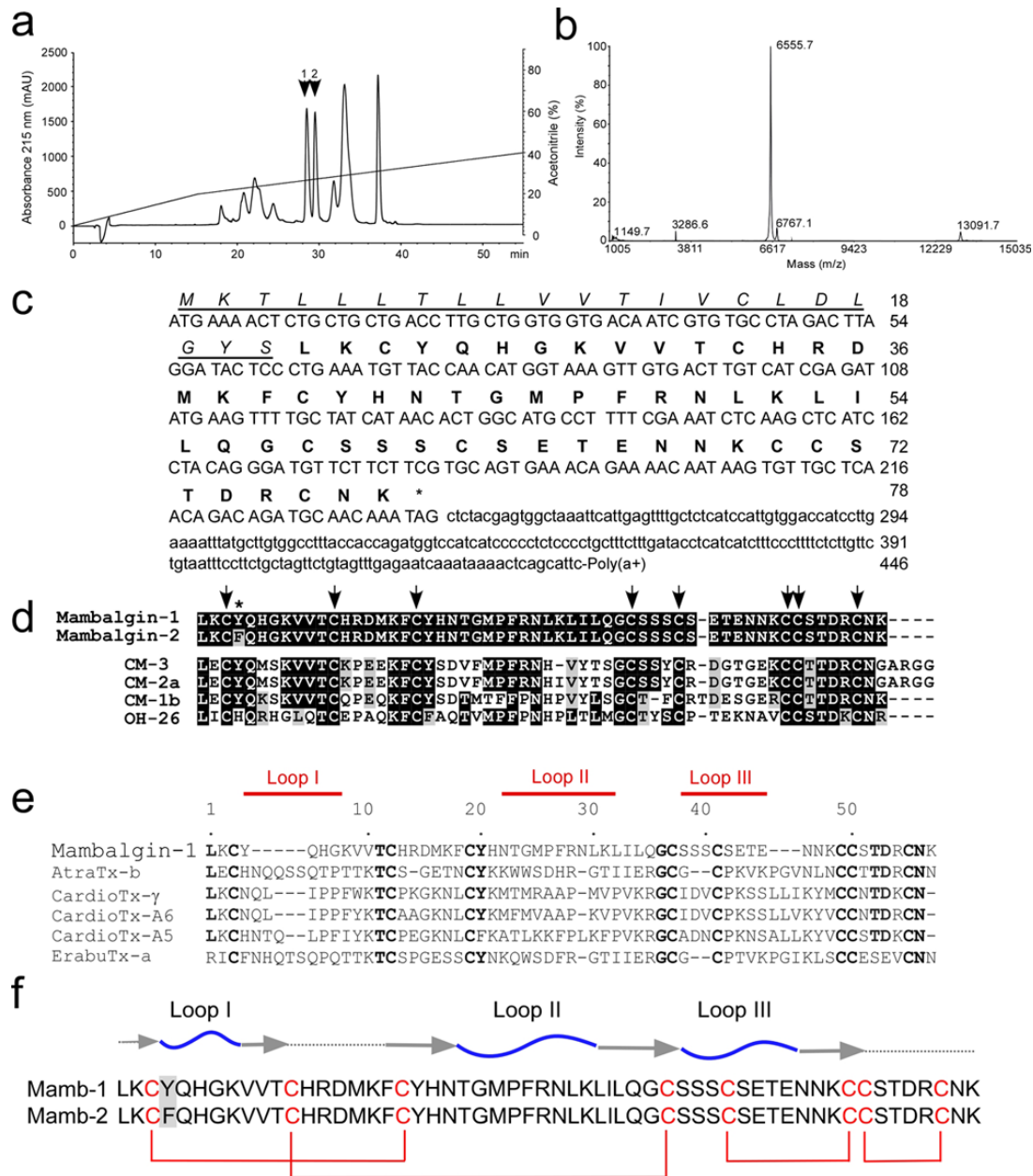


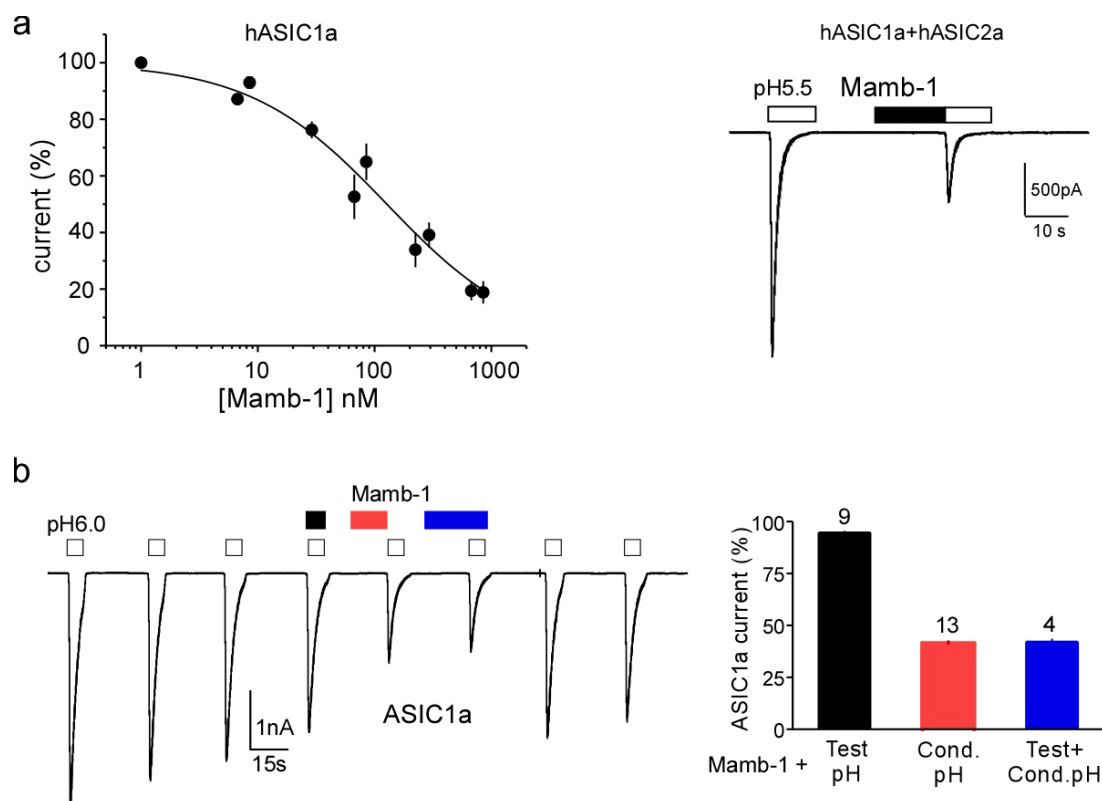
This set of 12 figures show the mambalgin-1 cDNA sequence, multiple protein sequence alignments with other toxins, including those used for the 3D-model and a secondary structure prediction (Suppl. Figure 1), the effects of mambalgin-1 on human ASIC channels and on ASIC currents depending on the protocol for application (Suppl. Figure 2), the inhibition of ASIC currents in mouse hippocampal neurons by mambalgins and the absence of inhibition of neuronal and recombinant ASIC1a+ASIC2a current by high concentration of PcTx1 (Suppl. Figure 3), the effect of mambalgin-1 on the excitability of spinal neurons in response to acidic pH without unspecific effect on evoked or spontaneous action potential triggering and on spontaneous postsynaptic currents (Suppl. Figure 4), the absence of effect of mambalgin-1 on ASIC2a and ASIC3-containing channels and the absence of inhibition of ASIC1a channels by other snake venoms (Suppl. Figure 5), the absence of unspecific effects of mambalgin-1 on a panel of voltage-activated and ligand-activated ion channels (Suppl. Figure 6), the lack of effect of mambalgin-1 on mouse motor behavior (Suppl. Figure 7), the analgesic effect of mambalgin-1 upon *i.c.v.* injection (Suppl. Figure 8), *in vitro* and *in vivo* validation of the siRNAs targeting ASIC1a+1b, ASIC2a+2b and ASIC2a (Suppl. Figure 9), the analgesic effect of *i.t.* injection of mambalgin-1 in mice deficient for the preproenkephalin gene (pEnk-knockout) (Suppl. Figure 10), the analgesic effect of central co-injection of mambalgin-1 and PcTx1 and the absence of interaction between the two toxins on recombinant ASIC channels (Suppl. Figure 11), and finally, a summary diagram (Suppl. Figure 12).



