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# Peptides originally derived from *Chilobrachys jingzhao* tarantula venom possess beneficial effects on pancreatic beta cell health and function

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ABSTRACT

Clinical approval of the glucagon-like peptide-1 (GLP-1) mimetic exenatide for the treatment of type 2 diabetes highlights the therapeutic effectiveness of venom-derived peptides. In the present study, we examined and characterised the glucose-lowering potential of synthetic Jingzhaotoxin IX and Jingzhaotoxin XI peptides, which were originally isolated from the venom of the Chinese earth tarantula *Chilobrachys jingzhao*. Following confirmation of lack of beta-cell toxicity of synthetic peptides, assessment of enzymatic stability and effects on *in vitro* beta-cell function were studied, alongside putative mechanisms. Glucose homeostatic and appetite suppressive actions of Jingzhaotoxin IX and Jingzhaotoxin XI alone, or in combination with exenatide, were then assessed in normal overnight fasted C57BL/6 mice. Synthetic Jingzhaotoxin peptides were non-toxic and exhibited a decrease in mass of 6 Da in Krebs-Ringer bicarbonate buffer suggesting inhibitor cysteine knot (ICK)-like formation, but interestingly were liable to plasma enzyme degradation. The Jingzhaotoxin peptides evoked prominent insulin secretion from BRIN BD11 beta-cells, with activity somewhat characteristic of Kv2.1 channel binding. In addition, Jingzhaotoxin peptides enhanced beta-cell proliferation and provided significant protection against cytokine-induced apoptosis. When injected co-jointly with glucose, the Jingzhaotoxin peptides slightly decreased blood-glucose levels but had no effect on appetite in overnight fasted mice. Whilst the Jingzhaotoxin peptides did not enhance exenatide-induced benefits on glucose homeostasis, they augmented exenatide-mediated suppression of appetite. Taken together, these data highlight the therapeutic potential of tarantula venom-derived peptides, such as Jingzhaotoxin IX and Jingzhaotoxin XI either alone or in combination with exenatide, for diabetes and related obesity.

## 1. Introduction

Type 2 diabetes (T2D) is characterised by a decrease in beta-cell mass and function as well as impaired insulin action leading to overt hyperglycaemia (Sakran et al., 2022). This unfortunately means individuals with T2D are at an increased risk of developing complications such as neuropathy, retinopathy, nephropathy and cardiovascular disease (Tomic and Shaw Je Magliano, 2022). Whilst there are many drugs available to help manage T2D, polypharmacy is commonplace due to drug failure (Lafferty et al., 2022). Thus, many patients still fail to achieve glycaemic targets, increasing the risk of diabetic complications (Sun et al., 2021). There is an urgent need to develop new and effective drugs for T2D that can aid in the regulation of glucose homeostasis and reduce complication risk (Mudaliar, 2023).

Peptides isolated from animal venom are recognised as possessing therapeutic potential, linked to enhanced enzymatic stability and high specificity for target channel or receptor (Coulter-Parkhill et al., 2021). This promise has been epitomised through the isolation, characterisation and subsequent clinical approval of exenatide for T2D, a peptide originally isolated from the venom of the Gila monster *Heloderma suspectum* lizard (Eng et al., 1992). Indeed, peptides with potential hypoglycaemic actions have already been discovered within the venom of cone snails, snakes, bees and tarantulas (Herrington et al., 2006; Safavi-Hemami et al., 2015; Moore et al., 2015; Gui et al., 2020). Despite these breakthroughs, the elusive insulin secretory venom-derived molecule, beyond exenatide, that can effectively treat human T2D has yet to be uncovered (Coulter-Parkhill et al., 2021). That said, novel agents to directly improve insulin resistance, as well as or instead of promoting insulin secretion, would also hold unquestionable promise for the treatment of T2D.

We have aimed to characterise the potential of peptides previously isolated from the venom of the Chinese earth tarantula *Chilobrachys*

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*jingzhao*, namely Jingzhaotoxin IX and Jingzhaotoxin XI (Liao et al., 2006; Deng et al., 2009) Jingzhaotoxin IX and Jingzhaotoxin XI have confirmed activity at ion channels known to be important for pancreatic beta-cell function, such as the delayed potassium rectifier channel (Kv2.1) and the voltage-gated sodium channel (NaV) (Liao et al., 2006; Deng et al., 2009). In addition, both peptides are believed to adopt a characteristic inhibitor cysteine knot (ICK) structure as a result of the six cysteine residues present within their amino acid sequences (Table 1). The ICK is considered to provide effective resistance against circulating enzymatic breakdown (Kimura, 2021), which should also increase therapeutic utility. To date, neither peptide has been explored in the context of diabetes.

The current study has confirmed lack of detrimental effects of Jingzhaotoxin IX and Jingzhaotoxin XI at the level of the pancreatic beta-cell, and then explored *in vitro* and *ex vivo* insulinotropic actions as well as their impact on beta-cell proliferation and protection against cytokine-induced apoptosis. Finally, the ability of Jingzhaotoxin peptides to improve glucose tolerance or reduce food intake was examined in mice, both alone and in combination with exenatide. The data reveal, for the first time, that peptides derived from the venom of the *C. jingzhao* tarantula possess beneficial effects on pancreatic beta-cell health and function as well as augmenting exenatide-induced appetite suppressive actions.

