Measurement of cortical, nerve, and muscle excitability in early phase clinical drug development

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CHAPTER 5

MUSCLE VELOCITY RECOVERY CYCLES AS PHARMACODYNAMIC BIOMARKER: EFFECTS OF MEXILETINE IN A RANDOMIZED DOUBLE-BLIND PLACEBO-CONTROLLED CROSS-OVER STUDY

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ABSTRACT

Measuring muscle velocity recovery cycles (MVRC) is a method to obtain information on muscle cell excitability, independent of neuromuscular transmission. The goal was to validate MVRC as pharmacodynamic biomarker for drugs targeting muscle excitability. As proof-of-concept, sensitivity of MVRC to detect effects of mexiletine, a voltage-gated sodium channel (Na_V) blocker, was assessed. In a randomized, double-blind, two-way crossover study, effects of a single pharmacologically active oral dose of 333 mg mexiletine was compared to placebo in 15 healthy male subjects. MVRC was performed pre-dose, 3- and 5-hours post-dose using QTrac. Effects of mexiletine vs. placebo were calculated using a mixed effects model with baseline as covariate. Mexiletine had significant effects on MVRC when compared to placebo. Early supernormality after five conditioning stimuli was decreased by mexiletine (estimated difference (ED) -2.78% (95% confidence interval (CI): -4.16, -1.40); p=0.0003). Moreover, mexiletine decreased the difference in late supernormality after five vs. one conditioning stimuli (5XLSN) (ED -1.46% (95% CI: -2.26, -0.65); p=0.001). These results indicate that mexiletine decreases the percentage increase in velocity of the muscle fiber action potential after five conditioning stimuli, at long and short interstimulus intervals, which corresponds to a decrease in muscle membrane excitability. This is in line with the pharmacological activity of mexiletine, which leads to use-dependent $Na_V1.4$ blockade affecting muscle membrane potentials. This study shows that effects of mexiletine can be detected using MVRC in healthy subjects, thereby indicating that MVRC can be used as tool to demonstrate pharmacodynamic effects of drugs targeting muscle excitability in early phase drug development.

INTRODUCTION

Neuromuscular diseases (NMDs) have received growing attention in preclinical and clinical research in recent decades, which has led to increased understanding of these disorders. However, significant progress is still to be made where it comes to developing treatment options for these patients. An essential part of advancing treatments through (pre) clinical drug development towards therapy is the use of biomarkers, especially for these often complex disorders.[1] Such biomarkers should be tailored to specific NMDs, as they are a collection of rare disorders with a broad spectrum of underlying pathophysiology. However, despite their heterogeneity, a common feature for many of these diseases is direct or indirect muscle pathology, resulting in symptoms of muscle weakness and other muscle pathology. A biomarker that can characterize these defects and allows quantification of pharmacological effects, would therefore be of great value in drug development for a relevant subset of NMDs.

Muscle velocity recovery cycle (MVRC) measurements could be such a pharmacodynamic biomarker, as they evaluate muscle cell excitability *in vivo* and are considered to be independent of neuromuscular transmission.[2] The physiological muscle action potential is followed by early and late depolarizing afterpotentials, resulting in two periods of increased excitability. By applying one or more conditioning pulses before the test pulse, MVRC can indirectly quantify these afterpotentials as periods of increased velocity (supernormality).[2] Previous studies showed that MVRC was able to distinguish different types of NMD from healthy controls, indicating that the method has analytical and clinical validity. Abnormalities in MVRC endpoints were detected in critical illness neuropathy, Anderson Tawil syndrome, channelopathies, erythromelalgia, myotonic dystrophies, inclusion body myositis, hypo- and hyperkalemic periodic paralysis, sodium channel myotonias and myotonia congenita.[3–12]

However, to our knowledge, sensitivity of MVRC to detect (acute) pharmacodynamic effects has not been evaluated. Therefore, the primary aim of this study was to investigate whether MVRC could detect pharmacologically induced changes in muscle excitability in healthy subjects. As a proof-of-concept, we selected mexiletine as pharmacological intervention. Mexiletine is a use-dependent voltage-gated sodium (Na_V) channel blocker, thought to influence muscle excitability through blocking Na_V channels subtype 1.4 in skeletal muscle fibers.[13–15] As a

secondary objective, this study was set up to evaluate the feasibility and repeatability of MVRC for use in an early phase clinical drug study.

MATERIALS AND METHODS

This trial was approved by the Foundation 'Beoordeling Ethiek Biomedisch Onderzoek', an independent Ethics Committee based in Assen, The Netherlands. The trial was executed between January 2020 and March 2020, in accordance with the Declaration of Helsinki. The study was registered in the Dutch Trial Registry (Nederlands Trial Register, registration number NL8084).

STUDY DESIGN This was a randomized, double-blind, placebo-controlled, two-way cross-over study in healthy subjects. Subjects received a single dose of mexiletine 333 mg and matching placebo in randomized order on two separate study visits. Drug administrations were separated by a wash-out period of seven days. MVRC measurements were performed pre-dose and at two post-dose timepoints based on the pharmacokinetic (PK) profile of mexiletine. The first post-dose measurement was performed three hours post-dose (approximately T_{max}), the second at five hours post-dose (another measurement at expected high plasma concentrations of mexiletine), maximizing the power to detect a pharmacodynamic effect. Measurement conditions and mealtimes were standardized, and measurements were performed at approximately the same clock time, to avoid interference of diurnal variation or effects of food. A follow-up visit was performed five to nine days after the last dose administration.

No important changes were made to the methods or trial outcomes after study commencement.

STUDY POPULATION All subjects signed written informed consent before participation in the study. To confirm eligibility and health status, subjects were screened before participation, based on an interview of medical history, physical examination (including vital signs and electrocardiogram), and laboratory tests. Subjects were aged between 18 and 45 years, with a BMI between 18 and 30 kg/m^2 and a minimum weight of 50 kg. Subjects with active or chronic disease that could interfere with the safety or conduct of the study were excluded, particularly history of trauma to the lower extremities or other conditions that could interfere with the MVRC measurements. The use of medication, dietary supplements, CYP-enzyme containing products, alcohol and caffeine were prohibited during the study. Subjects with history of addictive substance abuse were excluded, and drug- and alcohol tests were performed to determine current use of these substances. Excessive exercise was prohibited within 72 hours before dosing.

