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Rufinamide Attenuates Mechanical Allodynia in a Model of Neuropathic Pain in the Mouse and Stabilizes Voltage-gated Sodium Channel Inactivated State

--Manuscript Draft--

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Abstract:	Background: Voltage-gated sodium channels dysregulation is an important feature of hyperexcitability leading to pain persistence. Sodium channel blockers currently used for the treatment of neuropathic pain are poorly tolerated or not available orally. Getting new molecules to clinical use is an arduous process and we here propose to use a sodium channel blocker already marketed as anticonvulsant, rufinamide. Methods: We tested rufinamide on the Spared Nerve Injury model of neuropathic pain in mice. We compared its effect on mechanical allodynia to amitriptyline. The effect of rufinamide on sodium currents was tested using patch clamp in human embryonic kidney 293 cells expressing the voltage-gated sodium channel Nav1.7 isoform and on freshly dissociated dorsal root ganglion neurons and compared to amitriptyline and mexiletine. Results: In naive mice, amitriptyline (20mg/kg) increased withdrawal threshold to mechanical stimulation from 1.3g (0.6-1.9) (median (95% confidence interval)) to 2.3g (2.2-2.5) and latency of withdrawal to heat stimulation from 13.1s (10.4-15.5) to 30.0s (21.8-31.9), whereas rufinamide had no effect. Rufinamide and amitriptyline alleviated injury-induced mechanical allodynia-like behavior for 4 hours (maximal effect from 0.10±0.03g (mean±SD) to 1.99±0.26g for rufinamide and from 0.25±0.22g to 1.9±0.8g for amitriptyline). 24 hours later, the effect had worn off. All drugs reduced peak current and stabilized the inactivated state of voltage-gated sodium channel Nav1.7 in cell expression system, with similar effects in dorsal root ganglion neurons. Conclusions: At doses alleviating neuropathic pain, amitriptyline showed alteration of behavioral response possibly related to either alteration of basal pain sensitivity or/and sedative effect. Side effects and drug tolerance/compliance are major problems with drugs such as amitriptyline. Rufinamide seems to have a better tolerability profile. Our results suggest rufinamide could be a new alternative to explore for the treatment of neuropathic pain.

Dear Professor Brennan,

We are pleased to resubmit a fully revised version of our manuscript "**Rufinamide attenuates mechanical allodynia in a model of neuropathic pain in the mouse and stabilizes voltage-gated sodium channel inactivated state"** for consideration for publication. Following your decision letter of June 7th 2012, we revised the manuscript according to the constructive points raised by your statistical reviewer and addressed the missing parts.

Responses to the **Reviewer #4:**

Global response: We changed the description of the statistical method (p. 3, l. 19), according to the changes mentioned in the point-to-point response below.

Abstract

1. This is simply an opinion and the authors are encouraged to revise their abstract as they see fit. Although describing the results of the study using a narrative style can often convey the findings of the study, it is far more informative to report the actually observed effects using descriptive statistics and measures of effect size (e.g., mean differences, odds ratios, etc.).

Response: We thank the reviewer for the remark. We changed the abstract and added median with 95% confidence interval for withdrawal threshold and withdrawal latency in naïve animals as well as the mean values±SD of withdrawal threshold after SNI at the peak effect for the highest drug dose. We also added these values in the result section under point 1.1 to 1.3.
We did not add the values for the global effect on the stabilization effect of inactivation properties of Na channels (values of V1/2 inactivation, recovery from inactivation, and activity dependent block for each drug) because of word limitation of the abstract.

Introduction

2. When conducting inferences (i.e., significance testing using p values) it is important to formally state a hypothesis to test. If a p value is reported, a hypothesis is being tested. Explicitly state the hypothesis(es) in the Introduction section will provide a wealth of information to your readers concerning the expected results of the study.

Response: We added the null hypothesizes tested in our experiments at the end of the introduction (p. 6, l. 28)

Methods

3. It is important to define the measures of central tendency AND variability when they are reported in the methods section (e.g., mean +- SD). At present a value is reported in the methods before they are introduced in the stats section. Is this value a mean +- SD?

Response: We agree with the reviewer. The only value mentioned with variability in the method section is the room temperature and we changed it to 21°C (p. 7, l. 44). This is how it is set in the animal facility but we do not have the control measurement to give a precise range of temperature at that period of time. We apologize for mentioning variability in the previous version.

4. Please ensure that exact sample sizes are available for all comparisons. Of note is that reporting a sample size range (e.g., "n = 4 - 10") is insufficient in this regard.
Response: We apologize for this ambiguity which was corrected in the method section (p. 9, l. 37).

Statistical Methods
5. Several of the variables under study may violate the assumptions of parametric tests (e.g., normality, homogeneity of variances, level of measurement), were such assumptions considered prior to conducting the analysis? Several of the descriptive statistics appear to be quite skewed, leading to the fear that these data do not satisfy parametric assumptions. Please reconsider the approach.
Response:
We apologize if our assumptions of normality and homogeneity of variance of our variable was erroneous for some variables. As it is not clear which variables are questioned by reviewer 4, we go over all figures to hopefully clarify the analysis.
a) We considered the measurements of mechanical allodynia using logarithmic values for homogeneity of variance. Threshold responses are usually normally distributed. Sometimes the sample for one timepoint does not pass a normality test, but this was the exception and we assumed the whole population does. Mainly this occurs for baseline (BL) data which are skewed because the upper threshold is fixed at 2.56 g (with von Frey of higher values the paw of the mice is lifted passively) and the distribution is only one-sided. We therefore changed our analysis for the development of allodynia by using a non-parametric test (Wilcoxon matched-pairs signed rank test); this concerns only the comparison of BL versus preinjection (post nerve injury) values. The result reject the null hypothesis that the values are the same ($p<0.05$ with Bonferrroni's correction for multiple testing). The two-way repeated measures ANOVA was then used from preinjection to 24h post injection (precision below).

b) For measures of figure 2, some variables show a skewed distribution because of fixed thresholds. We changed the analysis to non-parametric Kruskal-Wallis test with Dunn's correction for multiple testings:

*For the **withdrawal threshold,** we obtain the following*

Kruskal-Wallis test			
P value	0.0456		
Number of groups	6		
Kruskal-Wallis statistic	11.31		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
RUF50vF vs DMSOvF	-4.438	No	ns
AMI10vF vs NaClvF10	13.69	No	ns
AMI20vF vs NaClvF20	16.88	Yes	*

*For the **withdrawal latency,** we obtain the following*

Kruskal-Wallis test			
P value	< 0.0001		
Number of groups	6		
Kruskal-Wallis statistic	28.31		

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
RUF50HP vs DMSO-HP	0.0	No	ns
AMI10HP vs NaCl-HP10	22.25	Yes	**
AMI20HP vs NaCl-HP20	19.63	Yes	*

*For the **total activity,** we obtain the following*

Kruskal-Wallis test			
P value	0.0192		
Exact or approximate P value?	Gaussian Approximation		
Kruskal-Wallis statistic	7.906		
Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
NaCl vs RUF50	0.3333	No	ns
NaCl vs AMI10	7.667	Yes	*
RUF50 vs AMI10	7.333	No	ns

We also changed figure 2 accordingly and present the data with Box-Plot (median, box for 25th and 75th percentile, whiskers representing the minimum and maximum data).

c) Electrophysiological measures in vitro:
Most datasets pass the Kolmogorov-Smirnov test for normality. Some n are too small to perform normality tests. We assume thus that the general population of data in this section can be analyzed with parametrical tests.
As asked by the reviewer we changed the results and figures to show the exact p values when adequate:
- for SSI: 0.0038 for RUF, 0.0019 for AMI and 0.0003 for MEX
-for RFI: <0.0001 for RUF (real value is 3.58E-10), 0.0011 for AMI and 0.0002 for MEX
-for use-dependent block the tables were changed in figure 6

d) Electrophysiological measures ex-vivo:
Peak currents, activation, SSI and recovery from inactivation data are normally distributed and paired t-tests were used for comparison. Exact p-values are 0.0084 for peak current, 0.17 for V1/2 of activation, 0.35 for slope of activation, <0.0001 (0.00009) for V1/2 of steady-state inactivation, 0.34 for slope of steady-state inactivation and 0.028 for recovery from inactivation. These values, if not present were added in the text.

6. Please report the software that was used to conduct the statistical analyses in the statistical analysis section.
Response: We apologize for the omission which was corrected (p. 14, l. 2) "Statistical analysis was performed using Prism 5 for windows, version 5.03, GraphPad Software, San Diego California USA"

7. Please report the nature of the hypothesis testing (i.e., one-tailed versus two-tailed testing).
Response: We apologize for the omission. This was corrected (p. 14, l. 0) "All hypotheses were challenged using two-tailed testing."

8. I had great difficulty following the analytical plan. As currently written, I could not replicate the analyses if given access to your data (which is the goal for this description). The elegant experimental

design naturally leads to two-way repeated measures ANOVA and this approach is introduced. However, there are confusing statements in the statistical methods section that indicate that a nonconventional approach was taken to the analysis. Statements such as "?but the statistical analysis was done for each group separately" indicate that a series of one-way repeated measures ANOVAs were conducted (and the results certainly read this way as well). This approach does not formally test group differences over time/dose and would not apply the proper control for multiple comparisons. The authors are encouraged to seek statistical consultation (if necessary) to revise either the description of the plan, the actual plan, and the reporting of the results (a group x time interaction would be extremely helpful here).

