch-clamp recordings were made from Purkinje cells in the cerebellum using conventional techniques. The pipette solution consisted of the following (in mM): 135 Cs-methanesulfonate, 6 CsCl, 2 MgCl₂, 0.15 CaCl₂, 0.2 EGTA, 10 HEPES, 4 Na₂-ATP, 0.4 Na₃-GTP, pH 7.2–7.3, and osmolality = 290 mosM. Pipette resistance varied from 1.5 to 2 MΩ. Cells were voltage clamped using a Multiclamp 700A amplifier (Molecular Devices; Sunnyvale, CA). Unless otherwise noted the command potential was −70 mV. Series resistance was <15 MΩ. Recordings of membrane current were filtered at 1 kHz, digitized at 5 kHz, and collected with pClamp 9 software (Molecular Devices). DISC was induced by a test stimulus consisting of five 10-ms-long depolarizing command pulses from −70 to 0 mV, delivered at 10 Hz.

The following agents were added to the recording bath solution: SR95531 (GABAζ) was purchased from Ascent Scientific (Princeton, NJ) and glibenclamide was purchased from Tocris (Ellisville, MO). All other chemicals were from Sigma (St. Louis, MO).

**Mice.** TRPM4 null mice were obtained from the laboratory of Pierre Launay, and TRPM5 null mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TRPM4 null mice and wild-type littermates were genotyped as previously described (Barbet et al. 2008). TRPM5 null mice and wild-type littermates were genotyped as described in The Jackson Laboratory genotyping protocol database. TRPM4 or TRPM5 null mice were created by breeding TRPM4 or TRPM5 heterozygotes to allow for littermate wild-type controls. TRPM4/5 double null mice were created by breeding TRPM4 or TRPM5 homozygous nulls to generate double heterozygotes. Double heterozygotes were then mated to nonsibling wild-type mice to create TRPM4/5 double nulls and wild-type controls.

**Analysis.** Patch-clamp data were analyzed offline using Clampfit (Molecular Devices), Origin (OriginLab), and Igor Pro (WaveMetrics) software. Group data were expressed as means ± SE. The Mann-Whitney U test was used to determine significance in pairwise statistical comparisons. The DISC charge transfer was measured from a 1-s-long segment centered at the DISC peak. Offline digital processing of traces was used to high-pass filter the traces at 10 Hz to extract the noise envelope, which tracks DISC amplitude and therefore allows for clear separation between the faster inward current (Ca-sensitive Cl current; Llano et al. 1991) and DISC (Shin et al. 2008). Western blot 72 h later. Endogenous TRPM4 protein levels were measured in tissue derived from 17-day-old C57BL/6 mice. These samples were isolated in cell lysis buffer [10 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1 mM vanadate] and homogenized to a known mg RNA was used for the nonconcentration of the concentrated lentivirus solution was adjusted using NaOH. To validate the efficacy and specificity of shRNA, lentiviruses carrying shRNA against mouse TRPM4 and control were transduced into a mouse adult neural progenitor cell line. These cells were harvested for Western blot week later. Endogenous TRPM4 protein levels were measured in tissue derived from 17-day-old C57BL/6 mice. Thirty samples were isolated in cell lysis buffer [10 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, and 1 mM vanadate] and homogenized to a known mg.
Quantification of the Western blots was performed using FluorChem8900 software.

**RESULTS**

To determine if the DISC conductance involves Ca flux, we performed simultaneous confocal Ca imaging and whole cell patch-clamp recording from Purkinje cells in cerebellar slices derived from juvenile mice. Previous work from our group had shown that a brief burst depolarization protocol (consisting of five 10-ms-long step depolarizations from −70 to 0 mV at 10 Hz) could reliably induce robust DISC in Purkinje cells of cerebellar lobules IX and X (Kim et al. 2009). Here we have repeated that protocol but have supplemented the pipette solution with the Ca indicator dye Fluo-5F (0.3 mM) and the cytosolic marker dye Alexa Fluor 594 hydrazide (0.3 mM), a cytosolic marker. A representative Purkinje cell in which brief burst depolarization produced DISC that peaked at 2.43 s after burst onset, similar to that previously reported (Shin et al. 2008; Crepel et al. 2011; Kim et al. 2009). The simultaneously recorded Ca transient in the distal dendritic region of this cell revealed a peak ΔF/Φ0 of 7.78 that was achieved 0.65 s after burst onset. The decay phase of this Ca transient was well fit by double exponentials with τfast = 0.66 s and τslow = 5.48 s. Importantly, at t = 2.53 s, which was the peak of DISC, the Ca transient had decayed to ΔF/Φ0 = 1.31.