STUDY DRUGS, RANDOMIZATION, AND BLINDING Mexiletine (Namuscla, 167 mg, Lupin Europe GMBH) and matching placebo were administered as capsules. The matching placebo was indistinguishable from the active drug. A dose of 333 mg mexiletine was chosen as it was thought to be pharmacodynamically active, because the recommended therapeutic dose for patients with myotonia congenita is between 200 and 600 mg mexiletine hydrochloride daily (167 – 500 mg mexiletine). Moreover, a dose of 333 mg mexiletine was considered safe for healthy subjects – doses up to 600 mg mexiletine have been administered.[16]

The randomization schedule was generated using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA) by an unblinded statistician, who was not involved in the clinical execution of the study. A balanced treatment allocation (two sequences, each for 6 subjects) was chosen to control for first-order carry-over effects. Blinded study staff enrolled subjects and assigned participants to interventions. All participants and study staff remained blinded during the study.

MUSCLE VELOCITY RECOVERY CYCLES Practical details of the MVRC procedure were described previously.[2,17] We performed the measurements in the distal tibialis anterior muscle. A monopolar needle electrode (Natus Dantec DCN, 25mmx26G) for stimulation was inserted approximately one centimetre proximal to the distal end of the muscle. The anode surface electrode (BlueSensor NF, Ambu, Ballerup, Denmark) was placed distal to-and in near proximity of-the monopolar needle. A concentric recording needle electrode (25mm×30G, TECA elite, Natus, Middleton, USA) was placed two cm proximal to the monopolar electrode. Needles were inserted perpendicular to the skin, to a depth of approximately one cm. A ground electrode (Red dot, 3M, St. Paul, USA) was placed on the medial malleolus. Stimulation was computer guided by QTracS software (protocol M3REC6, Institute of Neurology, London, UK). Pulses

were applied by an isolated bipolar constant-current stimulator (DS5, Digitimer, Hertfordshire, UK). The recordings were amplified (gain 1000, bandpass filter 3 Hz to 3 kHz) using an EMG amplifier (D440-2, DigiTimer, Hertfordshire, UK). An analog-digital convertor (NI-USB-6341, National Instruments, Austin, Texas) digitized the signal at a sampling frequency of 20 kHz. Hum Bug (Quest Scientific Instruments, North Vancouver, Canada) was used to minimize 50 Hz noise. Skin temperature was held between 32-36° Celsius by an infrared lamp (Daylight heat lamp, General Electronic). Skin temperature was recorded at the beginning and end of the measurement.

Two stimulation paradigms were applied: recovery cycles with one, two, and five conditioning stimuli; and frequency ramp. In the first paradigm, conditioning pulses are applied at interstimulus intervals (ISIs) of 10 ms. After the last conditioning pulse, a test pulse is applied at a decreasing ISI between 1000 and 1.8 ms in 33 steps: 1000, 900, 800, 700, 600, 500, 450, 400, 350, 300, 260, 220, 180, 140, 110, 89, 71, 56, 45, 35, 28, 22, 18, 14, 11, 8.9, 7.1, 5.6, 4.5, 3.5, 2.8, 2.2, and 1.8 ms. In the frequency ramp paradigm, a train of conditioning pulses is applied with a frequency ranging between 1 and 30 Hz.[11]

Moreover, 15-point repeated recovery cycles measurements before, during and after 5 minutes of ischemia induced by a blood pressure cuff around the upper leg. Execution of this complex measurement proved challenging which led to limited data quality; therefore, it is not reported.

DATA HANDLING MVRC variables were generated using QTracP (Institute of Neurology, London, UK), details described previously.[2]

From the recovery cycles recordings, latency from test stimulus to peak muscle action potential is measured. The effect of conditioning stimuli on the latency after the test pulse are estimated as the percentage change compared to an unconditioned test pulse.[8,11] As published previously,[11] the following endpoints were generated for recovery cycles with one, two and five conditioning stimuli. Muscle relative refractory period (MRRP): interpolated ISI at which the latency of the unconditioned response, and latency of the response after one conditioning stimulus, are the same. Early supernormality (ESN): peak percentual latency change induced by one conditioning stimulus at ISIs <15 ms. Early supernormality is also calculated for five conditioning pulses: 5ESN. Time to peak ESN (ESN@) is the ISI corresponding to ESN. SN20 is the supernormality at ISI 20 ms. Late supernormality (LSN) is defined as the mean percentage latency change due to one conditioning stimulus, at ISIs between 50 and 150 ms. XLSN: the difference in LSN between two and one conditioning stimuli, and 5XLSN: the difference in LSN between five and one conditioning stimuli. Residual supernormality (RSN) is the percentage latency change between ISIs 900 and 1000 ms, and 5XRSN is the difference in RSN between five and one conditioning stimuli.

For frequency ramp, latency change is calculated as the percentage of unconditioned action potentials recorded before the ramp.[11] Latency changes after stimulus trains with pulse frequencies of 15 Hz (Lat[15Hz]) and 30 Hz (Lat[30Hz]) were calculated, as well as percentage change in amplitudes of the action potentials after 15 Hz (Peak[15Hz]) and 30 Hz (Peak[30Hz]) trains. The minimal latency (expressed as percentage of the unconditioned pre-ramp potential) measured during the ramp is LatMin, the corresponding frequency when latency is minimal is FreqLatMin. Latency and amplitude changes are calculated for the first and last potential in each train, and these are indicated as 'First' and 'last'. Percentage change in amplitude between 30 and 15 Hz (Peak[30-15Hz]) is calculated, as well as percentage latency and peak change 30 seconds after the ramp (Lat[30Hz30s] and Peak[30Hz30s], respectively).

Before generation of the endpoints, raw data was visually inspected by blinded study staff, and interpolation of single datapoints was performed in case of single outliers with an abnormal muscle response. Additionally, a blinded data review was performed to remove measurements with technical abnormalities from analysis.

STATISTICAL ANALYSIS Statistical analysis was performed in SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Visual evaluation of normal distribution was performed during analysis, and no variables needed log-transformation to correct for log-normal distribution. Repeatedly measured MVRC data are analysed with a mixed effects model with fixed factors: treatment, period, time and treatment by time, random factors: subject, subject by treatment and subject by time, and the average pre-value as covariate. The contrast calculated within the model is placebo versus mexiletine. To indicate inter- and intrasubject variability of MVRC, coefficients of variation (CV%) were calculated from placebo measurements (within-day variability) and derived from the raw data as well as model covariate variables. Statistical significance was defined at the 5% level.

We used previously published variability data of MVRC in healthy subjects[18] to estimate the required sample size. Because no pharmacodynamic effects on MVRC had been reported previously in healthy subjects, expected effect sizes for this study were based on those observed with ischemia.[2] A sample size of twelve subjects in a cross-over design would be able to detect a difference in MRRP of 0.37 ms, and difference in ESN of 1.16%. with a power of 0.8.

RESULTS

A total of 15 subjects were enrolled, of which 14 subjects completed the study. This includes three replacement subjects enrolled due to insufficient quality of MVRC measurements in three of the first 12 subjects. Demographics are summarized in *Supplementary Table 1*.

