Original Paper

Tff3 is Expressed in Neurons and Microglial Cells

Ting Fu^a Anne Stellmacher^{b,c} Eva B. Znalesniak^a Daniela C. Dieterich^{b,c,d}
Hubert Kalbacher^e Werner Hoffmann^a

^aInstitute of Molecular Biology and Medicinal Chemistry, Otto-von-Guericke University Magdeburg, Magdeburg ^bInstitute for Pharmacology and Toxicology, Otto-von-Guericke University Magdeburg, Magdeburg ^cLeibniz Institute for Neurobiology, Magdeburg, ^dCenter for Behavioral Brain Science (CBBS), Magdeburg, ^eInterfaculty Institute of Biochemistry, Eberhard-Karls-University Tübingen, Tübingen, Germany

Key Words

TFF3 • TFF peptide • Trefoil factor • Brain • Neuron • Microglial cell

Abstract

Background/Aims: The trefoil factor family (TFF) peptide TFF3 is typically secreted by mucous epithelia, but is also expressed in the immune system and the brain. It was the aim of this study to determine the cerebral cell types which express Tff3. **Methods:** Primary cultures from rat embryonic or neonatal cerebral cortex and hippocampus, respectively, were studied by means of RT-PCR and immunofluorescence. Moreover, Tff3 expression was localized by immunocytochemistry in sections of adult rat cerebellum. **Results:** Tff3 transcripts were detectable in neural cultures of both the cortex and the hippocampus as well as in glial cell-enriched cultures. Tff3 peptide co-localized with Map2 indicating an expression in neurons *in vitro*. The neuronal expression was confirmed by immunofluorescence studies of adult rat cerebellum. Furthermore, Tff3 peptide showed also a clear co-localization with Iba-1 *in vitro* typical of activated microglial cells. **Conclusion:** The neuronal expression of Tff3 is in line with a function of a typical neuropeptide influencing, e.g., fear, memory, depression and motoric skills. The expression in activated microglial cells, which is demonstrated here for the first time, points towards a possible function for Tff3 in immune reactions in the CNS. This opens a plethora of additional possible functions for Tff3 including synaptic plasticity and cognition as well as during neuroinflammatory diseases and psychiatric disorders.

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Introduction

The peptide TFF3 is a member of the trefoil factor family (TFF). These peptides represent typical secretory products of mucous epithelia and play a key role in multiple protection and repair processes (for reviews, see refs. [1, 2]). For example, Tff3-deficient mice show extreme sensitivity in the dextran sodium sulfate (DSS) colitis model [3]. For

TFF3, a motogenic effect due to chemotaxis as well as both pro- and anti-apoptotic activities have been reported [4-6]. Thus far, specific TFF3 receptors have not been characterized. However, chemokine receptors are promising candidates [7]. A major expression site for TFF3 are intestinal goblet cells [8] where it forms mainly a disulfide-linked heteromer with IgG Fc binding protein (FCGBP) and few monomers and homodimers [9].

Besides mucous epithelia, TFF peptides are also synthesized in the brain (for reviews, see [2, 10]). Major expression sites for Tff3 are the murine cerebellum, the cortex, and the hippocampus [11]. Interestingly, Tff3 expression in the murine cerebellum is developmentally regulated with a maximum between P15 and P20 [11]. Weak Tff3 expression has also been reported to occur in the rat amygdala [12] and injections of synthetic Tff3 into this brain region showed fear modulating effects [13]. Furthermore, TFF3 is also synthesized in the rat and human hypothalamus [12, 14] where it localizes to oxytocin-producing neurons in the paraventricular and supraoptic nuclei [14]. Immunoelectron microscopy revealed that TFF3 and oxytocin are co-localizing within the same secretory vesicles in the neural (posterior) lobe of the porcine pituitary [15]. From there, systemic neurohypophyseal release of TFF3 occurs into the bloodstream. The concentration of TFF3 in human serum has been reported to be about 140 pmol/L [16]. However, the TFF3 targets are still unknown, but there might be surface receptors on the basolateral side of gastric mucous neck cells as well as antral and cardiac gland cells [17].

Thus far, the cellular localization and function of Tff3 in the cerebellum, cortex, and hippocampus are not known. In order to gain more insights into which cerebral cell types are capable of synthesizing Tff3 we studied various primary cell cultures from the embryonic or neonatal rat cerebral cortex and hippocampus, respectively by means of RT-PCR and immunofluorescence. In particular, two types of cultures were investigated, one from the cerebral cortex or the hippocampus, which contained both neurons and glial cells, and a second type from the cerebral cortex nearly devoid of neurons (glial cell-enriched culture). Furthermore, Tff3 was localized in the adult rat brain by means of immunocytochemistry.

