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Effects of S906T polymorphism on the severity of a novel borderline mutation I692M in $Na_v1.4$ cause periodic paralysis

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Running title: S906T polymorphism exacerbates phenotype of a novel I692M mutation

Key words: hyperkalemic periodic paralysis, novel borderline mutation, founder effect

Key Summary points:

Hyperkalemic periodic paralysis (HyperPP) is caused by missense mutations in $Na_v1.4$ channels. A clear genotype-phenotype relationship is not established yet.

Here we report a novel mutation I692M in 14 families out of 104 genetically identified HyperPP families in Neuromuscular Center Ulm. Interestingly, 13 of 14 families carrying the I692M mutation also have a known functional polymorphism S906T on the same allele suggesting a founder effect in central Europe.

Clinically, the one family carrying only I692M mutation had shorter weakness episodes, less affected muscles, no CK elevation and absence of permanent weakness.

Electrophysiological investigation showed S906T enhances I692M defects on 1) left shift in voltage gated activation, 2) stabilization of fast inactivated state, 3) destabilization of slow inactivated state.

Polymorphism S906T exacerbates the gating defects of borderline mutation I692M which underlying the severe clinical phenotypes.

Abstract

Hyperkalemic periodic paralysis (HyperPP) is a dominantly inherited muscle disease caused by mutations in *SCN4A* gene which encodes α subunit of skeletal muscle voltage gated $Na_v1.4$ channels. We identified a novel $Na_v1.4$ mutation I692M in 14 families out of the 104 genetically identified HyperPP families in the Neuromuscular Centre Ulm and is therefore as frequent as I693T (13 families out of 14 HyperPP families) in Germany. Surprisingly, in 13 families, a known polymorphism S906T was present on the affected allele suggesting a founder effect in central Europe. All affected members suffered from episodic weakness; myotonia was also common. Compared with patients with I692M-S906T, patients with I692M had shorter weakness episodes, less affected muscles, no CK elevation and also absence of permanent weakness. Whole-cell patch clamp was performed to investigate the functions of the I692M mutant with and without the polymorphism S906T. Compared with the wild type (WT), I692M and I692M-S906T both showed a hyperpolarizing shift of activation, stabilization of fast inactivation and impaired slow inactivation, whereby I692M-S906T exhibited more severe effects. The impaired slow inactivation of both I692M and I692M-S906T is consistent with most known HyperPP mutations. Together with the stabilization of fast inactivation, it accounts for the long periods of muscle weakness. The hyperpolarizing shift of activation facilitates the generation of action potentials and thus contributes to myotonia. Our results suggest that polymorphism S906T has effects on the clinical phenotypic and electrophysiological severity of a novel borderline $Na_v1.4$ mutation I692M, making the borderline mutation fully penetrant.

Abbreviations HyperPP: Hyperkalemic periodic paralysis; WT: wild type

Introduction

HyperPP is a dominantly inherited muscle disease characterized by episodes of flaccid weakness associated with high levels of serum potassium (Weber et al.1993). The prevalence of HyperPP is approximately 1/200,000, and its penetrance is high (> 90%) (Lehmann-Horn & Rudel, 1996). Triggers of HyperPP include potassium-rich food, rest after exercise and fasting. Clinically, HyperPP can present as isolated episodic weakness or be associated with clinical or EMG myotonia (Weber et al.1993; Charles et al. 2013).

Until now, more than 10 mutations in SCN4A gene encoding $Na_v1.4$ channels have been identified to cause HyperPP (Simkin & Bendahhou, 2011; Lossin et al. 2012; Jurkat-Rott et al. 2010). The coding region of SCN4A consists of 24 exons and 23 introns (George et al. 1993). Condon changes in exon 9, 13, 19, 23 and 24 have been reported to cause HyperPP. Four pathogenic substitutions have been identified in exon 13, leading to mutations including T704M, I693T and L689I/V which account for more than 2/3 of all HyperPP patients (Weber et al.1993; Simkin & Bendahhou, 2011; Miller et al, 2004).