A total of 85 measurements were performed in 15 subjects. One subject only underwent two measurements and was subsequently excluded. One measurement in another subject was not obtained for technical reasons. Additionally, the following measurements were excluded from analysis in a blinded data review (see chapter Data handling): for eleven measurements the recovery cycles were (partially or fully) excluded, for eight measurements frequency ramp was (partially or fully) excluded. Individual and mean plasma concentrations of mexiletine are shown in *Supplementary Figure 1*, mean concentrations per protocol time are in *Supplementary Figure 2*. Adverse events reported in the study were mild to moderate in intensity, and transient.

TEST-RETEST RELIABILITY Test-retest reliability, estimated in CV%s, of all MVRC variables is shown in *Supplementary Table 3*. Raw baseline MVRC endpoints and estimated means of measurements 3- and 5-hours post-dose, are shown in *Supplementary Table 4*.

EFFECTS OF MEXILETINE ON RECOVERY CYCLES Effects of mexiletine on recovery cycles are listed in *Table 1*. Mexiletine significantly decreased early supernormality after five conditioning stimuli (5ESN) compared to placebo (*Figure 1*). Moreover, difference in late supernormality after five versus one conditioning stimuli (5XLSN) was significantly decreased (*Figure 2*).

To visualize these treatment effects, average post-dose recovery cycles recordings with five conditioning stimuli are shown in *Figure 3*, for mexiletine and placebo. Average post-dose recovery cycles recordings with one conditioning stimulus and two conditioning stimuli are shown in *Supplementary Figure 2* and *Supplementary Figure 3*, respectively.

FIGURE 1 Effects of mexiletine versus placebo on early supernormality after five conditioning stimuli (5ESN), shown as the estimated mean change from baseline (CFB) at three- and five-hours post-dose. Error bars indicate the 95% confidence interval of the estimated mean.

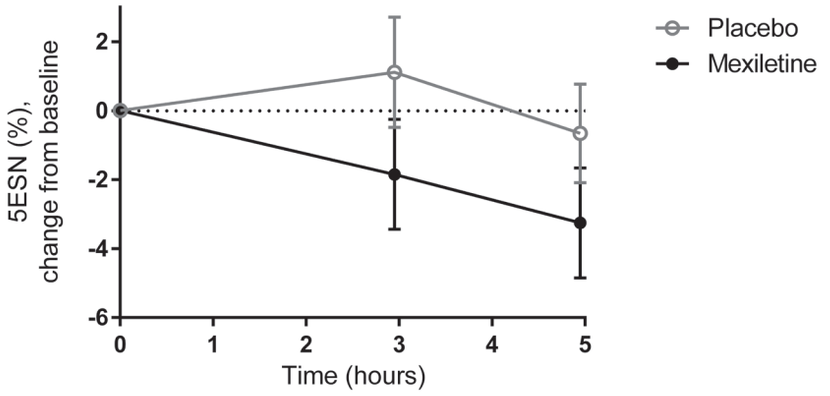


FIGURE 2 Effects of mexiletine versus placebo on the difference in late supernormality of five versus one conditioning stimuli (5XLSN), shown as the estimated mean change from baseline (CFB) at three- and five-hours post-dose. Error bars indicate the 95% confidence interval of the estimated mean.

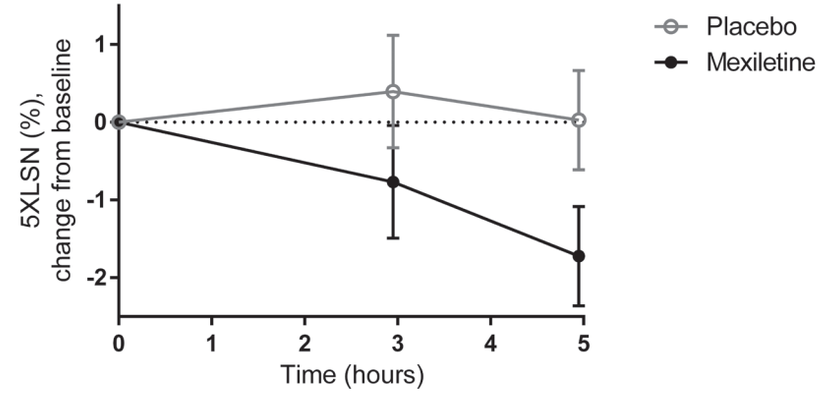


TABLE 1 Effects of mexiletine versus placebo on MVRC endpoints, shown as the estimated mean of the treatment period (post-dose) and the estimated difference of mexiletine versus placebo, reported with 95% confidence interval and p-value.

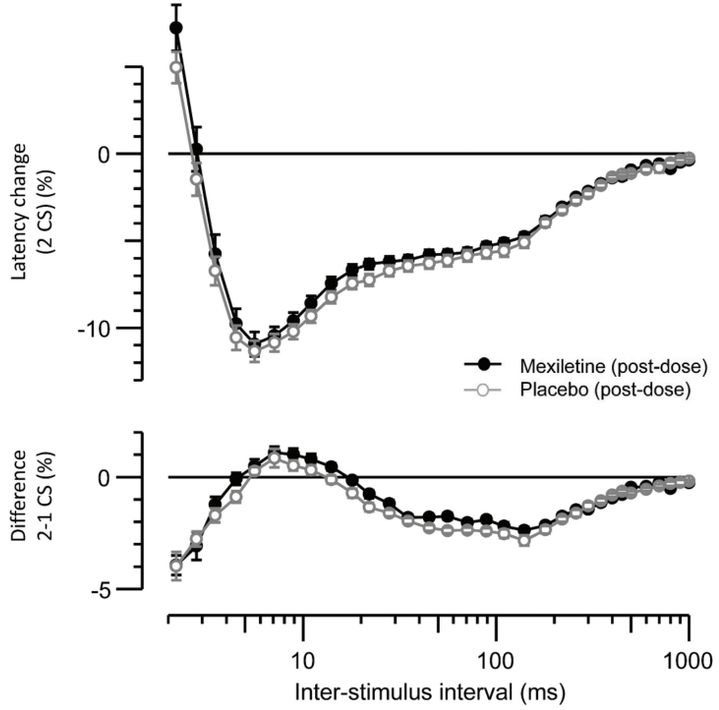
		Treatment	Estimated mean treatment period	Estimated difference	95% confidence interval	P value
RECOVERY CYCLES WITH 1,2 AND 5 CONDITIONING STIMULI	MRRP (ms)	Placebo	3.03			
		Mexiletine	3.09	0.058	(-0.250, 0.365)	0.702
	ESN (%)	Placebo	12.40			
		Mexiletine	11.55	-0.854	(-2.760, 1.051)	0.328
	ESN@ (ms)	Placebo	6.27			
		Mexiletine	6.62	0.34	(-0.48, 1.17)	0.401
	5ESN (%)	Placebo	13.41			
		Mexiletine	10.64	-2.78	(-4.157, -1.396)	<0.001*
	SN20 (%)	Placebo	6.42			
		Mexiletine	5.92	-0.497	(-1.33, 0.340)	0.230
	LSN (%)	Placebo	3.19			
		Mexiletine	3.26	0.075	(-0.527, 0.676)	0.797
	2XLSN (%)	Placebo	2.47			
		Mexiletine	2.08	-0.39	(-0.811, 0.032)	0.068
	5XLSN (%)	Placebo	6.95			
		Mexiletine	5.49	-1.46	(-2.258, -0.653)	0.001*
	RSN (%)	Placebo	0.166			
		Mexiletine	0.165	-0.001	(-0.331, 0.330)	0.997
	5XRSN (%)	Placebo	0.888			
		Mexiletine	0.717	-0.171	(-0.573, 0.231)	0.388

