1 **Analgesic treatment of ciguatoxin-induced cold allodynia**

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1 **Introduction**

2 Ciguatera is a perplexing neurological disease caused by consumption of highly lipophilic
3 polyether compounds known as ciguatoxins (CTX) that bioaccumulate in tropical and sub-
4 tropical fish. Clinically, ciguatera is associated with gastrointestinal disturbances of limited
5 duration, in particular nausea, diarrhoea and abdominal pain, with neurological disturbances
6 being the predominant presentation. The neurological symptoms of ciguatera include
7 distressing, often persistent sensory disturbances such as perioral and distal paraesthesias,
8 dysaesthesias, pruritus, headache and asthenia [18; 21]. Of these neurological disturbances,
9 temperature dysaesthesia, or cold allodynia, is considered pathognomonic and occurs in up to
10 95% of ciguatera victims [3; 21].

11 At the molecular level, ciguatoxin is the most potent known activator of voltage-gated
12 sodium channels (Na_v) [23]. Ciguatoxin also inhibits neuronal potassium channels [5],
13 resulting in further increased neuronal excitability. The pharmacological action of
14 ciguatoxins on Na_v in excitable cells results in a range of pathophysiological effects,
15 including spontaneous action potential discharge, release of neurotransmitters, increase of
16 intracellular Ca^{2+}, and axonal Schwann cell oedema (for review see [27]).

17 Importantly, clinical management of the peripheral sensory disturbances associated with
18 ciguatera, in particular cold allodynia, remains symptomatic and relies on the largely empiric
19 choice of analgesics. This reflects both difficulties in recruiting ciguatera patients in
20 sufficient numbers for systematic randomised controlled trials, as well as the absence of
21 suitable *in vitro* and *in vivo* models able to assess the therapeutic potential of various
22 compounds. We thus sought to establish a novel *in vitro* ciguatoxin assay to identify
23 inhibitors of acute ciguatoxin-induced Na_v activation at the cellular level. In addition, we
24 systematically evaluated the anti-allodynic effects of approved analgesic drugs and

1 medications modulating neuronal excitability in a novel animal model of ciguatoxin-induced
2 cold allodynia with the aim to identify new treatment strategies for this painful condition.
3
4 **Materials and Methods**
5 *Materials*
6 P-CTX-1, P-CTX-2 and P-CTX-3 were isolated from the viscera of moray eel and purified as
7 previously described [11]. Brevetoxin-A (BTX-A) was obtained from Latoxan (France).
8 Synthetic conopeptides CVID ($Ca_v2.2$ inhibitor), TIIIA ($Na_v1.2/Na_v1.1$ inhibitor) and GIIIA
9 ($Na_v1.1/Na_v1.6$ inhibitor) were a kind gift from Prof. Paul Alewood, Institute for Molecular
10 Bioscience, The University of Queensland. Veratridine was obtained from Ascent Scientific
11 (Bristol, UK), tetrodotoxin (TTX) was from Enzo Life Sciences (Farmingdale, NY, USA)
12 and ProTxII was from Peptides International (Louisville, KY, USA). All other reagents,
13 unless otherwise stated, were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). P-
14 CTX-1. P-CTX-2, BTX-A, ProTxII, and TIIIA were routinely diluted in 0.3–0.5 % bovine
15 serum albumin (BSA) solution to avoid adsorption to plastic surfaces.
16
17 *Ethics approval for human experiments*
18 The local human ethics committee of the University of Erlangen approved human studies that
19 were conducted in accordance with German law, the Declaration of Helsinki principles and
20 the Belmont Report. The dose of Pacific Ciguatoxin-1 (P-CTX-1) administered by shallow
21 intracutaneous injection (0.2-2 pg/kg) was extrapolated from the minimum pathogenic oral
22 dose (0.6 ng/kg)[2]. Independent academic volunteers (n=5) were recruited and a detailed risk
23 assessment and information sheet was provided to all participants. Informed written consent

was obtained prior to the experiment. All participants gave their consent voluntarily and could have withdrawn from the study at any time.

Assessment of ciguatoxin-evoked effects in human volunteers

P-CTX-1 was isolated from moray eel and purified to > 95% purity by HPLC using good laboratory practice [11]. The lyophilized non-pyrogenic material was reconstituted in sterile medical grade Ringer solution for intradermal injection. For injections, P-CTX-1 was prepared as 0.1 nM – 10 nM solution in sterile Ringer's solution and injected intracutaneously in a volume of 50 µl into the volar forearm of study participants (age 27-50). Thermal sensitivity was assessed by exposure of the injection site to a surface cooled using a vortex thermode [4] with temperatures ranging from 40 – 2 °C at a rate 0.5 °C/s. Pain sensations were rated verbally by the subjects (n = 5) in 2 °C intervals on a 11 point scale in which 0 is no pain, 3 pain threshold and 10 maximal imaginable pain. The ciguatoxin-induced axon reflex sweating was measured as previously described [14] using a custom-made sweat chamber, in which moisture accumulation of dry air passed over the skin was quantified using a humidity sensor (HygroClip-SC04, Rotronic GmbH, Germany). Sweat output is presented in mV from data recorded by the humidity sensor control unit (HygroLab 2, Rotronic GmbH, Germany). The ciguatoxin-induced neurogenic flare reaction was quantified using a Laser Doppler Imager (LDI; Moore, London, UK) as previously described [14]. Briefly, a rectangular area (158 x 102 pixels or $75 cm^2$) surrounding the P-CTX-1 injection site was scanned with a spatial resolution of 0·5 mm from a distance of 35 cm at baseline and at 2 minute intervals.

CGRP release

1 Male Wistar rats weighing 60-80 g were sacrificed by exposure to 100% CO_2 and the skin
2 covering both hindpaws was isolated through subcutaneous excision, sparing the toes, larger
3 vessels and saphenous and peroneal nerve stems. The resulting skin flaps (0.13-0.35 g) were
4 secured to acrylic rods and tied in place, corium side exposed. After equilibration for 30 min
5 in 32 °C synthetic artificial fluid (SIF; composition: NaCl 108 mM, $NaHCO_3$ 26·2 mM,
6 sodium gluconate 9·64 mM, glucose 5·55 mM, sucrose 7·6 mM, KCl 3·48 mM, NaH_2PO_4
7 1·67 mM, $CaCl_2$ 1·53 mM and $MgSO_4$ 0·69 mM, pH 7·3) gassed with carbogen, the flaps
8 were placed in glass vessels containing varying concentrations of P-CTX-1 and incubated for
9 5 min in a shaking bath at 32 °C. The CGRP content was determined as previously described
10 in detail using a commercial enzyme immunoassay kit (SPIbio, Montigny, France) and a
11 microplate reader (Dynatech, Channel Islands, UK) [1; 29].
12
13 *In vitro ciguatoxin assay in SH-SY5Y cells*
14 SH-SY5Y human neuroblastoma cells were routinely maintained in RPMI medium
15 (Invitrogen, Australia) supplemented with 15 % foetal bovine serum and L-glutamine [18].
16 To assess responses elicited by activation of endogenously expressed $Na_v1.2$, $Na_v1.3$ and
17 $Na_v1.7$, cells were plated on 384-well black-walled imaging plates (Corning) 48 h prior to
18 loading with Calcium-4 dye (Molecular Devices, Sunnyvale, CA) for 30 min at 37 ˚C.
19 Fluorescence responses (excitation 470–495 nm; emission 515–575 nm) to addition of P-
20 CTX-1, P-CTX-2, P-CTX-3 or BTX-A were assessed using a FLIPR^{TETRA} plate reader
21 (Molecular Devices). Raw fluorescence readings were converted to response over baseline
22 using the analysis tool of Screenworks 3.1.1.4 (Molecular Devices) and were expressed
23 relative to the maximum increase in fluorescence of control responses.
24
25 *Ciguatoxin-induced cold allodynia*

