Self-Limited Hyperexcitability: Functional Effect of a Familial Hemiplegic Migraine Mutation of the Na_v1.1 (SCN1A) Na⁺ Channel

Sandrine Cestèle,^{1,2} Paolo Scalmani,¹ Raffaella Rusconi,¹ Benedetta Terragni,¹ Silvana Franceschetti,¹ and Massimo Mantegazza^{1,3}

¹Department of Neurophysiopathology, Besta Neurological Institute, 20133 Milan, Italy, ²Inserm U836 (Equipe 3), Institute of Neurosciences, 38054 Grenoble, France, and ³Equipe Avenir Inserm, Institut Fédératif de Recherche 95-Saints-Pères, Université Paris 5–Descartes, 75006 Paris, France

Familial hemiplegic migraine (FHM) is an autosomal dominant inherited subtype of severe migraine with aura. Mutations causing FHM (type 3) have been identified in *SCN1A*, the gene encoding neuronal voltage-gated Na_v1.1 Na⁺ channel α subunit, but functional studies have been done using the cardiac Na_v1.5 isoform, and the observed effects were similar to those of some epileptogenic mutations. We studied the FHM mutation Q1489K by transfecting tsA-201 cells and cultured neurons with human Na_v1.1. We show that the mutation has effects on the gating properties of the channel that can be consistent with both hyperexcitability and hypoexcitability. Simulation of neuronal firing and long depolarizing pulses mimicking promigraine conditions revealed that the effect of the mutation is a gain of function consistent with increased neuronal firing. However, during high-frequency discharges and long depolarizations, the effect became a loss of function. Recordings of firing of transfected neurons showed higher firing frequency at the beginning of long discharges. This self-limited capacity to induce neuronal hyperexcitability may be a specific characteristic of migraine mutations, able to both trigger the cascade of events that leads to migraine and counteract the development of extreme hyperexcitability typical of epileptic seizures. Thus, we found a possible difference in the functional effects of FHM and familial epilepsy mutations of Nav1.1.

Key words: migraine; current; epilepsy; excitability; neuron; sodium channel

Introduction

Migraine is a common disease with a strong genetic component. Approximately 35% of migraine patients have migraine with aura, consisting of transient focal neurological symptoms (often visual disturbances) that precede the headache (Pietrobon and Striessnig, 2003; Kors et al., 2004; Silberstein, 2004).

Functional studies in patients have provided evidence that the visual aura coincides with cortical spreading depression (CSD) (Bowyer et al., 2001; Hadjikhani et al., 2001), a wave of neuronal depolarization that spreads slowly across the cerebral cortex and generates a transient intense firing activity followed by a long-lasting suppression (Pietrobon and Striessnig, 2003). The headache is caused by the stimulation of trigeminal fibers that innervate the blood vessels of the meninges and activate brain areas involved in the perception of pain (Pietrobon and Striessnig, 2003). Experiments in animal models have shown that CSD can activate this pain pathway (Bolay et al., 2002), but it is not clear

what is the trigger of CSD and what is the mechanism that links CSD to activation of nociceptors.

Causative genes have been identified for familial hemiplegic migraine (FHM), a rare severe autosomal dominant inherited subtype of migraine with aura characterized by hemiparesis during the attacks (Pietrobon and Striessnig, 2003; Kors et al., 2004; Pietrobon, 2007). FHM type 1 is caused by gain-of-function mutations of the $\alpha 1$ subunit of neuronal Ca_v2.1 Ca²⁺ channel (Ophoff et al., 1996) consistently with enhanced glutamate release (Pietrobon, 2007); facilitation of CSD was observed in a knock-in mouse model (van den Maagdenberg et al., 2004). FHM type 2 is caused by loss-of-function mutations of the $\alpha 2$ subunit of the Na⁺/K⁺ pump (ATP1A2) (De Fusco et al., 2003), consistently with reduced removal of K⁺ and glutamate from the extracellular space, and thus with inhibition of recovery from neuronal excitation, long-lasting depolarizations, and CSD (Pietrobon, 2007).

More recently, FHM (type 3) mutations have been identified in *SCN1A*, the gene encoding neuronal voltage-gated Na_v1.1 Na⁺ channel α subunit (Dichgans et al., 2005; Gargus and Tourney, 2007; Vanmolkot et al., 2007), but the functional studies have been done using the cardiac Na_v1.5 Na⁺ channel. However, Na_v1.1 is the major target of epileptogenic mutations, and the functional effects that have been observed for some of them are similar to those reported for migraine mutations studied with Na_v1.5 (Spampanato et al., 2001; Meisler and Kearney, 2005;

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Correspondence should be addressed to Dr. Massimo Mantegazza, Department of Neurophysiopathology, Besta Neurological Institute, Via Celoria 11, 20133 Milan, Italy. E-mail: mmantegazza@istituto-besta.it.

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Avanzini et al., 2007), complicating our understanding of the differential pathophysiological mechanism of the two diseases. It is important to study the functional effects of migraine mutations in the human Na_v1.1 clone. In fact, despite their high level of homology, cardiac and neuronal Na⁺ channels show several structural and functional differences (Fozzard and Hanck, 1996; Richmond et al., 1998; Mantegazza et al., 2001, 2005a; Catterall et al., 2005).

We introduced the FHM mutation Q1489K into hNa_v1.1 cDNA (Q1478K according to the nomenclature of the hNa_v1.1 clone that we have used; see Results), and we studied its functional effects in transfected human tsA-201 cells and rat cultured neurons, shedding some light on the possible pathophysiological mechanism that differentiates Na_v1.1 migraine mutations from epileptogenic mutations.

Materials and Methods

Site-directed mutagenesis. The human clone of the shorter splice variant isoform (1998 aa) of Na_v1.1 Na⁺ channel (Schaller et al., 1992) was provided by Dr. Jeff Clare (GlaxoSmithKline, Stevenage, Herts, UK). We subcloned it into the plasmid pCDM8 (Mantegazza et al., 2005b), which was propagated in Top 10/P3 bacteria (Invitrogen), grown at 30°C for ~48 h to minimize rearrangements. We used pCDM8 because in our experience it was able to reduce the rearrangements of several Na⁺ channel clones (Mantegazza et al., 2001, 2005a). In fact, pCDM8-hNa_v1.1 has a very low yield when propagated in bacteria; thus, when pCDM8 is “loaded” with hNa_v1.1, it becomes functionally a very low-copy-number vector that reduces the rearrangements and improves the efficiency of the mutagenesis. The mutation Q1478K was introduced into pCDM8-hNa_v1.1 by means of the Quick Change XL site-directed mutagenesis kit (Stratagene) with the following primers: 5′TTCAACCAGAGAAAAA-GAAGTTTGG (forward) and 5′TCTTTTCTTCTGGTTGAAATTATC (reverse). Colonies were screened by sequencing. We sequenced the whole open reading frame of hNav1.1 after each propagation to exclude the presence of spurious mutations.