(*Continuation Table 1*)

		Treatment	Estimated mean treatment period	Estimated difference	95% confidence interval	P value
FREQUENCY RAMP	$Lat[15Hz]_{first}$ (%)	Placebo	96.3			
		Mexiletine	96.5	0.20	(-0.69, 1.10)	0.650
	$Lat[15Hz]_{last}$ (%)	Placebo	86.6			
		Mexiletine	89.3	2.77	(0.99, 4.55)	0.004*
	$Lat[30Hz]_{first}$ (%)	Placebo	97.2			
		Mexiletine	98.2	0.98	(-0.75, 2.71)	0.252
	$Lat[30Hz]_{last}$ (%)	Placebo	87.4			
		Mexiletine	95.0	7.58	(3.80, 11.4)	<0.001*
	Lat[30Hz+30s] (%)	Placebo	101.6			
		Mexiletine	100.7	-0.90	(-2.30, 0.49)	0.190
	$Peak[15Hz]_{first}$ (%)	Placebo	110.5			
		Mexiletine	109.5	-1.02	(-9.24, 7.19)	0.801
	$Peak[15Hz]_{last}$ (%)	Placebo	107.5			
		Mexiletine	110.4	2.84	(-12.45, 18.14)	0.692
	$Peak[30Hz]_{first}$ (%)	Placebo	112.8			
		Mexiletine	112.6	-0.13	(-13.48, 13.21)	0.983
	$Peak[30Hz]_{last}$ (%)	Placebo	88.3			
		Mexiletine	89.5	1.20	(-19.45, 21.84)	0.903
	Peak[30-15Hz] (%)	Placebo	1.80			
		Mexiletine	4.49	2.69	(-3.49, 8.86)	0.376
	Peak[30Hz+30s] (%)	Placebo	98.1			
		Mexiletine	97.8	-0.23	(-7.28, 6.82)	0.948
	$LatMin_{first}$ (%)	Placebo	95.4			
		Mexiletine	95.9	0.45	(-0.80, 1.70)	0.435
	$LatMin_{last}$ (%)	Placebo	85.01			
		Mexiletine	88.76	3.75	(1.55, 5.95)	0.002*
	$FreqLatMin_{first}$ (Hz)	Placebo	20.12			
		Mexiletine	18.54	-1.57	(-5.48, 2.33)	0.412
	$FreqLatMin_{last}$ (Hz)	Placebo	21.61			
		Mexiletine	17.79	-3.82	(-6.09, -1.54)	0.002*

*Significant results are highlighted with *.* ESN, *early supernormality;* ESN@, *time to peak early supernormality;* LSN, *late supernormality;* MRRP, *Muscle relative refractory period;* MVRC, *muscle velocity recovery cycle;* RSN, *Residual supernormality;* SN20, *supernormality at interstimulus interval 20 ms.*

FIGURE 3 Mean post-dose recordings of recovery cycles with five conditioning stimuli, for mexiletine (black, filled) and placebo (grey, empty). Error bars show the standard error. The upper graph shows the percentual latency change after five conditioning stimuli at different interstimulus intervals. The lower graph shows the additional change in latency of five versus one conditioning stimuli. Variables with significant effects (mexiletine versus placebo) are visualized by indicating the name of the variable. Variable visualization is reproduced from[11]. Note this graph is meant to visualize treatment effects, but does not fully reflect the statistical analysis, because the statistical model includes baseline as a covariate which is not reflected in the graph.



EFFECTS OF MEXILETINE ON FREQUENCY RAMP Effects of mexiletine versus placebo on frequency ramp are listed in *Table 1*. Mexiletine significantly increased the percentual latency after the last pulse of a 15 Hz train ($Lat[15Hz]_{last}$) and a 30 Hz train ($Lat[30Hz]_{last}$), as shown in *Figure 4* and 5, respectively. Moreover, mexiletine increased the minimal latency during the ramp ($LatMin_{last}$) and decreased the frequency at which the latency was minimal ($FreqLatMin_{last}$) (*Supplementary Figure 4* and *Supplementary Figure 5*, respectively).

Average post-dose frequency ramp recordings (*Figure 6*) visualize these effects, showing that the latency decrease due to the 15 Hz and 30 Hz trains is reduced by mexiletine.

FIGURE 4 Effects of mexiletine versus placebo on the latency change after a 15 Hz train of stimuli ($Lat[15Hz]_{last}$), shown as the estimated mean change from baseline (CFB) at three and five hours post-dose. Error bars indicate the 95% confidence interval of the estimated mean.

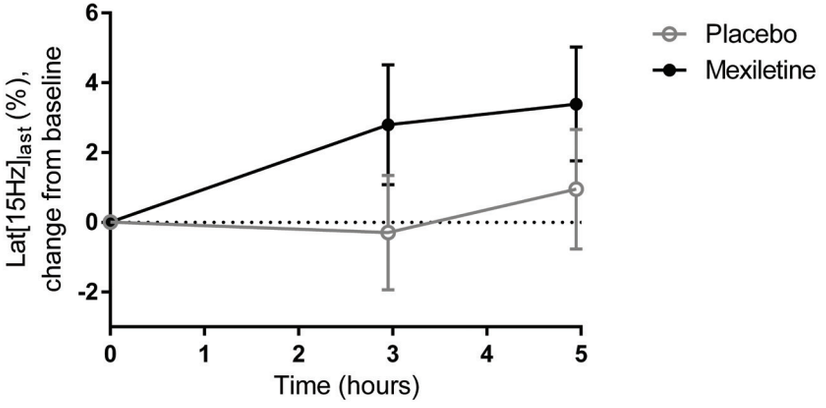


FIGURE 5 Effects of mexiletine versus placebo on the latency change at the end of a 30 Hz train of stimuli ($Lat[30Hz]_{last}$), shown as the estimated mean change from baseline (CFB) at three and five hours post-dose. Error bars indicate the 95% confidence interval of the estimated mean.

