IN VIVO ASSESSMENT OF MUSCLE MEMBRANE PROPERTIES IN THE SODIUM CHANNEL MYOTONIAS

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ABSTRACT

Introduction: The gain-of-function mutations that underlie sodium channel myotonia(SCM) and paramyotonia congenita(PMC) produce differing clinical phenotypes. We used muscle velocity recovery cycles (MVRCs) to investigate membrane properties.

Methods: MVRCs and responses to trains of stimuli were compared in patients with SCM (n=9), PMC (N=8), and normal controls (n=26).

Results: The muscle relative refractory period was reduced in SCM, consistent with faster recovery of the mutant sodium channels from inactivation. Both SCM and PMC showed an increased early supernormality, and increased mean supernormality following multiple conditioning stimuli, consistent with slowed sodium channel inactivation. Trains of fast impulses caused a loss of amplitude in PMC, after which only half of the muscle fibres recovered, suggesting that the remainder stayed depolarized by persistent sodium currents.

Discussion: The differing effects of mutations on sodium channel function can be demonstrated in human subjects *in vivo* using this technique.

KEY WORDS:
Sodium channel, Myotonia, Paramyotonia congenita; Membrane potential; Velocity recovery cycle, channelopathy

INTRODUCTION

Mutations in the transient sodium channel Nav1.4 underlie both sodium channel myotonia (SCM) and paramyotonia congenita (PMC).

Patients with SCM typically have myotonic stiffness following muscle contraction, which improves with repetition (the 'warm-up' phenomenon) and do not have exercise- or cold-induced weakness. These patients may sometimes demonstrate mild worsening of stiffness with repeated muscle contraction (paradoxical myotonia), particularly with forced eye closure, before subsequently showing the typical 'warm-up' phenomenon.

Patients with PMC typically have paradoxical myotonia as well as exercise-related weakness, particularly with muscle exertion at cold temperatures. With warming, this weakness does not resolve immediately, and when severe, may take several hours to recover. Some patients may also have exercise-induced weakness at room temperature.

The sodium channel mutations in both conditions produce a gain-of-function abnormality due to disrupted inactivation or enhanced activation. Slowed fast inactivation increases the action potential duration, which increases the potassium efflux into the t-tubules, resulting in a larger after-depolarisation following each action potential which, when sufficient, triggers spontaneous action potentials leading to a train of myotonic discharges[1] .

In some PMC mutations, there is often an additional problem disrupting the final extent of inactivation, as well as a cold-induced left shift of the activation curve causing a greater, and more persistent, inward sodium current leading to more marked depolarisation and consequent weakness[2]. It would be helpful if these membrane abnormalities could be reliably studied *in vivo,* particularly as a potential tool for assessing the efficacy of any treatment interventions. A convenient, though necessarily indirect, method of inferring changes in muscle fibre membrane properties *in vivo* is to measure how the velocity of a muscle action potential depends on the time interval after a preceding action potential, i.e.

the muscle velocity recovery cycle (MVRC)[3]. Immediately after an action potential the muscle fibre is refractory, and then, during the muscle relative refractory period (MRRP) a second impulse is conducted more slowly. Following the MRRP, there is a supernormal period, when action potentials are conducted faster than normal, which lasts at least a second. The supernormal period comprises two functional components: early supernormality (ESN), which is related to an early depolarizing afterpotential, and late supernormality (LSN), which is related to a late depolarizing afterpotential, and unlike ESN is increased by multiple conditioning impulses. Like superexcitability in nerve, ESN in muscle is strongly dependent on membrane potential and sodium channel gating, since they depend primarily on the excess of inward sodium charge movement over outward potassium charge movement during the action potential. LSN has no equivalent in nerve, and is thought to relate to potassium accumulation in the t-tubule system[3] .

MVRCs, comprising MRRP, ESN and LSN, can be reliably measured in human muscles by direct stimulation and recording of a bundle of adjacent muscle fibres with a sequence of paired electrical pulses of equal amplitude and varying interstimulus interval (ISI)[3-5]. They indicate a rapid onset of membrane depolarization during limb ischemia[3] and have been used to infer muscle membrane depolarization in renal failure[6] and to provide information about membrane abnormalities in critical illness myopathy[7], Andersen-Tawil syndrome[8], myotonia congenita[9] and myotonic dystrophy[10].

In this study we have used MVRCs, supplemented by two previously described repetitive stimulation protocols[11] , to investigate the muscle membrane abnormalities in SCM and PMC. Our aim was to determine if these new methods could provide new insights into the pathophysiology of these sodium channelopathies, or provide new measures that could be useful as biomarkers.

METHODS

Patients

There were 9 patients with SCM and 8 with PMC (Table 1). The SCM patients were aged 45.6 ± 13.6 years (mean ± SD, range 22-67) and the PMC patients were aged 46.8 ± 18.4 years (range 21 to 72). All were genetically confirmed. The patients with PMC all had the same common Thr1313Met mutation, and 5 of the SCM patients had the Val1589Met mutation.

Asymptomatic Controls

The MVRC studies were compared with recordings from 26 healthy volunteers, 10 men, 16 women, aged 44.2 ± 12.3 years (range 27-66) who served as normal controls (NC).

Consent

Informed written consent was obtained from all patients and controls according to the Declaration of Helsinki. This study was approved by the St Thomas' Hospital Research Ethics Committee, London, UK.

Study Protocol

All the patients had standard nerve conduction studies, muscle velocity recovery studies, the short exercise test at room temperature, and a blood sample for electrolytes and glucose taken on the same day.

Short Exercise Test

Short exercise tests (SETs) were performed by stimulating the ulnar nerve at the wrist and recording with surface electrodes over the abductor digiti minimi. Compound muscle action potentials (CMAPs) were recorded at baseline and every 10s during 3 short exercise trials (10s exercise followed by 60s rest). The amplitude changes from baseline were calculated

and plotted as described previously[12]. The SET Pattern was given a number as described by Fournier and colleagues[13] - Pattern 1 : Decrement without recovery during the 60s rest period, typically worsening with consecutive exercise trials; Pattern 2: Transient decrement with recovery during each 60s rest period; often becoming less marked with subsequent exercise trials; Pattern 3: no decrement.

