Original Paper

Increased Cerebral Tff1 Expression in Two Murine Models of Neuroinflammation

Eva B. Znalesniak^a Ting Fu^a Karina Guttek^b Ulrike Händel^c Dirk Reinhold^b
Werner Hoffmann^a

^aInstitute of Molecular Biology and Medicinal Chemistry, Otto-von-Guericke University Magdeburg,

^bInstitute of Molecular and Clinical Immunology, Otto-von-Guericke University Magdeburg, ^cInstitute of Medical Microbiology and Hygiene, Otto-von-Guericke University Magdeburg, Magdeburg, Germany

Key Words

Inflammation • Inflammasome • TFF peptide • Trefoil factor • *Toxoplasma gondii* • Encephalitis • Experimental autoimmune encephalomyelitis • Multiple sclerosis

Abstract

Background/Aims: The trefoil factor family (TFF) peptide TFF1 is a typical secretory product of the gastric mucosa and a very low level of expression occurs in nearly all regions of the murine brain. TFF1 possesses a lectin activity and binding to a plethora of transmembrane glycoproteins could explain the diverse biological effects of TFF1 (e.g., anti-apoptotic effect). It was the aim to test whether TFF expression is changed during neuroinflammation.

Methods: Expression profiling was performed using semi-quantitative RT-PCR analyses in two murine models of neuroinflammation, i.e. *Toxoplasma gondii*-induced encephalitis and experimental autoimmune encephalomyelitis (EAE), the latter being the most common animal model of multiple sclerosis. Tff1 expression was also localized using RNA *in situ* hybridization histochemistry. **Results:** We report for the first time on a significant transcriptional induction in cerebral Tff1 expression in both *T. gondii*-induced encephalitis and EAE. In contrast, Tff2 and Tff3 expression were not altered. Tff1 transcripts were predominantly localized in the internal granular layer of the cerebellum indicating neuronal expression. Furthermore, also glial cells are expected to express Tff1. Characterization of both experimental models by expression profiling (e.g., inflammasome sensors, inflammatory cytokines, microglial marker Iba1, ependymin related protein 1) revealed differences concerning the expression of the inflammasome sensor Nlrp1 and interleukin 17a. **Conclusion:** The up-regulated expression of Tff1 is probably the result of a complex inflammatory process as its expression is induced by tumor necrosis factor α as well as interleukins 1 β and 17. However on the transcript level, Tff1^{KO} mice did not show any significant signs of an altered immune response after infection with *T. gondii* in comparison with the wild type animals.

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Introduction

The peptide TFF1 (formerly pS2) is a member of the trefoil factor family (TFF) and a typical secretory product of the gastric mucosa (reviews: [1, 2]). Here, it is released from

surface mucous cells together with the mucin MUC5AC [3, 4]. In the gastric antrum, TFF1 is capable of forming disulfide-linked heterodimers with gastrokine 2 [5, 6]. TFF1 is also secreted from human conjunctival goblet cells [7] as well as the human urinary tract [8]. Furthermore, a very low level of TFF1 expression is found in nearly all regions of the murine and rat brain [9-14].

Tff1-deficient (Tff1^{KO}) mice show mainly a gastric phenotype, i.e., they all spontaneously develop antropyloric adenoma and about 30% progress to carcinoma [15, 16]. This tumor suppressor function of TFF1 is in line with the observation that TFF1 reduces mammary tumor development [17]. Generally, loss of TFF1 is accompanied with an inflammatory phenotype [18] and treatment of Tff1^{KO} mice with a COX-2 inhibitor suppressed tumor growth in the antrum [19]. TFF1 expression suppressed tumor necrosis factor (TNF)- α -mediated NF- κ B activation [18] and also suppressed *Helicobacter pylori*-induced inflammation in gastric carcinogenesis [20]. Tff1^{KO} mice also show dysregulated gastric self-renewal (expanded populations of surface mucous cells in the fundic [21] and antral units [22]).

