Distal extracellular teneurin region (teneurin C-terminal associated peptide; TCAP) possesses independent intracellular calcium regulating actions, in vitro: A potential antagonist of corticotropin-releasing factor (CRF)

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Teneurin C-terminal associated peptides (TCAP) are natural bioactive peptides that possess anxiety-reducing roles in animals, in vivo, and increase cell viability, in vitro. Although these peptides have some primary structural similarity to corticotropin-releasing factor (CRF), they are derived from the distal extracellular region of the teneurin transmembrane protein where they may act as separate soluble peptides after auto-catalytic cleavage from the teneurin protein following interaction with the cognate teneurin receptor, latrophilin (ADGRL), or expressed as a separate mRNA. However, although the signal transduction mechanism of TCAP in neurons has not been established, previous studies indicate an association with the intracellular calcium flux. Therefore, in this study, we have characterized the TCAP-mediated calcium response in hypothalamic cell lines using single-cell calcium methods with pharmacological antagonists to identify potential calcium channels, in vitro. Under normal circumstances, TCAP-1 reduces cytosolic calcium concentrations by uptake into the mitochondria and efflux through the plasma membrane independently of the teneurins. In doing so, TCAP-1 could inhibit the potential ‘stress’-inducing actions of CRF.

1. Introduction

The regulation of the stress response and metabolism is critical to the survival of all multicellular animals. Over the last couple of decades, the teneurin C-terminal associated peptides (TCAP) have emerged as a new peptide family associated with the stress response. The TCAPs [1–3] are present in all four of the vertebrate teneurin paralogues, are 40–41 residues in length and have primary sequence similarity to the secretin, calcitonin and corticotropin-releasing factor (CRF) families of peptides [3–5]. They are named accordingly (1–4) for each teneurin they are associated with. TCAP is derived from the distal extracellular tip of the teneurin transmembrane proteins [3,6,7] which may be liberated auto-catalytically [8,9] or by an independent mRNA [10]. Synthetic TCAP is highly efficacious, in vivo, in molluscs [11], protochordates [12], fish [13] and mammals [4,14–16] to regulate aspects of the stress response.

The physiological actions of TCAP are transduced, in part, by the latrophilins (LPHN) [17,18]. LPHNs are members of the adhesion G-protein coupled receptor (GPCR) family (ADGRL [19]); but possess a phylogenetically conserved hormone-binding domain (HBD) related to that of the CRF and Secretin family receptors [17,18,20–22], and are also structurally related to the Secretin family of GPCRs [19,23]. LPHNs are involved in cell-cell and cell-matrix adhesion [19] in a variety to tissue types. LPHN activation stimulates G-protein signalling and modulation of multiple intracellular signalling pathways via their interaction with the teneurins [24–27].

Alpha(α)-latrotoxin (αLTX), is a peptide component of the black widow venom that also possesses primary structure homology to the Secretin family of peptides [20]. By itself, αLTX, is associated with neurotransmitter release by a calcium (Ca²⁺)-dependent mechanism.

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[21,28–30], however, it is not clear how this mechanism occurs [31], although it has been also implicated in mitochondrial (MT) function [32]. LPHN-1, is one of the endogenous receptors of αLTX [33,34]. Previous studies with the TCAP region of teneurins indicate that it interacts with the HBD of the LPHNs [17,18]. Because TCAP also possesses structural similarity to the αLTX and Secretin families of peptides [5], it likely also plays a role in intracellular Ca\(^{2+}\) regulation. Moreover, TCAP has been also implicated in MT activity [14,35].

A number of studies indicate that TCAP regulates the energetic demands of stress physiology and behaviour. Synthetic TCAP-1 decreases CRF-associated stress behaviours in a variety of studies including acoustic startle response [2], elevated plus-maze and open-field test [36,37] and cocaine reinstatement models [38–40]. Moreover, TCAP-1 inhibits the CRF-mediated eicos expression in rat brain limbic regions [41]. Recent studies indicate that TCAP-1 regulates MT activity in fish [13] and rodents [14] and stress activity in oysters [11]. Together, these studies indicate that a fundamental mechanism of TCAP-1 is to regulate energy metabolism. We postulate that this may be due, in part, by regulation of intracellular calcium flux and potential antagonism with CRF afferent activity [35].

In vertebrates, there are two CRF receptor subtypes: the CRF receptor 1 (CRF1) and CRF receptor 2 (CRF2). These receptors belong to the Secretin (previously, class B) Family of GPCRs [23,42], but differ in their expression pattern and by their ligand selectivity [42–44]. Both receptor pathways elevate intracellular Ca\(^{2+}\) and contribute to Ca\(^{2+}\)-mediated vesicle release. Taken together, these studies indicate that TCAP-1 may interact with these receptors either through binding or regulating the Ca\(^{2+}\)-associated CRF-mediated activation in hypothalamic neurons that express both the CRF1 and LPHN-1 receptors. Our objectives for this study were, therefore, to assess: how TCAP-1 regulates intracellular Ca\(^{2+}\) metabolism; how TCAP-1 can antagonize the actions of CRF in neurons and; if TCAP-1 plays a role in CRF-associated energy metabolism using this route. In this study, we show that although TCAP does not act directly with the CRF receptors, it may antagonize the down-stream (signal transduction) CRF-mediated Ca\(^{2+}\) response, and in doing so, regulates MT (aerobic)-based energy metabolism in cells. This study also provides strong evidence that the intracellular actions of TCAP-1 are independent of the teneurins.

2. Materials and methods

2.1. Peptide synthesis and preparation

The sequence of the mouse TCAP-1 was determined by examining the carboxy-terminal exon region of mouse teneurin-1 (accession number: NM011855). TCAP-1 was synthesized at 95% purity on an automatic solid-phase peptide synthesizer (model Novasynt CTP, Cambridge, MA, USA). The peptide was synthesized on a Rink-amide resin (chloroform) that was desalted on a Sephadex G-10 column (Pharmacia, Uppsala, Sweden). The final peptide was purified with triethylamine, diisopropylamine, triisopropylsilane and bromoacetic acid (Calbiochem-Novabiochem Group, San Diego, CA, USA). Eight times excess diisopropyl ethyl amine (Sigma-Aldrich, Oakville, ON, Canada) and 4x excess Fmoc-amino acid activated with HATU (O-[7-azabenzo- triazol]-1-3, 3-tetramethyluronium hexafluorophosphate; Applied Biosystems, Foster City, CA, USA) at a 1:1 (mol/mol) ratio were used during the coupling reaction. The reaction time was 1 h. A solution of 20% piperidine (Sigma-Aldrich) in N,N-dimethylformide (DMF; Caledon Laboratories, Georgetown, ON, Canada) was used for the deprotection step in the synthesis cycle. The DMF was purified in-house and used fresh each time as a solvent for the synthesis. The cleavage/deprotection of the final peptide was carried out with trifluoroacetic acid (TFA), thioanisole, 1,2 ethanethiol, m-cresol, triisopropylsilane and bromotrimethyl silane (SigmaAldrich) at a ratio of 40:10:5:1:1:5. Finally, it was desalted on a Sephadex G-10 column (Pharmacia, Uppsala, Sweden) using aqueous 0.1% TFA solution and lyophilized. For use as a negative control, a scrambled TCAP-1 analogue, scrambled (sc) TCAP-1, was synthesized using the same process as described above. Both peptides were synthesized by the American Peptide Company (Thermo Fisher Scientific, Sunnyvale, CA, USA). The Sc-TCAP-1 peptide contained the same amino acids as rat/mouse TCAP-1 (sequence: pEQLGTLGVRQ- GYDGYFVLSVEQYLELSDANNHFRMQESEI-NH\(_2\)) except the amino acids were randomized into a different sequence: pETSSLRLVSLI-GEVQQFQIGYENQSDQNYGLAYFDRVGS-MH\(_2\). TCAP-1 and scTCAP-1 were dissolved at 1 mg/\(\mu\)l in water with an ammonium hydroxide vapor puff. The scTCAP-1 was added to the vehicle unless otherwise stated.

