The genetics of spinocerebellar ataxia and dystonia

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A missense mutation in CACNA1H causes autosomal dominant Writer's Cramp

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Abstract

Objectives To identify the genetic background of autosomal dominant inherited writer's cramp.

Methods Exome sequencing was used to identify the genetic cause followed by molecular genetic analysis and electrophysiological experiments to validate pathogenicity.

Results The family presented with dystonic posturing limited to writing. Through exome sequencing we identified a p.R481C mutation in *CACNA1H*. Whole cell patching showed that Cav3.2-R481C channels exhibited a significant negative shift in half-activation potential and a distinct increase in whole-cell current density compared to wild type Cav3.2 channels, that was not caused by altered Cav3.2-R481C protein plasma membrane levels.

Conclusions This study identified the first causal gene for writer's cramp. The p.R481C mutation in *CACNA1H* leads to a gain of channel function, fitting with enhanced muscle activation and a hyperkinetic movement disorder seen in writer's cramp. Channelopathies may be considered in dystonia that may co-exist with ataxia and epilepsy.

Keywords

Writer's cramp; dystonia; Exome sequencing; calcium channel; channelopathy

Introduction

Focal dystonia's are a group of neurological disorders that affects a muscle or group of muscles in a specific body part causing involuntary muscle contractions and abnormal postures. In some cases, the focal dystonia only occurs while performing a specific task, whereas other tasks are normal.[1]

Writer's cramp (WC) is a task-specific focal dystonia that occurs selectively in the hand and arm during considerable writing. The origin of the task-specificity is not yet determined but clearly is an interesting nature of the disorder. This group of focal dystonias are thought to have a multifactorial etiology given its increased familial occurrence. The low penetrance complicates gene hunt, although some genes have been identified associating with focal dystonia, including *GNAL, ANO3* and *COL6A3*.[2–4] However, to date, no gene has yet been identified for writer's cramp.

Here we report that autosomal dominant writer's cramp is caused by a c.1441C>T; p.R481C mutation in *CACNA1H,* which encodes the Cav3.2 voltage-gated calcium channel α1 subunit.

Methods

Patients

The patients were seen at the movement disorders clinic in the Academic Medical Center in Amsterdam (AMC), The Netherlands. They underwent neurological investigation.

Standard protocol approvals, registration and patient consents

The study was approved by the Medical Ethical Committee of the AMC (METC protocol 05/030 #05.17.0239), and all participants gave written informed consent.

Genetic analysis

Two patients (II-1 and IV-7) were included in exome sequencing analysis. Exome capture was performed using SureSelect Human all Exon V4 Kit (Agilent technologies, USA) and 100bp paired-end reads were generated on a HiSeq2000 platform (Illumina Inc., USA). Sequences were aligned to hg19 and variants identified through our in-house bioinformatics pipeline and interpreted using NGS Bench Lab software (Cartagenia, Belgium). We selected predicted pathogenic, protein-changing variants present in both patients and absent in dbSNP, 1000 genomes and NHLBI ESP databases and with minor allele frequencies <0.0001 in ExAC browser for follow-up. Variants were validated by Sanger sequencing and co-segregation analysis in the available family members; II-1, II-11, III-3,

III-5, and IV-7. Primers for PCR amplification of the exons containing the mutations are available upon request.

Cell culture and transfection

The mutation was introduced in the cDNA of human *CACNA1H* in pcDNA3.1 using the QuikChange site-directed mutagenesis kit (Agilent Technologies) as per manufacturer's instructions. Following mutagenesis, the clone was sequenced to verify the mutation and sequence fidelity. Human embryonic kidney tsA-201 cells were cultured and transfected as previously described.[5] For biotinylation experiments, cells were grown at 37°C for 72 hours. For electrophysiology experiments, cells were plated on glass coverslips and grown at 28°C for 72 hours.

Cell surface biotinylation

Transfected cells were washed with ice-cold HBSS (Hepes-based saline solution) and incubated on ice for 15 min to stop trafficking of proteins. Surface proteins were biotinylated for 1 hour on ice with 1 mg/ml of EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, USA) and the reaction was quenched with 100mM glycine for 15 min. Cells were lysed in modified RIPA buffer for 45 min and protein quantification was performed using the Bio-Rad protein assay dye. Two milligrams of each sample lysate were incubated with 100µl of neutravidin beads (Thermo Scientific) for 1.5 hours at 4°C, followed by washing with lysis buffer and elution with 2x Laemmli sample buffer. Biotinylated proteins and lysates were analyzed by Western blot using the mouse antibody α-Cav3.2 (1:500, Novus Biologicals, USA).