TFF1 has been reported to lower cell proliferation by delaying G1-S cell phase transition and to have an anti-apoptotic effect [23]. Furthermore, TFF1 is a motogenic factor, which probably has a function during rapid repair of the gastric mucosa by cell migration (restitution) and also during gastric self-renewal [17, 24, 25]. The motogenic effect would establish TFF1 as a chemokine, which could promote the recruitment of, e.g., immune cells as well as cancer cell invasion. Thus far, neither the precise molecular function of TFF1 has been elucidated nor a specific receptor has been characterized. However, a pH-dependent lectin activity has been reported for TFF1 responsible to bind to a *H. pylori* lipopolysaccharide [26]. Thus, TFF1 seems to play a major role in colonization of the gastric mucus by *H. pylori*. Furthermore, its lectin activity could also explain the diverse biological effects of TFF1 by binding to a plethora of transmembrane glycoproteins, as suggested for TFF2 [27, 28].

Pathologically, TFF1 is expressed in some adenocarcinomas such as breast cancer [1] and in retinoblastoma cells [29]. Furthermore, ectopic TFF1 expression has been reported during chronic intestinal ulceration [30] and other chronic inflammatory diseases; for example, TFF1 expression is induced in human chronic pancreatitis tissue [31], in the colon of infants with inflammatory bowel disease [32], in a murine asthma model in trans-differentiating Clara cells [33, 34], in the porcine colonic epithelium after infection with *Salmonella typhimurium* [35], and in the murine spleen after oral *Toxoplasma gondii* infection (ileitis model) [36]. Here, we report for the first time on a significant transcriptional induction of cerebral Tff1 expression in two murine models of neuroinflammation, i.e., *T. gondii*-induced encephalitis and experimental autoimmune encephalomyelitis (EAE), the latter being the most common animal model of multiple sclerosis (MS).

Materials and Methods

Murine T. gondii-induced encephalitis model

Two male mice heterozygous for Tff1 [15] (obtained from Dr. M.-C. Rio and Dr. C. Tomasetto, IGBMC, Illkirch, France) were crossed with 129/SvJ mice in order to establish a stable line. Homozygous offspring (i.e., wild type and Tff1^{KO} animals, respectively) were used for further experiments. Animal care and experimental procedures were performed according to legal regulations and *T. gondii* infection experiments were approved by the state authorities (Landesverwaltungsamt Sachsen-Anhalt, Halle, Germany). The animals were kept in standard cages under specific-pathogen free (spf) conditions at the animal facility of the Medical Faculty, maintained on laboratory food and tap water *ad libitum* in a regular 12 h dark/light cycle with a temperature of 22°C.

To obtain *T. gondii* cysts, NMRI mice (Harlan-Winkelmann, Borcheln, Germany) were orally infected with 5 cysts of the type II DX-strain at the age of 2-3 months, and the cysts in the brain homogenates were counted 3-5 months after infection as described previously [37]. Experimental mice (male Tff1^{KO} and corresponding wild type animals, respectively; age: 3-5 months) were intraperitoneally (i.p.) infected with a brain inoculum equivalent to 5 cysts per mouse. 4 weeks post-infection, the animals were anaesthetized

with isoflurane, transcardially perfused with 50 mL 0.9% NaCl and brain and stomach samples were collected. The left half of the brain was used for *in situ* hybridization histochemistry, whereas the right half was divided into cerebellum and remaining brain and used for RT-PCR analysis.

Murine EAE model

C57BL/6 mice were bred and housed in the animal facilities of the Medical Faculty. Animal care and experimental procedures were performed according to legal regulations and EAE induction experiments were approved by the state authorities (Landesverwaltungsamt Sachsen-Anhalt, Halle).

Induction of EAE was performed as described earlier [38]. Briefly, 5 male C57BL/6 mice (8 to 12 weeks old) were immunized subcutaneously in depots distributed over 4 spots across the flanks with 200 µg myelin oligodendrocyte glycoprotein peptide 35-55 (corresponding to mouse sequence MEVGWYRSPFSRVVHLYRNGK) in 0.2 mL emulsion consisting of equal volumes of PBS and complete Freund's adjuvant (Sigma, Taufkirchen, Germany), containing 4 mg/mL of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). 200 ng Pertussis toxin (List Biological Laboratories, Campbell, CA) were administered i.p. at days 0 and 2.

Mice were scored daily for clinical signs of EAE according to the following increasing severity scale: 0: no disease; 1: tail weakness (tail plegia); 2: hindlimb paraparesis and/or weak righting-reflex; 3: hindlimb paraplegia; 4: paraplegia with forelimb weakness or paralysis; 5: moribund animals. Mice with intermediate clinical signs were scored in 0.5 increments. The mice were killed after 21 days (4 animals reached a score of 1.5 and one animal a score of 3).

