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## Mutations in the sodium channel gene *SCN2A* cause neonatal epilepsy with late-onset episodic ataxia

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5 **Abstract**  
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7 Mutations in *SCN2A* cause epilepsy syndromes of variable severity including neonatal-infantile seizures. In one case, we  
8 previously described additional childhood-onset episodic ataxia. Here, we corroborate and detail the latter phenotype in  
9 three further cases. We describe the clinical characteristics, identify the causative *SCN2A* mutations and determine their  
10 functional consequences using whole-cell patch-clamping in mammalian cells. In total, four probands presented with  
11 neonatal-onset seizures remitting after five to 13 months. In early childhood, they started to experience repeated episodes  
12 of ataxia, accompanied in part by headache or backpain lasting minutes to several hours. In two of the new cases, we  
13 detected the novel mutation p.Arg1882Gly. While this mutation occurred *de novo* in both patients, one of them carries an  
14 additional known variant on the same *SCN2A* allele, inherited from the unaffected father (p.Gly1522Ala). Whereas  
15 p.Arg1882Gly alone shifted the activation curve by -4 mV, the combination of both variants did not affect activation, but  
16 caused a depolarizing shift of voltage-dependent inactivation, and a significant increase in Na<sup>+</sup> current density and  
17 protein production. p.Gly1522Ala alone did not change channel gating. The third new proband carries the same *de novo*  
18 *SCN2A* gain-of-function mutation as our first published case (p.Ala263Val). Our findings broaden the clinical spectrum  
19 observed with *SCN2A* gain-of-function mutations, showing that fairly different biophysical mechanisms can cause a  
20 convergent clinical phenotype of neonatal seizures and later onset episodic ataxia.  
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34 **Key words:** Epilepsy, genetics, ataxia, channelopathy, sodium channel  
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5 **Introduction**  
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8 Gene discovery in epilepsy and related neurological syndromes, such as migraine, paroxysmal dyskinesia and episodic  
9 ataxia, has revealed that mutations in ion channels, transporters, or synaptic proteins play a crucial role in their  
10 pathophysiology [1-3]. These mutations affect the excitability of different neuronal subtypes and compartments. The  
11 altered excitability, sometimes provoked by typical decompensating triggers, episodically induces a dysfunction of  
12 neuronal networks in distinct brain regions thereby causing well-defined paroxysmal clinical symptoms. Beside ‘pure’  
13 syndromes with only one characteristic clinical feature, several overlap syndromes with epilepsy as a core phenotype  
14 have been described [4,5].  
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17 The neuronal voltage-gated Na<sup>+</sup> channel Nav1.2, encoded by the *SCN2A* gene, is mutated in benign familial neonatal-  
18 infantile seizures (BFNIS), an autosomal dominant epilepsy syndrome characterized by transient seizures in the first  
19 weeks or months of life [6,7]. These mutations mainly lead to gain-of-function defects causing neuronal  
20 hyperexcitability [8,7]. More severe, non-familial phenotypes with neonatal onset seizures caused by *de novo* *SCN2A*  
21 mutations are increasingly described [9-14]. One loss-of-function mutation causes epileptic encephalopathy with later  
22 onset [15].  
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25 We previously described a single patient with neonatal-onset, relatively severe epilepsy resolving at 13 months of age,  
26 with episodes of ataxia, myoclonus and pain starting at 18 months of age. We identified a *de novo* missense mutation in  
27 *SCN2A* showing a gain-of-function defect [3]. Here, we describe three further independent cases with neonatal-onset  
28 seizures and episodic ataxia starting in early childhood. We reveal the common clinical features, the underlying *SCN2A*  
29 mutations and their pathophysiological mechanisms.  
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5 **Materials and methods**  
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7 **Patients**  
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9 Clinical evaluation of the first new case (patient #1) and follow-up of the previously described one (#4) were performed  
10 in Helsinki. The other two new patients were examined at the University Clinic Giessen (patient #2) or at the Epilepsy  
11 Center Kork (patient #3). All patients were selected from routine clinical presentations in the respective centers and  
12 underwent subsequent genetic testing due to clinical suspicion of an *SCN2A* defect, since they presented highly similarly  
13 as case #4.  
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18 **Molecular Genetics**  
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20 Patient #1 underwent direct (Sanger) sequencing of *SCN2A*. For patients #2 and #3 an epilepsy gene panel screening was  
21 performed as reported previously [16]. Confirmation of the mutations and segregation was performed by Sanger  
22 sequencing. Long range PCR was used to determine if the two variants detected in patient #2 were on the same allele.  
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24 For *SCN2A* sequencing genomic DNA was isolated from EDTA-blood. The coding exons and exon-intron boundaries of  
25 *SCN2A* in patients #2 and #3, and mutation-carrying exons in their parents, were amplified (primers available upon  
26 request). *SCN2A* in patient #4 and her parents was amplified (primers available upon request) by the Qiagen Multiplex  
27 PCR kit, and by the Expand Long Range PCR kit (Cat. No. 04829034001 Roche, Mannheim, Germany) on a Biometra  
28 T3 thermocycler (Biometra, Göttingen, Germany). Long range PCR products were cloned (TOPO<sup>®</sup> XL PCR Cloning  
29 Kit, Cat. No. K4700-10) and finally sequenced. For long range PCR, covering both detected variants chromosomal  
30 position 166242926-66246608 (forward: ATCGTGCCACTGCACTCCAACC, reverse:  
31 CTATCGTCTGAGTAGCCATTACGCC) was amplified resulting in a 3682 bp fragment. Sequencing reactions were  
32 performed using ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Weiterstadt,  
33 Germany). Capillary electrophoresis was conducted on an ABI PRISM<sup>®</sup> 3100 Genetic Analyzer (Applied Biosystems,  
34 Weiterstadt, Germany) or an ABI 3730 DNA Analyzer (Perkin Elmer, Foster City, CA, U.S.A.).  
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45 **Mutagenesis**

46 To engineer the mutations into the adult splice variant of the human Nav<sub>v</sub>1.2 channel, site-directed mutagenesis was  
47 performed using Quickchange<sup>®</sup> II XL (Agilent Technologies, Santa Clara, CA, USA; primers are available upon  
48 request) for the R1882G mutation and GoTaq<sup>®</sup> Long PCR Master Mix (Promega Corporation, Madison, WI, USA;  
49 primers are available upon request) for the G1522A and G1522A + R1882G mutations. Before used in experiments, the  
50 mutant cDNA was fully resequenced to verify the introduced mutations and exclude any additional sequence alterations.  
51 GlaxoSmithKline (Brentford, UK) kindly provided the human Na<sup>+</sup> channel subunits hβ<sub>1</sub> and hβ<sub>2</sub> in the pCLH vector. The  
52 hygromycin coding region in the vector was exchanged with the sequence coding for either enhanced green fluorescent  
53 protein (EGFP) or CD8 marker genes to obtain pCLH-hβ<sub>1</sub>-EGFP and pCLH-hβ<sub>2</sub>-CD8 [3,17].  
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### Transfection and expression in tsA201 cells

