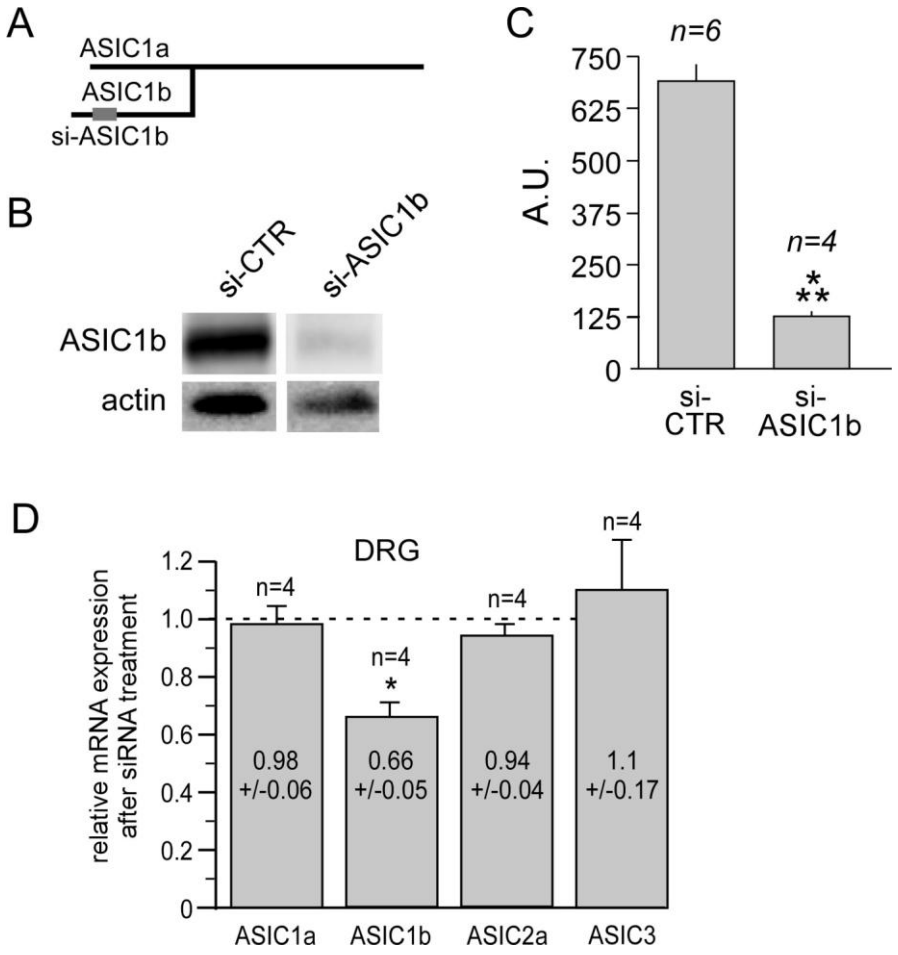
Analgesic effects of mambalgin peptide inhibitors of acid-sensing ion channels in inflammatory and neuropathic pain

Sylvie Diochot, Abdelkrim Alloui, Précillia Rodrigues, Mélodie Dauvois, Valérie Friend, Youssef Aissouni, Alain Eschalier, Eric Lingueglia & Anne Baron

