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Identification of a novel Brain Derived Neurotrophic Factor (BDNF)-inhibitory factor: Regulation of BDNF by Teneurin C-terminal Associated Peptide (TCAP)-1 in immortalized embryonic mouse hypothalamic cells

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ABSTRACT

The teneurins are a family of four large transmembrane proteins that are highly expressed in the central nervous system (CNS) where they have been implicated in development and CNS function. At the tip of the carboxyl terminus of each teneurin lies a 43-amino acid sequence, that when processed, could liberate an amidated 41-residue peptide. We have called this region the teneurin C-terminal associated peptide (TCAP). Picomolar concentrations of the synthetic version of TCAP-1 inhibit stress-induced cocaine reinstatement in rats. Because cocaine-seeking is associated with increased brain derived neurotrophic factor (BDNF) in the brain, we examined whether synthetic mouse TCAP-1 has the potential to regulate BDNF expression in immortalized mouse neurons. Immortalized mouse neurons (N38; mHypoE38) show strong FITC-labeled [K_8]-TCAP-1 uptake and BDNF labeling in the cytosol. Moreover, FITC-labeled [K_8]-TCAP-1 bound competitively to membrane fractions. In culture, the labeled TCAP-1 peptide could be detected on cell membranes within 15 min and subsequently became internalized in the cytosol and trafficked toward the nucleus. Administration of 10^{-8} M unlabeled TCAP-1 to cultures of the N38 cells resulted in a significant decrease of total cell BDNF immunoreactivity over 4 h as determined by western blot and ELISA analyses. Real-time PCR, utilizing primers to the various BDNF transcripts showed a significant decline of promoter IIB- and VI-driven transcripts. Taken together, these studies indicated that in vitro, TCAP-1 induces a significant decline in BDNF transcription and protein labeling in embryonic mouse immortalized hypothalamic neurons. Thus, TCAP-1 may act as a novel BDNF inhibitory factor.

1. Introduction

The teneurins are a family of four large transmembrane proteins that are highly expressed in the central nervous system where they have been implicated in development and neuronal function [18,19,44,45,51]. Previous studies indicate that they play a role with neuronal patterning, neurite outgrowth and process development [22,30,37], and interact with elements of the extracellular matrix [23]. The structure of the teneurins is complex, consis7ting of a number of subdomains in both intracellular and extracellular components [4,15,15,30–32,34]. As type II transmembrane proteins, the carboxyl termini are extracellular. At the tip of the carboxyl terminus of each teneurin lies a 43-amino acid sequence, that when processed, could liberate an amidated 41-residue peptide [26]. We refer to this region as the teneurin C-terminal associated peptide (TCAP).

The synthetic version of TCAP-1 has a number of bioactive effects. In vivo, administration of this peptide regulates stress-induced behavior in rats [2,39,46] inhibits corticotropin-releasing factor (CRF)-induced cFOS expression in the brain [40], and causes dendritic remodeling of CA3 neurons in the hippocampus [41]. In vitro, TCAP-1 regulates cytoskeletal elements [3] and reduces reactive oxygen species (ROS) by up-regulation of superoxide dismutase and catalase [43].

Recently, TCAP-1 was shown to inhibit the CRF-induced increase of cocaine-seeking in rats using the cocaine-reinstatement model [21]. In a previous and separate study, termination of cocaine administration in rats resulted in an increase of brain derived neurotrophic factor (BDNF) in the medial prefrontal cortex where disruption of the TrkB receptor reduces the locomotor hypersensitivity to cocaine. This suggests that the increased BDNF expression in this region may, in part, play a role in the increased motivation for cocaine following the termination of its administration [28]. In addition, a number of

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stressors, partly mediated by CRF, can also increase cocaine-seeking in this model. We have previously established that TCAP-1 can also inhibit CRF-induced cFOS labeling in this region of the brain [40]. Taken together, we hypothesize that these mechanisms may be linked and that TCAP-1 may exert its effects, in part, by inhibiting BDNF expression and release.

In order to investigate whether TCAP-1 affects BDNF regulation, we examined the relationship between BDNF expression and TCAP-1 action in a recently developed immortalized embryonic mouse hypothalamic cell line [7] as a model for neuronal cells. TCAP-1 is expressed throughout the brain notably in limbic and cortical regions [46; Chand and Lovejoy, manuscript in preparation] and, therefore, the mechanism of action may be similar throughout neurons. We have previously shown that TCAP-1 has a number of actions on this cell line [3,43,46]. We now show that TCAP-1 binds to these cells, whereupon it is internalized and significantly decreases the expression and intracellular concentration of BDNF.

2. Materials and methods

2.1. Peptide and chemicals

Mouse TCAP-1 was prepared by solid-phase synthesis by American Peptide Company (Sunnyvale, USA) and was dissolved in phosphate-buffered saline (PBS) to a stock concentration of 2×10^{-5} M. Stock mouse TCAP-1 was then diluted in medium to a final concentration of 10^{-8} M and stored at 4 °C until needed. The inactive peptide, $TCAP_{9–41}$, was diluted in medium to a final concentration of 10^{-8} M and used as a negative control. A concentration of 10^{-8} M was utilized as previous studies indicated that this concentration provided the clearest signal-to-background effects. Forskolin (Sigma Chemical Co.) was dissolved in DMSO (Sigma Chemical Co.) to a stock concentration of 10^{-2} M and was diluted with PBS to a final concentration of 10^{-5} M for treatment.

