

Contribution of the dopaminergic system in toxoplasmic encephalitis neuroimmunopathogenesis

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Summary. *Toxoplasma gondii* (*T. gondii*), a parasitic intracellular protozoan, can establish a chronic infection in the host brain and cause significant neuropathology. The current study aimed to determine the role of Tyrosine Hydroxylase (TH), Dopamine Receptor D1 (D1R), Nuclear Receptor Related-1 (Nurr1), and Dopamine Transporter (DAT) expression in the neuroimmunopathogenesis of toxoplasmic encephalitis (TE) at 15, 30, 45, and 60 days after infection with *T. gondii*. Additionally, the study investigated whether there was a correlation between the markers on these critical days, which had yet to be explored. The results showed that TH expression in brain tissue of BALB/c mice was significantly increased in all infected groups compared with healthy controls ($p < 0.05$). However, other striking findings of the study were that D1R, DAT, and Nurr1 expression were significantly decreased in all infected groups compared with healthy controls, in contrast to TH expression ($p < 0.05$). Study findings regarding behavioral changes in chronic *T. gondii*-infected laboratory animals and humans with TE provide important evidence of the relationship between neuropsychiatric diseases and *T. gondii* infection. By elucidating the pathogenesis of the disease in detail, treatment protocols that consider these coordinated changes in expression that vary from day to day can be developed.

Key words: DAT, D1R, Nurr1, *Toxoplasma gondii*, Tyrosine hydroxylase

Introduction

Toxoplasma gondii (*T. gondii*) is a protozoan parasite that significantly impacts human and animal health globally. It can infect humans and animals by the ingestion of undercooked or raw meat, food, or water contaminated with oocysts excreted by infected cats (Montoya and Liesenfeld, 2004; Dubey and Beattie, 1988). Following acute systemic infection, *T. gondii* tachyzoites multiply rapidly, cross the blood-brain barrier (BBB), transform into bradyzoites, and establish a chronic infection by forming cysts (Dubey and Beattie, 1988). During the progression of *T. gondii* infection, innate and adaptive peripheral immune cells must maintain a balance between pro- and anti-inflammatory cytokine production. However, an imbalance in this process can result in neuroinflammation, pathologies in neurons and glial cells, and vascular and BBB damage (David et al., 2016; Graham et al., 2021). Although the pathologies affecting the dopaminergic system during the transition from acute systemic infection to the chronic period in TE have not been fully elucidated, it is widely recognized that dopaminergic neurotransmission is disrupted (Dincel et al., 2023). Therefore, understanding the role of *T. gondii* in the pathogenesis of TE is crucial for developing effective therapeutic strategies.

Toxoplasma gondii has been shown to manipulate the behavior of its intermediate host to enhance its own reproduction, potentially increasing the rate of parasite transmission between hosts (Prandovszky et al., 2011; Wana et al., 2023). Experimental animal models infected with *T. gondii* have displayed unusual behavioral changes, including fearlessness, courage, or excessive anxiety under threat (Prandovszky et al., 2011; Wana et al., 2023). There is a growing body of evidence suggesting a close relationship between *T. gondii* and psychiatric disorders, such as schizophrenia and

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depression (Hermes et al., 2008; Kocak et al., 2012). Furthermore, studies have demonstrated that the behavior of *T. gondii*-infected animals treated with psychiatric drugs returns to normal (Henriquez et al., 2009). *T. gondii* has been found to manipulate the dopaminergic system, leading to alterations in host behavior (Groer et al., 2011). Although multiple studies have demonstrated that *T. gondii* causes behavioral changes, the precise mechanism by which it does so remains elusive (Henriquez et al., 2009; Groer et al., 2011). Further research must fully elucidate the complex interplay between *T. gondii* and the dopaminergic system and its impact on host behavior.

This study explored the potential influence of *T. gondii* infection on the pathophysiology of dopaminergic system-related behavioral changes in TE. The study examined the relationship between Tyrosine Hydroxylase (TH), Dopamine Transporter (DAT), Dopamine Receptor D1 (D1R), and Nuclear Receptor Associated-1 (Nurr1) expression and neuropathology during critical illness periods. Additionally, the study investigated the correlation between these markers and histopathology. Furthermore, the study identified the key parameters involved in the progression of TE according to cycles and determined whether they contribute to the development of severe neuropsychiatric outcomes in living organisms. This study aimed to investigate dopaminergic neurotransmission and pathway changes in chronic *T. gondii* infection, and their possible effects on the behavioral modulation of the parasite and the etiopathogenesis of neuropsychiatric diseases.