Response: We apologize for the misunderstanding in the analytical plan by using the word "separately". As the different rufinamide doses were not performed at the same time (to many groups) and in order to provide a similar environment to treated and control mice, we used a control group each time. We used a two-way repeated measures ANOVA for each experiment. That is:
*- RUF 25mg/kg, RUF 50mg/kg and DMSO30%, n=10, 10 and 9 respectively. Treatment comes as a significant source of variation with a p=0.0009, as well as time with a p<0.0001, Treatment x time interaction is also source of variation with a p at 0.0053. Bonferroni's posttests comparing each timepoint after injection to the pre-injection value are shown on figure 1 (*p<0.05, **p<0.01, ***p<0.001, the exact p values are not given for post-hoc testing).*
- RUF 5mg/kg, RUF 10mg/kg and DMSO30%, n=8 per group. Only time comes as a significant source of variation (p<0.0003). p=0.66 for treatment and 0.46 for treatment x time interaction. All pvalues for Bonferroni's posttests comparing each timepoint after injection to the pre-injection value are above 0.05.
*- AMI 10mg/kg, AMI 20mg/kg and saline, n=10, 10 and 9 respectively. Treatment, time and treatment x time interaction all contribute to variation with a p<0.0001. Bonferroni's posttests comparing each timepoint after injection to the pre-injection value are shown on figure 1 (*p<0.05, **p<0.01, ***p<0.001, the exact p values are not given for post-hoc testing).*

In fig 1, we averaged the two DMSO control groups for the clarity of the figure.

9. Related to the above, the practice of applying a series of unprotected paired t-tests in Figure 2 violates the Journal's family-wise error rate policy. Please revise this approach to account for the fact that multiple comparisons are being made.
Response: We thank the reviewer for this precision. The approach has been revised (see above, point 5).

Results
10. Although the reporting of p values using "p <" or "p >" is still acceptable to many journals, reporting the exact p values (e.g., p = 0.031) is preferred. This reporting practice even applies to p values that are statistically non-significant (e.g., p = 0.54)
*Response: We changed wherever necessary with exact p values, except for posttests when only the approximation is available in GraphPad (*p<0.05, **p<0.01, ***p<0.001). We also left "p<" when more than 4 digits were necessary.*

11. Unless explicitly stated, it can often be difficult to tell if any of the measurements were lost to observation or were missing in the analysis. Were any data missing for any of the variables? The methods state that certain cells were not used in the analysis. Some estimates of the lost data must be provided to your reader.
Response: No data were missing for behavior after drug delivery. For electrophysiology, once the cells were sealed, around 15% of cells were lost before any measurement with drug could be completed. This was added in the method (p. 10, l. 48)

General
12. The reporting of standard error of the mean (SEM or SE) as a measure of variability is certainly commonplace and is routinely encountered. However, as a measure of variability, perhaps there are better choices than SEM. When simply describing the sample, standard deviation (SD) provides a measure of variability that is less abstract and can be directly used for standard effect size calculations. When appealing to the sampling distribution, +-SEM is actually a 68% confidence interval that is of little use for conventional inference testing. A much more informative choice would be to report a 95% confidence interval in the text, tables, and figures.
Response: We changed figure 1 to show SD instead of SEM as suggested by the reviewer. On figure 2 as mentioned above we changed the graphical representation to box-plot. SD was already used for the other figures.

13. The use of the term "significant" has been found without an important modifier in numerous places in the manuscript. While discussing a "significant effect" is relatively common, it is not nearly as informative (or precise) as discussing a "statistically significant effect" or a "clinically significant effect". Please reconsider the use of 'significant" throughout the manuscript.
Response: We thank the reviewer for the comment and verified the use of "significant" throughout the manuscript.

Rufinamide attenuates mechanical allodynia in a model of neuropathic pain in the mouse and stabilizes voltage-gated sodium channel inactivated state

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Effect of rufinamide on gating properties of voltage-gated sodium channel Nav1.7, MR Suter, H Abriel, I Decosterd
European Society of Anaesthesiology meeting, Amsterdam, Netherland, June 2011
Rufinamide alleviates mechanical allodynia in a mouse neuropathic pain model

MR Suter, G Kirschmann, H Abriel, I Decosterd

Number of words in Abstract (285), in Introduction (543), and in Discussion (1661)

Running title:

Rufinamide reduces neuropathic pain in the mouse

Brief summary:

The anti-epileptic drug rufinamide alleviates mechanical allodynia in a neuropathic pain model with less side-effect than amitriptyline, one of the first line treatments for neuropathic pain. Rufinamide stabilizes sodium channels in their inactivated state.

Abstract:

Background: Voltage-gated sodium channels dysregulation is an important feature of hyperexcitability leading to pain persistence. Sodium channel blockers currently used for the treatment of neuropathic pain are poorly tolerated or not available orally. Getting new molecules to clinical use is an arduous process and we here propose to use a sodium channel blocker already marketed as anticonvulsant, rufinamide.

Methods: We tested rufinamide on the Spared Nerve Injury model of neuropathic pain in mice. We compared its effect on mechanical allodynia to amitriptyline. The effect of rufinamide on sodium currents was tested using patch clamp in human embryonic kidney 293 cells expressing the voltage-gated sodium channel Nav1.7 isoform and on freshly dissociated dorsal root ganglion neurons and compared to amitriptyline and mexiletine.

Results: In naive mice, amitriptyline (20mg/kg) increased withdrawal threshold to mechanical stimulation from 1.3g (0.6-1.9) (median (95% confidence interval)) to 2.3g (2.2-2.5) and latency of withdrawal to heat stimulation from 13.1s (10.4-15.5) to 30.0s (21.8-31.9), whereas rufinamide had no effect. Rufinamide and amitriptyline alleviated injury-induced mechanical allodynia-like behavior for 4 hours (maximal effect from 0.10±0.03g (mean±SD) to 1.99±0.26g for rufinamide and from 0.25±0.22g to 1.9±0.8g for amitriptyline). 24 hours later, the effect had worn off. All drugs reduced peak current and stabilized the inactivated state of voltage-gated sodium channel Nav1.7 in cell expression system, with similar effects in dorsal root ganglion neurons.

Conclusions: At doses alleviating neuropathic pain, amitriptyline showed alteration of behavioral response possibly related to either alteration of basal pain sensitivity or/and sedative effect. Side effects and drug tolerance/compliance are major problems with drugs such as amitriptyline. Rufinamide seems to have a better tolerability profile. Our

results suggest rufinamide could be a new alternative to explore for the treatment of neuropathic pain.

Introduction:

Pain is essential for survival as it serves as an alert to engage protective behavior. Neuropathic pain defined by the International Association for the Study of Pain as "pain caused by a lesion or disease of the somatosensory nervous system" affects 7% of the population[1] and possesses no protective purpose.

Sodium channels are major targets for the development of new drug to treat neuropathic pain[2]. Nerve injury changes the expression (number, subtypes) of sodium channels[3] which affects peripheral nerve hyperexcitability and ectopic discharges along the nerve, in the dorsal root ganglion or at the injury site where a neuroma forms[4;5]. They are composed of a α-pore forming subunit associated to one or two β-modulating subunits. Nine genes encodes for the α-subunits, Nav1.1-1.9[6].

Current therapy for neuropathic pain involves adjuvant medications - not primarily developed for this purpose - such as anticonvulsants, antidepressants or local anesthetics[7]. Tricyclic antidepressants are the most studied family of antidepressant in pain therapy and considered as first line treatment in different international guidelines[8]. Their mode of action does not seem to be linked to their antidepressant actions as acknowledged by their faster onset[9]. Among other members of that family, amitriptyline was shown to interact with sodium channels as exemplified by its cardiac toxicity and this target could also play a role in pain modulation[10]. Mexiletine, a sodium channel blocker and an oral analog of local anesthetics has been used in the treatment of neuropathic pain[11] but its tolerance on long term therapy raises considerable questions as shown by a median discontinuation of treatment of 43 days in a recent study[12]. Rufinamide is an antiepileptic drug licensed for a refractory type of epilepsy in the childhood, the Lennox-Gastaut syndrome[13]. Its principal mechanism of action is considered to be inhibition of sodium channels, stabilizing its inactive form and reducing the firing of sodium dependent action potentials[14].

Since the discovery that loss-of-function mutations in *SCN9A*, the gene encoding for Nav1.7 isoform, are associated with congenital insensitivity to pain[15], it has become a potential target for treatment. Moreover, gain-of-function mutations *SCN9A* are associated with familial pain syndromes (erythromelalgia and paroxysmal extreme pain disorder reviewed by Lampert[16]) and in subset of patients with idiopathic small nerve fiber neuropathy or generalized pain syndromes[17;18]. Nav1.7 is expressed in sensory, sympathetic and myenteric fibers[19-21]. It exhibits slower recovery from fast inactivation[22;23] compared to other tetrodotoxin-sensitive channels Nav1.4 and 1.6 and slower inactivation at potentials close to the membrane resting potential, thus contributing to the large ramp current during slow depolarization[24]. Nav1.7 is thought to play an important role in "boosting" the depolarization of small diameter nociceptive neurons.

In the present study, we investigated the analgesic effect of rufinamide on the spared nerve injury (SNI) model of neuropathic pain and amitriptyline was used as a positive control. Our null hypothesis was that treated and control groups show the same behavior. We also explored the effect of rufinamide on Nav1.7 channels heterogeneously expressed in human embryonic kidney 293 cells and used mexiletine and amitriptyline as control. We finally tested the effect of rufinamide on dorsal root ganglia neurons. In these electrophysiological experiments our null hypothesis was that the drugs do not change the measured parameters, which were $V_{1/2}$ of activation and steady-state inactivation and $t_{1/2}$ of recovery from inactivation.

Materials & Methods:

Drugs:

Rufinamide (R8404), amitriptyline (A8404) and mexiletine (M2727) were purchased from Sigma (Buchs, Switzerland). For behavioral experiment, rufinamide was dissolved in dimethylsulfoxide (DMSO) and then mixed with 1x phosphate buffered saline to the desired concentration. Control was 30% DMSO in 1x phosphate buffered saline. Doses (5, 10, 25, 50 mg/kg) were chosen corresponding to the therapeutic ones used in epilepsy models in mice (rufinamide was effective in the maximal electroshock test (ED 23.9 mg/kg orally) and in the pentylenetetrazol induced seizure test (54 mg/kg, intraperitoneally)[25]. Amitriptyline was dissolved directly in sterile 0.9% saline and doses were chosen according to previous studies in neuropathic pain models. Drugs were administered intraperitoneally.

Animal experiments:

All experiments were approved by the Committee on Animal Experimentation of the Canton de Vaud, Lausanne, Switzerland, in accordance with Swiss Federal law on animal care and the guidelines of the International Association for the Study of Pain[26]. Five-week-old C57BL/6 male mice (Charles River, l'Abresle, France) weighting 20-25g at the start of experiment were housed in the same room, 5 per cage, at constant temperature of 21°C and a 12/12 dark/light cycle. No other animals were housed in that room. Mice had ad libitum access to water and food.