Supplementary Figure 1. Purification of mambalbins from *Dendroaspis polylepis* venom, cDNA, sequence comparisons with other toxins and secondary structure prediction.

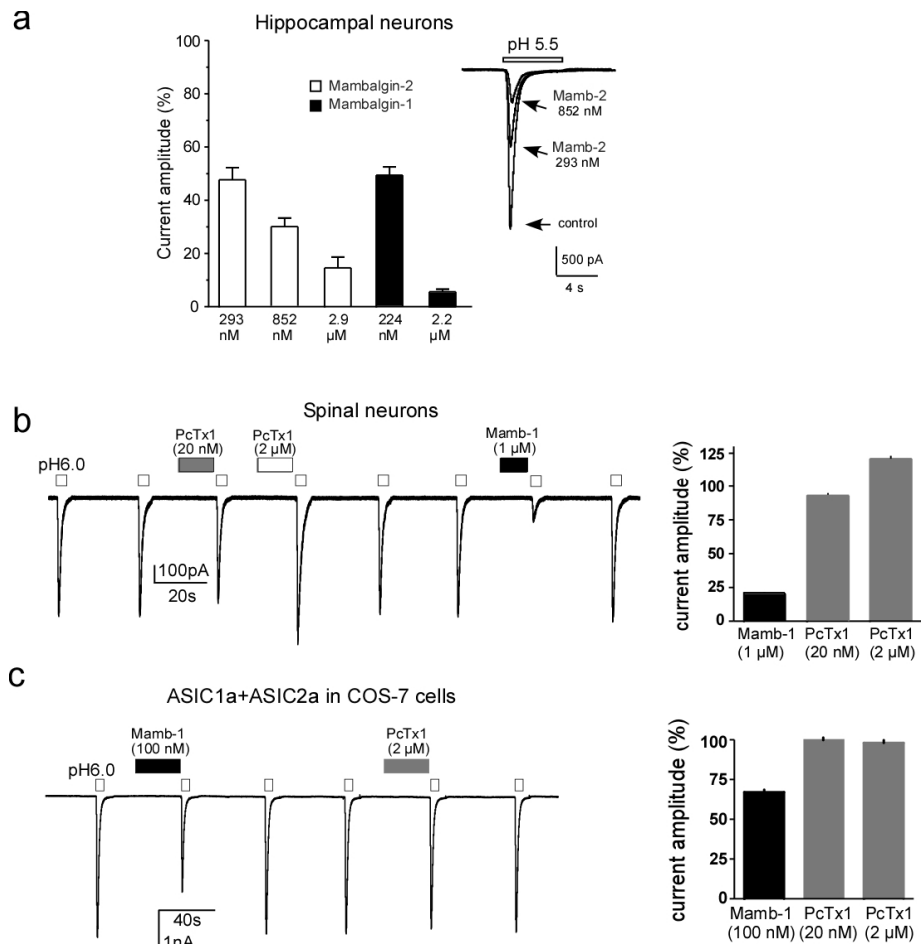
a- Reverse phase HPLC profile of the peptide fraction active on ASIC1 currents. Peak 1 and 2 eluted at 26 and 27% of acetonitrile correspond to mambalgin-1 and mambalgin-2, respectively. The line indicates the gradient (% of acetonitrile) **b-** Mass spectrum of mambalgin-1 (peak 1, measured average molecular mass of 6554.7 Da). Measurement was performed using MALDI-TOF/TOF-MS. Mass spectrum was acquired in mass range of

1,000–16,000 Da using positive linear mode. **c**, Sequence of mambalgin-1 cDNA and the corresponding protein. Signal peptide (in italics and underlined) and final toxin (in bold) are displayed on top of the corresponding nucleotide sequence. **d**, Multiple protein sequence alignment of mambalgin-1 and mambalgin-2 (π -Dp1 and π -Dp2 according to the nomenclature proposed by King et al.⁴⁴) with the most similar sequences from databases. CM-3 and CM-2a from *Naja haje annulifera*⁴⁵, CM-1b from *Hemachatus haemachatus*⁴⁶, the α -neurotoxin OH-26 from *Ophiophagus hannah*⁴⁷, all from cobra venoms, share 55%, 54%, 47% and 47% sequence identity with mambalgin-1, respectively (individual alignments with mambalgin-1, ClustalW). These peptides constitute a class of non-toxic peptides with no known target, which are immunochemically unrelated to the toxic “Naja type” cytotoxins and α -neurotoxins isolated from Elapid venoms. Amino acids that are identical or similar to mambalgin-1 are printed white on black or black on grey background, respectively. The eight highly conserved cysteine residues are shown with arrows. Position 4 that differs between mambalgin-1 and mambalgin-2 is marked by an asterisk. Note that the venom of *Naja haje annulifera*, which contains the CM-2a and CM-3 toxins, and the venom of *Ophiophagus hannah*, which contains the OH-26 toxin, have no significant effect on ASIC1a channels expressed in *Xenopus* oocytes (see Supplementary Fig. 5b). **e**, Multiple sequence alignment with other snake three-finger toxins of known 3D structures that share the highest sequence identities with mambalgin-1 and have been chosen as templates for 3D-modeling of the toxin. The three loops are indicated on the top of the sequences by red lines. Residues in black are highly conserved between mambalgin-1 and the templates. Atratoxin-b from *Naja atra* (AtraTx-b, PDB 1VB0,⁴⁸), Cardiotoxin- γ from *Naja nigricollis* (CardioTx- γ , PDB 1TGX,⁴⁹), Cardiotoxin-A6 from *Naja atra* (CardioTx-A6, PDB 1UG4,⁵⁰), Cardiotoxin-A5 from *Naja atra* (CardioTx-A5, PDB 1CVO,⁵¹), and Erabutoxin-a from *Laticauda semifasciata* (ErabuTx-a, PDB 3ERA,⁵²) show 31%, 28%, 28%, 29% and 25% sequence identity with mambalgin-1, respectively (multiple alignment, MUSCLE). Cardiotoxin-A5, Cardiotoxin-A6 and Cardiotoxin- γ are cytotoxins known to bind to and disrupt the organization, integrity, and function of the cell membrane, and Atratoxin-b and Erabutoxin-a are two short neurotoxins known to bind to acetylcholine receptors. **f**, Sequence alignment of mambalgin-1 (Mamb-1) and mambalgin-2 (Mamb-2) and secondary structure prediction showing the localization of loops, β -strands (gray arrows) and disulfide bridges between the eight highly conserved cysteine residues (in red). Position 4 differs between mambalgin-1 and mambalgin-2.