Muscle velocity recordings

Experimental setup

The recording technique was as described previously for tibialis anterior (TA)[8-10], and will be briefly summarized below. For details of the muscle velocity recovery cycle and ramp protocols, please see Tan et al 2014[9], and for details of the repetitive stimulation protocol, please see Tan et al 2012[8].

Recordings were performed from the distal third of TA. Stimuli consisting of 0.05 ms rectangular current pulses were delivered through an insulated monopolar needle electrode with a surface electrode as anode. Muscle activity was recorded by means of a concentric needle electrode approximately 20 mm proximal to the stimulating needle. Patients were studied in a heated room and kept warm in an effort to achieve a skin temperature as near as possible to 32 deg C at the start of the study. Surface temperature over TA was recorded at the end of the recording.

The signal was amplified (gain 300-1000, bandwidth 0.2 Hz to 3 kHz) and digitized (NIDAQ-6062E, National Instruments Europe Corp.) using a sampling rate of 20 kHz. The needle electrodes were adjusted to obtain a stable negative peak response with a stimulus of 3-10 mA. Stimulation and recording were controlled by Qtrac software (written by H. Bostock, Institute of Neurology, London, UK), using the 1200RCMQ.QRP recording protocol.

Muscle Velocity recovery cycles (MVRCs) at rest

MVRCs were recorded following 1, 2, and 5 conditioning stimuli (10 ms apart). Test stimuli were delivered every 2s. The inter-stimulus interval (ISI) between the last conditioning stimulus and the test stimulus was reduced from 1000 to 1.4 ms in 34 steps in an approximately geometric series.

Frequency ramp.

In order to characterize the effects of progressive muscle activation, a 1-sec train of stimuli was delivered every 2 sec, with the number of stimuli in the train increased (ramped up in frequency) by 1 from 2 to 31 in successive 2-sec cycles[11]. During the frequency ramp, the mean stimulation rate increased from 1 to 15.5 Hz over 1 minute, and responses were measured to the first and last stimuli in each train. Stimulus cycles with the test stimulus alone were recorded before the frequency ramp (10 cycles at 0.5Hz) and for 30 sec after the end of the ramp (15 cycles at 0.5 Hz).

Repetitive stimulation.

The effects of intermittent high frequency electrical stimulation on muscle membrane properties was studied by recording repeated short (18-step) MVRCs and then interleaving the conditioning and test stimuli with 1s trains of stimuli at 20 Hz as previously described in detail[8,11]. Four MVRCs were initially recorded at rest (each providing estimates of MRRP and ESN), then 5 MVRCs were measured during intermittent 20 Hz stimulation for 6 minutes, and further MVRCs were recorded during recovery for at least 5 minutes.

Data analysis

Recovery cycle data were analyzed by the QtracP program, as previously described[3,4]. After filtering the responses, latencies were measured from the start of the test stimulus to the negative peak of the muscle action potential. The effects of 1, 2, and 5

conditioning pulses on the latency of the test response were calculated as percentage differences compared to the responses to the test stimulus alone.

The following excitability measures were derived from the 3 recording protocols:

a) MVRCs at rest. The MRRP was defined as the earliest (interpolated) ISI at which the latencies of the conditioned and unconditioned test responses were identical. Early supernormality (ESN) was measured as the largest percentage decrease in latency for ISIs below 15 ms. Late supernormality (LSN) was the mean percentage decrease in latency for ISIs between 50 and 150 ms. We defined 'supernormality at 20 ms' (SN20) as the mean of supernormalities at 18 and 22 ms, 5ESN as the early supernormality after 5 conditioning impulses, and 'residual supernormality' (RSN) as the mean percentage decrease in latency at the end of the sweep, averaged for ISIs of 900 and 1000 ms. MSN is mean supernormality, i.e. average latency reduction between RRP and 1 sec, corresponding to area under curve when plotted with linear ISI axis. We also defined the 'extra' supernormalities 5XLSN, 5XMSN, and 5XRSN as the differences between the percentage latency decreases for 5 and 1 conditioning stimuli. Supernormality to 5 conditioning stimuli at 20ms' (5SN20) was defined as the mean of supernormalities at 18 and 22ms to 5 conditioning stimuli.

In our earlier study of MC patients, we found that those on mexiletine were distinguished by having significantly smaller values of 5ESN (early supernormality after 5 conditioning pulses) [9]. To check whether inclusion of these patients was materially affecting the results, we compared 5ESN values inclusive and exclusive of these patients.

b) Frequency ramp. We measured the latency of the negative peak of the muscle action potential, expressed as a percentage of baseline latency, at 15Hz [Lat(15Hz)] and 30Hz [Lat(30Hz)] during the ramp, and 30s after the end of the ramp Lat(30Hz+30s)%. During the ramp, responses to the first and last stimuli in the train were designated with the subscript $_{First}$ or $_{Last}$ respectively.

c) Repetitive stimulation. The MVRC measurements taken during intermittent 20 Hz stimulation were: changes in peak amplitude and latency, MRRP and ESN. These values were averaged for the first two (1,2) and the last two (4,5) cycles of intermittent 20 Hz stimulation, and for cycles 1-3 during recovery after the end of repetitive stimulation.

Statistics

We used the Welch rank test (non-parametric unequal variance t-test) for intergroup comparisons. When comparing groups with multiple comparisons or correlations, only $P<0.01$ was considered significant, but for discussion, $P<0.05$ is mentioned when relevant for individual tests.

RESULTS

Nerve conduction studies

None of the patients had evidence of a generalised large fibre neuropathy.

