CROATICA CHEMICA ACTA
CCACAA **81** (1) 113–117 (2008)

ISSN-0011-1643
CCA-3219
Original Scientific Paper

Novel Expression Patterns for Trefoil Peptides: Presence of Tff2 and Tff3 in Rodent Cochlea

Maria Lubka,[a,*] Carsten M. Pusch,[a] Nikolaus Blin,[a] and Mirela Baus-Lončar[b]

[a]*Division of Molecular Genetics, University of Tübingen, Wilhelmstr. 27, D-72074 Tübingen, Germany*

[b]*School of Medicine, Josip Juraj Strossmayer University of Osijek, Josipa Huttlera 4, HR-31000 Osijek, Croatia*

RECEIVED MARCH 1, 2007; REVISED JULY 12; ACCEPTED JULY 23, 2007

Trefoil peptides: Tff1, Tff2 and Tff3, assigned to the so-called trefoil factor family (TFF), are secretory proteins predominantly expressed by the gastrointestinal (GI) epithelial cells. Their multiple protective functions include an important role in immune response and apoptosis. Here, a novel localization of Tff2 and Tff3 in the cochlea of mouse inner ear is presented. *Tffs* expression and localization in mouse cochlea was analyzed by using the quantitative real time PCR (qPCR) and immunohistochemistry. *Tff2* and *Tff3* mRNA quantification by qPCR showed their presence in the cochlea. Immunohistochemistry demonstrated the presence of Tff2 in the spiral ligament and limbus and for Tff3 in the spiral ligament and the organ of Corti. These new and surprising findings of Tff's presence in the cochlea indicate a new role of TFFs connected with the organ of hearing which still needs to be physiologically evaluated.

Keywords
trefoil peptides
inner ear
cochlea
immune response
inflammation

INTRODUCTION

To hear, our ears must capture mechanical energy that is carried by sound, transmit it to the ear's receptive organ, and transduce it into electrical signals suitable for analysis by the nervous system. These three tasks are the functions of the external ear, the middle ear, and the inner ear (cochlea). Cochlea (Figure 1) is the snail-shaped receptor organ of the inner ear which transduces sound energy into electrical signals and forwards them to the brain.[1] Three types of epithelia are present in the cochlea: sensory epithelia (organ of Corti), ion transporting epithelia (stria vascularis and dark cell regions of the vestibular system) and relatively unspecialized epithelia (Reissner's membrane and epithelia forming permeability barriers).[2] Defense of the inner ear against infection occurs through immune response where inner cells begin to secrete chemical attractants and recruit inflammatory cells.[3,4] Low molecular weight proteins, secreted by mucin-producing epithelia cells, known as trefoil peptides (TFFs)[5] are involved in immune response in different ways. They participate in the mucosal immune response by stimulating immunocyte migration[6] and are considered as a novel class of cytokines that modulate immune system.[7,8] TFFs are predominantly expressed in GI tract but also in other tissues as the respiratory tract, salivary glands, uterus, conjunctiva and even brain.[9] The physiological functions of trefoils are multiple. They enhance mucosal healing by promoting migration of epithelial cells whereas their expression in the brain suggests their possible function as neuropeptides.[10] Their presence and possible role in the organ of hearing have not been analyzed so far due to

* Author to whom correspondence should be addressed. (E-mail: marialubka@yahoo.com)

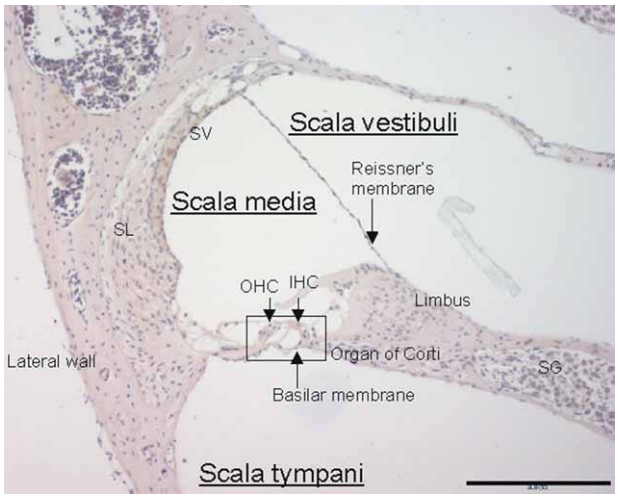

Figure 1. A paraffin section of the mouse cochlea. The three fluid-filled spaces of scala vestibuli, scala media and scala tympani are separated from each other by Reissner's membrane and basilar membrane. The stria vascularis (SV) and spiral ligament (SL) lie close to the lateral wall of the cochlea. The organ of Corti (in a frame) contains one inner hair cell (IHC), three outer hair cells (OHC) and supporting cells. Spiral ganglion (SG) is in the modiolus. Bar: 200 μm.