Transient expression. TsA-201 cells were maintained and transfected with CaPO₄, as done by Mantegazza and Cestèle (2005). We cotransfected pCDM8-hNa_v1.1 and hβ1 (provided by Dr. Al George, Vanderbilt University, Nashville, TN) using a 1:1 molar ratio. We subcloned hβ1 into the bicistronic plasmid pIRES-YFP (Clontech), which expresses both the protein of interest and yellow fluorescent protein (YFP) as reporter. We did control experiments without β1 (cotransfecting empty pEYFP-N1 as reporter), obtaining similar results (data not shown). Neurons were prepared, cultured, and transfected as by Scalmani et al. (2006). Briefly, neocortical neurons were isolated from postnatal day 1 (P1)–P3 CD rat pups (Charles River); rats were decapitated under ether anesthesia, the brain was quickly removed, and the cerebral cortex was isolated using fine tweezers and chopped into small pieces that were digested with protease type XIV (1 mg/ml; Sigma) for 15 min. The tissue was then mechanically dissociated using a series of fire-polished Pasteur pipettes. The dissociated neurons were plated in Petri dishes and cultured at 37°C and with 5% CO₂ in Neurobasal A culture medium (Invitrogen) supplemented with B27 (Invitrogen), glutamine (1 mM; Invitrogen), β-FGF (10 ng/ml; Invitrogen), penicillin G (50 U/ml), and streptomycin (50 μg/ml; Sigma). The neurons were transfected on the same day by magnetofection with Lipofectamine 2000 (Invitrogen) and CombiMag (OZ Biosciences) and used within 30 h.

Electrophysiological recordings and analysis. Transfected cells were selected visually by their fluorescence, and recordings were done at room temperature (22–25°C) using a Multiclamp 700A patch-clamp amplifier and pClamp 10.2 software (Molecular Devices). Signals were filtered at 10 kHz and sampled at 100 kHz.

We recorded Na⁺ currents from tsA-201 cells with the whole-cell configuration of the patch-clamp technique. Recordings were usually started 5 min after the rupture of the membrane patch, to allow intracellular dialysis with the pipette solution. External bath solution contained the following (in mM): 150 NaCl, 10 Cs-HEPES, 1 MgCl₂, 2 KCl, and 1.5

CaCl₂, pH 7.4; internal pipette solution was the following (in mM): 105 CsF, 35 NaCl, 10 Cs-HEPES, and 10 EGTA, pH 7.4. Cell capacitance and series resistance errors were carefully compensated (~85%) throughout the experiment. Pipette resistance was between 1.5 and 2.0 MΩ, and series resistance was between 2.5 and 4.5 MΩ; maximum accepted voltage-clamp error was 2.5 mV. The remaining linear capacity and leakage currents were eliminated online using a P/4 subtraction paradigm.

Macroscopic Na⁺ currents were recorded from transfected cultured neurons using the on-cell macropatch configuration of the patch-clamp technique to avoid the space-clamp errors caused by the long cellular processes (Scalmani et al., 2006). We used different recording solutions than with tsA-201 cells, to selectively record Na⁺ currents, blocking the endogenous contaminating currents that are present in neurons. The internal pipette solution contained the following (in mM): 110 NaCl, 35 tetraethylammonium-Cl, 1 CaCl₂, 2 MgCl₂, 0.3 NiCl₂, 0.4 CdCl₂, 0.4 BaCl₂, 1.5 4-aminopyridine, and 10 Na-HEPES, pH 7.4. The bath solution contained the following (in mM): 40 K-gluconate, 100 KCl, 5 EGTA, 10 K-HEPES, and 30 glucose, pH 7.4. Capacitative currents were minimized by means of the amplifier circuitry. Series resistance compensation was not used because the maximum peak amplitude of Na⁺ currents was <270 pA. The remaining transient and leakage currents were eliminated using P/4 subtraction. The diameter of the tip (~3 μm) and the shape of the pipettes were controlled for each pipette and kept constant to maintain the area of the membrane patch approximately constant. Pipette resistance was between 2.6 and 3.0 MΩ. The action potentials used as voltage stimuli in voltage-clamp macropatch recordings of transfected cultured neurons were recorded from layer V pyramidal neurons in rat neocortical slices as described previously (Mantegazza et al., 1998; Scalmani et al., 2006).

Current-clamp recordings of the firing of the transfected neurons were obtained with the whole-cell configuration of the patch-clamp technique. The internal pipette solution contained the following (in mM): 120 K-gluconate, 15 KCl, 2 MgCl₂, 0.2 EGTA, 20 phosphocreatine-Tris, 2 ATP-Na₂, 0.2 GTP-Na₂, 0.1 leupeptin, and 10 K-HEPES, pH 7.2. The bath solution contained the following (in mM): 129 NaCl, 1.25 NaH₂PO₄, 1.8 MgSO₄, 1.6 CaCl₂, 3 KCl, 10 Na-HEPES, and 35 glucose, pH 7.4. We obtained the seal, the measurement of the passive properties, and the recording of the total currents in voltage-clamp mode (for the latter, cell capacitance and series resistance errors were compensated at ~85%). Series resistance was between 6.5 and 15 MΩ. We then switched the amplifier to current-clamp mode, applied the bridge balance compensation, and held the resting potential at −70 mV by injecting the appropriate holding current. Neuronal firing was recorded injecting depolarizing current pulses of increasing amplitude. The neurons with unstable resting potential and/or unstable firing were discarded from the analysis.

All the recordings were corrected for junction potential errors (Barry, 1994) and analyzed using pClamp and Origin 7.5 (OriginLab). Conductance–voltage curves were derived from current–voltage (*I*–*V*) curves according to $G = I/(V - V_R)$, where *I* is the peak current, *V* is the test voltage, and *V_R* is the apparent reversal potential in tsA cells and the calculated Nernst potential in neurons. The voltage dependence of activation and voltage dependence of inactivation were fitted to a Boltzmann relationship of the form $1/[1 + \exp((V_{1/2} - V)/k)]$ plus a baseline, where *V_{1/2}* is the voltage of half-maximal activation (*V_a*) or inactivation (*V_i*) and *k* is a slope factor (in millivolts). Fits were achieved using the Levenberg–Marquardt algorithm with Origin. The fitting lines in the figures were obtained using mean parameters calculated by averaging the parameters of the fits of the single cells. Statistical analyses were made using Origin. The results are given as mean ± SEM, and statistical significance was at *p* = 0.05. Statistical comparisons were performed with the *t* test.

Results

In our study we used the human clone of the shorter splice variant isoform of Na_v1.1 Na⁺ channel α subunit (Noda et al., 1986; Schaller et al., 1992), which has a deletion of 11 aa and could be the predominant Na_v1.1 variant expressed in brain (Schaller et al., 1992). The clone has already been used for functional studies

Figure 1. Effects of Q1478K on activation properties and I_{NaP} in tsA-201 cells. **A**, Representative whole-cell Na⁺ currents (top, hNa_v1.1; bottom, hNa_v1.1-Q1478K) recorded with 75-ms-long depolarizing steps from -65 mV to $+55$ mV in 10 mV increments, from a holding potential of -100 mV. Calibration: 500 pA, 2.5 ms. The insets show the first 4 ms of the traces. **B**, Average normalized currents (solid line, hNa_v1.1; dashed line, hNa_v1.1-Q1478K) elicited with a depolarizing step to -15 mV. The error bars are the SEM of selected data points. Calibration: 500 μ s. The inset shows the final 5 ms of the 150 -ms-long traces, to compare I_{NaP} . **C**, Current density–voltage plots for hNa_v1.1 (black symbols; 174 ± 14 pA/pF; $n = 21$) and hNa_v1.1-Q1478K (white symbols; 124 ± 8 pA/pF; $n = 45$; $p = 0.009$) recorded 5 min after the rupture of the patch. **D**, Mean voltage dependence of activation; the lines are Boltzmann relationships whose parameters were calculated by averaging the parameters of the fits of the single cells: solid line, hNa_v1.1 ($V_a = -34.2 \pm 0.9$ mV; $K_a = 8.2 \pm 0.4$ mV); dashed line, hNa_v1.1-Q1478K ($V_a = -36.6 \pm 0.7$ mV; $K_a = 8.8 \pm 0.3$ mV). **E**, Mean current–voltage plots for I_{NaP} recorded after 5 min from the establishment of the whole-cell configuration: hNa_v1.1, $1.3 \pm 0.2\%$; hNa_v1.1-Q1478K, $5.3 \pm 1.0\%$ ($p = 0.002$). **F**, Mean current–voltage plots for I_{NaP} recorded after >17 min: hNa_v1.1, $0.9 \pm 0.3\%$ (20 – 60 min; $n = 7$); hNa_v1.1-Q1478K, $1.2 \pm 0.2\%$ (17 – 30 min; $n = 6$); the line with no symbols is the mean I_{NaP} recorded in the same cells after 5 min: hNa_v1.1, $1.2 \pm 0.2\%$; hNa_v1.1-Q1478K, $4.5 \pm 0.6\%$. The results are given as mean $\pm$ SEM.