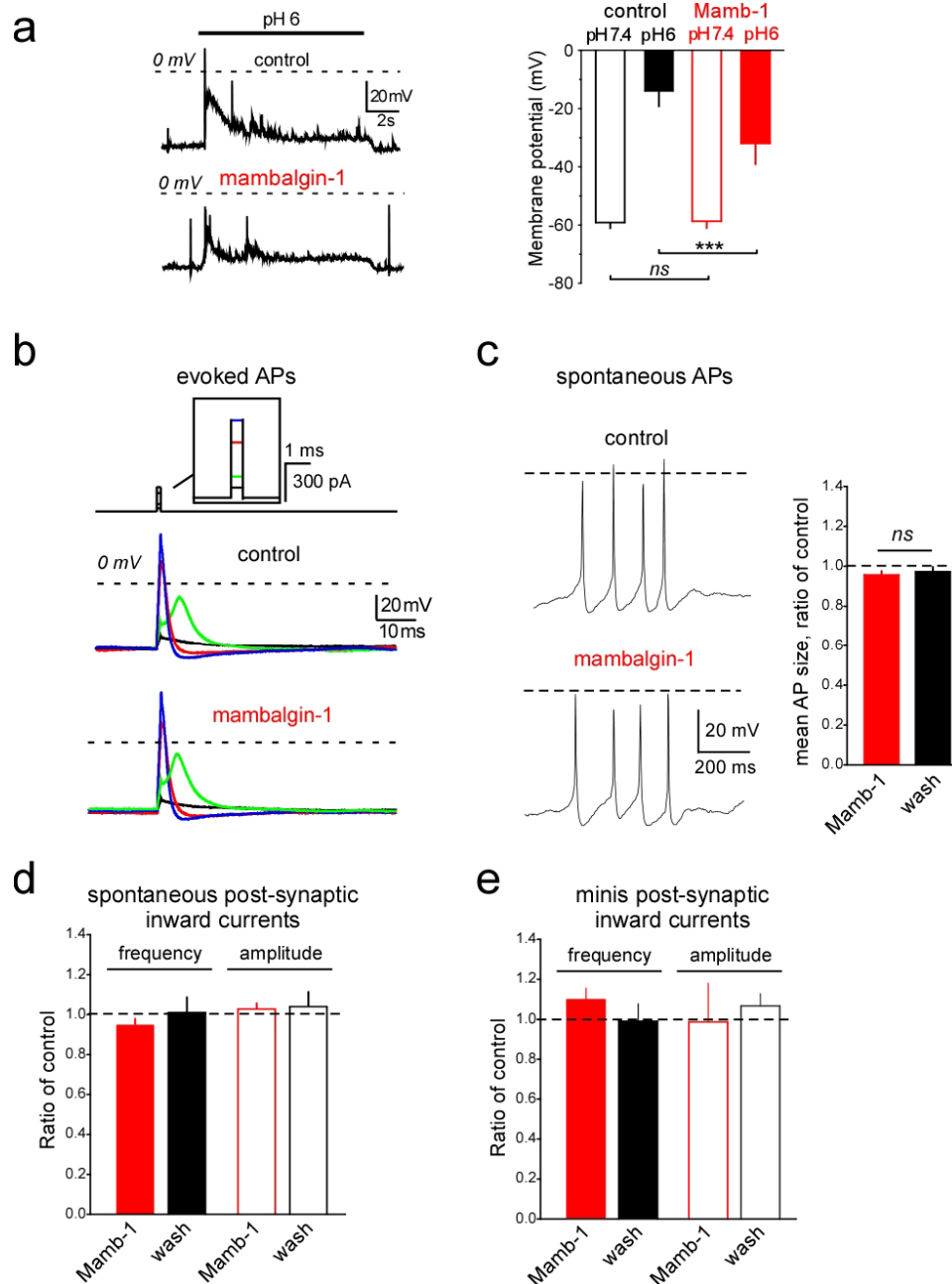
Supplementary Figure 2. Mambalgin-1 inhibits human ASIC1a and ASIC1a+ASIC2a channels and its effect on ASIC channels depends on the protocol for applying the toxin.

a, Inhibition by mambalgin-1 (Mamb-1) of homomeric hASIC1a current (left panel, $IC_{50}=127$ nM, mean $\pm$ SEM, $n=4-15$) and heteromeric hASIC1a+hASIC2a current (right panel, Mamb-1 674 nM) expressed in COS-7 cells. ASIC currents were activated by rapid changes of the external pH from 7.4 to 5.5 (holding potential -60 mV). **b**, When mambalgin-1 (Mamb-1, 85nM) was applied only during the acid pH drop to 6.0 (Test pH) from pH 7.4, the toxin did not produce any blockade of rat ASIC1a current expressed in COS-7 cells ($5.2\pm2.2\%$, $n=9$; Mean $\pm$ SEM, right panel). When mambalgin-1 was applied at pH 7.4 (conditioning pH, Cond. pH) 30s before the pH drop and kept during the stimulation (Test + Cond. pH), it produced an inhibition of the current similar to an application only at pH 7.4 (Cond. pH) (*i.e.*, without toxin during the pH drop) ($58.6\pm3\%$, $n=13$ and $57.8\pm3\%$, $n=4$, respectively; Mean $\pm$ SEM, right panel). Holding potential -50 mV. Control and washout responses to pH before and after application of the peptide have been done for the distinct ways of applying the peptide. These data indicate that mambalgin-1 does not bind to the open channel but it is very difficult to distinguish between pH dependence for toxin binding to the channel from state-dependent binding *per se*.