complicated procedure of cochlea preparation. Considering the role of trefoils in immune response regulation and the existence of highly specialized epithelium in the inner ear we wanted to analyse the presence of trefoils in the inner ear.

We have detected expression of Tff2 and Tff3 proteins in the mouse inner ear by demonstrating the presence of Tff2 mainly in the spiral ligament and Tff3 in the organ of Corti of the cochlea. The role of trefoils in the inner ear still needs to be evaluated. Growing evidence supports the concept that immune reactions occur in the cochlea, where they can function either in protection or as a source of inflammation.[11] Recently published data about the role of trefoils (mostly TFF2 and TFF3) in immune defense[7,8] may indicate their possible role for the inner ear immunity.

EXPERIMENTAL

RNA Isolation and Quantitative Real Time PCR (qPCR)

After micro-homogenisation (B. Braun Biotech International) of various mouse tissues from 5 animals, total RNA was isolated from colon and cochlea of *Tff3* wild-type/knock-out mice, from stomach and cochlea of *Tff2* wild-type/knock-out mice with the RNeasy Mini Kit (Qiagen), and transcribed into cDNA (iScript cDNA Synthesis Kit; Bio-Rad). Quantitative Real Time PCR (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) on an iCycler (Bio-Rad). Specificity and efficiency of PCR reactions were verified by using the melting curve method and gel electrophoresis respectively. Comparison of melting curve profiles confirmed that a weak signal in knock-out animals (cycle threshold, Ct, was about 32) is an artefact. PCR was performed using the specific primer pair for *Tff3* (mTff3_3F: 5'-AACCGTGGCTGCTGCTTT-3' and mTff3_3R: 5'-CCTGGAGCCTGGACAGCTT-3') and *Tff2* (mTff2_3F: 5'-CTTGGTGTTTCCACCCACTT-3' and mTff2_3R: 5'-CACCAGGGCACTTCAAAGAT-3'). Since the expression of *GAPDH* was stable in stomach, colon and cochlea we used it as an internal control detected by following primer pair: mGAPDH_4F: 5'-AACGACCCCTTCATTGAC-3' and mGAPDH_4R: 5'- TCCACGACATACTCAGCAC-3'. The PCR conditions were: denaturation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 25 s, 58 °C for 20 s and 72 °C for 10 s, ended with 95 °C for 1 min. Data were calculated by using the REST-mcs-beta program.[12]

Tissue Preparation

The cochleae were rapidly removed, fixed by immersion for 2 hours in 2 % paraformaldehyde and cryosectioned as described by Knipper *et al.*[13] Care and use of the animals and the experimental protocol were reviewed and approved by the animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

Immunostaining Procedure

Immunohistological analyses were performed from cochlea samples taken from three to five wild-type mice at day (P22). Formalin-fixed paraffin-embedded specimens of mouse cochleae were used. Five-micron sections were cut from the paraffin blocks, routinely processed through xylene and alcohol gradient, treated with methanolic hydrogen peroxide to eliminate the endogenous peroxidase activity, and then immunostained. We used polyclonal antibodies that were immunopurified to eliminate the unspecific staining. To reduce background staining sections were treated with normal goat serum 1:20 (Vector Laboratories). After being rinsed in PBS + 0.05 % Tween, sections were incubated overnight at +4 °C with primary antibody rabbit anti-mouse Tff2 (dil. 1:400) or rabbit anti-human Tff3 (dil. 1:10000). Sections were washed with PBS + 0.05 % Tween before biotinylated secondary goat anti-rabbit antibody was applied for 35 min at room temperature, followed by extensive PBS + 0.05 % Tween rinses and incubation with streptavidine and horse radish peroxidase (dil. 1:400) for 35 min at room temperature. Specificity of staining was confirmed by including controls were we preincubated antibodies with their specific peptides. Immunohistochemical signals were visualized with 0.5 mg ml^{-1} 3',3-diaminobenzidine (DAB, Sigma-Aldrich) in 0.1 mol dm^{-3} PBS and 0.01 % H_2O_2. Sections were imaged using an Olympus AX-70 microscope. Representative images were chosen for presentation.

