The $\alpha_1$-adrenoceptor inhibitor $\rho$-TIA facilitates net hunting in piscivorous Conus tulipa

Mriga Dutt$^{1,3}$, Jean Giacomotto$^{2,3}$, Lotten Ragnarsson$^1$, Åsa Andersson$^1$, Andreas Brust$^1$, Zoltan Dekan$^1$, Paul F. Alewood$^1$ & Richard J. Lewis$^{1,*}$

Cone snails use separately evolved venoms for prey capture and defence. While most use a harpoon for prey capture, the Gastriidium clade that includes the well-studied Conus geographus and Conus tulipa, have developed a net hunting strategy to catch fish. This unique feeding behaviour requires secretion of "nirvana cabal" peptides to dampen the escape response of targeted fish allowing for their capture directly by mouth. However, the active components of the nirvana cabal remain poorly defined. In this study, we evaluated the behavioural effects of likely nirvana cabal peptides on the teleost model, Danio rerio (zebrafish). Surprisingly, the conantokins (NMDA receptor antagonists) and/or conopressins (vasopressin receptor agonists and antagonists) found in C. geographus and C. tulipa venom failed to produce a nirvana cabal-like effect in zebrafish. In contrast, low concentrations of the non-competitive adrenoceptor antagonist $\rho$-TIA found in C. tulipa venom ($EC_{50}$ = 190 nM) dramatically reduced the escape response of zebrafish larvae when added directly to aquarium water. $\rho$-TIA inhibited the zebrafish $\alpha_1$-adrenoceptor, confirming $\rho$-TIA has the potential to reverse the known stimulating effects of norepinephrine on fish behaviour. $\rho$-TIA may act alone and not as part of a cabal, since it did not synergise with conopressins and/or conantokins. This study highlights the importance of using ecologically relevant animal behaviour models to decipher the complex neurobiology underlying the prey capture and defensive strategies of cone snails.

Cone snails are a group of ~800 species of marine molluscs endemic to Indo-Pacific and Atlantic coral reef systems. They possess a long venom duct that can differentially secrete compartmentalised predatory and defensive venoms. These molluscs prey on polychaete annelids (vermivores), molluscs (molluscivores) or fish (piscivores) using venom peptides injected intramuscularly (i.m.) through a hollow radula tooth harpooned into prey. The injected venom of an individual cone snail contains >1000 cysteine-rich peptides (conopeptides or conotoxins) that target a diverse range of neuronal ion channels and cell membrane receptors. Piscivorous cone snails mostly use a harpoon strategy that deploys excitatory (lightning-strike cabal) or inhibitory (motor cabal) conotoxins to paralyse fish. Additionally, the Gastriidium clade, including the well-studied Conus geographus and Conus tulipa, use a net hunting strategy where so called “nirvana cabal” peptides are secreted into the water to inhibit the escape response of teleost fish, allowing capture of large fish (see Supplementary Movie 1 of ref. 2). Early studies hypothesised the nirvana cabal comprises non-paralytic peptides including conantokins and conopressins. However, any potential involvement in the nirvana cabal was extrapolated from their identified physiological effects following their intracerebral injection in rodents and direct effects of most postulated nirvana cabal peptides on fish when added directly to aquaria water have not been shown.

Venomic studies of C. geographus revealed the presence of non-paralytic conantokins, conopressins, conotulakins and conoinsulins in the distal duct segment where the predatory-evoked venoms are secreted in this species. Recent venomic studies of C. tulipa support a similar distribution, with conantokins dominant in the distal duct section, the $\alpha_1$-adrenoceptor ($\alpha_1$-AR) antagonist $\rho$-TIA and vasopressin receptor (VR) antagonist conopressin-T dominant in the proximal central duct, whereas conoinsulins were only detected at low levels in the C. tulipa transcriptome. $\rho$-TIA is an allosteric antagonist of the mammalian $\alpha_1$-AR that binds to a well-characterised pharmacophore on the extracellular surface of this family A GPCR. While injection of...
\( \rho \)-TIA into fish did not reveal a phenotype\(^{16} \), previous studies examining the effects of prazosin on fish suggest \( \rho \)-TIA might also induce a sleep-like state in fish\(^{18} \). However, with the exception of the conoinsulins\(^{19} \), the behavioural effects of these potential nirvana peptides added to aquarium water housing fish has not been established to confirm their potential role in net hunting.