Human tsA201 cells were cultured at 37°C, with 5% CO<sub>2</sub> humidified atmosphere and grown in 89% Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) + 10% (v/v) foetal bovine serum (PAN-Biotech GmbH, Aidenbach, Germany) + 1% L-Glutamin 200 mM (Biochrom GmbH, Berlin, Deutschland). Transfections using Mirus TransIT®-LT1 reagent (Madison, WI 53711 USA) were performed for transient expression of wild-type or mutant Na<sup>+</sup> channel  $\alpha$ -subunits together with  $\beta_1$ - and  $\beta_2$ -subunits in tsA-201 cells. For co-expression of  $\alpha$ - and both  $\beta$ -subunits 2.4  $\mu$ g of total DNA (2.0  $\mu$ g  $\alpha$ -subunit, 0.2  $\mu$ g  $\beta_1$ -subunit and 0.2  $\mu$ g  $\beta_2$ -subunit) was transfected in a molar ratio of 1:1:1. Anti-CD8 antibody-coated microbeads (Dynabeads M450, Dynal, Norway) were suspended in phosphate buffered saline and added to the cells. Cells positive for both CD8 antigen and EGFP fluorescence were used for electrophysiological recordings.

### Western Blot

For protein identification we applied 35  $\mu$ g per line of total cell lysate on a 6% SDS gel. After separation and blotting, the Protran® Nitrocellulose Membranes was blocked with 5% skim milk powder/PBS/0,1% Tween 20 and incubated with mouse monoclonal anti-SCN2A antibody 1:500 (overnight, 4 °C) in 1% skim milk powder/PBS/0,1% Tween 20. As secondary antibody HRP conjugated goat anti-mouse serum 1:10.000 (1 h, RT) was used. Western Blots were developed using the Mini-PROTEAN® Tetra Cell according to the manufactures procedures. **Quantification of signals was performed using ImageJ software (NIH, Bethesda, MD). Expression of Nav1.2 proteins were normalized to corresponding Vinculin signals in total lysates and pooled from different experiments.**

### Electrophysiology

Standard whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier, a Digidata 1320A digitizer and pCLAMP 8 data acquisition software (Axon Instruments, Union City, CA, USA), as has been described before [7]. Leakage and capacitive currents were automatically subtracted using a pre-pulse protocol (-P/4). Currents were filtered at 5 kHz and digitized at 20 kHz. Cells were visualized using an inverted microscope (Axio-Vert.A1; Zeiss). All recordings were performed at room temperature of 21–23°C. Na<sup>+</sup> currents of 1-12 nA were recorded from transfected tsA201 cells 10 min after establishing the whole cell configuration. Borosilicate glass pipettes had a final tip resistance of 1-2 M $\Omega$  when filled with internal recording solution (see below). We carefully checked that the maximal voltage error due to residual series resistance after up to 95% compensation was always <5 mV. The pipette solution contained (in mM): 5 NaCl, 2 MgCl<sub>2</sub>, 5 EGTA, 10 (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 130 CsF (pH 7.4, 290 mOsm). The bath solution contained (in mM) 140 NaCl, 4 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 5 HEPES, 4 Dextrose (pH 7.4, 300 mOsm).

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5 *Voltage clamp protocols and data analysis:* The activation curve (conductance–voltage relationship) was derived from  
6 the current–voltage relationship obtained by plotting the peak current over various step depolarizations (7.5 mV steps  
7 from a holding potential of -140 mV) according to  
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$$10 \quad g(V) = \frac{I}{(V - V_{rev})}$$

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16 with  $g$  being the conductance,  $I$  the recorded peak current at test potential  $V$ , and  $V_{rev}$  the apparent observed  $\text{Na}^+$  reversal  
17 potential.  
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20 The voltage-dependence of activation was fit with the following Boltzmann function:  
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$$22 \quad g(V) = \frac{g_{max}}{\{1 + \exp[(V - V_{1/2})/k_V]\}}$$

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27 with  $g$  being the conductance,  $I$  the recorded current amplitude at test potential  $V$ ,  $V_{rev}$  the  $\text{Na}^+$  reversal potential,  $g_{max}$  the  
28 maximal conductance,  $V_{1/2}$  the voltage of half-maximal activation and  $k_V$  a slope factor. Steady-state inactivation was  
29 determined using 300 ms conditioning pulses to various potentials followed by the test pulse to -20 mV at which the  
30 peak current reflected the percentage of non-inactivated channels. A standard Boltzmann function was fit to the  
31 inactivation curves:  
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$$34 \quad I(V) = \frac{I_{max}}{\{1 + \exp[(V - V_{1/2})/k_V]\}}$$

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40 with  $I$  being the recorded current amplitude at the conditioning potential  $V$ ,  $I_{max}$  being the maximal current amplitude,  
41  $V_{1/2}$  the voltage of half-maximal inactivation and  $k_V$  a slope factor. For analysis of the time constants of fast inactivation,  
42 the cell membrane was depolarized to various test potentials from a holding potential of -140 mV to record  $\text{Na}^+$  currents.  
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44 A second-order exponential function was best fit to the time course of fast inactivation during the first 70 ms after onset  
45 of the depolarization, yielding two time constants. The weight of the second slower time constant was relatively small.  
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47 Only the fast time constant, named  $\tau_h$ , was therefore used for data presentation in the ‘Results’ section. Persistent  $\text{Na}^+$   
48 currents ( $I_{ss}$ , for the ‘steady-state’ current) were determined at the end of depolarizing pulses, lasting 95 ms, to different  
49 test potentials and are given relative to the initial peak current ( $I_{PEAK}$ ). Recovery from fast inactivation was recorded from  
50 holding potentials of -140 mV. Cells were depolarized to -20 mV for 100 ms to inactivate all  $\text{Na}^+$  channels and then  
51 repolarized to various recovery potentials (-80, -100 or -120 mV) for increasing duration. A second-order exponential  
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5 function with an initial delay was best fit to the time course of recovery from inactivation. The faster time constant with  
6 the much larger relative amplitude,  $\tau_{rec}$ , is shown for data evaluation.  
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### 9 **Data and statistical analysis**

10 Traces were displayed off-line with Clampfit software of pClamp 10.0 (Axon Instruments). Graphics were generated  
11 using a combination of Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), and Origin (version 6.1;  
12 OriginLab Inc., Northampton, MA, USA) software, statistics were performed using SigmaStat 3.1 (Statcon). All data  
13 were tested for normal distribution. For statistical evaluation t-test was used for comparing two groups. For comparing  
14 more than two groups, ANOVA on ranks (Kruskal-Wallis-Test) with Dunn's posthoc test for not normally distributed  
15 data or one-way ANOVA (Bonferroni posthoc test) was used when data sets were normally distributed. All data are  
16 shown as means  $\pm$  SEM, "n" gives the number of cells. For all statistical tests, significance with respect to control is  
17 indicated on the figures using the following symbols: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  
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5 **Results**  
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7 **Clinical characterization.**  
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10 All four identified patients showed very similar phenotypes with neonatal-onset focal or generalized seizures remitting  
11 almost completely at 5-13 months old, followed by episodes of ataxia **at least once a month** starting in early childhood.