(Oliveira et al., 2004; Mantegazza et al., 2005a,b; Rusconi et al., 2007). We introduced in hNa_v1.1 the Q1489K FHM3 mutation (Dichgans et al., 2005), which we denominated Q1478K according to the numeration of the clone that we used, and investigated its functional effects by patch-clamp recordings in transiently transfected tsA-201 cells and neocortical cultured neurons.

Voltage-gated Na⁺ channel α subunits are formed by four homologous domains (DI–DIV), each containing six transmembrane segments (S1–S6) connected by extracellular and intracel-

lular loops; the S4 of each domain constitute the voltage sensor. The mutation Q1478K is located in the cytoplasmic loop between DIII and DIV, the inactivation gate that plugs the pore during fast inactivation (Catterall, 2000).

Effect of Q1478K on the functional properties of hNa_v1.1 in tsA-201 cells Activation and persistent current

Fig. 1A shows representative whole-cell Na⁺ current traces recorded over a range of potentials in tsA-201 cells transfected with wild-type hNa_v1.1 or the migraine mutant hNa_v1.1-Q1478K. The time course of activation was not modified by the mutation, as shown by the comparison on a shorter time scale of the mean current traces elicited by depolarizing steps to -15 mV displayed in Fig. 1B. The current decay of both hNa_v1.1 and hNa_v1.1-Q1478K showed a large rapidly inactivating component (I_{NaT}), reflecting inactivation from the open state of the channel, followed by a slowly inactivating “persistent” component (I_{NaP}) that failed to inactivate by the end of long depolarizing steps of up to 150 ms (Fig. 1B, inset). The faster component of the decay was not modified by the mutation, but I_{NaP} was consistently larger for hNa_v1.1-Q1478K (see below).

The mean current density–voltage plots of I_{NaT} obtained applying test pulses to membrane potentials between -60 mV and $+80$ mV are shown in Figure 1C. I_{NaT} began to activate at approximately -50 mV, peaked at -15 mV, and inverted at approximately $+40$ mV both with wild type and hNa_v1.1-Q1478K, but the mutant showed a significant 29% reduction in maximum current density, which is consistent with reduced excitability in neurons expressing the mutant channel. Consistently with the results obtained with the current density–voltage plots, the analysis of the conductance–voltage plots (Fig. 1D) did not show any significant modifications of the activation curve of I_{NaT} .

We quantified I_{NaP} by measuring the average current between 45 and 55 ms after the beginning of the test pulse. Figure 1E shows the comparison over a range of potentials of the mean I_{NaP} recorded 5 min after the establishment of the whole-cell

configuration, expressed as percentage of maximum I_{NaT} . I_{NaP} began to activate at approximately -55 mV, peaked at -20 mV, and inverted at approximately $+40$ mV, both with wild type and hNa_v1.1-Q1478K. I_{NaP} in cells transfected with hNa_v1.1-Q1478K was 3.9-fold larger at -20 mV than with the wild type and significantly larger in the whole range of potentials (Fig. 1E), a modification that is consistent with increased neuronal excitability. The increase of I_{NaP} induced by the mutation was statistically significant also considering the reduction of the amplitude of I_{NaT}

Figure 2. Effects of Q1478K on fast inactivation properties in tsA-201 cells. **A**, Mean voltage dependence of fast inactivation (black symbols, $\text{hNa}_v1.1$; white symbols, $\text{hNa}_v1.1$ -Q1478K); the lines are Boltzmann relationships with the following mean parameters: $\text{hNa}_v1.1$ ($n = 20$), $V_h = -69.3 \pm 0.5$ mV; $K_h = 7.3 \pm 0.4$ mV; baseline, 0.017 ± 0.008 ; $\text{hNa}_v1.1$ -Q1478K ($n = 42$), $V_h = -65.2 \pm 0.5$ mV ($p = 0.006$); $K_h = 7.4 \pm 0.3$ mV; baseline, 0.057 ± 0.07 ($p = 0.01$). **B**, Mean kinetics of development of fast inactivation at -65 mV; the lines are mean fits of single-exponential relationships. **C**, Time constants of development over a range of potentials: -45 mV, $\text{hNa}_v1.1$ ($n = 9$), $\tau_{\text{DEV}} = 3.9 \pm 0.6$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 14$), $\tau_{\text{DEV}} = 2.6 \pm 0.2$ ms ($p = 0.03$); -55 mV, $\text{hNa}_v1.1$ ($n = 12$), $\tau_{\text{DEV}} = 14.9 \pm 2.9$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 16$), $\tau_{\text{DEV}} = 8.4 \pm 0.9$ ms ($p = 0.02$); -65 mV, $\text{hNa}_v1.1$ ($n = 13$), $\tau_{\text{DEV}} = 40.6 \pm 5.5$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 21$), $\tau_{\text{DEV}} = 17.6 \pm 1.9$ ms ($p = 6 \times 10^{-5}$); -75 mV, $\text{hNa}_v1.1$ ($n = 9$), $\tau_{\text{DEV}} = 35.7 \pm 2.9$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 9$), $\tau_{\text{DEV}} = 14.4 \pm 2.5$ ms ($p < 10^{-5}$). **D**, Mean kinetics of recovery at -95 mV from a 100 ms inactivating pulse to 0 mV; the lines are mean fits of single-exponential relationships to the data. **E**, Time constants of recovery over a range of potentials: -95 mV, $\text{hNa}_v1.1$ ($n = 11$), $\tau_{\text{REC}} = 6.6 \pm 0.4$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 20$), $\tau_{\text{REC}} = 3.4 \pm 0.2$ ms ($p < 10^{-5}$); -105 mV, $\text{hNa}_v1.1$ ($n = 9$), $\tau_{\text{REC}} = 4.2 \pm 0.4$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 17$), $\tau_{\text{REC}} = 2.2 \pm 0.1$ ms ($p < 10^{-5}$); -115 mV, $\text{hNa}_v1.1$ ($n = 8$), $\tau_{\text{REC}} = 3.0 \pm 0.1$ ms; $\text{hNa}_v1.1$ -Q1478K ($n = 13$), $\tau_{\text{REC}} = 1.8 \pm 0.1$ ms ($p < 10^{-5}$). The results are given as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$.

that we observed with $\text{hNa}_v1.1$ -Q1478K (2.9-fold larger at -20 mV). However, comparing I_{NaP} after >17 min from the establishment of the whole-cell configuration, the increase induced by the mutation was no longer observable (Fig. 1F). Thus, the increase of I_{NaP} is a labile property of the mutant that is inhibited by a long-lasting dialysis of the cytoplasm.