HyperPP mutations either affect the intracellular loops or the transmembrane segments of $Na_v1.4$ channels involved in inactivation (Jurkat-Rott et al. 2010; Jurkat-Rott & Lehmann-Horn, 2007). Fast and slow inactivation of $Na_v1.4$ channels work in concert to regulate the fraction of available sodium channels and control the membrane excitability of skeletal muscle cells. As two independent processes, fast inactivation occurs within milliseconds, terminating the generation of action potentials, while slow inactivation happens in seconds to minutes, governing the availability of excitable sodium channels (Vilin & Ruben, 2001; Ruff, 1997). Sustained inward sodium current due to disrupted fast inactivation with re-opening persistent current is

a common feature for several HyperPP mutations (Lossin, 2012; Bendahhou et al.1999). The persistent inward current may also be generated by an increased window current created by shifting of voltage dependence of either activation or both activation and inactivation (Bendahhou et al.1999; Rojas et al. 1999; Bendahhou et al.2002). Slight membrane depolarization as a result of the persistent current facilitates the generation of repeated action potentials, leading to myotonia (Simkin & Bendahhou, 2011; Jurkat-Rott & Lehmann-Horn, 2007). Further membrane potential depolarization beyond the threshold inactivates $Na_v1.4$ channels, leading to muscle paralysis (Simkin & Bendahhou, 2011; Jurkat-Rott & Lehmann-Horn, 2007). Defects in channel slow inactivation also contribute to clinical weakness (Ruff, 1994; Ruff, 1997), for example the impaired slow inactivation in L689I, I693T, T704M and M1592V (Bendahhou et al. 2002; Hayward et al. 1999; Cummins & Sigworth, 1996).

The polymorphism S906T, encoded by exon 14 and located in II-III loop of $Na_v1.4$ channel, has been described in 4% healthy German population and in 1.8% worldwide controls (Kuzmenkin et al. 2003) (http://exac.broadinstitute.org). Electrophysiological characterization of S906T revealed no effects on fast gating process of $Na_v1.4$ channels, but slowed entry into and recovery from slow inactivation (Kuzmenkin et al. 2003).

In the present study, we identified a C2076G base change in exon 13 leading to a novel mutation I692M in the $Na_v1.4$ channel. The mutation was found in 14 families, 13 of which had another G2717C base change in exon 14 predicting the known polymorphism S906T on the same allele. Clinical analysis and electrophysiological investigations showed that S906T exerted an influence on the severity of both clinical phenotypes and gating change of the borderline I692M mutation.

Materials and Methods

Genetics and Phenotype. 14 HyperPP families were enrolled into this study for mutation screening and clinical analysis. Informed consent was obtained from all participants, and all procedures were approved by the Ethics Committee of Ulm University and were in accordance with the Declaration of Helsinki. Direct sequencing of all SCN4A exons was performed using the Sanger method.

Electrophysiological studies. The human $Na_v1.4$ α subunit sub-cloned to the pEGFP vector was used for site-directed mutagenesis of both I692M and I692M-S906T mutations. Whole-cell patch clamp recordings were performed 24h after transient transfection of SCN4A in human tsA201 cells using the jetPEI kit. The pipette resistance was around 1.5 MΩ after filling with internal solution containing (in mM): NaCl 35, CsF 105, EGTA 10, HEPES 10. The external solution contained (in mM) NaCl 150, KCl 2, $CaCl_2$ 1.5, $MgCl_2$ 1, HEPES 10. pH was adjusted to 7.4 and osmolarity to ~300 mOsm. Before data acquisition, cells were allowed to stabilize for 10 min after establishment of the whole-cell configuration. Sodium currents were recorded at room temperature (21-23°C) with 4/P leak subtraction after partial series resistance compensation (~85%) using an Axopatch 200B amplifier (Molecular Devices). Data were filtered at 10 kHz and sampled at 50 kHz.

Data analysis. Data were analysed by a combination of pClamp (Molecular Devices), Excel (Microsoft), SPSS (IBM) and ORIGIN (Microcal software). Data are presented as mean ± standard error of the mean (SEM). Student's t-tests were applied for statistical evaluation with significance levels set to *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Results

Genetics and Phenotype

Sanger sequencing of all exons of SCN4A revealed 14 HyperPP families harbour a heterozygous G to C substitution at nucleotide 2076 in exon 13, corresponding to an

I692M mutation in the S4-S5 linker of domain II in $Na_v1.4$ channels (Fig. 1). This variant has not been reported in 60706 alleles included in the ExAC browser, however, a similar substitution, I692T, was found in 1 of those 60706 controls (http://exac.broadinstitute.org). 13 out of the 14 HyperPP families also had a G2717C base exchange in exon 14, predicting the known polymorphism S906T on the same allele (Fig. 1).

