Control of transient, resurgent, and persistent current by open-channel block by Na channel β4 in cultured cerebellar granule neurons

Jason S. Bant[a] and Indira M. Raman[b,1]

[a]Interdepartmental Biological Sciences Graduate Program and [b]Department of Neurobiology and Physiology, Northwestern University, Evanston, IL 60208

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Voltage-gated Na channels in several classes of neurons, including cells of the cerebellum, are subject to an open-channel block and unblock by an endogenous protein. The $Na_V\beta4$ (*Scn4b*) subunit is a candidate blocking protein because a free peptide from its cytoplasmic tail, the β4 peptide, can block open Na channels and induce resurgent current as channels unblock upon repolarization. In heterologous expression systems, however, $Na_V\beta4$ fails to produce resurgent current. We therefore tested the necessity of this subunit in generating resurgent current, as well as its influence on Na channel gating and action potential firing, by studying cultured cerebellar granule neurons treated with siRNA targeted against *Scn4b*. Knockdown of *Scn4b*, confirmed with quantitative RT-PCR, led to five electrophysiological phenotypes: a loss of resurgent current, a reduction of persistent current, a hyperpolarized half-inactivation voltage of transient current, a higher rheobase, and a decrease in repetitive firing. All disruptions of Na currents and firing were rescued by the β4 peptide. The simplest interpretation is that $Na_V\beta4$ itself blocks Na channels of granule cells, making this subunit the first blocking protein that is responsible for resurgent current. The results also demonstrate that a known open-channel blocking peptide not only permits a rapid recovery from nonconducting states upon repolarization from positive voltages but also increases Na channel availability at negative potentials by antagonizing fast inactivation. Thus, $Na_V\beta4$ expression determines multiple aspects of Na channel gating, thereby regulating excitability in cultured cerebellar granule cells.

Scn4b | siRNA | inactivation | Purkinje | voltage-gated

$Na_V\beta4$ (*Scn4b*), one of four β subunits of voltage-gated Na channels (1), is implicated in several pathologies: $Na_V\beta4$ is down-regulated in Huntington's disease (2), cleaved by enzymes activated in Alzheimer's Disease (3), and mutated in some long-QT syndromes (4), raising the question of how it modulates Na currents in neurons and other cells. Among the proposed roles for $Na_V\beta4$, based on studies of a peptide fragment of its cytoplasmic tail, is that it may act as an open-channel blocker of Na channels in neurons that produce resurgent current, i.e., reopening of Na channels upon repolarization from positive voltages (5).

Resurgent current is present in several neuronal classes, including cell types in the cerebellum, brainstem, subthalamic nuclei, and dorsal root ganglia (6–11). As in other cells, voltage-gated Na channels in these neurons are closed at negative voltages and open upon depolarization. After opening, however, channels are blocked rapidly by an endogenous protein that prevents the fast inactivation gate from binding. Upon repolarization, this blocker is expelled, and resurgent current flows as channels reopen before either inactivating or deactivating, depending on the voltage (12). In Purkinje cells, modeling studies (13, 14) and experiments on $Na_V1.6$ mutant mice, in which resurgent currents are reduced (14–16), have led to the proposal that Na channels that carry resurgent current facilitate repetitive firing.

Given the dependence of Purkinje firing rates on resurgent current, and the widespread incidence of the current in the nervous system, it is likely that open-channel block and unblock of Na channels regulates the intrinsic excitability of many neurons. If so, the blocking protein may serve as a molecular switch that enables neurons to produce specific patterns of activity, e.g., repetitive firing or bursting. Indeed Na channel mutations associated with pain syndromes and movement disorders can increase resurgent current amplitudes in affected cells, implicating the blocking protein in exacerbating certain channelopathies (17). An understanding of the molecular mechanism of resurgent current, as well as the ability to manipulate it under pathological conditions, however, relies on identification of the blocking protein(s).

Previous experiments have demonstrated that the blocking protein is distinct from the α subunit (15, 18), and that its action resembles open-channel block by compounds that produce hooked tail currents in Na channels (19). $Na_V\beta4$ emerged as a candidate blocking protein because part of its cytoplasmic tail, KKLITFILKKTREK, contains functional groups that resemble exogenous blocking compounds. Moreover, a free peptide comprising these amino acids (the β4 peptide) restores resurgent currents after enzymatic degradation of the endogenous blocker (20). Thus, the endogenous blocking protein must contain a domain whose interaction with the pore resembles that of the β4 peptide, raising the possibility that $Na_V\beta4$ is itself an open-channel blocker.