Surgery

SNI surgery[27;28] on mice[29] was performed under 1.5–2.5% isoflurane (Abott AG, Baar, ZG, Switzerland) anesthesia. Briefly, the left hindlimb was immobilized in a lateral

position and slightly elevated. Incision was made at mid-thigh level using the femur as a landmark and a section was made through the biceps femoris in the direction of point of origin of the vascular structure. The three peripheral branches (sural, common peroneal and tibial nerves) of the sciatic nerve were exposed without stretching nerve structures. Both tibial and common peroneal nerves were ligated using a 6.0 silk suture and transected together. The sural nerve was carefully preserved by avoiding any nerve stretch or nerve contact.

Behavior

For all the behavioral experiments, the observer was blinded to the treatment applied. Mechanical sensitivity: Animals were habituated to the testing environment daily for at least 2 days before baseline testing. The room temperature and humidity remained stable for all experiments. For testing mechanical sensitivity, animals were put under inverted plastic boxes on an elevated mesh floor and allowed 10 min for habituation before the threshold testing. Mechanical allodynia was tested using a series of von Frey hairs with logarithmically incrementing stiffness (0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, and 2.56 g). The filaments were applied perpendicularly to the plantar surface 1–2 s. The 50% withdrawal threshold was determined using Dixon's up–down method[30].

Heat sensitivity: The effect of rufinamide and amitriptyline on basal heat sensitivity was assessed with the Hot Plate assay. Briefly, the animals were placed on the hot-plate surface set at 52°C. The latency of response (in seconds) was determined as the time until a hindlimb lick or jump occurred. The cutoff was set at 30 s to avoid tissue damage.

Activity was quantified with the Activ-meter (Bioseb, France). The total activity (summation of immobile, slow and fast activity given by the software) of naive animals in their home cage was measured during the 4 hours following injection of rufinamide (50 mg/kg) and amitriptyline (10 mg/kg). It was compared to the activity after saline injection. All experiments for activity were performed between 5 and 9pm.

A 5 point sedation score from 0-4 points was used for rufinamide (50 mg/kg) and amitriptyline (10 mg/kg), 0=normal behavior, normal locomotion, 1=awake, slow locomotion, 2=no locomotion, eyes half closed, still responding to righting reflex, 3=asleep, eyes closed, still responding to righting reflex, 4=no righting reflex, adapted from Boast et al.[31].

Experimental design

For drug effect on naïve animals, 8 animals per group were used to assess mechanical withdrawal threshold and heat withdrawal latency. For the Activ-meter, 6 animals were used in a cross-over design for rufinamide and amitriptyline.

Normal mechanical threshold was assessed before surgery without difference between groups. SNI surgery was performed and one week later allodynia-like behavior was tested before intraperitoneal injection of rufinamide. Two series of experiments were done, the first one compared rufinamide 25 mg/kg and 50 mg/kg to DMSO 30% (n=10 per group, 9 for DMSO) and the second one compared rufinamide 5 mg/kg and 10 mg/kg to DMSO 30% (n=8 per group) at 20-40-60-120-240 min and 24 h. After a washout period of one week the animals of the first series were tested with amitriptyline 10 or 20 mg/kg or saline at 60-120-240 min and 24 h after intraperitoneal injection (n=9 per group for amitriptyline 20mg/kg and 10 per group for amitriptyline 10mg/kg and saline).

Plasma levels of the drug were assessed at 120 min after injection of 50 mg/kg rufinamide. Mice (n=3) were anesthetized with isoflurane and 1 ml of blood was collected intracardially. Drug levels were analyzed by the pharmaceutical monitoring laboratory of Lavigny, Switzerland (http://www.ilavigny.ch/html/hopital/laboratoire.php, last accessed June 21st 2012).

Electrophysiology:

Rufinamide was dissolved in DMSO at 10 μmol/l as stock solution and diluted daily at desired concentration in the extracellular medium. As control the same DMSO concentration was used (1% for 100 μg/ml, to 5% for 500 μg/ml). Higher concentration could not be achieved without increasing DMSO content. Amitriptyline and mexiletine were dissolved in extracellular medium directly.

Human embryonic kidney 293 cells stably expressing Nav1.7 were kindly provided by Simon Tate (PhD, Chief Scientific Officer, Convergence Pharmaceuticals, Cambridge, UK) and were cultured in DMEM-F12 + L-Glutamine (Invitrogen, Merelbeke, Belgium) supplemented with 5% fetal bovine serum and geneticin 0.4 mg/ml. Measurements were made at room temperature using pClamp software, version 10.2, and a VE-2 amplifier (Alembic Instruments, Montreal, Quebec, Canada). The sampling rate was 30 kHz. Data were smoothed and analyzed using Clampfit software version 10.2.0.12 (Axon Instruments, Union City, CA), and KaleidaGraph (Synergy Software, Reading, PA). Whole-cell Patch clamp recordings were conducted using an internal solution containing (in mmol/l) CsCl 60, Cesium aspartate 70, EGTA 11, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, and Na_2-ATP 5, pH adjusted to 7.2 with CsOH; and an external solution containing NaCl 50, n-methyl-D-glutamine-Cl 80, $CaCl_2$ 2, $MgCl_2$ 1.2, CsCl 5, HEPES 10, and glucose 5, pH adjusted to 7.4 with CsOH. Holding potential was -100 mV. The values were not corrected for liquid junction potential. Pipette resistance was ranging from 2 to 4 MOhm. Only data from cells having stable access resistance over the duration of the experiment were used. Cells for which signs of poor voltage-clamp control, such as delayed inflections of the current or discontinuities in the peak I_{Na} versus V_m curve were not analyzed. Around 15% of sealed cells were lost. Data were filtered after acquisition using Boxcar 9 points. Peak currents were measured with a single 10ms pulse protocol to -10mV from the holding potential. Percentage inhibition was calculated as ($peak_{vehicle}$-$peak_{drug}$)/$peak_{vehicle}$ x100 for each cell and then mean inhibition for each drug and

concentration was calculated. Other protocols are shown as inserts in the figures. The linear ascending segment of the current-voltage relationship was used to estimate the reversal potential for each trace before obtaining the voltage–dependent activation curve. Voltage dependence of activation and steady state inactivation curves were individually fitted with Boltzmann relationships, $y(V_m) = 1/(1 + \exp[(V_m - V_{1/2})/K])$ in which y is the normalized current or conductance, Vm is the membrane potential, $V_{1/2}$ is the voltage at which half of the channels are activated or inactivated and K is the slope factor. The value of $t_{1/2}$ of recovery from inactivation was calculated by interpolation from a linear relation between the 2 points juxtaposing half recovery ($y_1<0.5<y_2$), using the relation $x = [0.5-(y_1x_2-y_2x_1)/(x_2-x_1)]*(x_2-x_1)/ (y_2-y_1)$. For use-dependent block, the percentage of decrease of current was calculated between the 1st and 50th pulse.

For ex-vivo recordings, dorsal root ganglion neurons were collected from adult C57BL/6 mice (4-8 weeks old). Briefly, L4 and L5 dorsal root ganglion neurons were harvested and digested in Liberase blendzyme TM (Roche, Indianapolis, USA) 0.5U/dorsal root ganglion with 12 μM EDTA in 5 ml Complete Saline Solution (in mmol/l, NaCl 137, KCl 5.3, MgCl2-6H2O 1, Sorbitol 25, HEPES 10, CaCl2 3 and pH adjusted to 7.2 with NaOH) for 20 min at 37°C. Neurons were further digested with Liberase blendzyme TL with EDTA in Complete Saline Solution with papaïn (30U/ml) for 10 min. Finally neurons were suspended in dorsal root ganglion medium mix (89% DMEM/F-12, 10% BSA, 1% penicillin/streptomycin) supplemented with 1.5 mg/ml of trypsin inhibitor and 1.5 mg/ml of purified BSA. Mechanical dissociation was performed using a pipetman and neurons were plated on poly-D-lysine coated coverslips and incubated 12 hours before recording to allow recovery and adhesion of neurons. Neurons were only recorded for 12 more hours to prevent long-term culture phenotypic changes and neurite outgrowth that degrades space clamp. Small neurons (diameter <30 μm) were recorded using an EPC-10 amplifier (HEKA Electronics, Lambrecht, Germany) and Patchmaster/Fitmaster software

for data acquisition/analysis. The sampling interval was 20 µs and a 5 kHz filter was used in all experiments. Experiments were carried out in the whole-cell patch clamp configuration. Extracellular solution contained (in mmol/l) NaCl 30, TEA–Cl 110, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, Glucose 10, CdCl 0.1; pH was adjusted to 7.3 using Tris base, osmolarity was adjusted to 320 mosm/l with sucrose. The pipette solution contained CsF 140, NaCl 10, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 1.1, HEPES 10, pH was adjusted to 7.2 with CsOH and osmolarity was adjusted to 310 mosm/l. Pipettes were pulled from Borosilicate glass (World Precision Instruments, Sarasota, FL, USA) and had a resistance < 3 MΩ, when filled with the pipette solution. Capacity transients were cancelled and series resistance was compensated to around 90%. Leakage current was digitally subtracted online using hyperpolarizing control pulses, applied after the test pulse, of one-fourth test pulse amplitude (P/4 procedure). For current density measurements, membrane currents were normalized to the membrane capacitance which was calculated from the integral of the transient current in response to a brief hyperpolarizing pulse of 10 mV from the holding potential.

Once in whole cell configuration, cells were held at -60 mV for 5 minutes to dialyze the cell with CsF solution (fluoride shifts Nav1.8 steady-state activation and inactivation to hyperpolarized potentials) to reach Nav1.8 stable biophysical properties and to inactivate Nav1.9 current and was further clamped at -80 mV for 2 more minutes. Whole-cell Na currents were elicited by a series of 100 ms test pulses ranging from -80 mV to +40 mV in increments of 5 mV at a frequency of 0.33 Hz. Test pulses were preceded by a prepulse of 3 s at -120 mV. Normalized conductance (G/Gmax) was fitted as described for *in vitro* recordings and $V_{1/2}$ and slope factor were extracted from the equation. Steady state inactivation curves (SSI) were measured from a holding potential of −120 mV using 500 ms prepulses to the indicated potentials followed by a test pulse to 0 mV. Again, $V_{1/2}$ and slope factors were obtained as mentioned for *in vitro* recordings.