2.2. Cell lines

Immortalized mouse embryonic hypothalamic cells (mHypoE-N38 cells; N38) [45] were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco). Cells were cultured in 100 x 15 mm petri dishes and maintained at 60%–70% confluency in a humidified CO\(_2\) incubator at 5% CO\(_2\) and 37 °C. For fluorescence experiments, cells were grown in 6-well plates on poly-\(\lambda\)-lysine (50 μg/ml) coated 25 mm round No. 1 glass coverslips (Warner Instruments, Hamden, CT, USA) and cultured for 2–4 days prior to experimentation. Cells were serum-starved for 3 h (h) before the start of experimental treatments. The HEK-293T cell line was used for transfection of the CRF receptors and cultured in a similar manner as the N38 cells.

2.3. siRNA-mediated knockdowns of teneurin and TCAP

SiRNA sequences for TCAP (terminal exon) and the negative controls were synthesized by Genosys Inc., annealed, phosphorylated and cloned into pLKO.1 puro (Sigma) (Fig. 1). SiRNA 270 was designed to recognize TCAP 2,3,4 and siRNA 721 to TCAP 3. Both siRNAs were co-transfected with a puromycin resistant gene. The lentiviruses were packaged in the HEK-293T cells using Mission Lentiviral packaging mix plasmids (Sigma). 48 h later, filtered supernatants with viral particles were transferred to N38 cells and incubated with 8 μg/ml hexadimethrine bromide (Sigma) for 16 h. The effect of TCAP-1 siRNA 270 and 721 on cell morphology was investigated and compared to a non-transfected N38 cells. All 3 cell lines were grown in 6-well culture plates with 2 mL of DMEM containing high glucose, 1-glutamate, 25 mM HEPES buffer, pyridoxine hydrochloride in the absence of sodium pyruvate and 5 μl penicillin with 10% FBS at pH 7.4 (Gibco-Invitrogen, Burlington, Canada). The cells were washed and incubated in the media for 24 h before puromycin selection at 2 μg/ml (Sigma). The selection was monitored using the uninfected control cells. For higher stringency, another subset of the cells was subsequently treated with puromycinin (4 mg/ml) 28hrs post-incubation at 80% confluency. In passage 3, the antibiotic concentration was increased to 10 mg/ml and after 72 h, the cells were harvested at 80–90% confluency. The cells remained in culture, from P0 to harvest in P3 was 13d for control cells luciferase, 17d for 721 and 28d for 270. The cells were harvested and total RNA was isolated using the RNeasy Mini Kit (74124, Qiagen) followed by mRNA purification with Oligotex kit (70022, Qiagen). The quality and quantity of samples were evaluated by spectrophotometer at 260 and 280 nm. The mRNA was submitted to DNase treatment (Turbo DNA-free Kit, Ambion) in order to remove any persistent DNA contamination. Sets of primers were designed for TCAPs, teneurins and histone H3 (Table 1). About 60–80 ng of mRNA from cell cultures were submitted to one-step RT-PCR (OneStep RT-PCR kit, Qiagen) in a 50-μl final volume reaction, containing 400 μM of each dNTP, 10 μl of 5 × QiAGEN OneStep RT-PCR buffer, 10 μl of 5x Q-solution, 2 μl of QiAGEN OneStep RT-PCR enzyme mix and 0.2 μM each primer to TCAPs and teneurins or 0.1 μM to histones. The thermal cycler conditions were 30 m at 94 °C for reverse transcription, then 15 m at 95°C for initial denaturation followed by 20–33 cycles of: denaturation (1 m at 94 °C), annealing (1 m at 59 °C) and extension (1 m at 72 °C), followed with a final extension period of 10 m at 72 °C. The RT-PCR products were examined by 1.5% agarose gel electrophoresis using a UVP System 8000 digital imaging system with
the Laboratory Imaging and Analysis System software (Ver. 4) (UVP, Upland, CA, USA).

2.4. CRF receptor activity by TCAP

The mouse immortalized neurons, N38, which endogenously express the CRF1 and CRF2 receptors [45], were used to determine TCAP actions on these receptors. The pGL3 luciferase (Luc) expression vector (Promega Corp.) containing either the promoter sequence was used as a reporter gene containing the cAMP-responsive element (CRE) sequence (shown in bold), CAAATGGACCACTACACTTTTCATCAATTCAAGAGATTGATGAAGTTGATGTGTCCTTTTTG-3’

Fig. 1. siRNA oligonucleotides used to knockdown teneurin and TCAP expression. A. The 270 oligonucleotide is designed to knockdown mouse TCAPs 1–4 whereas the 721 oligonucleotide was designed to be more specific to mouse TCAP-3. C. Negative control oligonucleotides.

Table 1

| Table 1 | RT-PCR primers to amplify TCAPs and Teneurins. |
|---------|------------------------------------------------|
| Gene product | Forward primer | Reverse Primer |
| TCAP-1 | 5’-TTCATTTTCCGAGCTTTTTTCAGCTCTATG-3’ | 5’-AAACGCTGCTTTTCTCTGCTGCA-3’ |
| Teneurin 1 | 5’-GTTGTCACCTTACTAGATCAGCGAGAGCCACCCGATCAAGCTTCATCAGGTGGCTCCCGCTGAAATTGGAATCC-3’ |
| TCAP-2 | 5’-GGCCGCCGGACACACACTAATATTCATCAATTCAAGAGATTGATGAAGTTGATGTGTCCTTTTG-3’ |
| Teneurin 2 | 5’-AATTCAAAAAGGATTCCAACTCAGCGAGAGCCACCCGATCAAGCTTCATCAGGTGGCTCCCGCTGAAATTGGAATCC-3’ |
| TCAP-3 | 5’-ATCTTGACACCTACTTTCTGAGACAGGACGCTGAC-3’ |
| Teneurin 3 | 5’-ATCGAGGTTCTGCTGAGCAAGGACGCTGAC-3’ |
| TCAP-4 | 5’-TTCATTTTCCGAGCTTTTTTCAGCTCTATG-3’ |
| Teneurin 4 | 5’-GGCCGCCGGACACACACTAATATTCATCAATTCAAGAGATTGATGAAGTTGATGTGTCCTTTTG-3’ |
| Histone H3 | 5’-GCAAGAAGTGGGCGCCCTTACTG-3’ |

2.5. Intracellular calcium studies

Changes in intracellular Ca^{2+} were assessed using the membrane-permeable Ca^{2+}-sensitive fluorescent indicator Fluorophore-4 (Fluo-4) (F-14201; Invitrogen, Burlington, ON, Canada) (Table 2). Cells were loaded with Fluo-4 by incubating coverslips in DMEM containing 4 μM Fluo-4 (from a 1 mM stock solution in DMSO) for 30 min (37 °C) followed by a 15 min wash in physiological saline containing: 135 mM NaCl, 4.5 mM KCl, 1 mM MgCl₂, 1.2 mM CaCl₂, 10 mM HEPES, 10 mM glucose and 2 mM CaCl₂ (pH 7.4) with an osmolality of 300 mOsmol at room temperature (RT). Coverslips with cells were placed in a flow-through bath chamber (RC-40HP, Warner Instruments, Hamden, CT, USA) of an inverted microscope (Axio Observer Z1, Zeiss, Toronto, ON, Canada) equipped with
a 40x oil immersion objective. Cells were continuously perfused with saline via a gravity drip perfusion system at a rate of 2–3 ml/min at RT. Changes in Fluo-4 fluorescence were imaged using a green fluorescent protein (GFP) filter set (Semrock, Rochester, NY, USA) and an X-Cite 120 Rhodamine-123; RuR. Rhodamine red; SERCA, sarco-endoplasmic reticulum mitochondria; PM, plasma membrane; Rhod-2, Rhodamine-2; Rhod-123, calcium ATPase; TGX, thapsigargin; VDT, vanadate.