Fast inactivation

We studied the voltage dependence of fast inactivation (Fig. 2A) by applying 100-ms-long inactivating prepulses to potentials from -110 mV to -5 mV, followed by a test pulse to -5 mV. The inactivation curve of $\text{hNa}_v1.1$ -Q1478K showed a significant positive shift of 4.1 mV and, consistently with the increase of I_{NaP} , its baseline was larger than for the wild-type. Nevertheless, the pos-

itive shift of the voltage dependence of inactivation caused by Q1478K may be attributable to a slower development of fast inactivation of $\text{hNa}_v1.1$ -Q1478K, which could make the 100 ms inactivating prepulse that we used not sufficiently long to induce steady-state fast inactivation. Therefore, we studied the kinetics of development of fast inactivation by applying inactivating prepulses of increasing duration to potentials between -75 and -45 mV. The curves of both wild type and $\text{hNa}_v1.1$ -Q1478K were well fit by a single-exponential relationship, as shown by the mean curves obtained with depolarizing pulses to -65 mV, displayed in Figure 2B. However, the development of fast inactivation was significantly faster with the mutant over the whole range of potentials, and the largest difference was observed at -65 mV, where $\text{hNa}_v1.1$ -Q1478K showed a 2.3-fold faster kinetics than the wild-type (Fig. 2C). Thus, the shift of the inactivation curve is a real modification of the voltage dependence of the channel, because it cannot be caused by the acceleration of the rate of development of fast inactivation of $\text{hNa}_v1.1$ -Q1478K. These modifications are consistent with opposite effects on neuronal excitability: the positive shift of the inactivation curve is consistent with neuronal hyperexcitability, because at the typical resting potential of -65 mV, $\sim 50\%$ of $\text{hNa}_v1.1$ -Q1478K current is inactivated, compared with $\sim 65\%$ of wild-type current; the acceleration of the development of inactivation is consistent with hypoexcitability because $\text{hNa}_v1.1$ -Q1478K current can be inactivated by shorter depolarizations.

Because we observed modifications of the kinetics of development of fast inactivation, we investigated whether other kinetic properties were also modified. We studied the kinetics of the recovery from fast inactivation by applying a 100-ms-long inactivating pulse to 0 mV followed by recovery interpulses of increasing duration to potentials between -115 and -95 mV and by a test pulse to -5 mV. The recovery curves were well fit by a single-exponential relationship, as shown by the mean curves obtained with an interpulse potential of -95 mV, displayed in Figure 2D. The recovery at -95 mV was approximately twofold faster with $\text{hNa}_v1.1$ -Q1478K and significantly faster over the whole range of potentials tested (Fig. 2E). This effect is consistent with increased neuronal excitability, because $\text{hNa}_v1.1$ -Q1478K current can recover more rapidly after short depolarizations.

Thus, Q1478K modifies the voltage dependence of inactivation and the kinetics of development of fast inactivation and of recovery from fast inactivation. These properties depend on the transitions among the closed and the closed-inactivated states of the channel (they are studied at potentials at which the channel is closed). Therefore, Q1478K can modify the properties of the inactivation from the closed states but not those of the inactivation from the open state, because the decay of the current was not modified (see above).

Slow inactivation

Long depolarizations induce in Na^+ channels a process of slow inactivation that is kinetically and mechanistically distinct from fast inactivation (Goldin, 2003). The properties of the slow inactivation may be particularly important for the pathogenesis of migraine, because neurons undergo long-lasting depolarizations during CSD. We initially used a 1-s-long inactivating pulse to -5 mV to study the properties of both slow and fast inactivation. Recovery at -95 mV after this pulse clearly showed two phases well described by a double-exponential relationship, corresponding to recovery from fast and slow inactivation (Fig. 3A). Notably, the slow phase of recovery was $\sim 81\%$ of total recovery for $\text{hNa}_v1.1$ -Q1478K, but only 43% for the wild-type channel. This is

Figure 3. Effects of Q1478K on slow inactivation properties in tsA-201 cells. **A**, Mean recovery at -95 mV from a 1 s inactivating pulse to -5 mV (black symbols, hNa_v1.1; white symbols, hNa_v1.1-Q1478K); the lines are mean fits of double-exponential relationships with the following parameters: hNa_v1.1 ($n = 9$), $\tau_{\text{FAST}} = 6.9 \pm 1$ ms; $A_1 = 0.59 \pm 0.06$; $\tau_{\text{SLOW}} = 976 \pm 110$ ms; $A_2 = 0.41 \pm 0.05$; hNa_v1.1-Q1478K ($n = 10$), $\tau_{\text{FAST}} = 2.4 \pm 0.8$ ms ($p = 5 \times 10^{-5}$); $A_1 = 0.22 \pm 0.05$ ($p < 10^{-5}$); $\tau_{\text{SLOW}} = 1083 \pm 102$ ms; $A_2 = 0.78 \pm 0.06$ ($p < 10^{-5}$). **B**, Mean development of slow inactivation at -5 mV; the lines are mean fits of exponential relationships with the following parameters: hNa_v1.1 ($n = 6$), $\tau_{\text{DEV}} = 2090 \pm 240$ ms; baseline = 0.10 ± 0.05 ; hNa_v1.1-Q1478K ($n = 7$), $\tau_{\text{DEV}} = 938 \pm 91$ ms ($p = 0.02$); baseline = 0.06 ± 0.04 . **C**, Voltage dependence of development obtained with a 20 s inactivating prepulse; the lines are Boltzmann relationships with the following mean parameters: hNa_v1.1 ($n = 5$), $V_h = -75.8 \pm 1.3$ mV; $K_h = 6.2 \pm 0.5$; baseline = 0.06 ± 0.04 ; hNa_v1.1-Q1478K ($n = 6$), $V_h = -77.2 \pm 1.1$ mV; $K_h = 7.7 \pm 0.4$; baseline = 0.08 ± 0.04 . **D**, Mean recovery of slow inactivation at -95 mV; the lines are mean fits of exponential relationships with the following parameters: hNa_v1.1 ($n = 12$), $\tau_{\text{REC1}} = 1319 \pm 550$ ms; $A_1 = 0.35 \pm 0.16$; $\tau_{\text{REC2}} = 6403 \pm 1550$ ms; $A_2 = 0.65 \pm 0.17$; hNa_v1.1-Q1478K ($n = 14$), $\tau_{\text{REC1}} = 617 \pm 208$ ms ($p = 0.03$); $A_1 = 0.23 \pm 0.14$; $\tau_{\text{REC2}} = 5555 \pm 431$ ms; $A_2 = 0.77 \pm 0.16$. **E**, Voltage dependence of recovery obtained with a 20 s inactivating prepulse; the lines are Boltzmann relationships with the following mean parameters: hNa_v1.1 ($n = 5$), $V_h = -60.4 \pm 2.3$ mV; $K_h = 8.1 \pm 0.8$; hNa_v1.1-Q1478K ($n = 5$), $V_h = -65.8 \pm 4.3$ mV; $K_h = 7.1 \pm 0.7$. The results are given as mean $\pm$ SEM.

consistent with a faster entry of hNa_v1.1-Q1478K into a slow inactivated state during the 1-s-long inactivating pulse, and shows that after relatively long depolarizations the mutant channel can generate less current than the wild type, consistently with neuronal hypoexcitability. The fast phase of recovery was approximately threefold faster for Q1478K, consistently with the data obtained studying the recovery from fast inactivation, and slow recovery was not significantly different with this protocol of stimulation. However, the value of the time constants derived

from these data may not be accurate because of the commixture between recovery from fast and slow inactivation.