The University of Queensland Animal Ethics Committee approved experiments involving animals. Cold allodynia was assessed in adult male C57BL/6 mice as described [26]. $Na_v1.3^{-/-}$ animals were a kind gift from Prof John Wood (University College, London) and were back-crossed on C57/BL6 background for at least 6 generations. P-CTX-1 (5 nM), P-CTX-3 (15 nM), BTX-A (300 nM) and veratridine (50 µM) were diluted in sterile saline containing 0.3% serum albumin and administered in a volume of 40 µl by shallow intraplantar (i.pl.) injection under brief isoflurane (3%) anaesthesia. Drugs were administered in a volume of 500 µl by intraperitoneal injection 60 min prior to assessment of cold allodynia. To assess cold allodynia, the number of paw lifts, licks or shakes when exposed to a temperature-controlled (15 °C) Peltier plate (Ugo Basile, Comerio, Italy) was counted by a blinded investigator for 5 min.

Motor performance assessment

To assess the effect of drugs on motor performance, locomotor activity was assessed using a standard Rotarod test. In brief, 7 days prior to the test, animals were trained in 5 separate sessions to walk on a rotating drum (Rotarod, Ugo Basile, Italy), with the speed increasing gradually over 5 min from 8 to 40 rpm. On the day of the test, analgesic drugs were administered 60 min prior to the motor performance test, and the latency to fall was recorded by a blinded observer at 24 rpm with a cut-off of 5 min. The change in the latency to fall was determined for each animal respective to its pre-treatment performance.

Activity at heterologously expressed Na_v isoforms

Na_v responses were assessed in Chinese Hamster Ovary (CHO) cells heterologously expressing $hNa_v1.3$, $hNa_v1.6$, $hNa_v1.7$ and $hNa_v1.8$ (Chantest, Cleveland, Ohio) as previously described [24]. Cells were plated 24-48 h prior to the assay on 384-well black-walled

imaging plates at a density of 10,000-15,000 cells/well and were loaded with red membrane potential dye (Molecular Devices) according to the manufacturer's instructions for 30 min at 37 °C. Changes in membrane potential after pre-treatment with varying concentrations of small molecule Na_v inhibitors were assessed using the $FLIPR^{Tetra}$ (excitation 515-545 nm, emission 565-625 nm).

Data analysis and statistics

Data was plotted and analyzed using GraphPad Prism Version 4.00. Statistical significance was defined as $p < 0.05$ and was determined using One-way ANOVA analysis with Dunnett's post-test.

Results

Peripheral administration of P-CTX-1 elicits sensory disturbances consistent with the clinical symptomatology of ciguatera

Intracutaneous injections of P-CTX-1 into the volar forearm of human volunteers caused dose-dependent, strictly localized peripheral sensory disturbances consistent with the clinical symptomatology of ciguatera that were associated with a marked axon reflex flare and axon reflex sweating (Fig. 1A-E). Sub-nanomolar (0.1 nM) concentrations of P-CTX-1 elicited localized pruritus, while at higher concentrations (1 nM) intense, short-lasting burning pain and cold allodynia were experienced. The symptoms of cold allodynia persisted for several hours, with exposure of the ciguatoxin-injected region of the forearm to innocuous cool temperatures eliciting intense stabbing, pricking and burning pain (Fig. 1F) that was relieved immediately upon warming. The axon reflex erythema is generally caused by release of the vasodilatory neuropeptide calcitonin gene-related peptide (CGRP) from axons and nerve terminals [20]. Quantification of CGRP release from rat skin illustrated the exquisite potency

1 of P-CTX-1 to activate cutaneous nerve endings (Fig. 1G). In contrast, neither detectable
2 prostaglandins nor histamine were released by P-CTX-1 up to 10 nM (data not shown), which
3 supports a direct and specific action of P-CTX-1 on nociceptors and sympathetic fibers at low
4 nM concentrations. These findings are consistent with previous reports of preferential
5 activation of peptidergic, CGRP-positive sensory neurons by P-CTX-1, but little effect of
6 ciguatoxins on IB4-positive neurons [26].
7
8 *A novel assay for in vitro characterization of ciguatoxin-induced responses*
9 Clinical management of ciguatoxin-induced sensory neuropathies currently relies largely on
10 symptomatic treatment based on the empiric choice of analgesic compounds. However, little
11 is known about the *in vitro* and *in vivo* efficacy of clinically available analgesics for treatment
12 of ciguatera. To assess the ability of clinically available Na_v and potassium channel
13 modulators to inhibit acute ciguatoxin-induced responses, we established a novel *in vitro*
14 ciguatoxin assay in the human neuroblastoma cell line SH-SY5Y. These cells endogenously
15 express Na_v channels relevant for pain signalling, including $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$, and
16 are thus a suitable model for assessment of *in vitro* efficacy of Na_v inhibitors with analgesic
17 effect [25].
18
19 Consistent with the site 2 toxin veratridine eliciting Ca^{2+} responses in SH-SY5Y cells through
20 activation of Na_v, which in turn leads to activation of endogenously expressed Ca_v channels
21 and influx of Ca^{2+}, the site 5 toxins P-CTX-1, P-CTX-2, P-CTX-3 and BTX-A were able to
22 elicit concentration-dependent Ca^{2+} responses with an EC_{50} of 2.2 ± 0.6 nM, 9.3 ± 2.6 nM,
23 8.3 ± 2.4 nM and 160.7 ± 19.3 nM, respectively (Fig. 2A). The ciguatoxin-induced responses
24 were completely inhibited in the presence of 300 nM TTX (Fig. 2B and C). Similar to
25 veratridine-induced responses [18], P-CTX-1 responses were partially inhibited by nifedipine

1 (75.5 ± 4.3 % inhibition) and CVID (36.3 ± 3.6 % inhibition; Fig. 2D), consistent with
2 contribution of both L- and N-type channels to Ca^{2+} responses elicited by P-CTX-1.
3 Surprisingly, $Na_v1.7$ did not contribute substantially to P-CTX-1 responses, as ProTxII
4 blocked responses with an IC_{50} of 4.3 ± 3.1 μM (pIC_{50} 6.45 ± 0.65) and inhibited only 11.9 ±
5 1.6 % of responses at a concentration (100 nM) that fully inhibits $Na_v1.7$ (Fig. 2C). In
6 addition, the $Na_v1.2$-selective conopeptide TIIIA inhibited 25.4 ± 0.9 % of responses (pIC_{50}
7 7.23 ± 0.19; Fig 2C), suggesting that the majority of P-CTX-1-induced Ca^{2+} influx in SH-
8 SY5Y cells is mediated through $Na_v1.3$, the third sodium channel subtype present in these
9 cells.
10 Using this assay, we characterised the ability of clinically used analgesic compounds to
11 inhibit P-CTX-1 responses. Consistent with their reported pharmacological effects on Na_v
12 and K_v channels, all compounds tested, with the exception of topiramate, were able to
13 concentration-dependently inhibit ciguatoxin-induced responses (Fig. 3A). Amitriptyline was
14 most potent (pIC_{50} 4.91 ± 0.22), while lamotrigine (pIC_{50} 3.03 ± 0.05) and phenytoin (pIC_{50}
15 3.52 ± 0.57) were less potent than flupirtine (pIC_{50} 4.00 ± 0.25), mexiletine (pIC_{50} 3.99 ±
16 0.27) and carbamazepine (pIC_{50} 4.19 ± 0.51) (Fig. 3B).
17
18 In order to assess the therapeutic potential of these compounds, we assessed their analgesic
19 efficacy in a novel animal model of ciguatoxin-induced cold allodynia. Intraplantar
20 administration of P-CTX-1 induces cold allodynia [26], consistent with the clinical
21 presentation of ciguatera and our findings from intradermal injection of P-CTX-1 in human
22 volunteers (Fig 1). As expected, intraplantar injection of an equivalent concentration of P-
23 CTX-3, based on its *in vitro* potency in SH-SY5Y cells, also elicited cold allodynia (Fig. 4A).
24 However, intraplantar injection of the site 5 toxin BTX-A, or the Na_v activator veratridine,
25 surprisingly only elicited spontaneous nocifensive behaviour (data not shown) but no cold