### 2.6. Mitochondrial studies

#### 2.6.1. Mitochondrial calcium measurements

Changes in mitochondrial (MT) Ca\(^{2+}\) levels were assessed using the fluorescent indicator Rhodamine-2-AM (Rhod-2) (Table 2). N38 cells were loaded with Rhod-2 by incubating coverslips in DMEM containing 4 μM Rhod-2 (from a 1 mM stock solution in DMSO with 20% pluronic; Invitrogen - Pluronic™ F-127) for 30 min at 22 °C. It was subsequently washed in fresh dye-free physiological saline for 30 min at RT. Rhod-2 was excited with a wavelength of 552 nm for 100 ms every 30 s and fluorescence emission was measured at wavelength of 577 nm. Changes in MT Ca\(^{2+}\) were measured by assessing fluorescent change in a region of interest (ROI) taken from the cell body (not the nucleus). The average change in Rhod-2 fluorescence of 5 neurons per coverslip was used as a single replicate with 6 coverslips examined.

#### 2.6.2. Mitochondrial membrane potential measurements

Changes in MT membrane potential were assessed using the fluorescent indicator Rhodamine-123 (Rhod-123) (Table 2), used in quench mode. N38 cells were loaded with Rhod-123 by incubating coverslips in DMEM containing 5 μM Rhod-123 (from a 1 mM stock solution in DMSO) for 30 min at 37 °C followed by a 15 min wash in physiological saline at RT. Rhod-123 was excited with a wavelength of 480 nm for 100 ms every 30 s and fluorescence emission was measured at wavelength of 516 nm. MT membrane potential was measured by assessing the fluorescent change in the region of interest (ROI). The average change in Rhod-123 fluorescence of 5 neurons per coverslip was used as a single replicate where 6 coverslips were examined.

#### 2.7. Intracellular ATP assay

Live-cell ATP turnover was determined using a commercial ATP ELISA assay (Promega, Wisconsin, USA) following the manufacturer’s instructions. Briefly, N38 cells were seeded at 10,000/well in a 96-well plate. The next day, cells were either treated with vehicle or TCAP-1 (100 nM) and lysed at 0, 15, 30 and 60 min after treatment. Ultra-Glo™ recombinant luciferase was added to the media to determine ATP levels. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of magnesium, ATP and molecular oxygen. Thus, increases in luciferin fluorescence directly correlates to an increase in ATP levels. Fluorescence from blank wells was subtracted from all samples to account for any background noise. As the fluorescence signal naturally decays over the course of the experiment, TCAP-1-treated cells were compared relatively to the vehicle-treated cells for each time point (n = 8).

#### 2.8. Resazurin NADH assay

Cytosolic NADH concentration turnover in N38 cells was assessed using a resazurin assay. N38 cells were seeded at 10,000/well in 96-well plates. The resazurin assay began the following day by adding the resazurin solution (525 nM, Sigma) to all the wells. Cells were treated with either vehicle or TCAP-1 (100 nM). Fluorescent readings were measured every 5 min over 1 h, with excitation at 530 nm and emission read at 590 nm. Measurements of blank wells that contained no cells were subtracted from all readings.

#### 2.9. Statistical analyses

All summary data on graphs are represented as mean ± SEM unless otherwise indicated. All data were typically analyzed by a one-way
ANOVA followed by a Tukey’s post-hoc test, as described within each figure caption unless otherwise indicated. Mean values were obtained from a minimum of n = 4–6 independent experiments and data were considered statistically significant with an a priori hypothesis of P < 0.05 (***P < 0.001, **P < 0.01 or *P < 0.05).

3. Results

3.1. siRNA knockdown of teneurin and TCAP

Our initial experiments, using the N38 hypothalamic cell model, were designed to establish that the TCAPs were essential for normal cellular growth and viability. The siRNA oligonucleotides that were designed to attenuate TCAP gene expression and cell viability were assessed (Figs. 1 and 2). Both siRNA vectors (p270 and p721) downregulated TCAP and teneurin expression in N38 cells (Fig. 2A–C). The TCAP-3 and teneurin-3 messages were reduced to about 20% of the control values whereas the p270 vector was effective at reducing TCAP-4/teneurin-4 mRNA levels to about 50% of the control values. The p721 vector showed a similar effect at reducing TCAP-4 levels but only reduced teneurin-4 levels to 70% of the control value. Reduction of the TCAP-2 and teneurin-2 mRNA messages were the least effective with p270 reducing the TCAP-2 and teneurin-2 messages to about 75–90% of the control values, respectively. The p721 vector had no significant effect on the teneurin-2 mRNA but reduced the TCAP-2 level to about 80% of the control value. Although N38 cells do not express either TCAP-1 or teneurin-1 in significant amounts, we have previously shown that TCAP-1 can be used as a structural proxy for the other 3 TCAPs given the high level of sequence conservation among them.

3.2. Actions of TCAP on CRF receptors

Having established that the teneurins and TCAPs were required for cell viability, we examined whether TCAP-1 could directly modulate CRF receptor action. Although, we and others, have established previously that TCAP’s actions are likely due to its binding and activation of the LPHNS [17,18], we have not examined whether TCAP can have a direct effect on CRF receptors (Fig. 3). To assess this, we employed a CRF receptor cAMP-responsive element (CRE)-containing promoter luciferase reporter assay in which activation of the CRF receptor leads to activation of the CRE with an increase in luminescence. CRF treatment at 10⁻⁹ M significantly increased the activation of the cAMP response element above basal conditions via CRF receptor 1 (R1) (Fig. 3A) and CRF receptor 2 (R2) (Fig. 3B). The results were analyzed using a one-way analysis of variance (p < 0.0001). The results confirm the validity of the assay and that CRF activates the CRE via the CRF receptor. However, TCAP-1 alone, had no effect on CRE activation via CRF R1 or R2 compared to basal conditions and, moreover, when TCAP-1 and CRF were added together, did modify the CRF-induced CRE response. Similarly, TCAP-1 did not reduce the Ca²⁺ response with respect to the FLIPR analyses when administered to the CRF receptors. TCAP-1 showed no agonism of the CRF1 receptor with a value of –0.6 ± 0.1% and 0.1 ± 0.6% for the CRF1 receptor at 1 and 100 nm, respectively. Similarly, there were no significant TCAP-1-mediated agonist actions on the CRF2

![Fig. 2. Effect of TCAP and Teneurin expression on N38 cells transfected with TCAP-based siRNAs.](image_url)

A. N38 cells after transfection with either the pLuc negative control, or p270 and p721 siRNA containing plasmids. Scale bar: 50 μM. B. Expression product of histone H3, TCAP and teneurin after transfection. C. Quantification of the data provided in ‘B’. TCAP-1 and teneurin-1 is not expressed by N38 cells. The level of significance was determined using a one-way analysis of variance (ANOVA) using Tukey’s post hoc test. n = 4 for all analyses. (*p < 0.05; **p < 0.01; ***p < 0.001).
receptors (~1.6 ± 0.3% and 1.7 ± 0.1% activity at 1 and 100 nm, respectively). The potential antagonistic actions of TCAP-1 on these receptors rendered a similar insignificant action where the inhibition of the CRF1 response was only 2.3 ± 2.7% and 3.4 ± 1.9% for 1 and 100 nM respectively, and for the CRF2 response, a suppression of only 8.3 ± 0.6% and 6.6 ± 3.6%, respectively. This indicated that no significant inhibitory action on the CRF receptors occurred as a result of possible TCAP-1 interaction. Thus, together both the reporter gene and FLIPR studies indicates that it is unlikely that TCAP-1 binds directly to the CRF receptors.