SUPPLEMENTAL DATA

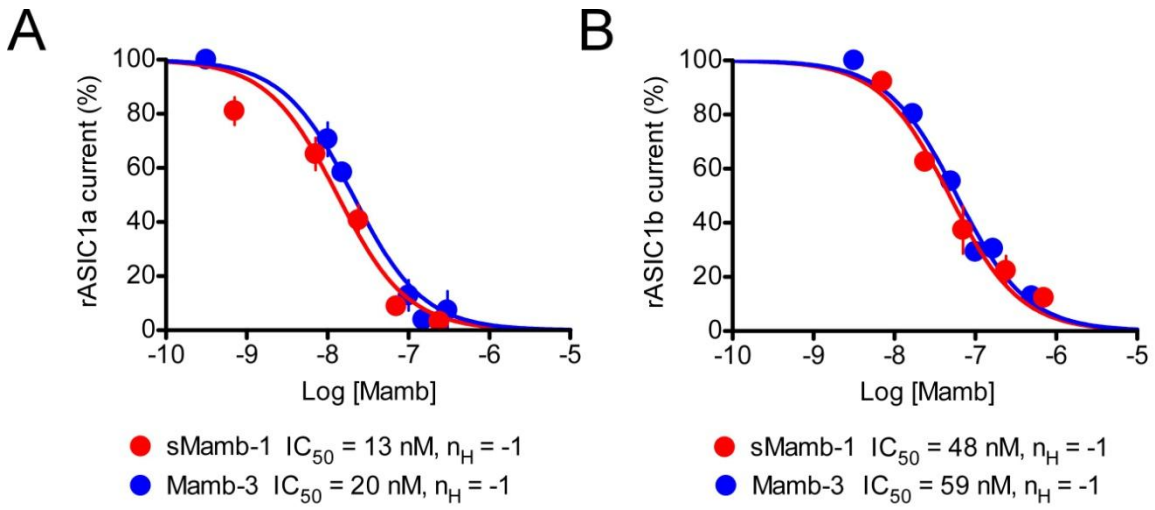


Suppl Fig. 1: *In vitro* and *in vivo* validation of the siRNA targeting ASIC1b.

A-C, siRNA targeting ASIC1b (si-ASIC1b**)** and control siRNA (si-CTR) were co-transfected in COS-7 cells with a plasmid coding for rat ASIC1b (pCI-ratASIC1b). The level of protein was assessed 48 hours after transfection by Western blot (in **B)** with an ASIC1-specific antibody. The blot was also probed with a monoclonal antibody against actin to normalize for protein loading. A densitometric quantification of the signal has been done in **C** and showed a 80% reduction of ASIC1b expression by si-ASIC1b treatment compared to si-CTR. A.U, arbitrary units. Mean ± S.E.M. *** : $p<0.001$ compared with si-CTR, unpaired *t*-test. **D**, *In vivo* siRNA treatment significantly reduced the level of ASIC1b transcripts in lumbar dorsal root ganglia (DRG) of WT mice but did not affect the level of ASIC1a, ASIC2a and ASIC3 transcripts. * : $p<0.05$ compared with ASIC1a, ASIC2a and ASIC3, paired t-test.

Methods: For validation of the *in vivo* effect of the siRNAs, lumbar DRG were removed after the last siRNA injection for total RNA isolation (RNeasy kit, Qiagen) followed by cDNA synthesis (High Capacity RNA-to-cDNA Kit, Applied Biosystems). The relative amounts of ASIC transcripts were evaluated by quantitative reverse-transcription PCR in a Light-Cycler