1 allodynia (Fig. 4A). This finding supports the relevance of our animal model to the clinical
2 presentation of ciguatera, and validates cold allodynia as a hallmark feature of the effects of
3 ciguatoxins on peripheral sensory neurons.
4
5 *Analgesic treatment of ciguatoxin-induced cold allodynia*
6 Based on these results, we used our novel animal model of ciguatoxin-induced peripheral
7 sensory disturbances [26] to assess the analgesic effect of these compounds tested at doses
8 approximately equivalent to ceiling doses used in humans. Surprisingly, only lamotrigine (10
9 mg/kg; 82.5 ± 6.1 % inhibition), flupirtine (10 mg/kg, 72.0 ± 5.0 % inhibition) and phenytoin
10 (10 mg/kg; 57.5 ± 6.3 % inhibition) significantly ($p < 0.05$) reduced ciguatoxin-induced cold
11 allodynia, while amitriptyline (3 mg/kg; 2.5 ± 13.2 % inhibition), carbamazepine (10 mg/kg;
12 34.9 ± 3.0 % inhibition), topiramate (50 mg/kg; 24.7 ± 14.1 % inhibition) and mexiletine (10
13 mg/kg; 30.7 ± 12.4 % inhibition) had no significant effect on ciguatoxin-induced cold
14 allodynia (Fig. 4B). The observed effects on cold allodynia were not due to impaired motor
15 performance, since only amitriptyline caused a significant ($p < 0.05$) decrease in locomotor
16 activity attributable to sedating effects that were apparent at the administered dose (Fig. 4C).
17 In addition, we assessed the effect of the two most efficacious compounds, lamotrigine and
18 flupirtine, on CGRP release. Only flupirtine (50 µM; 36.3 ± 5.3 pg/ml; control, 56.6 ± 4.6
19 pg/ml), but not lamotrigine (50 µM; 38.5 ± 6.7 pg/ml; control, 48.3 ± 9.9 pg/ml) significantly
20 ($p < 0.05$) decreased CGRP release induced by P-CTX-1 (3 nM), suggesting contribution of
21 non-peptidergic nociceptors to ciguatoxin-induced cold allodynia, and/or central analgesic
22 effects of these compounds.
23 *TTX-sensitive Na_v isoforms contributing to ciguatoxin-induced cold allodynia*

We have previously demonstrated that cold allodynia induced by local intraplantar injection of P-CTX-1 is mediated partially through $Na_v1.8$ [26]. However, TTX-sensitive channels, expressed on unmyelinated C- as well as myelinated A-fibers, also contribute significantly to cold-induced pain after local injection of ciguatoxin [26]. To assess the contribution of TTX-sensitive Na_v isoforms to ciguatoxin-induced cold allodynia *in vivo*, we assessed the effect of subtype-selective Na_v inhibitors on cold pain behaviours. Intraplantar injection of ProTxII (30 nM), a $Na_v1.7$-selective inhibitor, did not affect the development of ciguatoxin-induced cold allodynia (Fig. 5). Intraplantar administration of the $Na_v1.2$/$Na_v1.1$ inhibitor TIIIA (10 µM) [28] also did not affect cold allodynia, while pain behaviour was virtually abolished by concomitant intraplantar injection of A803467 (10 µM), a $Na_v1.8$ inhibitor, and the conotoxin GIIIA (10 µM), which inhibits $Na_v1.4$, $Na_v1.1$, $Na_v1.6$ and $Na_v1.2$, but not $Na_v1.7$ or $Na_v1.3$ [28]. In addition, ciguatoxin-induced cold allodynia and spontaneous pain was not significantly decreased in $Na_v1.3^{-/-}$ animals (98.5 ± 13.5 % of control). This provides further evidence that TTX-sensitive isoforms other than $Na_v1.7$ or $Na_v1.3$ contribute to ciguatoxin-induced cold allodynia, and suggests an important role for $Na_v1.6$ in peripheral cold pain pathways [8]. However, given the lack of subtype-selective inhibitors for these isoforms, and the profound effect on motor performance in mice with loss of function mutations of Scn8a and Scn1a, the precise role of these Na_v subtypes in pain pathways remains to be elucidated.

We next assessed the *in vitro* activity of adjuvant analgesics at heterologously expressed $Na_v1.3$, $Na_v1.6$, $Na_v1.7$ or $Na_v1.8$ (Fig. 6). The rank order of inhibition was similar to that observed in SH-SY5Y cells, with amitriptyline being most potent at all Na_v subtypes (pIC_{50} $Na_v1.3$, 4.85 ± 0.09; $Na_v1.6$, 4.89 ± 0.17; $Na_v1.7$, 4.85 ± 0.11; $Na_v1.8$, 4.51 ± 0.06), followed by flupirtine (pIC_{50} $Na_v1.3$, 3.88 ± 0.18; $Na_v1.6$, 3.86 ± 0.21; $Na_v1.7$, 3.99 ± 0.35; $Na_v1.8$, 3.53 ± 0.10) and mexiletine (pIC_{50} $Na_v1.3$, 3.73 ± 0.08; $Na_v1.6$, 3.65 ± 0.26; $Na_v1.7$, 3.93 ±

0.16; $Na_v1.8$, 3.60 ± 0.52). Carbamazepine (pIC_{50} $Na_v1.3$, 3.26 ± 0.13; $Na_v1.6$, 3.29 ± 0.15; $Na_v1.7$, 3.49 ± 0.17; $Na_v1.8$, 3.45 ± 0.16), lamotrigine (pIC_{50} $Na_v1.3$, 3.37 ± 0.32; $Na_v1.6$, 3.41 ± 0.39; $Na_v1.7$, 3.36 ± 0.10; $Na_v1.8$, 2.87 ± 0.19) and phenytoin (pIC_{50} $Na_v1.3$, 2.66 ± 0.14; $Na_v1.6$, 2.84 ± 0.13; $Na_v1.7$, 2.73 ± 0.12; $Na_v1.8$, 3.06 ± 0.25) were least potent. Overall, little subtype-selectivity for Na_v isoforms was apparent, suggesting that in addition to inhibition of Na_v in peripheral sensory neurons, alternative mechanisms such as activity at thermosensitive TRP or neuronal potassium channels, but also central analgesic effects, may contribute to the observed anti-allodynic effects.