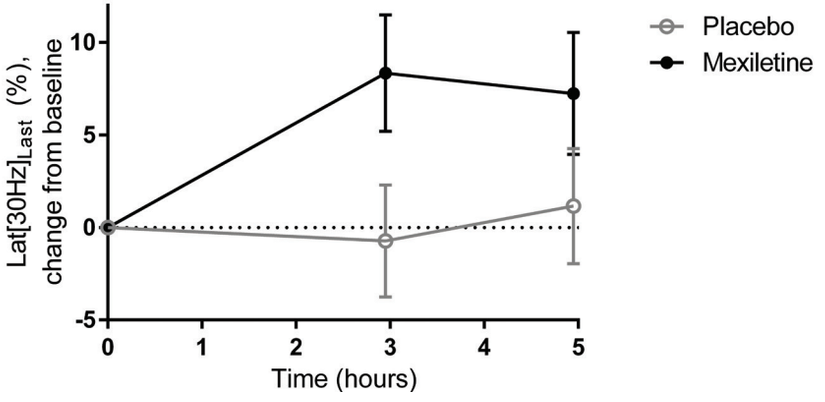
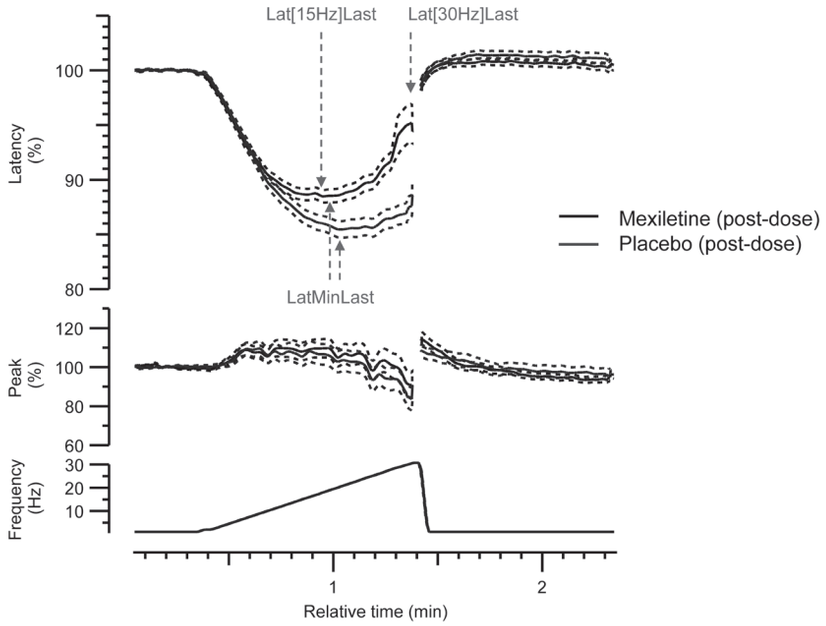


FIGURE 6 Mean post-dose recordings of frequency ramp, for mexiletine (black) and placebo (grey). Dotted lines show the standard error. The upper graph shows the percentual latency (compared to unconditioned latency) by a train of pulses (stimulation rate shown in the lowest graph). The middle graph shows the percentual amplitude change (compared to unconditioned amplitude values). Both graphs show the last-in-train values. Variable with significant effects (mexiletine versus placebo) is visualized by indicating the name of the variable. Variable visualization is reproduced from[11]. Note this graph is meant to visualize treatment effects, but does not fully reflect the statistical analysis, the statistical model includes baseline as a covariate which is not reflected in the graph.



DISCUSSION

The aim of this study was to investigate the use of MVRC as a tool to demonstrate pharmacodynamic effects on muscle excitability. As a proof-of-concept we compared effects of mexiletine to placebo in healthy subjects and were able to demonstrate significant effects of mexiletine on several MVRC variables. The recovery cycles variables 5ESN and 5XLSN were decreased by mexiletine, indicating that mexiletine decreases supernormality of the muscle action potential after five conditioning stimuli, at long and short ISIs. Moreover, we detected a significant increase of $Lat[15Hz]_{last}$, $Lat[30Hz]_{last}$, $LatMin_{last}$ and $FreqLatMin_{last}$ by

mexiletine using the frequency ramp paradigm. In this paradigm, a train of conditioning stimuli physiologically results in an increase in latency at the end of the train – we show that mexiletine suppresses this latency increase after a 15 Hz and 30 Hz stimulus train.

These results indicate that MVRC endpoints are sensitive to detect effects of pharmacological interventions on muscle excitability. The effects on 5ESN and 5XLSN, and $Lat[15Hz]_{last}$, $Lat[30Hz]_{last}$, $LatMin_{last}$ and $FreqLatMin_{last}$, can be explained by the mechanism of action of mexiletine. Mexiletine reduces muscle cell excitability through a use-dependent block of $Na_V1.4$, with higher affinity for Na_V channels in the open and inactivated state.[13–15] This pharmacological property may explain why mexiletine significantly reduces early and late supernormality after five conditioning pulses, as an increased number of $Na_V1.4$ channels will be in the open or inactivated state after previous activations shortly before the test pulse. Additionally, our finding that ESN is only affected by mexiletine after five conditioning stimuli, and not after one or two conditioning stimuli, may be explained by the use-dependence of the Na_V blockade, as fewer conditioning stimuli would result in a relatively lower availability of inactivated Na_V channels that can be bound by mexiletine. When observing effects of mexiletine on post-dose recovery cycles recordings of one (*Supplementary Figure 2*) and two conditioning stimuli (*Supplementary Figure 3*), there is no effect on recovery cycles with one conditioning stimulus, and a small (non-significant) effect on supernormality after two conditioning stimuli, in the same direction as the effect seen with five conditioning stimuli (*Figure 3*). This appears to indicate that the effect of mexiletine indeed increases with an increasing number of conditioning stimuli. The effects on frequency ramp – significant decrease in supernormality due to stimulus trains at high frequencies ($Lat[15Hz]_{last}$ and $Lat[30Hz]_{last}$) – also corresponds to effects expected from a use-dependent Na_V block: effects of mexiletine are larger after repetitive stimulation. Additionally, the difference between mexiletine and placebo is much larger after 30 Hz trains than 15 Hz trains, suggesting an increasing effect at higher stimulation frequencies.

To our knowledge, this is the first study to evaluate effects of Na_V blockers on muscle excitability using MVRC in placebo-controlled manner. An interesting report in this context however, evaluated effects of a gain-of-function mutation in $Na_V1.4$ channels on MVRC in patients with sodium channel myotonia.[9] This mutation results in slowed Na_V inactivation,[9] which should theoretically exhibit somewhat opposite

effects to mexiletine as $Na_V1.4$ blocker. Indeed, 5ESN and 5XLSN (amongst others) were significantly increased, and $Lat[15Hz]_{last}$ and $Lat[30Hz]_{last}$ significantly decreased in sodium channel myotonia, strengthening our results and confirming the mechanism involved in influencing MVRC.