In all patients, episodic paralysis was reported with onset at ~14 years old. Myotonia was also a common clinical sign, affecting about 61% of all patients. Frequency of episodic weakness varied from daily to monthly. Tendentially, paralytic attacks lasted longer in patients with I692M-S906T than patients with the single I692M mutation (Table 1).

During attacks, mostly extremity muscles were affected. Facial, laryngeal, and neck muscles were involved in the patients with I692M-S906T mutation but not in patients with I692M mutation. Typical triggers of paralytic attacks were fasting, sports, longer rest, eating and stress. About 28% of patients with I692M-S906T showed permanent muscular weakness whereas no permanent weakness was present in patients with only I692M mutation. Also, patients with I692M-S906T showed elevated CK (averaged value of 795.25u/l). Summarising it up, the shorter attack duration, the less affected muscles, the absence of permanent muscular weakness and the absence of CK elevation may indicate that patients carrying only I692M mutation are clinically less affected than patients with I692M-S906T (Table 1).

As a prophylactic treatment of paralytic episodes, hydrochlorothiazide had a beneficial effect on 9 of 11 patients with I692M-S906T mutation which was applied to. Acetazolamide was also able to reduce the attack frequency in 2 of 3 patients with I692M-S906T. During an acute episode, glucose intake or inhaled salbutamol may

be beneficial. Due to the small number of patients, these therapeutic results should be interpreted cautiously (Table 1).

Electrophysiology.

Heterologous expression of WT, I692M and I692M-S906T channels produced typical inward sodium currents (Fig. 2B inset). The corresponding conductance-voltage relationship revealed a significant -4mV and -7mV left shift for I692M and I692M-S906T compared with the WT, respectively (Fig. 2A, Table 2). S906T enhanced the left shift of voltage dependence of activation of I692M. Such a left shift of activation curves is reminiscent of the negative shifting of activation curves reported for the known HyperPP mutations L689I and I693T which are also located in S4-S5 linker of domain II (Bendahhou et al. 2002; Plassart-Schiess et al. 1998). While unlike most known HyperPP mutations (Bendahhou et al. 1999; Rojas et al,1999), such negative shifting of activation did not result in larger sustained sodium currents at negative potentials where window currents might occur. Left shift of activation could, however, lower the threshold of action potential and further facilitate the membrane depolarization. The facilitated activation observed in I692M and I692M-S906T at least will contribute to the repetitive action potentials and the myotonia experienced by the patients.

The time constant of fast inactivation which was fitted to a single exponential function revealed no difference for both I692M and I692M-S906T than the WT over the range of -30 to 40mV (Fig. 2B), except the faster current decay at potentials of -40 and -35mV which might result from the negative shift in the voltage dependence of activation and therefore larger currents in the mutations. Even though persistent current is a common feature for HyperPP mutations, neither I692M nor I692M-S906T produced such additional persistent current after both 20ms (Fig.2B inset) and

100ms (data not shown) depolarization which is in agreement with the undistinguishable time constant of fast inactivation between mutations and WT.

However, I692M and I692M-S906T both showed around -5mV left shift in voltage gated steady-state fast inactivation (Fig. 2A, table 2). Additionally, I692M and I692M-S906T exhibited enhanced extent of closed state inactivation with holding potential of -90mV, leading to 9% to 12% of inactivated channels compared with 5% for the WT (Fig. 2C, table 2), and S906T further increased I692M effects on the enhanced entry into closed state. Alterations in fast inactivation and the observed slower recovery from fast inactivation for both I692M and I692M-S906T mutations (Fig.2D) predict a stabilization of the fast inactivated state, which was unlike the mutations L689I and I693T (Bendahhou et al. 2002; Plassart-Schiess et al. 1998). This result indicates that S4-S5 linker of domain II is involved in the fast inactivation process of $Na_v1.4$ channels. In agreement with the stabilization of fast inactivated state, stimulations of repetitive depolarized pulses at different frequencies and from different holding potentials showed enhanced use-dependent block for both I692M and I692M-S906T (Fig. 3). Especially, S906T increased this effect and showed around 18% less residual channels than the WT with the simulation frequency of 125HZ from a holding potential of -90mV. The disturbed fast inactivation caused by both mutations might contribute to the pathogenesis of the muscle weakness due to sustained depolarization with $Na_v1.4$ inactivation.