Discussion

Ciguatera, the most common non-bacterial ichthyosarcotoxism, remains a significant clinical challenge, with the treatment standard, mannitol (IV, 0.5–1.0 g/kg), no longer recommended due to a reported lack of efficacy, especially in more prevalent milder forms of the disease [10; 21]. Thus, in the absence of effective treatment strategies validated through appropriate clinical trials, management of the neurological symptoms of ciguatera, including a number of painful neuropathies, remains predominantly symptomatic. The sensory neuropathies associated with ciguatera have been postulated to arise from a direct excitatory action of the toxin on peripheral sensory neurons. However, given the presence of centrally-mediated symptoms such as ataxia in human ciguatera patients, altered central processing could also contribute to the perception of pain and cold allodynia [18]. To address this issue, we have now shown for the first time that local intradermal administration of P-CTX-1 in humans elicits symptoms consistent with ciguatera, confirming a peripheral origin of cold allodynia.
To initially profile analgesics that might reverse ciguatoxin-induced activation of neurons, we established a novel ciguatoxin assay in the human neuroblastoma cell line SH-SY5Y. In SH-

SY5Y cells, ciguatoxin-induced Ca^{2+} responses are elicited as a result of membrane depolarisation which in turn activates Ca_v channels. In peripheral sensory neurons, this effect is amplified by cold-induced activation of TRPA1 in CTX-sensitive neurons, although P-CTX-1 does not directly activate or potentiate TRPA1 [26]. P-CTX-1 also causes *de novo* Ca^{2+} responses to cold in cultured sensory neurons [26] , and elicits Ca^{2+} increases in neurons as a result of Na_v-mediated membrane depolarisation (for review see [27]). Thus, neuronal cells that permit characterisation of the effects of CTX on Na_v channels, in particular TTX-sensitive Na_v, provide an elementary *in vitro* model of the cellular mechanisms underlying ciguatoxin-induced neuronal activation. SH-SY5Y cells express TTX-sensitive Na_v which have previously been suggested to be important in pain signalling, including $Na_v1.3$ and $Na_v1.7$, and were particularly sensitive to the effects of P-CTX-1 compared to other neuronal cell lines including ND7/23 and Neuro2a cells (data not shown). Activation of endogenously expressed $Na_v1.2$, $Na_v1.3$ and $Na_v1.7$ in SH-SY5Y cells leads to membrane depolarization, and subsequent Ca^{2+} influx through endogenously expressed voltage-gated Ca^{2+} channels. Thus, this assay provides an excellent signal-to-noise ratio and enables characterization of the effects of subtype-selective pharmacological modulators as well as clinically used analgesics on ciguatoxin-induced responses. Surprisingly, $Na_v1.2$ and $Na_v1.7$ contributed little to responses elicited by P-CTX-1, while these Na_v isoforms were recently shown to mediate the majority of veratridine-induced responses in this cell line. The lack of subtype-specific $Na_v1.3$ inhibitors prohibited direct characterization of the contribution of $Na_v1.3$ to ciguatoxin-induced responses. However, given that responses elicited by P-CTX-1 were entirely TTX-sensitive in this cell line, and both selective $Na_v1.2$ and $Na_v1.7$ inhibitors blocked only a minor portion of ciguatoxin responses in SH-SY5Y cells, it seems likely that $Na_v1.3$ is the major mediator of P-CTX-1 responses in this assay. The toxicological target of

1 CTX is thus distinct from veratridine, which induces Ca^{2+} responses in SH-SY5Y cells
2 through activation of $Na_v1.2$ and $Na_v1.7$ [25].

3 Based on their ability to pharmacologically antagonise the effects of ciguatoxin, compounds
4 with activity at Na_v, such as antiepileptics or tricyclic antidepressants, would be expected to
5 provide effective relief from ciguatera symptoms. Thus, we characterised the *in vitro* efficacy
6 of a number of clinically used adjuvant analgesics on the ciguatoxin-induced responses in this
7 assay. All compounds except topiramate were able to inhibit P-CTX-1 responses with
8 varying potencies. While topiramate has been reported to inhibit Na_v currents in rat cerebellar
9 granule cells [30], these cells express predominantly $Na_v1.2$ and $Na_v1.6$ [17], suggesting that
10 topiramate may have a preference for inhibition of Na_v isoforms other than $Na_v1.3$ [17].
11 Consistent with a lack of effect on P-CTX-1-induced *in vitro* ciguatoxin-induced responses,
12 topiramate did not decrease ciguatoxin-induced cold allodynia significantly. Similarly,
13 amitriptyline had no effect on cold allodynia in our animal model, consistent with previous
14 anecdotal reports that this tricyclic antidepressant was ineffective for the treatment of
15 ciguatera-associated cold allodynia [7; 19], despite it being the most potent inhibitor of
16 ciguatoxin-induced responses in SH-SY5Y cells. In contrast, both lamotrigine and flupirtine
17 provided nearly complete inhibition of cold allodynia, while phenytoin partially reversed
18 ciguatoxin-induced cold allodynia. The reason(s) for the lack of efficacy of amitriptyline,
19 mexiletine and carbamazepine are unclear, but may involve pharmacokinetic and
20 pharmacodynamic effects that result in insufficient local concentration at peripheral sensory
21 neurons at the doses selected in this study, which were chosen to be approximately equipotent
22 based on *in vitro* efficacy, *in vivo* dosing and tolerability in animals.

23 The weak correlation between our *in vitro* assay and *in vivo* efficacy suggests that CTX-
24 induced cold allodynia might not arise simply from activation of sodium channels. We have

previously shown that ciguatoxin-induced cold allodynia is mediated by TRPA1-expressing unmyelinated C- and myelinated A-fibers despite the lack of any direct effect of CTX on TRPA1 [26]. In addition, ciguatoxin is known to affect K^+ channels, some of which also have profound effects on excitability at cool temperatures [13; 16]. Thus, CTX-induced cold allodynia appears to arise from effects of CTX on both Na_v and K^+ channels expressed in TRPA1-expressing C fibers as well as myelinated A-fibers, with both TTX-resistant and TTX-sensitive sodium channels contributing to this effect.

The most effective analgesics in our model were lamotrigine and flupirtine, both of which affect Na_v and potassium channels [6; 9]. Thus, it is plausible that rather than a subtype-selective effect on a particular isoform of Na_v channels, the *in vivo* efficacy is a reflection of the combined pharmacological profile of a given compound on Na_v, K_v and perhaps TRP channels relevant for signalling in pain pathways. Such effects are too complex to be replicated well in *in vitro* systems, and it is thus not surprising that the potency at individual Na_v isoforms was a poor predictor of efficacy. In addition to inhibition of Na_v and K_v isoforms in peripheral sensory neurons, central effects of adjuvant analgesics tested here may also contribute to the observed *in vivo* anti-allodynic effect. Thus, the *in vitro* ciguatoxin assay described here is a poor model for ciguatoxin-induced cold allodynia and may more accurately reflect the acute activation of neurons by ciguatoxin. The weak correlation between *in vitro* and *in vivo* efficacy highlights the complex nature of cold allodynia and native nerve terminals and supports the need for appropriate *in vivo* models. Nonetheless, although not directly representative of the mechanisms underlying ciguatoxin-induced cold allodynia, the *in vitro* ciguatoxin assay presented here may be useful for high-throughput assessment of modulators of acute ciguatoxin-induced neuronal activation.