Materials and Methods

Neural cell cultures from the rat brain cortex and hippocampus, respectively

Primary hippocampal and cortical cultures were prepared according to Goslin et al. [18] from hippocampi and cortices, respectively, of E18 rat embryos (Sprague Dawley; Harlan, Rossdorf, Germany). Briefly, hippocampi or cortices were carefully dissected and then treated with 0.05% trypsin (Life Technologies GmbH, Darmstadt, Germany) for 20 min at 37°C. After incubation with trypsin inhibitor (0.5 mg/mL, from *Glycine max*; together with 0.24 mg/mL DNase I; both from Sigma-Aldrich Chemie GmbH, Munich, Germany), the hippocampi and cortices, respectively, were triturated in Hank's balanced salt solution plus 3 mg/mL bovine serum albumin (both from Life Technologies GmbH, Darmstadt, Germany) with needles of decreasing diameters and the resulting cell suspension applied to cell strainers (BD Biosciences, Heidelberg, Germany) until the cells were fully suspended. Then $2 - 4 \times 10^4$ cells were plated onto cover slips (diameter 12 mm, Gerhard Menzel, Braunschweig, Germany) coated with poly-D-lysine (Sigma-Aldrich Chemie GmbH, Munich, Germany) and cultivated in Neurobasal® medium supplemented with 0.8 mM glutamine and 1x B-27® supplement (all from Life Technologies GmbH, Darmstadt, Germany) at 37°C with 5% CO₂ (v/v). The cells were fed with 1/10 of fresh culture medium every week, i.e. 50 µL fresh medium was added to each well of a 24-well plate (Greiner Bio-One, Frickenhausen Germany). The cells were cultivated for 20 – 22 days *in vitro* to obtain mature neurons forming synaptic networks.

Glial cell-enriched cultures from the rat brain cortex

Glial cell-enriched primary cultures were prepared according to Guizzetti and Costa [19]. In brief, cortices of P2 rats (Sprague-Dawley; Harlan, Rossdorf, Germany) were dissected and trypsinized (0.25% v/v trypsin, 20 min at 37°C). Trituration was performed with needles and cell strainers as described above. Cells were cultivated in a flask in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10% fetal calf serum, and penicillin/streptomycin (all from Life Technologies GmbH, Darmstadt, Germany).

Table 1. Oligonucleotides used for RT-PCR analysis and calculated size of the products. Iba1: ionized calcium binding adapter protein 1; Gfap, glial fibrillary acidic protein; Map2: microtubule-associated protein 2

Genes	Accession No.	Primer No.	Primer Pairs	Nucleotide Positions	Tm	Cycles	Size (bp)	Intron Spanning
Iba1	NM_017196.3	MB2141	TTGGATGGGATCAACAAGCA	168-187	60°C	32	274	yes
		MB2142	ATCTCTTGCCCAGCATCATT	441-422				
Gfap	NM_017009.2	MB2145	AGGGACAATCTCACACAGGA	416-435	60°C	32	439	yes
		MB2146	GGCGATAGTCATTAGCCTCG	854-835				
Map2	NM_013066.1	MB2143	CATCATTCGCACTCCTCCAA	5031-5050	60°C	32	462	yes
		MB2144	GAGGAGACATTGCTGAGTCG	5492-5473				

The medium was changed every 3 - 4 days and the cells reached confluency after 10 - 12 days. Then, the cells were collected from the culture flask by trypsinization and plated onto cover slips (diameter 12 mm, Gerhard Menzel, Braunschweig, Germany) at a dilution of 1:12 and cultivated for 4 - 5 days.

RT-PCR analysis

After reaching confluency, cells from the different cultures were collected. RNA isolation, digestion with DNase, and RT-PCR analysis have been described previously [20]. As a control for the integrity of the cDNA preparations, β -actin transcripts were amplified in parallel reactions. The cDNA was also checked for contaminating chromosomal DNA by amplification of a promoter sequence from the β -actin gene. The specific primer pairs used in this study are listed in Table 1 or have been published previously [20, 21].