12 All clinical data are summarized in Table 1 and more detailed case descriptions are given in Online Resource 1.  
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16 Epileptic seizures in patient #1 (Fig. 1a), carrying only the novel de novo missense mutation p.Arg1882Gly (R1882G)  
17 started at two days old with bilateral tonic-clonic seizures with reduced oxygen saturation and unresponsiveness. The  
18 seizures were initially pharmacoresistant to phenobarbitone (PB) and valproate (VPA) but stopped by five months of age  
19 with no recurrences. Since the age of 3.7 years, episodes with slurred speech, ataxia, nausea and headache occur 1-2  
20 times per month lasting minutes to hours. In patient #2, who carries both R1882G and also the inherited variant  
21 p.Gly1522Ala (G1522A), multifocal clonic and tonic-clonic seizures lasting 10-120 seconds occurred in the fourth week  
22 of life, and were well controlled by PB. The girl's further course was uncomplicated until a series of generalized tonic-  
23 clonic seizures during a febrile infection at five months old. PB was changed to VPA and no further seizures occurred  
24 after the age of 5 months, also after VPA was finally discontinued at 7 years old. From 20 months of age on, episodes  
25 characterized by headache, slurred speech, impaired balance and ataxic gait occurred 1-10 times per week lasting 1-5  
26 minutes.  
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36 Patient #3 (Fig. 1c), carrying the same de novo missense *SCN2A* mutation as the previously described patient #4 [3]  
37 (p.Ala263Val, A263V), presented immediately after birth with a marked muscular hypotonia and sleepiness. His  
38 development was slightly retarded. A first cluster of bilateral tonic seizures occurred at 7 days of life which was resistant  
39 to different medications (see Table 1). Oxcarbazepine (OXC) and levetiracetam (LEV) were started at 4 months of age,  
40 and the patient remained seizure-free so far from 7 months on. At 22 months, the parents reported an episode of ataxia  
41 with mild symptoms starting in the morning and inability to walk or stand in the afternoon. Further similar attacks with  
42 ataxic gait occurred since then every 10 to 14 days, lasting hours to one day. Formerly described patient #4 presented  
43 with neonatal-onset seizures with hypomotor semiology followed by tonic-clonic seizures as described previously [3].  
44 During treatment with phenytoin (PHT), seizures became much less frequent at the age of 13 months. Since then, he has  
45 only suffered from three isolated generalized tonic-clonic seizures at the age of 3.5, 6.5 and 14.5 years. The habitual  
46 headache-ataxia episodes started at 18 months old and were pharmacoresistant [3] until now.  
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57 Episodic ataxia in all four patients responded poorly to any of the medications tried so far (see Table 1, Online Resource  
58 1 and discussion).  
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5 **Genetics.** In both patients #1 and #2 we identified the novel missense mutation c.5644C>G, p.Arg1882Gly (R1882G;  
6 GeneBank NM\_021007.2, NC\_000002.11). Sequencing of the parents revealed that the mutation occurred *de novo* in  
7 both patients. In addition, the missense variant c.4565G>C, p.Gly1522Ala (G1522A; rs147522594) was identified in  
8 patient #2. Rs147522594 was also detected in the healthy father (Fig. 1c) and occurs with an allele frequency of  
9 0.00073% (allele count: 89/121922) within the Exome Aggregation Consortium (ExAC). Both G1522 and R1882 are  
10 completely conserved in all other 28 studied vertebrates ([www.1000genomes.org](http://www.1000genomes.org)). R1882G is not known to be present in  
11 unaffected individuals ([www.1000genomes.org/](http://www.1000genomes.org/), <http://exac.broadinstitute.org/>, <http://www.ncbi.nlm.nih.gov/SNP/>).  
12 DNA Sequencing of a cloned long range PCR fragment revealed that both variants are present on the identical allele in  
13 patient #2.  
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17 Diagnostic gene panel sequencing was performed in patient #3 [16]. The same mutation (c.788C>T, p.Ala263Val) as  
18 previously described in patient #4 was detected in *SCN2A* [3]. Sequencing of both parents revealed that the mutation  
19 occurred *de novo*  
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22 **Functional studies.** Electrophysiological analysis was first performed for the wild type (WT) channel in comparison  
23 with the *de novo* R1882G mutant, which was detected as the only *SCN2A* mutation in patient #1 (Fig 2). This analysis  
24 revealed a significant hyperpolarizing shift of the activation curve for mutant channels (Fig. 2d). We did not find  
25 significant differences for any other recorded gating parameter including current density (Fig. 2, Table 2, Online  
26 Resource 1 Fig. S1).  
27

28 Since patient #2 carries both the *de novo* R1882G mutation and the inherited variant G1522A on the same allele, we  
29 additionally analyzed mutant channels carrying (i) G1522A alone, and (ii) both G1522A and R1882G (Fig. 3). Mutant  
30 G1522A alone did not reveal any significant changes compared to WT channels. Interestingly, the double mutant  
31 channel did not show changes of the activation curve (Fig. 3e), as described above for R1882G alone. In contrast, the  
32 most prominent effect for the double mutation was a 1.7-fold increase in current density compared to the other three  
33 clones, i.e. WT, G1522A and R1882G alone (Fig. 3d, Table 2). In addition, we found a significant shift of the steady-  
34 state fast inactivation curve towards more depolarized potentials only for channels carrying both variants (Fig. 3, Table  
35 2). Consistent with the increase in current density (as a measure for the number of functional channels in the membrane),  
36 **Western blot analysis of transfected tsA201 cells -to study the amount of protein expression- revealed that the total**  
37 **Nav1.2 protein amount was significantly increased only in channels carrying both variants in comparison to the WT** (Fig.  
38 4).  
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41 We thus found differential gain-of-function effects for Nav1.2 channels carrying either the R1882G mutation alone or  
42 both variants, R1882G and G1522A. In both cases (R1882G alone or in combination with G1522A), the changes predict  
43 an increase of membrane excitability in neurons expressing mutant Nav1.2 channels.  
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## Discussion