Short exercise test

The results of the SETs at room temperature are detailed in Table 1. The SET performed on the day of the muscle excitability studies showed a progressive decrement typical of PMC (Pattern 1) in 6 of 8 patients with PMC. Two patients with SCM showed a fluctuating decrement which did not correspond to Patterns 1 or 2. The remaining patients showed no abnormal decrement (Pattern 3).

Velocity recovery cycles

The results of the MVRCs with 1, 2 and 5 conditioning stimuli are illustrated in Figure 1, and the measurements are compared in Table 2. The most striking feature, which is common to both SCM and PCM patients, is an increase in supernormality which becomes

more pronounced and prolonged by additional conditioning stimuli, so that with 5 conditioning stimuli there are highly significant increases, compared with controls, in early supernormality (5ESN), and also in the extra late supernormality (5XLSN) and extra mean supernormality over 1s (5XMSN) compared with a single conditioning impulse. The differences between SCM and PMC on the one hand, and MC on the other, were most marked in the case of the extra residual supernormality at 900-1000ms (5XRSN: NC = 1.0%, SCM and PMC = 1.7%, MC = 2.8%).

The main difference between the recovery cycles of SCM and PMC patients was in their relative refractory period (MRRP), and time to peak supernormality (ESN@). In this respect the SCM patients showed a significantly quicker post-spike recovery of excitability than either the controls or the PMC patients.

In this study only one SMC patient and 1 PMC patient were under the influence of mexiletine at the time of the recordings. In both cases, 5ESN values for the patient on mexiletine were actually above the average of the others, and mean values were not appreciably affected: SMC 16.9 including and 16.7 excluding mexiletine; PMC 15.5 including and 15.2 excluding mexiletine; all values well above 13.0 for the normal controls. Because of the limited numbers of patients, and the absence of any clear effect of mexiletine, the two patients on mexiletine were included in the remaining analyses.

Frequency Ramp

The results of increasing the stimulation rate up to an average of 15.5 Hz, by adding intermittent 1s trains of conditioning stimuli at frequencies from 1 to 30 Hz are illustrated for an SCM patient in Figure 2A, for a PMC patient in Figure 2B, and the average changes in

latency and peak amplitude compared with the normal control subjects in Figure 3 and Table 3.

This stimulation protocol produced only modest changes in amplitude for the SCM patients, as for the controls (Figs. 2A and 3). In contrast there were marked changes in the PMC group, in all of whom the amplitude of the responses dropped during the ramp and only partially recovered afterwards, as shown in Figs. 2B and 3. Because of the almost complete loss of amplitude during the high-frequency trains, it was not possible to measure latencies for most of the PMC patient for the last action potential in each train for the latter part of the frequency ramp. There was, however, always some recovery by the time the next train started 1s later, so latencies for the first action potential in each train were always measurable.

In the SCM group, the main difference from the controls was a greater reduction in latency at 15Hz, especially at the end of the 15Hz train, indicating a greater than normal depolarisation.

Repetitive stimulation

The results of interspersing a 1 s train of 20 stimuli between recovery cycle measurements for a period of 6 m are illustrated in Figure 4 and measurements listed in Table 4. As with the frequency ramp, the most conspicuous abnormality was seen in the responses of the PMC patients, which suffered a severe loss of amplitude when the 20 Hz trains were started, which made the latencies of many of the subsequent responses unmeasurable.

In the SMC group, the abnormally short RRPs and high ESN values were maintained throughout the period of repetitive stimulation. The most distinctive feature was a failure to show the normal decrease in supernormality during the repetitive stimulation, so that the

most significant abnormality in our recordings from these patients was the value of ESN during cycles 4 and 5 .

Separation of myotonia subtypes

Supplementary Figure 1 explores the potential use of the various MVRC and other measurements to distinguish between the different types of myotonia studied so far. Supplementary Figure 1A shows that the drop in amplitude of the muscle action potentials during the frequency ramp separates the PMC patient from the other groups, including the previously studied 11 MC patients that were not on medication[9]. Combinations of MVRC and frequency ramp measurements provided separation between the 3 myotonia subtypes (Supplementary Figure 1B) or between PMC, MC and normal controls (Supplementary Figure 1C). In most measurements there was considerable overlap between the SMC and NC groups, and combinations of MVRC, frequency ramp and repetitive stimulation measurements were needed to separate them (Supplementary Figure 1D).

DISCUSSION

Conduction changes related to myotonia and weakness

We have previously reported that in myotonia congenita (MC) the lack of functional chloride channels results in increased early, late and residual supernormality, indicating an enhanced depolarizing afterpotential, which increases and becomes prolonged with trains of impulses[9]. These changes are in the direction expected to facilitate repetitive myotonic discharges, although such activity was not detected in the small columns of muscle fibres studied. Rather similar increases in early, late and residual supernormality were seen in both the SCM and PMC groups of patients. Although less pronounced than in MC, (e.g. 5XLSN: NC = 7.0%, SCM and PMC = 9.1%, MC = 10.1%; 5XMSN: NC = 3.5%, SCM and PMC = 4.4%, MC =

5.8%) it seems reasonable to conclude that the enhanced depolarizing afterpotentials that give rise to the supernormality provide the trigger for myotonia in these patients also.

Since MRRP and ESN@ are strongly temperature-dependent, it is important to note that the skin temperatures recorded in the SCM patients (30.2 ± 0.4˚C, mean ± SE) were no warmer than those in the controls (30.4 ± 0.2˚C) or the PMC patients (30.6 ± 0.7˚C).

In our previous study on MC patients[9], Mexiletine was associated with a reduced 5ESN. In this study, the SCM and PMC patient on Mexiletine each had 5ESN values above the average in their respective groups. One possible explanation for the difference being in the opposite direction to that expected compared with the patients not on Mexiletine, is that the two patients taking regular mexiletine were particularly clinically symptomatic, and it is possible that their symptoms reflected the degree of channel dysfunction, and therefore even treatment with mexiletine did not result in reduction of the 5ESN below the mean for the rest of the group.