$Na_v1.3$, the isoform contributing to the majority of P-CTX-1-induced responses in our *in vitro* assay, is expressed at low levels in adult rodent DRG neurons, and its contribution to pain remains controversial [12; 15]. Consistent with low expression levels in adult rodents, we found that $Na_v1.3$ plays a minor role in ciguatoxin-induced cold allodynia in mice, and had no effect on spontaneous pain behaviours. It is unclear whether $Na_v1.3$ is expressed at similarly low levels in humans, or whether this isoform contributes significantly to cold allodynia, acute pain or the axon reflex flare. Alternatively, it may be possible that this isoform contributes to ciguatera symptoms other than cold allodynia and spontaneous pain, such as central nervous system disturbances.

It is clear that ciguatoxin-induced cold allodynia in mice involves contribution of both $Na_v1.8$ and TTX-sensitive Na_v [26]. However, ciguatoxin-induced cold allodynia was not significantly affected in $Na_v1.3^{-/-}$ animals. Consistent with reports that IB4-negative sensory neurons, the population which we previously found to be particularly sensitive to P-CTX-1, express TTX-sensitive Na_v isoforms other than $Na_v1.7$ [22], we found that ciguatoxin-induced cold allodynia was not affected by intraplantar injection of the $Na_v1.7$-selective inhibitor ProTxII but was blocked almost completely when $Na_v1.8$ as well as $Na_v1.6$ were inhibited.

Interestingly, other Na_v activator toxins appear to target different sodium channel combinations, since local intraplantar injection of the Na_v activators BTX-A and veratridine failed to induce signs of cold allodynia using a similar protocol but caused spontaneous nocifensive behaviour, evidenced by lifting, licking, shaking and flinching of the ipsilateral hind paw. These findings validate cold allodynia as a pathognomonic symptom of ciguatera,

and support the relevance of our animal model to the clinical presentation of ciguatera. Based on our findings, as well as pharmacokinetic and safety considerations, lamotrigine and flupirtine appear to have potential in the treatment of ciguatoxin-induced cold allodynia. While these findings remain to be validated clinically, this is the first systematic evaluation of clinically used analgesics for the treatment of ciguatoxin-induced cold allodynia.

Acknowledgements

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Figure Legends

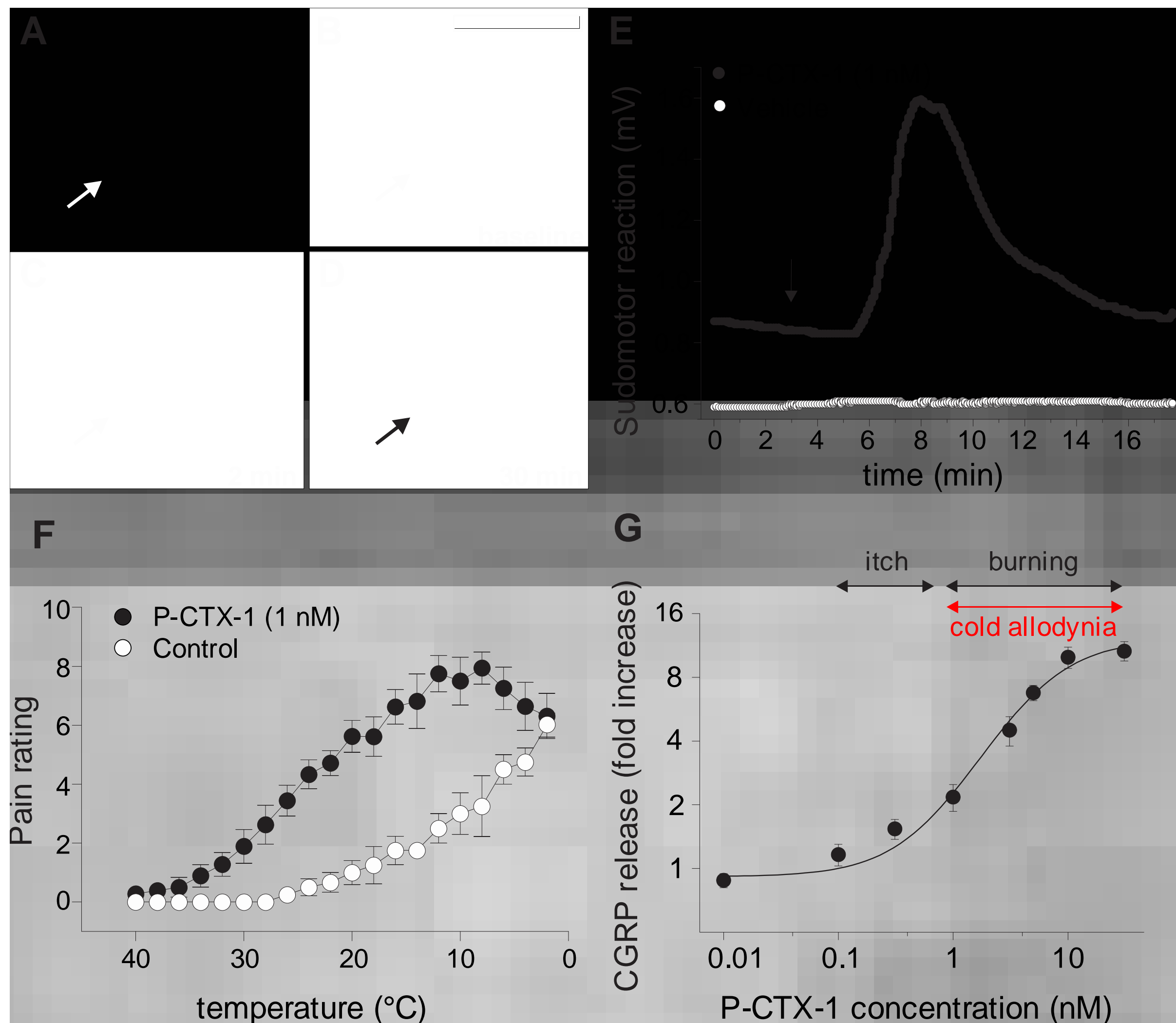

Figure 1. Peripheral sensory effects of P-CTX-1 in humans. (A-D) Subcutaneous injection of P-CTX-1 (1 nM; 50 µl) into the volar forearm of human subjects causes an axon reflex flare. Arrow; P-CTX-1 injection site. **(A)** Photographic image of the injection site at baseline. **(B-D)** Laser doppler image of the injection site at baseline **(B)**, 2 min **(C)** and 30 min **(D)** after injection of P-CTX-1. Scale bar; perfusion units. **(E)** Axon reflex sweating induced by P-CTX-1. Arrow; timepoint of P-CTX-1 injection. Sweat output is presented in mV from data recorded by the humidity sensor control unit (HygroLab 2, Rotronic GmbH, Germany). **(F)** Temperature-dependence of cold pain (control, prior to P-CTX-1 injection, white) and cold allodynia induced by P-CTX-1 (1 nM, black) in human volunteers (n=5). **(G)** P-CTX-1

increased CGRP release from rat skin with an EC_{50} of 2.3 nM. The intensity and nature of sensations elicited by intradermal injection of P-CTX-1 in human skin paralleled CGRP release in rat skin, with concentrations equal to and above the EC_{50} of ~2.3 nM for CGRP release causing pain and cold allodynia, while concentrations below 1 nM caused itch.