We here describe patients carrying two different *SCN2A* mutations and comprising neonatal -onset seizures and childhood-onset episodic ataxia as the two main features. When we published the first case in 2010, it was not clear if the *de novo* A263V mutation in *SCN2A* was responsible alone for the main clinical symptoms, as migraine was a common feature in both branches of the family which could have influenced the phenotype of patient #4 [3]. Detection of a second patient (#3), with the same clinical key symptoms carrying the same mutation occurring independently *de novo*, now strongly suggests that this mutation is causative for both the seizures and episodic ataxia. Patients #1 and #2, carrying another *de novo* mutation in the same gene (in patient #2 occurring in combination with an inherited known rare polymorphism), corroborate these findings that the phenotype is caused by *SCN2A* gain-of-function mutations. This widens the spectrum of disorders combining epilepsy with other paroxysmal neurological symptoms such as dyskinesia or ataxia, which could be attributed to the expression of respective genes in different brain areas inducing an episodic dysfunction of neuronal networks [18-23].

The clinical characteristics of these patients in comparison to other *SCN2A*-associated syndromes are that seizures recurred more frequently and persisted longer than in typical BFNIS and that those were more difficult to treat [24]. In contrast to more severe epileptic encephalopathies [9-15], the seizures still remitted after 5-13 months (except for few occasional later seizures in patient #4). Since drugs have been discontinued in patient #2, seizure freedom can be attributed to spontaneous remission, whereas this remains unclear in the other cases, as treatment has been continued (patients #1 and #3) or restarted after seizure relapse during a drug-free period (patient #4). Notably, seizures in patient #3 responded well to the Na<sup>+</sup> channel blocker oxcarbazepine. Attacks of episodic ataxia with onset ranging 15 months to 3.7 years of age were the most important symptom beside the seizures. Episodic headache was an additional feature occurring in all patients, although rare and uncertain in one, and could be accompanied by back pain, discomfort and vomiting. Treatment of episodic ataxia is more difficult. Patients #1 and #4 did not respond to acetazolamide. In addition, 4-aminopyridine, which works well in the clinically rather similar episodic ataxia type 2 [25,26], did not help patient #4. The effect of Na<sup>+</sup> channel blockers, which work very well in the clinically distinct episodic ataxia type 1 with much shorter attacks triggered by sudden movements after rest [27-29], is not yet entirely clear in our patients. Theoretically, this group of antiepileptic drugs should counteract a gain-of-function of *SCN2A* mutations. There is increasing evidence that patients with severe early-onset epilepsies due to *SCN2A* mutations do respond to Na<sup>+</sup> channel blockers [30]. However, network effects are difficult to predict and could influence the response to such drugs – also in a distinct way in different brain regions – as shown recently for the hippocampus [31].

Our previous and the here presented electrophysiological data clearly indicate that different gain-of-function effects of the detected mutations in *SCN2A* cause the clinical phenotype. Whereas the A263V mutation causes mainly an increased

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5 persistent Na<sup>+</sup> current [3], the *de novo* mutation R1882G found in patient #1 showed a hyperpolarizing shift of the  
6 activation curve. In contrast, the combination of both variants in the same channel subunit, as detected in patient #2,  
7 leads to a prominent increase in current density, confirmed by a larger amount of produced protein, and a depolarizing  
8 shift of the steady-state inactivation curve (but no shift of the activation curve). Until now, many BFNIS-associated  
9 *SCN2A* mutations – partly showing other gain-of-function mechanisms – rather exhibited a decrease in current density or  
10 cell surface expression, which however rarely reached statistical significance [3,7,17,32]. This confirms the peculiar  
11 importance of the increased current density of the combined variants found in patient #2. All observed effects predict an  
12 increased Na<sup>+</sup> inflow and a neuronal hyperexcitability, which can explain the dysfunction of cortical or cerebellar  
13 networks causing the clinical symptoms of our patients.  
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16 We have previously suggested that the age-dependent occurrence of seizures and remission in BFNIS could be due to the  
17 high Nav1.2 channel expression in axon initial segments of hippocampal and cortical pyramidal neurons early in  
18 development and their partial replacement by Nav1.6 channels with increasing maturation [7]. This mechanism could  
19 also contribute to seizure remission in the additional three cases. Although the episodic ataxia found in the studied  
20 patients might have many different causes, one hypothesis for later onset ataxia could be due to a delayed upregulation of  
21 Nav1.2 in cerebellar granule cells [7,33].  
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24 Interestingly, different substitutions of the arginine at position 1882 (R1882Q and R1882L) have been identified in two  
25 *de novo* cases exhibiting epileptic encephalopathy [11] and intractable seizures, optic atrophy, severe intellectual  
26 disability, brain abnormalities and muscular hypotonia [13]. Furthermore, the A263V mutation has also been described  
27 as occurring *de novo* in monozygotic twins with Ohtahara syndrome and other unique neuropathologic abnormalities  
28 [34], and a A263T mutation has been identified in a patient with a different type of early-onset epileptic encephalopathy  
29 [12]. These observations indicate that not only the mutation determines the clinical phenotype, but that other factors such  
30 as the genetic background have to play a role in genotype-phenotype relationships.  
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32  
33 In summary, we here identified four patients with a common clinical phenotype (neonatal seizures and later onset ataxia)  
34 caused by *SCN2A* mutations, which enlarges the spectrum of neurological *SCN2A*-related phenotypes.  
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**Conflicts of interest**

The authors declare that they have no conflict of interest.

**Ethical standards**

Informed consent was obtained from the parents of all four patients. All procedures were in accordance with the Declaration of Helsinki and were approved by the local ethical review boards.

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5 **Tables**  
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8 **Table 1** Main phenotypic characteristics of all four patients