### 3.3. TCAP and CRF modulates intracellular Ca$^{2+}$ flux

Because previous studies indicate that the CRF1 receptors are associated with anxiety-increasing properties, whereas TCAP-1 has an opposing action, we focused our attention toward the intracellular Ca$^{2+}$ response to determine if TCAP-1 could antagonize CRF at this signal transduction level. Using Fluo-4, the initial dose-response studies established that both TCAP-1 and CRF induced a Ca$^{2+}$ response at physiological concentrations although in opposite directions (Fig. 4A and B). Increasing CRF concentrations from 0.01 nM to 100 nM produced an increasing dose–response curve with a half-maximal effective concentration (EC$\text{_{50}}$) of 0.008 nM and a maximal effective concentration (EC$\text{_{100}}$) of 100 nM. In contrast, increasing TCAP-1 concentrations from 0.01 nM to 100 nM had the opposite action leading to decreases in intracellular Ca$^{2+}$ that produced an EC$\text{_{50}}$ of −0.1 nM and an EC$\text{_{100}}$ of −1 nM. We next assessed the interactive responses of intracellular Ca$^{2+}$ following CRF and TCAP-1 treatment (Fig. 4C–E). Cells were first perfused with artificial cerebral spinal fluid (ACSF) for 5 min until a steady-state fluorescence baseline was established (Fig. 4C–I). Next, cells were treated for 10 min with CRF (Fig. 4 C-II; 100 nM), scTCAP-1 (Fig. 4C-III; 100 nM), or TCAP-1 (Fig. 4C-IV; 100 nM). Treatment with either ACSF or Sc-TCAP did not change Fluo-4 fluorescence relative to pre-treatment levels (n = 6 each; P = 0.9184 and P = 0.39874, respectively, paired t-tests; Fig. 4C-I; -II; D,E). However, CRF induced a rapid (30s) increase in fluorescence. Compared to pre-treatment baseline, there was a 27.1 ± 2.4% increase in fluorescence intensity after 10 min (n = 5, p = 0.0011, Fig. 4 C-II; D,E). Conversely, TCAP-1 treatment decreased fluorescence by 12.1 ± 1.7% compared to pre-treatment baseline (n = 6, p = 0.0156, Fig. 4 C-IV; D,E). The time to onset of fluorescence decrease was between 30 and 60s.

### 3.4. TCAP-1 antagonizes CRF-mediated increases in intracellular Ca$^{2+}$

The previous experiments established that TCAP-1 and CRF having opposing actions of intracellular Ca$^{2+}$ when they were individually applied to N38 cells. However, TCAP-1 can antagonize CRF actions in both in vivo and in vitro studies leading to the possibility that cells treated with a combination of CRF and TCAP-1 could have a unique Ca$^{2+}$ response due to convergence of Ca$^{2+}$ signalling pathways (see Discussion). To investigate this potential interaction, cells were perfused with ACSF for 5 min, followed by CRF (1 nM) for 5 min, and then by a co-treatment with the second peptide. A double CRF treatment (CRF + CRF, bath and perfusion system, respectively) resulted in a 28.4 ± 1.4% increase in Fluo-4 fluorescence compared to pre-treatment baseline (p = 0.0001; n = 5; Fig. 5A-I; B,C). To confirm that TCAP-1 specifically antagonizes CRF, we next treated cells with CRF followed by a secondary application of TCAP-1. Compared to the CRF + CRF treatment, a secondary perfusion of TCAP-1 decreased the rate of fluorescence by 65% relative to the CRF response (P = 0.008 unpaired t-test; n = 5 each; Fig. 5A-II; B,C), and after 5 min, there was a significant decrease in intracellular Ca$^{2+}$ fluorescence (p = 0.0004), demonstrating an antagonistic effect of TCAP-1 on CRF-mediated Ca$^{2+}$ signalling. To confirm that TCAP-1-mediated decreases in Ca$^{2+}$ were not due to CRF wash-off, we also treated cells with CRF and the negative control peptide, scTCAP-1. Relative to baseline, fluorescence intensity increased by 25.2 ± 1.4%, which is not different from the CRF-mediated change fluorescence (p = 0.5786; n = 5; Fig. 5A-II; III; B,C).

The previous set of experiments showed that TCAP-1 inhibits CRF-induced intracellular Ca$^{2+}$ concentrations. The next set of experiments assessed if a pre-treatment with TCAP-1 inhibits the subsequent CRF-mediated Ca$^{2+}$ actions. Control experiments showed that a TCAP-1 + TCAP-1 experimental protocol led to a 9.14 ± 0.94% decrease in Fluo-4 fluorescence compared to pre-treatment baseline (n = 5; Fig. 6 A-I; B,C). A secondary treatment with scTCAP-1 also did not change fluorescence compared to the TCAP-1 + TCAP-1 regimen (p = 0.4044) (Fig. 6 A-II; B, C). These studies indicate that CRF does not antagonize the TCAP-1-mediated decrease in intracellular Ca$^{2+}$ when the cells are pre-treated with TCAP-1. However, when a 5 min perfusion of TCAP-1 was followed by a 5 min perfusion of CRF, there was only a 10.48 ± 2.1% decrease in Fluo-4 fluorescence compared to baseline (n = 4; Fig. 6 A-III; B,C). This is not different from the effect of TCAP-1 + TCAP-1 (P = 0.4997; unpaired t-test), indicating TCAP-1 treatment prevents the CRF-mediated Ca$^{2+}$ increase. Thus, whereas TCAP can inhibit the CRF-mediated rise in intracellular Ca$^{2+}$, CRF does not inhibit the TCAP-1-mediated decrease in intracellular Ca$^{2+}$. These data indicate that TCAP-1 and
Fig. 4. Intracellular calcium flux by TCAP-1 and CRF in immortalized neurons. A. Dose-response curves showing the acute effect of increasing concentrations of CRF or TCAP-1 on intracellular Ca2+ as established by Fluo-4 in mHypoE-38 cells where CRF shows dose-dependent increase in cytosolic Ca2+ concentrations and TCAP has an opposite action. Data represent the mean ± SEM (n = 4–5 replicates per treatment dose) B. Cells showed normal behaviour after treatment. Representative differential interference contrast (DIC) (left) and Fluo-4 fluorescence images of N38 neurons (right). The scale bar indicates 20 μm. C-E: TCAP-1 and CRF induce opposing calcium responses in embryonic mouse hypothalamic neurons. C. Superimposed Fluo-4 calcium responses following treatment with I) ACSF, II) CRF (100 nM); III) sc-TCAP (100 nM), and IV) TCAP (100 nM). The red trace represents the mean of the individual grey traces (n = 5–6 each), and each grey trace represents each of the average calcium response of 5 cells from the same experiment. D. Overlay of summary data shown in C. E. Summary graph showing percent change from baseline from data obtained in B and C, data assessed at the 15 min time point. Data represent the mean ± SEM (n = 5–6 replicates per experiment). **P < 0.01, ****P < 0.0001. One-way ANOVA with Tukey’s post-hoc test was used. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 5. TCAP-1 antagonizes CRF pre-treatment-mediated increases in cytosolic Ca2+ concentrations. A. Fluo-4 measurements in response to a 5 m CRF (1 nM) pre-treatment followed by a 5 m treatment with I) CRF (1 nM); II) TCAP-1 (100 nM); III) sc-TCAP (100 nM). The red trace represents the mean of the individual grey traces (n = 5 each), and each grey trace represents the mean Ca2+ response of 5 cells from the same experiment. B. Overlay of summary data shown in A. C. Quantification showing percent change from baseline from data obtained in B and C, summary data assessed at the 15 min time point. Data represent the mean ± SEM (n = 5–6 replicates per experiment). **P < 0.01, ***P < 0.001. One-way ANOVA with Tukey’s post-hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
CRF are not competing on the same set of Ca\(^{2+}\)-associated channels.