Recovery from inactivation curves were obtained with a standard two-pulse protocol consisting of a depolarizing pulse from a holding potential of -120 mV, to 0 mV for 50 ms to inactivate the channels, followed by a variable duration step (from 0.05 ms to 3276.8 ms) back to -120 mV to promote recovery. The availability of the channels was assessed with a second test pulse at 0 mV and the ratio of the second pulse versus the first was plotted against the recovery interval. The $t_{1/2}$ of recovery was calculated as mentioned previously.

Statistics:

-Behavioral statistics: For the time course and drug effect on mechanical allodynia after nerve injury 3 experiments were done separately: i) rufinamide 25mg/kg, rufinamide 50mg/kg and DMSO30%, ii) rufinamide 5mg/kg, rufinamide 10mg/kg and DMSO30%, iii) amitriptyline 10mg/kg, amitriptyline 20mg/kg and saline. The log values of withdrawal thresholds were assessed for each experiment using an Anova two-ways with Bonferroni correction for repeated measures from preinjection to 24h after injection. For the development of allodynia, baseline and preinjection were compared by using the Wilcoxon matched-pairs signed rank test (Bonferroni's correction for multiple testing) because baseline values are skewed. For clarity purposes on figure 1, a mean value of both DMSO groups is used and values are presented as mean±SD also for baseline. For the drug effect on naïve animals, data were analyzed with Kruskal-Wallis test and Dunn's correction for multiple testing. The numerical data are presented as median with 95% confidence interval.

-Electrophysiological statistics: Data are presented as mean±SD and were analyzed using paired student's *t* tests for drug effect.

All hypotheses were challenged using two-tailed testing and $p<0.05$ was used as the level of significance. Statistical analysis was performed using Prism 5 for windows, version 5.03, GraphPad Software, San Diego California USA.

Results:

1. Behavior

1.1 Rufinamide reduces mechanical allodynia following SNI

All animals developed allodynia one week after surgery ($p<0.05$, preinjection versus baseline for all groups). Rufinamide significantly and dose dependently alleviated SNI-induced allodynia (Fig. 1A), with maximal effect from 0.10±0.03g (mean±SD) to 1.99±0.26g. The effect was seen already 20 minutes following injection, peaked at 60 min, lasted for at least 4 hours, but had faded 24 hours after drug administration. At the highest dose of rufinamide, allodynia-like behavior was completely reversed. The vehicle DMSO showed a tendency for anti-allodynic effect but the values did not reach statistical significance in multiple testing.

1.2 Amitriptyline reduces mechanical allodynia following SNI

All animals showed allodynia before injection of amitriptyline ($p<0.05$ preinjection versus baseline for all groups). Amitriptyline alleviated the allodynic behavior from 60 to 240 min after injection and the effect had disappeared at 24h (Fig. 1B) with maximal effect from 0.25±0.22g to 1.9±0.8g. There was no difference between 10 and 20 mg/kg.

1.3 Amitriptyline but not rufinamide affects basal sensitivity

Rufinamide (50mg/kg) did not modify basal mechanical sensitivity of naive animals or heat withdrawal latency. We therefore did not test lower doses (Fig. 2A-B). On the other hand, amitriptyline at 20 mg/kg increased withdrawal threshold for innocuous mechanical stimulation with von Frey hair from 1.3g (0.6-1.9) (median and 95% confidence interval) to 2.3g (2.2-2.5) and increased withdrawal latency on heat stimulation compared to saline from 13.1s (10.4-15.5) (median, 95%CI) to 30.0s (21.8-31.9). We therefore tested amitriptyline at 10 mg/kg and also observed antinociceptive effect on heat

stimulation (withdrawal threshold from 10.5s (7.2-11.7) to 25.3s (16.4-27.7)), but no statistically significant difference on non-noxious mechanical stimulation (Fig. 2A-B). Animals injected with rufinamide 50 mg/kg did not lower their total activity measured over 4 hours after injection with the Activ-meter as compared to saline injected controls. Amitriptyline decreased total activity statistically significantly compared to saline injected controls (Fig. 2C).

Amitriptyline increased the score of sedation from 0 (saline group) to 2[0-3] (median, [range], n=8). Rufinamide did not change the score (0).

1.4 Rufinamide plasma level corresponds to therapeutic level for epileptic patients

At peak effect for mechanical allodynia, the range of plasma level for rufinamide was 68-86 μmol/l.

2. Effect of rufinamide on Nav1.7 channel compared to amitriptyline and mexiletine

2.1 Rufinamide reduces Nav1.7 peak current

Rufinamide reduced peak sodium current (INa) induced by a single pulse depolarization using human embryonic kidney 293 cells stably expressing Nav1.7 (Fig. 3). The most substantial reduction obtained with rufinamide was 28.3%, at a concentration of 500 μmol/l. The drug could not be dissolved at higher concentration. A concentration of 100 μmol/l was used for the rest of the testing to avoid the high DMSO concentration used for 500 μmol/l. With high concentration of amitriptyline and mexiletine a complete inhibition of I_{Na} could be obtained and EC50 were used for the following experiments (Fig. 3).

2.2 Rufinamide shifts steady-state inactivation of Nav1.7

The voltage dependence of activation was examined using a series of 10 ms depolarizing test pulses from -80 to +85 mV from a holding potential of -100 mV. Rufinamide had no

effect on voltage-dependency of activation for Nav1.7 sodium channel, nor did amitriptyline and mexiletine. No statistically significant changes were seen in $V_{1/2}$ of activation. Slopes were slightly altered by rufinamide and mexiletine (Fig. 4). For the steady-state inactivation experiments, cells were given a 500 ms conditioning pulse at voltages between −130 mV and −10 mV from a holding potential of -100 mV followed by a 20 ms test pulse. Normalized sodium currents (I_{Na}/Imax) measured during test pulses were plotted against conditioning voltage. Rufinamide shifted the steady-state inactivation relationship to more hyperpolarized value with a $V_{1/2}$ of inactivation shifting from -81.0±4.4 to -87.6±4.9 mV. The control drugs had a similar effect with shift of $V_{1/2}$ of inactivation, from -81.8±2.8 to -88.4±1.1 mV for amitriptyline and from -80.0±3.0 to -91.4±2.6 mV for mexiletine. The slopes of steady-state inactivation curves were not influenced by any of the tested drugs (Fig. 4).

2.3 Rufinamide prolongs the recovery from fast inactivation of Nav1.7

Effects on the recovery from fast inactivation was examined with a standard double-pulse protocol consisting of a depolarizing pulse to -10 mV to inactivate the channels followed by a variable duration (0.25 to 2000 ms) step to the holding potential of -100 mV to promote recovery. The availability of the channels at the end of the recovery interval was assessed with a standard test pulse. The ratios of response of $2^{nd}/1^{st}$ pulse were plotted versus the recovery interval. The $t_{1/2}$ of recovery was interpolated. It was statistically significantly prolonged for the 3 tested drugs (Fig. 5).

2.4 Rufinamide shows use-dependent inhibition of Nav1.7

Frequency-dependent or use-dependent blocking refers to the accumulation of channels in inactivated state when subjected to a train of depolarizing pulses at high frequency. We applied a series of 50 pulses at varying frequencies (2, 5, 10, 25, 50Hz) and plotted

the normalized current against the pulse number. Rufinamide at 100 μmol/l increased the use-dependent block at all frequencies tested, except 2Hz. Amitriptyline and mexiletine also increased the use-dependent block, even at 2 Hz (Fig. 6).

3. Rufinamide influences INa in dorsal root ganglion neurons

We then wanted to validate the effect of rufinamide using dissociated mouse dorsal root ganglion neurons which contain also other Nav channels and the β-subunits. We first observed that rufinamide at 100 μmol/l consistently induced a statistically significant 10.1% mean reduction in peak sodium current densities from 956±396 to 850±339 pA/pF ($p<0.05$) despite a great variability in absolute values of current density (Fig. 7A). We then assessed voltage-dependence of activation and inactivation of the sodium current on the dorsal root ganglion with step protocols. The global effect of rufinamide on dorsal root ganglion was similar to the one observed using human embryonic kidney 293 cells expressing only Nav1.7. The voltage-dependence of activation was unchanged and the inactivation curve was shifted with statistical significance toward more hyperpolarized potentials, from a $V_{1/2}$ of inactivation of -64.4±16.8 mV to-69.4±17.1 mV ($p<0.0001$) (Fig. 7B). Finally we observed that rufinamide also delayed $t_{1/2}$ of recovery from inactivation from 2.58±2.12 to 6.24±5.04 ms ($p<0.05$) (Fig. 7C).

Discussion:

We here demonstrate that rufinamide alleviates mechanical allodynia-like behavior in the SNI model of neuropathic pain in mice. Its effect is comparable to amitriptyline, but with no interference on basal sensitivity and activity tests. We also show that rufinamide modulates Nav1.7. It stabilizes the channel in its inactivated state similarly to amitriptyline and mexiletine, and delays its recovery from inactivation. By the observation of rufinamide effect on total sodium currents recorded in dorsal root ganglion neurons, we finally validated a potential peripheral mechanism of action of rufinamide for the treatment of neuropathic pain.

Effect of rufinamide on mechanical allodynia following SNI in mice

We present a robust effect of amitriptyline and rufinamide on SNI-induced mechanical-allodynia in mice. To our knowledge, this is the first trial testing rufinamide in a model of neuropathic pain.

Amitriptyline is a first line treatment for clinical neuropathic pain[8]. Amitriptyline alleviates neuropathic pain-like behavior in the chronic constriction injury[32;33] and spinal nerve ligation models[34]. In other studies it failed to affect mechanical allodynia in these models[35;36] or on paw pressure hypersensitivity in a rat diabetes-related pain model[37]. In the SNI model, in rats, amitriptyline decreased mechanical allodynia in the early 3-5 days post injury[38] whereas it failed to show an effect 2 to 4 weeks after nerve injury[39]. When administered perisurgically for 1 week, amitriptyline failed to prevent the development of mechanical allodynia in rodents[40].

