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Research article

Expression of serotonin 2A, 2C, 6 and 7 receptor and IL-6 mRNA in experimental toxoplasmic encephalitis in mice

Hasan Tarik Atmaca *

Balikesir University, Faculty of Veterinary Medicine, Department of Pathology, Balikesir, Turkey

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ABSTRACT

The neurotropic pathogen *Toxoplasma gondii* infects about one-third of the human population. Both acute and chronic (latent or life-long) forms of toxoplasmosis are associated with specific neurologic and neuropsychiatric symptoms. In the present study, swiss albino mice were inoculated intraperitoneally with 15–20 tissue cysts of the ME-49 strain of *Toxoplasma gondii*. The brain samples were collected on the days 10, 20, and 30 for determining the histopathological scores and the number of cysts. Furthermore, a real-time quantitative polymerase chain reaction (RT-PCR) was conducted to find out the gene expression levels of the serotonin 2A receptor (5-HTR2A), serotonin 2C receptor (5-HTR2C), serotonin 6 receptor (5-HTR6), serotonin 7 receptor (5-HTR7), and interleukin-6. The results were compared to the histopathological findings of encephalitic toxoplasmosis. The expression levels were observed to increase for all receptors; however at different time points of infection. This experimental model demonstrates that the expression of serotonin receptors was induced in *Toxoplasma gondii* infections, displaying unique findings for each of the individual receptors.

1. Introduction

Toxoplasma gondii is a neurotropic protozoan that is found worldwide. According to the statistical data, almost one-third of human beings in the world are infected with *Toxoplasma gondii*. After acquiring *Toxoplasma gondii*, overt disease symptoms may not develop at all or they may become manifest in patients with a compromised immune system. When the parasite is ingested by an immediate host, it moves from the intestines to other organs in the body. Finally, the parasite lodges in the brain and muscles. In the brain, the parasites hide within neurons and glial cells as tissue cysts. These cystic structures tend to have reduced exposure to cellular, as well as molecular, mediators of the immune system; which fight the infection with success, but fail in its removal. Latent chronic infections commonly occur in persons with a weak immune system. Several studies have recently revealed that; in a population of individuals with a strong immune system, *Toxoplasma gondii* seropositivity is linked with mental and behavioral disorders including schizophrenia and suicidal attempts [1, 2, 3]. Results of animal experiments led to the development of the behavioral manipulation theory due to *Toxoplasma gondii*, proposing that the *Toxoplasma gondii* cysts in the central nervous system (CNS) control behaviors of the host to enhance the rates of transmission [4, 5, 6, 7]. The associated behavioral changes with the *Toxoplasma gondii* infection include enhanced novelty seeking, delayed reaction time, and attraction to predator odors. These alterations in the behavior are linked to chronic latent *Toxoplasma gondii* infections in the neurons, manifested with glial cysts, disruptions in the serotonin, norepinephrine, and dopamine systems, and local inflammatory processes in the brain [6]. The host's immune responses to *Toxoplasma gondii* infections result in the synthesis of proinflammatory cytokines like Tumor Necrosis Factor (TNF) and IL-6, which activate the T helper (Th) cells. Th cells secrete IFN-Gamma, which inhibits the growth of *Toxoplasma gondii* via induction of IDO enzyme activity. Consequently, this activation leads to depletion of tryptophan reserves, causing a decrease in serotonin synthesis in the brain [1, 8, 9, 10].

Specific analyses of cDNA libraries based on conserved sequences in the known receptors have disconcertedly demonstrated that the number of established 5-HT receptors was on a significant rise. A variety of fourteen receptor subtypes of serotonin (5-HT), classified in seven families, has been established [11], where the receptors do not include proteins synthesized via alternative splicing single gene transcripts. Among the available neurotransmitters, 5-HT is known to act on a highly diversified class of receptors. Even though 5-HT is produced only by a small group of neuronal cells within the brain stem's raphe nuclei, this small group of cells sends a number of both ascending and descending

* Corresponding author.
E-mail addresses: tarik.atmaca@balikesir.edu.tr, ht_atmaca@yahoo.com.