Weakness is a feature of PMC but not of SCM. Whereas very little difference is seen between these two groups in the effects of 1 to 5 conditioning stimuli, the biophysical basis for the weakness in PMC is clearly seen in the effects of the frequency ramp and repetitive stimulation in Figs. 2 - 4. In all 8 patients there was a similar rapid decline in amplitude of the muscle action potentials at frequencies above about 10 Hz. The lack of full recovery in the amplitude after the end of the ramp accounts for the weakness that can continue in PMC patients following exercise. Since the latency returned to normal, it appears that the population of muscle fibres was split by the frequency ramp: about half the fibres recovered to normal resting potential, while the remainder stayed sufficiently depolarized to be inexcitable. This bistability of the membrane potential can be explained by the sodium channel mutation, as discussed below.

Unlike the SET at room temperature, where 2 of the 8 PMC patients failed to show an abnormality, the frequency ramp demonstrated a clear abnormality in all 8 of these patients, as did the later intermittent stimulation at 20 Hz.

Relationship of conduction changes to sodium channel mutations

The larger ESN, indicating an increased depolarizing afterpotential, in both SCM and PMC groups is consistent with a lengthened duration of the muscle action potential due to a slower rate of entry into inactivation (SCM and PMC), and slowed deactivation in SCM[14-16]. In spite of this, the relative refractory period (MRRP) was significantly shorter than normal in the SCM group. This can be explained by the faster recovery of the mutant sodium channel from inactivation, which has been found for Val1589Met mutations (the most common among our SCM patients) expressed in human embryonic kidney cells *in vitro*7. In the case of the PMC patients, their Thr1313Met mutation has been reported to induce both slowed inactivation[18] and faster recovery from inactivation[16] and these effects appear to largely cancel out.

The most striking differences between the SCM and PMC mutations was in their effects on repetitive stimulation in the frequency ramp. At high frequencies the PMC muscle fibres became depolarized to inexcitability, and after the train only about half the fibres recovered their membrane potential, as discussed above. In the fibres that remained depolarized, a continuous source of depolarizing current, sufficient to outweigh the repolarizing potassium and chloride currents, was presumably provided by the mutant sodium channels. Consistent with this, all the PMC patients in this study had the Thr1313Met mutation, which has been shown by *in vitro* heterologous expression experiments to generate unusually large persistent (non-inactivating) sodium currents[16]. The persistent sodium currents are regenerative, and enable the muscle fibres to exist in two

stable states: either at a normal resting potential with the currents switched off, or held depolarized and inexcitable by strongly activated persistent currents. It seems that the muscle fibres that recovered from the frequency ramp protocol were not inherently resistant to this regenerative depolarization, since when they were subjected to a further bout of repetitive stimulation with the intermittent 20 Hz protocol (which always followed the frequency ramp), there was a further substantial drop in amplitude, which was again irreversible for the duration of the experiment (Fig. 4C).

In conclusion, although it is of interest that multiple measurements are able to separate different myotonia subtypes, we do not advocate the use of these techniques as a diagnostic utility. Muscle channelopathy gene panels have become increasingly more widely available and affordable, and there is a decreasing requirement for electrodiagnostic guides to genetic testing. Instead, the strength of these techniques is in providing a means of studying the effects of the muscle ion channel mutations in vivo. In this study, we have found that abnormalities in mutant sodium channel function can be demonstrated *in vivo* in human subjects using muscle excitability studies. In earlier publications[8-10] we have shown that alterations in chloride and potassium channel function are also demonstrable using this stimulation paradigm. The method is minimally invasive (the stimulation setup is similar to stimulated single fibre EMG studies), relatively painless, and can be easily repeated in the same individual over time. It is therefore suitable for use as a biomarker in future treatment trials.

Abbreviations

CMAP: compound muscle action potential

ESN: early supernormality

ISI: inter-stimulus interval

LSN: late supernormality

MC: myotonia congenita

MRRP: muscle relative refractory period

MSN: mean supernormality

MVRC: muscle velocity recovery cycle

PMC: paramyotonia congenita

RSN: residual supernormality

SET: short exercise test

SCM: sodium channel myotonia

SN20: Supernormality at 20ms (mean of supernormalities at 18 and 22 ms)

TA: tibialis anterior

5ESN: early supernormality after 5 conditioning stimuli

5SN20: Supernormality at 20ms after 5 conditional stimuli

5XMSN: extra mean supernormality after 5 conditioning stimuli

5XLSN: extra late supernormality after 5 conditioning stimuli

5XRSN: extra residual supernormality after 5 conditioning stimuli

REFERENCES

1. Cannon SC. Pathomechanisms in channelopathies of skeletal muscle and brain. Annual review of neuroscience 2006;29:387-415.

2. Mohammadi B, Mitrovic N, Lehmann-Horn F, Dengler R, Bufler J. Mechanisms of cold sensitivity of paramyotonia congenita mutation R1448H and overlap syndrome mutation M1360V. The Journal of physiology 2003;547(Pt 3):691-698.

3. Z'Graggen W J, Bostock H. Velocity recovery cycles of human muscle action potentials and their sensitivity to ischemia. Muscle & nerve 2009;39(5):616-626.

4. Z'Graggen WJ, Troller R, Ackermann KA, Humm AM, Bostock H. (2011) Velocity recovery cycles of human muscle action potentials: repeatability and variability. Clinical Neurophysiology 2011;122:2294-9.

5. Bostock H, Tan SV, Boërio D, Z'Graggen WJ. Validity of multi-fiber muscle velocity recovery cycles recorded at a single site using submaximal stimuli. Clinical Neurophysiology 2012;123: 2296-305.

6. Z'Graggen WJ, Aregger F, Farese S, Baumann C, Bostock H. Velocity recovery cycles of human muscle action potentials in chronic renal failure. Clinical Neurophysiology 2010;121:874-81.