DNA and RNA extraction, PCR analysis

For genotyping the Tff1^{KO} or corresponding wild type animals, genomic DNA was isolated from tail clippings as described [36]. The primer pairs used for PCR analysis are listed in Table 1 (Neo, Tff1).

Furthermore, isolation and purification of total RNA as well as RT-PCR analysis and semi-quantitative evaluation of relative expression levels of selected genes including statistical analysis have been described [Super Script II reverse transcriptase (Thermo Fisher Scientific, Invitrogen) was used for the *T. gondii* model, whereas RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific, Fermentas Walldorf, Germany) was used for the EAE model] [36]. The cDNA was also checked for contaminating chromosomal DNA by amplification of a β-actin promoter sequence [36]. The specific primer pairs used here have been published (Actb, MB2166/MB2167; Iba1; Il1β; Il10; Il12a; Tff1; Tff3; Tnfα; [36]) or are listed in Table 1.

RNA in situ hybridization histochemistry

The brain halves were fixed in 10% neutral buffered formalin solution (Sigma Aldrich, Munich, Germany) at room temperature for 24 hours, washed with PBS followed by dehydration in an increasing series of ethanol (in nuclease free water; Ambion/Thermo Fisher Scientific, Dreieich, Germany) and finally in xylol, and embedded in paraffin.

Tissue sections (6 µm) were mounted on Superfrost Plus slides (Thermo Scientific, Dreieich, Germany), deparaffinized, rehydrated, treated with Pretreat 1, -2, -3 (Cat.-Nos. 320044, 320043, 320045) and stained with RNAscope 2.0 Kit-Red (Cat.-No. 310034; Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's protocol. Tff1 detection was performed with probes against mouse Tff1 mRNA (Cat.-No. 406041). The housekeeping gene ubiquitin C (Ubc; Cat.-No. 310771)

Table 1. Oligonucleotides used

Genes	Accession No.	Primer No.	Primer Pairs	Nucleotide Positions	Tm	Size (bp)	Intron Spanning
Cd4	NM_013488.2	MB2117 MB2118	ACCCCTTGACAGAGTGCAAA TCCTGATGCAGTGTCCCTTT	630-649 1101-1082	60°C	472	yes
Cd8a	NM_001081110.2	MB2119 MB2120	CCCGAAGTCCGAATCTTTCC CAACACGCTTTCGGCTC	200-239 806-789	60°C	587	yes
Epdr1	NM_134065.4	MB1890 MB1891	CCTGGTGTCTACGATGGTC GTCTCTGGACCGGATAACA	463-482 801-782	60°C	339	yes
Il-17a	NM_010552.3	MB2620 MB2621	ACCTCACACGAGGCACAAG GGGCCAAGGGAGTTAAAGAC	5-23 233-214	60°C	229	yes
Mnda	NM_001033450.4	MB2600 MB2601	GCCAGTGCTGCTCAAGAAAT TCTCTGTTACTTCTGATCTCTCT	49-68 528-505	60°C	480	yes
Nlrp1a	NM_001004142.2	MB2576 MB2577	CAGGCAGGTCTCCTATCAC CTTGCTTGTACGTGCTCCT	53-73 399-380	60°C	347	yes
Nlrp3	NM_145827.3	MB2584 MB2585	GGCGAGACCTCTGGGAAAAA CGTGTAGCGACTGTTGAGGT	459-478 721-702	60°C	263	yes
Nlr4	NM_001033367.3	MB2382 MB2383	CTAGGAACGTGGGACGCTTT TGGGCCAAAACATTGAGGTC	186-205 524-505	60°C	339	yes
Nlr5	NM_001033207.3	MB2608 MB2609	GGACCAAAGGTAGACTGGGC AGGCTCCCCATAGTAGGCAA	1658-1677 2156-2137	60°C	499	yes
Tff2	NM_009363.3	MB2306 MD6	CTGGTAGAGGGCGAGAAACC AATGCTGTGTCTAGCAACTG	92-111 551-532	59°C	460	yes
Neo/Tff1	Z93724.1 NC_000083.4	MB988 MB990	AGGATCTCCTGTCATCTCACCT CCCCTACTGTGCTGAGAGATG	4021-40223 2376-2396	60°C	505	targ. vect. intron2
Tff1	NC_000083.4	MB989 MB990	CCATGACTCACCTGCTTTT CCCCTACTGTGCTGAGAGATG	2182-2202 2376-2396	60°C	215	intron1 intron2

was used as a positive control, whereas the bacterial gene dihydrodipicolinate reductase (DapB; Cat.-No. 310043) was used as a negative control. The incubation and hybridization steps were performed in an HybEZ™ Oven (Advanced Cell Diagnostics, Hayward, CA, USA). The sections were counterstained with Gill 1 hematoxylin (Richard Allan Scientific/Thermo Scientific, Dreieich, Germany).