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projections through the CNS across several areas [12]. Due to these widespread innervations, 5-HT has been linked with many vital physiological and pathophysiological phenomena such as cycles of sleep-wakefulness and psychiatric symptoms [11].

There is no detailed information on the function of serotonin receptors in Toxoplasmic encephalitis. Furthermore, detailed receptor expression responses of the brain to *Toxoplasma gondii* infections remain poorly understood. To elucidate the interaction of *Toxoplasma gondii* with serotonin receptors including, serotonin 2A receptor (5-HTR2A), serotonin 2C receptor (5-HTR2C), serotonin 6 receptor (5-HTR6) and serotonin 7 receptor (5-HTR7); we investigated the gene expression profile of those 5-HT receptors in mice on an experimental model of chronic *Toxoplasma gondii* encephalitis.

2. Materials and methods

2.1. Infection model

For inducing an experimental model of toxoplasmic encephalitis; 6–8 weeks old female Swiss albino mice were inoculated intraperitoneally (IP) with 15–20 tissue cysts of the ME-49 strain of *Toxoplasma gondii*, suspended in 0.25 mL sterile physiologic saline as previously described by Atmaca et al., 2014 [13]. All experimental procedures and animal manipulations in the present study were approved by The Animal Care Committee of the University of Kirikkale.

2.2. Experimental procedures and tissue processing

On each of the 10th, 20th, and 30th days after the infection (d.a.i.), groups of six mice were anesthetized with pentobarbital by IP injection and then they were euthanized by cervical dislocation. Their brains were collected for tissue cyst analysis, histopathological examination, and real-time quantitative polymerase chain reaction (RT-PCR) analyses.

After harvesting the brain tissues for histopathological analysis, the tissues were fixed in 10% buffered formalin and processed for paraffin embedding and sectioning. Tissue sections of 4–5 μm in thickness were obtained from the brain tissue of each mouse in the study and were mounted onto slides for histopathological examination.

2.3. Determination of the tissue cysts

Half of the sagittally sectioned brain tissue taken at necropsy was used in identifying and examining the cysts. The brain tissue was homogenized with 2 ml distilled water. The mean numbers of cysts were determined by counting under light microscopy in three different samples (25 μl each) of brain tissue homogenate obtained from each mouse.

2.4. Histopathology

Digital images were captured using an Olympus microscope (BX51) attached with a DP25 digital microscopy camera (Japan). The total number of focal or diffuse inflammatory foci was counted in a sagittal section of brain previously described [14]; blood-vessel cuffing and inflammatory cell infiltrates in the meninges were also analyzed. The inflammatory score was represented as numeral units: 0–2, mild; 2–4, moderate; 4–6, severe; and above 6, very severe. All analyses were performed at 20x or 40× magnification.

2.5. Total RNA extraction and cDNA synthesis

Brain tissue (20 mg) was harvested as sagittal sections and it was immediately stabilized in the RNA Stabilization Reagent (RNAlater, Qiagen, Hilden, Germany). The tissue samples were initially homogenized for 2 min using the TissueLyser II (Qiagen). The total RNA was purified using RNeasy Mini Kit Qiagen according to the manufacturer's instructions in Qiaqube (Qiagen). The RNA samples were reverse-transcribed into complementary DNA by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). A total of 10μl RNA was treated with 2 μl 10 X RT Buffer, 0.8 μl 25 X dNTPs mix, 2 μl 10X RT Random Primers, 1 μl MultiScribe Reverse Transcriptase, and 4.2 μl DEPC-H2O. Reverse transcription was first carried out at 25 °C for 10 min, then repeated at 37 °C for 120 min, and finally repeated again at 85 °C for 5 min using a Veriti 96-Well Thermal Cycler (Applied Biosystem). The cDNA concentration and quality were assessed and quantified by the Epoch Spectrophotometer System and Take3 Plate (Biotek).