Coexpressing $Na_V\beta4$ with Na channel α subunits in expression systems, however, modulates Na channels without reconstituting resurgent current (21, 22). Thus, either the $Na_V\beta4$ subunit does not behave as a blocker, or it requires neuron-specific modulation to enable open-channel block. To investigate the functional role of $Na_V\beta4$, we recorded from cultured cerebellar granule neurons, which normally express an endogenous blocker, and tested how silencing *Scn4b* transcripts with small interfering RNA (siRNA) affects Na current kinetics and action potential firing. Knockdown of $Na_V\beta4$ correlated with reduced resurgent but not transient current, demonstrating that $Na_V\beta4$ is indeed necessary for normal resurgent current. Loss of the subunit had the additional effects of reducing persistent current, stabilizing fast inactivation, and decreasing repetitive firing. With $Na_V\beta4$ knocked down, the β4 peptide restored not only resurgent current but also all other aspects of Na current kinetics and spiking to control values. These data suggest that $Na_V\beta4$ is an endogenous blocking protein, and that it normally promotes excitability in cerebellar granule cells by regulating multiple components of Na channel gating.

Results

To explore the functional role of $Na_V\beta4$ in neurons, we studied cerebellar granule cells, which express resurgent current (7) and survive well in culture, making them amenable to molecular manipulation. Voltage-clamped, TTX-sensitive Na currents were evoked in neurons 7–14 d in vitro (DIV) by a 15-ms depolarization

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[1]To whom correspondence should be addressed. E-mail: i-raman@northwestern.edu.

from –90 mV to +30 mV, followed by a 200-ms repolarization to –30 mV. In untransfected cells, resurgent current evoked by repolarization was 11.7 ± 1.1% of the peak transient current evoked at +30 mV ($n = 9$). This fraction is near published values (7), confirming that the channel blocking machinery is retained in cultured neurons.

Next, we used siRNA to test whether expression of the $Na_V\beta4$ subunit influences the amplitude of any component of Na current. Control cells were transfected with a pool of four "non-target" siRNAs that target no mouse transcripts, as well as GFP as a marker of transfection. Recordings were repeated in neurons transfected with a pool of four *Scn4b*-targeted siRNAs and GFP. Relative to untreated cells, the peak transient Na current was reduced equivalently in both "non-target cells" and "on-target cells" (by 52% and 49%, $P > 0.7$; Fig. 1*F*). This observation suggests either that smaller cells are more readily transfected or that Lipofectamine incorporation leads to a reduction in current density independently of the siRNAs applied. We therefore considered non-target cells as the control group, with which all on-target effects were compared. Non-target cells expressed both transient and resurgent current proportionate to that that in untreated cells (9.0 ± 1.0% resurgent-to-transient ratio, $n = 9$, $P = 0.1$ vs. untreated cells). In contrast, resurgent current was undetectable in 9 of 18 cells, and the mean resurgent-to-transient ratio across all 18 cells fell to 3.7 ± 0.5% ($P < 0.0001$; Fig. 1 *A* and *B*). Thus, resurgent current is indeed sensitive to knockdown of $Na_V\beta4$.

The decrease, however, may result either directly, from a loss of the endogenous blocking protein, or indirectly, from modulation of α subunits, other proteins, and/or their interactions, such that channels are no longer subject to block. We therefore repeated recordings in on-target cells with the β4 peptide (200 μM), which blocks open channels much like the endogenous blocking protein (20), in the pipette. The peptide rescued the amplitude and kinetics of resurgent current to close to non-target levels (mean current rise time and τ_{decay}: control, 6.4, 48 ms; rescue, 6.9 ms, 41 ms; Fig. 1 *A* and *B*). Thus, α subunits remain susceptible to open-channel block in on-target cells, supporting the idea that decreased $Na_V\beta4$ expression disrupts the endogenous blocker itself.

Nevertheless, the reduction of resurgent current varied across cells, suggesting either that knockdown itself was variable or that open-channel block in some neurons might be independent of $Na_V\beta4$. We reasoned that if multiple blocking proteins existed, they might have distinct affinities for the channel, such that the kinetics of resurgent current might differ in the apparently knockdown-resistant cells. The rise time and τ_{decay} of the average residual resurgent current across all on-target cells, however, were 6.9 and 46 ms, much like control. Thus, the blocker that was lost, the blocker that remained, and the β4 peptide all interact similarly with the channel.