Voltage clamp recordings

Whole cell patch clamping recordings were performed 72 hours after transfection using an Axopatch 200B amplifier linked to a computer with pCLAMP 9.2 software. Series resistance was compensated by 85% in all experiments and data were filtered at 5kHz. Cells were held at -100 mV and pulsed in 10 mV increments from -90 mV to 60mV for a period of 1s to determine the current/voltage (I/V) relationship. The external recording solution consisted of [in mM]: 20 $BaCl_2$, 114 CsCl, 1 $MgCl_2$, 10 HEPES, and 10 Glucose, pH adjusted to 7.4 with CsOH. The internal patch pipette solution contained [in mM]: 108 $CsMeSO_4$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, and pH adjusted to pH 7.3 with CsOH. GTP (0.6 mM) and ATP (2 mM) were added to the internal solution before use.

Results

Clinical features

The index patient (III-3) was born in a family of Dutch origin (Figure 1). He developed writer's cramp in his early twenties, which gradually worsened over months, after which it stabilized. Dystonic posturing was limited to writing. His medical history has been unremarkable, otherwise. At age 50, he showed severe mobile flexion dystonia in the thumb of the right hand combined with extension in the wrist during writing, requiring assistance of the left index finger to press the pen on the paper. His Arm Dystonia Disability Scale (ADDS) score was 3.[6] No sensory tricks were detected and mirror dystonic movements occurred in the right hand when he was writing with the left. Additional, laboratory investigations showed normal thyroid, ceruloplasmin, and copper levels. Notably, trials of botulinum toxin injections did not lead to functional improvement.

His mother (II-1) noticed difficulties with writing from the age of 54. Previous medical history includes a fracture of the right lower arm at age 61. At examination at age 88, she showed a mobile (predominant flexion) dystonia with tremor of the right hand (ADDS 3) during writing. She had no sensory tricks; while writing with her left hand, mirror movements were present with flexion and trembling of the right thumb. The sister of the index patient (III-5) exhibited right-sided writer's cramp characterized by a tremulous writing pattern (ADDS 2) from the age of 36. Additionally, her medical history is unremarkable except for a bilateral carpal tunnel syndrome, requiring neurolysis at age 42. Her son (IV-7) suffered from writer's cramp from the age of 18. He showed dystonic posturing of the right thumb during writing. Mirror dystonia, with flexion of the

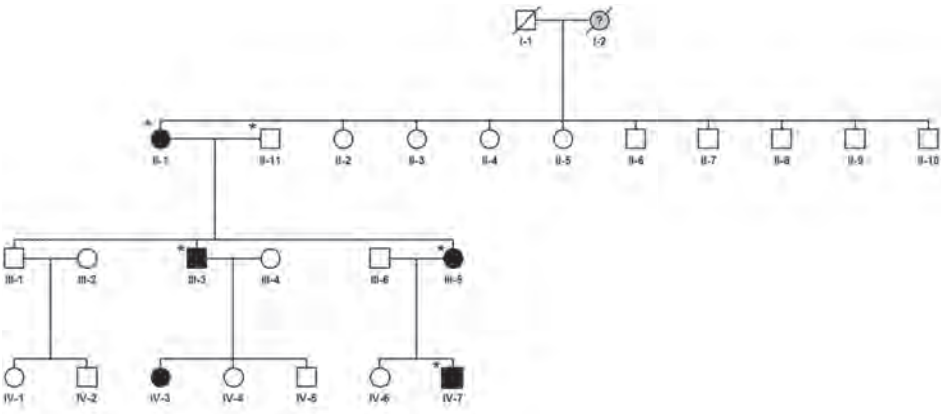

Figure 1. Pedigree of the Dutch family with the *CACNA1H* c.1441C>T; p.R481C mutation
Open symbols indicate unaffected family members, and solid black symbols indicate affected members. Individuals marked with an asterisk were clinically examined. The index patient is marked by double asterisks.

right thumb was present when writing with the left hand. The daughter of patient III-3 is also reported to have difficulties with writing, however she has not been examined or included in the genetic analysis. A summary of the clinical findings is presented in Table 1.