Population measurements in the distal dendritic compartment revealed a peak ΔF/Φ0 of 6.94 ± 1.31 that was achieved at t = 0.73 ± 0.03 s after burst onset (n = 5). The decay phase was well fit with τfast = 0.61 ± 0.06 s and τslow = 3.87 ± 0.67. At the peak of DISC, at t = 2.50 ± 0.14 s, Ca transients had decayed to ΔF/Φ0 = 1.34 ± 0.12, which is ~19% of the Ca transient peak. In the somatic compartment, the peak ΔF/Φ0 was lower and slightly slower: peak ΔF/Φ0 of 3.85 ± 1.17 that was achieved at t = 0.91 ± 0.19 s after burst onset (n = 5). The decay phase was well fit with τfast = 1.02 ± 0.31 s and τslow = 3.67 ± 0.50 s. In the proximal dendrite, Ca transients were intermediate between the distal dendrite and the soma: peak ΔF/Φ0 of 5.41 ± 1.48 that was achieved at t = 0.95 ± 0.24 s after burst onset (n = 5). The decay phase was well fit with τfast = 0.77 ± 0.12 s and τslow = 3.97 ± 0.99 s. These findings indicate that, while depolarization-evoked Ca influx is important as a trigger for DISC (Shin et al. 2008), the DISC conductance does not flux substantial amounts of Ca.

If Ca influx is important in triggering DISC (Shin et al. 2008) but Ca influx does not appear to mediate the DISC conductance (Fig. 1), then what cation(s) do underlie it? Na influx is an obvious candidate. To address this possibility, we recorded baseline DISC responses and then briefly switched from normal external saline [total extracellular Na concentration of ~151 mM] to an external saline in which NaCl was substituted with N-methyl-d-glucamine-NaCl (NMDG; total extracellular Na concentration of ~27 mM). NMDG is impermeant for most monovalent cation channels such as Ca-blockable monovalent cation channel in the ectoderm of the chick embryo as well as Ca-impermeable AMPA and kainate receptors (Sabovcik et al. 1995; Burnashev et al. 1996). This
treatment produced a near-complete blockade of DISC that could be recovered upon washout (Fig. 2). The population measures showed that DISC charge transfer was reduced to 9 ± 3% of baseline by Na substitution with NMDG (n = 7 cells). When depolarizing burst-evoked Ca transients were measured in a separate set of cells, NMDG substitution caused a modest increase in the amplitude of the evoked Ca transient (Fig. 2C; 116 ± 7% of baseline, n = 5), possibly as a result of attenuating extracellular Na/intracellular Ca exchange. In any case, this observation suggests that the blockade of DISC by Na substitution with NMDG is not secondary to a block of depolarization-evoked Ca influx.

The DISC conductance is triggered by Ca but appears to involve Na influx but not significant Ca influx. It is likely that K efflux also occurs but that was not specifically tested here. This weakly Ca-permeable cation current matches the properties of the TRPM4 conductance studied in various cell types and locations, as well as heterologous expression systems (Launay et al. 2002; Vennekens and Nilius 2007). It also closely matches the properties of the TRPM5 conductance (Hofmann et al. 2003; Guinamard and Simard 2011). As such, we wished to determine if TRPM4 and TRPM5 proteins are expressed in those cerebellar Purkinje cells where robust DISC can be recorded. To this end, we performed floating section immunohistochemistry using a polyclonal antiserum directed against a 17 amino acid peptide from human TRPM4 and a 22 amino acid peptide from mouse TRPM5 (Fig. 3). Sections of juvenile mouse cerebellum showed clear immunoreactivity for both TRPM4 and TRPM5 in the soma and dendrites of Purkinje cells from lobule IX, where robust DISC is routinely recorded and much lower levels in lobule VI where DISC is weak (Kim et al. 2009). For TRPM4 staining, mean pixel intensity for the Purkinje cell layer was 134.23 ± 7.06 in lobule IX and 45.47 ± 0.44 in lobule VI (arbitrary units, n = 4). For TRPM5 staining, mean pixel intensity for the Purkinje cell layer was 119.69 ± 15.20 (n = 4) in lobule IX and 32.75 ± 1.53 in lobule VI. In the cerebellar molecular layer, which contains the Purkinje cell dendrites, TRPM4 staining yielded a mean pixel intensity of 76.70 ± 4.82 in lobule IX and 44.28 ± 0.55 in lobule VI. For TRPM5 staining, mean pixel intensity for the molecular layer was 82.80 ± 10.47 (n = 4) in lobule IX and 29.84 ± 1.44 in lobule VI. The specificity of these antibodies was confirmed by experiments in which TRPM4 and TRPM5 antibodies were applied to tissue from their corresponding null mice, yielding only background levels of immunoreactivity (Fig. 3). Controls with no primary antibody also showed background levels of immunoreactivity.