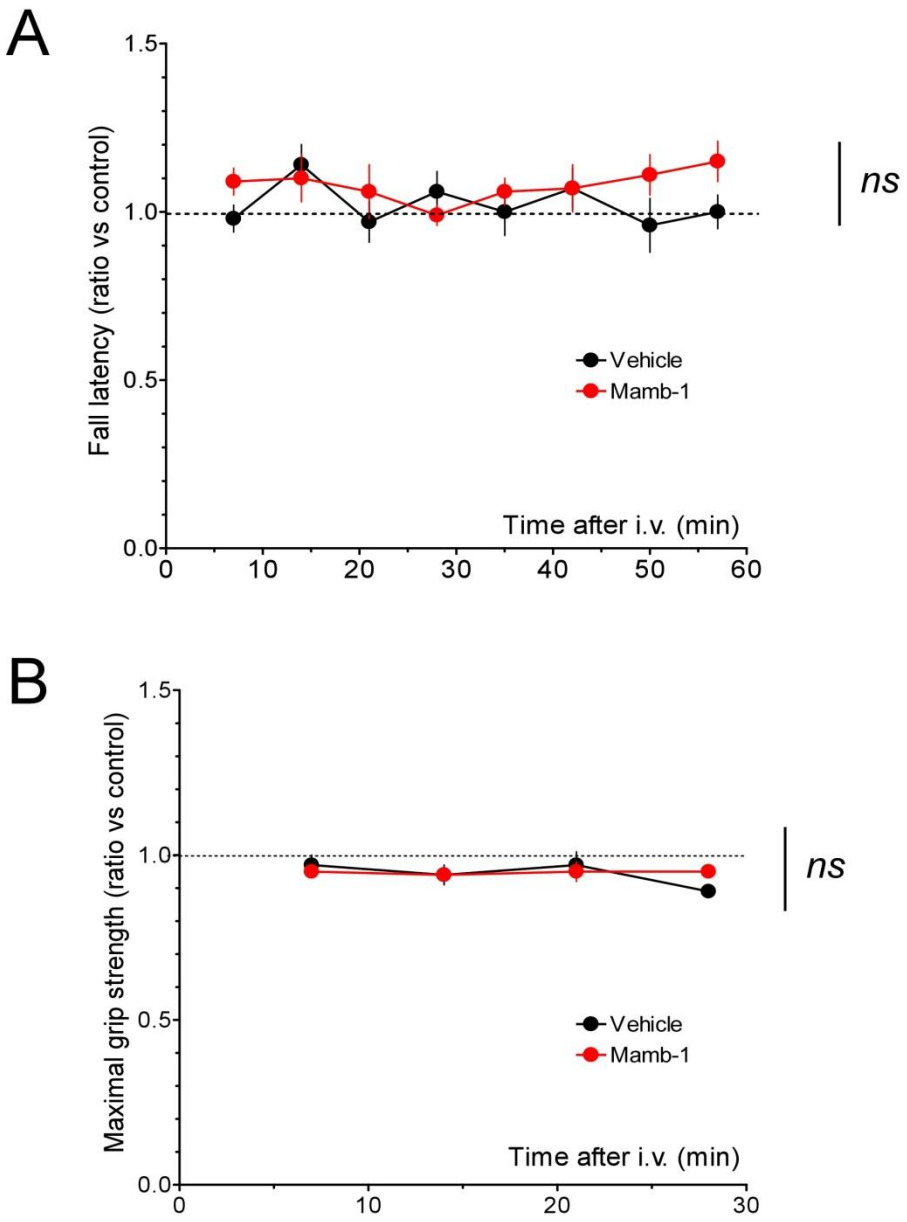
480 (Roche Products). Primers used are as follows: 18S aagtccctgccctttgtacaca/gatccgagggcctcactaaac; ASIC1a gccctgctcaacaacaggta/ tacggaagttggccttgtcc; ASIC1b cgcacgtgactttgctagat/gaaggtgacagctgggaaga; ASIC2a aagttcaaggggcaggagtg/tgtccagcatgatctccagc; ASIC3 cacccaatgacttgcactgg/taggcagcatgttcagcagg. Each cDNA sample was run in triplicate and results were normalized against 18S and converted to fold induction relative to control siRNA treatment.



Suppl. Fig 2: Inhibition by synthetic mambalgin-1 of recombinant rat ASIC1a and rat ASIC1b channels expressed in *Xenopus* oocytes.

Dose-reponse curves of inhibition (% of control current) of homomeric ratASIC1a current (**A**) and homomeric rat ASIC1b current (**B**) heterologously expressed in *Xenopus* oocytes by synthetic mambalgin-1 (sMamb-1) compared to native purified mambalgin-3 (Mamb-3). Mean ± SEM, n=3 to 7. Curves were fitted according the sigmoidal dose-response equation: Y= current % / (1+10^((LogIC_{50}-X)*n_H)), with n_H the Hill slope number and IC_{50} the concentration that inhibits 50% of the control current.

Methods: Electrophysiology in* Xenopus *oocytes. ASIC currents were recorded using the two-electrode voltage-clamp (TEVC) technique in *Xenopus laevis* oocytes injected into the nucleus with 30 nl of pCI-rat ASIC1a (5µg/µl) or pCI ratASIC1b (100µg/µl) plasmids, as previously described [19]. Briefly, oocytes were kept at 19 °C in ND96 solution containing 96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES (pH 7.4 with NaOH) supplemented with penicillin (6 µg/ml) and streptomycin (5 µg/ml). Expression of ASIC channels was tested 24 to 48 hours after injection using a Roboocyte2 automated workstation (MultiChannelSystems MCS, Reutlingen, Germany). Oocytes were clamped at -60 mV and ASIC currents were activated by rapid pH changes from 7.4 to 5.5 in external ND96 solutions. All experiments were performed at 19 to 21 °C in ND96 solution supplemented with 0.05% fatty acid- and globulin-free bovine serum albumin (Sigma) to prevent nonspecific adsorption of the toxins to tubing and containers. Mambalgins were applied 30 seconds before the acid stimulation.