Another relevant paper in this context describes muscle excitability in myotonia congenita patients. Patients with myotonia congenita carry a mutation in ClC-1, resulting in an increase in muscle excitability. The authors compared MVRC of myotonia congenita patients off-treatment, to patients using Na_V blockers (mainly mexiletine).[11] Tan *et al.* showed that the presence of myotonia congenita (in patients who are not on treatment) results in an increase in ESN, 5ESN, LSN and 5XLSN compared to healthy subjects. The authors showed that patients on-treatment with Na_V blockers have a significant decrease in all these variables (a change in the direction of normal controls). This suggests a (partial) reversing of the effects of myotonia congenita by Na_V blockers. Although the results cannot directly be compared to our study because Tan *et al.* did not measure the effects within a patient on- and off-drug, but between patients using or not using Na_V blockers chronically, their findings do corroborate the decrease of 5ESN and 5XLSN due to mexiletine that we found. Moreover, although no significant difference in $Lat[15Hz]_{last}$ was found between myotonia congenita and healthy subjects, patients using Na_V blockers did have a significant increase in $Lat[15Hz]_{last}$, in line with our results. $FreqLatMin_{last}$ is significantly decreased in patients using Na_V blockers when compared to patients without these drugs, in line with our findings for mexiletine.

MVRC AS A BIOMARKER IN DRUG DEVELOPMENT Our study shows that MVRC endpoints are suitable to detect drug effects on muscle excitability, even in a small number of healthy subjects, with a limited number of post-dose measurements. The sample size used here is a typical sample size used in phase I studies. Additionally, the MVRC measurement was safe and well-tolerated in this study. The duration of one measurement allows for pre-dose and multiple post-dose measurements: the stimulation protocol used in this study takes approximately 7 minutes. In addition, the intra-subject variability derived from the model is acceptable, reflected by CV%s below 20% for 17 of 25 variables, which supports the use of MVRC as a biomarker in a cross-over study design. As these test-retest reliability results are based on the data in the placebo treatment, this indicates that the endpoints were rather stable under placebo, i.e. there was no apparent placebo response. These properties are a prerequisite for a valuable biomarker in early phase clinical trials. Whether effects of compounds developed for various NMDs can be detected using MVRC will have to be confirmed in future studies. However, we propose the use of MVRC as a biomarker for target engagement of drugs developed to influence muscle excitability, such as novel (subtype-specific) Na_V blockers,[19,20] or existing sodium- or potassium channel modulating therapies proposed as new treatments for myotonia.[21–23] This biomarker may therefore be used for proof of target engagement but may also facilitate an informed choice of the dose level in the translation from Phase 1 studies in healthy subjects to Phase 2 and 3 studies in patient populations. Furthermore, MVRC may also be used in the translational phase between preclinical and clinical studies because the measurement can also be performed in animal studies.[24,25]

For further development of MVRC as pharmacodynamic biomarker, it would be of interest to explore concentration effect relationships on MVRC. The current study is not set up to reliably evaluate this, because the spread in plasma concentrations is insufficient: we only performed two post-dose PD measurements, both at high plasma concentrations.

LIMITATIONS Due to potential effects of oedema or bleeding around the needle electrodes on consecutive measurements, the insertion location of the needle varied slightly (approximately 0.5 cm) between measurements on the same day. This may influence the conduction distance slightly between measurements performed on the same day. However, intra-subject variability was low, suggesting that this was not a major problem. Moreover, a previous variability study did not report a significant effect of conduction distance on the MVRC endpoints calculated as percentage latency change.[18]

A potential limitation of MVRC is that it can be challenging to find suitable muscle responses to perform the MVRC measurement. This can lead to technically aberrant measurements that have to be removed from analysis, although this occurred rarely in our dataset (see section Data handling).

The analyses presented here were not corrected for multiple testing, due to the exploratory nature of the study.

CONCLUSION The aim of this study was to evaluate MVRC as a biomarker for pharmacodynamic effects on muscle excitability. We demonstrated significant effects of the use-dependent Na_V channel blocker mexiletine on MVRC in healthy subjects. The results indicate a reduction of muscle excitability by mexiletine, in line with its suggested mechanism of action. Whether MVRC can detect pharmacodynamic effects of other (novel) treatments for NMDs remains to be determined in future work. However, this study encourages the use of MVRC as a tool to demonstrate pharmacodynamic effects of drugs targeting muscle excitability in early phase clinical drug development.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1 Demographics of the study population.

	N	15
Age (years)	Mean (SD)	24 (5)
	Median	22
	Range	19-41
Height (cm)	Mean (SD)	179 (8)
	Median	179
Weight (kg)	Mean (SD)	74 (12)
	Median	73
BMI (kg/m²)	Mean (SD)	23 (2)
	Median	22

BMI = *body mass index*; SD = *standard deviation*.

SUPPLEMENTARY TABLE 2 Mean, standard deviation (SD), and median plasma concentrations of mexiletine at each protocol time.

Time after dosing	Mean concentration (mg/L)	SD	Median concentration (mg/L)
2h 39m	1.34	0.27	1.27
4h 39m	1.16	0.27	1.16

SUPPLEMENTARY TABLE 3 Coefficients of variation (CV%) of MVRC endpoints. Intrasubject CV% is calculated within-day (placebo occasion), from the raw data as well as the estimated values from the model (corrected for baseline). Intersubject CV% is calculated from estimated values from the model.

		Raw Intrasubject CV%, within-day	Model Intrasubject CV%, within-day	Model Intersubject CV%
RECOVERY CYCLES WITH 1, 2 AND 5 CONDITIONING STIMULI	MRRP	13.80%	13.80%	13.80%
	ESN	18.90%	16.80%	20.40%
	ESN@	15.30%	15.60%	15.60%
	5ESN	18.80%	18.80%	21.90%
	SN20	18.0%	16.0%	16.0%
	LSN	28.60%	25.60%	25.60%
	2XLSN	25.10%	22.90%	22.90%
	5XLSN	16.40%	15.50%	15.50%
	RSN	429.10%	277.30%	287.90%
	5XRSN	57.10%	79.40%	79.40%
FREQUENCY RAMP	$Lat[15Hz]_{first}$	1.20%	1.50%	1.50%
	$Lat[15Hz]_{last}$	2.70%	3.00%	3.00%
	$Peak[15Hz]_{last}$	23.20%	20.60%	21.10%
	$Peak[15Hz]_{first}$	12.40%	12.10%	12.30%
	$Lat[30Hz]_{first}$	1.70%	1.70%	1.70%
	$Lat[30Hz]_{last}$	6.0%	4.4%	4.4%
	$Peak[30Hz]_{first}$	12.90%	15.10%	15.10%
	$Peak[30Hz]_{last}$	31.90%	27.30%	27.30%
	Peak[30-15Hz]	507.20%	385.90%	385.90%
	Lat[30Hz+30s]	1.50%	1.00%	1.00%
	Peak[30Hz+30s]	13.50%	11.80%	11.80%
	$LatMin_{first}$	1.8%	1.6%	1.7%
	$LatMin_{last}$	3.4%	3.6%	3.6%
	$FreqLatMin_{first}$	25.3%	28.6%	28.6%
	$FreqLatMin_{last}$	14.7%	17.8%	187%