As a first test of the hypothesis that TRPM4 and/or TRPM5 underlie the DISC conductance, we used a series of TRPM4 and TRPM5 blocking drugs: glibenclamide (100 μM), flufenamic acid (100 μM), and 9-phenanthrol (100 μM; Fig. 4). A control group, which was simply recorded for 20 min after stable DISC was achieved, showed a mean DISC charge transfer amplitude of 0.61 ± 0.02 nC (n = 5). All three of these drugs produced strong attenuation of DISC charge transfer (Fig. 4). The mean DISC charge transfer after application of glibenclamide was 0.13 ± 0.06 nC, which was 22 ± 10% of predrug baseline (n = 5; P < 0.01, compared with control). For flufenamic acid it was 0.04 ± 0.01 nC and 6 ± 2% of predrug baseline (n = 5; P < 0.01), and for 9-phenanthrol it was 0.06 ± 0.01 nC and 10 ± 3% (n = 5; P < 0.01). While these drugs have been used to block TRPM4 or TRPM5 in heterologous expression systems (Ulrich et al. 2005; Grand et al. 2008) or various cardiac cells including sinoatrial node cells and ventricular cardiomyocytes (Guinamard et al. 2006; Demion et al. 2007), they also have nonspecific effects on other ion channels and transporters. Glibenclamide affects the cystic fibrosis transmembrane conductance regulator Cl channels, other Cl channels, and ATP-sensitive K channels (Ashcroft and Gribble 1999; Pompermayer et al. 2007). Flufenamic acid has side-effects on Ca-activated current, K channels, and other currents (Takahira et al. 2005; Gardam et al. 2008). 9-Phenanthrol, which has been reported to block TRPM4 but not TRPM5 (Grand et al. 2008), also blocks the cystic fibrosis transmembrane conductance regulator and other ATP-binding cassette proteins.

Because of the lack of specific blockers of TRPM4 and TRPM5, we turned to previously characterized null mice...
Fig. 3. Transient receptor potential cation channel, subfamily M, member 4 (TRPM4) and TRPM5 are strongly expressed in Purkinje cells of those posterior cerebellar regions where DISC is largest. Representative confocal images from different subregions of the cerebellum are shown. Immunohistochemistry using polyclonal antibodies directed against the human TRPM4 and the mouse TRPM5 proteins was performed using free-floating sagittal slices of cerebellar vermis. Strong immunoreactivity for TRPM4 and TRPM5 proteins was seen in the soma, primary, and secondary dendrites of Purkinje cells located in lobule IX, where robust DISC is routinely recorded (top). By contrast, very weak immunoreactivity for both ion channels was seen in Purkinje cells in the more anterior lobule VI, where DISC is typically small (middle). Bottom: controls with either antibodies applied to the corresponding null mouse (left) or without primary antibody (right). Scale bar: 50 μm. KO, knockout.

(TRPM4: Barbet et al. 2008; TRPM5: Riera et al. 2009), singly and also crossed to produce double nulls. Because the genetic background of each of these 3 mutant mice is different, each must be compared with its own wild-type littermate (Fig. 5). Purkinje cells in brain slices derived from TRPM4 null mice showed DISC charge transfer that was 46 ± 7% of that in age-matched wild-type littersmates (TRPM4 KO: 0.27 ± 0.06 nC, n = 40; WT control: 0.60 ± 0.07 nC, n = 31; P < 0.001). By contrast, DISC in TRPM5 nulls was not significantly attenuated at 80 ± 15% of control (TRPM5 KO: 0.49 ± 0.06 nC, n = 19; WT control: 0.62 ± 0.08, n = 15). Finally, Purkinje cells from TRPM4/5 double null mice expressed DISC at levels 35 ± 9% of their age-matched wild-type littersmates. (TRPM4/5 DKO: 0.23 ± 0.04 nC, n = 28; WT control: 0.64 ± 0.04 nC, n = 46; P < 0.001).