Disruption of slow inactivation, another common feature among HyperPP mutations, was present in both I692M and I692M-S906T. I692M and I692M-S906T showed reduced extent of entry into slow inactivation with values of 72% to 68% compared with 89% for WT (Fig. 4A, Table 2). I692M showed comparable rate to enter slow inactivated state compared with the WT, while I692M-S906T had a significant faster

entry into slow inactivation state. Also, I692M and I692M-S906T had a right shift of slow inactivation curves that did not reach significant levels. This was accompanied by impaired slow inactivation for both I692M and I692M-S906T at potentials more depolarized than -60mV after 30s depolarization pulse at -10mV, leading to 22% to 26% non-inactivated sodium channels for I692M and I692M-S906T compared with 11% for WT (Fig. 4B, Table 2). The incomplete slow inactivation for I692M and I692M-S906T mutations is similar to the impaired slow inactivation caused by the known HyperPP mutations L689I, I693T, T704M and M1592V which predominant symptom is episodic weakness (Bendahhou et al. 1999; Rojas et al. 1999; Bendahhou et al. 2002; Hayward et al. 1999; Cummins & Sigworth, 1996). Additionally, all three channels recovered in a bi-exponential way from slow inactivation. I692M and I692M-S906T showed significant faster recovery rates compared with the WT, with decreased fast and slow time constants (Fig. 4C, Table 2). These alterations destabilized the slow inactivated state which was in agreement with the right shift in steady state slow inactivation, although the later difference was not significant. S906T increased I692M effects of reduced entry into slow inactivation, enhanced fraction of non-inactivated sodium channels and faster recovery from slow inactivation.

Altogether, S906T enhances I692M defects on 1) left shift in voltage gated activation, 2) stabilization of fast inactivated state, 3) destabilization of slow inactivated state.

Discussion.

The I692M mutation accounted for 13.5% of the 104 genetically clarified HyperPP families of the Neuromuscular Centre Ulm and is as frequent as I693T in Germany (in 13 of 104 families). The fact that 13 of 14 HyperPP families carrying the I692M mutation which also had an additional polymorphism S906T on the same allele

suggests a founder effect in central Europe. The one family carrying the I692M without S906T showed shorter weakness episodes, less muscle affected, no CK elevation and absence of permanent weakness indicating that S906T exacerbates the phenotype. Such a mild phenotype might explain why this novel I692M mutation was not identified before because it should have been found since exon 13 is always sequenced for HyperPP diagnosis as it accounts for 2/3 of causative mutations.

Clinical symptoms are determined by the degree of membrane depolarization. When the membrane is slightly depolarised by elevated extracellular potassium due to ingestion or exercise (Jurkat-Rott & Lehmann-Horn, 2007), due to the left-shift of activation, opening of mutant channels could lower the threshold of action potentials and contribute to the generation of repetitive action potentials underlying the phenotype of myotonia. During action potentials, influx of sodium ions and release of potassium ions out of skeletal muscles further depolarise membrane potentials and both mutant and WT sodium channels become inactivated leading to muscle paralyzed, especially both I692M and I692M-S906T exhibited stabilized fast inactivated state. Over seconds to minutes, slow inactivation will occur due to the sustained depolarization. Sodium channels will enter into the slow inactivation state, leading to decreased number of excitable channels and terminate the enhanced channel activation, resulting in abolishment of sodium ion influx and the repolarization of membrane potentials (Ruff, 1997; Ruff, 1994; Ruff, 1997). The incomplete slow inactivation for I692M and I692M-S906T mutations could facilitate the long-time membrane depolarization and explain weakness episodes experienced by our patients.

Compared with gating alterations of I692M alone or S906T alone (Kuzmenkin et al, 2003), the double mutation I692M-S906T did not exhibit additive effects; instead

S906T just enhanced I692M defects which would agree with the more pronounced clinical phenotype we observed in patients. This supports the idea of potential interaction of S4-S5/II and II-III loop involved in regulation of $Na_v1.4$ channels gating. This is the first report of a functional polymorphism exacerbating the phenotype of an allelic ion channelopathy leading to founder effects in central Europe.