Results

Expression profiling in mouse brain after i.p. *T. gondii* infection (encephalitis model)

Cerebral Tff expression was compared between *T. gondii*-infected and non-infected animals using RT-PCR analyses and semi-quantitative evaluation (Fig. 1). As a control, the stomach of these animals was investigated. Tff1 expression was significantly induced in the cerebellum and also the remaining brain after *T. gondii* infection. In contrast, Tff2 and Tff3 expression did not change.

Fig. 1. RT-PCR analyses. (A) Tff1, Tff2, Tff3, and β -actin (Actb) expression in the cerebellum, remaining brain, and stomach of non-infected control mice and i.p. *T. gondii*-infected mice (5 wild type/WT and 5 Tff1^{KO} mice each, respectively). The number of amplification cycles is given in parentheses. (B) Semi-quantitative evaluation (standardized against β -actin; significancies are indicated by asterisks).

Fig. 2. RT-PCR analyses. (A) Nlrp3, Nlr4, Nlr5, Mnda, Iba1, Tnfa, Il1b, Il10, Il12a, Cd4, Cd8a, Epr1, and β -actin (Actb) expression in the cerebellum of non-infected control mice and i.p. *T. gondii*-infected mice (5 wild type/WT and 5 Tff1^{KO} mice each, respectively). The number of amplification cycles is given in parentheses. (B) Semi-quantitative evaluation (standardized against β -actin; significancies are indicated by asterisks).

Fig. 3. RNA *in situ* hybridization histochemistry (red) and counter-staining with hematoxylin (blue). (A) Staining of cerebellum of non-infected control mouse with probe against Tff1. (B, C, D) Staining of cerebellum of i.p. *T. gondii*-infected mouse with probes against Tff1, DapB, and Ubc, respectively. (E, F, G) Staining of stomach of non-infected control mouse with probes against Tff1, DapB, and Ubc, respectively. Scale bars: 200 μ m.

Fig. 4. RT-PCR analyses. (A) Tff1, Tff2, Tff3, and β -actin (Actb) expression in the cerebellum, remaining brain, and spinal cord of 4 control animals and 4 or 5 EAE animals, respectively (C57BL/6). The sample for spinal cord of EAE animal 3 is lacking (grey box). The scores for the clinical signs of EAE were 1.5 (animals 1-4) and 3 (animal 5), respectively. The number of amplification cycles is given in parentheses. (B) Semi-quantitative evaluation (standardized against β -actin; significancies are indicated by asterisks).

Furthermore, the expression of typical inflammatory marker genes was analyzed in the cerebellum of wild type and Tff1^{KO} mice (Fig. 2). The transcripts analyzed encode the inflammasome constituents Nlrp1a (data not shown), Nlrp3, Nlrp6 (data not shown), Nlrp4, Nlrp5, and Mnda, the ionized calcium binding adapter protein 1 (Iba1), the cytokine Tnf α , the interleukins Il1 β , Il10, Il12a, and Il17a (data not shown) as well as the differentiation antigens Cd4 and Cd8a. Additionally, the expression of ependymin related protein 1 (Epdr1) was evaluated, because it is down-regulated in a murine asthma model [33]. With exception of Epdr1, all genes outlined in Fig. 2 showed a significant increased expression after *T. gondii* infection. However, there was no significant difference in the expression levels between wild type and Tff1^{KO} mice. Nlrp1a was not detected, Nlrp6 did not change significantly, and Il17a was barely detected after *T. gondii* infection.