2.6. Relative quantification of gene expression

Relative quantification analyses to determine the expression of 5-HTR2A, 5-HTR2C, 5-HTR6, 5-HTR7, and interleukin-6 (IL-6) mRNA levels were performed with StepOne Plus Real-Time PCR System (Applied Biosystem) using cDNA synthesized from HepG2 RNAs. Real-Time PCR was performed by using primers generated for mice. The sequences of the specific primers are shown in Table 1 (Primer Design Ltd., Southampton, UK). The results were expressed in relative-folds and they were compared to the control groups. Expression levels of β-actin in each cell group were used as endogenous controls. For each cell group, the tests were performed in triplicates for the targets in a 96-well optical plate, using 9 μl of cDNA (100 ng), 1 μl of Primer Perfect Probe mix, and 10 μl of QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany); making a total volume of 20 μl in each well. After the plates were heated for 2 min at 50 °C, and then for 10 min at 95 °C; they were heated for 15 s at 94 °C and 60 s at 60 °C for subsequent 40 cycles. All levels of expression quantities were expressed in fold-changes, comparing the animal groups using the 2-ΔΔCt method [15].

3. Results

3.1. Detection of tissue cysts

Toxoplasma gondii tissue cysts were detected on the experimental groups in the histopathological examination and in the examination of the brain homogenates under a light microscope. *Toxoplasma gondii* tissue cysts and the comparison of the total number of tissue cysts are shown in Figs. 2a,b, and 1a, respectively.

3.2. Histopathology

In the brain, an inflammatory reaction was observed on the 10th d.a.i. The lesions were characterized by perivascular mononuclear cell infiltrations (Fig. 2g-h), focal mononuclear cell infiltrations in the meninges (Fig. 2c-E), and glial proliferation (microglia/blood-borne macrophages (Fig. 1f). The histopathological scores are presented in Fig. 1b. Tissue cysts were observed in all *Toxoplasma gondii* -infected groups. Inflammatory lesions in the brain were more pronounced at the beginning of the infection and during the established chronic infection (20th and 30th d.a.i.). The lesions were severe on the 20th and 30th d.a.i.

Table 1
Primer sequences used for RT-PCR.

Gene	Primer sequence	
5-HTR2A	F	5′-AGAAGCCACCTTGTGTGTGA -3′
	R	5′-TTGCTCATTGCTGATGGACT-3′
5-HTR2C	F	5′-CTATTTTCAACTGCGTCCAT-3′
	R	5′-ATTCACGAACACT1TGCTTT-3′
5-HTR6	F	5′-CTGAGCATGTTCT1TGTCAC-3′
	R	5′-CATGAAGAGGGGATAGATGA-3′
5-HTR7	F	5′-GTTAGTGTCACGGACCTCAT-3′
	R	5′-ATCATTTTGGCCATACATTT-3′
IL-6	F	5′-GAGGATACCACTCCCAACAGACC-3′
	R	5′-AAGTGCATCATCGTTGTTCATACA-3′
β-actin	F	5′-GGCACCACACCTTCTACAATG-3′
	R	5′-GGGGTGTTGAAGGTCTCAAAC-3′