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Conflict of interest

All authors declare no conflict of interest.

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Table 1: Clinical features of HyperPP families

	I692M-S906T	I692M
Number of families/Individuals	13/29	1/2
Onset age	14.6	14
Frequency: Daily/Weekly/Monthly/Yearly	23%/55%/14%/5%	50%/0/50%/0
Duration: Minutes/Hours/Days	14%/55%/32%	100%/0/0
Myotonia	61%	50%
Permanent weakness	28%	0
CK (%/u/l)	28%/795.25	100%/187.50
Muscle affected		
Proximal/Distal	87%/59%	100%/100%
Face/Neck/Larynx	86%/34%/28%	0/0/0
Trunc/Abdominal	4%/4%	0/0
Triggers		
Fasting/Sport/Rest	33%/46%/42%	100%/50%/100%
Stress/Eating/Cold/K^+	38%/21%/8%/8%	50%/0/0/0
Medication		
Hydrochlorothiazide	41%	100%
Acetazolamid	10%	100%
Glucose/Flupirtin	10%/4%	0/50%
Salbutamol/Potassium	17%/4%	0/0

Table 2: Gating parameters of WT and both mutations

Parameters	WT	I692M	I692M-S906T
Activation	n=9	n=10	n=10
$V_{0.5}$ (mV)	-28.03±2.45	-32.89±0.95*	-35.27±1.29*
k	-5.89±0.44	-6.81±0.35	-6.09±0.14
Fast inactivation	n=10	n=10	n=14
$V_{0.5}$ (mV)	-71.65±1.15	-77.40±1.06**	-76.56+1.12**
k	-5.23±0.17	-5.28±0.12	-5.28±0.09
Entry into closed inactivation	n=10	n=11	n=10
τ (ms)	14.84±3.88	22.63±5.80	16.46±3.90
A	0.05±0.01	0.08±0.01**	0.11±0.02**
Recovery from fast inactivation	n=9	n=10	n=14
τ (ms)	1.81±0.16	2.60±0.24*	2.46±0.12**
Entry into slow inactivation	n=5	n=6	n=7
τ (ms)	2355.6±344.7	1566.7±223.2	968.71±92.3**
c	0.11±0.02	0.28±0.01***	0.32±0.03***
Slow inactivation	n=6	n=8	n=9
$V_{0.5}$ (mV)	-58.07±3.78	-49.87±2.77	-52.59±2.86
k	-12.50±0.96	-13.86±0.95	-14.90±1.50
c	0.11±0.02	0.22±0.04*	0.26±0.03**
Recovery from slow inactivation	n=8	n=6	n=6
τ_{fast} (ms)	96.36±25.22	25.22±6.34*	26.73±3.7*
τ_{slow} (ms)	1100.98±306.64	350.0±53.0*	309.49±44.0*

Significance levels set to *p<0.05, **p<0.01 and ***p<0.001

Legends of Figures

Fig. 1: Pedigrees of 14 HyperPP families with mutation status of I692M or S906T were given
Affected individuals are shown as solid symbols. Squares represent male and circles represent female. Question marks indicate that no clinical information was available at the age indicated. Family 14 is the one having only I692M mutation.