RESULTS

TFF2 *and* TFF3 *Expression in the Cochlea*

Quantitative real time PCR (qPCR) revealed that *Tff2* and *Tff3* are expressed in the cochlea (Ct value was 27 and

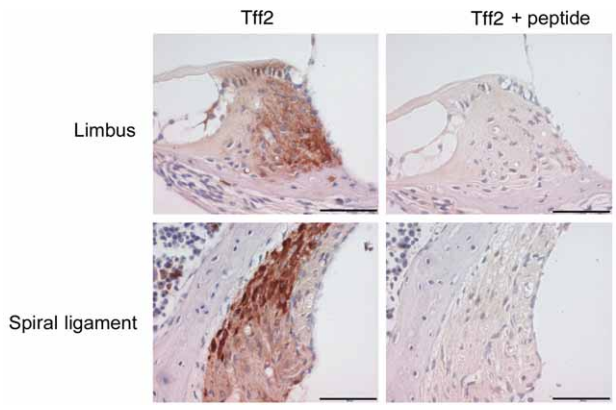

Figure 2. Immunohistochemical localization of Tff2. A positive staining for Tff2 protein was noticed in fibrocytes of spiral ligament and limbus (left). Immunostaining was blocked with the specific peptide (right). Bar: 50 μm.

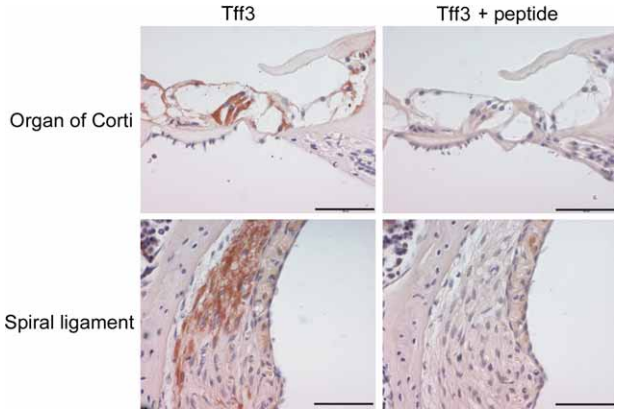

Figure 3. Immunohistochemical localization of Tff3. Tff3 was expressed in the organ of Corti and spiral ligament (left). Immunostaining was blocked with the specific peptide (right). Bar: 50 μm.

28 respectively) of adult mouse beside their presence in control tissues: stomach and colon (Ct value was 13 and 15 respectively). No expression was found in tissues of corresponding knock-out animals (Ct value ≈ 32). The expression of *GAPDH* was stable in the stomach, colon and cochlea (Ct values were 15–16) (Table I).

Localization of Tff2 and Tff3 Proteins in the Cochlea

Our immunohistochemical data demonstrated for the first time an expression pattern for Tff2 and Tff3 proteins in the cochlear tissues of adult mice. Immunochemistry revealed Tff2 (Figure 2) and Tff3 staining in various areas of the cochlea (Figure 3). The highest expression for Tff2 was detected in the spiral ligament and limbus and for Tff3 in the spiral ligament and in the organ of Corti. The protein-antibody interaction was completely abolished upon pre-incubation with synthetic Tff2 and Tff3 peptide (Figure 2 and Figure 3).

DISCUSSION

TFFs are multifunctional peptides and since 1989 it has been well documented that TFFs are expressed along the length of the normal GI tract where they maintain epithelial surface integrity[5] and in various other tissues.[9] Here for the first time we have identified the Tffs' presence in the specific sections of the mouse inner ear. Tff2 is present in the spiral ligament and limbus while Tff3 is located in the spiral ligament and the organ of Corti of the mouse inner ear. Similar situation was noted for the rat inner ear (data not shown). Tffs have multiple roles in the organism. They can serve as motogens, are linked to anti-apoptosis, can trigger chemotaxis and could be involved in neoplastic development.[5] Their presence in the inner ear could have an immune response related role.