Materials and methods

Animal infection model and experimental procedures

The experimental protocols and applications were approved by the Animal Experimentation Ethics Committee of Kirikkale University Hüseyin Aytemiz Experimental Research and Application Center (Approval Report No: 23/06/21).

Mice were examined for general health criteria. During the experimental period, animals had restricted access to commercial pellet chow and water. Lighting conditions were maintained at 12 hours light and 12 hours dark. Ambient temperature was maintained at 20–23°C and humidity at 40–60%. Cages, food, water, and the environment were consistently cleaned and disinfected throughout the study until termination.

The study involved 10-week-old female BALB/c mice weighing 20±2 g. The survival, health, and weight of the mice were monitored regularly until tissue collection. Mice were housed in individual cages with full access to food and water. The low virulence ME-49 cystogenic strain of *T. gondii* was obtained from the Dr. Alvaro Freyre Toxoplasma Research Lab, Uruguay, in 2009 under legal permits and was regularly passaged every three months. The researchers followed all relevant guidelines and regulations in the conduct of the

study.

In this experiment, *T. gondii* tissue cysts were extracted from the brains of mice pre-inoculated with 20 cysts. In the infection model, 28 mice (divided into four groups of seven) were inoculated intraperitoneally with 15 cystogenic ME-49 tissue cysts suspended in 0.25 mL of sterile saline (Subauste and Hubal, 2023). Notably, the mice did not show any obvious signs of acute toxoplasmosis after infection, such as lethargy, ruffled fur, or hunched posture. The BALB/c mice were divided into four groups and sacrificed at 15, 30, 45, and 60 days post-infection (DPI), with seven healthy mice in each group. In addition, seven mice received an intraperitoneal injection of sterile saline as a control. None died prior to euthanasia.

Necropsy and rapid determination of tissue cysts

Mice were sacrificed using a CO₂ euthanasia chamber and brain samples were collected. The brains were immediately extracted in preparation for further analysis. The rapid and accurate identification of *T. gondii* tissue cysts was performed using the 'squash smear cyst detection technique'. The squash smear cyst detection technique was employed as reinterpreted and developed by Dincel in 2017 (Dincel, 2017). Five small samples of freshly harvested brain tissue were randomly selected from the infected group and placed on poly-L-lysine-coated glass slides. The sections were immediately immersed in a 4% neutral buffered paraformaldehyde solution for 1 min, followed by a sequential rehydration process in various alcohols for 45 s. The sections were then subjected to Mayer's hematoxylin and eosin (H&E) staining for 35 and 45 s and then rinsed with distilled water. The sections were then desiccated through a series of alcohol solutions of increasing concentration for 1 minute. They were treated with xylene for three min to remove any remaining contaminants and then mounted on glass slides. The unstained and H&E-stained tissues were examined by light microscopy. The analysis was performed in two ways: unstained tissues and H&E-stained tissues were both evaluated.

Histopathological examination

The brain sections were fixated in a solution of 4% neutral buffered paraformaldehyde for 48 hours and washed with tap water overnight. After undergoing conventional tissue preparation methods, the tissue samples were dehydrated using a sequence of alcohol and xylene (Leica TP1020, Germany) and then placed into paraffin blocks (Leica 1150H, Germany). The paraffin serial sections were cut (using the Leica HistoCore AUTOCUT) to a 4–5 µm thickness and placed on glass slides. The cerebral cortex was sliced into sections with a thickness of 5 µm and subjected to staining using H&E. The slices were further analyzed using a light microscope (Olympus BX51 microscope

fitted with a DP25 camera, Tokyo, Japan). This research evaluated sagittal cross-sections of the brains of both healthy controls and infected groups. In this study, we performed a semi-quantitative evaluation of the histological features of the brain sections. We focused on perivascular mononuclear cell infiltration, gliosis, neuronal degeneration/necrosis, and encephalitis. We utilized a rating scale, as suggested by Hermes et al. (2008), to assess the severity of each histological characteristic. The rating system is as follows: 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). Additionally, we examined the number of tissue cysts present in the region of interest.