In this study, we analysed behavioural effects on the teleost zebrafish induced by synthetic candidate nirvana cabal conotoxins added to their surrounding water to characterize their potential to contribute to net hunting. Zebrafish larvae behaviour was monitored using an automatic tracking system (Zebrabox Revolution) that allows real-time measurement of swim speed, scoot distance and angle of turn behaviours\(^{20-27} \). Surprisingly, conopressins and conantokins had no detectable effect on the fish ‘fight or flight’ response either alone or in combination, whereas \( \rho \)-TIA potently blocked the zebrafish escape response to mechanical touch stimuli. N-terminal truncations of \( \rho \)-TIA and site-directed mutagenesis of the zebrafish \( \alpha_1 \)-AR confirmed that \( \rho \)-TIA acted at a related allosteric site in the zebrafish \( \alpha_1 \)-AR. This study directly implicates \( \rho \)-TIA as an antagonist at the zebrafish \( \alpha_1 \)-AR that may contribute to the nirvana cabal, allowing fish capture directly by mouth without harpooning.

### Results

#### Systemic effects of dissected C. tulipa venom in adult zebrafish.

To determine the venom duct localisation of peptides contributing to harpoon prey capture, we extracted \( C. \) tulipa dissected venom from four duct sections, proximal (P), proximal central (PC), distal central (DC) and distal (D), and administered 0.1 \( \mu \)g of each intramuscularly (i.m.) and monitored for any behavioural changes. Venom from each of the duct sections reduced the swimming ability of fish, with the P section being most potent and causing an 80% reduction in total swim distance relative to the control (Fig. 1A). Dissected venom from each section produced iclectric paralysis that drastically slowed swimming movements, consistent with a motor cabal effect. Fish injected i.m. with the P dissected venom displayed an immediate and almost complete loss of motor activity that was irreversible over 15 min. In contrast, fish administered with the DC dissected venom had a delayed onset of activity, while PC and D had similar but weaker effects, reducing the total swim distance by 50% relative to the control.

Dissected \( C. \) tulipa venom did not induce a nirvana cabal effect in larval zebrafish. In an attempt to establish the duct localisation of nirvana cabal peptides, we administered dissected venoms to the fish water column and monitored for any change in fish behaviour using 5-day post fertilisation (5-dpf) zebrafish larvae.
Figure 2. Synthetic conantokins and conopressins are ineffective on the zebrafish larval escape response. The four nirvana peptides, conantokin-G (circle), conantokin-T (square) (0.003–1 μM), conopressin-G (inverted triangle) and conopressin-T (triangle) (0.003–5 μM) were dosed in water and touch-evoked larval escape responses plotted. All four peptides at maximal concentrations failed to elicit any effect on the larvae, as they constantly displayed an escape response score of around 9. Untreated larvae exhibited an average escape response score of 9.2 (dotted line). Data are expressed as the mean ± SEM of three independent experiments.

in multi-well assay plates. Addition of dissected C. tulipa duct venom (0.001–100 ng/μl) from each of the duct sections failed to alter the fish escape response (Fig. 1B) and all larvae displayed normal swimming behaviour.

Conantokin and conopressin have no effect on fish escape responses. Administration of dissected venom to larval water failed to produce nirvana cabal-like activity in zebrafish larvae, perhaps because the relevant peptides are secreted only on demand. We thus decided to test the hypothesised synthetic nirvana peptides individually and in combination on larval zebrafish. Neither the VR antagonist conopressin-T nor the NMDAR antagonist conantokin-T from C. tulipa affected the larval escape response at concentrations up to 1 μM (Fig. 2). Similarly, neither the VR agonist conopressin-G nor conantokin-G from C. geographus affected the larval escape response at concentrations up to 5 μM (Fig. 2). In all treatment groups, exposed larvae displayed normal swimming behaviour indistinguishable from the control group.