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	<b>Patient #1 (*2004)</b>	<b>Patient #2 (*2006)</b>	<b>Patient #3 (*2011)</b>	<b>Patient #4 (*1999)</b>
<b>Mutation</b>	c.5644C>G/p.R1882G	c.4565G>C/p.G1522A c.5644C>G/p.R1882G	c.788C>T/p.A263V	c.788C>T/p.A263V
<b>Gender</b>	male	female	male	male
<b>Exam at birth</b>	EEG at 42 con- ceptual weeks slightly abnormal (see text)	suspected amniotic infection syndrome	muscular hypotonia and sleepiness	hypomotor activity followed by TCS on alternating sides with contralateral ictal EEG discharges
<b>Age at seizure onset</b>	2 d	24 d	7 d	1 d
<b>Seizure type at onset</b>	bilateral TCS with reduced oxygen saturation and unresponsiveness	multifocal CS and TCS lasting 10 s to 2 min.	bilateral TS	TCS on alternating sides
<b>Other seizure types</b>	TCS, tendency to clustering	GTCS during febrile infection	TS evolving into secondary GTCS	GTCS
<b>Treatment (duration)</b>	PB (7 mo); Acetazolamide (4 mo); VPA (10 y)	PB (5 mo); CLZ+PHT (2 weeks); VPA (6 ½ y)	Vit B6 (brief); PB (brief); TPM (brief); LEV (14 mo); OXC (3 y); LTG (5 mo);	After Liao et al, 2010[3]: 4-AP (12 d); Propranolol (7 mo); TPM (10 mo); LTG (7 mo)
<b>Seizure outcome (age)</b>	seizure-free (since age 5 mo)	seizure-free (since age 5 mo)	seizure-free (since age 7 mo)	seizure-free (since age 13 mo), isolated sei- zures (3.5, 6.5, 14.5 y)
<b>Symptoms during childhood-onset episodic attacks</b>	paroxysmal dizziness and poor balance, slurred speech, ataxia, nausea, headache without vomiting	slurred speech, impaired balance, ataxic gait, headache, rarely vomiting	dizziness, unsteady gait, inability to walk, possibly rare painful episodes	poor balance, ataxia, slurred speech, inter- mittent myoclonic jerks, severe distress with headache, back pain, hypermotor acti- vity, hyperventilation, retching or vomiting
<b>Age at onset, freq. and duration of episodes</b>	3.7 y ; 1–2/mo; a few min to several h	20 mo; 1–10/w; 1-5 min (rarely repeating over 2h)	15 mo; 2-3/mo; 1 d	1.5 y; 1-3/mo; several h

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5 \*: year of birth; 4-AP: 4-aminopyridine; CLZ: clonazepam; d: day(s); CS: clonic seizure; GTCS: generalized tonic-  
6 clonic seizure; h: hour(s); LTG: Lamotrigine; LEV: levetiracetam; min: minute(s); mo: month(s); OXC: oxcarbazepine;  
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8 PB: phenobarbitone; PHT: Phenytoin; s: second(s); TS: tonic seizure; TCS: tonic clonic seizure; TPM: topiramate; VPA:  
9 valproate; w: week(s), y: year(s)  
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13 **Table 2** Current density and gating parameters for Nav1.2 WT and the mutations  
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	CD [pA/pF]	Steady-state activation		Steady-state inactivation			T <sub>rec</sub> [ms]	
		V <sub>1/2</sub> [mV]	k	V <sub>1/2</sub> [mV]	k	N	at -100 mV	N
<b>SCN2A-WT</b>	-450.9±54.8	-28.2±1.0	-5.7±0.2	-69.4±0.7	5.2±0.1	16	5.2±0.4	10
<b>R1882G</b>	-356.7±57.2	-31.8±1.0 <sup>§</sup>	-5.8±0.3	-67.8±0.8	5.7±0.3	12	5.8±0.6	12
<b>G1522A</b>	-448.0±78.1	-29.0±1.3	-5.3±0.3	-68.5±0.8	5.1±0.2	12	5.0±0.5	12
<b>G1522A/ R1882G</b>	-757.4±136.3 <sup>#</sup>	-28.8±1.2	-4.8±0.3 <sup>*</sup>	-64.3±0.9 <sup>&amp;</sup>	5.2±0.1	11	4.0±0.4	11

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31 Data are presented as means ± s.e.m. k: slope factor.

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33 <sup>§</sup>the R1882G mutant is significantly different from the WT (p<0.05, t-test).

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35 <sup>#</sup>the G1522A/R1882G double mutant is significantly different from the three other clones (p<0.05, one way ANOVA  
36 with Bonferroni posthoc test).

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38 <sup>\*</sup>the G1522A/R1882G double mutant is significantly different from the R1882G mutant (p<0.05, one way ANOVA with  
39 Bonferroni posthoc test).