3.5. TCAP-1 stimulates calcium efflux through the plasma membrane

Given these findings, the next goal was to identify the Ca\(^{2+}\) channels targeted by TCAP. The general identification of the Ca\(^{2+}\) channels targeted by TCAP-1 was established using a number of pharmacological blocking agents (Table 2). The first inhibitor used was a general Ca\(^{2+}\) channel inhibitor, ruthenium red (RuR) (Fig. 7 A-I; B,C). Similar to previous experiments, N38 cells were loaded with Fluo-4 for 30 min followed by a 15 min wash in ACSF. In cells pre-treated with RuR, although the 10 min TCAP-1 exposure (100 nM) increased slightly (1.3 ± 0.4%) relative to the pre-treatment baseline (p = 0.0424; paired t-test; n = 5; Fig. 7 A-I; B,C), indicating a minor inhibition of the RuR-mediated Ca\(^{2+}\) response, it was vastly reduced from the TCAP-1 response (Fig. 6) indicating that the reduction of cytosolic Ca\(^{2+}\) by TCAP-1 was due to the impact of TCAP-1 on Ca\(^{2+}\) channels in the PM, MT and possibly the ER.

Next, the possibility that TCAP-1 may be acting on plasma membrane (PM) Ca\(^{2+}\) channels was investigated by treating the cells with benzamil (BZ) which primarily targets the Na\(^{+}/Ca\(^{2+}\) exchanger (NCX) found in the neural PM. To determine if NCX plays a role in such TCAP-1-mediated Ca\(^{2+}\) actions, we inhibited cellular NCX activity with BZ (10 μM), using the same protocol as the RuR experiments. Again, treatment with TCAP-1 did not display a cytosolic Ca\(^{2+}\) decrease (p =

Fig. 6. Teneurin TCAP-1 pre-treatment prevents CRF-mediated increases in cytosolic Ca\(^{2+}\) concentrations. A. Superimposed Fluo-4 Ca\(^{2+}\) measurements in response to a 5 min TCAP-1 (100 nM) treatment followed by a 5 min treatment with I) TCAP (100 nM), II) Sc-TCAP (100 nM); III) CRF (1 nM). The red trace represents the average of the individual grey traces (n = 5 each), and each grey trace represents the average calcium response of 5 cells from the same experiment. B. Summary graph of data shown in B for direct comparison of mean traces. C. Quantification indicating percent change from baseline from data obtained in B and C, where the mean data was assessed at the 15 min time point. Data represent the mean ± SEM (n = 5–6 replicates per experiment). ns = not significant. One-way ANOVA with Tukey’s post-hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
0.9169; paired t-test; n = 6 each; Fig. 7A–II, B,C) as compared with the profile indicated in Fig. 6A–II, where the cytosolic TCAP-mediated Ca\(^{2+}\) response was not statistically different from that of the controls. These BZ-mediated data confirmed that, in the RuR studies, TCAP-1 acted on the PM and possibly MT Ca\(^{2+}\) channels, although we could not discount the possibility that TCAP-1 may also act on PM Na\(^{+}\) channels.

The endoplasmic reticulum (ER) plays a critical role in cell Ca\(^{2+}\) regulation and a high ER Ca\(^{2+}\) concentration can be the result of reduced activity of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump. Conceivably, TCAP-1 could decrease intracellular Ca\(^{2+}\) by stimulating SERCA pump activity. To test this, cells were treated with the SERCA pump inhibitor, thapsigargin (TGX), for a total of 20 m prior to TCAP-1 treatment (Fig. 7A–III; B,C). When cells were treated with TCAP-1 for 10 m, there was a 21.7 ± 2.6% decrease in intracellular Fluo-4 fluorescence, a 2-fold larger decrease than when TCAP-1 was used alone (Fig. 7B and C). This is likely due to the combined actions of the initial cytosolic depletion of Ca\(^{2+}\) by the pre-treatment of TGX, and by the TCAP-1-mediated removal of inhibition of PM-associated Ca\(^{2+}\) efflux and MT-mediated Ca\(^{2+}\) influx suggested by the RuR and BZ studies described above. Thus, this experiment indicates that TCAP-1 does not...
have a direct action on the SERCA pumps (see Discussion).

Subsequently, the effect of inhibiting Ca\textsuperscript{2+}-ATPases (PMCA) using vanadate (VDT) (500 \textmu M) was examined. It should be noted that VDT can also inhibit the Na\textsuperscript{+}/K\textsuperscript{+} ATPase (see Discussion). In cells pretreated with VDT, a 10 m TCAP-1 treatment resulted in a 19.9 \pm 5.5\% increase in intracellular Fluo-4 fluorescence, relative to baseline (p = 0.0362; paired t-test; n = 4; Fig. 7A–IV; B,C), indicating that inhibition of PMCAs also partially prevents the TCAP-1-mediated decrease in intracellular Ca\textsuperscript{2+} by possibly inhibiting the TCAP-1 mediated PMCA action to increase Ca\textsuperscript{2+} efflux out of the cell.

3.6. TCAP-1 antagonizes CRF actions in mitochondria

The studies presented above show that TCAP-1 may also act on MT Ca\textsuperscript{2+} channels in addition to PM sites. To determine whether MT Ca\textsuperscript{2+} levels change with TCAP-1, the MT-associated Ca\textsuperscript{2+} dye, Rhod-2 was used. Rhod-2 fluorescence increases in intensity when bound to Ca\textsuperscript{2+}.

We first confirmed that MT-bound Rhod-2 fluorescence was stable under the 15 m experimental timeline (Fig. 8 A; B–I). Following the establishment of the ACSF baseline, a sham switch to a second ACSF treatment did not change Rhod-2 fluorescence (p = 0.1019; n = 4, Fig. 8B–I). Next, we assessed the effect of TCAP-1 on MT Ca\textsuperscript{2+} levels. A 10 m treatment with TCAP-1 (100 nM) increased Rhod-2 fluorescence by 12.5 \pm 3.1\% relative to baseline (p = 0.0423; n = 4; paired t-test; Fig. 8 B-II; C, D). A 10 m application of CRF (1 nM) by itself led to a 30 \pm 4.3\% decrease in Rhod-2 fluorescence relative to pre-treatment baseline (p = 0.003; n = 4; paired t-test; Fig. 8 B-III; C,D). To determine if TCAP-1 can prevent CRF-mediated changes in MT Ca\textsuperscript{2+} levels, cells were pre-treated with TCAP-1 prior to a 10 m CRF treatment. A pre-treatment of TCAP-1 followed by a 10 m CRF treatment resulted in a 3.5 \pm 2.2\% insignificant increase in Rhod-2 fluorescence relative to baseline (p = 0.1580, n = 5; paired t-test; Fig. 8 B-IV; C,D) indicating that TCAP-1 can prevent CRF-mediated changes in MT Ca\textsuperscript{2+} and demonstrating that there is an opposing interaction between these two peptide signalling systems at the level of the MT.