Might the attenuated DISC in TRPM4 KO and TRPM4/5 DKO Purkinje cells be a consequence of attenuated voltage-evoked Ca transients? To address this possibility, we performed Ca imaging experiments in conjunction with DISC-inducing depolarizing stimulation. Measurements in distal dendritic compartments revealed no significant difference in either peak or integrated Ca signals when compared with C57BL/6 WT controls. The peak Ca transients were ΔF/Fl0 = 6.94 ± 1.31 in WT (n = 5; these are the same cells as shown in Fig. 1 and described in the accompanying text), 6.66 ± 1.11 in TRPM4 KO (n = 5), and 5.75 ± 0.97 in TRPM4/5 DKO (n = 5). In addition, no significant differences emerged when somatic or proximal dendritic regions of interest were measured (Fig. 6C).

We next considered the possibility that the reason that DISC was only partially attenuated in the TRPM4 null Purkinje cells (Fig. 5) was a result of some form of compensation in this mutant mouse: perhaps knockout of TRPM4 from the earliest stages of development causes the expression of another ion channel that can mediate DISC but does not normally do so. Therefore, to further test the hypothesis that TRPM4 is required for DISC, we constructed an shRNA directed against a unique sequence in the mouse TRPM4 gene and packaged it in the lentiviral vector FUGW, which also drives expression of the fluorescent marker EGFP. To test the efficacy of knockdown with the FUGW-shTRPM4 virus, we infected neural progenitor cells, which express endogenous TRPM4, and performed a Western blot using rabbit TRPM4 polyclonal antiserum (Fig. 7). Densitometric analysis of the TRPM4 band revealed that FUGW-shTRPM4 treatment reduced TRPM4 protein levels to 20% of those in uninfected cells. Treatment with empty FUGW yielded levels that were 96% of uninfected cells. Finally, treatment with FUGW engineering to express a nonsilencing shRNA yields TRPM4 protein levels that were 113% of uninfected cells.

 Lentivirus-containing solutions were injected into the posterior cerebellar vermis of anesthetized juvenile mice using stereotaxic methods, and 1 wk was allowed for recovery and transduction. Slices of cerebellar tissue were prepared, and whole cell patch-clamp recordings were made from EGFFP-positive Purkinje cells. Purkinje cells treated with the FUGW empty virus or the FUGW-nonsilencing shRNA virus expressed DISC with charge transfer of 0.57 ± 0.09 nC (n = 18) and 0.56 ± 0.1 nC (n = 24), respectively, which was not significantly different from each other. By contrast, treatment with FUGW-shTRPM4 produced a mean DISC charge transfer of 0.21 ± 0.06 nC (n = 31), a reduction to 37 ± 1% of the FUGW alone control values (P < 0.01). Control electrophysiological parameters such as membrane input resistance (Rm), whole cell capacitance (Cm, a measure of plasma membrane surface area, and thus cell size), and holding current (Ih) did not differ significantly among FUGW alone, FUGW-nonsilencing control, and the FUGW-shTRPM4 Purkinje cells. Thus shRNA-mediated knockdown of TRPM4 produced a partial attenuation of DISC (~37% of control) that was similar to that observed in the TRPM4 null (~46% of control) or TRPM4/ TRPM5 double null Purkinje cells (~35% of control).

**DISCUSSION**

The main finding of these experiments is that the DISC conductance has many hallmarks of TRPM4 and TRPM5 channels, being Ca-triggered, strongly permeant to Na and K but weakly Ca permeant, and strongly attenuated by a series of nonspecific TRPM4/5 blockers. Yet, it was only partially blocked by TRPM4 deletion, TRPM4 knockdown, or TRPM4/
TRPM5 double deletion. The observation that 35–46% of DISC remained with these treatments indicates that, while TRPM4 may have some role in the DISC conductance, neither it nor TRPM5 are absolutely required.

Ca imaging experiments showed that DISC triggering, but not expression, was associated with a large Ca transient (Fig. 1). When combined with the low Ca permeability of the DISC conductance (Fig. 1), the observation that DISC was near-completely and reversibly blocked by substitution of external Na with the impermeant cation NMDG (Fig. 2) indicated that the DISC conductance was predominantly carried by Na influx. It is likely that K efflux also underlies this current but this was not tested here.