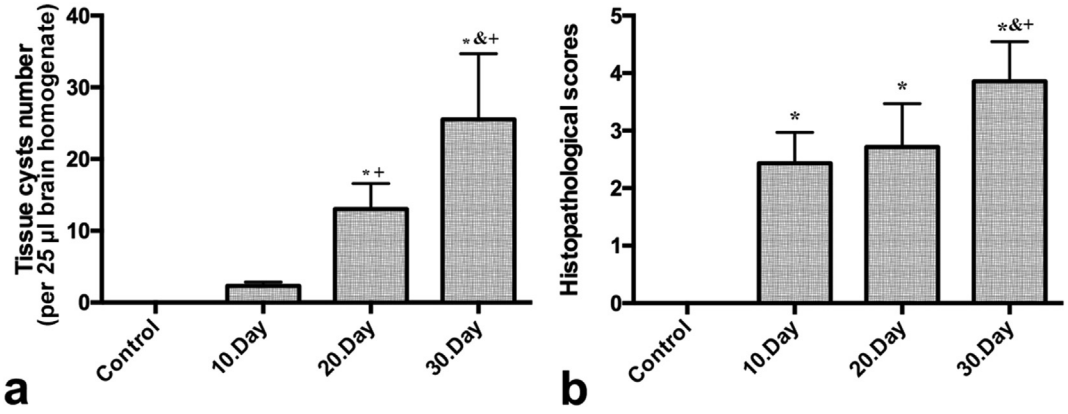

Fig. 1. Comparison of tissue cysts number and histopathological scores. a) Comparison of tisue cysts number. The number of tissue cysts number per brain (per 25 µl brain homogenate) in 10, 20, 30 days after infection.* Indicates tissue cyts number that are significantly greater than control group. + indicates tissue cyts number that are statistically greater than 10 d.a.i. & indicates tissue cyts number that are statistically greater than 20 d.a.i. Tissue cysts number comparison was evaluated from total tissue cysts number counted in 25 µl brain homogenate. $P < 0.05$ and lower was considered statistically significant. b) Comparison of histopathological scores. * Indicates histopathological score that are significantly greater than control group. & indicates histopathological score that are statistically greater than 10 and 20 d.a.i. + indicates histopathological score that are statistically greater than 20 d.a.i. $P < 0.05$ and lower was considered statistically significant.

The severity of the *Toxoplasma gondii* -induced histopathological findings improved in a time-dependent manner.

3.3. IL-6 mRNA expression

In all experimental groups, inflammatory foci and tissue cysts were present. The level of IL-6 expression was determined to be high based on the histopathological findings.

IL-6 activity was prominently higher in *Toxoplasma gondii* -infected mice brains on the 10th, 20th, and 30th d.a.i. compared to the healthy mice in the control group ($p < 0.05$). The highest level of expression was detected on the 30th d.a.i (Fig. 3).

3.4. HTR2A mRNA expression

HTR2A mRNA levels were found to be increased on the 10th and 20th d.a.i. in the brains of *Toxoplasma gondii* -infected mice compared to the expression levels in the controls. The differences between the two groups were statistically significant ($p < 0.05$). The highest level of expression was detected on the 10th d.a.i. While there was a slight decrease in the level of HTR2A expression on the 30th d.a.i., the levels remained to be higher compared to those of the controls (Fig. 3).

3.5. HTR2C mRNA expression

HTR2C mRNA levels increased on the 10th d.a.i. in the brains of the *Toxoplasma gondii*-infected mice compared to the expression levels of the controls. The difference between the two groups was statistically significant ($p < 0.05$). The highest level of expression was detected on the 10th d.a.i. There was a reasonable decrease in the levels of HTR2C expression on the 20th and 30th d.a.i., and these levels were lower compared to those found in the controls (Fig. 3).

3.6. HTR6 mRNA expression

HTR6 mRNA levels increased on the 10th and 20th d.a.i. in the brains of the *Toxoplasma gondii*-infected mice compared to the expression levels in the controls, and the difference between the two groups was statistically significant ($p < 0.05$). The highest level of expression was detected on the 20th d.a.i. On the 30th d.a.i., there was a reasonable decrease in the HTR6 expression levels and they were found to be lower compared to those in the controls (Fig. 3).

3.7. HTR7 mRNA expression

Interestingly, HTR7 mRNA levels decreased on the 10th, 20th, and 30th d.a.i. in the brains of the *Toxoplasma gondii*-infected mice, compared to the expression levels of the controls. Only the levels in the 20th d.a.i. group were statistically significantly different from the levels found in the controls ($p < 0.05$). The lowest level of expression was detected in the controls and in the 20th d.a.i group. On the 30th d.a.i., there was a slight increase in the levels of HTR7 expression; however, these levels were still lower than those found in the control group (Fig. 3).