Antisera

The following commercial polyclonal antisera were used for immunofluorescence studies: goat anti-Iba-1 (1:500 dilution, Abcam, Cambridge, UK), chicken anti-Gfap (1:1000, Abcam, Cambridge, UK), mouse anti-Map2 (1:500, Sigma-Aldrich Chemie GmbH, Munich, Germany); secondary antibodies: donkey anti-goat Cy3 (1:500, Dianova, Hamburg, Germany); sheep anti-rabbit Cy3 (1:300, Sigma-Aldrich Chemie GmbH); Alexa Fluor™ 488 goat anti-rabbit (1:1000), and Alexa Fluor™ 488 donkey anti-mouse (1:500, all from Invitrogen, Life Technologies GmbH, Darmstadt, Germany); donkey anti-chicken Cy3, DyLight™ 649 donkey anti-chicken, Alexa Fluor™ 647 donkey anti-chicken (1:500), and donkey anti-mouse Cy3 (1:1000, all from Dianova).

Furthermore, a polyclonal rabbit antiserum was generated against synthetic full length rat Tff3 (59 amino acid residues; Genbank accession No. NP037174.2). Peptide synthesis was analogous as reported previously [13] except Cys-57 was not modified here. The peptide was coupled to keyhole limpet hemocyanin via glutaraldehyde and used for immunization. The corresponding antiserum was termed anti-rTff3-2 and used for immunofluorescence studies (1:200 or 1:500).

Immunofluorescence studies

All procedures were performed at room temperature. The primary cultures grown on glass cover slips were fixed with 4% paraformaldehyde, 1.35% glucose, 0.1 M lysine-HCl and 0.01 M NaIO₄ in phosphate buffer/pH 7.4 for 30 min. Cells were washed 3 times with phosphate-buffered saline (PBS) and blocked with 10% horse serum, 2% BSA, 5% sucrose and 0.2 mg/mL saponin in PBS/pH 7.4 for 1 h. Subsequently, cells were incubated with the primary antibody diluted 1:500 in the blocking solution for 1 h. After 3 times washing with PBS, cells were incubated with the secondary antibodies diluted in blocking solution for 1 h. Then, the cell nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 μ g/mL; Sigma) for 3 min. Cells were washed 2 times with PBS, once with water, and finally mounted in Mowiol 4-88.

Cells were photographed with the microscope Axio Observer.Z1 equipped with an AxioCam MRm digital camera and the AxioVision software (Release 4.8; Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Images were processed using Adobe Photoshop 7.0.

Alternatively, brains of adult female Wistar rats were embedded in O.C.T. Compound, shock frozen in liquid nitrogen, 20 μ m serial sections were obtained with a cryomicrotome and processed similarly as described previously [22]. In brief, the dried sections were fixed in absolute methanol (-20°C), encircled with a hydrophobic barrier pen (ImmEdge™ Pen, Vector Laboratories) after removing O.C.T. with a forceps, equilibrated with PBS/0.1% Triton-X-100, blocked with 3% horse serum, incubated with anti-rTff3-2 (1:200)

in 1% horse serum, and washed with PBS. The secondary antibody was a Cy3-labeled F(ab')₂ fragment of sheep anti-rabbit IgG (1:200 dilution; Sigma-Aldrich Chemie GmbH). The sections were mounted with Dako Fluorescence Mounting Medium (Dako Deutschland GmbH, Hamburg, Germany). The specificity of the Tff3 staining was tested by competition with synthetic Tff3 [13], i.e., 200 µL anti-rTff3-2 (1:200 dilution) were pre-adsorbed with 20 µg peptide overnight at 4°C. Brain slices were imaged using a confocal LSM 710 microscope (objective: Plan-Apochromat 10x/0.45 M27; z-stack: 5 slices; resolution: 1.2044 pixels per µm; voxel size: 0.8303 x 0.8303 x 5.3775 µm³). The images are maximum intensity projections processed with ZEN 2012 software (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

Results

RT-PCR analysis of rat brain primary cell cultures

First, neural as well as glial cell-enriched cultures were characterized by expression profiling using established markers for microglial cells (Iba1, ionized calcium binding adapter molecule 1) [23], astrocytes (Gfap, glial fibrillary acidic protein), and neurons/dendrites (Map2, microtubule-associated protein 2), respectively (Fig. 1). Clearly, cerebral cultures from both cortex and hippocampus contained neurons, astrocytes, and microglial cells. In contrast, glial cell-enriched cultures from cortex are nearly devoid of neurons and are particularly enriched in microglial cells. Then, Tff3 expression was tested, which was detectable in both neural as well as glial cell-enriched cultures (Fig. 1).