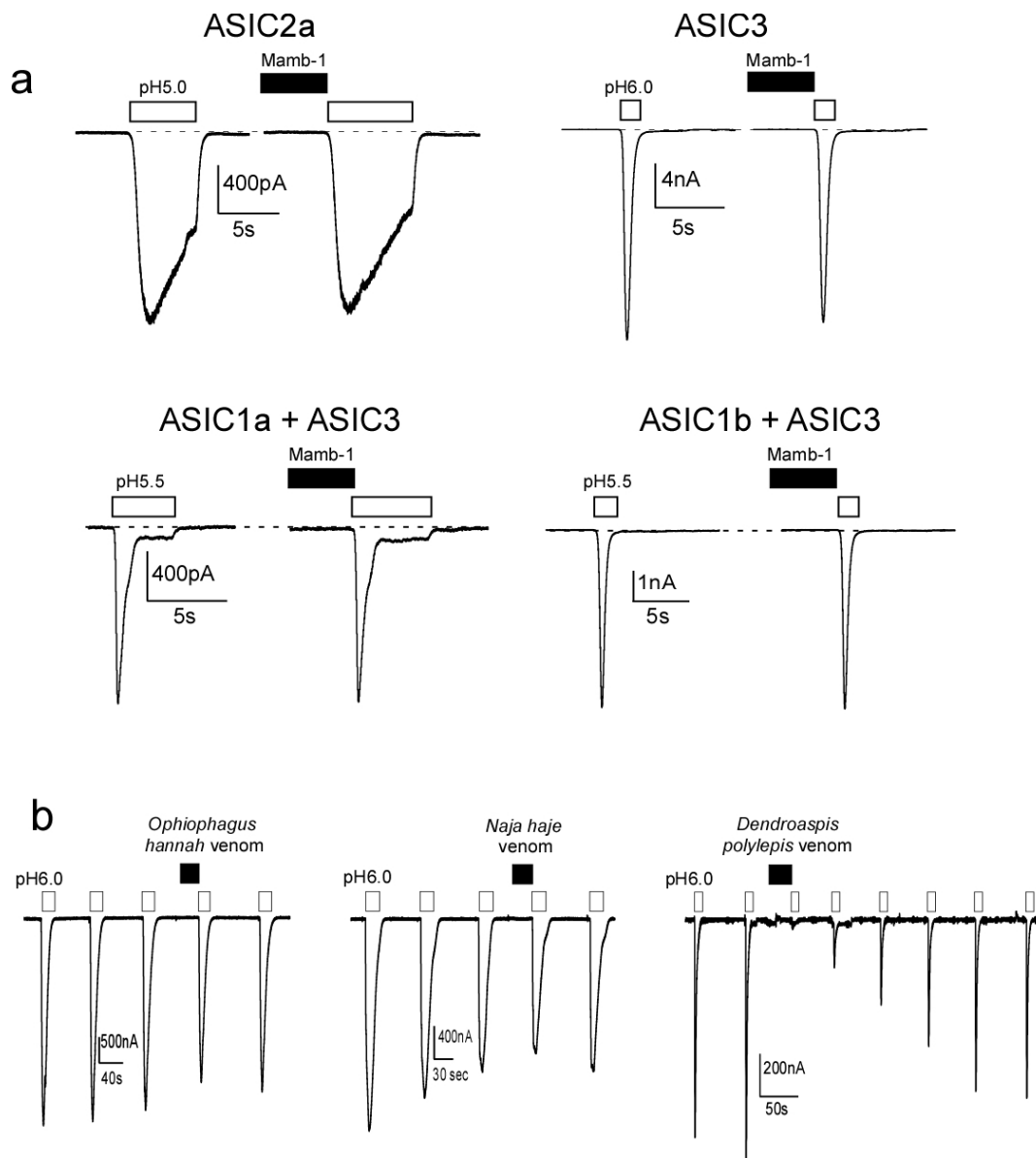
Supplementary Figure 3. Mambalgins inhibit native ASIC currents in hippocampal neurons, while a high concentration of PcTx1 does not inhibit native ASIC1a+ASIC2a like currents in spinal cord neurons or ASIC1a+ASIC2a channels expressed in COS cells.

a, Inhibition (% of control current) of ASIC current in mouse hippocampal neurons by mambalgin-1 (black bars) and mambalgin-2 (white bars). Mean $\pm$ SEM (n=26). Inset: currents recorded at -50 mV and activated by a drop in pH from 7.4 to 5.5. **b**, The native acid stimulated current evoked by a pH drop from 7.4 to 6.0 in the sub-population of spinal cord neurons expressing ASIC1a+ASIC2a channels (*i.e.*, neurons insensitive to PcTx1, 20 nM) is blocked by mambalgin-1 (Mamb-1, 1 μ M) but not by PcTx1 at a similar concentration (2 μ M, *i.e.*, ~100-fold the concentration used to block homomeric ASIC1a channels) (right panel, mean $\pm$ SEM, n=6-7). **c**, High concentration of PcTx1 (2 μ M) is also without effect on ASIC1a+ASIC2a heteromeric channels expressed in COS-7 cells. Currents evoked by a pH drop from 7.4 to 6.0 (right panel, mean $\pm$ SEM, n=4-5). Holding potential -60 mV.



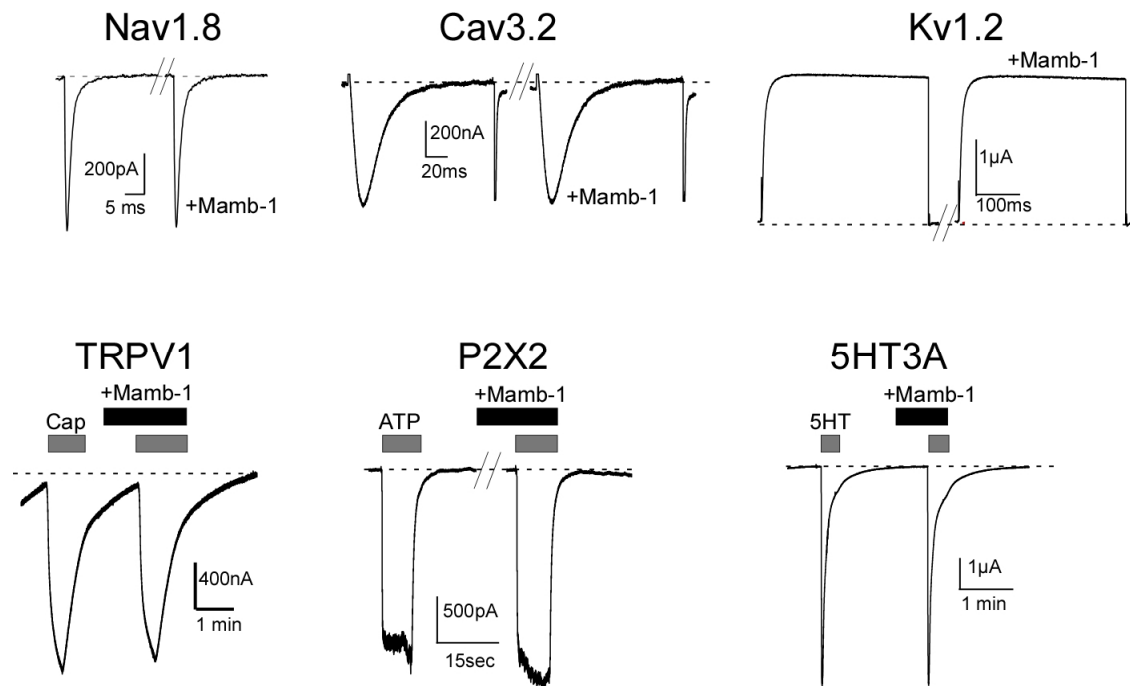
Supplementary Figure 4. Mambalgin-1 reduces the excitability in response to acidic pH without unspecific effect on basal neuronal excitability, on the threshold and the shape of evoked or spontaneous action potentials, and on spontaneous postsynaptic currents in primary cultured dorsal spinal neurons. **a**, Mambalgin-1 (674 nM) reduced the mean depolarisation ($n=10$) triggered by a pH drop from 7.4 to 6.0 without affecting the resting membrane potential in dorsal spinal cord neurons. Action potentials were excluded from membrane potential measurements. A typical recording is shown on the left. Mean $\pm$ SEM.