The effects of $Na_V\beta4$ knockdown were not limited to reducing resurgent current, however, as steady-state inactivation curves also depended on $Na_V\beta4$ expression. Cells in which we recorded resurgent current were held at negative potentials for 200 ms and availability was assessed with a step to 0 mV. Relative to non-target cells, the mean half-maximal inactivation voltage ($V_{1/2}$) in on-target cells was hyperpolarized by 7.7 mV ($n = 16$ on-target, $n = 9$ non-target, $P < 0.05$; Fig. 1 *C–E*), indicating that $Na_V\beta4$ normally increases the availability of Na channels at moderately negative voltages.

Like the change in resurgent current, the magnitude of the shift in the inactivation curve varied in on-target cells. Across cells, however, the $V_{1/2}$ correlated strongly with the percent resurgent current, with the most negative values in cells with the least resurgent current (Fig. 1*G*). In these cells, resurgent current was undetectable, and the $V_{1/2}$ was ~20 mV hyperpolarized to control values. In addition, the persistent current at the end of the repolarization to −30 mV dropped from 2.3 ± 0.6% in control to 1.0 ± 0.2% in on-target cells ($P < 0.05$), and the amplitude of persistent and resurgent currents in on-target cells were likewise correlated ($P < 0.05$). These data suggest that the efficacy of siRNA differed across cells, with successful knockdown modulating availability, persistent current, and resurgent current. Consequently, the effect of complete $Na_V\beta4$ loss is likely underestimated by the mean data.

To test which aspects of $Na_V\beta4$ modulation could be replicated by the β4 peptide, we repeated the experiments with the β4 peptide in the pipette. Strikingly, the peptide restored normal inactivation parameters ($n = 7$, $P = 0.74$ vs. control; Fig. 1 *D* and *E*), as well as the persistent current amplitude (2.8 ± 1.0%; $P = 0.65$ vs. control), demonstrating that the free peptide itself can not only block open channels but also oppose fast inactivation and increase Na current at negative voltages. Notably, heterologous expression of $Na_V\beta4$ with Na channels neither depolarizes the $V_{1/2}$ of inactivation nor generates resurgent current, although it can increase persistent current in the absence of $Na_V\beta1$ (1, 20, 21), supporting the idea that the effects of $Na_V\beta4$ on gating are modulated in neurons that normally express resurgent current. Together, the simplest interpretation is that the $Na_V\beta4$ subunit is itself a blocking protein in cultured granule cells, and serves to generate resurgent current, facilitate persistent current, and reduce steady-state inactivation of transient current at negative potentials.

Although in situ hybridizations yield low labeling of *Scn4b* transcripts in granule cells (1), immunohistochemistry suggests that these neurons indeed express $Na_V\beta4$ (23). Therefore, to verify that transcripts were present in cultured granule cells and reduced by on-target siRNA, we sought a direct measure of *Scn4b* mRNA. Because single-cell quantification of granule cell transcripts reveals

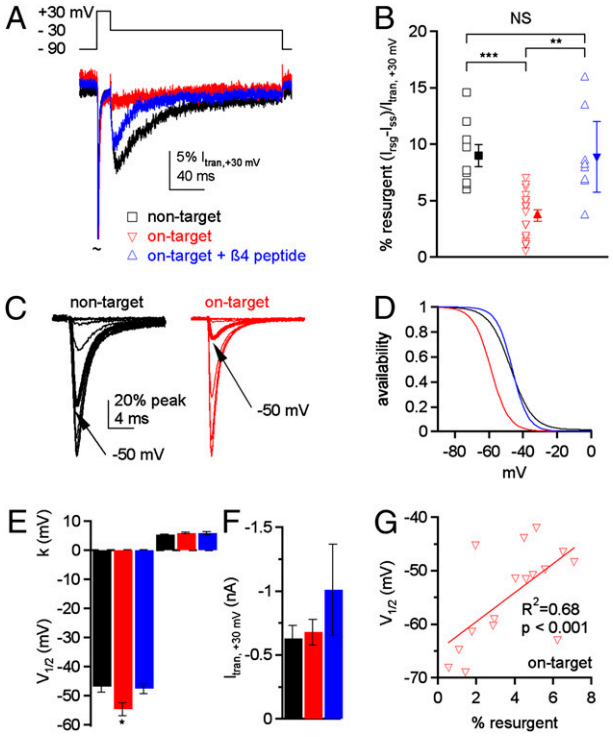

Fig. 1. Knockdown of $Na_V\beta4$ reduces resurgent current and shifts inactivation. (*A*) Transient and resurgent TTX-sensitive Na currents in a non-target cell, an on-target cell, and an on-target cell with the β4 peptide. (*B*) Relative resurgent current for all cells. (*C*) Transient currents evoked at 0 mV after 200-ms conditioning pulses at different voltages. (*D*) Availability curves from representative cells for data obtained as in *C*. (*E*) Mean $V_{1/2}$ and k availability parameters. (*F*) Transient current amplitudes in each condition. (*G*) $V_{1/2}$ vs. percent resurgent current for all on-target cells.