To more accurately characterize the properties of slow inactivation, we used other stimulation protocols. We studied the development of slow inactivation using inactivating prepulses to -5 mV of increasing duration, followed by 15 ms repolarizations to -95 mV to allow complete recovery from fast inactivation. The curves were well fit by a single-exponential relationship and reached the steady state after approximately 20 s (Fig. 3B). As inferred from the analysis of recovery from a 1-s-long inactivating prepulse (see above), hNa_v1.1-Q1478K showed indeed a 2.1-fold faster entry into the slow inactivated state than wild type. The voltage dependence of the development of slow inactivation was studied with 20-s-long prepulses at various potentials followed by 15 ms repolarizations to -95 mV, and was not modified by the mutation (Fig. 3C). We studied the recovery from slow inactivation by applying 20-s-long inactivating prepulses to -5 mV followed by recovery interpulses at -95 mV of increasing duration (Fig. 3D). The recovery curve was well fit by the sum of two exponentials and was faster for hNa_v1.1-Q1478K: the faster time constant of recovery of the wild type was ~ 2.2 -fold larger than that of hNa_v1.1-Q1478K, whereas the slower time constant did not show significant modifications. Thus, these experiments disclosed the acceleration of the faster phase of recovery from slow inactivation caused by Q1478K, which is consistent with neuronal hyperexcitability because hNa_v1.1-Q1478K current can recover more rapidly from long depolarizations. The voltage dependence of the recovery was studied by applying a 20 s inactivating prepulse to -5 mV followed by a 20 s recovery interpulse at various potentials, and was not significantly modified by the mutation (Fig. 3E).

Our results indicate that, in contrast with the findings obtained with Na_v1.5 (Dichgans et al., 2005), Q1478K has several effects on hNa_v1.1 properties. We observed a reduction of the current density, a positive shift of the voltage dependence of inactivation, a larger I_{NaP} , and faster development and recovery from fast and slow inactivation. These results are intriguing because some of the modifications are consistent with neuronal hyperexcitability and others with hypoexcitability. Therefore, we furthered our study by applying stimuli that can disclose the overall effect of the mutation.

Overall effect of Q1478K in tsA-201 cells: use dependence of wild-type and mutant channels

We simulated neuronal firing by applying trains of 2-ms-long depolarizing steps to -5 mV at different frequencies. Figure 4A shows normalized currents elicited by steps applied from a holding potential of -105 mV. At lower stimulation frequencies (10 and 50 Hz), the mutant channel showed similar or more pronounced use dependence than the wild type. At higher stimulation frequencies (100 and 200 Hz), the current elicited with hNa_v1.1-Q1478K was larger in the initial part of the train, but the decay during the stimulation was more pronounced. Thus, at the end of the train, hNa_v1.1-Q1478K current was smaller than the wild-type current. Figure 4B shows the results obtained by applying the trains of stimulation from a more physiological holding potential of -70 mV. At a lower frequency (10 Hz), the use dependence of hNa_v1.1-Q1478K was similar to that of the wild type. At higher frequencies (50, 100, and 200 Hz), the mutant had larger current at the beginning of the train than the wild type [this difference was larger than in the experiments done with holding potential of -105 mV (Fig. 4A)], but in the final part of

Figure 4. Overall effect of Q1478K studied with trains of depolarizing steps in tsA-201 cells. **A**, Use dependence induced by trains of 200 depolarizing steps 2 ms long to -5 mV from a holding potential of -105 mV, at the frequencies indicated (black symbols, hNa_v1.1; white symbols, hNa_v1.1-Q1478K); the arrows indicate the intersections between the curves of wild-type and mutant channels (hNa_v1.1: 10 Hz, $n = 16$; 50 Hz, $n = 15$; 100 Hz, $n = 19$; 200 Hz, $n = 17$; hNa_v1.1-Q1478K: 10 Hz, $n = 21$; 50 Hz, $n = 19$; 100 Hz, $n = 22$; 200 Hz, $n = 19$). **B**, Similar stimuli as in **A** but applied from a holding potential of -70 mV (hNa_v1.1: 10 Hz, $n = 14$; 50 Hz, $n = 12$; 100 Hz, $n = 13$; 200 Hz, $n = 8$; hNa_v1.1-Q1478K: 10 Hz, $n = 14$; 50 Hz, $n = 13$; 100 Hz, $n = 17$; 200 Hz, $n = 8$); the double arrows highlight the large difference between wild-type and mutant channels at the beginning of the train.

the train the curves converged, and thus the two channels elicited similar current at the end of the 200-pulse train.

These results suggest that hNa_v1.1-Q1478K can sustain high-frequency firing better than hNa_v1.1. However, during prolonged high-frequency discharges, the mutant becomes less effective than the wild type or comparable with it, probably because during these discharges the loss of function caused by the faster development of inactivation of the mutant counteracts the gain of function caused by other modifications. Interestingly, these modifications have not been described for Na⁺ channel epileptogenic mutations; thus, they may be typical of migraine mutations.

Functional study in transfected neurons

Voltage clamp

Na⁺ channel properties are sensitive to the cell background (Baroudi et al., 2000; Chen et al., 2000; Cummins et al., 2001; Mantegazza et al., 2005a); thus, we tested whether the effects that we have observed in tsA-201 cells were conserved in a neuronal cell background. We transfected neocortical neurons in short-term primary cultures (30 h after plating) obtained from P1–P3 rats. The neocortex is a brain structure involved in the generation of

Figure 5. Effects of Q1478K in transfected neurons. **A**, Average normalized macropatch currents elicited with a depolarizing step to -5 mV from a holding potential of -95 mV in neurons transfected with hNa_v1.1 (solid line) and hNa_v1.1-Q1478K (dashed line). Calibration: 5 ms. The inset shows the first 3.2 ms of the traces. **B**, Macropatch current density–voltage plots for hNa_v1.1 (black symbols; $n = 12$) and hNa_v1.1-Q1478K (white symbols; $n = 14$); hNa_v1.1 mean maximum current density was significantly higher: hNa_v1.1, 173 ± 22 pA; hNa_v1.1-Q1478K, 112 ± 14 pA ($p = 0.008$). **C**, Mean voltage dependence of activation; the lines are Boltzmann relationships whose mean parameters are as follows: hNa_v1.1, $V_a = -24.5 \pm 1.4$ mV; $K_a = 7.9 \pm 0.8$ mV; hNa_v1.1-Q1478K, $V_a = -24.8 \pm 1.0$ mV; $K_a = 9.1 \pm 0.6$ mV. **D**, Mean voltage dependence of inactivation; the lines are Boltzmann relationships with the following mean parameters: hNa_v1.1 ($n = 9$), $V_h = -65.1 \pm 1.1$ mV; $K_h = 6.0 \pm 0.5$ mV; hNa_v1.1-Q1478K ($n = 12$), $V_h = -60.1 \pm 0.8$ mV ($p = 0.03$); $K_h = 7.4 \pm 0.5$ mV ($p = 0.04$). The results are given as mean $\pm$ SEM.

migraine, thus appropriate for studying the effects of FHM mutations. Moreover, we have previously shown that these cultured neurons have relatively small endogenous Na⁺ current, making possible the selective study of exogenous transfected Na⁺ channel subunits, which are expressed at much higher levels than the endogenous ones (Scalmani et al., 2006). However, cultured neurons develop processes that do not allow, using the whole-cell configuration of the patch-clamp technique, adequate voltage- and space-clamp control of the neuronal membrane required for the analysis of the gating of Na⁺ channels. Thus, we used the on-cell macropatch configuration to record macroscopic Na⁺ currents from a relatively large patch of membrane under good voltage- and space-clamp conditions, as in our previous study (Scalmani et al., 2006).