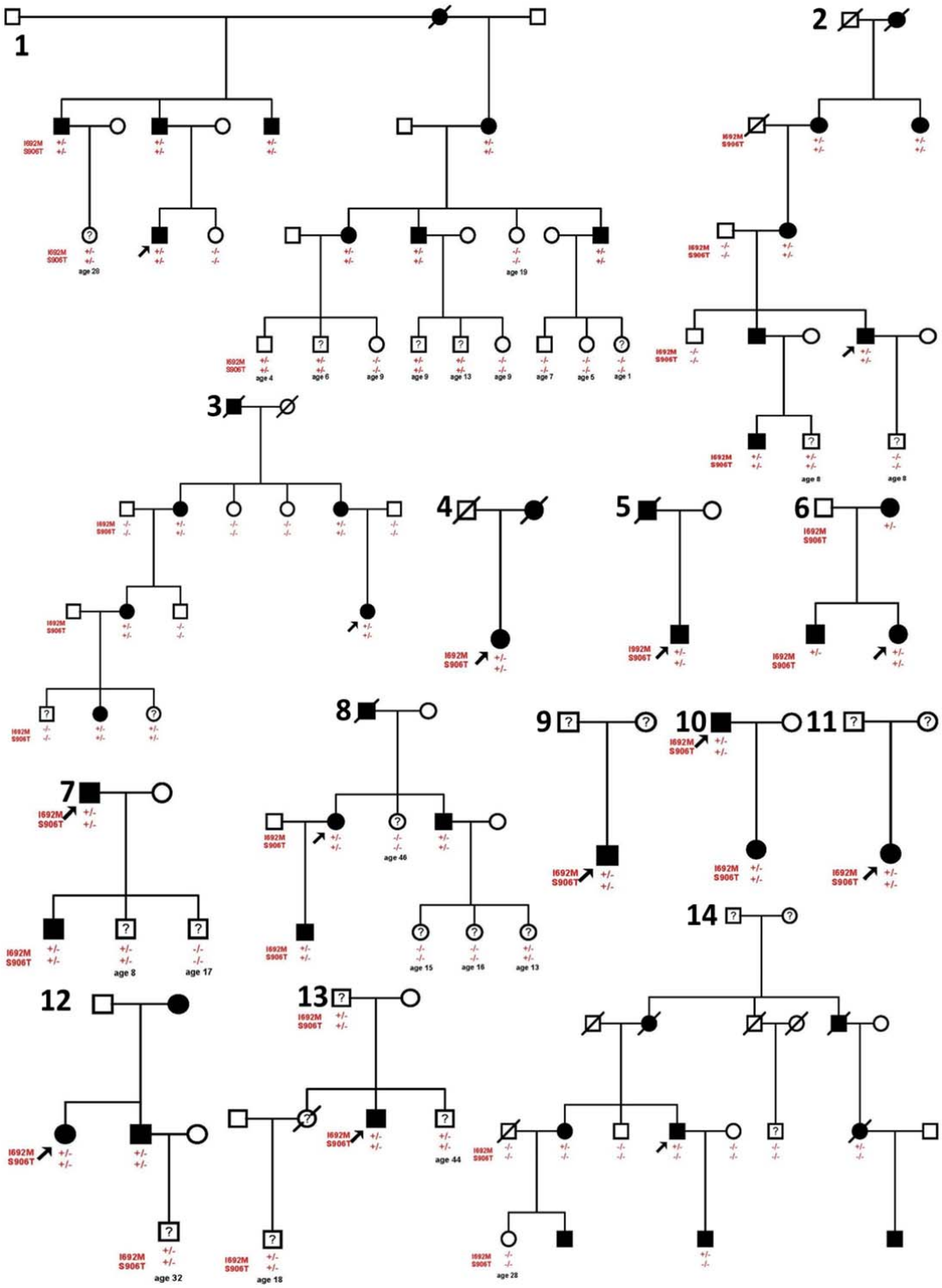
Fig.2: Functional characteristics of WT (filled circles), mutant I692M (open squares) and I692M + S906T (open triangles) channels expressed in tsA201 cells
(A) Steady-state fast inactivation curves and conductance-voltage curves. Inactivation curves were determined from a holding potential of -120mV using a series of 100ms pre-pulses to potentials between -140 to -10mV in 10mV steps followed by a -10mV test pulse (WT: n=10, I692M: n=10 and I692M + S906T: n=14). Activation curves were elicited from a holding potential of -120mV by voltage steps between -80 and +65mV in 5mV intervals for 20ms pulse (WT: n=9, I692M: n=10 and I692M + S906T: n=10). (B) Voltage-dependence of time constants of fast inactivation of WT and mutant channels (WT: n=9, I692M: n=10 and I692M + S906T: n=10). Typical whole-cell currents responding to activation protocols are shown in insets. (C) Time course of closed state inactivation for durations from 0.06 to 280ms

at −90mV from a holding potential of −120mV whereby a test pulse to −10mV determined the fraction of non-inactivated channels (WT: n=10, I692M: n=11 and I692M + S906T: n=10) (D) Recovery from fast inactivation with a two-pulse protocol: a 100ms-lasting depolarization pre-pulse to -10mV was used to inactivate sodium channels, a second -10mV test pulse followed after an increasing interval from 0.16 to 280ms from a holding potential of -120mV (WT: n=9, I692M: n=10 and I692M + S906T: n=14). Protocols are shown in insets. Data are shown as means ± SEM. Lines are fits to corresponding equations. Fitting parameters are listed in Table 2. Significance levels were set to *p<0.05.