## 2. Materials and methods

### 2.1. Peptide synthesis

C-terminally amidated Jingzhaotoxin IX and Jingzhaotoxin XI were commercially synthesised (>95% purity; Synpeptide Ltd., Shanghai, China). The amino acid sequence of Jingzhaotoxin IX is ECTKLLGGCTKDSECCPHLGCRKKWPYHCGWDGTF, and for Jingzhaotoxin XI is ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF (Liao et al., 2006; Deng et al., 2009, Table 1). Peptides were further characterised in-house using reversed-phase high-performance liquid chromatography (RP-HPLC) to confirm purity and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-ToF MS) to corroborate molecular mass, as described previously (Lafferty et al., 2019). To validate the presence of the ICK structural motif, the expected molecular mass of Jingzhaotoxin IX in linear form, 3953.7 Da, was compared to that of the folded peptide 3947.7 Da. An identical process was employed for Jingzhaotoxin XI which has a linear mass of 3726.5 Da and a folded mass of 3720.5 Da (Table 1).

### 2.2. Plasma stability

To determine the enzymatic stability of Jingzhaotoxin IX and Jingzhaotoxin XI, test peptides (1 mg/ml) were incubated at 37 °C with 10 μl of overnight fasted C57BL/6 murine plasma in 380 μl triethanolamine-HCl (50 mmol/l, pH 7.8) for 0, 30 and 60 min. Reactions were terminated by addition of 50 μl of 10% (v/v) trifluoroacetic acid (TFA)/water. Degradation products were separated using rp-HPLC with identification of peaks by MALDI-ToF MS, as described previously (Lafferty et al., 2018). To help identify possible enzymatic cleavage sites for both peptides, the molecular mass of the degradation fragments along with the known amino acid sequences were manually processed using a peptide mass calculator that is freely available at https://www.peptidesynthetics.co.uk/tools.

### 2.3. Beta-cell toxicity

The lactate dehydrogenase (LDH) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were used to assess the cytotoxic effects of Jingzhaotoxin IX and Jingzhaotoxin XI in BRIN BD11 beta-cells. LDH activity was measured using the CytoTox 96® non-radioactive cytotoxicity kit (Promega, UK), using test samples generated from the insulin secretory assay described below. For MTT assay, BRIN BD11 cells (10,000 cells per well) were incubated in 96-well plates with RMPI-1640 media for 24 h in the absence and presence of test peptides ($10^{-8}$–$10^{-6}$ M). After incubation, cells were supplemented with 20 μl MTT solution (5 mg/ml) and incubated for 2 h at 37 °C. Media was then removed, and formazan crystals dissolved using 100 μl DMSO with plate agitation for 10 min. Absorbance was read on a spectrophotometer at excitation and emission wavelengths of 570 nm and 630 nm, respectively.

### 2.4. In vitro insulin secretion and related putative mechanisms

The *in vitro* insulin secretory activity of test peptides was examined in BRIN BD11 cells which were cultured and maintained as previously described (McClenaghan et al., 1996). This cell line was derived and characterised in-house following electrofusion of New England Deaconess Hospital rat islet beta-cells with immortal RINm5F cells (McClenaghan et al., 1996). For experimentation, BRIN BD11 cells were seeded in 24-well plates at a density of 150,000 cells/well and allowed to attach overnight at 37 °C. Following pre-incubation with Krebs–Ringer bicarbonate buffer (KRBB) (pH 7.4) supplemented with 0.5% (w/v) BSA and 1.1 mM glucose (40 min; 37 °C), cells were then incubated with test peptides ($10^{-12}$–$10^{-6}$ M) at 1.1-, 5.6- or 16.7-mM glucose, as appropriate for 20 min. Aliquots of assay buffer (200 μl) were collected and stored at −20 °C prior to the assessment of insulin concentrations by radioimmunoassay (RIA). Furthermore, the effects of Jingzhaotoxin peptides on BRIN BD11 beta-cell membrane potential and intracellular calcium $[Ca^{2+}]_i$ were also assessed using a FLIPR membrane or calcium assay kit (Molecular Devices, USA), as previously described (Musale et al., 2020). Briefly, BRIN BD11 cells ($1 \times 10^5$) were incubated with KRBB containing 5.6 mM glucose and test peptides ($10^{-6}$ M), with membrane potential or calcium mobilisation data collected and analysed using Softmax Pro software.

### 2.5. Beta-cell proliferation and protection against apoptosis

To assess beta-cell proliferative effects, BRIN BD11 cells (40,000 cells per chamber slide) were incubated for 24 h in RPMI media with test peptides ($10^{-8}$ and $10^{-6}$ M). The media was discarded, and cells were washed with phosphate buffer solution (PBS) and fixed using 4% paraformaldehyde. Antigen retrieval was achieved using citrate buffer (90 °C for 20 min) and slides subsequently cooled for 20 min. Cells were then

**Table 1**
Amino acid sequences as well as predicted and experimental molecular masses of linear and folded Jingzhaotoxin IX and Jingzhaotoxin XI peptides.

| Peptide | Amino acid sequence | % purity | Theoretical linear mass (Da) | Experimental linear mass (Da) | Theoretical folded mass (Da) | Experimental average mass (Da) |
|---|---|---|---|---|---|---|
| Jingzhaotoxin IX | ECTKLLGGCTKDSECCPHLG CRKKWPYHCGWDGTF | >95% | 3953.7 | 3953.7 | 3947.7 | 3947.7 |
| Jingzhaotoxin XI | ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF | >95% | 3726.5 | 3726.6 | 3720.5 | 3720.6 |

Peptide sequences are shown using one letter amino acid abbreviations, with cysteine residues that form the characteristic ICK in red text. Percentage purity was determined by rp-HPLC. Jingzhaotoxin IX and Jingzhaotoxin XI adopted a folded structure in KRB buffer at pH 7.4, with linear mass detected in deionised $H_2O$. MALDI-ToF MS was employed to determine peptide masses.