b, c Action potentials (APs) evoked by increasing current pulses (**b**) and spontaneous APs recorded in the absence of CNQX and kynurenic acid (**c**), showing no change in the shape and threshold of APs before and during local extracellular perfusion of mambalgin-1 (Mamb-1, 674 nM) at pH 7.4. Two membrane potential traces evoked by infraliminar current pulses and two evoked by supraliminar current pulses triggering action potentials are superimposed in **b**. Inset in **c** illustrates the lack of change in AP amplitude in the presence of Mamb-1 and after wash-out of the peptide (wash) expressed as a ratio of control. Mean $\pm$ SEM, $n=3$, paired t -test. **d**, No change in spontaneous post-synaptic inward currents amplitude and frequency in the presence of mambalgin-1 (674 nM) and after wash-out of the peptide (wash) expressed as a ratio of control. Holding potential -50 mV. Mean $\pm$ SEM, $n=4$, not significantly different by paired t -test. **e**, No change in miniature post-synaptic inward currents amplitude and frequency in similar conditions. Holding potential -50 mV. Mean $\pm$ SEM, $n=3$, not significantly different by paired t -test. Miniature currents were recorded in the presence of 0.3 μ M tetrodotoxin (TTX). Analysis was performed with WinEDR and WinWCP programs of the Strathclyde Electrophysiology Software (courtesy of John Dempster, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK).



Supplementary Figure 5. Mambalgin-1 has no effect on ASIC2a, ASIC3, ASIC1a+ASIC3 and ASIC1b+ASIC3 channels, and venoms of *Naja haje annulifera* and *Ophiophagus hannah* do not inhibit ASIC1a channels.

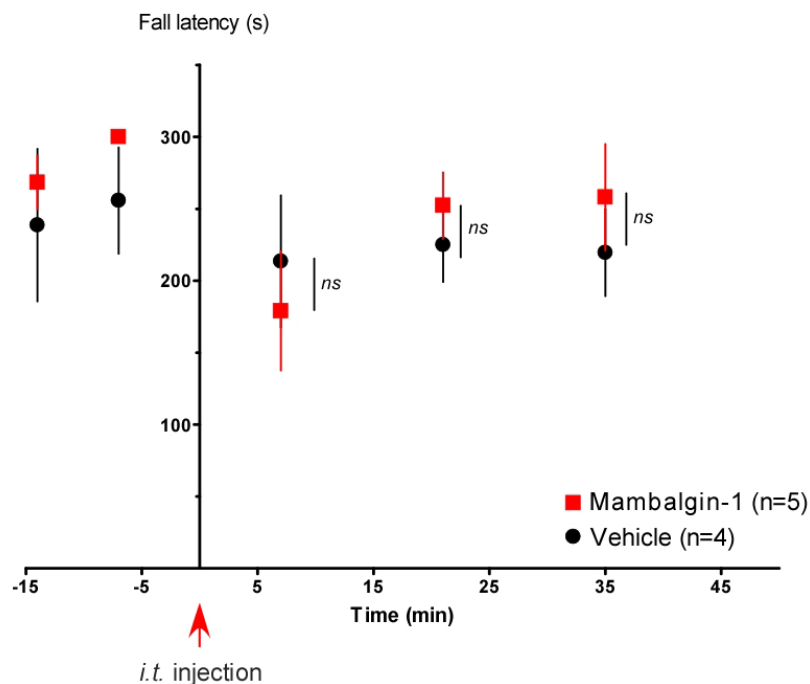
a, Effect of mambalgin-1 (Mamb-1, 674nM) on homomeric and heteromeric ASIC channels expressed in COS-7 cells (4-13 cells tested). **b**, The venom of egyptian cobra *Naja haje annulifera* (0.01 mg ml⁻¹), which contains the CM-2a and CM-3 toxins, and the venom of king cobra *Ophiophagus hannah* (0.05 mg ml⁻¹), which contains the OH-26 toxin, have no significant effect on ASIC1a channels expressed in *Xenopus* oocytes contrary to the venom of black mamba *Dendroaspis polylepis* (0.01 mg ml⁻¹) (n=3-5). Each venom was perfused during 30-45s before the pH drop. Holding potential -60mV, conditioning pH 7.4.



Supplementary Figure 6. Mambalgin-1 has no effect on a panel of different ligand-operated and voltage-activated channels.