To assess if the TCAP-1-mediated increase in MT Ca\textsuperscript{2+} modulates MT polarization, we next assessed MT membrane potential (\(\Psi_m\)) using rhodamine 123 (Rhod-123). For these experiments, Rhod-123 was loaded into cells and analyzed in quench mode [46]. Therefore, an increase in Rhod-123 fluorescence indicates depolarization of the MT.
membrane potential. In ASCF control experiments, Rhod-123 fluorescence remained unchanged over 15 m (p = 0.3424, paired t-test; n = 4, Fig. 9 B-I; C). A 10 m treatment with TCAP-1 decreased Rhod-123 fluorescence, indicating hyperpolarization of the MT matrix. A maximal decrease of $14.8 \pm 2.0\%$ occurred 4.5 m after TCAP-1 application started, and was significantly different from baseline values (p = 0.013; paired t-test; n = 5, Fig. 9 B-II; C,D). Conversely, CRF (1 nM) increased Rhod-123 fluorescence, relative to baseline, with a maximal increase of $15 \pm 2.3\%$, 5.5 min after the start of CRF perfusion (p = 0.0114; paired t-test; n = 4, Fig. 9 B-III; C,D). Treatment with scTCAP-1 did not change fluorescence intensity significantly relative to pre-treatment values (p = 0.0654; paired t-test; n = 4, Fig. 9 B-IV; C,D). Repeating this experiment with a 20-m TCAP-1 pre-treatment (100 nM) resulted in a fluorescence increase of only $3.6 \pm 0.9\%$ at 5.5 m, which is not different from baseline (p = 0.1058; paired t-test; Fig. 10B-V). However, this effect is significantly different from the CRF-only treated cells (p = 0.0123; unpaired t-test) (Fig. 9 B-V), indicating that a pre-treatment with TCAP-1 prevented the CRF-induced increase in fluorescence.

3.7. TCAP-1 enhances cellular energy production

Increased MT Ca$^{2+}$ uptake and the resulting hyperpolarization should enhance energy production. To test this, we first assessed cytosolic NADH concentration turnover using a resazurin assay. Application of TCAP-1 (100 nM) significantly increased resorufin (RSF) fluorescence compared to vehicle-treated cells after about 20 m (p < 0.01), and after 60 m, RSF fluorescence was two-fold higher (p < 0.0001) in TCAP-1 controls.
treated cells (Fig. 10A). CRF also increased RSF fluorescence with a 1.5-fold increase \( (p < 0.0001) \) over the vehicle baseline at 60 m (Fig. 10A), however, the rate of NADH production was significantly higher in TCAP-1-treated cells than in CRF- or vehicle-treated cells (Fig. 10B). TCAP-1 increases the rate of RSF fluorescence increase over both CRF and vehicle within 20 m, where the rate of NADH production remained elevated for more than 50 m. To determine whether TCAP-1-induced NADH turnover translates into increased cellular ATP energy production, we measured intracellular ATP concentration using an ATP ELISA assay. Both peptides showed a maximal ATP increase around 30 m where the TCAP-1 mediated response was about twice that of CRF \( (p < 0.05) \) compared to the vehicle response (Fig. 10C) (see Fig. 11).

4. Discussion

The rationale of this study was based on two main premises arising from previous TCAP research. The first aspect was that TCAP-1 showed antagonism of CRF action \textit{in vitro} and \textit{in vivo}. The second element was that CRF, teneurins and αLTX are all implicated in intracellular Ca\(^{2+}\) flux. Together, these past studies indicated that the TCAP region of the teneurins may have an independent Ca\(^{2+}\) regulating ability, separate from the teneurin proteins. We examined this hypothesis using a synthetic version of TCAP-1 based on the genomic structure of teneurin-1 that has been used successfully in the past. In this study, we showed that TCAP-1 is important for cell vitality as it inhibits the Ca\(^{2+}\) response stress-inducing peptide of CRF; and has a specific action on regulating the PM-, ER- and MT-based Ca\(^{2+}\) channels and transporters. Together, these data show that TCAP-1 has a biological action independent from the teneurins and may play a role in the regulation of cell and organismal stress.

Given that intracellular Ca\(^{2+}\) regulation is essential for cell survival and vitality, we attenuated teneurin and TCAP mRNA expression using a set of oligonucleotides designed to target the conserved elements of the mRNA associated with the TCAP region of the teneurin transcripts. To establish that the teneurins and TCAPs play a role in cell vitality in the immortalized hypothalamic N38 cells in this study, we inhibited the expression of the endogenously expressed TCAPs using siRNA oligonucleotides. This resulted in significantly reduced growth characteristics of the cells (Fig. 2). However, although the oligonucleotides, inhibited the expression of the TCAP transcript, they also had an attenuating action on the teneurins. These studies indicated that the TCAP region of the teneurins was important for cell vitality that we have previously posited regarding early evolving peptide and protein systems in animals [35] and the importance of teneurin gene in development [6,7].

Previous studies indicated that TCAP-1 inhibits CRF action \textit{in vivo}. Intracerebroventricular (ICV) TCAP-1 administration into rats ablates the CRF-mediated cFos protein expression response in the limbic regions [41] and significantly reduces the CRF-mediated actions on the acoustic startle response (ASR) [2,37], open-field, elevated plus-maze [36,37,48] and cocaine-seeking [38–40] models of behaviour [49]. CRF has been implicated an increase of these behaviours via cAMP and Ca\(^{2+}\) mediated intracellular responses [42–44,49–51]. We have previously shown that TCAP-1 and -3 has a minor action on cAMP [1,2,36]. In this current study, however, we did not see any TCAP-1- mediated cAMP
response due directly to TCAP-1 actions on the CRF receptors. However, past studies of the teneurins and their cognate receptors, latrophilins, indicate that a cAMP response occurs [52]. Although it can be speculated that TCAP-1 has cAMP production activity upon activation of the latrophilins, this was not the goal of this study. We acknowledge, however, that a TCAP-1-mediated cAMP response arising from potential TCAP-1–latrophilin interaction may have a bearing on the possible antagonism of TCAP-1 and CRF.

Together, these current studies indicated that TCAP-1 was acting on a receptor system distinct from that of the CRF receptors. Thus, the TCAP-1 antagonism of CRF may be related to the attenuating interaction of TCAP-1 and CRF intracellular Ca\(^{2+}\) pathways [14] rather than direct interaction at the receptor level. Interestingly, TCAP has some sequence similarity to the CRF peptide family and also the calcitonin and secretin peptide families [3, 5]. The Secretin Family of peptides have a major primary sequence similar to the α-latrotoxins (αLTX) [20] indicating that they may be phylogenetically related. This toxin achieves its actions by inducing a robust intracellular Ca\(^{2+}\) response. The endogenous vertebrate receptor for αLTX was identified as the latrophilins [53] a G-protein coupled receptor (GPCR) originally thought to be part of the Secretin Family of GPCRs but was established to be part of the Adhesion Family of GPCRs (ADGRL) [19, 23] where the Secretin family of receptors likely evolved from an Adhesion-like GPCR ancestor. Since then, it has been established that the teneurins are likely cognate receptors of the latrophilins [18, 52, 53]. The latrophilins possess a conserved hormone-binding domain (HBD) originally defined in CRF receptors [22] and found in most Secretin family of GPCRs [19, 23].