blocked using 4% bovine serum albumin (BSA) for 45 min, before being incubated with Ki-67 primary antibody (1:500; Abcam, ab15580), followed by Alexa Fluor® 488 secondary antibody (1:400, Invitrogen, A-11008). Chamber slides were washed with PBS, mounted for viewing using a fluorescent microscope (Olympus System Microscope) and photographed by a DP70 camera adapter system. Proliferation frequency was expressed as a percentage of total cells analysed. For analysis of the ability of Jingzhaotoxin peptides to protect against cytokine-induced apoptosis, BRIN BD11 cells were seeded as above. However, cells were also exposed to a cytokine cocktail (IL-1β 100 U/mL, IFN-γ 20 U/mL, TNF-α 200 U/ml, all purchased from Sigma-Aldrich) in the presence or absence of test peptides ($10^{-8}$ and $10^{-6}$ M) for 24 h. TUNEL staining (Roche Diagnostics Ltd, UK) was employed to quantify beta-cell apoptosis, as previously described (Lafferty et al., 2019). Apoptosis was expressed as a percentage of total cells analysed. Approximately 150 cells were analysed per group.

### 2.6. Acute *in vivo* experiments

Acute*in vivo* studies were conducted in 12-week-old adult male C57BL/6 mice (Envigo Ltd, UK). Mice were single-housed and kept in a temperature-controlled environment (22 ± 2 °C) under a 12-h light/dark cycle, with *ad libitum* access to drinking water and rodent maintenance diet (10% fat, 30% protein and 60% carbohydrate, per cent of total energy 12.99 kJ/g; Trouw Nutrition, UK). All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 as well as being approved by Ulster University Animal Ethics Review Committee (AWERB) committee (January 20, 2021) and protected by the UK Home Office Animal project license number PPL2902, approved on April 26, 2021. To assess the effects of Jingzhaotoxin peptides on food intake, overnight (16 h) fasted mice (n = 7) were administered an intraperitoneal (i.p.) injection of saline vehicle (0.9% [w/v] NaCl), Jingzhaotoxin IX, Jingzhaotoxin XI or exenatide (each at 25 or 75 nmol/kg bw) and cumulative food consumption recorded at regular intervals over a 180 min observation period. Specifically, mice were provided with pre-weighed rodent maintenance diet in their food hopper at t = 0 min, that was then re-weighed at each timepoint with all due care taken to account for any wastage or crumbling of the food pellets. For assessment of glucose homeostatic and insulin secretory benefits, blood glucose and plasma insulin concentrations were determined immediately prior to and 15, 30 and 60 min after i. p. injection of glucose alone (18 mmol/kg bw) or in combination with test peptides (each at 25 or 75 nmol/kg bw) in 16 h fasted mice. To investigate the potential additive effects of Jingzhaotoxin peptides and exenatide on appetite suppression or glucose homeostasis, mice were administered Jingzhaotoxin peptide (25 nmol/kg bw) in combination with exenatide (2.5 nmol/kg bw) and experiments repeated as described above. Reduced doses of exenatide were employed for these combined peptide administration experiments so as not to preclude additive effects of the peptides, given the prominent efficacy of exenatide in our experimental systems at doses of 25 or 75 nmol/kg (Coulter-Parkhill et al., 2023).

### 2.7. Biochemical analyses

Blood samples were obtained from conscious mice via the cut tip on the tail vein, and blood glucose was immediately measured using an Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, UK). Blood was also collected in chilled heparin/fluoride coated microcentrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged for 15 min at 12,000 rpm using a Beckman microcentrifuge (Beckman Instruments, Galway, Ireland) to separate plasma. Plasma insulin was then measured by RIA (Flatt and Bailey, 1981).

### 2.8. Statistical analyses

Statistical tests were conducted using GraphPad PRISM software (Version 5.0). Values are expressed as mean ± SEM. Comparative analyses between groups were performed using a one-way or two-way ANOVA with Bonferroni's post hoc correction for multiple comparisons or a student's unpaired *t*-test, as appropriate. Differences were deemed significant if $P < 0.05$.

## 3. Results

### 3.1. Peptide characterisation

Jingzhaotoxin IX and Jingzhaotoxin XI were confirmed as having greater than 95% purity and initially characterised in their linear form, with an average molecular mass of 3953.7 Da and 3726.6 Da, respectively (Table 1). Jingzhaotoxin IX and Jingzhaotoxin XI both appeared to fold into their characteristic ICK structure in KRBB at pH 7.4 (Table 1), with a decrease in mass of 6 Da corresponding to disulphide bond formation, although further confirmation of ICK folding is required.

### 3.2. Peptide stability

Interestingly, both Jingzhaotoxin peptides were degraded within 30 min following incubation in overnight fasted murine plasma (Table 2). The predicted but unconfirmed amino acid sequences of the plasma degradation products for Jingzhaotoxin IX and Jingzhaotoxin XI are presented within Table 2.

### 3.3. Effects of Jingzhaotoxin peptides on beta-cell function

Neither Jingzhaotoxin peptide impacted BRIN BD11 cell viability or production of LDH (Fig. 1A and B). However, both peptides ($10^{-12}$ - $10^{-6}$ M) evoked significant ($P < 0.05$-$P < 0.001$) insulin release from BRIN BD11 cells at 1.1-, 5.6- and 16.7-mM glucose concentrations (Fig. 1C–E). In terms of related impact on cell membrane potential and $[Ca^{2+}]_i$ (Fig. 2), the Jingzhaotoxin peptides increased intracellular calcium influx in BRIN BD11 beta-cells, with Jingzhaotoxin IX inducing a significant ($P < 0.05$) elevation when compared to 5.6 mM glucose control (Fig. 2A). Neither Jingzhaotoxin IX nor Jingzhaotoxin XI had any impact on beta-cell membrane potential (Fig. 2B). As expected, exenatide ($10^{-8}$ and $10^{-6}$ M) induced a significant ($P < 0.001$) increase in beta-cell proliferation when compared to media alone control (Fig. 3A). Jingzhaotoxin IX evoked a similar effect on beta-cell growth, but Jingzhaotoxin XI had no significant impact on this parameter (Fig. 3A). All peptides ($10^{-8}$ and $10^{-6}$ M), barring Jingzhaotoxin XI at $10^{-8}$ M, exerted significant ($P < 0.001$) protective effects against cytokine-induced beta-cell apoptosis (Fig. 3B).