7. Z'Graggen W, Bander L, Tuchscherer D, Scheidegger O, Brunello AG, Passath C, Takala J, Bostock H. Muscle membrane dysfunction in critical illness myopathy assessed by velocity recovery cycles. Clinical Neurophysiology 2011;122:834-41.

8. Tan SV, Z'Graggen W J, Boerio D, Rayan DL, Howard R, Hanna MG, Bostock H. Membrane dysfunction in Andersen-Tawil syndrome assessed by velocity recovery cycles. Muscle & nerve 2012;46(2):193-203.

9 . Tan SV, Z'Graggen WJ, Boerio D, Rayan DR, Norwood F, Ruddy D, Howard R, Hanna MG, Bostock H. Chloride channels in myotonia congenita assessed by velocity recovery cycles. Muscle & nerve 2014;49(6):845-857.

10. Tan SV, Z'Graggen W J, Boerio D, Turner C, Hanna MG, Bostock H. In vivo assessment of muscle membrane properties in myotonic dystrophy. Muscle & nerve 2016;54(2):249-257.

11. Boerio D, Z'Graggen WJ, Tan SV, Guetg A, Ackermann K, Bostock H. Muscle velocity recovery cycles: effects of repetitive stimulation on two muscles. Muscle & nerve 2012;46(1):102-111.

12. Tan SV, Matthews E, Barber M, Burge JA, Rajakulendran S, Fialho D, Sud R, Haworth A, Koltzenburg M, Hanna MG. Refined exercise testing can aid DNA-based diagnosis in muscle channelopathies. Annals of neurology 2011;69(2):328-340.

13. Fournier E, Arzel M, Sternberg D, Vicart S, Laforet P, Eymard B, Willer JC, Tabti N, Fontaine B. Electromyography guides toward subgroups of mutations in muscle channelopathies. Annals of neurology 2004;56(5):650-661.

14. Lerche H, Heine R, Pika U, George AL, Jr., Mitrovic N, Browatzki M, Weiss T, Rivet-Bastide M, Franke C, Lomonaco M, et al. Human sodium channel myotonia: slowed channel inactivation due to substitutions for a glycine within the III-IV linker. The Journal of physiology 1993;470:13-22.

15. Mitrovic N, George AL, Jr., Lerche H, Wagner S, Fahlke C, Lehmann-Horn F. Different effects on gating of three myotonia-causing mutations in the inactivation gate of the human muscle sodium channel. The Journal of physiology 1995;487(1):107-114.

16. Hayward LJ, Brown RH, Jr., Cannon SC. Inactivation defects caused by myotonia-associated mutations in the sodium channel III-IV linker. The Journal of general physiology 1996;107(5):559-576.

17. Mitrovic N, George AL, Jr., Heine R, Wagner S, Pika U, Hartlaub U, Zhou M, Lerche H, Fahlke C, Lehmann-Horn F. K(+)-aggravated myotonia: destabilization of the inactivated state of the human muscle Na+ channel by the V1589M mutation. The Journal of physiology 1994;478 Pt 3:395-402.

18. Yang N, Ji S, Zhou M, Ptacek LJ, Barchi RL, Horn R, George AL, Jr. Sodium channel mutations in paramyotonia congenita exhibit similar biophysical phenotypes in vitro. Proceedings of the National Academy of Sciences of the United States of America 1994;91(26):12785-12789.

Table 1. Myotonias due to sodium channel mutations: list of patients

Pt	SET Pattern	SET RT Abnormal (Y/N) #	Max amp decrement (%)	Diag	Gene	Amino acid change	Gender	Age	Rx	Rx
1	1	Y	77	PMC	SCN4A	Thr1313Met	M	55	Mexiletine	+
2	1	Y	64	PMC	SCN4A	Thr1313Met	F	42	None	-
3	1	Y	28	PMC	SCN4A	Thr1313Met	M	68	(Mexiletine) ‡	-
4	1	Y	49	PMC	SCN4A	Thr1313Met	M	72	None	-
5	3	N	9	PMC	SCN4A	Thr1313Met	M	49	(Mexiletine)	-
6	1	Y	31	PMC	SCN4A	Thr1313Met	M	41	(Mexiletine)	-
7	1	Y	78	PMC	SCN4A	Thr1313Met	F	21	None	-
8	3	N	9	PMC	SCN4A	Thr1313Met	M	25	None	-
9	3	N	0	SCM	SCN4A	Val1589Met	M	45	None	-
10	3†	?	16	SCM	SCN4A	Val1293Ile	M	40	(Mexiletine)	-
11	3	N	0	SCM	SCN4A	Gly1306Ala	F	36	(Mexiletine)	-
12	3	N	1	SCM	SCN4A	Gly1306Ala	M	67	Mexiletine	+
13	3	N	0	SCM	SCN4A	Leu128Pro	F	56	(Mexiletine)	-
14	3	N	2	SCM	SCN4A	Val1589Met	F	57	None	-
15	3†	?	40	SCM	SCN4A	Val1589Met	M	37	Quinine	+
16	3	N	0	SCM	SCN4A	Val1589Met	F	50	None	-
17	3	N	0	SCM	SCN4A	Val1589Met	M	22	None	-

Pt: patient; SET RT: short exercise test at room temperature; Y:yes; N:no; max: maximum; amp: amplitude; Diag: diagnosis; TW: transient weakness; PMC: paramyotonia congenita; SCM: sodium channel myotonia. # Upper limit of normal for amplitude-only decrement at room temperature = 11.5% (Tan et al, 2011). † Fluctuating decrement with no clear pattern of change (did not fit patterns 1 or 2). ‡Medication in parentheses and Rx designated '-' if the medication had been omitted for >5 times the half life ($T^{½}$) of the drug at the time of the study ($T^{1/2}$ mexilitine 10-12 h, $T^{1/2}$ quinine sulfate 12-18 h,).

Table 2. Velocity recovery cycle measurements compared between the three groups.