Immunoperoxidase examination

The study utilized immunohistochemistry to examine *T. gondii* antigen expression, TH, D1, DAT, and Nurr1, in brain tissue sections. After affixing tissue sections onto adhesive slides, they were deparaffinized in three xylene series for five min each and rehydrated in a series of graduated alcohol and distilled water. Antigen retrieval was accomplished by subjecting glass slide sections to a 20 min simmering process in citrate buffer (pH 6.0). Endogenous peroxidase activity was inhibited for seven minutes at room temperature in 3% hydrogen peroxide in absolute methanol. Phosphate-buffered saline (pH 7.4) was used to cleanse sections three times for 5 min between each experiment stage. Following this, sections were incubated for 5 min in blocking serum to inhibit non-specific binding. After that, sections were incubated with the primary antibody (TH, D1, DAT, and Nurr1) in a humidified chamber at room temperature for 60 min. At room temperature, sections were treated for 15 min with a biotin-labeled secondary antibody and streptavidin-peroxidase enzyme. The color reaction was induced by incubating sections in 3,3'-Diaminobenzidine (DAB) chromogen for 5-10 min. Mayer's hematoxylin was utilized as a counterstain for one to two min. The sections were then subjected to DAB treatment for 10 min under a microscope in a controlled environment. The slides were dehydrated in alcohol and cleared in xylene series and coverslipped using Entellan® mounting medium (Merck Millipore, Germany). As a control for non-specific endogenous peroxidase and biotin activities in each test, the primary antibody step was omitted. The sections were examined promptly utilizing a binocular microscope (specifically, an Olympus BX51 microscope outfitted with a DP25 camera, Japan) with an objective of 20x.

Antibodies

In this study, TH (sc-14007, Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1/100, D1R (Abcam; ab78021, Cambridge, UK) at a dilution of 1/100, DAT (Abcam; ab5990, Cambridge, UK) at a dilution of 1/500, and Nurr1 (sc-376984, Santa Cruz Biotechnology, Dallas, TX, USA) at a dilution of 1/100,

were used. These are commercial anti-mouse antibodies (Table 1).

Histomorphometric analysis and statistics

Using a computed image system comprised of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK) coupled to a Leica DM4000 B microscope (Leica Microsystems Imaging Solutions, Ltd.), the density of positive staining was determined. ImageJ analysis software (Java-based image processing software developed by the Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin, USA) was also utilized to examine the identical photographs. Five representative fields were selected for 20x objective magnification, and consecutive images were acquired using a 20x objective and Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions). All transparencies utilized identical configurations. All samples were treated with similar settings for each antibody. Using Leica Qwin Plus software, the integrated optical density of all positive stainings of TH, D1, DAT, and Nurr1 expression in each photograph was determined. Using Leica Qwin Plus, the mean TH-, D1-, DAT-, and Nurr1-positive area/total area was calculated for the images. Each image was captured using identical illumination conditions. To mitigate observer bias, each section was quantified by a blinded investigator. The statistical analyses of the data included the area's mean and standard deviation (mean±SD). Once the proportion (in percent pixels) of the stained area to the entire field had been computed, each slide's mean (in percent pixels) staining area was calculated.

Statistical analysis of TH, D1, DAT, and Nurr1 immunohistochemical results was performed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The data are presented as mean±SD. All statistical analyses and graphs were prepared using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). A p-value less than 0.05 was considered statistically significant.

Results

Histopathological findings

The architecture of the H&E-stained brain sections from the healthy control animals appeared normal. However, prominent neurohistopathological changes

Table 1. Primary antibodies.

Antibody	Manufacturer and Catalog Number	Dilution
TH	Santa Cruz; sc-14007	1/100
D1R	Abcam; ab78021	1/100
DAT	Abcam; ab5990	1/500
Nurr1	Santa Cruz; sc-376984	1/100