Tff1 in situ hybridization of cerebellum after i.p. T. gondii infection (encephalitis model)

In situ hybridization was performed in order to localize the *T. gondii*-induced Tff1 expression in the cerebellum on a cellular level (Fig. 3). Tff1 transcripts were predominantly localized in the internal granular layer of *T. gondii*-infected animals (Fig. 3B); in contrast, the molecular layer and the fiber tracts showed a much weaker Tff1 expression (Fig. 3B). Tff1 expression was not detectable in non-infected control animals (Fig. 3A).

The specificity of the *in situ* hybridization was tested by using the bacterial gene DapB as a negative control and Ubc as a positive control. As expected, DapB was negative (Fig. 3C), whereas the Ubc probe showed a positive signal in all regions of the cerebellum (Fig. 3D). As additional control for the specificity of the Tff1 staining, stomach tissue was hybridized. In agreement with previous data, Tff1 expression was detectable in the surface epithelium (Fig. 3E). The negative control DapB did not result in a signal (Fig. 3F), whereas the positive control Ubc stained all gastric cells (Fig. 3G).

Fig. 5. RT-PCR analyses. (A) Nlrp1a, Nlrp3, Nlrp4, Nlrp5, Mnda, Iba1, Tnfa, Il1b, Il17a, Cd4, Cd8a, Epr1, and β -actin (Actb) expression was monitored in the cerebellum and spinal cord of 4 control animals and 4 or 5 EAE animals, respectively (C57BL/6). The number of amplification cycles is given in parentheses. (B) Semi-quantitative evaluation (standardized against β -actin; significancies are indicated by asterisks).

Expression profiling in mouse brain and spinal cord after EAE induction

RT-PCR analysis and semi-quantitative evaluation of Tff1 expression levels revealed a significant increase in the cerebellum, the remaining brain and the spinal cord of EAE animals when compared with the controls (Fig. 4). Furthermore, the expression of typical inflammatory marker genes (similar as in Fig. 2) was significantly increased in the cerebellum as well as the spinal cord of EAE animals (Fig. 5). Only Epr1 expression did not change.

Discussion

Cerebral Tff1 expression is induced in two models of neuroinflammation

Here, we show for the first time that Tff1 expression is significantly induced in the murine brain during neuroinflammatory processes. In sharp contrast, Tff2 and Tff3 are not up-regulated. The increase in Tff1 expression is demonstrated in models of *T. gondii*-induced encephalitis as well as EAE.

In the *T. gondii* model, induced cerebellar Tff1 expression is localized predominantly to the internal granular layer (Fig. 3B) which is a strong indication that Tff1 is transcribed in neurons. This would be in line with reports on neuronal Tff1 expression in the mouse [14] and rat [13]. A neuronal Tff1 expression is also reminiscent to that of Tff3 in the rat cerebellum [39]. Furthermore, to lower extent, induced widespread cerebellar Tff1 expression is also detectable in the molecular layer and the fiber tracts (Fig. 3B). This points towards a Tff1 expression in astrocytes and activated microglial cells, the latter representing the primary immune cells of the CNS [40], which are known for their up-regulated synthesis of ionized calcium binding adapter protein 1 (Iba1) [41]. Indeed, Iba1 is one of the strongest up-regulated genes after *T. gondii* infection (Fig. 2). Of note, also Tff3 is expressed in activated microglial cells [39]. Tff1 expression by astrocytes would be in line with previous reports [9, 42].

The up-regulated Tff1 expression after *T. gondii* infection is probably the result of a complex inflammatory process. A primary response of the CNS is the formation of inflammasomes (e.g., in microglia, but also in neurons and astrocytes), the activation of caspase-1, and the release of IL1b and IL18 (reviews: [43, 44]). Then, IL1b and IL18 signal through their respective receptors (present on microglia, astrocytes and neurons)