Figure 5A shows average macropatch traces recorded applying a depolarizing step to -15 mV. The mutation did not have any significant effects on the kinetics of activation or inactivation (Fig. 5A, inset). The Na⁺ current began to activate around -55

mV and peaked at -5 mV in both the conditions, as displayed by the I - V plot in Figure 5B. The current amplitude in macropatch recordings could be affected by experimental factors that determine the area of the patch of membrane and by clustering of channels in specialized plasma-membrane regions. To have consistent results, we maintained the area of the patch approximately constant controlling for each experiment the shape of the pipette and the diameter of its tip and applying gentle suction, and we patched always the center of the soma. Comparing the peak Na⁺ current amplitude, we observed on average a 36% reduction in neurons transfected with Q1478K (Fig. 5B), which is similar to the reduction observed in tsA-201 cells. The voltage dependence of activation was not modified (Fig. 5C), but the voltage dependence of inactivation, studied by applying 100-ms-long inactivating prepulses at the indicated potentials, showed a 5 mV positive shift (Fig. 5D), consistently with the results obtained in tsA-201 cells. Notably, I_{NaP} was virtually absent in the whole range of potentials both with hNa_v1.1 and hNa_v1.1-Q1478K (Fig. 5A). I_{NaP} may have been too small to be resolved in some recordings, where I_{NaT} was particularly small (smallest peak currents were 64 pA for hNa_v1.1-Q1478K and 95 pA for hNa_v1.1); however, in some recordings, I_{NaT} was large enough for good resolution of I_{NaP} in the range of 3–4% of I_{NaT} (maximum peak currents were 226 pA for hNa_v1.1-Q1478K and 263 pA for hNa_v1.1). Thus, in macropatch recordings from transfected neurons, we did not observe a Q1478K-induced increase of I_{NaP} .

It is extremely challenging to study with classical stimulation protocols the properties of slow inactivation in transfected cultured neurons, because recordings can last in general just some minutes, a duration that is not sufficient for the application of the long slow inactivation stimulation protocols. Thus, to disclose the overall effect of the mutation, we applied as voltage stimulus a physiological neuronal discharge recorded in neocortical slices from a regular spiking adapting pyramidal neuron as in the study by Mantegazza et al. (1998). This stimulus reproduces the physiological dynamic conditions of a firing neuron and can disclose the modifications of both the fast and the slow gating properties of Na⁺ channels. The discharge was characterized by an initial frequency of 208 Hz, gradually decreasing to 37 Hz (Fig. 6A). Figure 6B displays mean macropatch Na⁺ currents recorded in pyramidal neurons transfected with wild-type or mutant channels, normalized for each patch to the maximum I_{NaT} derived from the I - V plot of the patch. The current elicited with the mutant channel was larger than with the wild type, as shown in the insets. To have a measurement representative of the whole time course of the current elicited by an action potential (action current), we integrated the action currents and compared their areas. Figure 6C shows the comparison of the areas subtended by normalized wild-type and mutant action currents. Most hNa_v1.1-Q1478K areas resulted significantly larger, consistently with a potentiation of the firing of the neurons that express hNa_v1.1-Q1478K.

To investigate the effect of long-lasting depolarizations, we applied the discharge preceded by a 1 s depolarizing prepulse to -5 mV. Using this stimulation, the action currents were quite small both with hNa_v1.1-Q1478K and wild-type channel. However, wild-type currents were significantly larger for the first action potential of the discharge and for some action potentials later on, as shown by the comparison of the areas of the action currents displayed in Figure 6D. This result is consistent with an enhanced inhibition of hNa_v1.1-Q1478K and a reduced ability to sustain neuronal firing after long depolarizations.

Figure 6. Overall effect of Q1478K studied with action potential clamp in transfected neurons. **A**, Action potential discharge recorded with sharp microelectrodes in a layer V neuron in neocortical slices while injecting a 380 ms depolarizing current step of 300 pA. **B**, Superimposed average macropatch currents elicited in neurons transfected with hNa_v1.1 (solid line; $n = 9$) or hNa_v1.1-Q1478K (dashed line; $n = 13$) by applying as voltage stimulus the action potential discharge shown in **A** from a holding potential of -65 mV; the insets show the enlargement of the underlined parts of the traces. **C**, Average area subtended by each action current in the train for hNa_v1.1 (black symbols) and hNa_v1.1-Q1478K (white symbols). **D**, Average area subtended by each action current when the train was preceded by a 1 s inactivating prepulse (prep.) to -5 mV. * $p < 0.05$.

Current clamp

To have a more direct evidence of the effect of Q1478K on neuronal excitability, we recorded the firing of the transfected neurons by current-clamp patch-clamp experiments in whole-cell configuration. We measured the passive properties of the cells and recorded the total voltage-gated currents in voltage clamp, and then we switched to current-clamp mode and recorded the firing induced by injections of depolarizing current steps. The membrane capacitance and the cell input resistance were not significantly different between untransfected ($C_m = 17.5 \pm 1.6$ pF; $R_m = 502 \pm 66$ M Ω ; $n = 6$), hNav1.1-transfected ($C_m = 16.7 \pm 1.7$ pF; $R_m = 592 \pm 70$ M Ω ; $n = 16$), and hNa_v1.1-Q1478K-transfected ($C_m = 20.6 \pm 1.8$; $R_m = 420 \pm 52$ M Ω ; $n = 12$) neurons.

We recorded the total currents to have an estimate of the amplitude of Na⁺ and K⁺ currents, and to compare the relative amplitudes of the Na⁺ currents with those obtained with macropatch measurements. Figure 7A shows total voltage-dependent currents recorded applying depolarizing voltage pulses of increasing amplitude to representative neurons transfected with hNa_v1.1 (left) and hNa_v1.1-Q1478K (right). Three distinct com-

ponents were present in these recordings: the inward Na^+ current, a peak outward K^+ current, and a steady-state outward K^+ current. Transfection of the wild-type channel induced on average a 5.2-fold increase in Na^+ current density compared with control untransfected neurons (510 ± 59 pA/pF with $\text{hNa}_v1.1$, 98 ± 16 pA/pF in untransfected neurons); Q1478K induced on average a 38% reduction in current density (315 ± 30 pA/pF) compared with $\text{hNa}_v1.1$, which was similar to the reduction observed in tsA-201 cells (Fig. 1C) and in macropatch recordings of transfected neurons (Fig. 5B). Conversely, K^+ current densities measured with a depolarizing step to +10 mV were not significantly different (peak component: 53.7 ± 10.1 pA/pF with $\text{hNav}1.1$, 59.2 ± 12.2 pA/pF with $\text{hNa}_v1.1\text{-Q1478K}$; steady-state component measured 100 ms after the beginning of the depolarizing step: 23.8 ± 5.5 pA/pF with $\text{Nav}1.1$, 29.3 ± 4.6 pA/pF with $\text{hNa}_v1.1\text{-Q1478K}$).