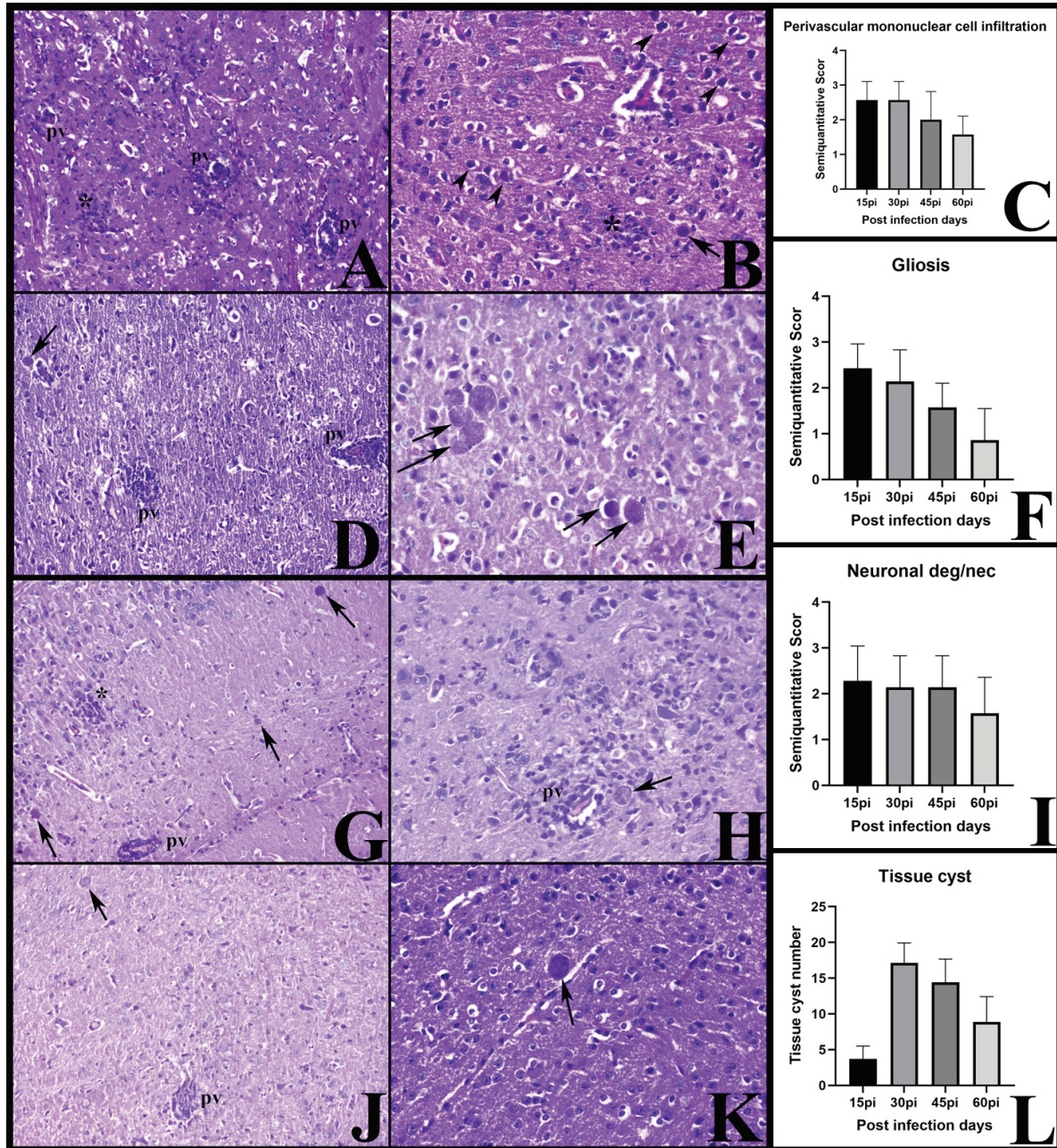


Fig. 1. Histopathological findings and graphs of statistical results were shaped according to days in the study groups. **A, B.** 15 DPI. Severe perivascular mononuclear cell infiltration (pv) and gliosis (asterisk) were observed in the striatum and cortical region. Also, moderate-to-severe neuronal shrinkage, hyperchromasia, and necrosis (arrowhead) in the somatomotor areas of the cerebral cortex, along with a small tissue cyst (arrow). **C.** Bar-graph of perivascular mononuclear cell infiltration, which is quite severe at 15 and 30 DPI, decreases as the infection becomes chronic and regresses to mild-to-moderate levels. **D, E.** 30 DPI. Perivascular mononuclear cell infiltrations (pv) consist of 3-4 layers in the thalamus and cerebral cortex, somatomotor area, and numerous tissue cysts (arrow) of varying sizes in all brain regions. **F.** The semi-quantitative score graph shows the gradual decrease in gliosis between 15 and 60 DPI. **G, H.** 45 DPI. Perivascular mononuclear cell infiltration (pv) and gliosis (asterisk) were observed in the hippocampal CA1 region and midbrain, with varying severity and different sizes of tissue cysts (arrow). **I.** The bar graph represents similar scores between days 15 and 45 DPI, with a slight decrease in neuronal changes at 60 DPI. **J, K.** 60 DPI. A few quite large tissue cysts (arrow) formed in the striatum and midbrain, and 1-2 layers of mononuclear cells (PV) around a blood vessel near the cyst in the striatum. **L.** Average tissue cyst graph on different days post-infection. The x-axis indicates days post-infection, and the Y-axis indicates the Tissue Cyst Number. Hematoxylin and Eosin Staining. A, D, G, J, x 200; B, E, H, K, x 400.