a low number and high variance of transcripts (24), single-cell studies were not suited for detecting knockdown of a rare transcript such as *Scn4b*. We therefore performed quantitative RT-PCR on samples from whole-culture RNA extracts. *Scn4b* transcripts were indeed present in neuron-glia cultures (identical to those from which recordings were made), whereas 8-fold fewer transcripts were detected in glia-only cultures, in which neurons had been killed by excitotoxicity ($P < 0.05$; Fig. 2*A*). Transcripts of *Scn2a* and *Scn8a*, which encode the primary Na channel α subunits of granule neurons ($Na_V1.2$ and $Na_V1.6$) (25), were reduced by 68% and 80% in glia-only cultures, confirming that the decrease in *Scn4b* resulted from the loss of neurons. These results not only verify that granule cells express $Na_V\beta4$ but also validate measuring neuronal transcripts from whole culture extracts.

Next, we estimated the degree of siRNA transfection directly, by omitting GFP and applying fluorescently tagged siRNA. Inspection of cultures indicated that, 1 d after transfection, fluorescence was detectable in about half of the neurons and many glia in each plate, suggesting that siRNA transfection was more efficient than indicated by GFP fluorescence. Although incorporation of tagged siRNA does not provide a guarantee of knockdown (26), these observations suggested that transfection might be efficient enough for knockdown of *Scn4b* in a subset of cells to be measurable, although underestimated, by whole-culture analysis.

Finally, we quantified *Scn4b* relative to the neuronal transcripts *Scn2a* and *Scn8a* in on-target and non-target cultures without GFP cotransfection. On-target and non-target cells had the same ratio of *Scn8a* to *Scn2a* transcripts (40% at 6 and 9 DIV and 60% at 12 DIV; $P > 0.5$, on-target vs. non-target), consistent with measurements of transient currents. These data support the conclusion from electrophysiological studies that transfection with either pool of siRNA had indistinguishable effects on α subunit transcripts, and verify that these transcripts were appropriate for relative quantification. At 6 DIV, however, the estimate of *Scn8a* transcripts was variable, so we used only *Scn2a* as the reference for this age group. *Scn4b* transcripts were decreased by 43 ± 10% at 6 DIV ($P < 0.005$) and by 66 ± 10% or 66 ± 15% at 9 DIV (relative to *Scn2a* or *Scn8a*, $P < 0.005$ for both; Fig. 2*B*). The reduction fell to 28 ± 15% or 21 ± 14% at 12 DIV ($P = 0.09$ for *Scn2a*, $P = 0.17$ *Scn8a* vs. control), falling just short of statistical significance. Nevertheless, as only ~50% of neurons per dish incorporate siRNA, knockdown in transfected cells is likely to be underestimated at all ages. The time course of the drop in siRNA efficacy is similar to that predicted by kinetic studies of gene-silencing by siRNA (26, 27). Thus, *Scn4b* is indeed measurably reduced in on-target cultures. The changes in Na currents recorded in voltage-clamp can therefore reasonably be attributed to changes in expression of $Na_V\beta4$.

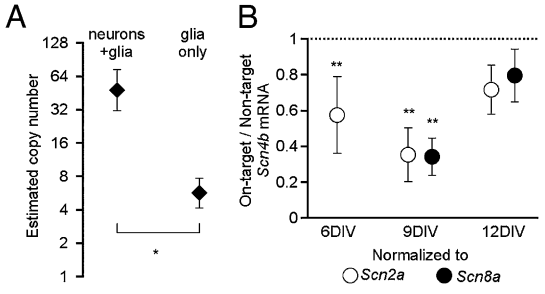

Fig. 2. On-target siRNA reduces neuronal *Scn4b* transcripts. (*A*) Number of *Scn4b* transcripts per sample estimated from the C_Q for mixed neuron–glia cultures and glia-only cultures. (*B*) Ratio of *Scn4b* transcripts in on-target to non-target cultures, normalized to *Scn2a* or *Scn8a*. Dotted line indicates no knockdown.