Despite diverging results explained by the different sensory modalities tested, timing, dose and administration route or species/genetic background[41;42], the SNI model remains a robust neuropathic pain model in rodents. In rats, mechanical allodynia following SNI

does not respond to moderate doses of morphine, gabapentin, carbamazepine, MK-801[39], lidocaine, lamotrigine[43] or rofecoxib[44]. Other groups showed a transient effect of high dose of morphine (6 mg/kg, effect < 3hours), mexiletine (37 mg/kg, < 1 hour) or gabapentin (100 mg/kg, < 5 hours)[45] and tocainide[43]. Side effects and sedation are rarely mentioned but with high doses, many of the tested drugs in SNI could impair basal sensitivity[39].

Rufinamide alleviates dose-dependently mechanical allodynia in this model, without inducing any changes in sedation or affecting basal sensitivity. Amitriptyline reduced allodynia, but also modified basal pain sensitivity and sedation score, which could participate in its anti-allodynic effect. Amitriptyline has been shown previously to change locomotor activity in rodents due to sedation, ataxia, changes in nociception, depression or anxiety[46-50]. In one study amitriptyline did not change locomotor activity in the chronic constriction injury model despite reducing allodynia. We are in agreement with others who showed an increase in thermal latency after acute amitriptyline treatment[46;51].

Rufinamide has the potential of a new treatment for neuropathic pain

As first line therapy for the treatment of neuropathic pain, clinical guidelines propose tricyclic antidepressants (amitriptyline), serotonin and norepinephrine reuptake inhibitors (duloxetine and venlafaxine) or anticonvulsants targeting α2-δ subunit of calcium channels (gabapentin and pregabalin)[8;52]. The most effective antidepressants in the treatment of neuropathic pain have sodium channel blocking properties[53], which may contribute to their analgesic activity[10;54]. The use of sodium channel blockers as a first-line evidence-based treatment recommendation has not yet been suggested except for two specific conditions: carbamazepine in trigeminal neuralgia[52] and topical lidocaine in postherpetic neuralgia with irritable nociceptor[11]. The systemic delivery of a sodium channel blocker is limited by the poor tolerability (and restricted availability in many countries) of mexiletine as well as the high risk of drug interaction with carbamazepine[55].

Rufinamide offers a valuable alternative to tricyclic antidepressants / serotonin and norepinephrine reuptake inhibitors and calcium channel α2-δ ligand for the pharmacologic management of neuropathic pain.

In clinical practice, the efficacy of amitriptyline on neuropathic pain is variable[56;57]. Amitriptyline is well known for its side effects, predominantly sedation, hypotension and anti-cholinergic effects, considerably reducing patient's compliance[58]. In particular, sedation has been known for a long time even at "light" dosage (50 mg)[59;60]. For rufinamide, in a study for Lennox-Gastaut syndrome, the incidence of adverse events for somnolence or vomiting was more common in the rufinamide treated group[13], but causing only 2 respectively 3 patients out of 74 to withdraw from the study.

Drug interaction is also a major issue for pain therapy. Rufinamide presents favorable pharmacokinetic parameters; it is well absorbed orally and is not a substrate of cytochrome p450 system, thereby reducing its potential interactions. It is however a mild inducer of CYP3A4[61]. Rufinamide may be a mood-stabilizing molecule with anxiolytic properties[62] that could be an added value considering the large proportion of psychiatric mood-disorders encountered in chronic pain patients[63]. The toxicity studies in rodents show a greater safety ratio than other anticonvulsants[25]. Na channels are still a major target in the development of new analgesic drugs[23;64], but rufinamide already being on the market, might offer a new treatment opportunity in the pain field, while other drugs trying their way through clinical trials have failed[65;66].

Site of action of Rufinamide

Rufinamide is a sodium channel blocker but its exact mechanism and site of action are unknown. Its effects on biophysical properties of sodium currents are similar to amitriptyline and mexiletine. Amitriptyline apparently interacts with residues on the

DIVS6 segment[67] but also DIS6, DIIIS6 and DIVS6 segments, which may jointly form parts of the amitriptyline/local anesthetic receptor[68].

The importance of DIVS6 segment in mexiletine binding to sodium channel has also been demonstrated[69]. Further studies are needed to investigate the site of action of rufinamide on Nav1.7 and other channel isoforms, but a similar site can be hypothesized from the properties of stabilization of the inactivated state shown here. Following the recent report of the crystal structure of the voltage-gated sodium channel we hope new mechanistic knowledge will be gained in drug-channel interactions[70].

We demonstrated the action of rufinamide on the peripherally expressed Nav1.7 isoform of sodium channel but we do not intend to show any specific Nav1.7 blocking properties. Indeed the drug is used in the treatment of epilepsy and therefore should also act on centrally expressed sodium channels. Rufinamide showed no relevant interaction with monoaminergic binding sites in radioligand binding studies and no interactions with benzodiazepine or gamma-aminobutyric acid (GABA) receptors, 5-HT1 and 5HT2 receptors, α- or β-adrenoceptors, or human recombinant metabotropic glutamate receptor subtypes 1b, 2, or 4 (mGluR1b, mGluR2, mGluR4). However, an inhibitory effect of rufinamide at the mGluR5 subtype was observed at 100 μmol/l[61]. mGluR5 is upregulated in the dorsal root ganglia and spinal cord after spinal nerve ligation (but not after partial sciatic nerve ligation)[71] and peripheral mGluR5 agonists can produce thermal hyperalgesia[72]. Trials on mGluR5 antagonists mostly show an effect on thermal sensitivity in neuropathic pain models but not on mechanical allodynia[71;73]. Some groups show an effect on spinal nerve ligation model or chronic constriction injury in rats[74], but the magnitude of effect on mechanical allodynia is below 40% of recovery toward baseline values for systemic administration. Dogrul et al showed a 66% reduction in mechanical allodynia with a shallow dose-response curve following spinal nerve ligation after intrathecal delivery of mGluR5 antagonist[75]. Fisher et al could show a preventive effect

when an antagonist of mGluR5 was administered over 8 days after constriction injury of the sciatic nerve but no effect was seen on established mechanical allodynia[76;77]. Altogether, the effects of mGluR5 antagonists are indeed not as potent as the complete reversal of established mechanical allodynia through rufinamide. Therefore, we suggest mGluR5 is not the major target for rufinamide.

Therapeutic plasmatic concentration for epilepsy (20-200 μmol/l)[13] and plasmatic concentration in our study at the time of anti-allodynic effect (range 68-86μmol/l) are in the range of concentration used for *in vitro* testing (100 μmol/l). The interest of rufinamide at the concentration we used is that the current is not completely blocked but globally the channel is less excitable. Nav1.7 is well expressed in basal condition but Nav1.7 transcripts or TTX-S currents are not upregulated after SNI or other peripheral nerve injuries[3]. After nerve injury hyperexcitability and ectopic discharges at the neuroma or in the dorsal root ganglion[4] might be affected by the modulation of Na channel properties by rufinamide whereas there is no effect on nociception on a naïve nerve. We therefore suggest the anti-allodynic effect of rufinamide is related to its Na channel blocking properties.

Limitations of the study

Differential effect of Rufinamide, amitriptyline and mexiletine on Nav1.7 sodium channel

We used the ED50 of amitriptyline and mexiletine, 10 μM and 100 μM respectively. The plasma concentrations of these 2 drugs are typically around 0.3 μmol/l[78] and 2.3-9.3 μmol/l[58]. Rufinamide was used at 100 μmol/l, due to its low solubility in patch clamp solution. Our study is not intended to compare the effect size of the drugs on the different biophysical properties. The low solubility of rufinamide impeded a comparison of the 3 drugs at their ED50 values. The effect on peak current on Nav1.7 as well as on

dorsal root ganglion neurons is low but nonetheless statistically significant and reproducible.

Effect of DMSO as control

DMSO was used to dissolve rufinamide despite the potential neurotoxicity with prolonged administration at high dose[79]. It was also used as a treatment option in osteoarthritis[80] but only with relative efficacy on pain scores. We did not see any effect of DMSO on naïve animal sensitivity behavior regarding toxicity and compared the anti-allodynic of rufinamide to DMSO.

Conclusion and future directions

We here show that rufinamide dose-dependently alleviates neuropathic pain behavior in the SNI model in mice. We show *in vitro* electrophysiological data that rufinamide induces a hyperpolarizing shift in the steady-state inactivation curve, a use dependent–block and a delay in recovery from inactivation from Nav1.7-mediated current and *ex-vivo* data that the same stabilizing effect on inactivation is also present in dorsal root ganglion neurons. Sodium channels blockers still belong to the potential targets to treat neuropathic pain but often do not come on the market for toxicity or side effects issues. Rufinamide is currently on the market and could therefore be used in clinical studies in the pain field rapidly. With the low rate of success from current chronic pain therapy, a new drug would be highly valued.

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Figure Legends

Fig. 1: Rufinamide (RUF) and amitriptyline (AMI) alleviate mechanical allodynia after SNI A) RUF dose-dependently alleviates neuropathic behavior following SNI from 20 to 240 min after injection with a peak at 60 min and a loss of effect at 24h, B) AMI alleviates neuropathic behavior following SNI from 60 to 240 min after injection and losts its effect at 24h, (*p<0.05, **p<0.01, ***p<0.001 vs PreInj). BL=baseline, Preinj=pre-injection (1 week after SNI for RUF, 2 weeks for AMI), SNI=spared nerve injury, DMSO=dimethylsulfoxide. Values are presented as mean±SD.