Table 1. Summary of clinical details of affected family members

Patient, sex	Age at examination	Age at onset	Clinical description	site**	mirror movements	sensory tricks	ADDS
III-3, M*	55	21	mobile dystonia	wrist, dig 1	Yes	No	3
II-1, F	88	54	mobile dystonia, tremor	wrist; dig 2, 3	Yes	No	3
III-5, F	53	36	mobile dystonia, tremor	wrist, dig 1, 2	No	No	2
IV-7, M	23	18	mobile dystonia	dig 1	Yes	No	2

* index patient; ** punctum maximum of dystonic movement; M=male, F=female, ADDS=Arm Dystonia Disability Scale

Genetic investigation

Exome sequencing identified 34 heterozygous variants that were shared between both patients (II-1 and IV-7) that were absent in dbSNP and 1000 genomes, and NHLBI ESP databases. Of these 34 variants 18 were predicted to be damaging by either SIFT or PolyPhen 2.0. Fifteen variants fulfilled our follow-up criteria, of which seven segregated with the disease phenotype in the other affected family members (Table 2). None of variants were present in unaffected family members.

Table 2. Variants in genes that co-segregated with the disease phenotype

Gene	HGNC	Transcript	cDNA change	Amino Acid change	Co-segregation with phenotype
CACNA1H	1395	NM_021098	c.1441C>T	p.R481C	Yes
TRNT1	17341	NM_182916	c.194C>T	p.A65V	Yes
LAMB4	6491	NM_007356	c.2353G>A	p.G785R	Yes
GPER1	4485	NM_001039966	c.505C>T	p.R169C	Yes
CLIP1	10461	NM_002956	c.11T>G	p.L4R	Yes
SPTBN5	15680	NM_016642	c.8572C>T	p.H2858Y	Yes
NUBP2	8042	NM_012225	c.296C>T	p.P99L	Yes

However, mutations in *TRNT1* were already reported to cause congenital sideroblastic anemia with immunodeficiency, fevers, and developmental delay (SIFD),[7] *CLIP1* mutations were recently linked to autosomal recessive intellectual disability,[8] and somatic

LAMB4 mutations were identified in cancerous tissues.[9] Because mutations in *CACNA1H* encoding the Cav3.2 voltage-gated calcium channel are known to cause childhood absence epilepsy (CAE) and idiopathic generalized epilepsy (IGE)[10,11] that overlaps with paroxisomal movement disorders such as focal dystonia, we further investigated the variant in *CACNA1H*. Moreover, T-type channel dysfunction is also linked to various other neurological disorders such as sleep disorders, neuropathic pain, Parkinson's disease-associated tremor, and neuropsychiatric disorders.[12]

The c.1441C>T; p.R481C variant occurred in exon 9 of *CACNA1H* and changed the highly conserved amino acid arginine 481 to cysteine on the intracellular loop connecting domains I and II. To elucidate the effect of p.R481C on Cav3.2 function, we performed electrophysiological analysis on the human Cav3.2 channel in HEK tsA-201 cells as reported before.[13] The Cav3.2-R481C channel was well transfected in HEK tsA-201 cells and showed increased whole-cell peak current densities compared with wild-type (WT) channels (R481C: 64.78 ± 6.53 pA/pF; WT: 43.90 ± 6.47 pA/pF; $p<0.05$, t-test) (Figure 2).

In addition, Cav3.2-R481C channels showed a significant hyperpolarizing shift in the half-activation potential by ~6mV (R481C: -40.09 ± 1.360; WT: -34.47 ± 0.8316; $p<0.01$), indicating that the p.R481C variant promotes channel opening in response to membrane depolarization (Figure 3A). As the half-inactivation voltage of Cav3.2-R481C channels was not changed (Figure 3B), the effect on the half activation potential leads to an overall increase in the size of the window current. Overall, these data suggest increased tonic activity of Cav3.2-R481C channels near typical neuronal resting membrane potentials.