Changes in Na channels that reduce resurgent current disrupt repetitive firing by Purkinje neurons (14, 15), and resurgent current is likewise proposed to facilitate spiking by granule cells (28). We therefore tested whether knockdown of $Na_V\beta4$ altered the excitability of cultured granule cells. Because protein expression is expected to lag the decay of siRNA efficacy, we restricted recordings to cells 8–12 DIV, in which loss of $Na_V\beta4$ protein should be substantial and in which Na current density should be relatively constant. To further minimize variance, we analyzed data only from cells with input resistances between 0.7 and 2.1 GΩ (>90% of cells, Fig. 3*A*). Action potentials were evoked from −70 mV with 300-ms current injections in 5-pA increments. Under these conditions, rheobase was 13 ± 1 pA (n = 13). In non-target cells, the maximal spikes/step ranged from 7 to 28 (mean, 17 ± 2). This diversity is consistent with the dependence of granule cell firing rates on K current properties, which can vary widely in these cells. Nevertheless, nearly all control cells fired throughout the 300-ms step for several amplitudes of current injection (Fig. 3 *B* and *C Left*). To define the duration of repetitive firing across cells, we aligned spike rasters to rheobase and plotted the median time of the last spike for each current injection, which describes the envelope of the rasters for the population (Fig. 3*D Left*). This analysis illustrates that non-target cells fired throughout the step for approximately six steps (30 pA).

Maximal firing rates in on-target cells also ranged widely (2–31 spikes/step, mean 15 ± 2, n = 19), but rheobase was raised to 25 ± 4 pA ($P < 0.05$ vs. control). Moreover, firing persisted through a narrower range of current injections. Most cells failed to fire steadily with depolarizations >20 pA above rheobase, such that the median firing duration across cells fell near the lower 25th percentile for control cells ($P < 0.0005$; Fig. 3 *B*, *C,* and *D Middle*). This decreased repetitive firing during depolarizations is predicted from a loss of resurgent current, because it is the dissociation of the blocker that restores Na channel availability at moderate interspike potentials, at which fast-inactivated channels remain unavailable. Moreover, the disruption of firing will be exacerbated by the hyperpolarized availability curve and is likely to be further affected by the smaller persistent current. Nevertheless, reduced excitability might also result from changes in other channels, secondary to the loss of $Na_V\beta4$. We therefore tested whether the β4 peptide could increase the dynamic range of repetitive firing in on-target cells. Indeed, the peptide restored the duration of repetitive firing ($P > 0.05$; Fig. 3 *B, C,* and *D Right*), as well as rheobase (16 ± 2 pA, $P = 0.37$ vs. control), to control values. Maximal firing rates ranged from 8 to 33 spikes per step (mean, 16 ± 2, n = 17). These results suggest that the disruption of repetitive firing does not depend on changes downstream of the loss of $Na_V\beta4$. Instead, the simplest interpretation is that it can be attributed largely to the loss of the portion of the $Na_V\beta4$ cytoplasmic domain that generates an open-channel block and unblock and that maintains Na channel availability throughout action potential trains.

Discussion

By directly manipulating the $Na_V\beta4$ protein and examining the effect on Na channel gating in cultured cerebellar granule neurons, we found evidence that $Na_V\beta4$ is necessary for normal resurgent Na current: Although knockdown of *Scn4b* by siRNA was incomplete across neurons, resurgent current was absent only from cells treated with on-target siRNA. Moreover, the loss of resurgent current correlated with a hyperpolarized availability curve and drop in persistent current, suggesting that when knockdown did occur, all three parameters were affected. Finally, because the voltage-dependence and kinetics of all components of Na current in on-target cells were restored by a peptide from the $Na_V\beta4$ tail that behaves as an open Na-channel blocker, it is likely that $Na_V\beta4$ is itself an endogenous blocking protein in granule neurons.