Fig. 2: Rufinamide (RUF) and amitriptyline (AMI) differentially affect basal sensitivity and activity of naïve animals. A) RUF at 50 mg/kg does not affect withdrawal threshold to mechanical stimulation with von Frey filaments as compared to AMI which significantly increased the threshold at the dose of 20 mg/kg (not statistically significant for 10mg/kg), n=8. B) RUF at 50 mg/kg does not affect withdrawal latency to heat stimulation as compared to AMI which significantly increased the latency at the dose of 10 and 20 mg/kg, n=8. C) The total activity (in hours) of the animals was measured using the Activ-meter system over a 4 hours period following drug injection and compared to activity following saline. AMI (10mg/kg) but not RUF (50mg/kg) significantly reduces the activity compared to control, n=6. Data are expressed as median (*horizontal line*) and *box* and *whiskers* with first and third quartiles (*box*), and minimum and maximum (*whiskers*), ns=non-significant, *p<0.05, **p<0.01 versus control (CTRL).

Fig. 3: Drugs inhibit voltage-gated sodium channel Nav 1.7 peak current. A) Percentage reduction of peak current after single pulse stimulation B) Example of traces with the drug concentrations used afterwards in the biophysical properties testing, respectively

100, 10 and 100 μmol/l for rufinamide (RUF), amitriptyline (AMI) and mexiletine (MEX). Transients were blanked.

Fig. 4: Drugs induce a shift of inactivation properties of voltage-gated sodium channel Nav1.7. Rufinamide (RUF), amitriptyline (AMI) and mexiletine (MEX) (at respectively 100, 10 and 100 μmol/l) induce a hyperpolarizing shift in steady state inactivation (SSI) without changing activation (ACT) properties of the voltage-gated sodium channel Nav1.7. $V_{1/2}$ of activation/inactivation, slopes, p values and n values are summarized in the tables. Insert: stimulation protocols. CTRL=control, values are mean±SD.

Fig. 5: Drugs induce a prolongation of recovery from inactivation of voltage-gated sodium channel Nav1.7. Rufinamide (RUF), amitriptyline (AMI) and mexiletine (MEX), at respectively 100, 10 and 100 μmol/l, prolonged in a statistically significant way the half time ($t_{1/2}$) of recovery from inactivation of Nav1.7 channel. Values of interest are summarized in the table. Insert: stimulation protocol. CTRL=control, values are mean±SD.

Fig. 6: Drugs induce a use-dependent block of voltage-gated sodium channel Nav1.7 Rufinamide (RUF), amitriptyline (AMI) and mexiletine (MEX), at respectively 100, 10 and 100 μmol/l, all induced a statistically significant use-dependent block with stimulation frequencies from 2 to 50 Hz (except RUF at 2 Hz). All frequencies are shown in tables but for clarity purposes only 10 and 25 Hz are shown graphically. CTRL=control, values are mean±SD.

Fig. 7: Effects of rufinamide on freshly dissociated dorsal root ganglion neurons

A) Rufinamide (RUF) at 100 μmol/l induced a 10% reduction in sodium peak current density (p=0.0084, n=7, horizontal bars represent mean values). B) It significantly shifted the steady-state inactivation (SSI) curve to a hyperpolarizing direction ($V_{1/2}$ of inactivation from -64.4±16.8 to -69.35±17.1mV, p<0.0001, n=6) without changing activation properties ($V_{1/2}$ of activation from -40.6±8.4 to -43.4±5.1mV, p=0.17, n=7). C) RUF also prolonged recovery from inactivation with half-time ($t_{1/2}$) for CTRL and RUF of respectively 2.58±2.12 and 6.24±5.04ms, p=0.0028, n=6. CTRL=control, values are mean±SD.

Figure1
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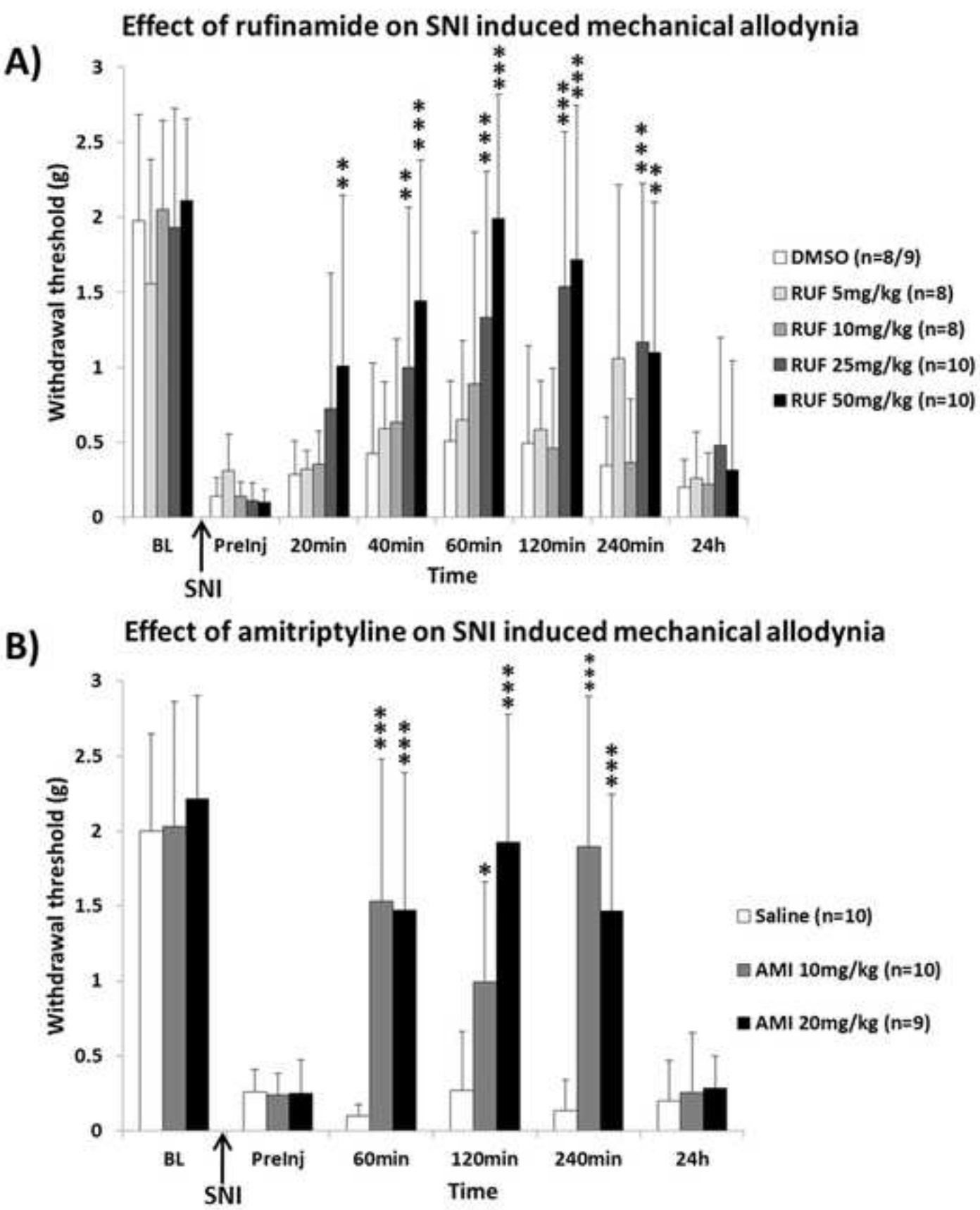

Figure2
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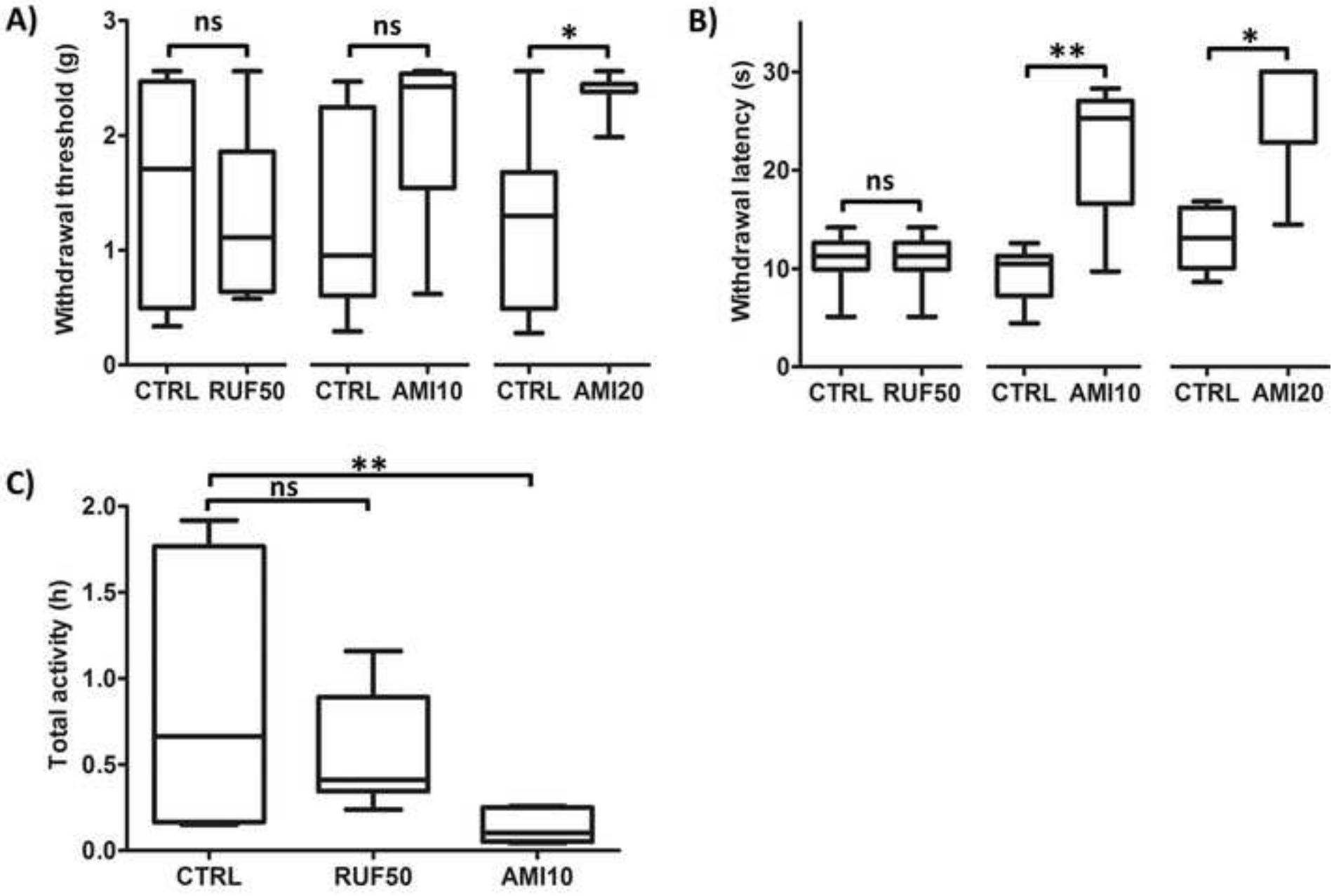