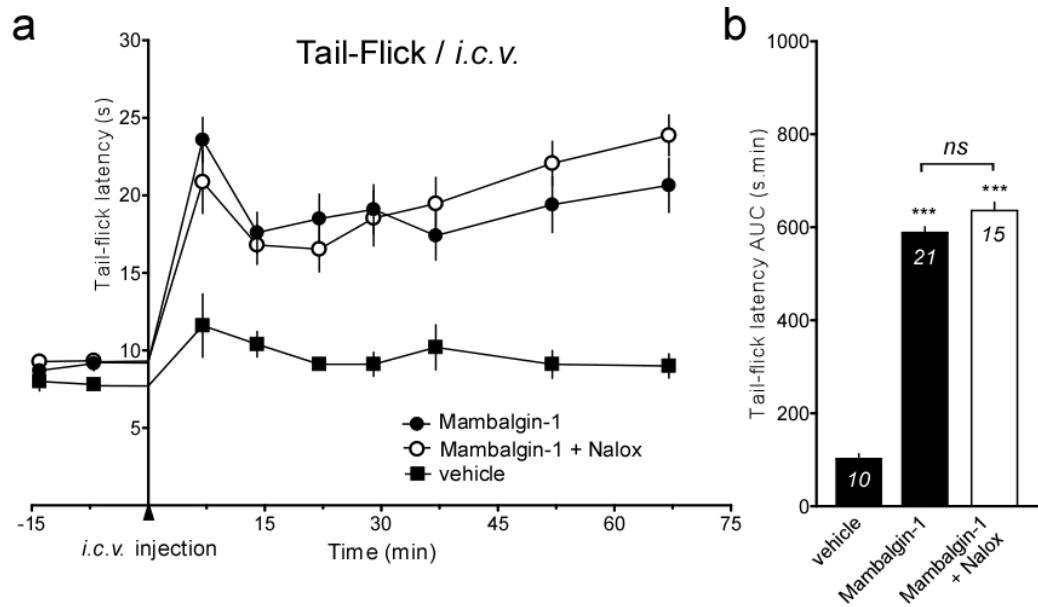
Voltage-gated currents were recorded by depolarization every 10s to -20mV (hCav3.2 and Kv1.2 expressed in *Xenopus* oocytes) or to 0mV (Nav1.8 expressed in HEK 293 cells) from a holding potential of -80mV. Mambalgin-1 (Mamb-1, 3μM) was perfused continuously during depolarization steps and a representative trace is shown for each channel before and after 50-60s toxin application (n=4-5).

TRPV1, 5-HT3a (expressed in *Xenopus* oocytes) and P2X2 (expressed in COS-7 cells) were activated by their respective ligands (1μM capsaicin, 10μM ATP and 30μM 5-HT, respectively). Mambalgin-1 (3μM) was perfused continuously before (45s) and during the pH stimulation. No significant inhibition was observed (n=4-5).



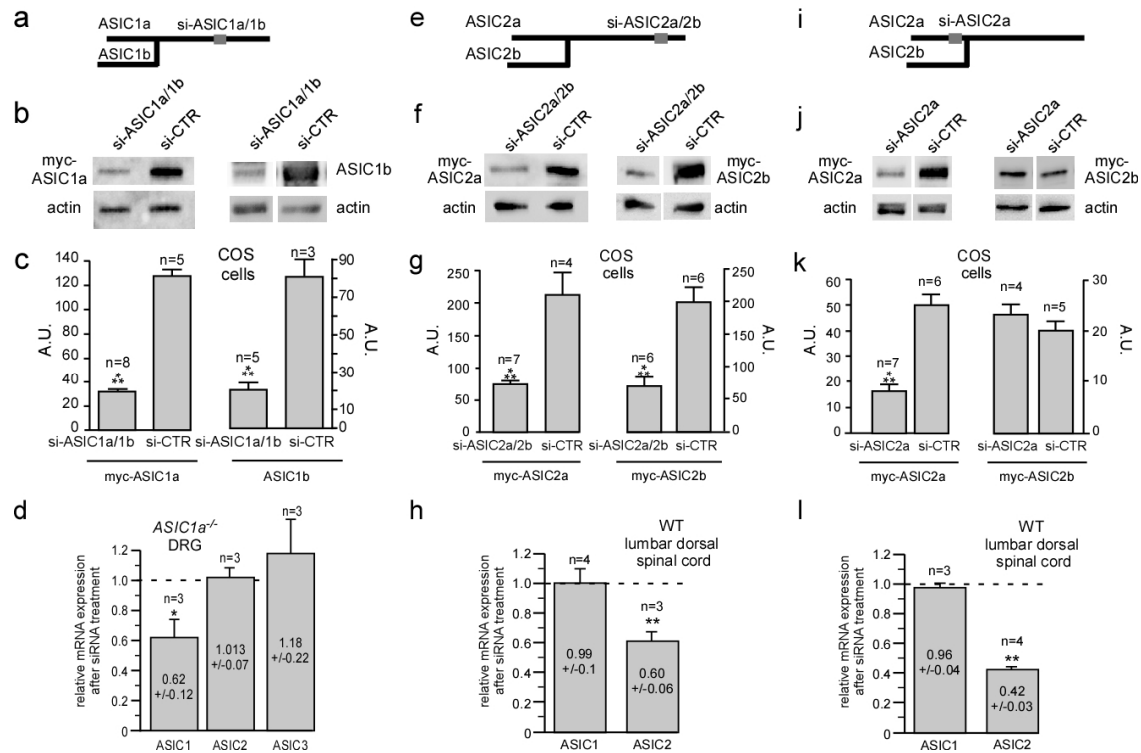
Supplementary Figure 7. Intrathecal injection of mambalgin-1 has no effect on mice motor behavior evaluated by the Rotarod test.

Time-course of fall latency (s) before and after an *i.t.* injection mambalgin-1 (34 μ M, 10 μ l) or vehicle. The mouse was placed onto the dowel with the accelerating Rota-rod treadmill (Ugo Basile) rotating at 4 rpm and then accelerating at a constant rate of 5 rpm min⁻¹ up to 40 rpm⁵³. The latency to fall to the floor was automatically recorded, with a maximum cut-off latency of 300 s. Two control trials were performed before *i.t.* injection, and test trials were then performed 7, 21 and 35 minutes after injection. Mean $\pm$ SEM. Intrathecal injection of mambalgin-1 induced no significant change in motor activity (unpaired *t*-test).



Supplementary Figure 8. Analgesic effect of intracerebroventricular injections of mambalgin-1 on acute thermal nociception.

a, Time-course of the effect of *i.c.v.* injection of mambalgin-1 (68 μ M, 5 μ l) on tail-flick latency (mean $\pm$ SEM, $n=10-21$). The naloxone sensitivity of the effect was assessed by *s.c.* injection of naloxone (Nalox, 2 mg kg^{-1}) ten minutes before the *i.c.v.* injection. **b**, Total analgesic effect calculated on each mouse as the Area Under Curve (AUC; s.min) (mean $\pm$ SEM, $n=10-21$). Comparison with vehicle unless specified. The *i.c.v.* injections were stereotactically performed into the lateral ventricle of deeply anesthetized mice (1.5% isoflurane).

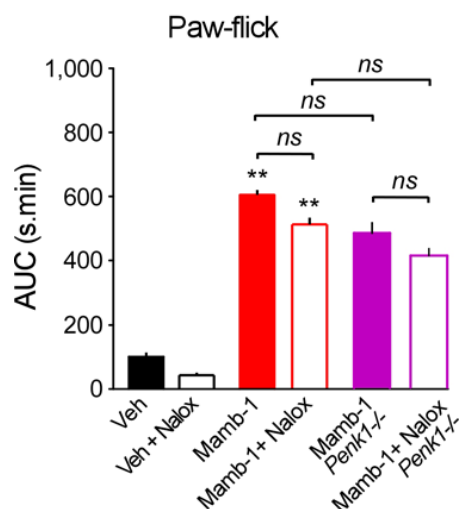


Supplementary Figure 9. *In vitro* and *in vivo* validation of the siRNAs targeting ASIC1a+1b, ASIC2a+2b and ASIC2a.