### 3.4. Effects of Jingzhaotoxin peptides on food intake and glucose tolerance in mice

When injected to overnight fasted mice at doses of 25 or 75 nmol/kg, exenatide induced significant ($P < 0.001$) appetite suppressive actions at all observation time points during the 180-min experimental period (Fig. 4A and B). In contrast, neither Jingzhaotoxin IX nor XI displayed any significant effects on food intake at either peptide dose (Fig. 4A and B), being appreciably less efficacious ($P < 0.05$-$P < 0.001$) than exenatide (Fig. 4A and B). As anticipated, exenatide improved ($P < 0.05$-$P < 0.001$) glucose tolerance in mice at doses of 25 or 75 nmol/kg that was linked to augmented glucose-induced insulin secretion (Fig. 5A and B). Jingzhaotoxin IX and Jingzhaotoxin XI also improved ($P < 0.05$-$P < 0.01$) glucose disposal at 25 and 75 nmol/kg, but were significantly ($P < 0.001$) less effective than exenatide in this regard (Fig. 5A and B). In terms of related plasma insulin concentrations, neither Jingzhaotoxin

**Table 2**
Jingzhaotoxin IX and Jingzhaotoxin XI predicted but unconfirmed plasma degradation products.

| Peptide | Time fragment appeared | Mass detected (Da) | Potential but unconfirmed sequence of degradation product |
|---|---|---|---|
| Jingzhaotoxin IX | 0 h | 3953.6 | ECTKLLGGCTKDSECCPHLGCRKKWPYHCGWDGTF |
| Jingzhaotoxin IX | 0.5 h | 2285.7 | ECTKLLGGCTKDSECCPHLGCRKKWPYHCGWDGTF |
| Jingzhaotoxin IX | 0.5 h | 1883.3 | ECTKLLGGCTKDSECCPHLGCRKKWPYHCGWDGTF |
| Jingzhaotoxin IX | 1 h | 1941.3 | ECTKLLGGCTKDSECCPHLGCRKKWPYHCGWDGTF |
| Jingzhaotoxin IX | 1 h | 2706.1 | ECTKLLGGCTKDSECCPHLGCRKKWPYHCGWDGTF |
| Jingzhaotoxin XI | 0 h | 3726.3 | ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF |
| Jingzhaotoxin XI | 0.5 h | 2212.6 | ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF |
| Jingzhaotoxin XI | 0.5 h | 1863.8 | ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF |
| Jingzhaotoxin XI | 1 h | 2414.8 | ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF |
| Jingzhaotoxin XI | 1 h | 1687.0 | ECRKMFGGCSVDSDCCAHLGCKPTLKYCAWDGTF |

Enzymatic stability of peptides (40 μg) was determined following incubation in fasted murine plasma (10 μl of C57BL/6). Profiles were followed on rp-HPLC. Molecular masses were acquired on the AB Sciex 4800 instrument in positive ion mode. The predicted amino acid sequence of degradation products are shown in red text and were investigated manually using https://www.peptidesynthetics.co.uk/tools/.

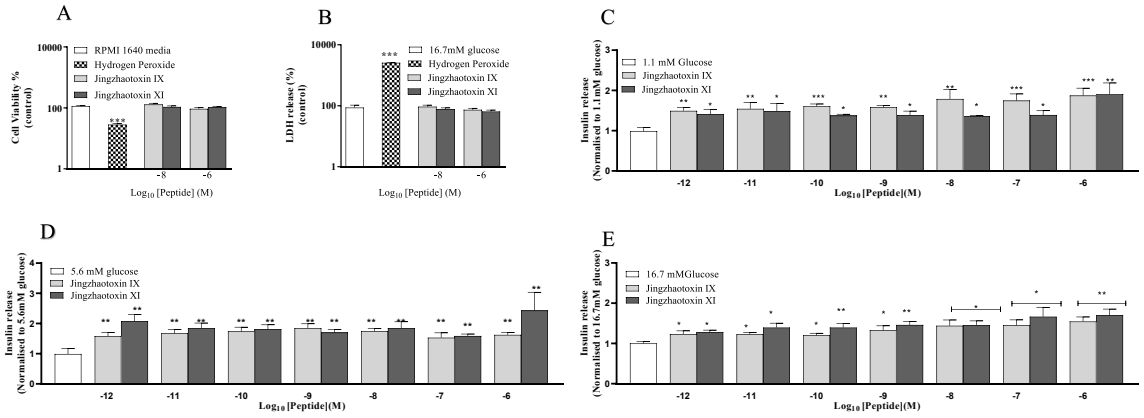


**Fig. 1.** Effects of Jingzhaotoxin IX and Jingzhaotoxin XI on cell viability and insulin secretion from BRIN BD11 cells. (A) BRIN-BD11 cells (n = 8) were exposed to Jingzhaotoxin IX and Jingzhaotoxin XI ($10^{-8}$ and $10^{-6}$ M) for 24 h and cell viability was assessed by MTT assay. (B) BRIN-BD11 cells (n = 3) were incubated with 16.7 mM glucose-supplemented Jingzhaotoxin IX and Jingzhaotoxin XI ($10^{-8}$ and $10^{-6}$ M) for 4 h and LDH cell secretion was determined using LDH assay. (C–E) BRIN BD11 cells (n = 8) were incubated for 20 min with (C) 1.1 (D) 5.6 or (E) 16.7 mM glucose supplemented with Jingzhaotoxin IX or Jingzhaotoxin XI ($10^{-12}$–$10^{-6}$ M) and insulin secretion determined by radioimmunoassay. Values are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to (A) media control or (B–E) respective glucose control.