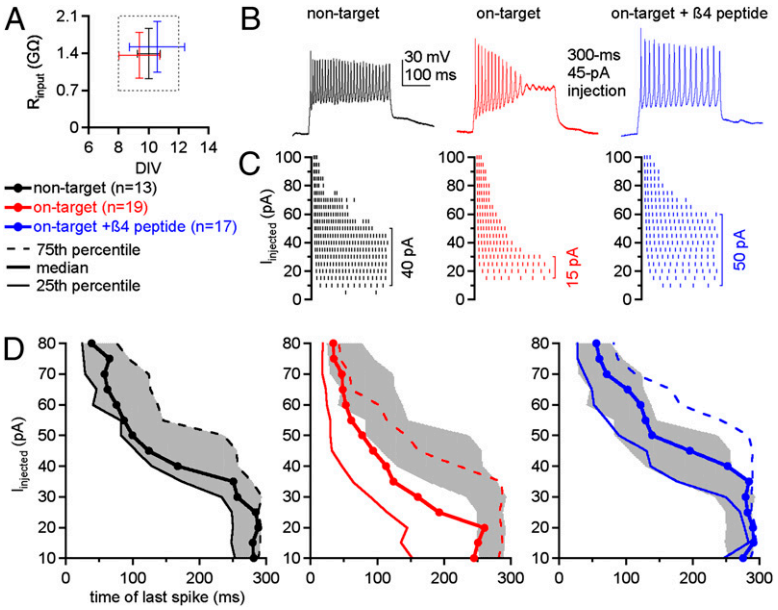

Fig. 3. $Na_V\beta4$ is required for normal excitability in granule cells. (*A*) Mean ± SD of input resistances and ages of cells recorded in current clamp. (*B*) Responses to 45-pA current injections in each condition. (*C*) Spike rasters evoked by range of currents for cells in *B*. (*D*) Median time of last spike (symbols) with upper and lower quartiles (thin solid and dashed lines) for each population of cells. Middle quartiles for non-target controls (gray) are superimposed on the on-target ± β4 peptide conditions for comparison.

These data also reveal a competition between $Na_V\beta4$ and the fast inactivation gate at negative voltages. Previous work has shown that resurgent and persistent Na current amplitudes are both reduced in neurons from mice lacking $Na_V1.6$, an α subunit that is a particularly good target for the endogenous blocker in some but not all cell types (9, 15, 29, 30). More precisely, α subunits with rapid inactivation kinetics (non-$Na_V1.6$ channels in Purkinje cells) fail to produce large resurgent currents because the endogenous blocker cannot compete successfully with fast inactivation (18). To the extent that rapid inactivation correlates with stable inactivation, such channels are likely to produce little persistent current as well. The present results, however, offer an additional explanation for why resurgent and persistent current amplitudes covary: The depolarization of the availability curve by $Na_V\beta4$, as well as by the β4 peptide, raises the interesting possibility that channels equilibrating among open and closed states are delayed from entering fast-inactivated states by a rapid but unstable binding and unbinding of the blocker at negative voltages (31). Alternatively, the β4 peptide may exert an additional, separate effect on the channel, independent of open-channel block, which reduces fast inactivation and increases persistent current.

Nevertheless, in cells in which $Na_V\beta4$ acts as an endogenous blocker, resurgent current, persistent current, and steady-state availability cannot be separated: The subunit antagonizes inactivation at negative voltages, promoting subthreshold persistent current flow, and occludes channels at positive voltages, setting the stage for resurgent current upon repolarization. Therefore, except near the peak of the action potential, $Na_V\beta4$ actually increases availability and activation, promoting depolarization and spike initiation, and, if K currents permit, rapid and repetitive spiking.

Indeed, $Na_V\beta4$ also measurably influences excitability. Consistent with the decreased subthreshold availability, rheobase is increased in on-target cells. The most salient change, however, is that the duration of repetitive firing during depolarizations is reduced. In granule cells, loss of $Na_V\beta4$ subunits may well lead to changes in the expression or properties of multiple ion channels and/or neuron morphology (2), but the observation that the β4 peptide rescues repetitive spiking rules out such changes as the primary basis for reduced firing. Instead, the data support the idea that excitability of cultured granule neurons is regulated by $Na_V\beta4$ acting at least in part as an open-channel blocker of Na channels. Thus, with all other aspects of intrinsic excitability held constant, the expression of $Na_V\beta4$ may act as a switch that permits sustained firing, particularly in the face of depolarizing inputs.

$Na_V\beta4$ may not be unique as an endogenous blocking protein across neurons, however. The distribution of resurgent current indeed correlates well with that of $Na_V\beta4$, which is expressed strongly in the cerebellum, brainstem, subthalamic nuclei, and dorsal root ganglia (1), but some brain regions that express $Na_V\beta4$ only weakly also contain cells with resurgent Na current (30, 32). Despite weak labeling, some of these cells may nevertheless express $Na_V\beta4$, as is the case for cerebellar granule neurons. Alternatively, because most ion channel proteins exist in multiple isoforms, many of which are tissue or cell specific, other proteins with structural and functional similarity to $Na_V\beta4$ may well be identified in the future.