Immunofluorescence studies of rat brain primary cell cultures

In the next step, Tff3 peptide expression was determined in different primary cultures by the use of immunofluorescence. In order to characterize the different cell types, markers were used for microglial cells (Iba1), astrocytes (Gfap), and neurons (Map2), respectively.

A double immunofluorescence study revealed that Tff3 co-localizes with Map2 in both neural cultures from cortex (Fig. 2A) and hippocampus (Fig. 2C). This indicates that Tff3 is clearly a product of neurons. In contrast, Tff3 and Gfap did not stain corresponding cells in

Fig. 1. RT-PCR analysis. Tff3, Iba1, Gfap, and Map2 expression was monitored in neural cell cultures from cortex and hippocampus, respectively, as well as from glial cell-enriched cultures from cortex (3 parallel experiments each). The integrity of the cDNA was tested by monitoring the transcript levels for β-actin (Actb).

Fig. 2. Localization of Tff3, Map2 and Gfap in neural cell cultures from the cortex (first row) and hippocampus (second row). (A, C) Double immunofluorescence with monoclonal anti-Map2/Cy3-label (red) and polyclonal anti-rTff3-2/Alexa Fluor 488-label (green). (B, D) Double immunofluorescence with chicken anti-Gfap/Cy3-label (red) and polyclonal anti-rTff3-2/Alexa Fluor 488-label (green). Nuclei were counterstained with DAPI (blue). Scale bars: 20 µm.

Fig. 3. Localization of Tff3, Iba1 and Gfap in glial cell-enriched cultures from the cortex. Triple immunofluorescence with goat anti-Iba1/Cy3-label (red), polyclonal anti-rTff3-2/Alexa Fluor 488-label (green), and chicken anti-Gfap/DyLight 649-label (magenta). Scale bar: 20 μ m.

Fig. 4. Localization of Tff3 in sagittal sections of the adult rat cerebellum. Triple immunofluorescence with polyclonal anti-rTff3-2/Cy3-label (red), monoclonal anti-Map2/Alexa Fluor 488-label (green), and chicken polyclonal anti-Gfap/Alexa Fluor 647 (cyan). (A) immunofluorescence with anti-rTff3-2; (B) immunofluorescence of parallel section after competition with synthetic Tff3. (C, D) staining with anti-Map2 and below double staining with anti-rTff3-2. (E, F) staining with anti-Gfap and below double staining with anti-rTff3-2. The insets in A and C-F show magnifications of the rectangular areas marked. FT, fiber tract; IGL, internal granular layer; PC, Purkinje cell. Scale bar: 100 μ m.

neural cultures from cortex (Fig. 2B) and hippocampus (Fig. 2D), respectively. Thus, Tff3 is not synthesized by astrocytes.

Furthermore, a triple immunofluorescence study with glial cell-enriched cultures showed a clear co-localization of Tff3 and Iba1 (Fig. 3); whereas Gfap staining was not congruent with Tff3 staining (Fig. 3). This clearly points to a synthesis of Tff3 also in microglial cells and not in astrocytes.

Immunofluorescence studies of adult rat brain

Tff3 peptide was also localized in sections of rat brain in order to investigate the *in vivo* situation in adult animals (Fig. 4). Here, the characteristic localization in the cerebellum is shown because Tff3 transcripts are known to accumulate in this region [11]. Tff3 localizes

in the internal granular layer (IGL) of the cerebellum (Fig. 4A), which is typically recognized by strong Map2 staining of neurons (Fig. 4C). Double immunofluorescence clearly revealed co-localization of Tff3 and Map2 (Fig. 4D). Generally, Tff3 staining is more diffuse than Map2 staining, the latter typically recognizing neuronal soma and dendrites. In particular, Tff3 staining is more pronounced in the fiber tract (FT) when compared with Map2 staining. In contrast, Tff3 and Gfap do not co-localize (Fig. 4F). Thus, Tff3 is localized in cerebellar neurons of the internal granular layer but not in astrocytes. The specificity of Tff3 staining was confirmed by competition with the synthetic peptide (Fig. 4B). Staining of the internal granular layer and the fiber tract was strongly reduced, whereas the faint Tff3 staining of the Purkinje cells was not reduced after competition. This is an indication for non-specific Tff3 staining of Purkinje cells.