It is known that expression of TFFs is modulated (mainly up-regulated) in response to inflammation, injury and repair.[5] TFF2 is expressed in immune response relevant tissues like spleen, lymph nodes, bone marrow,[6] peritoneal exudate cells (PECs), and primarily macrophages.[7] Recent data showing that Tff2 deficiency affects expression of the immune response relevant genes indicates that Tff2 is involved in some aspects of immune response.[8] TFF2 functions also as an anti-inflammatory peptide with specificity for IL-1β signaling and immune cell cytokine secretion. These data raised the possibility that Tff2 could regulate inflammation by directly affecting mononuclear cells (lymphocytes and macrophages) in inflammatory infiltrates.[7]

TABLE I. Expression of *Tff2* and *Tff3* mRNA in mouse tissues studied by quantitative real time PCR[(a)]

Tissue	*Tff2* Fold change	Tissue	*Tff3* Fold change
Stomach wildtype	1	Colon wildtype	1
Stomach knockout	–322737	Colon knockout	–24833
Cochlea wildtype	–9674	Cochlea wildtype	–3717
Cochlea knockout	–1123835	Cochlea knockout	–114104

[(a)] Expression is given with respect to the expression level in stomach or colon of normal mice (set to 1).

Uncontrolled prolonged inflammatory reactions cause tissue damages as a consequence of biologically active molecules released by activated inflammatory cells. Inflammation occurs also in the inner ear where may have been responsible for the hearing loss caused by degeneration of the organ of Corti, stria vascularis, spiral ganglion cells, spiral ligament and limbus.[14] Fibrocytes of the spiral ligament play an important role in the inner ear homeostasis.[15] They are also involved in the innate immune response of the inner ear by producing chemoattractants for recruiting inflammatory cells such as neutrophils and monocytes.[16] *In vitro*, fibrocytes secrete chemokines after stimulation by the pro-inflammatory cytokines TNF-α and IL-1β demonstrating their ability to respond to signals used by leukocytes for cell-cell signaling.[17] The same cytokines that are produced during wound healing (TNF-α, IL-1β and IL-6)[18] are also produced by cochlear cells in the early response to acoustic trauma (noise-induced hearing loss).[19] Macrophages contribute to wound healing through the secretion of cytokines and growth factors such as TNF-α, IL-1β, IL-6, TGF-α and TGF-β, and insulin like growth factor-1.[18] It seems that fibrocytes and macrophages function together to mediate cochlear repair following trauma. Immune response crucial modulating transcription factor NF-κB is commonly found in the inner ear, demonstrating the capability of the cochlea to respond to immunostimulation.[20] TFFs expression is strongly controlled by NF-κB[21] and downregulated in case of prolonged inflammation. The presence of Tff2 protein in the fibrocytes together with the knowledge about possible role of fibrocytes and Tff2 in immune regulation may indicate that TFFs may play an important role in the inner ear immune response.

A number of studies suggest that nitric oxide (NO) plays an essential role in the pathophysiology of the inner ear including inflammation and immune response.[22] TFFs are able to modulate production of NO.[23,24] In acute injury NO is produced by constitutively active nitric oxide synthase (cNOS) and it has beneficial effect by increasing blood flow and releasing various local repair mediators.[25] In chronic inflammatory states, NO is generated by inducible nitric oxide synthase (iNOS) within infiltrating macrophages and neutrophils[26] leading to production of highly damaging reactive nitrogen species that induce further inflammation, leading to ongoing tissues injury.[27] It is shown that TFF3 stimulates NO production[23] while TFF2 is able to inhibit LPS-induced NO production in a monocyte cell line.[24] In addition to its role in immune response[28] NO can also regulate intracellular Ca^{2+}-concentrations in the inner and outer hair cells, thus influencing their mechanical properties as well as neurotransmission at synapses of the auditory nerve. It may also interfere with the metabolism of secretory cells in the stria vascularis by influencing ionic pumps. The findings that NO can induce apoptosis in the outer hair cells of the organ of Corti suggests the possibility that the cochlea is affected when extra NO release occurs there, leading to cochlear dysfunction.[29] Apoptosis appears to play a critical role in the pathogenesis of hearing loss. It is known that caspases 8, 9, and 3 are involved in the apoptosis of physically damaged hair cells[30] and caspases 5 and 6 are suggested to participate[31] while caspases 7 and 10 are thought to be involved in the process of apoptosis of physical trauma-damaged hair cells.[32] It is known that trefoil peptides play an anti-apoptotic role[15,33,34] what may suggest their role in preventing apoptosis in the organ of hearing.