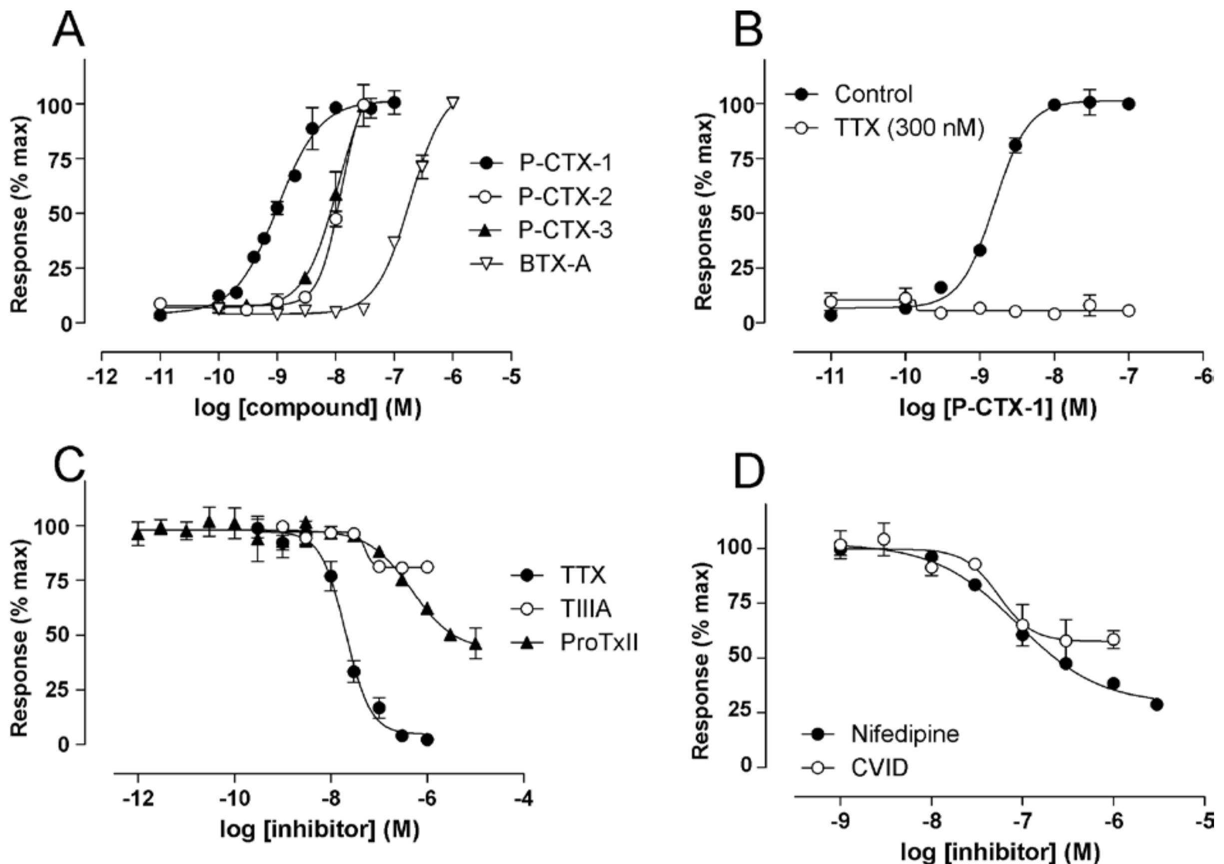

Figure 2. Characterisation of ciguatoxin-induced responses in the human neuroblastoma cell line SH-SY5Y. (A) In SH-SY5Y cells loaded with Calcium-4 dye, stimulation with P-CTX-1 (EC_{50} 2.2 ± 0.6 nM), P-CTX-2 (EC_{50} 9.3 ± 2.6 nM), P-CTX-3 (EC_{50} 8.3 ± 2.4 nM) as well as BTX-A (EC_{50} 160.7 ± 19.3 nM) caused concentration-dependent increases in intracellular Ca^{2+}. **(B)** P-CTX-1 responses were mediated through TTX-sensitive Na_v isoforms endogenously expressed in SH-SY5Y cells, as responses were completely abolished in the presence of TTX (300 nM). **(C)** TTX completely inhibited P-

CTX-1 responses with an IC_{50} of 12.9 ± 2.2 nM, while the $Na_v1.2$ inhibitor TIIIA caused partial (25.4 ± 0.9 %) inhibition with an IC_{50} of 49.9 ± 14.9 nM. The $Na_v1.7$ inhibitor ProTxII caused a small inhibition (11.9 ± 1.6 %) at concentrations (100 nM) which fully inhibit $Na_v1.7$, and blocked P-CTX-1 responses with an IC_{50} of 4.3 ± 3.1 µM. **(D)** The Ca_v inhibitors nifedipine and CVID partially blocked P-CTX-1 responses with IC_{50}s of 59.1 ± 16.4 nM and 33.6 ± 8.8 nM, respectively. Data is presented as mean +/- SEM and is representative of 3-9 independent experiments

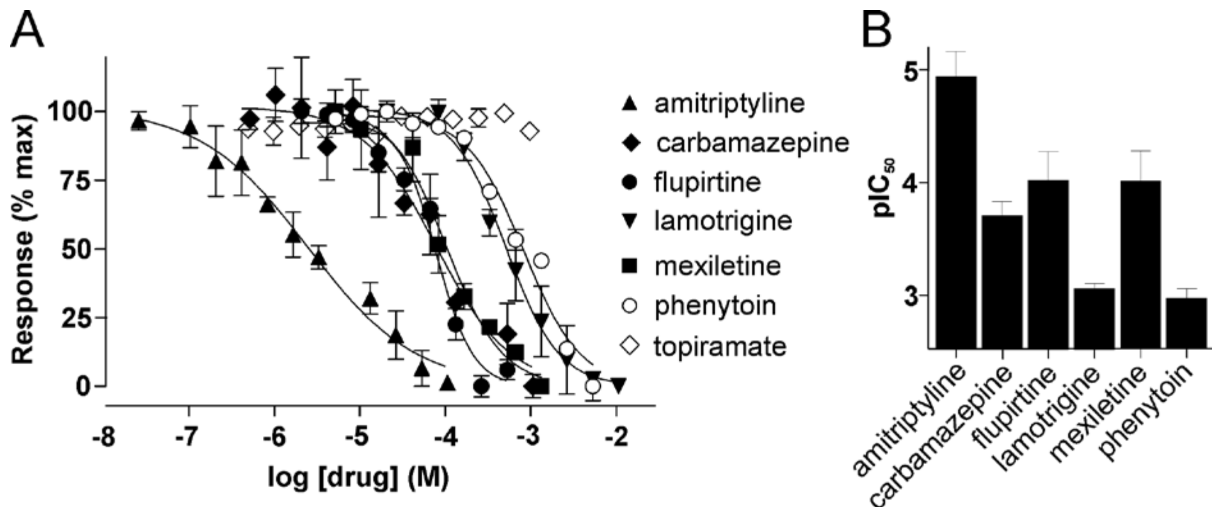

Figure 3. Inhibition of ciguatoxin-evoked responses in the human neuroblastoma cell line SH-SY5Y. (**A**) Amitriptyline, carbamazepine, flupirtine, lamotrigine, mexiletine and phenytoin concentration-dependently inhibited responses elicited by addition of P-CTX-1 (3 nM), while topiramate did not affect ciguatoxin-mediated responses at concentrations up to 1 mM. (**B**) The *in vitro* potency (pIC_{50}) of amitriptyline (pIC_{50} 4.91 ± 0.22), carbamazepine (pIC_{50} 4.19 ± 0.51), flupirtine (pIC_{50} 4.00 ± 0.25), lamotrigine (pIC_{50} 3.03 ± 0.05), mexiletine (pIC_{50} 3.99 ± 0.27) and phenytoin (pIC_{50} 3.52 ± 0.57) for inhibition of CTX-mediated responses. Data are presented as mean ± SEM of n = 3 independent experiments.