	(Mean ± SE)			(*P* for Welch rank test)		
	NC (*n* = 26)	SCM (*n* = 9)	PMC (*n* = 8)	NC v SCM	NC v PMC	SCM v PMC
MRRP (ms)	3.70 ± 0.12	3.05 ± 0.13	3.83 ± 0.11	**0.0045**	0.11	**0.0007**
ESN (%)	11.19 ± 0.44	13.37 ± 0.61	12.25 ± 0.48	**0.0076**	0.087	0.18
ESN@ (ms)	7.98 ± 0.23	6.94 ± 0.24	8.92 ± 0.35	**0.0088**	0.038	**0.00015**
5ESN (%)	13.01 ± 0.53	16.91 ± 0.52	15.53 ± 0.65	**1.7×10^{-5}**	**0.0068**	0.17
SN20 (%)	6.44 ± 0.28	7.57 ± 0.34	8.25 ± 0.28	0.018	**6.4×10^{-6}**	0.40
5SN20 (%)	12.25 ± 0.52	14.88 ± 0.71	15.39 ± 0.72	**0.0063**	**0.0018**	0.71
LSN (%)	3.68 ± 0.17	3.91 ± 0.25	4.46 ± 0.31	0.40	0.052	0.18
5XLSN (%)	7.03 ± 0.31	9.12 ± 0.50	9.14 ± 0.32	**0.0016**	**0.00060**	0.89
MSN (%)	1.28 ± 0.05	1.29 ± 0.08	1.362± 0.10	0.89	0.015	0.023
5XMSN (%)	3.40 ± 0.15	4.39 ± 0.32	4.36 ± 0.12	**0.0056**	**9.7×10^{-5}**	1.00
RSN (%)	0.13 ± 0.04	-0.11 ± 0.08	0.23 ± 0.79	**0.0096**	0.037	**0.0029**
5XRSN (%)	0.99 ± 0.08	1.72 ± 0.29	1.71 ± 0.24	0.019	**0.0017**	0.89

NC = normal controls. SCM = sodium channel myotonia. PMC = paramyotonia congenital. MRRP = muscle relative refractory period. ESN = early supernormality (up to 15 ms). ESN@ = inter-stimulus interval for maximum ESN. SN20 = supernormality at 20 ms. 5SN20 = SN20 after 5 conditioning stimuli. LSN = late supernormality (50-150 ms). MSN = mean supernormality (up to 1 s). RSN = residual supernormality (900-1000 ms). 5XLSN etc = extra supernormality after 5 conditioning stimuli compared with 1 conditioning stimulus. P values of <0.01 are in bold type. Values listed are means ± standard errors of the mean.

Table 3. Frequency ramp measurements compared between the three groups.

	(Mean ± SE)			(*P* for Welch rank test)		
	NC (*n* = 25)	SCM (*n* = 9)	PMC (*n* = 8)	NC v SCM	NC v PMC	SCM v PMC
$Lat(15Hz)_{First}$%	94.0 ± 0.6	92.2 ± 0.6	93.0 ± 1.1	0.014	0.35	0.35
$Lat(15Hz)_{Last}$%	84.8 ± 0.7	80.7 ± 0.6	81.8 ± 1.1	**0.00015**	0.039	0.75
$Lat(30Hz)_{First}$%	94.9 ± 0.7	93.5 ± 1.3	104.4 ± 2.4	0.35	**6.2×10^{-7}**	**9.3×10^{-6}**
$Lat(30Hz)_{Last}$%	89.0 ± 1.1	83.7 ± 1.7	-	0.032	-	-
Lat(30Hz+30s)%	101.7 ± 0.3	102.2 ± 0.7	101.4 ± 1.9	0.85	0.78	0.89
$Pk(15Hz)_{First}$%	111.5 ± 2.4	109.2 ± 5.9	96.6 ± 9.1	0.71	0.18	0.35
$Pk(30Hz)_{First}$%	115.0 ± 2.9	116.5 ± 10.5	39.6 ± 6.4	0.73	**4.6×10^{-10}**	**9.5×10^{-6}**
$Pk(30\text{-}15Hz)_{First}$%	3.4 ± 1.8	7.3 ± 5.3	-57.0 ± 11.6	0.79	**1.2×10^{-9}**	**9.5×10^{-6}**
$Pk(30Hz)_{Last}$%	87.5 ± 5.1	86.1 ± 18.4	3.5 ± 5.4	0.66	**1.2×10^{-9}**	**6.0×10^{-5}**
Pk(30Hz+30s)%	104.5 ± 2.2	110.3 ± 6.3	50.5 ± 7.6	0.70	**2.6×10^{-9}**	**9.5×10^{-6}**

$Lat(15Hz)_{First}$% = percentage change in latency for first muscle action potential of 15 Hz train. $Pk(30Hz)_{Last}$% = peak amplitude for last action potential in 30Hz train as percentage of control. Pk $(30\text{-}15Hz)_{First}$% = difference in peak amplitude of first action potentials at 15 and 30 Hz. Pk(30Hz+30s)% = peak amplitude 30s after end of 30Hz train. Gaps for $Lat(30Hz)_{Last}$% are because some of the PMC responses were too small to measure. Values and statistics are as in Table 2.

Table 4. Repetitive stimulation measurements compared between the three groups.