4. Discussion

The pathophysiological mechanism involved in the development of psychiatric symptoms in *Toxoplasma gondii* infections has not been elucidated clearly yet. In 2009, Zhu [16] suggested that psychotic symptoms observed in *Toxoplasma gondii* infections might be linked with the direct effects of the infection on the functioning of neurons, and also suggested that several immune-mediated alterations might be involved, too in serotonin and dopamine production. The immune responses in the host during the *Toxoplasma gondii* infection result in the generation of proinflammatory cytokines like TNF and IL-6. IL-6 induces the synthesis of IFN-gamma by stimulating the Th cells. This results in the inhibition of the growth of *Toxoplasma gondii* via induction of the IDO enzyme activation. Consequently, this activation leads to the depletion of tryptophan reserves, causing a decrease in the production of serotonin in the brain [1, 8, 9, 10].

Psychiatric symptoms observed in *Toxoplasma gondii* infections are associated with the inflammatory changes in CNS. The inflammation appears to be mediated by a number of proinflammatory cytokines including TNF-α, IFN-γ, IL-1, and IL-6 [17]. IL-6, being a product of monocytes as well as macrophages, is one of the immune parameters that have attracted most of the focus in research. The majority of study reports indicate markedly higher levels of in vitro generation of IL-6 [18] or increased levels of IL-6 in patients suffering from depression [19, 20].

These findings are in line with the findings of a study, reporting that there is a correlation between increased levels of in vitro IL-6 production and decreased tryptophan levels in depressed patients. This result emphasizes the influence of IL-6 on serotonin metabolism [21]. In the present study, the levels of IL-6 expression was found higher in all infection groups compared to the levels found in the control group, demonstrating an increase in inflammatory changes during the infection. Atmaca et al. (2014) [13] showed that the degree of inflammation and the number of