Previous work from our group had shown that DISC is weak in lobule VI of the cerebellar vermis but strong in more posterior regions such as lobule IX (Kim et al. 2009). Thus immunohistochemical experiments performed using slices of cerebellar vermis were encouraging when they showed TRPM4 and TRPM5 immunoreactivity in Purkinje cells of lobule IX but not VI (Fig. 3) as were results showing strong attenuation of DISC by three different nonspecific TRPM4/TRPM5 blockers, glibenclamide, flufenamic acid, and 9-phenanthrol (Fig. 4). As no specific blockers of TRPM4 and TRPM5 are presently available, we obtained null mice to test the hypothesis that these ion channels underlie DISC. While TRPM4 null Purkinje cells showed a significant but partial attenuation of DISC, DISC measured in TRPM5 null Purkinje cells was not significantly different from age-matched wild-type littermates. Double null Purkinje cells also showed partially attenuated DISC (Fig. 5). The attenuation of DISC in TRPM4 and TRPM4/5 DKO Purkinje cells is not likely to result from a side-effect on depolarization-evoked Ca transients as these were not significantly reduced (Fig. 6). Wild-type Purkinje cells transfected with a lentivirus engineered for shRNA-mediated knockdown of TRPM4 also showed partial attenuation of DISC (Fig. 7) consistent with the effect of TRPM4 deletion. Thus we believe...
that TRPM4 contributes to the DISC conductance but that neither it nor TRPM5 are absolutely required.

DISC was attenuated by the mGluR1 antagonist CPCCOEt, both in our hands (Shin et al. 2008) and in those of another group (Duguid et al. 2007). DISC was not blocked by either the NMDA receptor antagonist CPP or the AMPA/kainate receptor antagonist NBQX (Shin et al. 2008). This led us to suggest that the glutamate released from Purkinje cells by strong depolarization could also act in an autocrine fashion on mGluR1 to evoke DISC, mediated by previously described mGluR1-operated TRPC channels (Kim et al. 2003; Hartmann et al. 2008). However, further work in our group has invalidated this model (Shin et al. 2009). CPCCOEt, but not three other specific mGluR1 antagonists (JNJ16259685, 3-MATIDA, and Bay 36-7620), blocked DISC. This occurred even though all of these drugs produced near-complete blockade of inward currents evoked by application of the mGluR1/5 agonist DHPG. Most importantly, DISC was present in Purkinje cells derived from mGluR1 KO mice and mGluR1/mGluR5 DKO mice.

Recently, it has been shown that muscarinic receptor-modulated slow afterdepolarization in layer 5 pyramidal neurons of the medial prefrontal cortex shows a similar, partial attenuation in brain slices derived from TRPM4/5 DKO mice (Lei YT, Launay P, Margolskee R, Kandel ER, Siegelbaum SA, Thuault SJ, unpublished observations). Thus TRPM4 and TRPM5 may play similar roles in mediating slow, Ca-triggered conductances in different neuronal types.

ACKNOWLEDGMENTS

We thank Devorah Vanness for technical assistance, Richard Huganir for providing FUGW lentiviral vector and lentivirus, Robert Margolskee for Fig. 7. Short hairpin (sh)RNA directed against the mouse TRPM4 channel produces attenuation of DISC. A: diagram of the bicistronic lentiviral vector (FUGW) used for in vivo genetic manipulation. B: TRPM4 expression levels are shown in various brain regions extracted from P17 mouse brain by Western blot analysis using polyclonal rabbit TRPM4 antibody directed against human TRPM4. C: in vitro validation of the efficacy of shRNA directed against the mouse TRPM4 gene. Adult neural progenitor cells (NPC) were infected with lentiviral constructs expressing shRNA directed against a unique sequence in mouse TRPM4, shRNA-TRPM4 (NPC, FUGW-shTRPM4), control (NPC, no virus), empty vector (NPC, FUGW), and nonsilence shRNA (NPC, FUGW-nonsilence) were tested. Equal amounts of cell lysate samples were subjected to western blot analysis. D: population analysis of the shRNA-TRPM4 knockdown effect on DISC amplitude. shRNA-TRPM4-containing lentivirus or control lentivirus was injected into the posterior cerebellar vermis of anesthetized juvenile mice using stereotaxic methods. About 1 wk later, cerebellar slices were prepared and DISC was recorded from EGFP-positive Purkinje cells derived from lentivirus transduced mice. The mean DISC charge transfer recorded from FUGW alone (n = 18), FUGW-shTRPM4 (n = 31), and FUGW-nonsilence (n = 24) conditions are plotted. Representative single, unaveraged DISC traces are shown. Scale bar: 200 pA, 2 s for DISC current trace and 23.8 pA, 2 s for noise envelope. *P < 0.01 by Mann-Whitney U-test for DISC charge transfer comparing FUGW-shTRPM4 to FUGW-nonsilence or FUGW-shTRPM4 to FUGW.
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AUTHOR CONTRIBUTIONS


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