There is compelling evidence that TCAP-1 activates the LPHNs [14, 17, 18] resulting in the regulation of cytosolic Ca\(^{2+}\) concentrations. LPHN-1 was originally discovered as a high-affinity receptor for αLTX [53]. Given that the primary structure of the TCAP family of peptides is also similar to that of the Secretin-related peptides [3] and also regulates Ca\(^{2+}\) in neurons [14], we postulated that TCAP-1 may regulate CRF actions by antagonizing the CRF-associated Ca\(^{2+}\) response. However, given the sequence similarity among the TCAP and CRF paralogues, we could not discount the possibility that TCAP may act as a partial competitive antagonist of CRF at the receptor level under physiological conditions. Because an increase in intracellular Ca\(^{2+}\) is an important downstream response following activation of the CRF1 receptor, it is likely that there is an interaction between TCAP and CRF at the level of intracellular Ca\(^{2+}\) flux.

Based on these previous studies, we examined the interaction of TCAP-1 and CRF with respect to intracellular Ca\(^{2+}\) flux in the N38 cell model using Fluo-4 as an indicator of cytosolic Ca\(^{2+}\). Importantly, the cell line shows the validity of this model given that both TCAP-1 and CRF show an EC\(_{50}\) activity within a physiological range (Fig. 4), albiet, in opposite directions. Thus, whereas CRF increases cytosolic Ca\(^{2+}\) and TCAP significantly reduces its cytosolic concentrations. However, it was not clear from these studies as to how TCAP-1 could block the CRF-mediated cytosolic Ca\(^{2+}\) decrease. Thus, the cells were pretreated with either CRF or TCAP-1 (Figs. 5 and 6.). In both situations, the presence of TCAP-1 could significantly reduce the CRF-associated rise in cytosolic Ca\(^{2+}\).

Previously, we showed that TCAP-1 activates an IP3-DAG signal transduction pathway associated with Ca\(^{2+}\) mobilization, although it was not clear as to which Ca\(^{2+}\) channels and transporters were affected. Therefore, in this current study, we utilized a number of pharmacological antagonists to probe the identity of the key Ca\(^{2+}\) regulating mechanisms (Table 2; Fig. 11). Ruthenium red (RuR) [54], has a number of actions including inhibition of the MT Ca\(^{2+}\) uniporter, and blocking Ca\(^{2+}\) uptake and release from the MT. In addition, RuR inhibits Ca\(^{2+}\) release from ER, prevents Ca\(^{2+}\) release from ryanodine-sensitive intracellular ER and MT stores, and inhibits PM Ca\(^{2+}\) channels, including channels of the Transient Receptor Potential Vanilloid (TRPV) family [55, 56]. In our study, prior treatment with RuR inhibited the TCAP-1-induced reduction in cytosolic Ca\(^{2+}\) concentrations indicating...
that it was likely inhibiting the Ca\(^{2+}\) efflux sites from the cytosol such as extrusion from the PM, or importation into the organelles such as the ER or MT.

An essential cellular mechanism for removing Ca\(^{2+}\) from the cytosol involves the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) [57]. NCX exchangers, exchanging 1 Ca\(^{2+}\) ion for 3 Na\(^+\) ions and, under typical baseline conditions, this removes Ca\(^{2+}\) from cells, but depending on the Na\(^+\) gradient, can work in both a forward or reverse direction. The NCX works with the PM Ca\(^{2+}\) ATPase (PMCA) to keep cytosol concentrations of Ca\(^{2+}\) low. The exchanger is expressed in its highest concentration in the PM (NCX transports Ca\(^{2+}\) out of the cell) and MT (NCX transports Ca\(^{2+}\) out of the MT matrix where it helps maintain low MT Ca\(^{2+}\)) of excitable cells. To examine this potential action, the cells were treated with benzamil (BZ), a synthetic blocker of NCX and epithelial sodium channels (ENaC) [58-60]. In this study, this antagonist again inhibited the TCAP-1 reduction in cytosolic Ca\(^{2+}\) thus corroborating the actions of RuR, suggesting that the reduction of cytosolic Ca\(^{2+}\) by TCAP-1 occurred via PM and MT Ca\(^{2+}\) channel associated efflux. However, given the action of BZ on ENaC channels, we could not discount the possibility that TCAP-1 may also affect Na\(^+\) channels (see below).

These studies indicated that, although TCAP-1 may act via PM and MT Ca\(^{2+}\) channels, both RuR and BZ have limited actions on the Ca\(^{2+}\) channels of the ER and, therefore, we could not discount this possibility that TCAP-1 may induce Ca\(^{2+}\) uptake into the ER. Thapsigargin (TGX) is a non-competitive inhibitor of the SERCA pumps [61] that increases cytosolic Ca\(^{2+}\) concentrations by blocking the SERCA-mediated uptake of Ca\(^{2+}\) [62]. In our study, the N38 cells were first treated with TGX to prevent ER Ca\(^{2+}\) uptake, then treated with TCAP-1. If we assume that TCAP-1 reduces cytosolic Ca\(^{2+}\) concentrations by Ca\(^{2+}\) influx into the MT and efflux through PM Ca\(^{2+}\) channels, then this may explain the enhanced reduction of cytosolic Ca\(^{2+}\) of TGX and TCAP-1 together in comparison to TCAP-1 alone as TCAP-1 was acting, in part, to target Ca\(^{2+}\) uptake in the MT and likely other sites. Previously, ER-stress generated by increased cytosolic Ca\(^{2+}\) concentrations in mouse embryonic fibroblasts increased teneurin-4 (DOC4; downstream of CHOP) transcription [63] suggesting that teneurins and, hence, TCAP-1, may act to protect the cells against high cytosolic Ca\(^{2+}\) by reducing cytosolic Ca\(^{2+}\) concentrations via PM efflux and MT influx. perhaps in the manner indicated by our current data.

PM Ca\(^{2+}\) ATPases (PMCA) also play a role with respect to cytosolic Ca\(^{2+}\) efflux. Vanadate (VDT) is anionic vanadium complex that inhibits PM Ca\(^{2+}\) ATPases and Na\(^+\)/K\(^+\) ATPases, but not SERCA pumps [64-66]. We postulated that if TCAP-1 did not affect SERCA pumps, but potentiated the decrease in cytosolic Ca\(^{2+}\), then the treatment of VDT may reduce intracellular Ca\(^{2+}\), where the TCAP-1 treatment may affect the PMCA. The treatment of VDT not only inhibited the TCAP-1 cytosolic depression of Ca\(^{2+}\) concentrations but induced a significant rise above the control levels indicating that VDT was blocking the TCAP-1-mediated Ca\(^{2+}\) via PMCA channels. Thus, the VDT data corroborates the RuR, BZ and TGX studies that TCAP-1 reduces cytosolic Ca\(^{2+}\) concentrations by regulating PM and MT Ca\(^{2+}\) channels but not SERCA pumps.