It is interesting to note that our knowledge about these primarily gastrointestinal factors is growing and bringing new, important facts about their function. We present new data about the surprising presence of trefoil peptides in the inner ear. The role of TFFs is evolving from that simple secretory proteins interacting with mucins towards more complex proteins involved in various aspects of inflammatory reactions, anti-apoptotic mechanism, and possibly in physiology of cells in specialized organs like cochlea. The exact role of Tffs in the cochlea still has to be elucidated and the present data about their antiapoptotic and immunomodulating[7,13] role in the gastrointestinal tract as a predominant site of expression could be a good guide.

Acknowledgements. – We thank the Ministry of Science, Education and Sports of the Republic of Croatia for PhD fellowship awarded to M. Baus-Lončar and to Leonardo da Vinci Programme for scholarship awarded to Maria Lubka. Part of the work was supported by bilateral Croatian-German program supported by the Croatian Ministry of Science, Education and Sports and German Academic Exchange service (Project number: D/06/04760).

REFERENCES

1. R. Nagashima, C. Sugiyama, M. Yoneyama, and K. Ogita, *J. Pharmacol. Sci.* **99** (2005) 301–306.
2. A. Forge and T. Wright, *Br. Med. Bull.* **63** (2002) 5–24.
3. I. Ichimiya, K. Yoshida, T. Hirano, M. Suzuki, and G. Mogi, *Int. J. Pediatr. Otorhinolaryngol.* **56** (2000) 45–51.
4. E. R. Kandel, J. H. Schwartz, and T. M. Jessell, *Principles of Neural Science*, McGraw Hill Professional, International Edition, 2000, pp. 590–613.
5. N. Blin. *Cell. Mol. Life Sci.* **62** (2005) 2907–2909.
6. G. A. Cook, M. Familari, L. Thim, and A. S. Giraud, *FEBS Lett.* **456** (1999) 155–159.
7. E. A. Kurt-Jones, L. Cao, F. Sandor, A. B. Rogers, M. T. Whary, P. R. Nambiar, A. Cerny, G. Bowen, J. Yan, S. Takaishi, A. L. Chi, G. Reed, J. Houghton, J. G. Fox, and T. C. Wang, *Infect. Immun*. **75** (2007) 471–480.
8. M. Baus-Loncar, T. Kayademir, S. Takaishi, and T. Wang, *Cell Mol. Life. Sci.* **62** (2005) 2947–2955.
9. W. Hoffmann, W. Jagla, and A. Wiede, *Histol. Histopathol.* **16** (2001) 319–334.