peptide elevated glucose-induced insulin secretion when compared to glucose alone control (Fig. 5B). When administered in combination with exenatide, the Jingzhaotoxin peptides were unable to augment the substantial glucose-lowering and insulin secretory actions of exenatide (Fig. 6A). However, both Jingzhaotoxin IX and Jingzhaotoxin XI enhanced (P < 0.05-P < 0.001) the ability of exenatide to curb appetite in overnight fasted mice (Fig. 6B).

## 4. Discussion

The stimulation of insulin release is a complex and intricate cell signalling process, with modulation of various beta-cell ion channels intrinsically involved (Thompson and Satin, 2021). Arguably, one of the most characterised and best-understood ion channels of the beta-cell is the $K_{ATP}$ channel (Ashcroft et al., 1984), which was later cloned in 1995 (Aguilar-Bryan et al., 1995). These discoveries ultimately led to a more precise understanding of the mechanism of action of the sulfonylurea class of drugs, that are widely adopted for the treatment of T2D (Shyng, 2022). However, the action of sulfonylurea drugs is not glucose-dependent and therefore can increase the risk of hypoglycaemia (Costello et al., 2022). Furthermore, sulfonylurea efficacy depends on the presence of functioning beta-cells, and unfortunately, the degenerative nature of T2D often results in a decrease in beta-cell mass over time (Sanada et al., 2022). As a result, attention has focused on identifying and utilising other ion channels and target receptors on beta-cells, that can stimulate insulin secretion and impart therapeutic benefits in T2D without these drawbacks (Sanada et al., 2022).

The current study aimed to utilise two known Kv2.1 channel inhibitors Jingzhaotoxin IX and Jingzhaotoxin XI, originally derived from the venom of the Chinese earth tarantula *Chilobrachys jingzhao* and examine potential actions at the level of the pancreatic beta-cell. The proposed mechanism of Kv2.1 channel inhibition relates to delaying of beta-cell repolarisation, thus extending action potential duration and subsequently increasing insulin release (Zhou et al., 2016). This was borne out in our studies with BRIN BD11 cells, where both peptides significantly augmented insulin secretion. Interestingly, akin to the activity of sulfonylureas but not related Kv2.1 channel inhibitors (Ashcroft and Rorsman, 1990), the insulinotropic actions of Jingzhaotoxin IX and Jingzhaotoxin XI were not glucose-dependent. This would suggest a more detailed study of the exact target and mechanism of Jingzhaotoxin IX and Jingzhaotoxin XI is required, which is unfortunately outside the scope of the current study. Despite this, our mechanistic studies revealed that Jingzhaotoxin peptides did not directly induce beta-cell depolarisation but did increase intracellular calcium concentrations. Such a biological action profile is highly characteristic of Kv2.1 channel activity (MacDonald et al., 2002) and not equivalent to that of sulfonylurea drugs that directly depolarise the membrane by inhibiting the $K_{ATP}$ channel (Ashcroft and Rorsman, 1990).

In addition to this, modulation of Kv2.1 channel activity has previously been associated with proliferative actions and protection against apoptosis within beta-cells (Zhou et al., 2016). For example, SP6616 is a small molecular weight compound that is thought to act on the Kv2.1