ρ-TIA reduces the fish escape response in larval zebrafish. Following a preliminary screen of synthetic conotoxins, we identified that ρ-TIA (0.03–10 μM) added to the water column produced a striking loss of zebrafish larval escape response to light mechanical touch on their trunk or tail. This effect was dose-dependent, with an EC50 of 0.19 ± 0.62 μM. ρ-TIA at concentrations above 3 μM produced an acute reduction in swimming speed, with the larvae exhibiting an apparent rigid body with minimal gill movements and abnormal escape responses (escape response score 2–3 vs. a control score of 8.9 of 10). At 1 μM, reduced escape responses developed over the initial 5 min of exposure to ρ-TIA, allowing larvae to be easily pushed around with the Teflon prod without showing escape or avoidance responses. (Fig. 3, Supplementary Video). The effects of 1 μM ρ-TIA lasted beyond the 15 min observation period, while prolonged exposure to ρ-TIA (>3 μM) was lethal to larvae. Additionally, ρ-TIA induced bursts of erratic swimming, similar to a stress response but with a stronger swimming response (Supplementary Fig S1). This altered swimming behaviour was observed for ~1 min after addition of ρ-TIA at concentrations above 0.01 μM, and was most pronounced at 0.3 μM ρ-TIA.

Assessing synergistic effects of ρ-TIA with presumed nirvana cabal conotoxins. To determine if the observed nirvana cabal effect results from synergy between different peptide classes, we treated the larvae with different combinations of conopressins, conantokins and ρ-TIA, each dosed at 1 μM. Co-exposure of zebrafish to conopressins and conantokins from C. geographus (Fig. 4A) or C. tulipa (Fig. 4B) had no effect on larval zebrafish escape responses, and exposed zebrafish instantly displayed the characteristic “C-start” response when presented with the mechanical stimulus. The escape responses for ρ-TIA in the presence of the conantokin/conopressin combinations were also indistinguishable from the responses observed for ρ-TIA alone (Fig. 4).

ρ-TIA N-terminal tail is critical for the induced phenotypic effect. Sequential deletion of the four N-terminal residues (TIA2–19, TIA3–19 and TIA4–19) reduced ρ-TIA potency to inhibit the zebrafish escape response (Fig. 3, Table 1), with deletion of the entire N-terminal tail FNWR (TIA5–19) abolishing inhibition of the escape response (Fig. 3).

ρ-TIA affinity at zebrafish α1B adrenergic receptors (ZF α1Bb-AR). ρ-TIA affinity at WT and mutant ZF α1Bb-AR was determined using a [3H]-prazosin radioligand binding assay (Fig. 5A,C). E327D/S330F-ZF α1Bb-AR, mutated to introduce the ρ-TIA pharmacophore in mammalian α1B ARs (Table 2, Supplementary Fig S2) had 36-fold enhanced affinity for ρ-TIA (IC50 = 88.51 ± 11.40 nM) compared with WT ZF α1Bb-AR (Fig. 5A, Table 3). ρ-TIA was a partial inhibitor of prazosin binding at the zebrafish α1Bb-AR (Fig. 5A), as previously reported at hamster α1B-AR12.

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Figure 3. ρ-TIA induced escape response on zebrafish larvae. The potential of ρ-TIA and its analogues (0.003–10 µM) to affect the escape response of treated zebrafish larvae, when presented with a light mechanical stimulus, was analysed using the manual touch-evoked assay. Larvae treated with ρ-TIA displayed a loss of escape response in a concentration dependent manner, where maximal ρ-TIA concentration (10 µM) completely deprived the larvae of their escape abilities. Sequential truncation of the N-terminal residues of ρ-TIA produced analogues (TIA2–19, TIA3–19, TIA 4–19) that induced a reduction in escape responses to the mechanical touch stimulus, with lower potencies compared to ρ-TIA. Absence of the N-terminal sequence “FNWR” from the peptide (TIA5–19) and the amino acid sequence “FNWRCC” alone (TIA1–6) had no significant effect on the larvae flight response at up to 10 µM. Larvae exposed to negative control (E3) exhibited normal escape response behaviour, with an average score of 8.9 (dotted line). Data is expressed as ± SEM and is the mean of values obtained from six independent experiments, each consisting of ten individual touch-evoked scores.

Figure 4. Simulation of the nirvana cabal on zebrafish larvae. The prey capture environment was simulated on the zebrafish larvae using mixtures of the nirvana peptides. Equimolar concentrations (1 µM) of synthetic conantokins and conopressins from (A) C. geographus and (B) C. tulipa were applied to the zebrafish larvae to observe for effects on their sensory capabilities. In both experiments, co-addition of conopressins and conantokins did not induce any synergistic effect on the escape response of the larvae, whereas a reduction in the larval flight response was observed when 1 µM ρ-TIA was added to the mixture (simulated Geo/Tulipa cabal). The simulated cabal from C. geographus peptides produced an average escape response score of 3.06, while the C. tulipa peptides produced an average escape response score of 4.07. In both experimental conditions, this observed effect was parallel to that elicited by single addition of 1 µM ρ-TIA (black solid bar), thus demonstrating that the hypothesised nirvana peptides do not affect the sensory capabilities of larval zebrafish. Single additions of the conopressins, conantokins and E3 (negative control) were ineffective on larval escape behaviour. Data is expressed as ± SEM and is the mean of values obtained from five independent experiments, each consisting of ten individual touch-evoked scores.
Table 1. N-terminal truncated analogues of ρ-TIA. The amino acid sequence, disulfide connectivity and IC50 ± SEM to inhibit zebrafish escape response. *C-terminal amidation.