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41  
42 <sup>&</sup>the G1522A/R1882G double mutant is significantly different from WT (p<0.001), from G1522A (p<0.01) and R1882G  
43 (p<0.05, one way ANOVA with Bonferroni posthoc test)  
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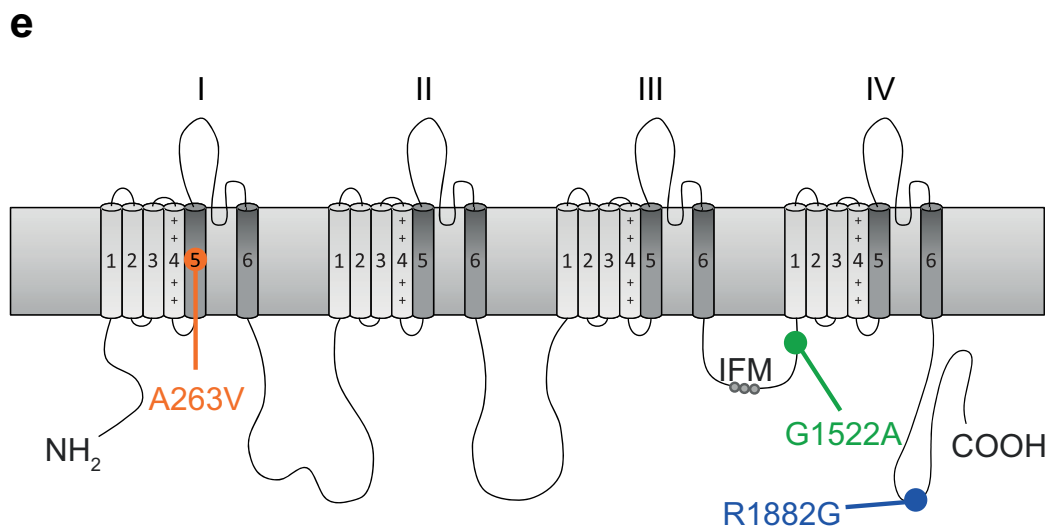
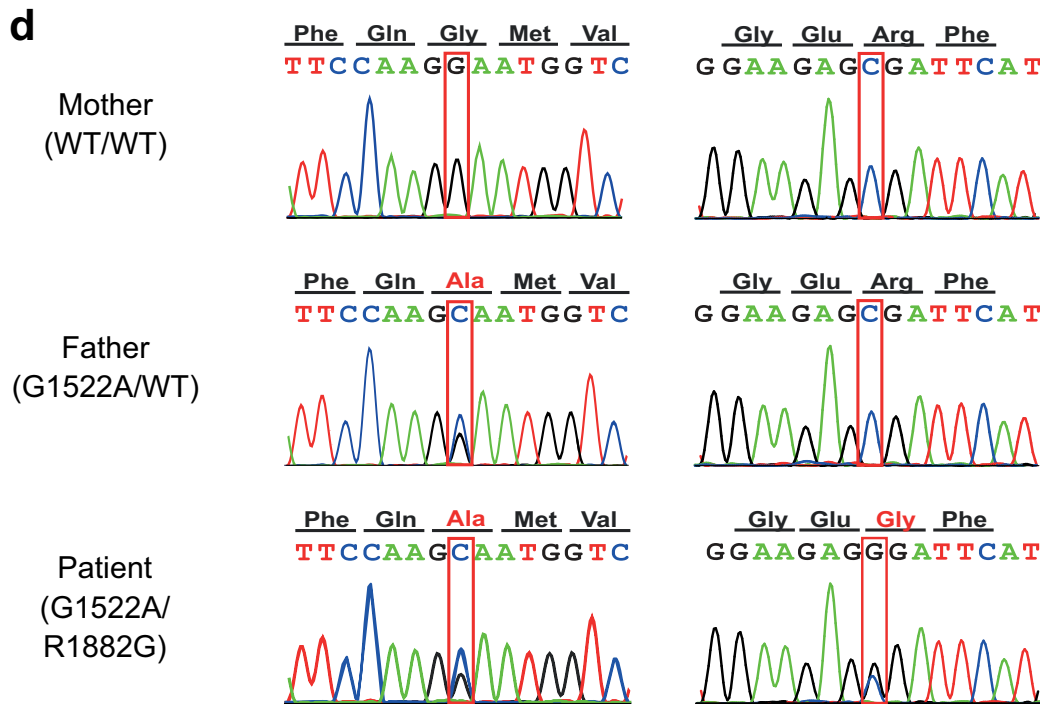
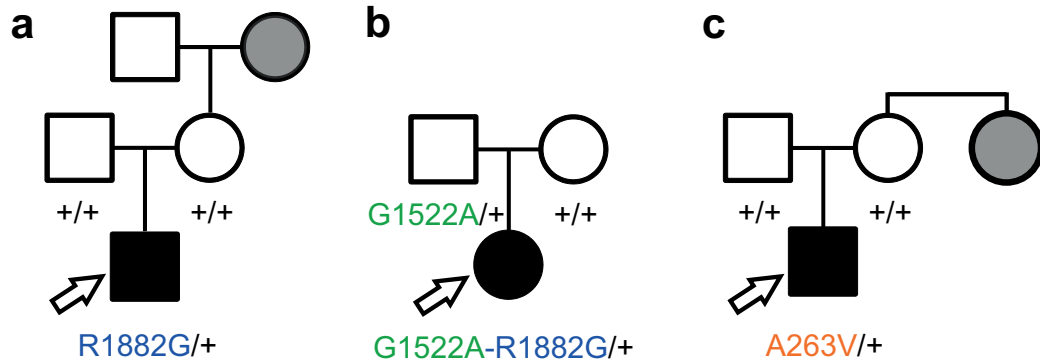
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5 **Figure legends**  
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7 **Fig.1** Pedigrees and *SCN2A* mutations in three additional cases with neonatal epilepsy and late-onset episodic ataxia. **(a-c)** Pedigrees of patients #1 (a), #2 (b) and #3 (c). The index patients are indicated by arrows. “+” denotes a wild type  
8 allele. A grandmother with reported Menière’s disease (a) and an aunt with reported neonatal-onset seizures (c) are  
9 marked in gray. Patients #1 and #3 carry one *de novo* *SCN2A* mutation each (a, c), whereas patient #2 carries both an  
10 inherited variant and a *de novo* *SCN2A* mutation on the same allele (b). **(d)** Sequence chromatograms of patient #2 and  
11 the unaffected parents showing a previously described, heterozygous c.4565G>C variant in the father and the patient,  
12 and an additional c.5644C>G mutation only in the patient. **(e)** Structure of the human Na<sub>v</sub>1.2 Na<sup>+</sup> channel α subunit  
13 showing the locations of all three mutations (A263V: orange circle, G1552A: green circle, R1882G: blue circle). **(f)**  
14 A263V, G1522 and R1882 (red boxed) and the surrounding amino acids show high evolutionary conservation  
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25 **Fig.2** Functional studies reveal a gain-of-function for the R1882G mutation. **(a-b)** Families of whole-cell Na<sup>+</sup> currents  
26 recorded from tsA-201 cells transfected with either *SCN2A*-WT (a) or R1882G (b) mutant channels. Sodium currents  
27 were elicited by 24 ms long step depolarizations ranging from -105 to + 97.5 mV from a holding potential of -140 mV.  
28 **(c)** The current density revealed no significant change in mean whole-cell peak current for R1882G mutant channels. **(d)**  
29 Voltage-dependence of steady-state Na<sup>+</sup> channel activation and inactivation revealing a significant hyperpolarizing shift  
30 in the activation curve for R1882G mutant channels compared with the WT (\*p<0.05, t-test). Lines represent fits of  
31 Boltzman functions. **(e)** The time course of recovery from fast inactivation determined at -100 mV showed no significant  
32 changes between WT and mutant channels. Lines represent fits of exponential functions yielding the time constant T<sub>rec</sub>.  
33 All values of electrophysiological results, numbers and p-values are listed in Table 2 and are shown as mean ± SEM  
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43 **Fig.3** Functional studies reveal a different gain-of-function mechanism when both mutations G1522A/R1882G are  