To exclude the possibility that the increased whole-cell peak current densities were due to increased Cav3.2-R481C channel trafficking to the plasma membrane, we also performed a surface biotinylation assay (Figure S1). The p.R481C variant did not exhibit significant alterations in Cav3.2 cell surface expression compared to WT channels, suggesting that the observed increases in whole-cell current amplitudes are mediated by gains in channel function (such as increased open probability).

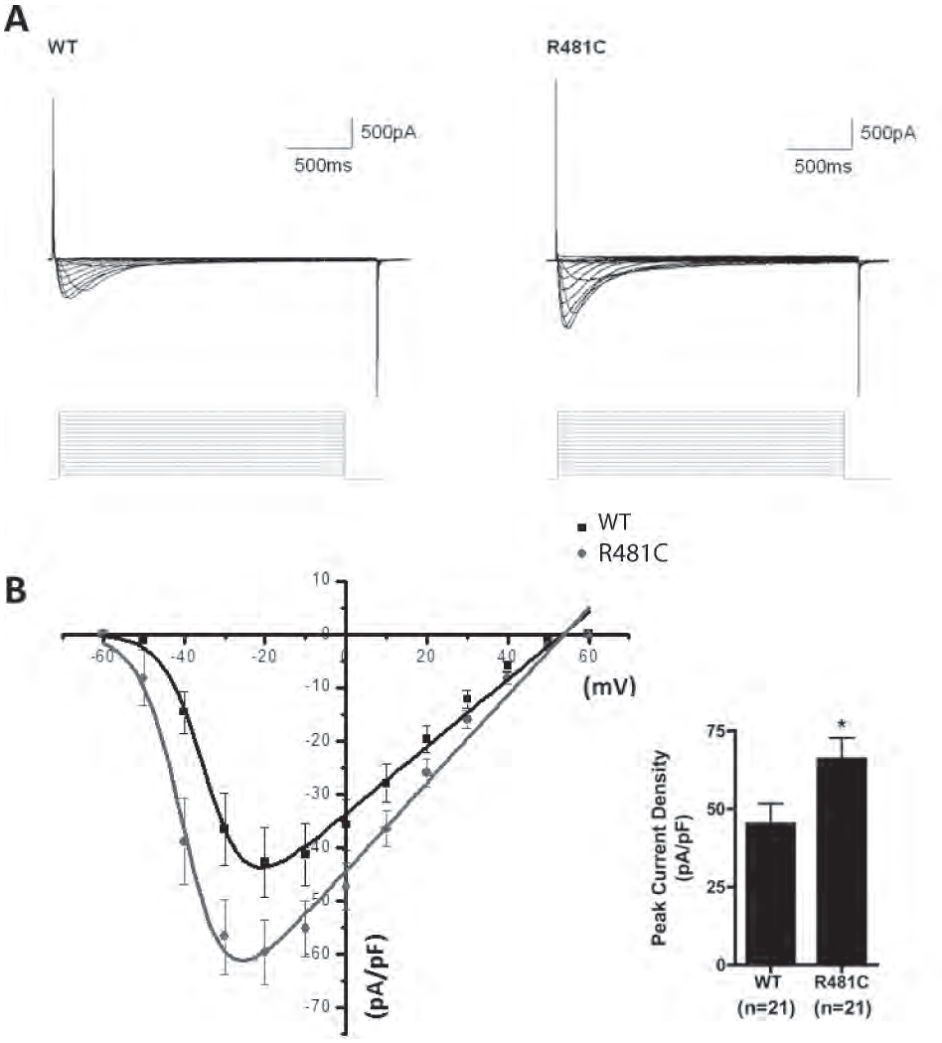

Figure 2. Patch-clamp studies show a gain of function effect of the mutation
(A) Raw current traces obtained with wild-type and Cav3.2-R481C channels. The currents were elicited by stepping from a holding potential of -100 mV to various test potentials as indicated by the pulse protocols below the current tracings. (B) Ensemble of whole-cell current-voltage relations obtained with WT and Cav3.2-R481C mutant channels. Values are represented as means +/- S.E. The solid lines are fits with the Boltzmann equation. Inset: Mean peak current density. Numbers of cells recorded are denoted in parentheses. Cells were recorded from at least 3 different transfections.