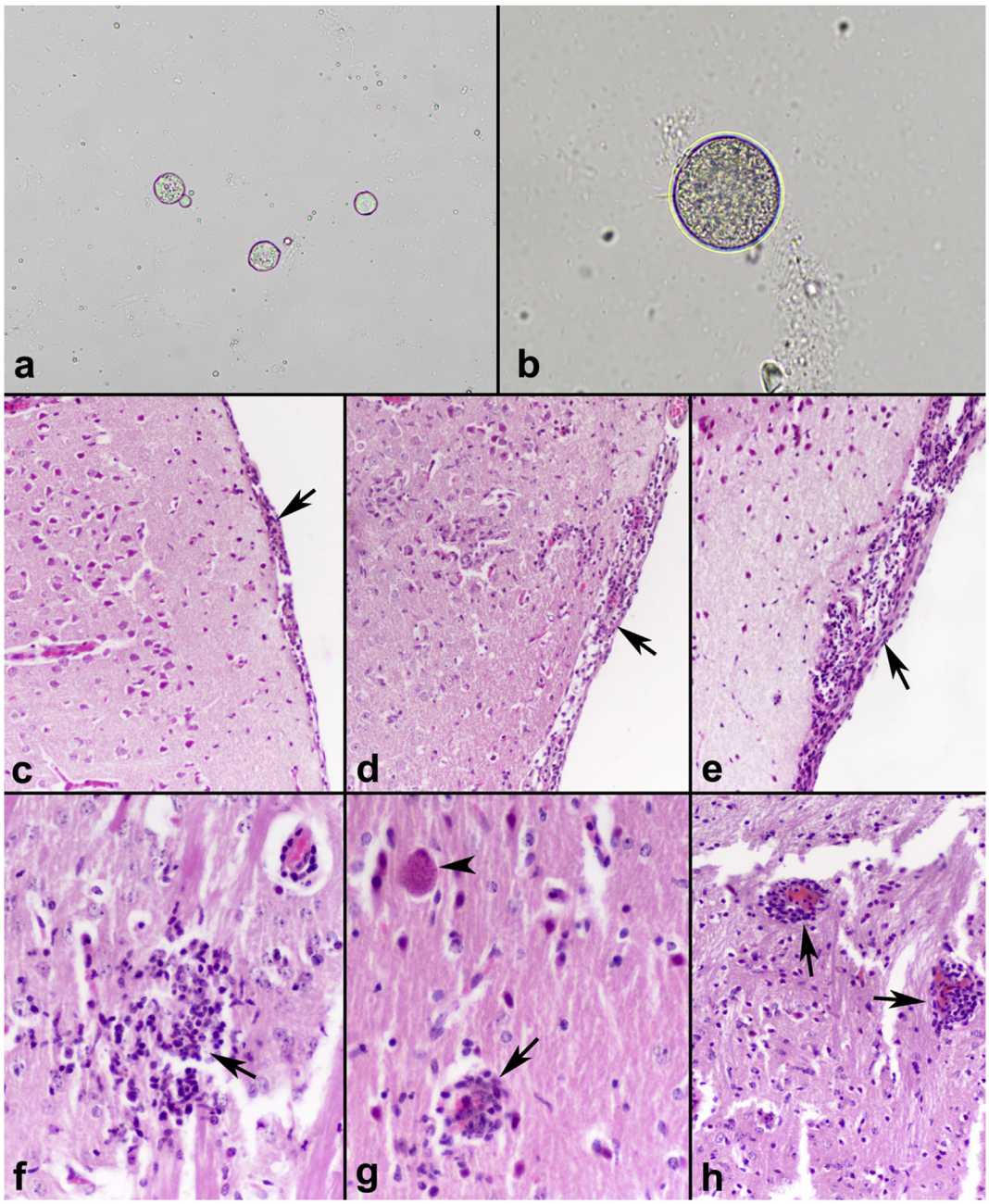

Fig. 2. Tissue cysts in brain homogenate a) 10x magnification, b) 40x magnification. Mononuclear cell infiltration in meninges, mild (c), moderate (d), severe (e) infiltration at 10,20 and 30 d.a.i. respectively (H&E, 10x magnification). Gliosis (arrow) (f), focal gliosis (arrow) and tissue cyst (g) (arrow head), severe perivascular mononuclear cell infiltrations (h) (arrows) (H&E, f and g, 40x magnification, h, 20x magnification).

tissue cysts increased in toxoplasmic encephalitis (TE). These results indicate that IL-6 may take part in the mechanisms of encephalitis and may have a protective role against developing TE. We used the Me49 strain of *Toxoplasma gondii* because this strain is associated with a high number of tissue cysts formed in the brain. Suzuki and Joh (1994) [22] showed that significantly greater numbers of cysts were formed with the ME49 strain in the experimental brain tissue samples compared to the other strains, which were Beverley and C56.

The infection may affect the function of neurons directly [23], thus explaining the neurophysiological deficits. Today, it is assumed that the cysts remain in the brain tissue in infected humans during their lifetime, even though the reports in the literature indicate that these are reported only on rare occasions after routine post-mortem investigations. In individuals with a compromised immune system, encephalitis is the normal presentation of *Toxoplasma gondii* infections. In these cases, reactivation of a substantial number of cysts may be found in the tissue. Research in particular mice strains shows that the cysts may lower in number with time, indicating a presence of a lifetime of individual cysts [13, 24]. This suggests a constant turnover of the cysts in the brain, causing the death of neuronal cells, which would, therefore, lead to neurological injury. Neurological changes have been shown in mice infected with *Toxoplasma gondii*. During an acute infection, a 40 percent rise in the levels of homovanillic acid levels and a reduction in the levels of noradrenaline in comparison with the controls have been reported. It was also reported that; during the acute infection, the levels of dopamine remained unchanged, but increased in mice with chronic *Toxoplasma gondii* infections. The levels of 5-HIAA and serotonin were found to be unchanged [25]. It is still unclear whether these variable results are linked to