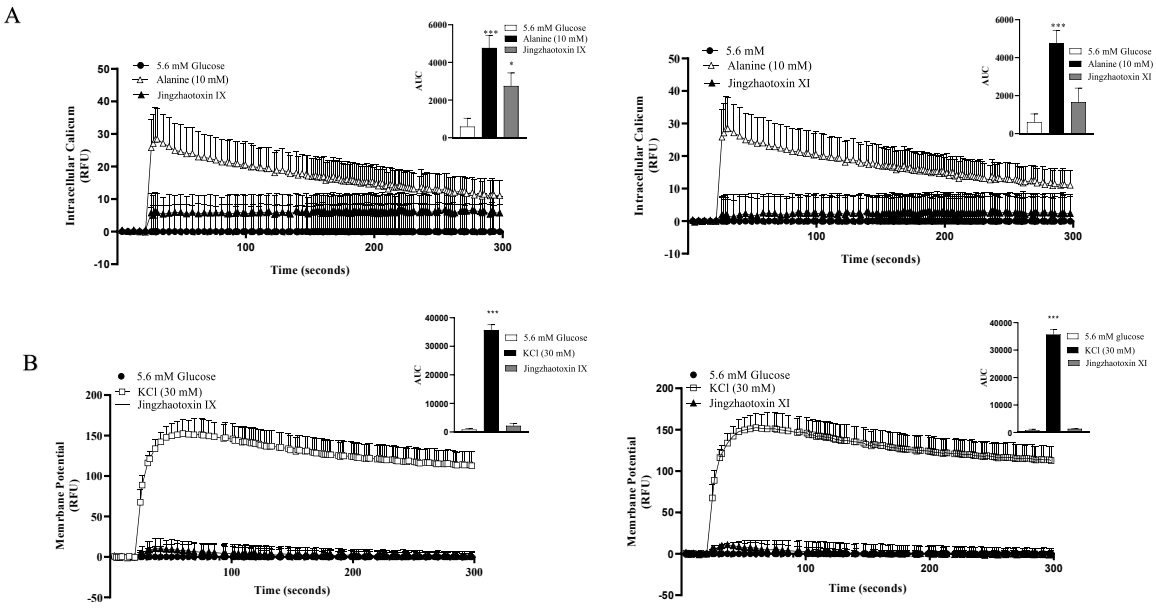


**Fig. 2.** Effects of Jingzhaotoxin IX and Jingzhaotoxin XI on BRIN BD11 cell intracellular calcium and membrane potential. (A,B) BRIN BD11 cells (n = 6) were incubated for 60 min with (A) calcium assay dye or (B) membrane potential dye (FLIPR membrane or calcium assay kit; Molecular Devices, USA) at 37 °C prior to the addition of 5.6 mM glucose supplemented with Jingzhaotoxin IX, Jingzhaotoxin XI ($10^{-6}$ M) as well as positive control (A) alanine (10 mM) or (B) KCl (30 mM). Treatments were added to the wells 20 s after the start of data acquisition at a rate of ~62 μl/s and RFU recorded. Values are mean ± SEM (n = 6). *P < 0.05 and ***P < 0.001 compared to respective glucose control.

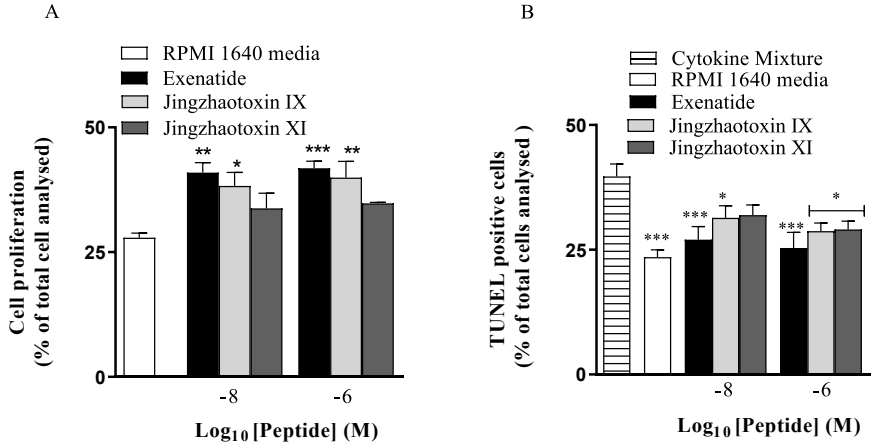


**Fig. 3.** Effects of Jingzhaotoxin IX and Jingzhaotoxin XI on BRIN BD11 beta-cell proliferation and protection against cytokine-induced apoptosis. (A) BRIN-BD11 cells (n = 3) were cultured (24 h) with test peptides ($10^{-8}$ and $10^{-6}$ M) and proliferation detected using Ki-76 staining. (B) BRIN-BD11 cells (n = 3) were cultured (24 h) with test peptides ($10^{-8}$ and $10^{-6}$ M) in the presence of a cytokine cocktail and apoptosis detected using the TUNEL assay. All values are mean ± SEM (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to (A) RPMI 1640 media alone or (B) cytokine cocktail.

channel and was observed to protect against beta-cell apoptosis in high fat-fed and genetically obese *db/db* diabetic mice, possibly through inhibition of caspase activity (Zhou et al., 2016). Moreover, inhibition of Kv2.1 channel activity has also been implicated in neuronal cell survival (Sun et al., 2022). As such, the ability of Jingzhaotoxin IX and Jingzhaotoxin XI to induce BRIN BD11 cell proliferation and protect against cytokine-induced apoptosis was not overly unexpected. Taken together, a drug target that can both augment insulin secretion whilst concomitantly exerting beta-cell protective actions would represent an ideal treatment paradigm for T2D, recognised as a disease where circulating insulin concentration are reduced alongside a loss of beta-cell mass and function (Weir et al., 2020).

Unfortunately, the highly relevant bioactive profile of both Jingzhaotoxin peptides *in vitro* was not fully replicated *in vivo*. Whilst both peptides did moderately improve glucose handling in mice, this effect was substantially less than observed with exenatide and intriguingly not associated with an obvious enhancement of glucose-induced insulin secretion. It would also have been interesting to assess the impact of the Jingzhaotoxin peptides on insulin sensitivity, but unfortunately we do not possess the expertise to perform hyperinsulinaemic-euglycaemic clamp studies. Moreover, investigating bioactivity of Jingzhaotoxin IX and Jingzhaotoxin XI in established rodent models of diabetes (King and Bowe, 2016) represents another relevant paradigm of potential study. Given the recent upsurge of interest in dual and triple-acting peptides for T2D treatment (Lafferty et al., 2023), we also investigated additive glucose homeostatic effects alongside exenatide. Although Jingzhaotoxin peptides and exenatide are likely to have complementary mechanisms of action at the level of the beta-cell (Sukma Rita et al., 2015), no discernible additive benefits were observed. The lack of translation between cell and animal models could well be due to the relatively rapid plasma degradation of both Jingzhaotoxin IX and Jingzhaotoxin XI. Thus, despite the peptides possessing a characteristic ICK structure in KRB buffer, that is believed to enhance enzymatic stability (Kikuchi et al., 2015), plasma degradation was noted within 30 min in our *ex vivo*