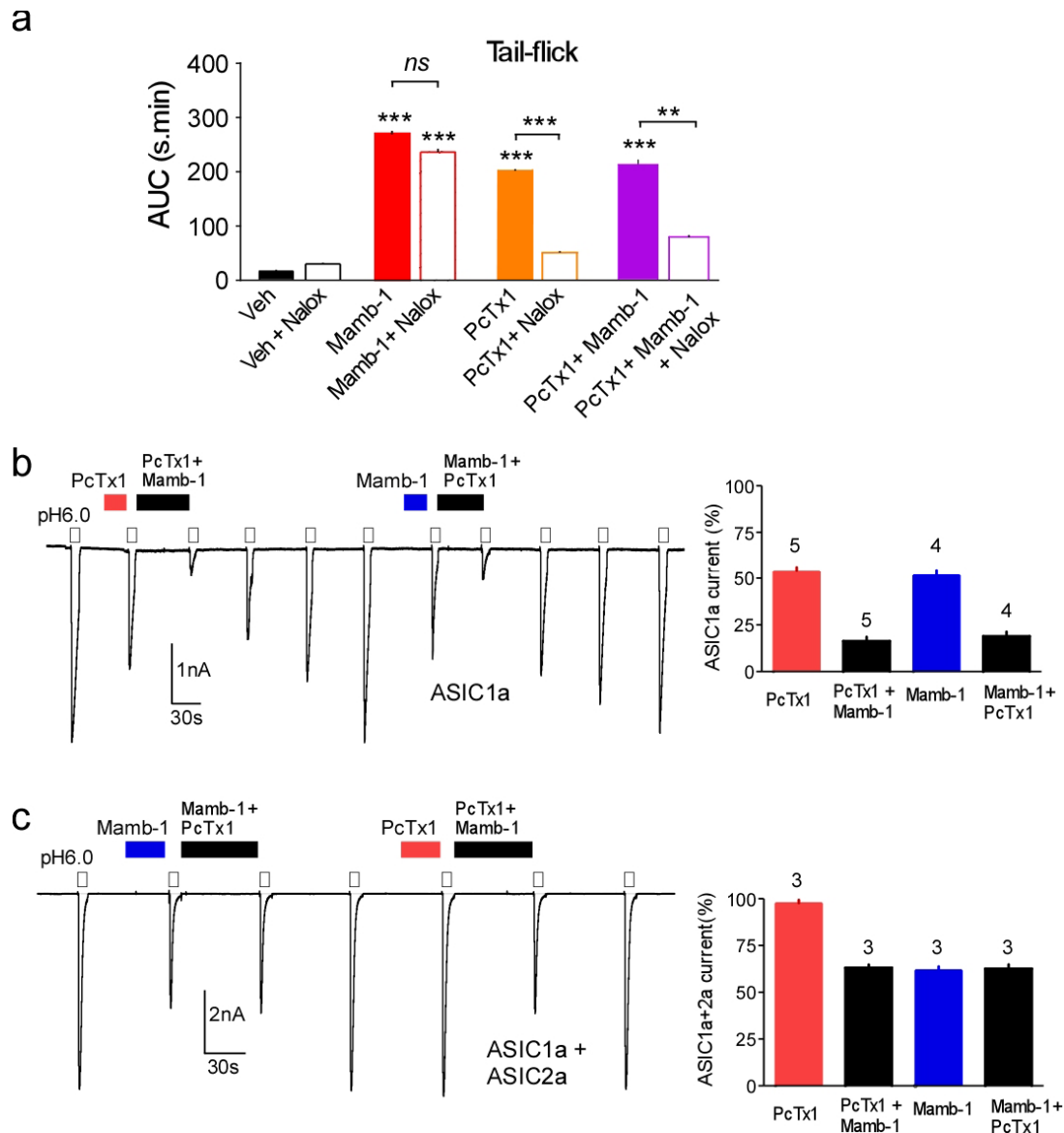
a-d, siRNA targeting both ASIC1a and ASIC1b (si-ASIC1a/1b) or control siRNA (si-CTR) were co-transfected in COS cells (25 nM) with a plasmid coding for myc tagged rat ASIC1a (myc-ASIC1a) or coding for rat ASIC1b. The level of protein was assessed 48h after transfection by Western blot with a myc antibody or an ASIC1-specific antibody (**b**). The blot was probed with an antibody against actin to demonstrate equal loading of proteins. A densitometric quantification of the signal shown in **b** has been done in **c** and showed a significant reduction of both ASIC1a and ASIC1b expression in COS-7 cells. *In vivo* siRNA treatment significantly reduced the level of ASIC1 transcripts in lumbar DRGs of *ASIC1a*^{-/-} mice but did not affect the level of ASIC2 and ASIC3 transcripts (**d**). Since ASIC1a is lacking in these mice (the ASIC1a mRNA was not detected in similar experimental conditions), the decrease in ASIC1 expression represents the specific knockdown of ASIC1b in lumbar DRGs. **e-h**, similar experiments performed with a siRNA targeting both ASIC2a and ASIC2b (si-ASIC2a/2b), showing a significant knockdown of the expression of ASIC2a and ASIC2b in COS-7 cells (**f,g**). *In vivo* siRNA treatment against ASIC2 in wild-type mice significantly reduced the level of ASIC2 transcripts in the lumbar dorsal spinal cord but did not affect the level of ASIC1 transcripts (**h**). The ASIC3 mRNA was not detected in similar experimental

conditions. **i-l**, similar experiments performed with a siRNA targeting ASIC2a but not ASIC2b (si-ASIC2a), showing a significant knockdown of the expression of ASIC2a but no effect on the expression of ASIC2b in COS-7 cells (**j,k**). *In vivo* siRNA treatment against ASIC2a significantly reduced the level of ASIC2 transcripts in the lumbar dorsal spinal cord of wild-type mice but did not affect the level of ASIC1 transcripts (**l**), consistent with the fact that ASIC2a represents the predominant ASIC2 mRNA in the spinal cord ¹⁶.

A.U, arbitrary units. Mean $\pm$ S.E.M. **c,g,k**, *** : $p < 0.001$ compared with si-CTR, unpaired *t*-test. **d,h,l**, *, $p < 0.05$ and ** $p < 0.01$, compared with ASIC2 and ASIC3 (**d**) or ASIC1 (**h,l**), paired *t*-test.