| Peptide         | Amino acid sequence | Disulfide connectivity | IC50 (μM)  |
|-----------------|---------------------|------------------------|------------|
| ρ-TIA           | FNWRCCPLICACRRNHKKFC* | 1–3, 2–4               | 0.19 ± 0.62|
| TIA2-19         | NWRCPLICACRRNHKKFC*  | 1–3, 2–4               | 1.34 ± 0.18|
| TIA3-19         | WRCLIPACRRNHKKFC*    | 1–3, 2–4               | 4.09 ± 1.12|
| TIA4-19         | RCLIPACRRNHKKFC*     | 1–3, 2–4               | 3.16 ± 1.01|
| TIA5-19         | CCLIPACRRNHKKFC*     | 1–3, 2–4               | >10        |
| TIA1-6          | FNWRCC*              | 1–2                    | >10        |

Table 2. ρ-TIA pharmacophore residues in hamster α1-AR17 and corresponding residues in human and zebrafish α1-ARs. *Sequence confirmed from NCBI database.

| Receptor | Residues influencing ρ-TIA binding |
|----------|-----------------------------------|
| Hamster α1B | D327    F330    V197    S318    E186 |
| Human α1B   | D326    F329    V196    S317    E185 |
| ZF α1AA     | D318    F321    I192    S309    E181 |
| ZF α1AB     | D317    F320    V188    S308    E177 |
| ZF α1AA     | E320    F323    I199    S311    E183 |
| ZF α1BB     | E327    S330    I194    S318    E188 |

Figure 5. ρ-TIA is a functional antagonist of the zebrafish α1-AR. (A,C) ρ-TIA displayed weak binding affinity (IC50 = 3198.9 ± 46.26 nM) to the zebrafish wild type α1BB-AR, while a 36-fold improvement in binding affinity was observed at the zebrafish α1BB-E327D/S330F double mutant (IC50 = 88.51 ± 11.14 nM). (B,D) Inhibition of NE induced IP3 accumulation in cells expressing the ZFα1BB-E327D/S330F-AR by ρ-TIA was determined using the IP-one HTRF assay. A 6-fold improvement in ρ-TIA potency was observed at the double mutant (IC50 = 2.61 ± 0.22 μM) when compared to the zebrafish WTα1BB-AR (IC50 = 15.26 ± 1.19 μM). Data has been expressed as ± SEM and is presented as an average of three to six independent experiments, each performed in triplicate.
Table 3. ρ-TIA potency at hamster and zebrafish α1-adenoreceptors. IC50 ± SEM values (n = 3–6) are shown.

| Receptor | Radioligand binding assay (nM) | IP-one functional assay (μM) |
|----------|-------------------------------|-----------------------------|
| Hamster α1A | 4.39 ± 0.14 (3) | 1.56 ± 0.17 (3) |
| Zebrafish α1A | 3198.9 ± 46.26 (3) | 15.26 ± 1.19 (6) |
| Zebrafish α1A, E327D/S330F | 88.51 ± 11.14 (3) | 2.61 ± 0.22 (4) |