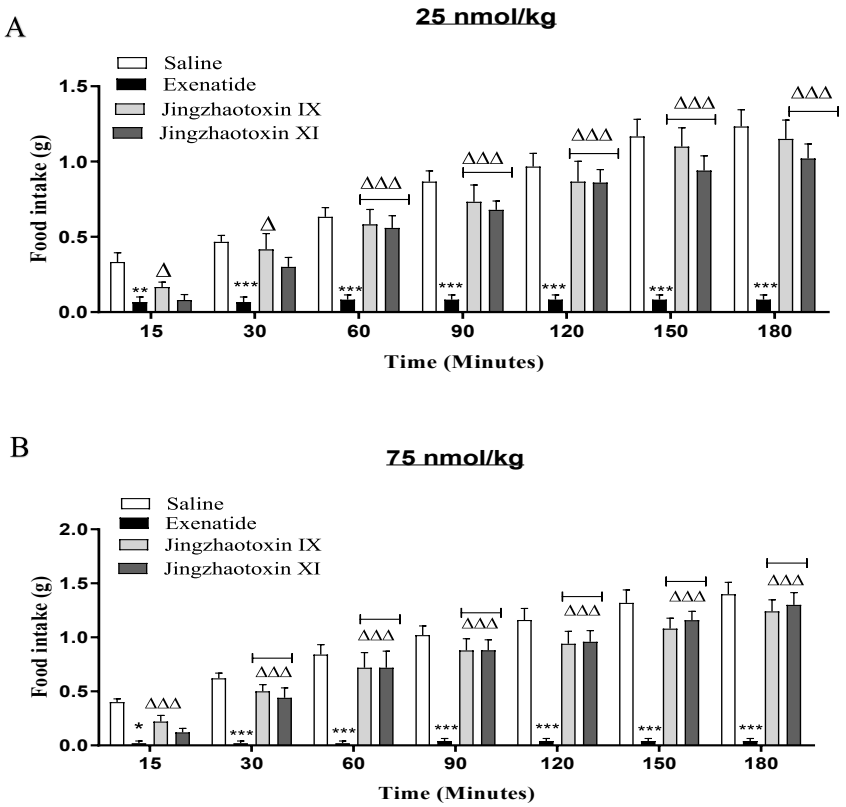


**Fig. 4.** Effects of Jingzhaotoxin IX and Jingzhaotoxin XI on food intake in mice. (A,B) Overnight (16 h) fasted C57BL/6 mice were administered Jingzhaotoxin IX, Jingzhaotoxin XI or exenatide at (A) 25 nmol/kg or (B) 75 nmol/kg in saline vehicle (0.9% (w/v) NaCl). Food intake was monitored over 180 min. Values represent mean ± SEM (n = 7). *P < 0.05, **P < 0.01, ***P < 0.001 compared to saline control. $^{\Delta}$P < 0.05 and $^{\Delta\Delta\Delta}$P < 0.001 compared to exenatide.

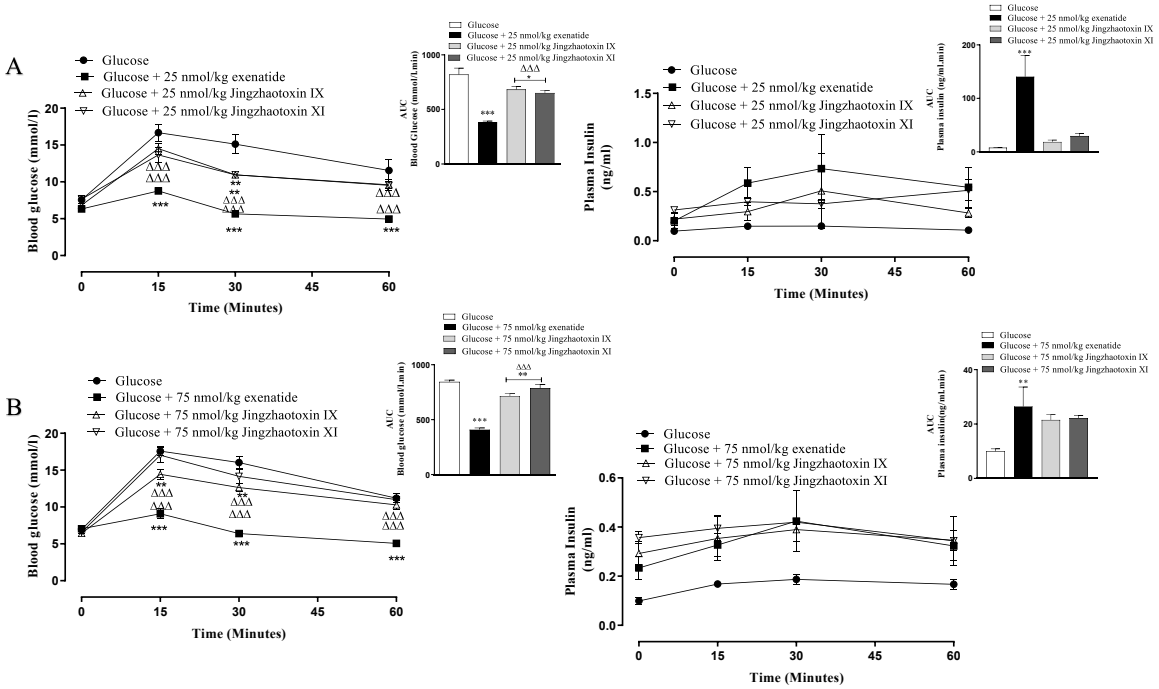


**Fig. 5.** Effects of Jingzhaotoxin IX and Jingzhaotoxin XI on glucose tolerance and insulin secretion in mice. (A,B) Blood glucose and plasma insulin concentrations were assessed following administration of glucose alone (18 mmol/kg) or in combination with Jingzhaotoxin IX, Jingzhaotoxin XI or exenatide at (A) 25 nmol/kg or (B) 75 nmol/kg in overnight (16 h) fasted C57BL/6 mice. Associated 0–60 min AUC data are also displayed. Values are mean ± SEM (n = 7). *P < 0.05, **P < 0.01 and ***P < 0.001 compared to glucose alone control. $^{\Delta\Delta\Delta}$P < 0.001 compared to exenatide.