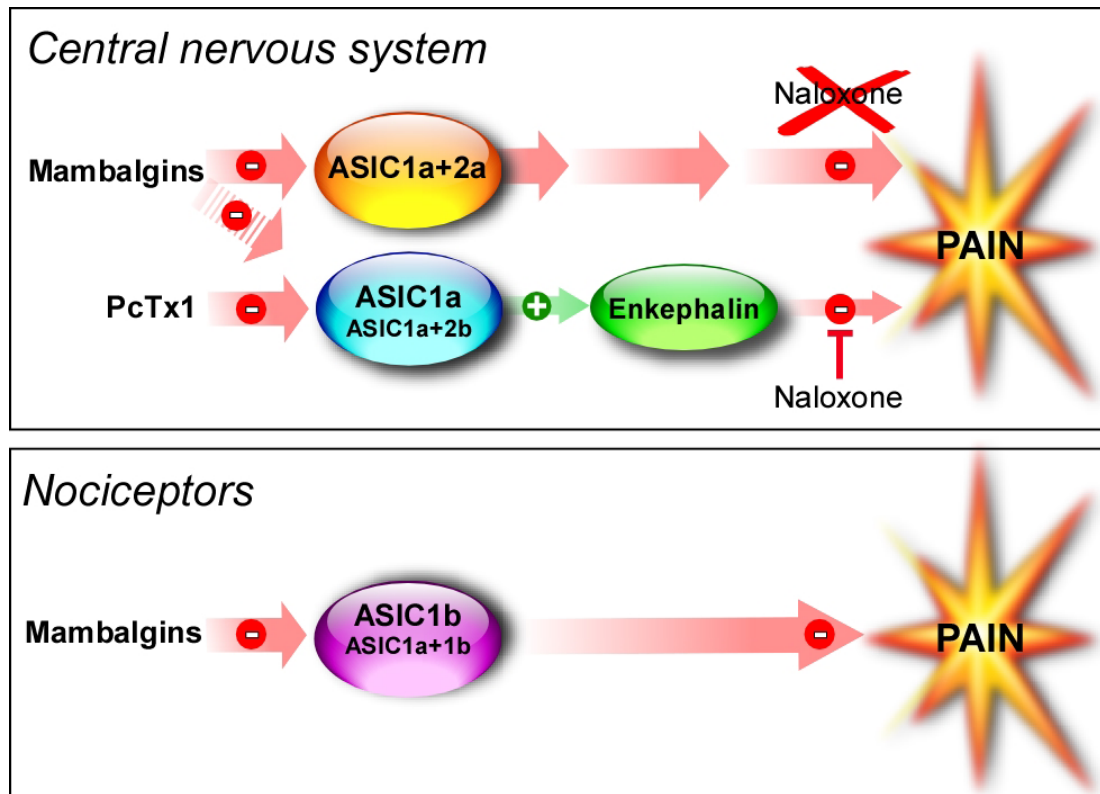
Supplementary Figure 10. Intrathecal injection of mambalgin-1 still produces a potent analgesia in mice deficient for the preproenkephalin gene. Effects on acute thermal pain (46°C) determined using the paw-immersion test showing a potent naloxone-insensitive analgesic effect in mice deficient for the preproenkephalin gene (*Penk1*^{-/-}, n=6), where the effect of PcTx1 was lost ⁵. Area Under Curve (AUC) calculated from each mouse over the first 37 min. Data in WT mice shown for comparison are the same as in Fig. 2b. Naloxone (Nalox) was injected *s.c.* 10 minutes before *i.t* injection of the peptide. Mamb-1, mambalgin-1; Veh, vehicle, comparison with vehicle unless specified. Mean ± SEM.



Supplementary Figure 11. Pain behavior experiments after central injection of mambalgin-1+PcTx1 and interaction between mambalgin-1 and PcTx1 on ASIC1a and ASIC1a+ASIC2a channels *in vitro*.

a, Effects on acute thermal pain (46°C) determined using the tail-immersion test showing a potent naloxone-sensitive analgesic effect similar to the effect of PcTx1 alone when mambalgin-1 (Mamb-1, 0.34 nmole) and PcTx1 (0.1 nmole) are intrathecally injected together (PcTx1 + Mamb-1, n=12-13). Data with vehicle, mambalgin-1 and PcTx1 alone are shown for comparison and are the same as in Fig. 2a. Area Under Curve (AUC) calculated from each mouse over the first 37 min. The opioid-dependent effect evoked by *i.t.* injection of PcTx1 is therefore dominant over the opioid-independent effect evoked by *i.t.* injection of

mambalgin-1. **b**, Lack of interaction between mambalgin-1 (Mamb-1) and PcTx1 on ASIC1a channels. ASIC1a currents stimulated by a pH drop from pH 7.4 to 6.0 show half-maximal inhibition by PcTx1 (1nM, *i.e.*, equivalent to IC_{50}) ($46.0 \pm 4.9\%$, $n=5$), subsequent co-application of Mamb-1 (60nM, *i.e.*, equivalent to IC_{50}) + PcTx1 (1nM) induces additive inhibitory effect ($83.7 \pm 4.9\%$, $n=5$). Similarly, a first application of Mamb-1 (60nM) inhibits ASIC1a ($48.4 \pm 5.8\%$, $n=4$) and subsequent co-application of PcTx1 (1nM) + Mamb-1 (60nM) also induces an additive inhibitory effect ($81.1 \pm 4.8\%$, $n=4$). **c**, Lack of interaction between mambalgin-1 (Mamb-1) and PcTx1 on ASIC1a+ASIC2a channels. ASIC1a+ASIC2a currents evoked by a pH drop from pH 7.4 to 6.0 are half-inhibited by Mamb-1 (300nM, *i.e.*, equivalent to IC_{50}) ($38.1 \pm 4.9\%$, $n=3$) but subsequent application of PcTx1 (10nM) has no additional effect ($37.4 \pm 3.3\%$, $n=3$). Inversely, application of PcTx1 (10nM) does not inhibit ASIC1a+ASIC2a ($2.3 \pm 2.3\%$, $n=3$), as also shown in Supplementary Figure 3c and in previous work¹⁴, while subsequent application of PcTx1 (10 nM) + Mamb-1 (300nM) blocks the current ($36.9 \pm 2.9\%$, $n=3$). Holding potential -60 mV. **b**, **c**, Original currents traces (left panel) and mean $\pm$ SEM (right panel).



Supplementary Figure 12. Mambalgins have analgesic effects by targeting both central neurons and primary nociceptors, but through different ASIC subtypes.

Mambalgins are able to block all the ASIC channel subtypes expressed in the central nervous system (*i.e.*, ASIC1a, ASIC1a+ASIC2b and ASIC1a+ASIC2a) but the peptides essentially evoke a naloxone-independent effect upon central injection. This suggests that *in vivo* and at the concentration that has been used, mambalgins only efficiently target the opioid-independent pain pathway involving ASIC1a+ASIC2a channels, but not the opioid-dependent pathway previously associated with ASIC1a and probably ASIC1a+ASIC2b channels and targeted by PcTx1. This is consistent with the fact that the opioid-dependent pathway prevails when PcTx1 and mambalgins are centrally injected together (see supplementary Figure 11). In nociceptors, ASIC1b-containing channels, but not ASIC1a, are targeted by mambalgins to evoke analgesia, demonstrating a role for ASIC1b in triggering cutaneous pain.

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