Fig. 3: Use dependence of WT (filled circles), mutant I692M (open circles) and I692M +S906T (open triangles) channels
Currents were elicited by 200 consecutive trains of 2ms-long depolarizing pulses to -10mV from different holding potentials (-120mV (left graphs) and -90mV (right graphs)) at different frequencies (50Hz (top graphs), 100Hz (middle graphs) and 125Hz (bottom graphs)). Each trace was normalized to the initial transient peak sodium current. Data are shown as means ± SEM. Significance tested for 2^{th}, 50^{th}, 100^{th} and 200^{th} pulse were set to **p<0.01 and ***p<0.001.

Fig. 4: Slow inactivation properties of WT (filled circles), mutant I692M (open circles) and I692M + S906T (open triangles) channels
(A) Entry into slow inactivation was elicited from a holding potential of -120mV by a depolarizing pulse to -10mV for an increasing time period (0.02 - 60s). An interval of 50ms at -120mV followed to recover the fast inactivated but not slow inactivated channels. Then the fraction of slow inactivated channels was determined by the following 20ms test pulse to -10mV (WT: n=5, I692M: n=6 and I692M + S906T: n=7). (B) Steady-state slow inactivation was determined by a 30s conditioning pulse between -140 to -10 mV, followed by a 50ms recovery period at -120mV prior to the -10mV test pulse (WT: n=6, I692M: n=8 and I692M + S906T: n=9). (C) Recovery from slow inactivation was measured by a 30s depolarizing pulse to -10mV followed by increasing recovery duration (from 0.001- 60s) from holding potential of -120mV. The test pulse to -10mV was then employed to record the recovered sodium currents (WT: n=8, I692M: n=6 and I692M + S906T: n=6). For all slow kinetics investigation cells were held at -120 mV between trials for 30s to allow the recovery from slow inactivation. Protocols are shown in insets. Data are shown as means ± SEM. Lines are fits to corresponding equations. Fitting parameters are listed in Table 2.

Fig. 1: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age 29
age 19
age 4
age 6
age 9
age 9
age 13
age 9
age 7
age 5
age 1
age 8
age 8
age 46
age 15
age 16
age 13
age 8
age 17
age 32
age 18
age 44
age 28

Fig.2: Functional characteristics of WT, mutant I692M and I692M + S906T channels expressed in tsA201 cells