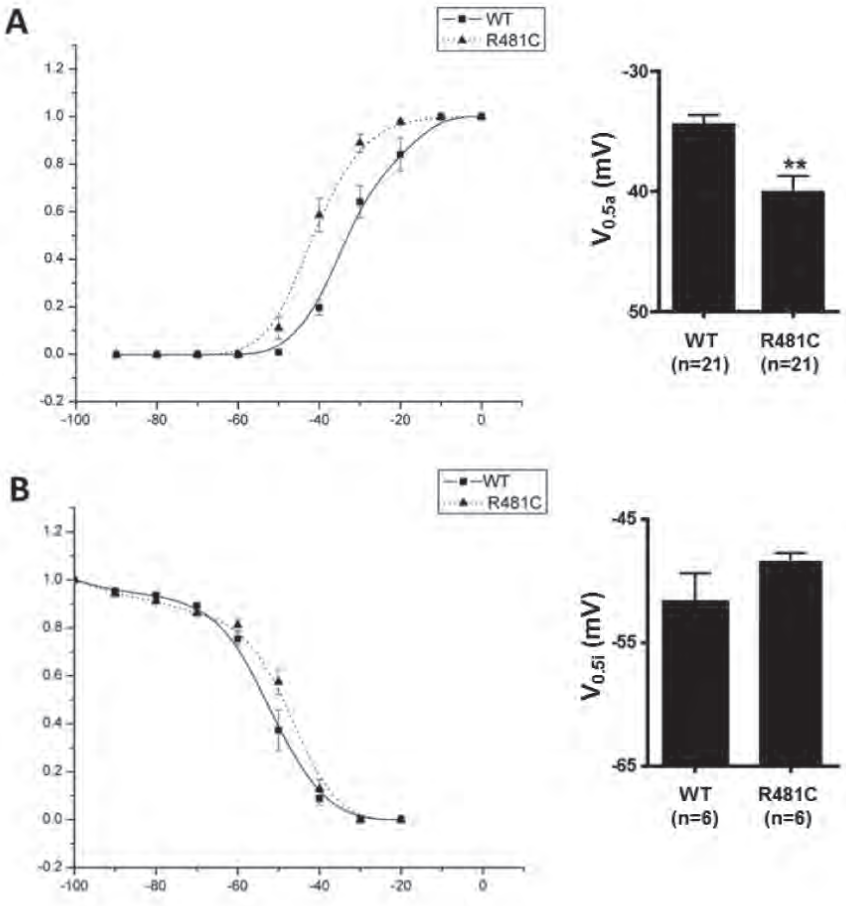

Figure 3. Patch-clamp studies show increased channel opening and no effect on closing
(A) Ensemble activation curves obtained with WT and Cav3.2-R481C channels extracted from the current voltage data shown in figure 2B. The lines are fits with the Boltzmann relation. Inset: Mean half-activation potentials. The half-activation potentials were determined via the Boltzmann equation fitting to individual whole-cell current-voltage relations. (B) Ensemble steady-state inactivation curves obtained with WT and Cav3.2-R481C channels. The lines are fits with the Boltzmann equation. Inset: Mean half-inactivation potentials determined via the Boltzmann equation from fits to individual state inactivation curves. Asterisks denote statistical significance relative to wild-type (*p<0.05, **p<0.01 , Student's t-test). Numbers of cells recorded are denoted in parentheses. Cells were recorded from at least 3 different transfections.

Discussion

The pathophysiology underlying task-specific hand dystonia, writer's cramp is yet to be determined. This is mainly the cause since the disease mostly occurs sporadically, exhibits low penetrance, or includes polygenetic abnormalities.[14] Due to the autosomal dominant inheritance pattern of WC in this family, we were able to unravel a piece of the genetic etiology, yielding new insights into the heritability and pathophysiology of WC.

This work revealed a pathogenic role of the *CACNA1H* p.R481C mutation causing WC including *in silico* approaches and assays to determine Cav3.2-R418C channel function. Recently, Mencacci et al. used weighted gene co-expression network analysis to strengthen their findings on *KCTD17* in the etiology of myoclonus dystonia.[15] This approach implies that genes that function in similar pathways are more likely to be co-expressed than genes operating in distinct pathways. Remarkably, *CACNA1H* was directly connected to *KCTD17* in this gene network and this further strengthens our finding that mutations in *CACNA1H* can cause dystonia.