Figure3
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A)

RUF (µM)	Inhibition (%)
50	12.5
100	21.2
500	28.3

AMI (µM)	Inhibition (%)
10	43.9
30	64.7
100	89.7

MEX (µM)	Inhibition (%)
30	21.7
100	43.4
160	67.7
300	77.1

B)

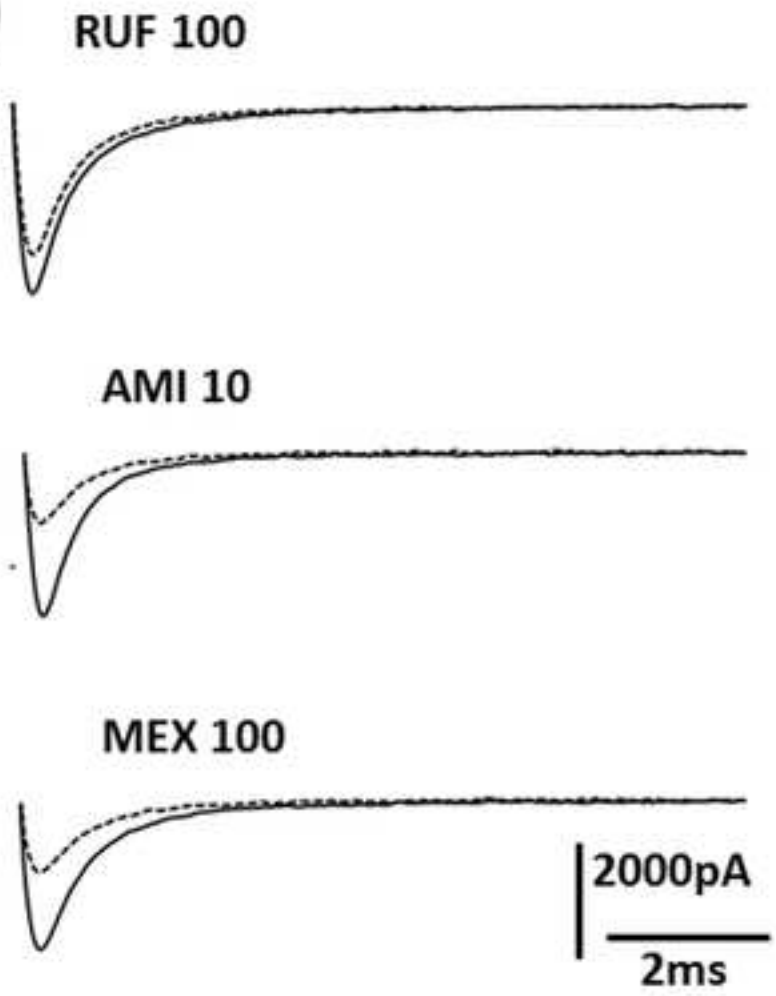

Figure4

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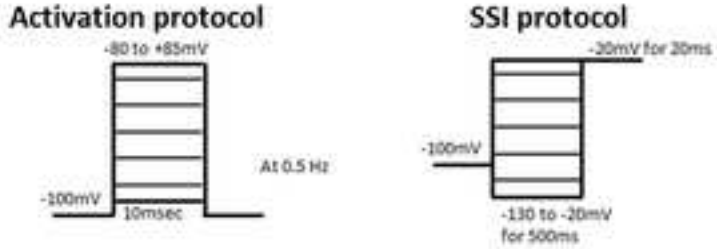
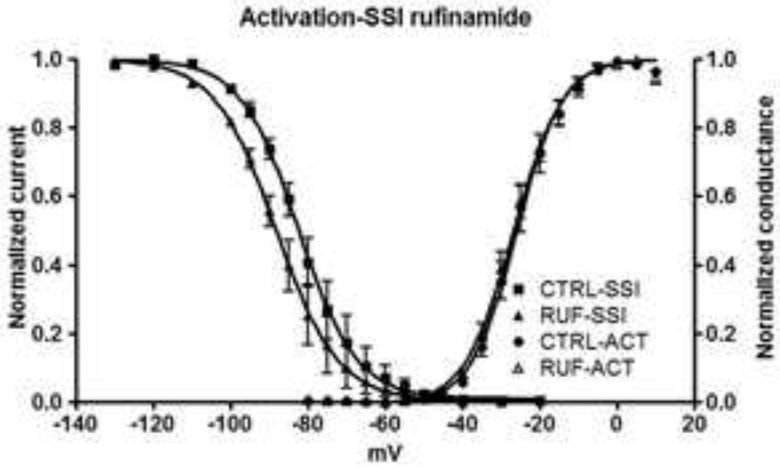
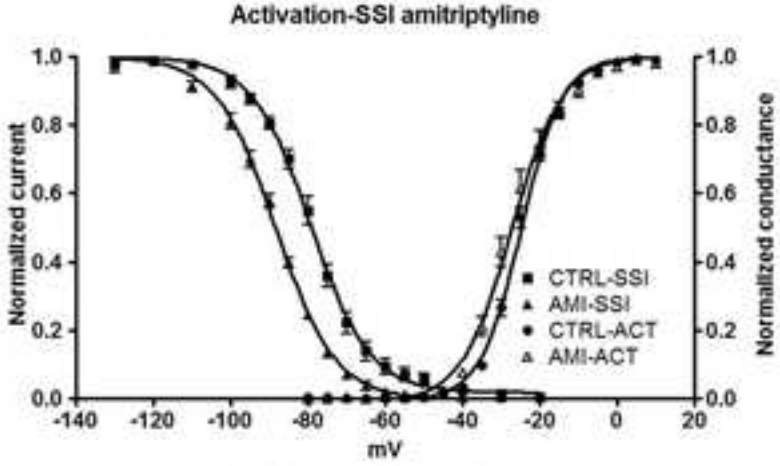
Activation	$V_{1/2}$ ± SD (mV)		Slope ± SD	
CTRL	-25.8 ± 3.3	p=0.28	5.5 ± 0.8	p=0.02
RUF	-26.6 ± 2.8		6.1 ± 1.0	n=5
Inactivation	$V_{1/2}$ ± SD (mV)		Slope ± SD	
CTRL	-81.8 ± 4.4	P=0.0038	7.3 ± 2.4	p=0.45
RUF	-87.6 ± 4.9		7.6 ± 2.0	n=4

Activation	$V_{1/2}$ ± SD (mV)		Slope ± SD	
CTRL	-24.8 ± 1.2	p=0.21	5.2 ± 0.5	p=0.11
AMI	-27.1 ± 2.8		6.1 ± 1.0	n=5
Inactivation	$V_{1/2}$ ± SD (mV)		Slope ± SD	
CTRL	-78.9 ± 2.8	P=0.0019	7.8 ± 1.0	p=0.52
AMI	-88.4 ± 1.1		7.7 ± 0.9	n=5

Activation	$V_{1/2}$ ± SD (mV)		Slope ± SD	
CTRL	-25.3 ± 2.9	p=0.61	5.7 ± 0.8	p=0.03
MEX	-25.1 ± 3.6		6.5 ± 1.2	n=5
Inactivation	$V_{1/2}$ ± SD (mV)		Slope ± SD	
CTRL	-79.8 ± 3.0	p=0.0003	7.4 ± 0.7	p=0.2
MEX	-91.4 ± 2.6		9.0 ± 1.2	n=4







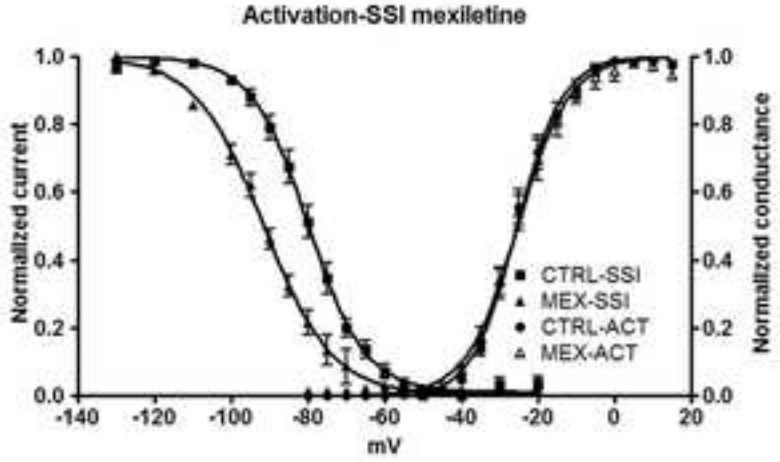


Figure5
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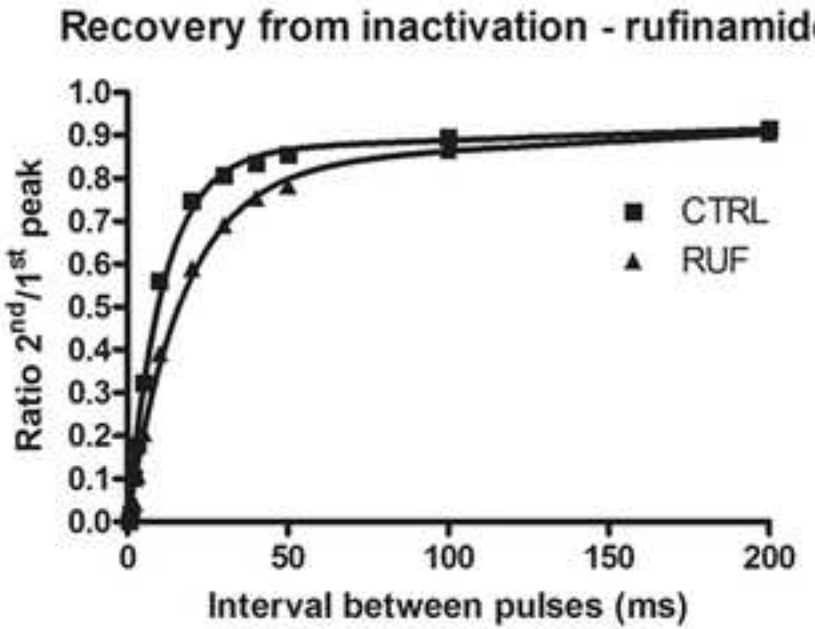