Conversely, it is likely that not all cells that express $Na_V\beta4$ produce resurgent current. Indeed, over-expressing $Na_V\beta4$ in CA3 neurons does not yield resurgent current (22), suggesting that cell-type-specific modulation of α or β subunits is required to enable open-channel block. Such modulation seems plausible, as resurgent current can be disrupted by broad-spectrum phosphatases (33); $Na_V\beta4$ is the target of enzymatic cleavage (3); and disease mutations change the susceptibility of α subunits to open-channel block (17). Even under conditions that "enable" block, however, $Na_V\beta4$ may have diverse effects on current, depending on its affinity for different α subunits. A high-affinity might require strong repolarization for expulsion, with little enhancement of subthreshold Na current, whereas a low affinity may effectively antagonize inactivation and permit persistent current without yielding an obvious resurgent component. In fact, resurgent current kinetics vary across cell types, consistent with diversity of blocker–channel interactions (29). Regardless of whether $Na_V\beta4$ turns out to be unique, it emerges as the first endogenous, open-channel blocking protein that generates resurgent current. That this subunit is highly expressed in several neurons with resurgent

current makes it seem likely that this function is not restricted to one cell type.

Materials and Methods

Culture Preparation. In accordance with Institutional Animal Care and Use Committee guidelines, cerebella were dissected from P0-P1 C57BL6 mice in cold D1 saline (mM: 140 NaCl, 5 KCl, 0.1 Na_2HPO_4, 2.2 KH_2PO_4, 5 Hepes, 4 sucrose, 30 glucose, 10 μL/mL penicillin/streptomycin stock, and 0.001% phenol red). After 30 min in D1 with 20 U/mL papain (Worthington Biochemicals), 1.7 mM cysteine, 100 μM $CaCl_2$, and 50 μM EDTA (pH 7.3, NaOH) at 37 °C, tissue was washed in minimal essential medium (MEM) (Invitrogen) with 5% heat-inactivated FCS (HyClone), 20 mM glucose, 0.5 mM Glutamax (Invitrogen) and 2.5 mg/mL each of BSA and trypsin inhibitor. After trituration, cells were plated onto coverslips coated with poly-L-lysine and collagen (Invitrogen) and grown to confluence. One week later, neurons were killed by excitotoxicity by (mM) 0.1 glutamate, 165 NaCl, 5 KCl, 2 $CaCl_2$, and 5 Hepes (30 min). Fresh neurons and glia were plated onto glial beds, and after 24–48 h, glial proliferation was prevented with 5 μM cytosine arabinofuranoside.

Transfections. A premixed pool of four on-target siRNA duplexes targeted to four sites in the *Scn4b* gene, and a premixed pool of four non-target negative-control siRNA duplexes were obtained from Dharmacon. On-target antisense siRNA sequences were CAAACAAGAAGUCCAAUGAUU, AAUUUAUCAACCACUUGGAUU, CAAGAGGAUGGAGAUGUUAUU, and UUAUUGUAGGACCACUUGAUU, designed by Dharmacon as described elsewere (34). Non-targeting sequences ("on-target plus siControl non-targeting pool"), which are proprietary to Dharmacon, do not target transcripts from the mouse genome, as previously confirmed (35). Simultaneous use of multiple on-target siRNAs maximizes the probability that at least one siRNA sequence will knock down the desired mRNA; the non-target pool serves as a control. Indeed, when siRNA pools were applied to HEK cells transfected with rat *Scn4b* (22), *Scn4b* expression by quantitative RT-PCR was reduced by 90% in on-target relative to non-target cells ($P < 0.005$; β-actin as reference gene). Each coverslip of granule cells (1–2 DIV) was transfected with 0.13 μg (10 pmol) of the four siRNAs, 0.15 μg GFP plasmid, and Lipofectamine 2000 (Invitrogen) at 1 μL (recording) or 4 μL (quantitative RT-PCR). Cells were incubated with reagent in serum-free OPTI-MEM (Gibco) for 2 h and then washed with serum-containing medium before feeding. To combat the tendency of siRNA efficacy to decrease over a period of a few days (26), we used siRNA concentrations that were ~100-fold in excess of those predicted to yield maximal knockdown (36). Experimental observations were consistent with knockdown of protein on the time scale reported elsewhere (27).