	(Mean ± SE)			(*P* for Welch rank test)		
	NC (*n* = 24)	SCM (*n* = 9)	PMC (*n* = 8)	NC v SCM	NC v PMC	SCM v PMC
Peak (% baseline, start of 20 Hz)						
Cycles 1,2	106.3 ± 1.6	102.5 ± 6.7	62.1 ± 8.0	0.84	**6.2×10^{-9}**	**0.00070**
Cycles 4,5	99.1 ± 2.9	100.8 ± 10.3	39.8 ± 6.7	0.91	**8.3×10^{-10}**	**2.8×10^{-5}**
Recovery	86.8 ± 3.6	86.9 ± 7.0	33.6 ± 7.3	0.67	**1.0×10^{-7}**	**2.8×10^{-5}**
Latency (% baseline, start of 20 Hz)						
Cycles 1,2	98.6 ± 0.3	98.7 ± 0.6	-	0.90	-	-
Cycles 4,5	103.1 ± 0.6	102.6 ± 1.1	-	0.82	-	-
Recovery	102.6 ± 0.4	100.3 ± 0.8	-	0.047	-	-
MRRP (ms)						
Baseline	3.94 ± 0.16	3.24 ± 0.09	4.64 ± 0.26	**0.0010**	**0.0020**	**8.8×10^{-6}**
Cycles 1,2	5.91 ± 0.29	4.59 ± 0.33	-	0.016	-	-
Cycles 4,5	5.48 ± 0.26	4.16 ± 0.23	-	**0.0012**	-	-
Recovery	4.04 ± 0.11	3.59 ± 0.14	-	0.013	-	-
ESN (%)						
Baseline	10.56 ± 0.45	12.87 ± 0.59	8.56 ± 0.86	**0.0098**	0.079	**0.00032**
Cycles 1,2	7.13 ± 0.46	11.99 ± 1.07	-	**0.00064**	-	-
Cycles 4,5	9.03 ± 0.42	12.70 ± 0.63	-	**1.4×10^{-6}**	-	-
Recovery	10.91 ± 0.41	11.07 ± 0.59	-	0.81	-	-

MRRP = muscle relative refractory period. ESN = early supernormality. Gaps occur where some PMC responses were too small to measure latencies. Values and statistics are as in Table 2.

Figure Legends

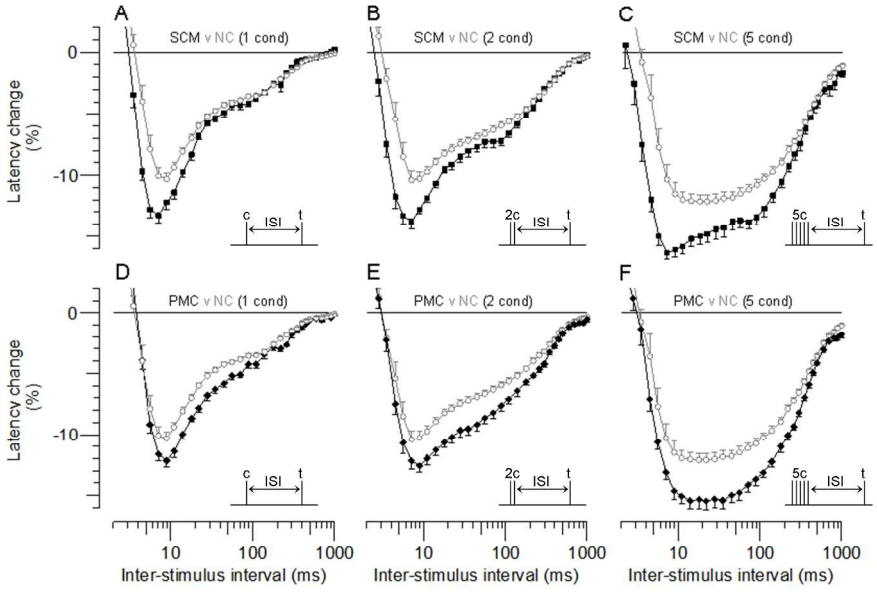
FIGURE 1. Muscle velocity recovery cycles (MVRCs) with 1, 2 and 5 conditioning stimuli. A: MVRCs in sodium channel myotonia (SCM, n=9, filled black squares) compared with normal controls (NC, n=26, open grey circles). Percentage change in latency is plotted against ISIs from 2 to 1000 ms (logarithmic scale). Inset shows relationship between single conditioning (c) and test (t) stimuli. Data points show means with single standard error bar. B,C: As A, but with 2 (2c) and 5 (5c) conditioning stimuli 10ms apart. D-F: corresponding plots comparing paramyotonia congenita patients (PMC, n=8, filled black circles) and normal controls.

FIGURE 2. Frequency ramp recordings in a patient with sodium channel myotonia (SCM) (A) and a patient with paramyotonia congenita (PMC)(B). In each part the main panel shows the responses during the 1s period of stimulation, delivered at intervals of 2s. The number of stimuli in 1s increases from 2 in the top trace to 31 in the penultimate trace, and drops back to 1 in the last trace. To the right of the main panel the responses to the first and last stimuli in each train are plotted on expanded time scales. In these traces the stimulus artefact (a) is visible as well as the muscle action potential (m). In the main panels the stimulus artefacts are suppressed for simplicity and to reveal the changing amplitudes of the muscle responses in the PMC patient more clearly.

FIGURE 3. Frequency ramp responses of sodium channel myotonia (SCM) and paramyotonia congenita (PMC) patients compared with those of normal controls (NC). Solid lines are means, dotted lines are means ± standard error. (A) Normal controls (n=26), (B) Sodium channel myotonia patients (n=9) plotted in black with controls in gray (means only), (C) Paramyotonia congenita patients (n=8) plotted in black with controls in gray. *Top row*: latency as percent of baseline for the last response in each train; *second row*: latency as % baseline for the first response in each train; *third row:* peak amplitude as % baseline for the

last response in each train; *fourth row*: peak amplitude as % baseline for the first response in each train; *bottom row*: stimulation rate during the train. Asterisk in C indicates time when some muscle action potentials became too small to measure latencies.

FIGURE 4. Effects of repetitive stimulation on muscle action potential amplitudes, latencies, and muscle velocity recovery cycles. **A**: Part of sequence of stimulating pulses, showing alternation between 1s trains at 20 Hz, conditioning(c) and test(t) pairs, and test stimuli alone. **B-D**: Average recordings from 26 normal control subjects (NC)(B), 9 patients with sodium channel myotonia (SCM)(C), and 8 patients with paramyotonia congenita (PMC)(D). Data are reported as mean (continuous line) ± standard error (broken lines). Comparisons in (C) and (D) are against mean control values in gray. *Top two panels*: Amplitude changes as a percentage of baseline of the first and last responses in the train respectively. *Third panel*: Latencies, expressed as % baseline value. *Fourth panel*: Relative refractory periods. *Bottom panel*: Peak early supernormality, expressed as % reduction in latency. Bars indicate 6m periods of intermittent 20 Hz stimulation. Asterisks in D indicate times when muscle action potentials in some PMC patients became too small to measure latencies.