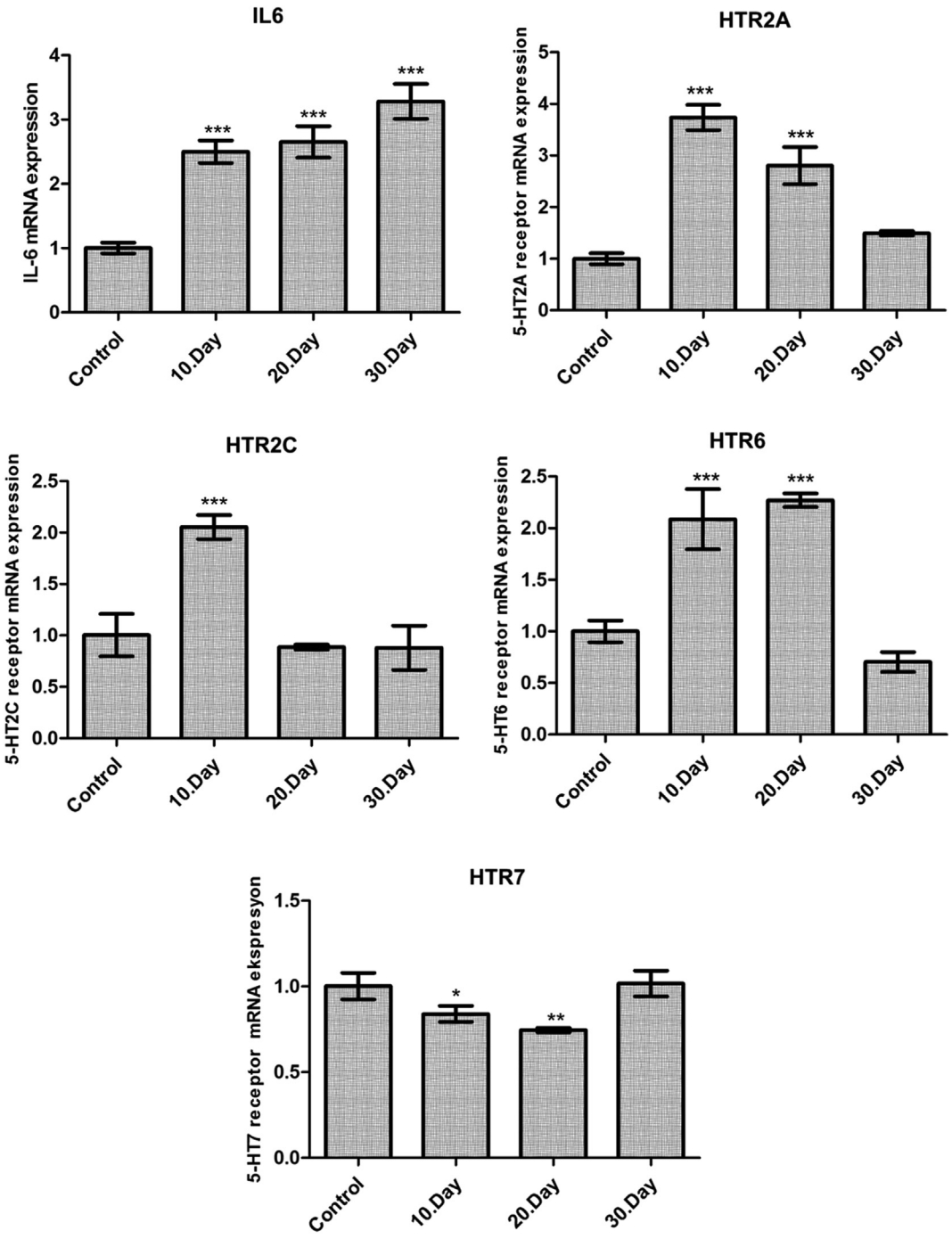

Fig. 3. Relative mRNA expression level of IL-6, serotonin 2A receptor (5-HTR2A), serotonin 2C receptor (5-HTR2C), serotonin 6 receptor (5-HTR6) and serotonin 7 receptor (5-HTR7) in the brains of healthy or *T. gondii*-infected mice. Gene expression was detected by quantitative real-time PCR. β-Actin was used as a reference gene. The gene-specific primers are listed in the Material and Methods. The relative expression levels were calculated by the 2 (-ΔΔCT) method. Statistical analysis was performed using a one-way analysis of variance and Tukey's multiple comparison test. The values represent means ± S.D. ***$p < 0.0001$. **$p < 0.01$, *$p < 0.05$.