and trigger NF- κ B-dependent complex transcriptional events [44]. Major components of inflammasomes are certain intracellular pattern recognition receptors (PRRs), which function as inflammasome sensors. In agreement with this model, the PRRs Nlrp3, Nlr4, Nlr5, and Mnda are significantly up-regulated after *T. gondii* infection (Fig. 2). In contrast, Nlrp6 is not up-regulated (data not shown). Of note, the PRRs Nlrp1a, Nlrp1b, and Nlrp1c are not detectable in Tff1^{KO} mice and the corresponding wild type animals (data not shown). The induction of a complex set of pro- and anti-inflammatory cytokines such as Tnf α , IL1 β , IL10, and IL12a is a clear sign for the ongoing severe inflammatory process after *T. gondii* infection. Induction of Iba1 is a clear sign for the activation of microglial cells [41]. Increased expression of the anti-inflammatory cytokine IL10 is due to stimulation of astrocytes, whereas Cd4 and Cd8a expression probably result from infiltrating T cells. Induction of cerebral Tff1 expression in the *T. gondii*-infected mice could well be a consequence of Tnf α and IL1 β secretion because these two cytokines are capable of inducing transcriptional Tff1 expression via NF- κ B [45]. A similar up-regulated expression of Tff1 has also been reported in cultured mouse astrocytes after induction with TNF α , but also IL6 and IL7 [42]. The up-regulation of Tff1 could also result from a FoxA (formerly: hepatocyte nuclear factor 3) binding site (motif IV), which is conserved in human and mouse near the TATA box [46-48]. The expression of FoxA is known to be up-regulated particularly by inflammatory cytokines [49]. Of note, FoxA1 and FoxA2 bind predominantly to the site in TFF1 and to lesser extent to sites in TFF2 and TFF3 [46].

A slightly different picture emerges in the EAE model. Here, the PRRs Nlrp3, Nlr4, Nlr5, and Mnda are significantly up-regulated again in EAE animals (Fig. 5). However, and in contrast to the *T. gondii* model, also Nlrp1a is significantly up-regulated in the EAE model. The reason is probably the variation in Nlrp1 in the different mouse strains [44] used for the two models (129/SvJ versus C57BL/6). Nlrp1a and Nlrp1c expression is lacking in certain 129S1 mouse strains (when compared with C57BL/6) and allelic variants in Nlrp1b control susceptibility to anthrax lethal toxin [50]. Of special interest, at least in the rat Nlrp1 controls not only susceptibility to the anthrax lethal toxin, but also to *T. gondii* [51]. Furthermore, several inflammatory cytokines such as Tnf α , IL1 β , IL10, and IL12a are induced in EAE animals similar to *T. gondii*-infected animals. Again, Iba1 is one of the strongest up-regulated genes indicative of activated microglial cells [41]. Of special note, a characteristic difference to the *T. gondii*-induced encephalitis model is observed because only the EAE animals show significant expression of the pro-inflammatory cytokine IL17a (Fig. 5). This result is in line with the observation that the occurrence of Th17 cells and expression of IL17 is a characteristic feature of inflammation in MS [52]. As a consequence, also IL17 might trigger transcriptional activation of the TFF1 gene via the transcription factors NF- κ B, ATF2, and AML1 [53].

In both neuroinflammatory models cerebral Epdr1 expression is not significantly changed when compared with the controls (Figs. 2, 5). In contrast, in the murine lung Epdr1 is down-regulated during *Aspergillus fumigatus*-induced inflammation [33]. Epdr1 (previously termed Merp2) probably encodes a lysosomal protein homologous to human UCC1/MERP1 [54]. MERPs/EPDRs share similarity to piscine endopodins, which represent meningeal secretory proteins involved in neuroplasticity and neuronal regeneration (review: [55]).

In conclusion, both neuroinflammatory models share similarities concerning the expression of certain inflammasome sensors (induction of Nlrp3, Nlr4, Nlr5, Mnda), several cytokines and particularly induction of Tff1. A major difference is the specific expression of IL17 in the EAE animals only. In the two neuroinflammatory models, Tff1 (and not Tff2 and Tff3) expression is specifically induced probably due to up-regulation of TNF α , IL1 β , and (in the EAE model) IL17a. This specific induction of TFF1 expression is in line with other inflammatory models [33, 35, 36] and might be regulated at least partially by FoxA. The function of TFF1 in the murine brain is not known thus far and a protective effect is within the limits of expectation. For example, the anti-apoptotic effect of TFF1 [23] might reduce inflammation-induced neurodegeneration [52]. Cerebral TFF1 expression could also suppress TNF α -mediated NF- κ B activation and could act as an anti-inflammatory

agent in the brain [20]. However, Tff1^{KO} mice did not show any obvious signs of an altered inflammatory response after infection with *T. gondii* in comparison with wild type animals (Fig. 2). For the future, it would be interesting to investigate whether TFF1 is induced also in human neuroinflammatory diseases such as MS.

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Disclosure Statement

The authors declare no conflict of interest.

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