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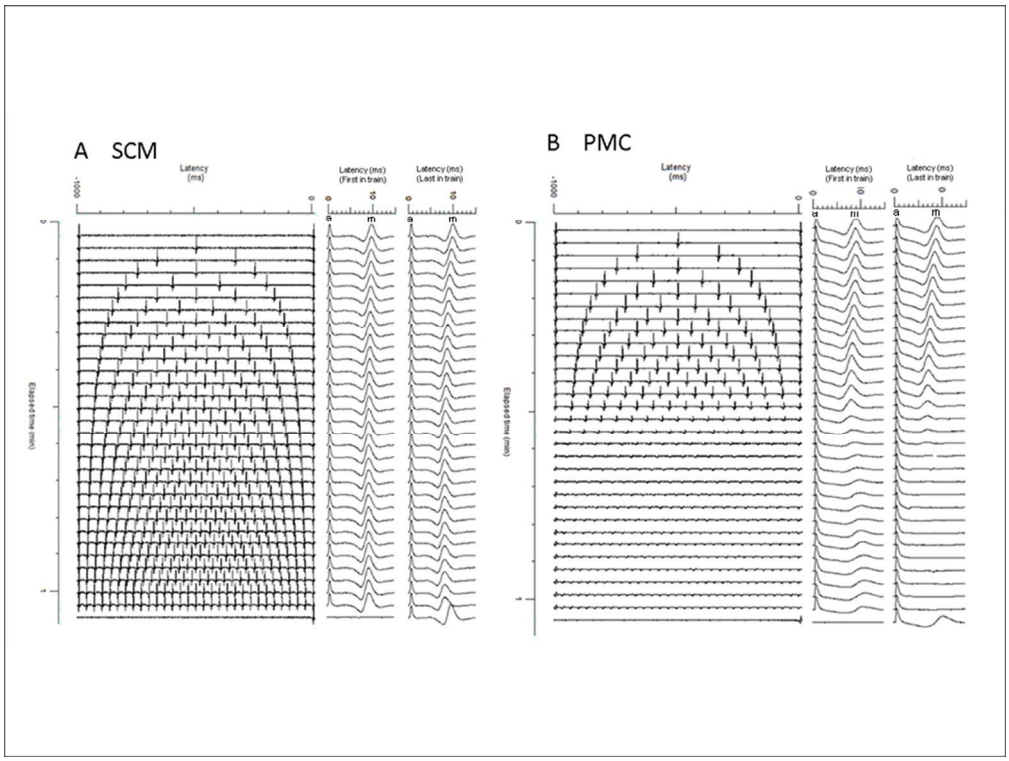

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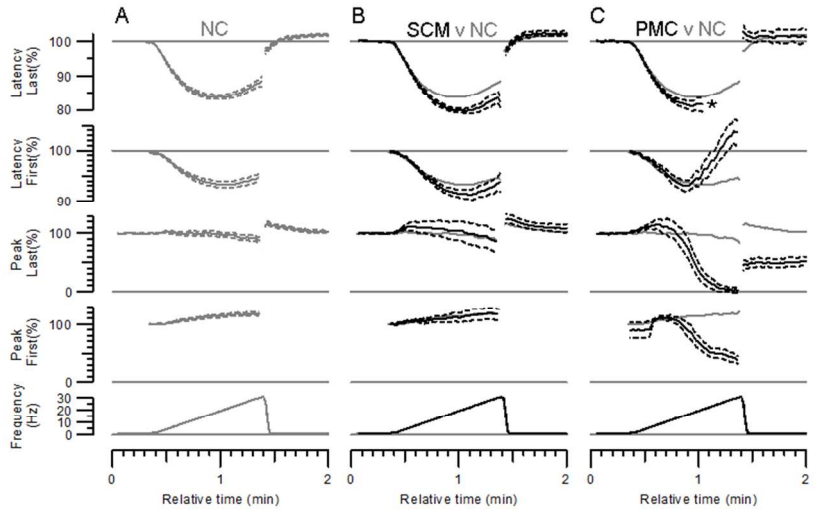

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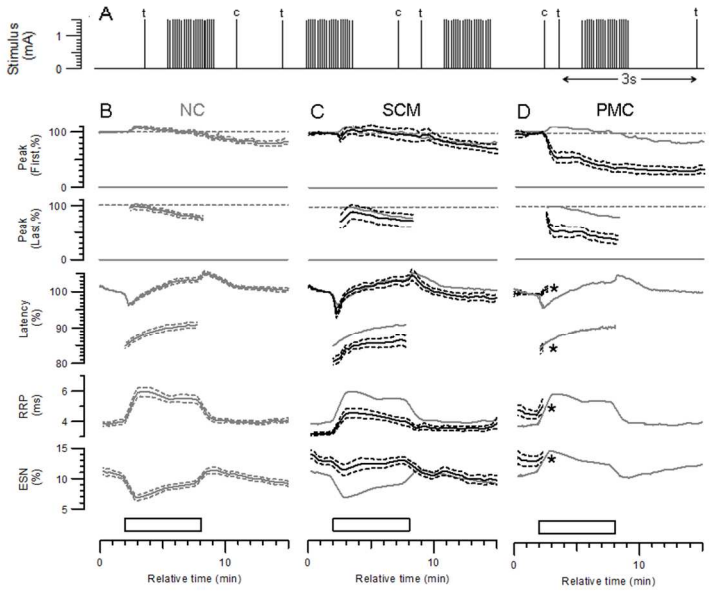

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