Electrophysiology. Recordings were made with an Axopatch 200B amplifier and pCLAMP software (Molecular Devices; 5 kHz filtering, 50 kHz sampling) from 6 to 15 μm diameter phase-bright cells with one or more neurites. Transfection was verified under fluorescence. Cells were bathed in Tyrode's solution containing (mM) 150 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, and 10 glucose (pH 7.36, NaOH). For voltage-clamp studies, patch electrodes (3.5–6.5 MΩ) were filled with the following (mM): 108 $CsCH_3SO_3$, 9 NaCl, 1.8 $MgCl_2$, 9 Hepes, 0.9 EGTA, 43 sucrose, 14 Tris-creatinePO_4, 4 MgATP, and 0.3 TrisGTP (pH 7.4, CsOH). Whole-cell series resistance was compensated to ~70%. With 100 nM tetrodotoxin (TTX, Alomone) in the bath to improve space clamp, somata were perfused through flow pipes containing the following (mM): 154 NaCl, 10 TEACl, 10 Hepes, 10 glucose, 0.3 $CdCl_2$, and 0.4 $BaCl_2$ ± 900 nM TTX. TTX-sensitive Na current was isolated by subtraction. Cells with escaping spikes or leak changes were rejected. For current-clamp studies, 5–10 MΩ electrodes contained the following (mM): 120 K-gluconate, 2 Na-gluconate, 2 $MgCl_2$, 0.9 Hepes, 1 EGTA, 20 sucrose, 14 Tris-creatinePO_4, 4 MgATP, and 0.3 TrisGTP (pH 7.4 with CsOH). Cells were bathed in Tyrode's solution.

Quantitative RT-PCR. The methods and standards proposed by Bustin et al. (37) were used as a guide. Primers, kits, and enzymes were from Invitrogen. Primers (5′ to 3′, forward then reverse) were as follows: *Scn4b*, GGGCTTTTGGGTCTCTTC, GAGGTTCTCAAAGCCATAACA; *Scn2a*, AAGTGGATTGTTCCATCAGG, AAAGAGCAGGGATTTCTTCC; *Scn8a,* GTCTTCACCTACATCTTCATCCTG, CCTAAGGGACTTTATGGCACC; *Scn8a neonatal,* GTCTTCACCTACATCTTCATCCTG, ACCCCCTCGCCCTTTAC; *Scn8a Δ18,* TGGAGATGTTGCTCAAATGG, ATGAGACACACCAGCAGCAC. Amplified products were run on gels to verify size and melt curves confirmed a lack of primer-dimers. Neonatal and Δ18 *Scn8a* (38) did not differ in on-target and non-target cells.

Cultured neurons (1 DIV) or glia after excitotoxicity were transfected with on-target or non-target siRNA. At 6, 9, and 12 DIV, RNA was extracted with TRIZOL. RNA samples with 260/280 absorbance ratio <1.8 (Nanodrop ND-1000) were rejected. RNA was digested with DNase, reverse transcribed (Superscript III kit), digested with RNase H, mixed with SYBR green SupermixUDG for quantitative PCR, and loaded onto 384-well plates in quadruplicate. Quantification cycle (C_Q) values (PCR cycles to reach fluorescence threshold) were obtained with the standard cycling program on a 7900HT-Fast Real-Time PCR System and SDS software (Applied Biosystems). Calibration curves for primers were obtained (38) by plotting C_Q for serially diluted cDNA samples vs. log[relative cDNA]. Efficiency, E, was calculated as $10^{-1/m}$ where m is the calibration curve slope. The change in cDNA was calculated (39) as $(E_{target}{}^{\Delta CQ(ctrl\text{-}sample)})/(E_{reference}{}^{\Delta CQ(ctrl\text{-}sample)})$. Efficiencies were 2.00 (*Scn4b*), 1.98 (*Scn2a*), and 2.00 (*Scn8a*). Copy number was estimated from qPCR of known quantities of rat *Scn4b* (22).

Data Analysis. Data were analyzed with IGOR-Pro (Wavemetrics) with NeuroMatic functions and are reported as mean ± SEM except as noted. Resurgent current was measured as the mean current for 1 ms about the peak, less the mean persistent current in the last 10 ms of the 200-ms step. Inactivation curves were normalized to peak current and fit as $I = F_{ss}+(1-F_{ss})/(1+\exp(V-V_{1/2})/k)$, where I is current, $V_{1/2}$ is the half-maximal inactivation voltage, k is the slope factor, and F_{ss} is the noninactivating fraction. Spike rasters were obtained with a 15 V/s threshold. Median rather than mean time of last spike was measured to account for the ceiling value of 300 ms. Statistical significance ($P < 0.05$) was assessed with Student's two-tailed *t* tests, confirmed with one-way ANOVAs for Na currents, or with univariate ANOVAs and two-sample Kolmogorov–Smirnov tests for spikes. Capacitative artifacts are reduced.

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