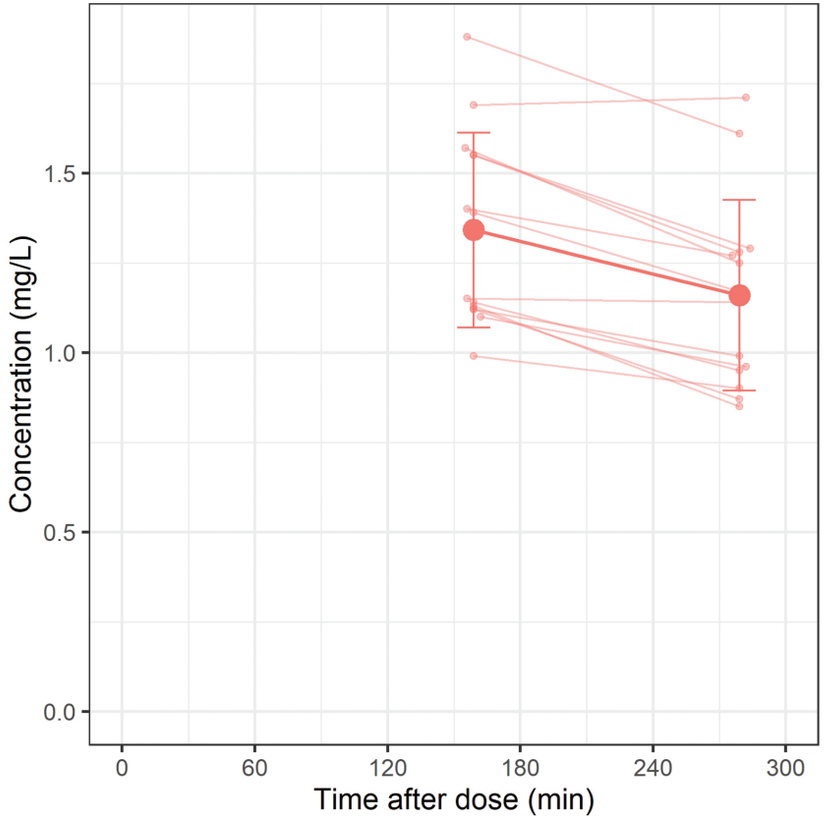
SUPPLEMENTARY TABLE 4 Raw mean baseline (pre-dose) values of MVRC endpoints, and estimated means of post-dose measurements at three- and five-hours post-dose, are listed.

		Treatment	Raw mean baseline	Estimated mean 3h post-dose	Estimated mean 5h post-dose
RECOVERY CYCLES WITH 1, 2, AND 5 CS	MRRP (ms)	Placebo	3.03	2.84	3.23
		Mexiletine	3.42	2.99	3.19
	ESN (%)	Placebo	13.1	13.1	11.7
		Mexiletine	12.9	12.1	11.0
	ESN@ (ms)	Placebo	6.57	5.98	6.57
		Mexiletine	7.57	6.52	6.72
	5ESN (%)	Placebo	13.3	14.3	12.5
		Mexiletine	12.8	11.3	9.9
	SN20 (%)	Placebo	6.60	6.77	6.07
		Mexiletine	6.52	6.39	5.46
	LSN (%)	Placebo	3.49	3.55	2.83
		Mexiletine	3.32	3.55	2.98
	2XLSN (%)	Placebo	2.28	2.49	2.44
		Mexiletine	2.37	2.29	1.86
	5XLSN (%)	Placebo	6.58	7.13	6.77
		Mexiletine	6.93	5.97	5.02
	RSN (%)	Placebo	0.01	0.12	0.22
		Mexiletine	0.31	0.19	0.14
	5XRSN (%)	Placebo	1.11	0.98	0.80
		Mexiletine	1.26	0.87	0.56

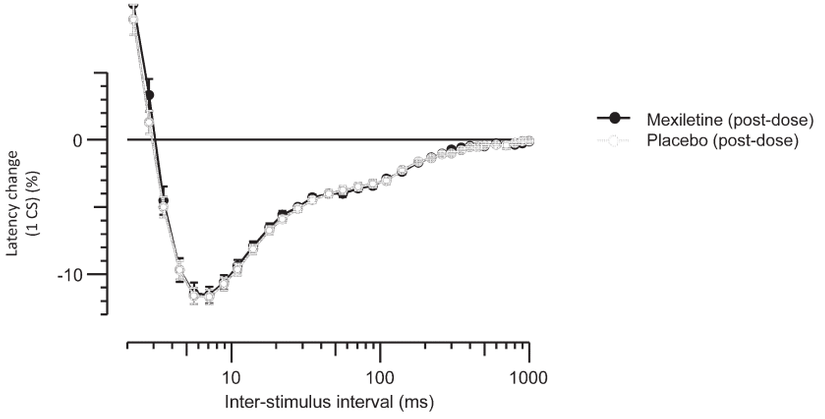
(*Continuation Supplementary Table 4*)