Discussion

The venom of piscivorous snails has been extensively studied for its pharmacological potential and more recently to understand the evolution of their predatory and defensive strategies. The Gastridium clade, including the well-studied C. geographus and C. tulipa, have developed a net hunting strategy to catch teleost fish directly by mouth using venom secreted directly into the surrounding water. However, the conotoxins contributing to this unique net hunting strategy remain unclear. In this study, we developed a zebrafish larval assay using an automated screening platform to quantify behavioural changes. Surprisingly, the dissected C. tulipa venom from each of the four duct sections added to the water column had no effect on the zebrafish escape response, suggesting nirvana cabal peptides are normally present in the venom duct at low concentrations and presumably upregulated and secreted into the water during net hunting. In contrast, i.m. injection of these dissected venoms produced flaccid paralysis in zebrafish that dramatically reduced their ability to swim, especially the proximal duct venom. While defensive venom peptides are produced in the closely related C. geographus, the proximal duct venom retained its antagonistic properties towards the fish α1-AR receptor antagonists. ρ-TIA added to aquarium water dramatically reduced the ability of the zebrafish to respond to mechanical stimuli, reminiscent of the hyperactivity described for excitatory kA-conotoxins in adult zebrafish. However, the excitatory effects observed with ρ-TIA lasted ~1 min and were fully reversible, with larvae resuming normal swimming after the hyperactive state, in contrast to the irreversible paralysis produced by kA-conotoxins. Neurotransmitter function is an important regulator of locomotion in adult fish, and disruption by antipsychotic drugs such as fluphenazine and haloperidol produces similar erratic swimming motion in zebrafish. It is possible that upon initial exposure, ρ-TIA may temporarily interfere with the lateral line sensory neurons, disrupting normal function.

Through a combination of behavioural and pharmacological assays on zebrafish, we have identified that ρ-TIA present in C. tulipa venom retains its antagonistic properties towards the fish α1-ARs and reverses the stimulatory effects observed with fluphenazine and haloperidol.
effects of norepinephrine on fish to induce a “sleep-like” quiescent state. This observed biological effect appears to be parallel to the sensory deprivation effect induced during net predation, which might facilitate fish capture by mouth by these nocturnal feeders. Thus, we hypothesise that ρ-TIA plays a direct biological role in net hunting by C. tulipa. The low levels of ρ-TIA identified in the venom duct suggest that secretion of ρ-TIA is stimulated only when C. tulipa starts net hunting. In contrast, conopressins and conantokins appear to play little role in the net hunting strategy of C. tulipa, raising questions about their biological function in this venom. Conantokins in particular have a dominant distal venom expression and yet there is no definitive study implicating their potential role in prey capture and/or defence. Given conantokins are expressed in several piscivorous venoms, their involvement in the hook and line prey capture strategy warrants further investigation. Similarly, the inactivity of conopressin-T in the behavioural assays further supports its potential role in the defensive venom of C. tulipa. Although ρ-TIA may act alone to facilitate net hunting in C. tulipa, we cannot exclude the possibility that other conotoxins, yet to be identified, may act in synergy. In contrast, net hunting in C. geographus may be facilitated by conoinsulins, which alone have been shown to affect fish behaviour at 25 μM, and/or contulakins, which are both prominent in C. geographus but not C. tulipa venom. Finally, development of a milking strategy for the net hunters of the Gastridium clade that allows direct isolation and sequencing of the “nirvana cabal” proteome is required to further characterise the peptides responsible for this unique predatory strategy that profoundly alters fish behaviour. Such peptides are expected to provide new research tools that are useful in the elucidation of the neurobiology of escape responses and sleep in fish.

Methods

Zebrafish husbandry. Adult and embryos zebrafish (TU strain) were maintained in accordance with the standards and guidelines approved by the University of Queensland Animal Ethics Committee (UQ AEC; AEC approval number IMB/158/15/NHMRC). Embryos were collected through mating pairs in specialised mating tanks (Tecniplast, USA), maintained at 28°C in E3 embryo water (2.9 g NaCl, 0.13 g KCl, 0.48 g CaCl2, 0.82 g MgSO4·7H2O, 0.01% v/v methylene blue in 1 L MilliQ water). For the larvae behaviour assays, healthy 5-dpf animals were selected and distributed into 24-multi-well plates (Corning® USA) supplemented with 500 μL of E3 medium. All described zebrafish behaviour assays were designed and performed according to the animal ethics guidelines approved by the UQ AEC (AEC approval numbers IMB/158/15/NHMRC and IMB/130/15/NHMRC).

Venom peptides. A single specimen of C. tulipa was sacrificed and the venom duct was dissected. The duct was divided into four equal parts: proximal (P), proximal central (PC), distal central (DC) and distal (D) section and dissected venom extracted separately from each section, as described previously. Conantokin-G, conantokin-T, ρ-TIA and its N-terminal truncated analogues were chemically synthesized as described below. All peptides were made up to the required stock concentrations in E3 medium and stored at 4°C prior to use.