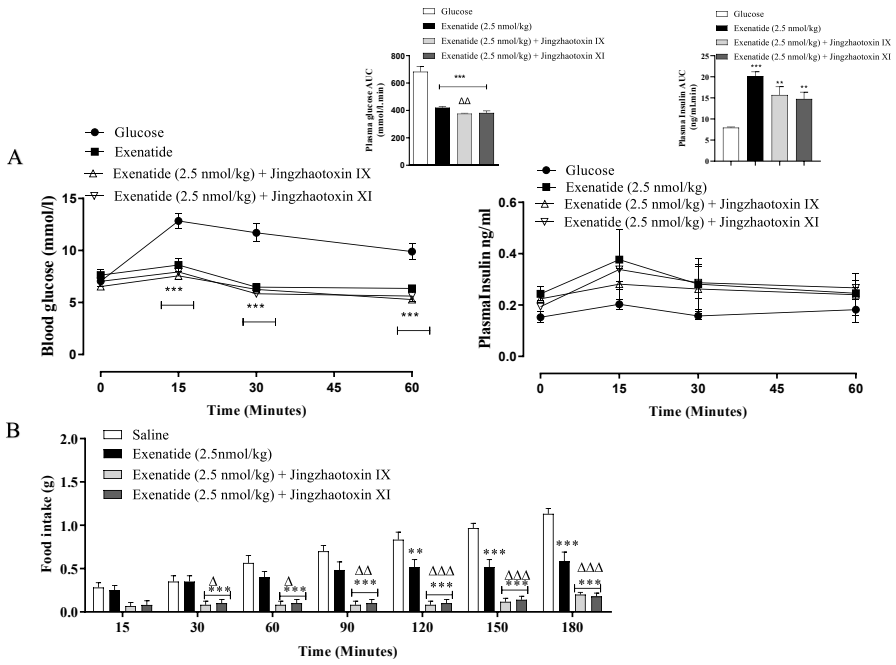


**Fig. 6.** Effects of Jingzhaotoxin IX and Jingzhaotoxin XI in combination with exenatide on food intake and glucose tolerance in mice. (A) Blood glucose and plasma insulin concentrations were assessed following administration of glucose alone (18 mmol/kg), glucose together with exenatide alone (2.5 nmol/kg) and in combination with Jingzhaotoxin IX or Jingzhaotoxin (both at 25 nmol/kg) in overnight (16 h) fasted C57BL/6 mice. (B) Overnight (16 h) fasted C57BL/6 mice were administered exenatide (2.5 nmol/kg) alone and in combination with Jingzhaotoxin IX or Jingzhaotoxin XI (both at 25 nmol/kg) using saline vehicle (0.9% (w/v) NaCl). Food intake was monitored over 180 min. Values represent mean ± SEM (n = 7). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to (A,B) glucose or (C) saline control, as appropriate. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ and $^{\Delta\Delta\Delta}P < 0.001$ compared to exenatide.

system. In this respect, it has been previously demonstrated that tarantula venom-derived peptides may not fold correctly within the *in vivo* environment (McCarthy et al., 2020), and chemical induction of the correct peptide tertiary structure may be required. Whether this is the case for Jingzhaotoxin IX and Jingzhaotoxin XI remains to be ascertained, but it represents one plausible explanation for the significant plasma-mediated degradation of the peptides. Notwithstanding this, it is important to note that the acute *in vivo* peptide treatment regimens employed for the current study have been utilised previously for other enzymatically liable peptides such as glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), Peptide YY (PYY) or cholecystokinin (CCK), with effects on glucose homeostasis and/or appetite clearly observable (Green et al., 2003; Irwin et al., 2005, 2012; Lafferty et al., 2018), confirming appropriateness of this experimental system for the Jingzhaotoxin peptides.

We were unable to formally identify degradation fragments of Jingzhaotoxin IX and Jingzhaotoxin XI, but unconfirmed predictions of enzymatic cleavage sites based on molecular masses of the fragments was deliberated (Table 2), with no obvious enzyme candidates recognised. Despite this, and the fact that the Jingzhaotoxin peptides had no direct independent effect on food intake, both peptides augmented the appetite-suppressive actions of exenatide. Thus, Jingzhaotoxin peptide degradation products may be biologically active or pass through the blood-brain barrier more easily, although further detailed studies would be needed to confirm this. That said, some regulatory peptide hormones, such as Peptide YY, appear to possess their most important biological function following enzymatic cleavage (Lafferty et al., 2021), which could also be the case for Jingzhaotoxin peptides, although confirmation of this would require further detailed study.

Whilst tarantula-derived peptides have great therapeutic potential, a unique structure can also bring about limitations in their application (McCarthy et al., 2020). Nonetheless, we have highlighted an exciting biological action profile of Jingzhaotoxin IX and Jingzhaotoxin XI in BRIN BD11 beta-cells. Although this was not entirely manifested in the *in vivo* setting, issues with peptide stability and/or tertiary structure are likely explanations. Further work to prolong the pharmacokinetic profile of Jingzhaotoxin peptides could help fully expose the clear potential of these peptides and Kv2.1 channel modulation for the treatment of diabetes.

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## Ethics statement

All animal studies were conducted in compliance with the UK Animals (Scientific Procedures) Act 1986 and the EU Directive 2010/63EU. Specifically, animal experiments were authorised by the local Ulster Animal Welfare and Ethical Review Body (AWERB) committee (January 20, 2021) as well as being protected by the UK Home Office Animal project license number PPL2902, approved on April 26, 2021.

## CRediT authorship contribution statement

**A. Coulter-Parkhill:** Methodology, Validation, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **V.A. Gault:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **S. McClean:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **N. Irwin:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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