44 combined. **(a-c)** Families of whole-cell Na<sup>+</sup> currents recorded from tsA-201 cells transfected with either *SCN2A*-WT (a),  
45 G1522A mutant channels (b) or G1522A/R1882G mutant channels (c). Sodium currents were elicited as in Fig. 2. **(d)**  
46 The current density was significantly (1.7-fold) increased for G1522A/R1882G mutant channels (\*p<0.05, one way  
47 ANOVA with Bonferroni posthoc test, Table 2). **(e)** Voltage-dependence of steady-state Na<sup>+</sup> channel activation and  
48 inactivation revealing a significant depolarizing shift of the inactivation curve for G1522A/R1882G mutant channels  
49 (from WT \*\*\*p<0.001, from G1522A \*\*p<0.01, one way ANOVA with Bonferroni posthoc test, Table 2). Lines  
50 represent fits of Boltzman functions. **(f)** Time course of recovery from fast inactivation at -100 mV, as described in Fig.  
51 2, did not reveal significant changes between WT and mutant channels. All values of electrophysiological results,  
52 numbers and p-values are listed in Table 2 and are shown as mean ± SEM  
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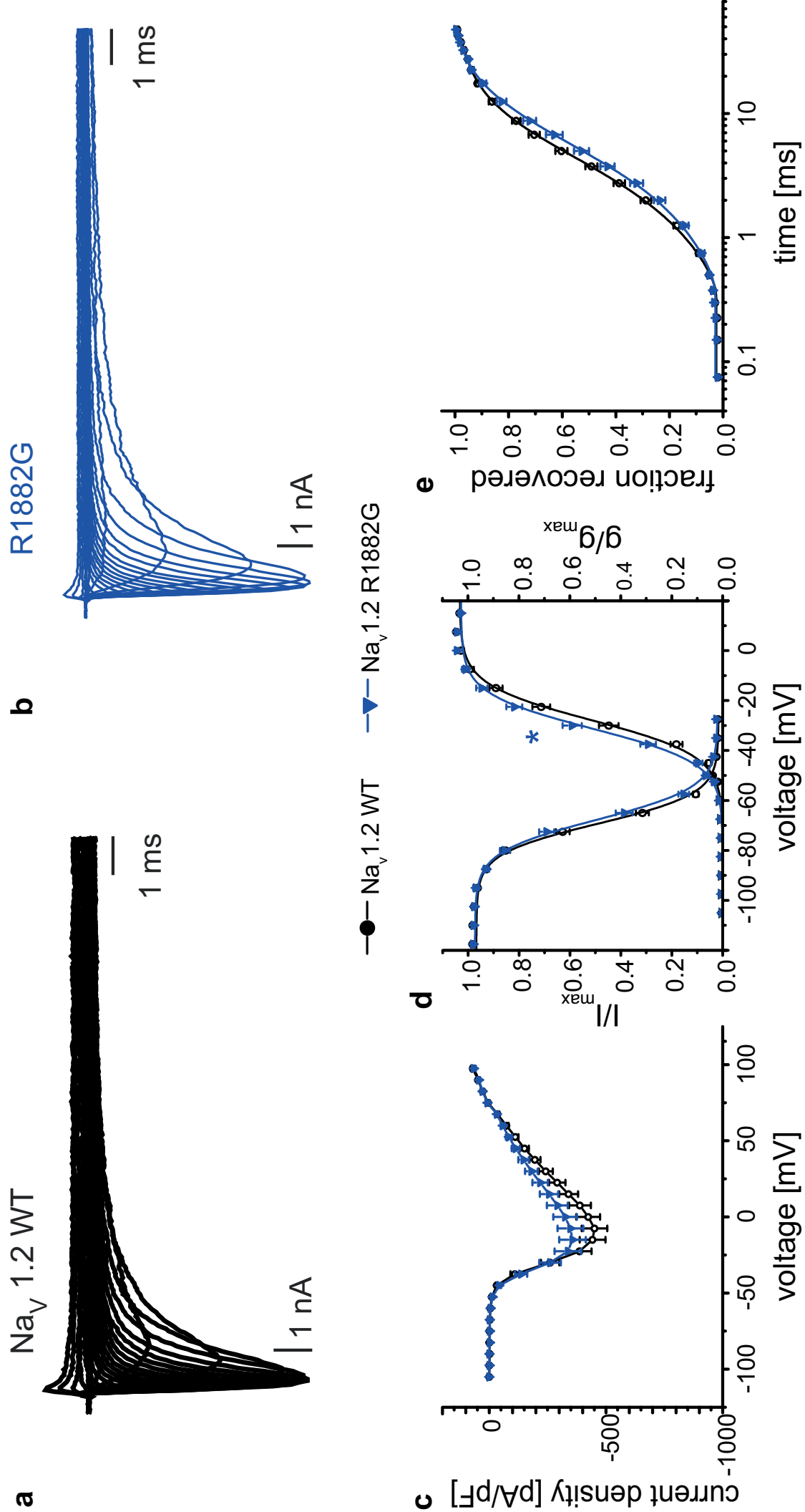
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7 **Fig.4** Western Blot Analysis shows a significant increase in expression level only for the G1522A/R1882G mutation. **(a)**  
8 Representative experiment illustrating total Nav1.2 protein expression of tsA201 cells transfected with: only transfection  
9 reagent as Mock control, WT-Nav1.2, only G1522A, only R1882G or both G1522A/R1882G and detected with anti- pan  
10 sodium channel antibody. The immunoreactive bands of Mock-control, Nav1.2-WT or mutant proteins were normalized  
11 to the amount of the endogenous membrane-cytoskeletal protein Vinculin. **(b)** Quantification of six independent  
12 experiments by normalizing the signals of Nav1.2 protein bands to the corresponding Vinculin signals in total lysates  
13 demonstrated that each mutation exposed a higher level of Na<sup>+</sup> channel expression, but only G1522A/R1882G presented  
14 a significant increase of the expression level compared to the WT (\*\*p<0.01; N=6, one way ANOVA with Bonferroni  
15 posthoc test). All values are shown as mean ± SEM  
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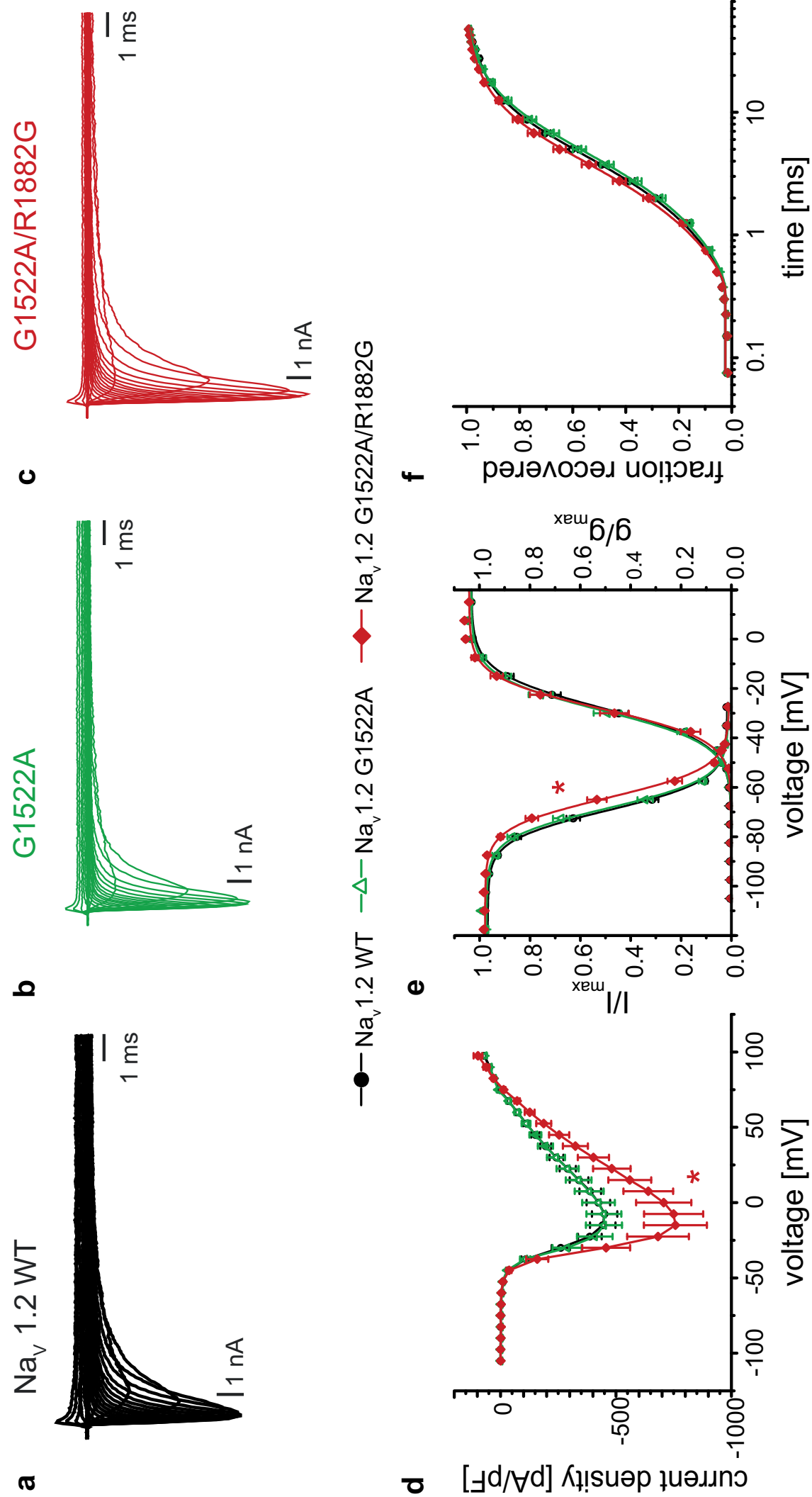
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	A263V	G1522A	R1882G
<i>Homo sapiens</i>	LSVF <b>A</b> LIGL	NKFQ <b>G</b> MVFD	QMEE <b>R</b> FMAS
<i>Pan troglodytes</i>	LSVF <b>A</b> LIGL	NKFQ <b>G</b> MVFD	QMEE <b>R</b> FMAS
<i>Macaca mulatta</i>	LSVF <b>A</b> LIGL	NKFQ <b>G</b> MVFD	QMEE <b>R</b> FMAS
<i>Mus musculus</i>	LSVF <b>A</b> LIGL	NKFQ <b>G</b> MVFD	QMEE <b>R</b> FMAS
<i>Gallus gallus</i>	LSVF <b>A</b> LIGL	NKFQ <b>G</b> MVFD	QMED <b>R</b> FMAS
<i>Danio rerio</i>	LSVF <b>A</b> LIGL	NKFQ <b>G</b> LVFD	QMED <b>R</b> FMAS