In current-clamp experiments, we maintained the resting membrane potential at -70 mV and recorded the firing injecting 2.5-s-long depolarizing current steps of increasing amplitude. Only two of the six untransfected neurons were excitable (they fired a single action potential and two action potentials, respectively), and the action potentials did not overshoot. Transfected neurons generated trains of action potentials. Figure 7B shows firing traces recorded in representative neurons transfected with $\text{hNa}_v1.1$ (left) or $\text{hNa}_v1.1\text{-Q1478K}$ (right). The firing threshold was on average 4.2 mV more negative in neurons expressing wild-type channels (-53.3 ± 1.2 mV for $\text{hNa}_v1.1$; -49.1 ± 1.5 mV for $\text{hNa}_v1.1\text{-Q1478K}$; $p = 0.042$), consistently with the larger Na^+ current amplitude observed in these neurons.

In all of the neurons, the input–output relationship (number of action potentials vs injected current) showed an initial monotonic rising phase followed by a falling phase that was attributable to a depolarizing block of the firing caused by the injection of large depolarizing current. However, the data points of the input–output relationship had a large scattering, and we did not observe statistically significant differences (data not shown). Thus, to quantitatively compare the firing properties of the transfected neurons, we selected for each neuron the firing trace obtained in response to the largest current pulse capable of consistently evoking a train of overshooting action potentials and that did not induce a depolarizing block (the traces selected for the representative neurons in Fig. 7B are indicated by arrows). Figure 7C shows the comparison of the

Figure 7. Effect of Q1478K on the firing of transfected neurons. **A**, Total current traces recorded in whole-cell configuration from representative neurons transfected with $\text{hNa}_v1.1$ (left) and $\text{hNa}_v1.1\text{-Q1478K}$ (right) while applying depolarizing voltage steps from -40 mV to $+10$ mV, from a holding potential of -80 mV. Calibration: 1 nA, 20 ms. **B**, Representative firing traces recorded from neurons transfected with $\text{hNa}_v1.1$ (left) and $\text{hNa}_v1.1\text{-Q1478K}$ (right) during application of 2.5 s injections of depolarizing current from a holding potential of -70 mV; the injected current was 10 pA for the top trace with 10 pA increments (left traces) and 50 pA for the top trace with 25 pA increments (right traces). The arrows indicate the traces selected for the analysis, and the dotted lines indicate the 0 mV level for each trace. Calibration: 50 mV, 500 ms. **C**, Mean instantaneous firing frequency of the trace preceding the depolarizing block (see Results for details). We plotted only the part of the curves in which all the considered cells were firing, disregarding the final part of the curves in which the scattering was too high because of the smaller number of cells contributing to the mean data points. Black symbols, $\text{hNa}_v1.1$; white symbols, $\text{hNa}_v1.1\text{-Q1478K}$. * $p < 0.05$; ** $p < 0.01$.

mean instantaneous firing frequency of the selected traces (the frequency of each action potential pair in the train): the mutant showed significantly higher frequency at the beginning of the discharge than the wild type, but in the final part of the discharge, the curves tended to converge and the frequency was not significant.

cantly different. We also compared the number of action potentials in the selected traces but did not find a significant difference (35.2 ± 2.4 action potentials for hNa_v1.1, 48.6 ± 7.2 for hNa_v1.1-Q1478K). Also, the action potential first latency was not significantly different (hNa_v1.1, 42.7 ± 4.6 ms; hNa_v1.1-Q1478K, 39.7 ± 8.8 ms). Moreover, the analysis of the action potential amplitude and of the maximum rise slope of each action potential in the train for the same traces selected for Figure 7C did not reveal significant differences (not shown).

Therefore, the overall effect of Q1478K in transfected neurons was a self-limited hyperexcitability evidenced in the analysis of the instantaneous firing frequency, which was consistent with the results obtained with tsA-201 cells. The other parameters considered were not significantly different. Thus, in neurons the effect appeared more subtle than in tsA-201, both on the action currents and on the firing, suggesting at least a quantitative difference between neurons and tsA-201 cells. Moreover, we did not observe I_{NaP} in the macropatch recordings from transfected neurons, indicating $I_{NaP} < \sim 3\%$ of I_{NaT} both for hNa_v1.1 and hNa_v1.1-Q1478K, and consistently with a possible increased sensitivity of the mutant to a modulation of I_{NaP} that was evidently not active in the cultured neurons (see Discussion).

Discussion

The Na_v1.1 FHM mutations Q1478K (Q1489K in the longer isoform) and L1638Q (L1649Q) have been previously studied using Na_v1.5 Na⁺ channel. Q1478K caused an accelerated recovery from fast inactivation of Na_v1.5 (Dichgans et al., 2005). L1638Q, which is located in the transmembrane segment S4 of DIV (a voltage sensor that is particularly important for fast inactivation), caused an acceleration of the recovery from fast inactivation and a decrease of the rate of fast inactivation (current decay) of Na_v1.5 (Vanmolkot et al., 2007). These effects induce a gain of function and are consistent with increased neuronal excitability and accumulation of extracellular K⁺ and glutamate; thus, they may facilitate CSD. However, the reported effects are similar to those observed for some epileptogenic mutations of Na_v1.1 (Spampanato et al., 2001).

Our results show that the effects of Q1478K in hNa_v1.1 are different from in hNa_v1.5 and can account for the different pathophysiological mechanism of migraine mutations in comparison with epileptogenic mutations. We observed modifications of several of the properties of fast and slow inactivation, both in tsA-201 cells and in transfected neurons. In tsA-201, the rate of recovery from inactivation was accelerated similarly to hNa_v1.5, but we observed also an increase in the rate of development of fast inactivation and a positive shift of its voltage dependence, thus a modification of the properties of the transitions occurring among the closed and the inactivated states of the channel. The rate of decay of the current (the transition from the open state to the open-inactivated state) was not modified, but I_{NaP} was significantly increased. The rate of development of slow inactivation and of recovery from slow inactivation was also accelerated. Moreover, current density was significantly reduced, but activation properties were not modified.

Thus, consistently with its position within the inactivation loop (DIII–DIV linker) (Catterall, 2000), Q1478K affected several properties of fast inactivation but did not alter activation. Interestingly, the mutation modified also the kinetic properties of slow inactivation, even if experimental evidences have shown that slow inactivation does not directly depend on DIII–DIV linker (Goldin, 2003). This points to a role of amino acids in DIII–DIV linker in setting the properties of slow inactivation.

In transfected neurons, we studied the properties of hNa_v1.1-Q1478K both in current clamp and in voltage clamp, using as voltage stimuli both classical voltage steps and trains of action potentials. We recorded Na⁺ currents with the on-cell patch-clamp configuration, thus with no rupture of the plasma membrane and no dialysis of the cytoplasm. The overall effects were consistent with those observed using tsA-201 cells, but they were less pronounced and there were some discrepancies. Remarkably, we did not observe I_{NaP} in the recordings from transfected neurons. I_{NaP} can be potentiated by a G-protein-mediated modulation (Mantegazza et al., 2005a), and thus the initial difference in I_{NaP} amplitude between wild-type and mutant channels, which was present in tsA-201, may be attributable to a Q1478K-dependent sensitization of the mutant to a modulation, rather than to an increase of I_{NaP} caused by the modification of an intrinsic property of the α subunit. Consistently, the run down of I_{NaP} may have been caused by the inhibition of the modulation induced by the fluoride-based intracellular solution used in our whole-cell recordings. Notably, in the present study the amplitude of wild-type I_{NaP} at the beginning of the recordings (5 min after the rupture of the patch) was much lower than that obtained using a non-fluoride-based intracellular recording solution (Mantegazza et al., 2005a; Rusconi et al., 2007). In neonatal cultured neurons, I_{NaP} may have been virtually absent because Na⁺ channels were in a nonmodulated state, consistently with data that show an increase of I_{NaP} during postnatal development in cortical neurons (Huguenard et al., 1988). In general, in neurons I_{NaP} is very small in comparison with tsA-201 cells, and probably its increase is tightly controlled, because even small enhancements would cause major modifications of neuronal function.