Discussion

Here we show for the first time that Tff3 is synthesized in different cerebral cell types *in vitro*, i.e. in neurons as well as in microglial cells. Tff3 expression in rat brain primary cultures was demonstrated on the mRNA as well as on the protein level. However, it is not known by now in which molecular form Tff3 is secreted (e.g., as a monomer, modified monomer, homodimer, or heteromer with Fcgbp etc.). However, these cultures expressed Fcgbp transcripts. It should be mentioned, that Tff1 and Tff2 transcripts were not detectable in the three different primary cultures used in this study (data not illustrated).

Tff3 is expressed in rat neurons in vitro and in vivo

The results clearly show that Tff3 peptide is localized in neurons grown in different cultures *in vitro* (E18, P2; Fig. 2) as well as, for example, in neurons of the adult cerebellum *in vivo* (Fig. 4). In the latter, Tff3 is specifically localized in neurons concentrated in the internal granular layer but not in Purkinje cell neurons, which sometimes show non-specific staining. The more diffuse Tff3 staining when compared with Map2 staining is probably due to the fact that Tff3 is a secretory peptide which eventually also becomes a component of the extracellular matrix. Such an association with the extracellular matrix could be of particular functional importance. The cerebellar expression pattern is perfectly in line with the previous RT-PCR analysis from murine brain regions [11]. Synthesis of TFF3 in neurons is also in agreement with its synthesis in magnocellular neurons of the human, rat and porcine hypothalamus and release by the neural lobe of the pituitary [12, 14, 15].

The neuronal synthesis of Tff3 could explain various behavioral effects, e.g., dose dependent anxiolytic and anxiogenic effects after injections into the rat amygdala [13], effects on object recognition memory [24], and anti-depressant-like effects [25]. Additionally, Tff3-deficient mice showed differences in their motoric skills when compared with wild type animals [26]. This could result from absence of Tff3 in the cerebellum (particularly in the internal granular layer, Fig. 4), which is the major expression site of cerebral Tff3 [11]. Taken together, Tff3 is expected to act as a neuropeptide influencing, e.g., fear, memory, depression, and motoric skills.

Tff3 is expressed in rat microglial cells in vitro

To our knowledge, this is the first report on microglial expression of Tff3. Synthesis in microglial cells is in line with its expression in lymphoid organs [27] because microglial cells represent the primary immune cells of the CNS [28-30]. Microglia have a ramified appearance in the healthy mature CNS ("resting" state); whereas after activation they adopt an amoeboid morphology [28, 29]. In cell culture, microglial cells generally enter an activated state [30]. In the brain, Iba1 is specifically expressed in microglia and is strongly upregulated in activated microglia [23]. As seen in Fig. 3, the Tff3-expressing microglial cells have an amoeboid morphology and strongly stain for Iba1 which both is consistent with an activated state. Thus, Tff3 seems to be expressed in so called activated microglial cells,

at least *in vitro*. This result is confirmed by the observation that the murine microglial cell line BV-2 also expresses Tff3 transcripts (data not illustrated). However, Tff3 expression was not inducible with lipopolysaccharide in both primary cultures and BV-2 cells (data not illustrated). Furthermore, the highly concentrated expression pattern of Tff3, e.g., in the internal granular layer of the cerebellum (Fig. 4) is not only an indication for its neuronal synthesis but also indicates that the resting microglial cells lack Tff3.

For example, Tff3 expression in activated microglial cells could be triggered after stroke and hypoxia via the hypoxia-inducible factor (Hif)-1 because the Tff3 promoter contains an Hif-responsive element [31]. Here, enhanced Tff3 expression could have a neuroprotective function when microglia is activated in the peri-ischemic area [32]. Enhanced Tff3 expression in activated microglial cells could also be controlled by the Toll-like receptor (Tlr) 2 similar as demonstrated for intestinal goblet cells [33]. TLR2 is particularly important for microglial activation following damage of sensory neurons and it is also involved in the uptake of amyloid β -protein by microglial cells [29].

Microglial cells influence numerous physiological processes including neural development, synaptic plasticity, and cognition [29, 34]. Also activated microglial cells are known to interact with neurons and participate in the events of synaptic pruning [28, 30]. Generally, all CNS diseases involve microglia [28, 30], in particular inflammatory diseases such as stroke, multiple sclerosis, neurodegenerative diseases, and after cerebral infection, e.g., with *Toxoplasma gondii*. Microglial cells also contribute fundamentally to psychiatric disorders such as the autism spectrum disorders [34]. Thus, Tff3 expression in activated microglia opens a plethora of different functions for this peptide in the brain and it will be an important aim for the future to test if Tff3 is expressed also in activated microglial cells *in vivo*.

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