Peptide synthesis. Peptides were assembled by SPPS on a Symphony automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) by Fmoc chemistry on 0.1 mmol scale and Fmoc-Rink-amide polymeric resin (Protein Technologies Inc., Tucson, AZ, USA) by Fmoc chemistry on 0.1 mmol scale and Fmoc-Rink-amide polymeric resin (Protein Technologies Inc., Tucson, AZ, USA). Protected Fmoc amino acids and O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU/DIEA) were used. Cleavage from the resin and removal of side chain protecting groups was achieved by treatment with 95% TFA/2.5% TIPS/2.5% H2O for 2 h at room temperature. The crude product was precipitated with cold diethyl ether (Et2O), washed with Et2O, redissolved in 50% acetonitrile/0.1% TFA/250 mM HCl and lyophilised. RP-HPLC solvent A was 0.05% TFA/H2O and solvent B was 0.043% TFA/90% acetonitrile/H2O. Analytical HPLC was performed on a Shimadzu LC20AT system using a Hypersil Gold C18 2.1 × 100 mm column and a gradient of 0–40% B over 40 min at a flow rate of 0.3 mL/min. Mass spectra were acquired in positive ionisation mode on an API 2000 triple quadrupole mass spectrometer (SCIEX, Framingham, MA, USA). Protected Fmoc amino acids and O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) were from Iris Biotech (Markredwitz, Germany), N,N-dimethylformamide (DMF) and N,N-diisopropylethylamine (DIEA) were from Auspep (Melbourne, Australia). All other reagents were obtained from Sigma Aldrich.

Simulation of the nirvana cabal. To evaluate the effect of venom/peptides on the behaviour and motor function of the zebrafish larva, we analysed both the response of the animals to a manual mechanical touch assay and their spontaneous swimming behaviour using the Zebabox Revolution automatic tracking/screening platform (ViewPoint, France). Briefly, 5-dpf larvae and adult fish (500–600 mg) were exposed to dissected venom (0.001–100 ng/μl), ρ-TIA and its analogues (0.001–10 μM), conopressins (0.003–1 μM) and conopressins (0.003–5 μM). The larvae and adults were tested in a 24-multiwell plate and a 0.5 L tank (Tecniplast, USA) respectively. The plates/tank were incubated in the Zebabox Revolution and the animals were automatically tracked with a user-defined programme. Experiments were performed in light conditions and the swimming distance was defined as small (less than 4 cm), medium (4 cm to 8 cm) and large (more than 8 cm). Zebrafish were monitored for a 5 min pre-treatment period and a 10 min post-treatment period, and data binned in 2 s intervals. Raw data and video were exported and processed in silico to export total swim distance for each group. At 15 min, treated fish were subjected to the touch response assay, as described below.

Touch response assay. Larvae escape response was evaluated using a manual mechanical touch using a Drummond PCR micropipet plunger (SDR scientific #5-000-1001-X10). Touch-evoked responses were scored...
1–10 (1, absence of movement; 2, fin vibration without movement; 3, one body coil without movement; 4, several body coils with slight movement; 5–9, gradual increment in swim distance; and 10 normal escape response with larvae reaching edge of the well). For each concentration, six larvae were tested individually, with each test comprising 10 separate manual stimulus scores. Untreated negative control responses (E3 medium only) were recorded in parallel using the same protocol.

**Systemic effects of conotoxins.** Adult fish were administered 0.1 μg of dissected venom from each of the four venom duct sections by intramuscular (i.m.) injections (5–10 μL) using a Hamilton syringe (cemented point style 2, 26 g gauge needle) (Sigma-Aldrich no. 20795-U). Injected animals were placed in 1 L tanks under ambient light conditions, and monitored using the Zebrabox revolution (Viewpoint) imaging platform. Baseline swimming readings were obtained for 15 min before administering 5 μL of the dissected venom and the resulting behavior was recorded for further 15 min. Zebrashipe were analysed for alterations in swimming pattern, and swimming distance relative to baseline quantified for each fish.

**Clone design and site-directed mutagenesis.** The ZF α1BB-AR cDNA (NCBI Reference Sequence: NM_001007358.2) was designed and cloned into pcDNA3.1 (Genescript, USA), whereas the hamster α1BB-AR cDNA was cloned into the pMT2’ vector (kind gift from Professor Bob Graham; Victor Chang Cardiac Research Institute, Sydney, Australia). The ZF α1BB-AR cDNA was subjected to *in vitro* site-directed mutagenesis to generate the double mutant E327D/S330F α1BB-AR cDNA, using specific primer pairs (Sigma Aldrich, Australia) and following the QuikChange mutagenesis kit (Stratagene). TOP10 Escherichia coli (Invitrogen) were transformed with WT or mutant cDNA and subsequently used for plasmid preparation using a PureLink® Quick Plasmid Miniprep Kit (Invitrogen) or High Speed Maxi kit (Qiagen). Purified cDNA was used to confirm all mutations by sequencing by the Australian Genome Research Facility.