Combined functional and structural imaging studies in patients with WC showed impairment of the anterior basal ganglia loops and structural abnormalities were observed in the cerebellum.[16,17] Additionally, abnormal associative learning in an eye blink classical conditioning model was observed in focal hand dystonia cases suggestive of a cerebellar motor learning deficit.[18] Cav3.2 is involved in pace-making and rebound burst activity, and widely expressed throughout the CNS including the basal ganglia circuitry and cerebellum.[19–21] Recently, T-type calcium channels including Cav3.2 were shown to contribute to rebound bursts in deep cerebellar nuclei (DCN) neurons determining the nature of plasticity at the Purkinje cell-DCN synapse.[19] In this context, the observed gain of function in the p.R481C variant is expected to lead to an increase in DCN neuron firing. Furthermore, maladaptive plasticity due to disruption of synaptic transmission is suggested to be an important factor in the pathogenesis of task specific focal dystonia.[22] Notably, work of others has strengthened the involvement of the cerebellum in the pathophysiology of various dystonia types.[23] Therefore, the identification of *CACNA1H* as WC gene might be another piece of evidence for the involvement of the cerebellum in the pathophysiology of dystonia.

The domain I-II linker region of the channel, where p.R481C is located, is apparently a hotspot for mutations causing absence seizures.[10,24] Our gain of function p.R481C mutation flanks the reported IGE missense mutation p.A480T that did not obviously alter channel function[13], whereas the majority of epilepsy mutations do lead to a gain of Cav3.2 channel function.[10] The domain I-II linker region is known as the "gating brake" since removal of the first part of this loop results in channel opening at more negative

voltages.[25] Similar to our findings, deletion of amino acids surrounding residue 481 led to negative shifts in half-activation potential and a marked increase in whole-cell current density.[26] Notably, the p.R481C mutant opened at more negative voltages coinciding with increased calcium currents, but that was not caused by increased cell surface expression of the mutant channels as has been shown for several other I-II linker domain mutations associated with childhood absence epilepsy.[26] Instead, it is likely that the mutation altered the maximum open probability of the channel, thus increasing the maximum slope conductance in the current voltage relation. It will be interesting to test specific Cav3.2 channel blockers, such as 5,6-EET, which has been reported to selectively inhibit Cav3.2,[27] as potential therapies for WC.

The role of voltage-gated calcium channels and other ion channels in the pathophysiology of dystonias was underscored by the recent identifications of a mutation in *CACNA1B* encoding Cav2.2 causing myoclonus dystonia and mutations in the calcium-gated chloride channel *ANO3* causing craniocervical dystonia.[3,28] As such, dystonia can be a manifestation of a channelopathy that may co-exist with epilepsy and ataxia, and we speculate that mutations in other ion channels may be involved in the etiology of different forms of dystonia.

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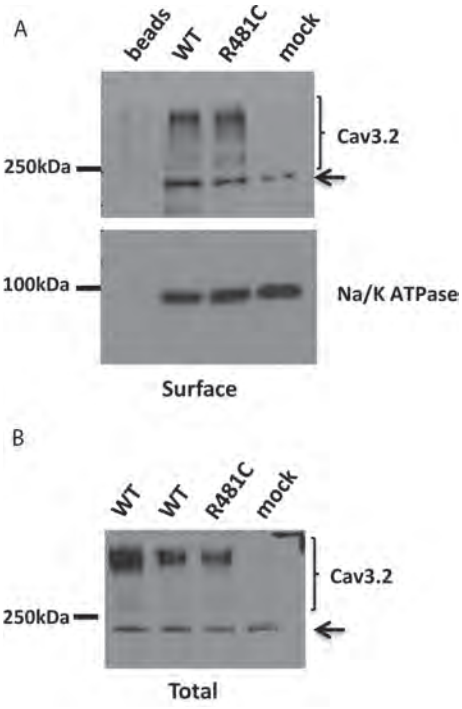

Figure S1. Expression and localization of the mutant channel is not affected

(A) Biotinylation experiment showing surface expression of WT and Cav3.2-R481C channels expressed in tsA201 cells. Image below is the same membrane stripped and reprobed for Na/K-ATPase as loading control. (B) Total expression of WT and R481C channels. Mock transfection was used as a negative control for the antibody. Arrows show a non-specific band that appears in mock transfected cells.