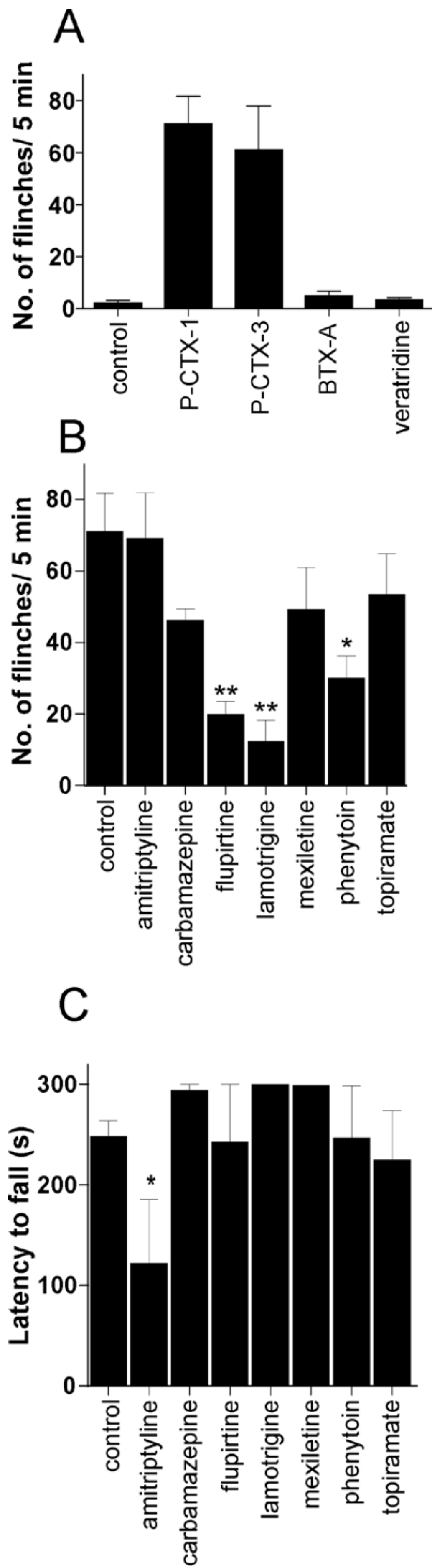

Figure 4. Anti-allodynic treatment of ciguatoxin-

induced cold allodynia. **(A)** Intraplantar injection of P-CTX-1 and P-CTX-3, but not the Na_v activators BTX-A or veratridine, elicits cold allodynia **(B)** Lamotrigine (10 mg/kg), flupirtine (10 mg/kg) and phenytoin (10 mg/kg) significantly decreased cold allodynia elicited by intraplantar injection of P-CTX-1 (5 nM), while amitriptyline (3 mg/kg), mexiletine (10 mg/kg), carbamazepine (10 mg/kg) and topiramate (50 mg/kg) had no significant anti-allodynic effect. Data are presented as mean ± SEM of n = 5-16 animals. **(C)** The anti-allodynic effects of lamotrigine, flupirtine and phenytoin were not due to impaired locomotor activity, as only amitriptyline significantly ($p < 0.05$) affected latency to fall in a Rotarod test. Data are presented as mean ± SEM of n = 5 animals. Statistical significance was determined using ANOVA with Dunnett's post-test; *, $p < 0.05$; **, $p < 0.01$.

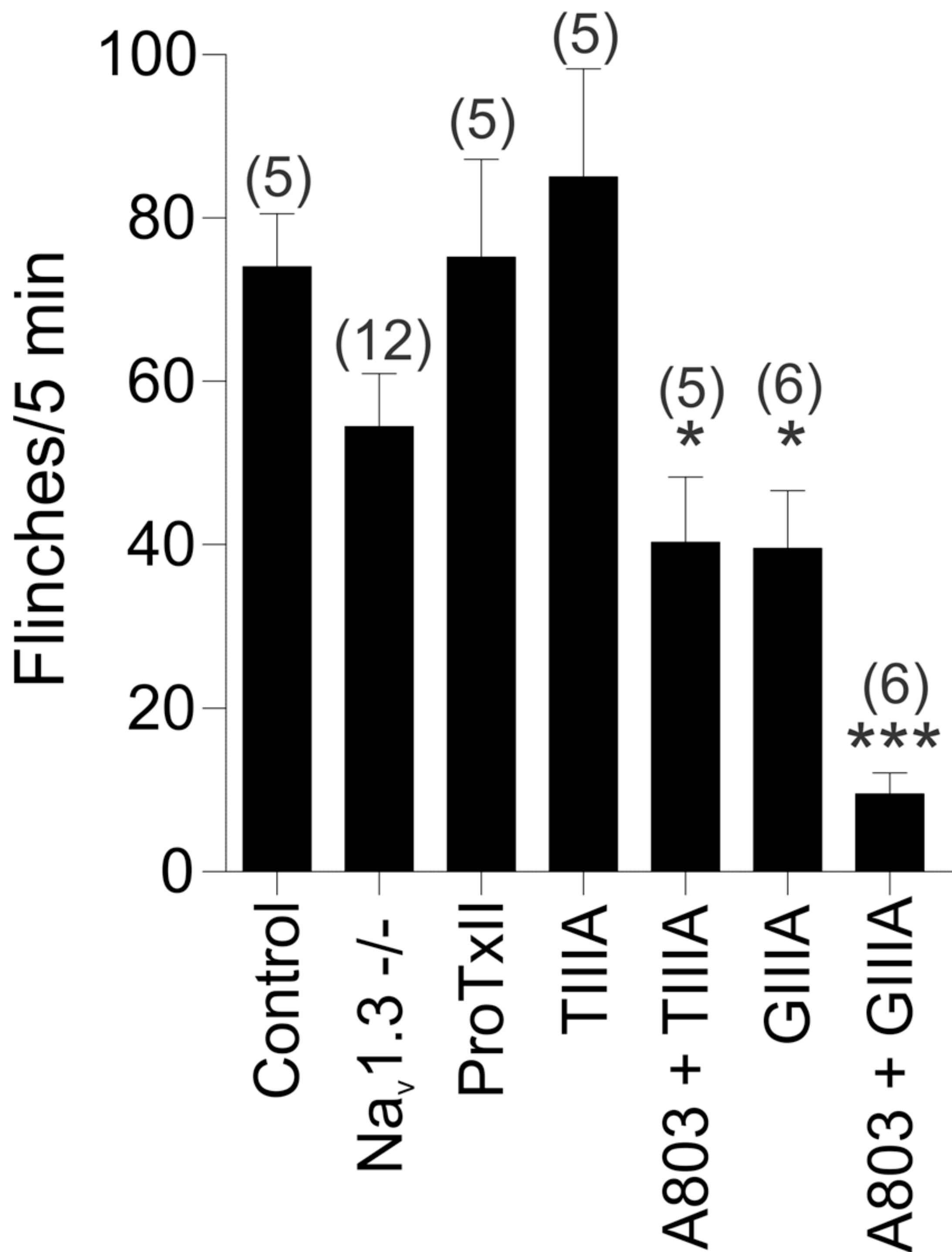

Figure 5. Na_v isoforms contributing to ciguatoxin-induced cold allodynia. Ciguatoxin-induced cold allodynia was not significantly inhibited in $Na_v1.3^{-/-}$ animals, or after intraplantar administration of the $Na_v1.7$-specific inhibitor ProTxII (10 nM) and the $Na_v1.2$/$Na_v1.1$ inhibitor TIIIA (10 μM). Co-administration of TIIIA (10 μM) and the $Na_v1.8$-inhibitor A803467 (10 μM) partially decreased cold allodynia elicited by i.pl. administration of P-

CTX-1. Intraplantar administration of the $Na_v1.1/Na_v1.6$ inhibitor GIIIA (10 µM) inhibited cold allodynia by 46.6 ± 8.9 % and was additive to inhibition of $Na_v1.8$, with co-administration of GIIIA and A803467 reducing cold pain behaviour by 87.2 ± 3.3 %. Data are presented as mean ± SEM of n = 5 – 12 animals. Statistical significance was determined using ANOVA with Dunnett's post-test; *, $p < 0.05$; ***, $p < 0.001$.