10. M. Hinz, H. Schwegler, C. E. Chwieralski, G. Laube, R. Linke, W. Pohle, and W. Hoffmann, *Peptides* **25** (2004) 827–832.
11. B. Gloddek, D. Bodmer, D. Brors, E. M. Keithley, and A. F. Ryan, *Audiol. Neuro-otol*. **7** (2002) 317–23.
12. M. W. Pfaffl, G. W. Horgan, and L. Dempfle, *Nucleic Acids Res.* **30** (2002) E36.
13. M. Knipper, I. Kopschall, K. Rohbock, A. K. E. Koepke, I. Bonk, U. Zimmermann, and H. P. Zenner, *Cell Tissue Res.* **287** (1997) 23–41.
14. S. J. Ono, T. Nakamura, D. Miyazaki, M. Ohbayashi, M. Dawson, and M. Toda, *J. Allergy Clin. Immunol.* **111** (2003) 1185–1199.
15. S. Spicer and B. A. Schulte. *Hearing Res.* **56** (1991) 53–64.
16. H. Yamane, M. Takayama, K. Sunami, H. Iguhi, A. Kanazawa, Y. Tokuhara, H. Nishiura, K. Nishimura, and S. D. Tao, *Acta Otolaryngol.* **554** (2004) Suppl., 6–11.
17. J. E. Park and A. Barbul, *Am. J. Surg.* **187** (2004) 11S–16S.
18. M. Fujioka, S. Kanzaki, H. J. Okano, M. Masuda, K. Ogawa, and H. Okano, *J. Neurosci. Res.* **83** (2006) 575–583.
19. B. A. Ghaheri, J. B. Kempton, D. A. Pillers, and D. R. Trune, *Laryngoscope* **117** (2007) 22–29.
20. M. Takahashi and J. P. Harris, *Laryngoscope* **98** (1988) 1133–1138.
21. M. B. Loncar, E.-D. Al-azzeh, P. S. M. Sommer, M. Marinovic, K. Schmehl, M. Kruschewski, N. Blin, R. Stohwasser, P. Gött, T. Kayademir, *Gut* **52** (2003) 1297–1303.
22. R. Popa, M. Anniko, M. Takumida, and W. Arnold, *Acta Otolaryngol.* **121** (2001) 454–459.
23. A. S. Giraud, P. M Pereira, L. Thim, L. M. Parker, and L. M. Judd, *Peptides* **25** (2004) 803–809.
24. E. N. Lalani, R. Williams, Y. Jayaram, C. Gilbert, K. S. Chaudhary, L. S. Siu, A. Koumarianou, R. Playford, and G. W. Stamp, *Lab. Invest.* **79** (1999) 537–546.
25. J. L. Wallace and M. J. Miller, *Gastroenterology* **119** (2000) 512–520.
26. J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman, *Proc. Natl. Acad. Sci. USA* **87** (1990) 1620–1624.
27. X. D. Tan, Q. P. Liu, W. Hsueh, Y. H. Chen, H. Chang, and F. Gonzalez-Crussi, *Biochem. J.* **338** (1999) 745–751.
28. K. Gosepath, U.R. Heinrich, U. Ecke, J. Maurer, R. Amedee, and W. J. Mann, *Eur. Arch. Otorhinolaryngol.* **257** (2000) 418–424.
29. D. R. Taupin, K. Kinoshita, and D. K. Podolsky, *Proc. Natl. Acad. Sci. USA* **97** (2000) 799–804.
30. K. Do, K. Baker, M. Praetorius, and H. Staecker. *Internat. Cong. Ser.* **1273** (2004) 167–170.
31. T. R. Van De Water, F. Lallemend, A. A. Eshraghi, S. Ahsan, J. He, J. Guzman, M. Polak, B. Malgrange, P. P. Lefebvre, H. Staecker, and T. J. Balkany, *Otol. Neurotol.* **25** (2004) 627–632.
32. S. K. Moon, R. Park, H. Y. Lee, G. J. Nam, K. Cha, A. Andalibi, and D. J. Lim. *Acta Otolaryngol.* **126** (2006) 564–569.
33. Y. H. Chen, Y. Lu, I. G. De Plaen, L. Y. Wang, and X. D. Tan, *Biochem. Biophys. Res. Commun.* **274** (2000) 576–582.
34. H. Mashimo, D. C. Wu, D. K. Podolsky, and M. C. Fishman. *Science* **274** (1996) 262–265.

SAŽETAK

Novootkrivena lokalizacija TFF proteina s domenom trostruke omče: prisutnost Tff2 i Tff3 u pužnici glodavaca

Maria Lubka, Carsten M. Pusch, Nikolaus Blin i Mirela Baus-Lončar

TFF proteini: Tff1, Tff2 i Tff3 pripadaju takozvanoj TFF obitelji proteina (engl. *Trefoil Factor Family*) sa strukturnom domenom sličnom listu djeteline (engl. *trefoil*). Tff proteini su predominantno prisutni u probavnom traktu kao sekretorni produkt epitelnih stanica probavnoga trakta. Zaštitna je uloga Tff proteina višestruka i uključuje sudjelovanje u imunosnom odgovoru i apoptozi. U ovom je radu prikazana novodokazana prisutnost Tff proteina u senzornom dijelu unutrašnjeg uha miša, tj. u pužnici. Prisutnost i raspodjela Tff proteina u pužnici praćena je pomoću kvantitative reakcije lančane polimeraze (qPCR) i imunohiostokemijski. qPCR metoda pokazala je prisutnost Tff2 i Tff3 glasničke RNA u pužnici, a imunohistokemijskom metodom je pokazana prisutnost Tff2 u spiralnom ligamentu i limbusu, tj. za Tff3 u spiralnom ligamentu i Kortijevu organu. Ova iznenađujuća prisutnost Tff proteina u pužnici upućuje na novu ulogu Tff proteina povezanu s organom sluha koja zahtijeva daljnje fiziološko pojašnjenje.