**Transient expression of α1-ARs and membrane preparation.** COS-1 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing glutamine and 5% fetal bovine serum. Cells were transiently transfected with purified plasmid DNA encoding WT or mutant α1-ARs using FuGENE HD (Promega) following the manufacturer’s protocol. 48 h post-transfection, cells were harvested and homogenized using a Polytron homogenizer (Brinkmann Instruments) in HEM buffer (20 mM HEPES, 1.5 mM EGTA, 12.5 mM MgCl2, pH 7.4) containing complete protease inhibitor (Roche Diagnostics). The homogenate was centrifuged at 484 × g (2000 rpm) for 10 min and the resulting supernatant was centrifuged at 23,665 × g (14000 rpm) for 30 min. The pellet was resuspended in HEM buffer containing 10% v/v glycerol and stored at −80°C prior to use. Protein concentration was determined using the BCA protein assay kit (Pierce) following the manufacturer’s protocol.

**Radioligand binding assay.** The affinity of p-TIA at the α1-ARs was determined using the radiolabelled α1-AR antagonist [3H]-prazosin (0.5 nM) and FlashBlue GPCR Scintillating Beads (PerkinElmer Life Sciences). Reactions containing radioligand, membranes from α1-AR-transfected COS-1 cells (5 μg protein), FlashBlue GPCR Scintillating Beads (100 μg/well) and increasing concentrations of p-TIA (100 pM–100 μM) in HEM buffer, were established in 96-well white polystyrene plates with clear flat bottoms in triplicate in a total reaction volume of 80 μL. After incubation for 60 min at room temperature with shaking, radioligand binding was detected using a Wallac 1450 MicroBeta scintillation counter (PerkinElmer Life Sciences).

**IP-one HTRF functional assay.** COS-1 cells (ATCC) were cultured and transiently transfected with WT or mutant α1-AR DNA using FuGENE HD (Promega) following the manufacturer’s protocol. Assays measuring IP3-accumulation were performed 48 h post-transfection following the manufacturer’s instructions (IP-one HTRF assay kit, Cisbio international). In brief, the effect of increasing concentrations of norepinephrine (NE) (100 pM–100 μM), or p-TIA (100 pM–100 μM) were determined in the presence of 10 μM NE for WT and mutant ZF α1BB-AR or 1 μM NE for WT hamster α1BB-AR. The assay was performed on 30,000 transfected cells in stimulation buffer in white 384 multiwell plates (Optiplate, PerkinElmer Life Sciences) incubated for 1 h at 37°C with 5% CO2. Cells were then lysed by the addition of HTRF reagents before europium cryptate-labeled anti-IP3 antibody and the d2-labeled IP3 analog were added (IP-one HTRF assay kit, Cisbio International), followed by incubation for 1 h at room temperature. Emissions at 590 nm and 665 nm were recorded after excitation at 340 nm using a microplate reader (Tecan).

**Statistical analysis.** Data were analysed using GraphPad Prism software, v7.0a (San Diego, USA). *In vivo* dose-response data was fitted to non-linear regression curves to establish EC50s. IP-one data was expressed as the HTRF ratio \( R = \left( \frac{\text{fluorescence}_{665\text{nm}}}{\text{fluorescence}_{590\text{nm}}} \right) \times 10^4 \) normalized to the response elicited without ligand (negative control). For both the IP-one and the radioligand binding assay, non-linear regression curves were used to establish EC50 and IC50 values. All IC50 and EC50 values are expressed as the mean ± SEM averaged from six independent experiments, unless stated otherwise.

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Author contributions
R.J.L. conceived the project; M.D., J.G. and R.J.L. designed the experiments; M.D. and J.G. performed the behaviour assays; L.R. and A.A. performed the functional assays; Z.D. and A.B. performed peptide synthesis; M.D., J.G. and L.R. did data analysis; M.D., J.G., L.R., P.F.A. and R.J.L. wrote the manuscript. All authors proofread the final manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to R.J.L.

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