were observed in all infection groups (15, 30, 45, and 60 DPI). Neuronal degeneration, perivascular mononuclear cell infiltration, and focal gliosis were typically observed

at the sites of tissue cysts (Fig. 1A,D,J). Mild to moderate neuronal degeneration and necrosis were observed at 15, 30, and 45 DPI. Mild degeneration and

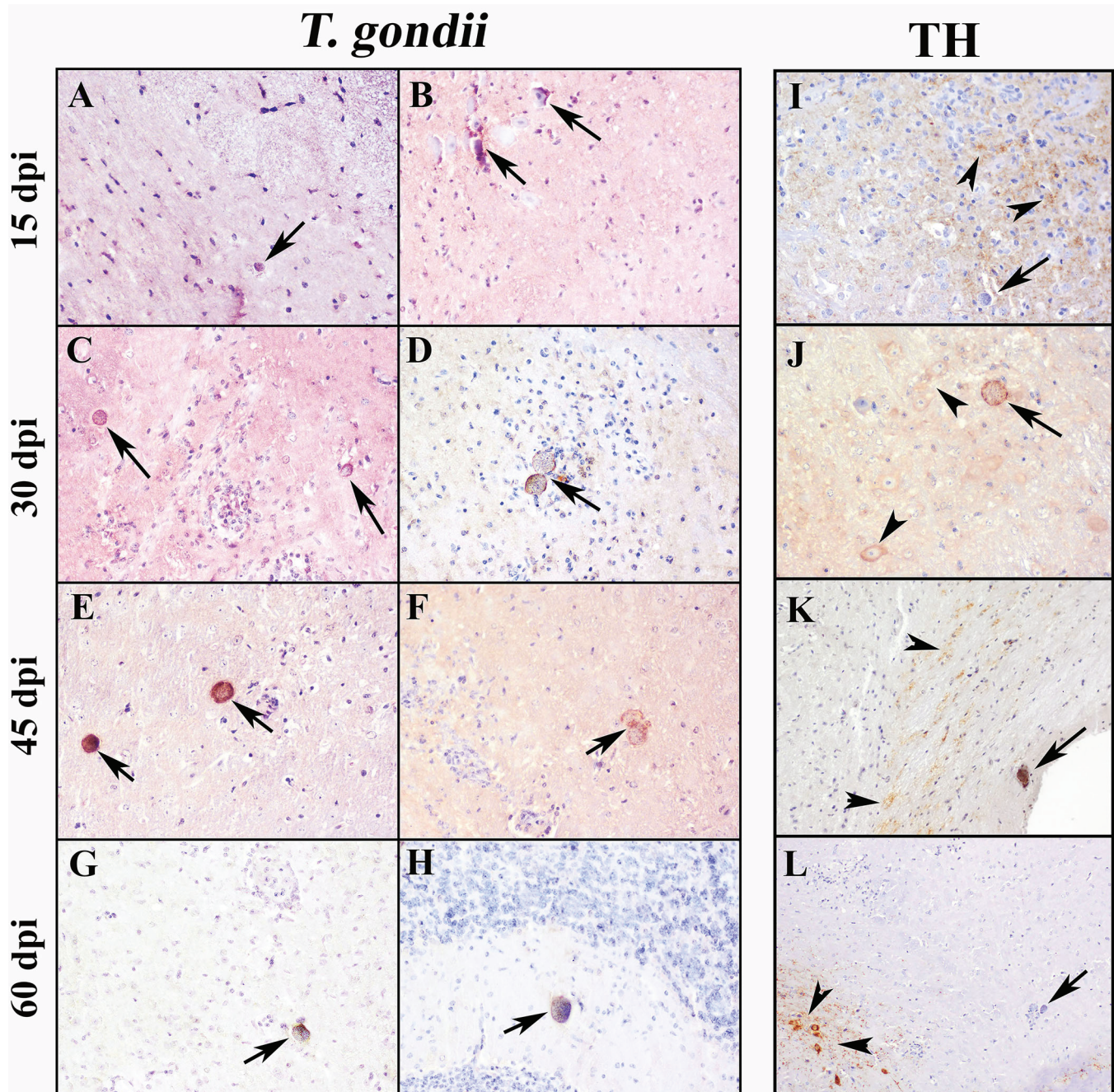


Fig. 2. Tissue cysts of *T. gondii* on different days and their anti-*T. gondii* and anti-TH immunoreactivities. **A-B.** 15 DPI. Successively, a considerably small immunopositive tissue cyst in the striatum and immunopositive neurons in the midbrain. **C-D.** 30 DPI. Strong immunopositive tissue cysts are in the striatum and thalamus, respectively. **E-F.** 45 DPI. Strong immunopositive tissue cysts in the striatum and motor-related part of the midbrain, respectively. **G-H.** 60 DPI. Mild immunopositive tissue cysts are in the striatum and hippocampus (CA3). **I and L.** Intense immunopositivity in dopaminergic neurons (arrowheads) and tissue cysts with no anti-TH immunoreactivity (arrow) in the 15th and 60th days postinfections, respectively. **J-K.** Both intense anti-TH immunoreactivity in dopaminergic neurons (arrowhead) and tissue cysts (arrow) in the 30th and 45th days after infections, respectively. **A-H:** *T. gondii* immunoperoxidase, immunoperoxidase, DAB chromogen with hematoxylin counterstain. **I-L:** TH IHC. DAB chromogen with hematoxylin counterstain. **A-J, L,** x 400; **K,** x 200.

necrosis were also observed at 60 DPI, however, this difference was not statistically significant (Fig. 1I). Perivascular mononuclear cell infiltration and gliosis showed similar patterns in all brain regions at days 15 and 30. From day 45, the decrease in lesions was significant. At 60 DPI, a statistically significant difference in lesions was observed between day 60 and days 15 and 30 (Fig. 1C,F).

Tissue cysts were observed in all *T. gondii*-infected mice brains, and tissue cysts were localized mainly in the striatum, motor, and behavioral regions of the midbrain, and cortical somatomotor and somatosensory areas at 15 DPI (Fig. 1B,L). However, tissue cyst numbers increased significantly at 30 and 45 DPI ($p < 0.0001$) and were evenly distributed throughout the brain and cerebellum without a specific preferential localization. The cysts had different sizes and various associations (Fig. 1D,E,G,H,L).