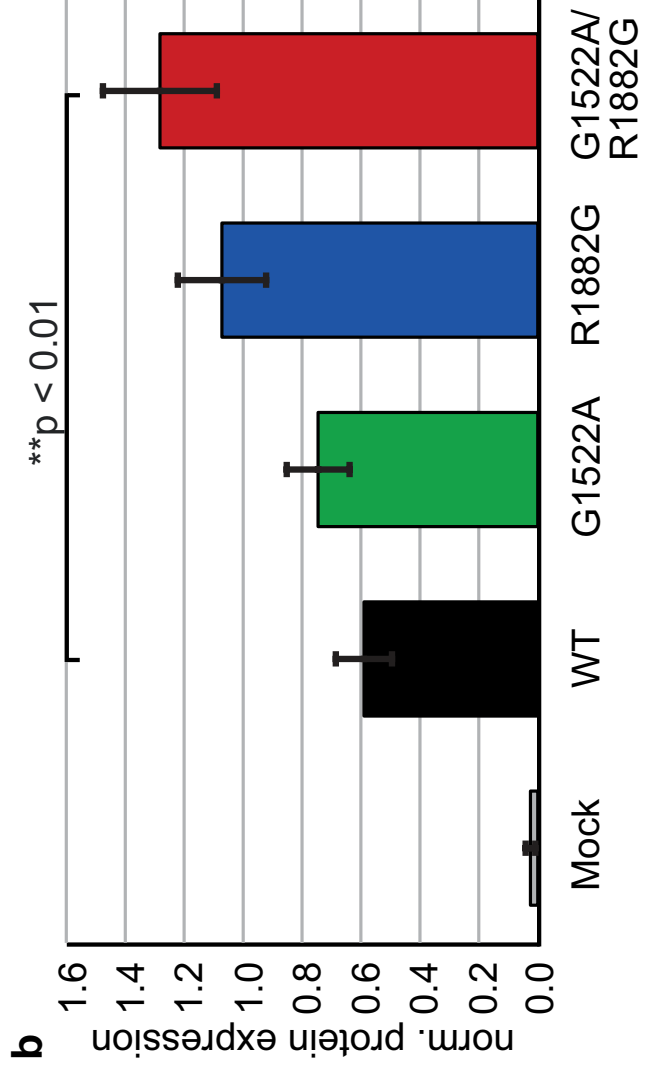
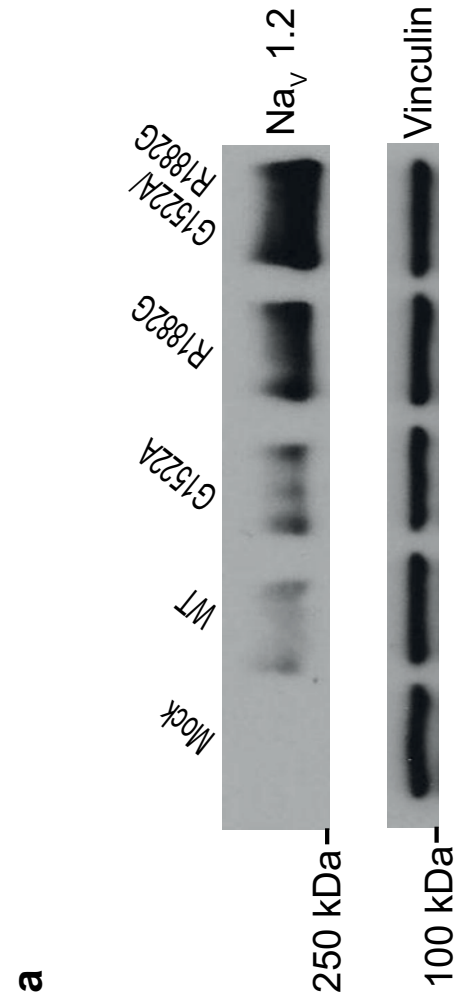
Figure



Figure









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