Interestingly, some of the effects that we have observed cause a loss of function of the channel, others a gain of function. Thus, some of them are consistent with cellular hyperexcitability, others with hypoexcitability. We showed that the overall effect is an initial enhanced capacity to sustain high-frequency neuronal discharges and, notably, that this capacity is retained for a limited period during long-lasting discharges, evidently because the modifications that are consistent with a loss of function become dominant during the discharge. Thus, Q1478K can cause a pronounced but self-limited neuronal hyperexcitability, and in some conditions may lead to hypoexcitability. In fact, during some long-lasting trains of stimulation in tsA-201 cells (which reproduce excessive neuronal firing), the elicited current became smaller than the wild-type current, and Q1478K generated less current than the wild-type channel also after long-lasting depolarizations, a key event in the pathogenesis of migraine. Current-clamp recordings from transfected neurons substantiated the results obtained with voltage-clamp recordings, showing a self-limited hyperexcitability induced by Q1478K. However, the effects were much smaller than in tsA-201 cells, and we could not induce very high firing frequency because of the development of a depolarizing block of the firing both with hNa_v1.1 and hNa_v1.1-Q1478K.

Na_v1.1 is the major target of epileptogenic mutations (Meisler and Kearney, 2005; Avanzini et al., 2007), but, interestingly, just few cases of seizures have been reported in FHM patients (Dichgans et al., 2005; Vanmolkot et al., 2007). Thus, FHM mutations should modify Na_v1.1 function differently than epileptogenic mutations.

Figure 8 illustrates the effects of Q1478K (we considered those observed in tsA-201 cells because they have been better characterized) in the context of a current scheme of pathogenic mechanism of migraine (Pietrobon and Striessnig, 2003). Some of the

Figure 8. Effects of Q1478K in the context of a current scheme of pathogenic mechanism of migraine. The scheme is based on that of Pietrobon and Striessnig (2003). The effects considered are those observed in tSA-201 cells because they have been better characterized; in neurons, the effects may be quantitatively and/or qualitatively different, but the overall effect that we observed is similar (see Results). In migraine, the genetic background is important in determining an intrinsic migraine threshold that is modulated by internal and external factors (trigger stimuli); emotional stress and minor head trauma are among the most common triggers of FHM attacks (Dichgans et al., 2005; Pietrobon, 2007). FHM triggers are thought to induce excessive neuronal firing and consequently lead to extracellular K^+ and neurotransmitter accumulation. The classical neurotransmitter involved is glutamate, but also GABA (or other neurotransmitters or neuromodulators) may play a major role (see Results). This accumulation can eventually lead to enhanced long-lasting neuronal depolarization and silencing of firing caused by inactivation of Na^+ channels, thus producing CSD, which causes aura and, through still unclear mechanisms, activation of trigeminal pain afferents and headache (Pietrobon and Striessnig, 2003; Pietrobon, 2007). Some of the effects of Q1478K can facilitate the development of FHM attacks: the positive shift of the inactivation curve, the acceleration of the recovery from fast and slow inactivation, and the increase of I_{NaP} may lead to excessive neuronal firing subsequently to a trigger stimulus, and thus to accumulation of extracellular K^+ and neurotransmitters. Decreased peak current amplitude could reduce excessive firing. The increase of I_{NaP} might show cell type variability because it may be attributable to an enhanced modulation of the mutant channel. In general, excessive firing may be cell type specific (in particular, the effects could be more pronounced in interneurons; see Discussion), but also, an increase of neuronal excitability in a subset of neurons would lead to accumulation of extracellular K^+ . Subsequently to the induction of hyperexcitability, the acceleration of the development of slow inactivation may facilitate the silencing of firing and the development of CSD. Importantly, some of the effects that we have observed can counteract neuronal hyperexcitability: the acceleration of the development of fast and slow inactivation can limit excessive firing during prolonged discharges, as we have shown applying trains of high-frequency stimulations, and thus effectively counteract the development of epileptic seizures. The acceleration of the recovery from slow inactivation can counteract the silencing of firing, but it should be more effective during the repolarization after a long depolarization, and thus in the recovery from CSD and not in the development of the migraine attack.

modifications induced by Q1478K may cause hyperexcitability and facilitate the development of CSD, thus generating aura and ultimately triggering migraine. However, other modifications can limit these effects and may be able to counteract the genera-

tion of seizures. For instance, acceleration of the development of fast and slow inactivation can limit the induction of excessive firing. The reduction in peak current amplitude is a loss-of-function effect that can limit hyperexcitability and that we excluded from the analysis of the overall effect in voltage-clamp recordings (Figs. 4, 6), because the analysis was done with normalized traces, but it did not impair the self-limited hyperexcitability effect that we observed in current-clamp recordings, a more physiological experiment in which all the effects of the mutation concurred in setting firing properties. Interestingly, the increase of I_{NaP} is an effect that can induce hyperexcitability, but, as pointed out above, it may be attributable to a modulation and thus it could be cell type and/or age specific, or even depend on the functional state of the brain (which is regulated by neuromodulations).

The self-limited hyperexcitability that we have observed with Q1478K has not been reported thus far for epileptogenic mutants and may be a specific characteristic of $\text{Na}_v1.1$ migraine mutations, able to both trigger the cascade of events that leads to migraine and block the development of extreme hyperexcitability that generate epileptic seizures. Gain-of-function effects have been reported for some epileptogenic mutations of $\text{Na}_v1.1$ (Spampanato et al., 2001; Lossin et al., 2002; Rhodes et al., 2004), but most of the described mutations cause a loss of function that is consistent with a reduction of whole-cell Na^+ currents and thus a decrease in cell excitability (Meisler and Kearney, 2005; Avanzini et al., 2007). GABAergic interneurons are particularly sensitive to the loss of $\text{Na}_v1.1$ in gene-targeted mice (Yu et al., 2006; Ogiwara et al., 2007), probably because $\text{Na}_v1.1$ is expressed at higher levels in GABAergic interneurons than in glutamatergic pyramidal neurons. Thus, epileptogenic mutations may cause seizures because of decreased inhibition in neuronal circuits.

Therefore, also the effects of Q1478K and in general of $\text{Na}_v1.1$ migraine mutations could be more pronounced in interneurons. Hyperexcitability of hippocampal interneurons can induce accumulation of extracellular K^+ and development of spreading depression episodes (Avoli et al., 1996). Interestingly, GABA can have excitatory actions in several adult brain areas (Marty and Llano, 2005). Thus, a self-limited hyperexcitability of interneurons may lead to a transient network hyperexcitability and to the development of CSD. Moreover, accumulation of extracellular GABA may potentiate CSD, depolarizing neurons in conditions of depletion of the Cl^- gradient.

However, the effect of the mutation may be more widespread. In particular, a moderate increase in I_{NaP} induced by a modulation can have large effects on neuronal firing (Mantegazza et al., 1998), and this could influence the excitability also of pyramidal neurons even if $\text{Na}_v1.1$ is expressed at low levels in these neurons.

In conclusion, we have shed some light, at the level of channel properties and single-cell excitability, on the difference in the functional effects of epileptogenic and FHM mutations of $\text{Na}_v1.1$. The actual effect on the fine properties of the excitability of neuronal networks should be studied in brain circuits, and development of gene targeted animal models will provide experimental systems for this purpose.

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