The largest size of tissue cysts was observed at 60 DPI when they were measured in comparison with the other groups. The midbrain (substantia nigra (SN), motor-related areas, and ventral tegmental area), striatum, hippocampal CA1, CA3, and dentate gyrus (DG) areas, cortical areas (frontal cortex, somatomotor, somatosensory, and visual areas), and the thalamus were the most commonly affected regions. The number of tissue cysts was found to be significantly reduced at 60 DPI in comparison with the other infected groups. The statistical significance is as follows: a comparison of day 15 versus day 60 yielded a p -value of 0.0157, day 30 versus day 60 yielded a p -value < 0.0001 , and day 45 versus day 60 yielded a p -value of 0.0083. Furthermore, additional inflammatory alterations were discerned at the locations of tissue cysts (Fig. 1J,K,L). Additionally, mild to moderate non-purulent meningitis and mild vasculitis were observed in the brains of some animals within the

groups, particularly at 30 and 45 DPI.

Immunoperoxidase findings

Toxoplasma gondii immunoreactivity and tissue cyst distribution

Anti-*T. gondii* immunoreactivity was observed in infected neurons, glial cells, and tissue cysts in all infection groups (Fig. 2A-H). The distribution of tissue cysts in the brain regions of infected animals is depicted in Figure 3. *T. gondii* immunopositivity was detected in the striatum, cortex, and distal parts of the midbrain, particularly in infected neurons and tissue cysts, at 15 DPI (Figs. 2A,B, 3A). Similar numbers and locations of tissue cysts were found at 30 and 45 DPI (Fig. 3B,C). In these groups, tissue cysts were smaller than those found at day 60 but larger than those detected at day 15 (Figs. 2C-F, 3B,C). Tissue cysts were present in almost all brain regions and showed intense immunopositivity in infected neurons and tissue cysts at 60 DPI (Figs. 2G,H, 3D). However, the number and size of cysts were limited, and only a few infected neurons and tissue cysts showed mild to moderate *T. gondii* immunopositivity in bradyzoites, particularly in the cortex, hippocampus (mainly in CA1 and DG), striatum, and midbrain (mainly in the ventral tegmental area (VTA) and SN) (Figs. 2G,H, 3D).