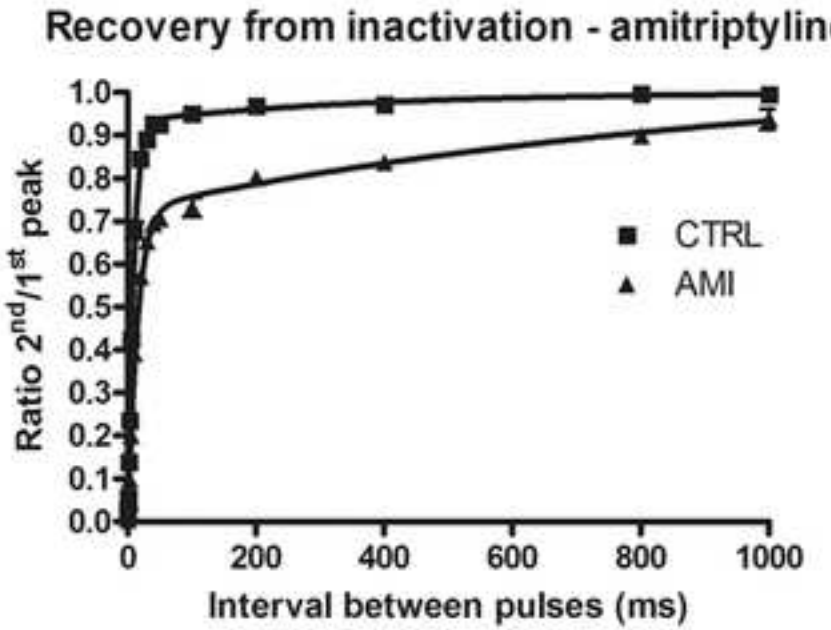

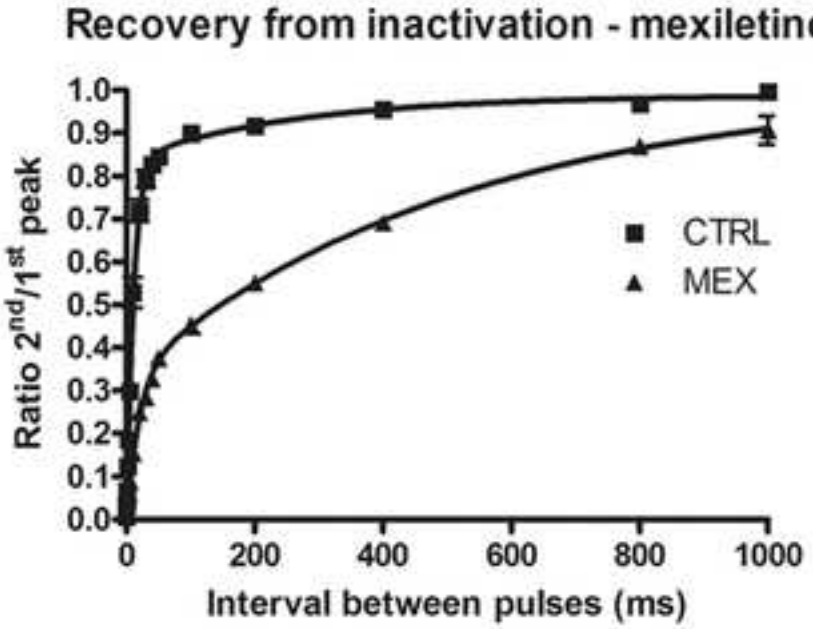

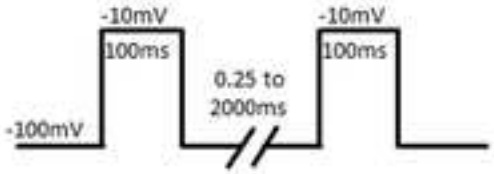

	RUF	AMI	MEX
	$t_{1/2}$ recovery (ms)	$t_{1/2}$ recovery (ms)	$t_{1/2}$ recovery (ms)
CTRL	8.89±1.64	6.48±0.64	7.73±3.13
Drug	15.41±2.37	16.08±1.06	152.4±22.4
t-test	p<0.0001	p=0.0011	p=0.0002
n	15	3	5

Figure6
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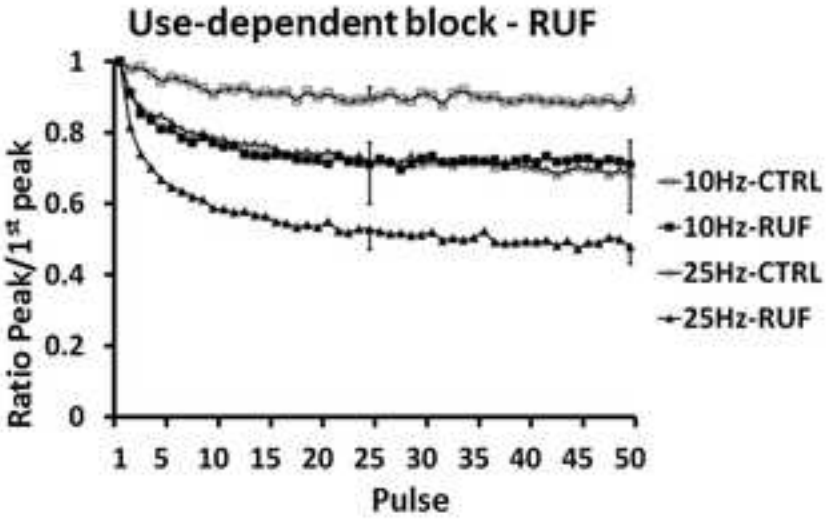
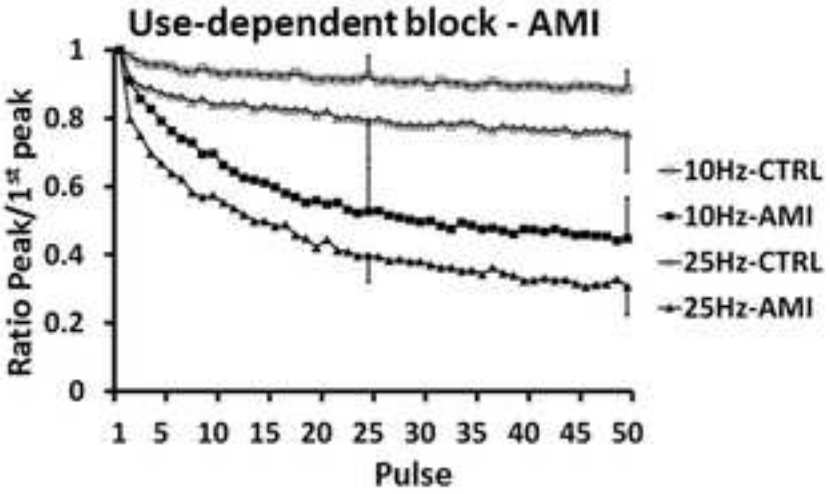
Normalized current after 50 pulses				
	CTRL	RUF		p value
2Hz	1.04	0.93	n=5	0.11
5Hz	0.92	0.80	n=4	0.0032
10Hz	0.89	0.71	n=3	0.033
25Hz	0.69	0.48	n=4	0.017
50Hz	0.23	0.10	n=3	0.033



Normalized current after 50 pulses				
	CTRL	MEX		p value
2Hz	1.01	0.74	n=5	0.0085
5Hz	0.96	0.44	n=4	0.0002
10Hz	0.92	0.25	n=5	0.0006
25Hz	0.81	-0.06	n=4	0.0017
50Hz	0.30	-0.16	n=3	0.038



Normalized current after 50 pulses				
	CTRL	AMI		p value
2Hz	0.99	0.76	n=5	0.028
5Hz	0.93	0.59	n=5	0.0005
10Hz	0.89	0.45	n=5	0.0011
25Hz	0.76	0.31	n=5	0.0009
50Hz	0.26	0.09	n=5	0.0007

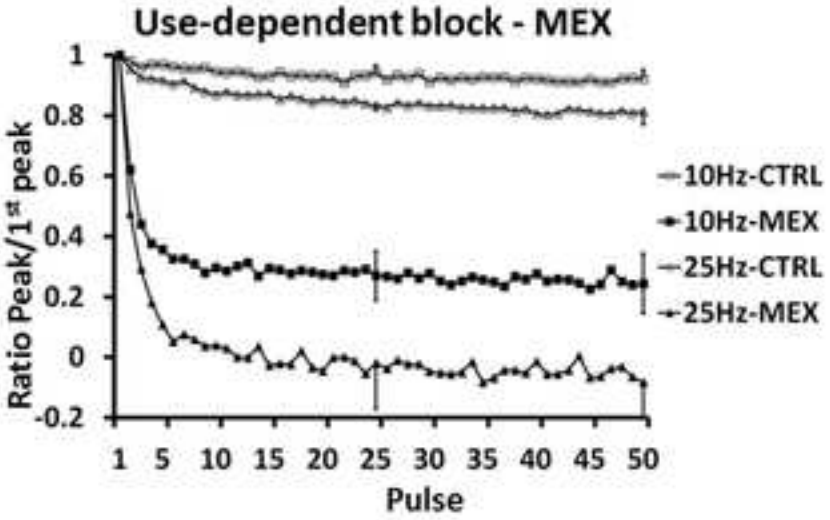


Figure7
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