TCAP-1 can increase glucose uptake and oxidative energy production in the CNS and N38 cells [14]. In neurons, this glucose increase coincides with a decrease in intracellular lactate and increase in pyruvate, indicating that MT activity is likely increased with TCAP-1. Because MT play a major role as a Ca\(^{2+}\) storage and signalling organelle, where the uptake and release are associated with increased cellular energy production, it is possible that TCAP-1 activates MT Ca\(^{2+}\) uptake. Therefore, we utilized rhodamine-2 (Rhod-2), an organic dye that can be used to localize Ca\(^{2+}\) in the MT [67]. This study showed that, whereas TCAP-1 increased Ca\(^{2+}\) into the MT, CRF reduced Ca\(^{2+}\) influx into the MT. When TCAP-1 was pre-treated with the cells, it ablated the CRF-mediated decrease in MT Ca\(^{2+}\) concentrations (see Fig. 6) likely leading to an increase in MT Ca\(^{2+}\)-stimulated electron transport chain (ETC) enzyme activity, resulting in increased MT proton (H\(^+\)) pumping and the subsequent generation of a hyperpolarized MT membrane potential [68]. To test the hypothesis that TCAP-1, by increasing Ca\(^{2+}\) concentrations could also regulate MT membrane potential, we utilized Rhod-123, a cationic dye that is used as a measure of MT membrane polarization [69] as Rhod-123 concentrates in membranes in a polarization-dependent manner [70]. In our experiments, TCAP-1 treatment decreased Rhod-123-associated fluorescence indicating hyperpolarization of the MT matrix whereas CRF, increased the fluorescence showing that it induced depolarization. However, when the cells were pretreated with TCAP-1, the CRF-induced depolarization action was ablated (see Fig. 9). These studies corroborate with those indicated in Fig. 8 (discussed above) that TCAP-1 acts to import Ca\(^{2+}\) into the MT whereas CRF inhibits this uptake. Thus TCAP-1 could increase MT Ca\(^{2+}\) uptake and hyperpolarization of MT membrane to potentiate energy production in N38 cells by stimulation of the ETC. This was examined first using a resazurin assay to measure NADH production. In this assay, non-fluorescent resazurin is reduced to fluorescent resorufin (RFN) by NADH to NADPH [71,72]. Although both TCAP-1 and CRF increased RFN-based fluorescence, the TCAP-1 response was greater and more rapid than the CRF response (Fig. 10A and B). A similar situation occurred with ATP production (Fig. 10C). Although it is not clear how this action is translated directly under in vivo conditions, it does further indicate antagonism between TCAP-1- and CRF-mediated actions.

Problematically, however, there are few studies to link CRF, and its paralogues, directly on MT function. In one such study, CRF induced CRF1 receptor activation related MT morphology through an NF-kB associated mechanism [73] although CRF may have a direct role on MT ROS production [74]. This could also lead to the dose-dependent increase in ATP production previously reported in the N38 cells [14]. Urocortin, a direct paragolpe of CRF [75-78], can inhibit Ca\(^{2+}\) uptake in cardiac muscle MT by inhibiting the MT permeability transition pore (MPTP) although the exact mechanism by which this occurs is not known [79]. Thus, our data indicate that further studies on the role of CRF and its paralogues on MT function is warranted.

The goals of this current were to identify the cellular targets associated with TCAP-1 action, and secondarily to develop a plausible mechanism to understand the TCAP-1 induced inhibition of the CRF response, in vivo. Discovered in 1994, ablation of the teneurin gene was shown to be embryonic-lethal in Drosophila [6,7]. Since then, numerous studies indicated that the teneurins are required for the development and maintenance of the CNS in metazoans and vertebrates [80-82]. At the distal extracellular tip, comprising the carboxy-terminus, lies a peptide sequence termed the ‘teneurin C-terminal associated peptide’ (TCAP) [1-3]. Synthetic TCAP-1 plays a similar but independent role as the teneurins on neuronal development as indicated by its ability to increase neuronal outgrow, axon development and axon fasciculation in vivo and in vitro [36,47,48].

CRF is the seminal neuropeptide responsible for the regulation of the organismal stress response in the CNS, and for initiation of the hypothalamic-pituitary-adrenal/inter-renal (HPA/IR) axis in vertebrates [14,76,77,83,84]. Elevated CRF signalling and the consequent dysregulation of the HPA/1 axis has been implicated in the onset of affective disorders, including major depression and anxiety, panic disorder, and post-traumatic stress disorder [49-51]. Phylogenetically, CRF peptides are found throughout the Metazoa where they are present in four paralogous forms in vertebrates as well as orthologues in arthropods and molluscs [35,76,77,85]. Their primary structure is conserved throughout the Metazoa, suggesting the search for a peptide candidate that may be representative of an earlier evolving peptide lineage. TCAP may represent such a candidate lineage.

Despite the findings entailed within this study, many peripheral questions regarding the actions of TCAP remain unanswered and are outside of the scope of our study. Although we have established that TCAP-1 has the potential to inhibit the CRF-directed cytosolic Ca\(^{2+}\) response, it is not clear how this interaction occurs with respect to specific Ca\(^{2+}\) channels and transporters in the various organelles. We
have concentrated on the PM, ER and MT with respect to TCAP-1 action, but have not examined the specific interactive relationship between CRF and TCAP-1 for each of these targets, nor have we examined the Ca$^{2+}$ uptake ability of the cytoskeletal elements, lysosomes or peroxisomes for example. We have used CRF as a proxy for its paralogues (urocortins 1–3 in mammals), and TCAP-1 as a model for its paralogues (TCAPs 2–4). We focused on the CRF and the CRF1 receptor as this peptide-ligand system the primary stress-inducing pathway in the CNS and HPA axis [44,84,86, 87]. Urocortins –2 and –3, represent a secondary lineage from the CRF-urocortin line [78,88] but possess anti-anxiety via interaction with the CRF2 receptor [89–91]. The interactive role of TCAP-1 and urocortins –2 and –3 has not been studied in vitro or in vivo, thus, it is not clear whether such interactions will be agonistic or antagonist with respect to stress-associated behaviours. With respect to TCAP-1 as an independent peptide, this current study and previous studies have shown that this region of the teneurins possesses numerous biological actions in vivo and in vitro actions independent from the teneurins (see above) although we have yet to show the presence of the free peptide in vertebrates and mammals. It is assumed that the other paralogues of TCAP-1 (TCAP-3 and TCAP-4) may be similarly active, but the method of liberation may differ. Further studies on how TCAP is liberated should provide evidence of this.

In summary, we show that CRF and TCAP-1 have opposing actions on the cytosolic Ca$^{2+}$ response and are due to antagonizing actions of their receptors given our experimental design. We also show that the potential TCAP-1 antagonism of the CRF response is independent of the CRF receptors but occurs downstream by its interaction with the regulation of intracellular Ca$^{2+}$ flux to ultimately affect the action of MT activity. This study supports the hypotheses that: TCAP can modulate intracellular Ca$^{2+}$ flux; it can inhibit CRF-associated activity by regulating in part the intracellular Ca$^{2+}$ response and may regulate MT activity, and therefore, cellular energy metabolism by intracellular Ca$^{2+}$ mobilization. Specifically, we have established that TCAP modulates PM and MT Ca$^{2+}$ flux by utilizing pharmacological blockers of Ca$^{2+}$ channels. Moreover, we have provided novel evidence that this mechanism is antagonistic to the actions of CRF with respect to intracellular Ca$^{2+}$ mobilization. Finally, we show additional evidence to support the actions of the MT-associated activation by TCAP-1 to induce increased energy production. Overall, this study provides new information to understand previous studies on cell metabolism by teneurin-latrrophilin coupling and is consistent with our theory that TCAP may represent an early evolving peptide lineage associated with the regulation of energy and ion exchange.

Author contributions

Dr. D. Hogg developed and performed all calcium imaging and wrote the initial draft of this manuscript. Dr. D. Barsyte-Lovejoy developed the methodology for the siRNA knock-downs of teneurins and TCAPs. Prof., C. Casatti developed and performed the cell culture and expression of the siRNA knock-down cells. Prof. D. Belsham provided the N38 cells and provided guidance on their culture. Prof. D. Lovejoy oversaw the entire program and the final drafts of the manuscript.

Declaration of competing interest

Prof. D. Lovejoy is a co-founder of Protagenic Therapeutics, Inc. (PTI; New York, USA) which has a commercial interest in TCAP peptides for clinical purposes. Although not employed by the PTI, he holds a small equity position in the company and is the inventor of several TCAP-related patents currently licensed to PTI. Both Prof. Lovejoy and Dr. Dalia Barsyte-Lovejoy currently act as unpaid scientific advisors for PTI.

Data availability

Data will be made available on request.

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