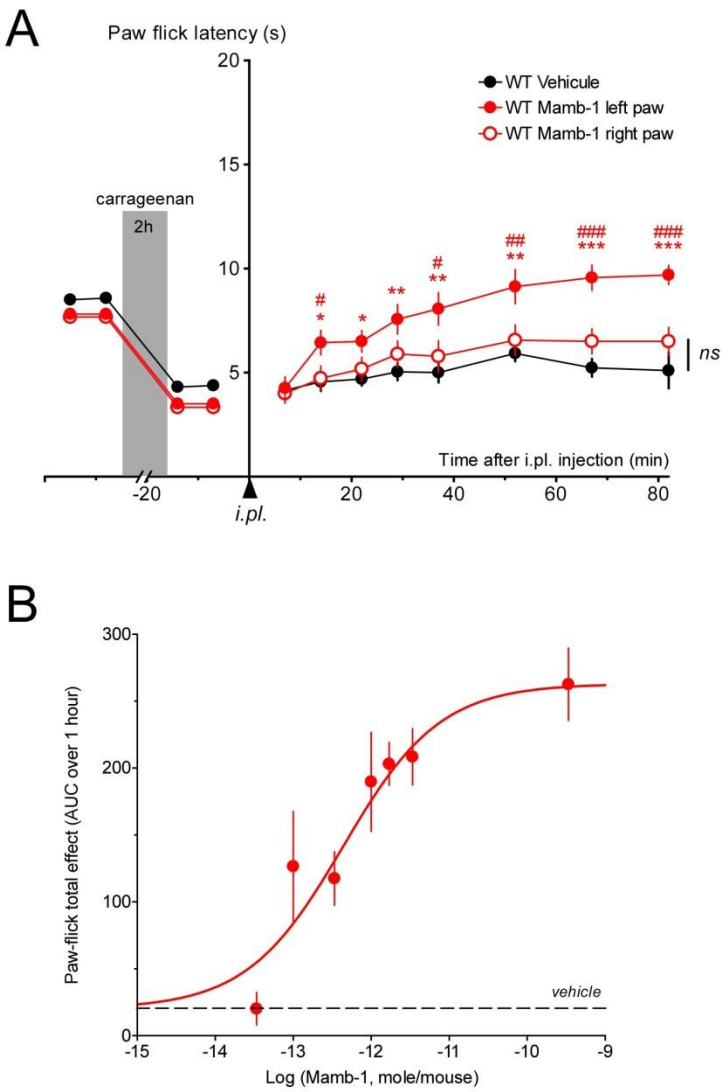


Suppl. Fig 3: Systemic *i.v.* injection of mambalgin-1 showed no motor impairment.

A, Accelerated rotarod test. Time-course of fall latency (seconds) after an *i.v.* injection of Mamb-1 (0.34 nmol/mouse) or vehicle, expressed as a ratio of the control latency before injection. Mean ± SEM, n=11 to 12. p>0.005, unpaired *t*-test. The control fall latency was 133 ± 7 seconds (n=11). **B**, Grip Test. Time-course of maximal grip strength (g) of all four limbs after an *i.v.* injection of Mamb-1 (0.34 nmol/mouse) or vehicle, expressed as a ratio of the control strength before injection. Mean ± SEM, n=13 to 17. p>0.05, unpaired *t*-test. Control maximal grip strength was 130 ± 6 g (n=13).

Methods: Motor behaviour tests.