2.2. Preparation of fluoresceinisothiocyanate (FITC)-labeled TCAP-1

The TCAP-1 variant with lysine in position 8 ([K_8]-TCAP-1) was labeled with FITC as previously reported [3]. Briefly, the lyophilized peptide was dissolved in 50 mM sodium borate buffer (pH 8.5) to a final concentration of 1 mg/ml. TCAP-1 peptide was labeled with FITC (EZ-Label FITC Protein labeling kit; Pierce Chemical Co.). FITC was dissolved in dimethylformamide (Sigma Chemical Co.), mixed with the TCAP-1 at a 24:1 excess molar ratio and incubated for 2 h at room temperature (RT) in the dark. FITC-TCAP-1 was purified using a dextran column (Thermo Scientific). The 4 fractions with the highest absorbance at 280 nm were combined and sterilize-filtered using an Acrodisc Syringe 0.2 mm Super low protein binding filter (Pall Life Sciences). The filtrate was concentrated using a Microsep 1 K Omega centrifugal device (Pall Life Sciences) and stored at 4–8 °C. The amounts used of the FITC-tagged TCAP-1 was first determined by titration in cytological preparations. The concentration that generated the highest signal to noise ratio as determined by the LabWorks 4.0 Image Aquisition and Analysis Software (Ultra-Violet Products Ltd.) was used for subsequent studies. In order to determine the affinity that TCAP-1 binds to membrane extracts, a displacement binding assay was performed (see below).

2.3. Primary embryonic hippocampal cell cultures

All procedures were approved by the University of Toronto Animal Care Committee in accordance with the guidelines from the Canadian Council on Animal Care. Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared as previously described [2]. Briefly, E18 pregnant Sprague Dawley rats were exposed to CO_2 for several minutes and cervically dislocated. The E18 rat embryonic hippocampi were isolated and treated with trypsin for 15 min at 37 °C. Cells were dissociated by gentle trituration and plated at a density of 50,000 cells/mL on poly-D-lysine-coated 25-mm glass coverslips in 35-mm petri dishes. Cells were plated in Neurobasal medium (Invitrogen) and supplemented with 2% B-27 (Invitrogen). Every 3 days, one-third of the medium was replaced with fresh Neurobasal medium supplemented with B-27.

2.4. Binding of FITC-[K_8]-TCAP-1 to cell membranes

To detect FITC-TCAP-1 binding to cell membranes, membrane preparations were prepared as previously published [25]. Displacement of the FITC-TCAP-1 tracer was achieved using increasing concentrations of synthetic TCAP-1. The decrease in fluorescence in membranes was determined digitally using a UVP Gel Doc System with LabWorks 4.0 Image Acquisition and Analysis Software (Ultra-Violet Products Ltd.). The size of the binding protein or receptor was determined by incubating the labeled TCAP-1 with N38 cell extracts. N38 cells were extracted and incubated with 0, 5 or 10 μl of TCAP-FITC for 1.5 h on a shaker at 37 °C. The samples were then prepared for a native PAGE without SDS and separated on a 10–20% Tris-Tricine gel (BioRad) for 2 h at 100 V.

2.5. Bifunctional crosslinking to TCAP-binding proteins

Potential binding proteins were identified by cross-linking TCAP-1 to binding partners using a bifunctional crosslinker. To attach TCAP-1 to the crosslinker reagent p-AzidoPhenyl Glyoxal (APG), 1 mM solutions of TCAP and APG in PBS, pH 7.4, was reacted in 1:10 ratio (v/v) in the dark for 1 h. A microsep (1 K) device was used to remove unconjugated APG from the solution. The TCAP-APG was diluted in PBS to an effective concentration of 320 μm, 32 μ, 6.4 μm and 3.2 μm and used to treat N38 cultured cells for 10 min after removal of the DMEM culture medium. The cells were then washed with PBS and treated with UV light at 254 nm for 10 min before extracting the cell proteins. The cell proteins were stored at −80 °C. Control cell cultures were treated with PBS or TCAP-1 solution instead of TCAP-APG and exposed to UV light as well.

2.6. Immunofluorescence confocal microscopy

Immortalized mouse embryonic hypothalamic N38 cells were grown on a 25 mm cover glass (Warner Instruments) in 6-well tissue culture plates (Corning Inc.) for 24 h in N38 medium DMEM (Invitrogen) containing 10% FBS (Invitrogen) and penicillin/streptomycin (Sigma Chemical Co). When the cells were approximately 50% confluent, cells were treated with 10^{-8} M TCAP-1, inactive 10^{-8} M $TCAP_{9–41}$, or vehicle in N38 medium for 0, 1, 2, 4 and 8 h. Immunocytochemistry was conducted on immortalized N38 cells incubated with FITC-[K_8]-TCAP-1 for 90 min as previously reported [3]. Rabbit polyclonal BDNF primary antibody (Santa Cruz; 1:500) and anti-rabbit Texas red TM-conjugated secondary antibody (Abcam; 1:200) were used to observe BDNF immunoreactivity. For controls, cells were incubated with primary antibody alone, secondary antibody alone, or unconjugated FITC. Images were acquired with a Zeiss AxioObserver (Zeiss, Germany) inverted microscope equipped with a confocal light path (WaveFx; Quorum) based on a modified Yokogawa CSU-10 head (Yokogawa Electric Corp.).

2.7. Western blot analysis of BDNF

Immortalized N38 cells were grown in 6-well tissue culture plates at a density of 400,000 cells/9 cm^2 wells for 24 h in N38 medium. When the cells were approximately 70–80% cell confluent, they were treated with either 10^{-8} M TCAP-1, inactive 10^{-8} M $TCAP_{9–41}$, vehicle, or 10^{-5} M forskolin. Cells were harvested at 0, 1, 2, 4 and 8 h after treatment. Cells were washed with sterile PBS at pH 7.4 and then harvested