Toxoplasma gondii infections in the brain cells or they occur as a result of the sophisticated neuroimmunoendocrinological interactions. Nevertheless, it is known that the *Toxoplasma gondii* genome contains two aromatic amino acid hydroxylases (GenBank Acc. No. ACB99414), which may potentially have a direct effect on the synthesis of serotonin and/or dopamine.

The objective of this study was to demonstrate the features of the serotonin receptor gene expression during an inflammatory reaction in *Toxoplasma gondii* infections.

The results of the study reveal primarily that the hosts have increased the IL-6 expression consistently since the beginning of infection (10th day) as expected. 5HT2A, 5HT2C, and 5HT6 receptor gene expression levels also show an increase in the first 10 days, parallel to the inflammatory response. After ten days, although the level of IL-6 expression increases, the expression of these three receptors begins to decrease and this continues until the 30th day.

In the literature, an emerging inflammatory response is an expected consequence in the host as soon as it encounters a pathogen. Especially the role of 5HT2A receptors in stress has been documented well [26, 27]. The blockade of the 5HT2A receptors shows therapeutic effects in cases

of anxiety and depression [28]. The effects of the SSRI type of antidepressants used for the treatment of anxiety and depression are considered to occur via the 5HT2A receptors [29]. The 5HT2C and 5HT6 receptors are thought to have similar characteristics with the 5HT2A receptors. The 5HT2C receptor is also associated with stress, especially with emerging vegetative symptoms (sleep, appetite, etc.) [30]. Finally, the 5HT6 receptor is known to play a role in responding to anxiety [31].

In the light of all this information, it can be thought that; in the early period of infection, the stress response emerging in *Toxoplasma gondii* infections may cause an increase in the expression of 5HT2A, 5HT2C, and 5HT6 receptors. However, the increased receptor levels and consequently increased serotonergic tonus possibly trigger the downregulation process in these receptors. This process might have caused, in return, the decrease in the receptor expression on the 20th and 30th days. The 5HT2A expression was reported to be weakened by interleukin-6. Thus, it could be argued that the increased IL-6 level might play a role in the decrease of the 5HT2A levels at a critical point [31].

An interesting result is that the 5HT7 receptor expression follows a course completely in the opposite direction compared to the other three receptors. This result is consistent with the comment with respect to the course of the other receptors. The 5HT7 receptor was shown to be a heterodimer of the 5HT1A receptor [32]. The 5HT1A agonists also show a strong affinity to the 5HT7 receptors [31]. It was also reported that the 5HT7 receptor contributed to the inflammatory response through IL-6 [33]. 5HT1A is a receptor, displaying functionally opposite features compared to the 5HT2a receptor [34, 35]. In general, it has anxiety-reducing effects, and as an autoreceptor, it reduces serotonin release [28, 34]. In relation with alleviating the elevated serotonergic tone, an increase in its expression can be expected.

The 5-HT7 receptor is a focus of research currently as a probable therapeutic target for a number of psychiatric conditions. Even though this receptor has been researched to a little extent with respect to addictive behaviors; aspects of its physiological, neuroanatomical, pharmacological, biochemical, and behavioral traits reveal that it may have a crucial function in the development of addictive behaviors [36]. Further studies may be required for determining the specificity of these receptors and their respective substrates, whether any alterations in their levels of expression or their functions are associated with *Toxoplasma gondii* infections.

5. Conclusion

It is known that *Toxoplasma gondii* can cause specific behavioral changes in its host. But the underlying mechanisms still remain poorly studied and clarified. There is a possibility that abnormalities in the neurotransmitter and neuropeptide systems perceived in Toxoplasmosis able to contribute to *Toxoplasma*-related alterations. These observations raise important implications for the direction of future research. Further studies are required to find out the potential of the serotonin receptors as probable molecular targets for decreasing the number of tissue cysts and the degree of inflammation in the brain.

Declarations

Author contribution statement

Hasan Tarik Atmaca: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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