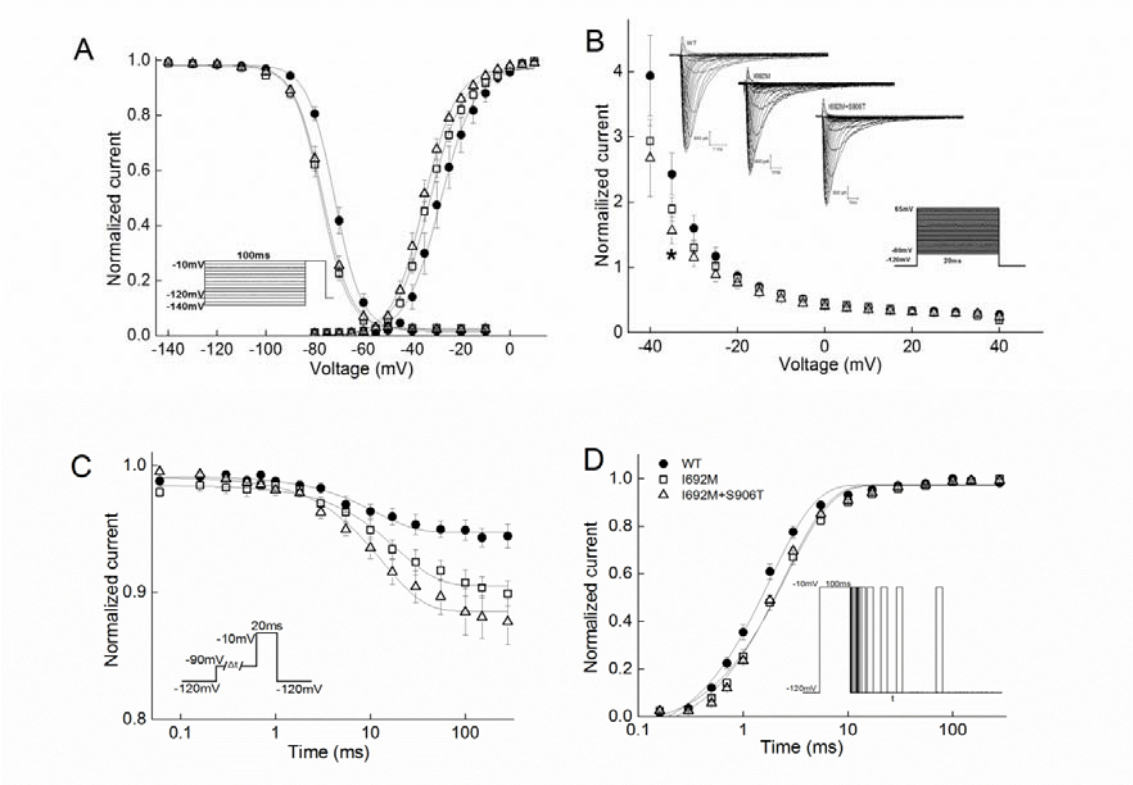

Fig. 3: Use dependence of WT, mutant I692M and I692M +S906T channels.

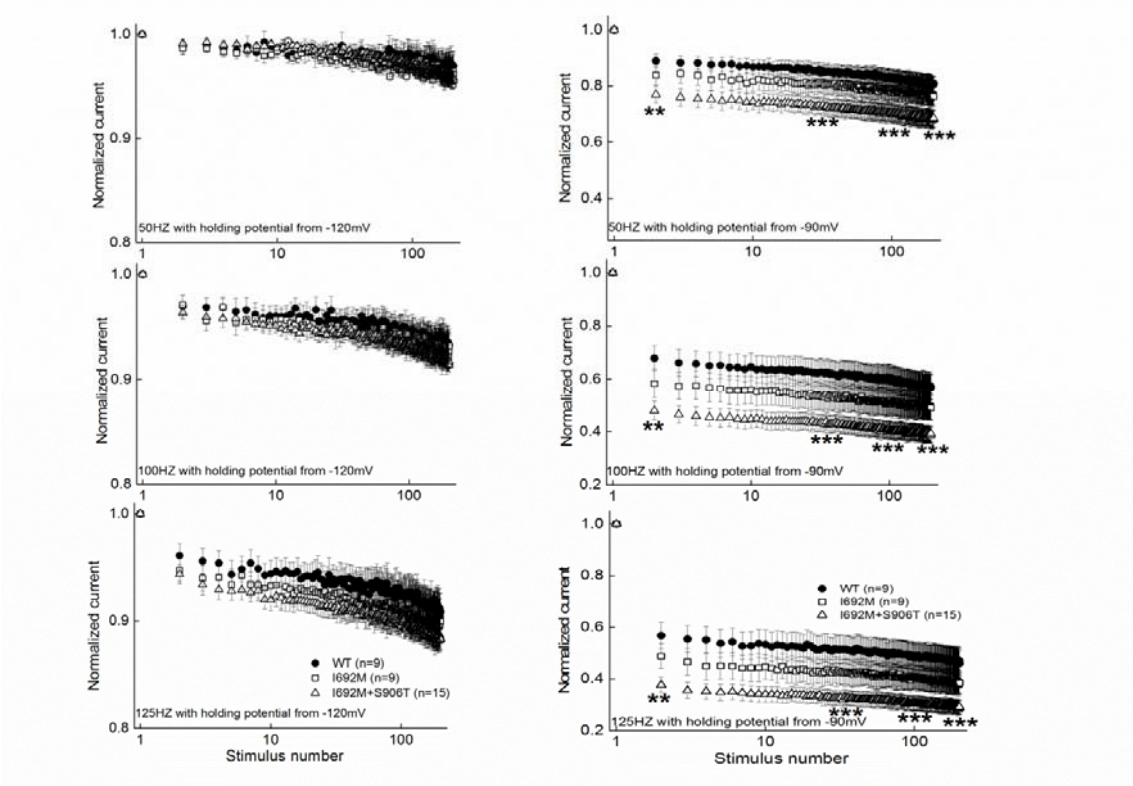

Fig. 4: Slow inactivation properties of WT, mutant I692M and I692M + S906T channels