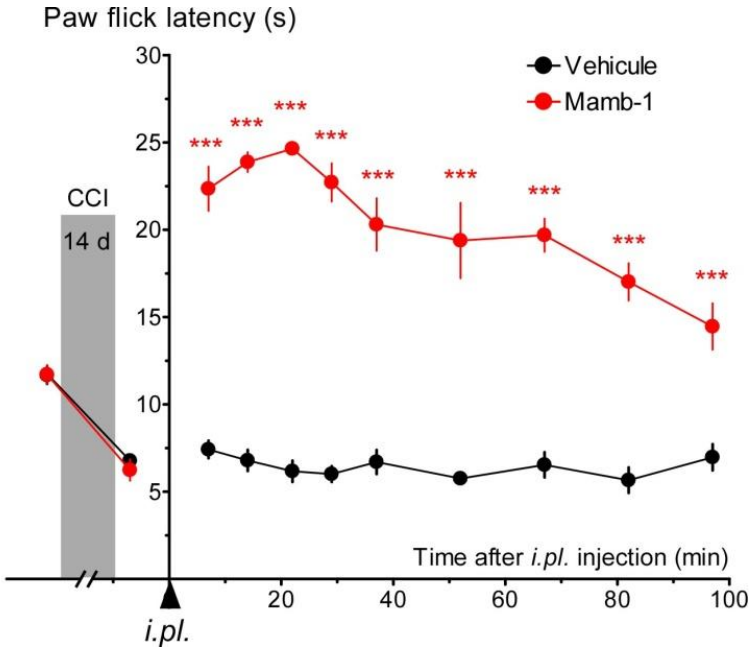
Accelerated Rotarod test. Mice were placed onto the dowel of an accelerating Rotarod treadmill (Bioseb, France) rotating at 4 rpm and then accelerating at a constant rate of 5 rpm up to 40 rpm [18]. The latency to fall to the floor was recorded, with a maximum cutoff latency of 300 sec. Mice were trained during two days before being tested, and each trial was done in duplicate. The time-course of fall latency (s) was measured before and after an injection of mambalgin-1 or vehicle. ***Grip strength test.*** After training, mice were allowed to grab a metal grid and then pulled backwards so that the maximal muscular grip strength developed by the four limbs was measured just before they lost grid (Bioseb, France). The time-course of grip force (g) was measured before and after an injection of mambalgin-1 or vehicle. Each trial was done in duplicate.



Suppl Fig. 4: Local analgesic effect of mambalgin-1 on thermal inflammatory hyperalgesia after intraplantar (*i.pl.*) injection.

A, Effect of *i.pl.* injection of mambalgin-1 (0.34 nmole/mouse) in the left or the right hindpaw (vehicle: saline + 0.05% BSA) on carrageenan-induced heat hyperalgesia when injected two hours after *i.pl.* carrageenan (2%, 20 µl) in the left hindpaw. Mean ± SEM, n=15 to 22. *, $p<0.05$; **, $p<0.001$; ***, $p<0.005$, ns, $p>0.05$ not significant, Anova + Newman-Keuls post-test *vs* vehicle. #, $p<0.05$; ##, $p<0.001$; ###, $p<0.005$, Anova + Newman-Keuls post-test *vs i.pl.* injection into the right hindpaw. **B**, Dose-dependent effect of *i.pl.* Mamb-1 on inflammatory heat hyperalgesia (carrageenan 2%, 2 hours) determined using the paw-immersion test (46°C). Area under curve (AUC, seconds x minutes) calculated over 1 hour from each mouse after *i.pl.* injection of the indicated quantity of Mamb-1 (mole/mouse). Data were fitted by a sigmoidal curve with an EC_{50} of 0.44 pmol/mouse and a Hill slop number of 0.7. Mean ± SEM, n=6 to 16.

Methods: For *i.pl.* injections, mambalgin-1 (sMamb-1, 34 µM, unless otherwise mentioned) was dissolved in 10 µl saline (NaCl 0.9%), and injected in the left hind paw (unless otherwise mentioned) with a 26G needle. Bovine serum albumin (0.05%) was added in all final dilutions to prevent toxin non-specific adsorption.



Suppl Fig. 5: Effect of *i.pl.* mambalgin-1 on pain behavior in the neuropathic model.

Effect of *i.pl.* Mamb-1 (0.34 nmol/mouse) on thermal hyperalgesia in the CCI model of neuropathic pain (paw-immersion test). Mean ± SEM, n=9 to 11. ***, $p<0.005$, Anova + Newman-Keuls post-test *vs* vehicle.

Methods: For *i.pl.* injections, mambalgin-1 (sMamb-1, 34 µM) was dissolved in 10 µl saline (NaCl 0.9%), and injected in the left hind paw with a 26G needle. Bovine serum albumin (0.05%) was added in all final dilutions to prevent toxin non-specific adsorption.