		Treatment	Raw mean baseline	Estimated mean 3h post-dose	Estimated mean 5h post-dose
FREQUENCY RAMP	$Lat[15Hz]_{first}$ (%)	Placebo	95.6	96.2	96.4
		Mexiletine	95.9	96.3	96.7
	$Lat[15Hz]_{last}$ (%)	Placebo	86.2	85.9	87.2
		Mexiletine	86.2	89.0	89.6
	$Lat[30Hz]_{first}$ (%)	Placebo	95.8	96.8	97.5
		Mexiletine	97.0	98.2	98.2
	$Lat[30Hz]_{last}$ (%)	Placebo	88.3	86.5	88.4
		Mexiletine	87.4	95.6	94.5
	Lat[30Hz+30s] (%)	Placebo	102	101	101
		Mexiletine	102	102	100
	$Peak[15Hz]_{first}$ (%)	Placebo	113	113	111
		Mexiletine	114	108	108
	$Peak[15Hz]_{last}$ (%)	Placebo	119	113	102
		Mexiletine	114	119	102
	$Peak[30Hz]_{first}$ (%)	Placebo	116	116	115
		Mexiletine	121	109	111
	$Peak[30Hz]_{last}$ (%)	Placebo	95.6	92.6	84.1
		Mexiletine	102.6	93.0	86.0
	Peak[30-15Hz] (%)	Placebo	2.73	2.13	1.48
		Mexiletine	5.76	4.98	4.00
	Peak[30Hz+30s] (%)	Placebo	98.3	101	97.6
		Mexiletine	100	95.0	98.1
	$LatMin_{first}$ (%)	Placebo	94.4	95.4	95.5
		Mexiletine	94.8	95.6	96.2
	$LatMin_{last}$ (%)	Placebo	84.9	84.3	85.7
		Mexiletine	84.7	88.7	88.9
	$FreqLatMin_{first}$ (Hz)	Placebo	20.6	18.6	21.7
		Mexiletine	21.9	18.4	18.7
	$FreqLatMin_{last}$ (Hz)	Placebo	20.8	21.2	22.1
		Mexiletine	20.7	17.7	17.9

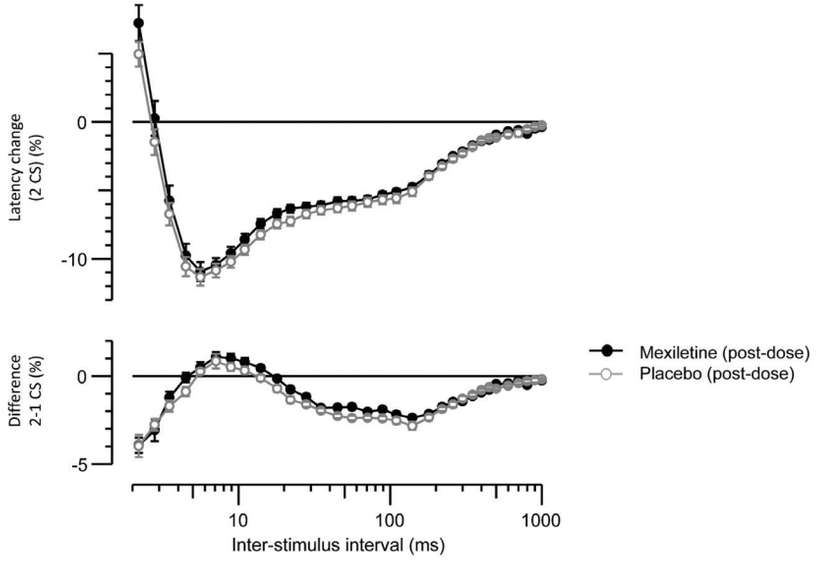
SUPPLEMENTARY FIGURE 1 Individual and mean ± SD plasma concentration of mexiletine, before the start of the post-dose MVRC measurements (2 hours and 39 minutes post-dose, and 4 hours and 39 minutes post-dose).



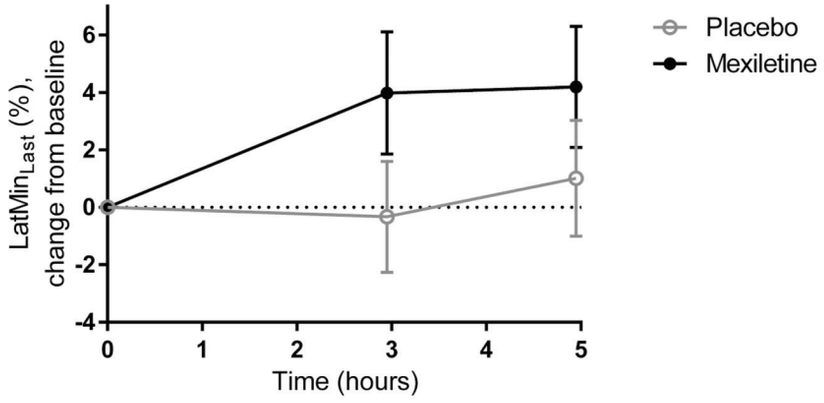
SUPPLEMENTARY FIGURE 2 Mean post-dose recordings of recovery cycles with one conditioning stimulus, for mexiletine (black, filled) and placebo (grey, empty). Error bars show the standard error. The graph shows the percentual latency change after one conditioning stimuli at different interstimulus intervals. Note this graph is meant to visualize treatment effects, but does not fully reflect the statistical analysis, because the statistical model includes baseline as a covariate which is not reflected in the graph.



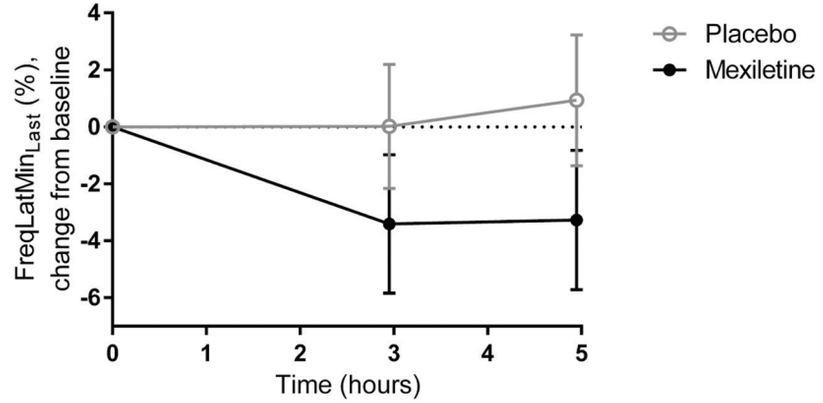
SUPPLEMENTARY FIGURE 3 Mean post-dose recordings of recovery cycles with two conditioning stimuli, for mexiletine (black, filled) and placebo (grey, empty). Error bars show the standard error. The upper graph shows the percentual latency change after two conditioning stimuli at different interstimulus intervals. The lower graph shows the additional change in latency of two versus one conditioning stimuli. Note this graph is meant to visualize treatment effects, but does not fully reflect the statistical analysis, because the statistical model includes baseline as a covariate which is not reflected in the graph.



SUPPLEMENTARY FIGURE 4 Effects of mexiletine versus placebo on the minimal latency (last in train) recorded during the ramp ($LatMin_{last}$), shown as the estimated mean change from baseline (CFB) at three- and five-hours post-dose. Error bars indicate the 95% confidence interval of the estimated mean.



SUPPLEMENTARY FIGURE 5 Effects of mexiletine versus placebo on the frequency at which the minimal latency (last in train) was recorded during the ramp ($FreqLatMin_{last}$), shown as the estimated mean change from baseline (CFB) at three- and five-hours post-dose. Error bars indicate the 95% confidence interval of the estimated mean.



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