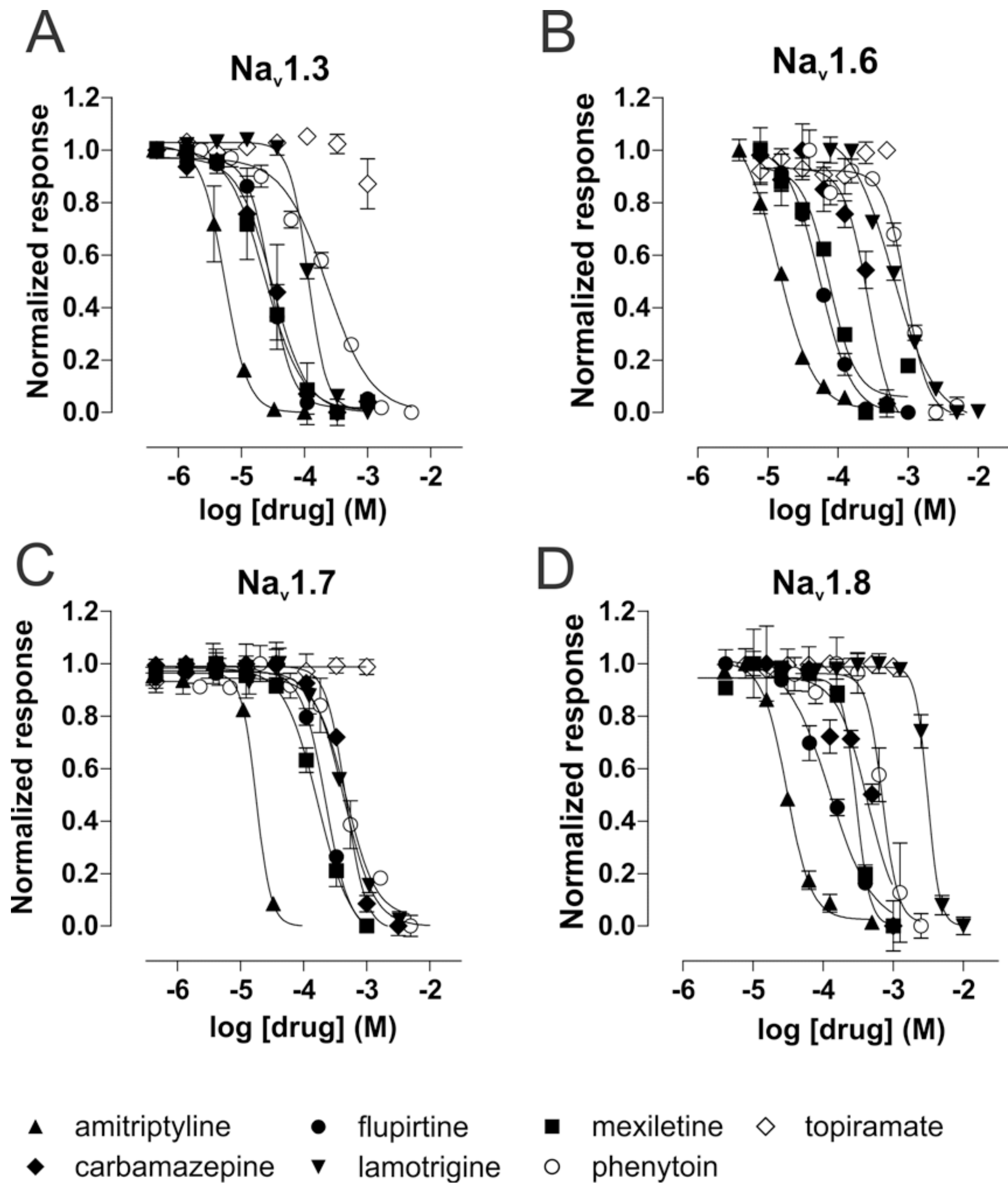

Figure 6. Inhibition of heterologously expressed Na_v isoforms by adjuvant analgesics. Inhibition of heterologously expressed $Na_v1.3$, $Na_v1.6$, $Na_v1.7$ and $Na_v1.8$ was assessed using a high-throughput FLIPR[Tetra] membrane potential assay. Amitriptyline (▴), carbamazepine (◆), flupirtine (●), lamotrigine (▾), mexiletine (▪) and phenytoin (○) concentration-dependently inhibited $Na_v1.3$ **(A)**, $Na_v1.6$ **(B)**, $Na_v1.7$ **(C)** and $Na_v1.8$ **(D)** mediated responses, while topiramate (◇) did not inhibit any Na_v isoform assessed. **(A)** The *in vitro* potency (pIC_{50}) for inhibition of heterologously expressed $Na_v1.3$ by amitriptyline (pIC_{50} 4.85 ± 0.09),

carbamazepine (pIC_{50} 3.26 ± 0.13), flupirtine (pIC_{50} 3.88 ± 0.18), lamotrigine (pIC_{50} 3.37 ± 0.32), mexiletine (pIC_{50} 3.73 ± 0.08) and phenytoin (pIC_{50} 2.66 ± 0.14). **(B)** The *in vitro* potency (pIC_{50}) for inhibition of heterologously expressed $Na_v1.6$ by amitriptyline (pIC_{50} 4.89 ± 0.17), carbamazepine (pIC_{50} 3.29 ± 0.15), flupirtine (pIC_{50} 3.86 ± 0.21), lamotrigine (pIC_{50} 3.41 ± 0.39), mexiletine (pIC_{50} 3.65 ± 0.26) and phenytoin (pIC_{50} 2.84 ± 0.13). **(C)** The *in vitro* potency (pIC_{50}) for inhibition of heterologously expressed $Na_v1.7$ by amitriptyline (pIC_{50} 4.85 ± 0.11), carbamazepine (pIC_{50} 3.49 ± 0.17), flupirtine (pIC_{50} 3.99 ± 0.35), lamotrigine (pIC_{50} 3.36 ± 0.10), mexiletine (pIC_{50} 3.93 ± 0.16) and phenytoin (pIC_{50} 2.73 ± 0.12). **(D)** The *in vitro* potency (pIC_{50}) for inhibition of heterologously expressed $Na_v1.8$ by amitriptyline (pIC_{50} 4.51 ± 0.06), carbamazepine (pIC_{50} 3.45 ± 0.16), flupirtine (pIC_{50} 3.53 ± 0.10), lamotrigine (pIC_{50} 2.87 ± 0.19), mexiletine (pIC_{50} 3.60 ± 0.52) and phenytoin (pIC_{50} 3.06 ± 0.25). Concentration-response curves are representative of 3-4 independent experiments, with pIC_{50} data presented as mean ± SEM from n = 3-4 independent experiments.