Tyrosine Hydroxylase expression

Anti-TH immunoreactivity was most prominent in the SN and VTA, weaker in the thalamus, and mild to moderate in the striatum, particularly in the nucleus accumbens (NA), in all infected groups (Fig. 4A-E,U). The % immunoreaction intensity and the ratio of reactive

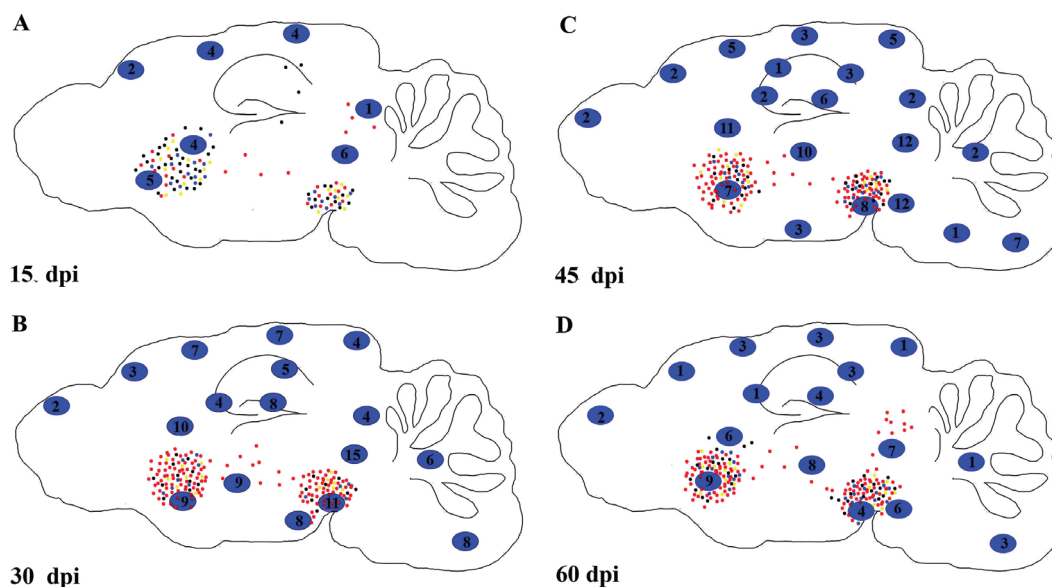


Fig. 3. Localization and numbers of *T. gondii* tissue cysts and expression of TH, D1R, DAT, and Nurr1 in the brain on different days. The number inside the blue circle represents the average of tissue cysts in the region where tissue cysts are located and dopaminergic system positivities are presented as colored pin-points, Red: TH, Green: D1R, Yellow: DAT, Blue: Nurr1.

areas to total area were significantly higher at 30, 45, and 60 DPI compared with healthy controls ($p<0.0001$). At 15 DPI, the values were also significantly higher compared with healthy controls ($p=0.0043$) (Fig. 4A-E,U). Additionally, a significant increase in TH immunoreactivity was observed at 30, 45, and 60 DPI compared with 15 DPI ($p<0.0001$) (Fig. 4A-E). While statistically similar levels of TH immunopositivity were observed at 30, 45, and 60 DPI, regional differences were noted (Fig. 3). Specifically, TH immunoreactivity was observed in the midbrain's VTA and SN and proximal motor-related regions at 15 and 60 DPI.

However, at 30 and 45 DPI, more intense staining over a larger area of the VTA and SN was highly conspicuous (Fig. 4A-E). Interestingly, TH immunopositivity was found in *T. gondii* tissue cysts of moderate to high density in addition to dopaminergic neurons at 30 and 45 DPI (Fig. 2I-L). However, no TH immunoreactivity was observed at 15 and 60 DPI.

Dopamine receptor D1 (D1R), dopamine transporter (DAT), nuclear receptor related-1 (Nurr1) expression

The expression levels of D1R, DAT, and Nurr1 were

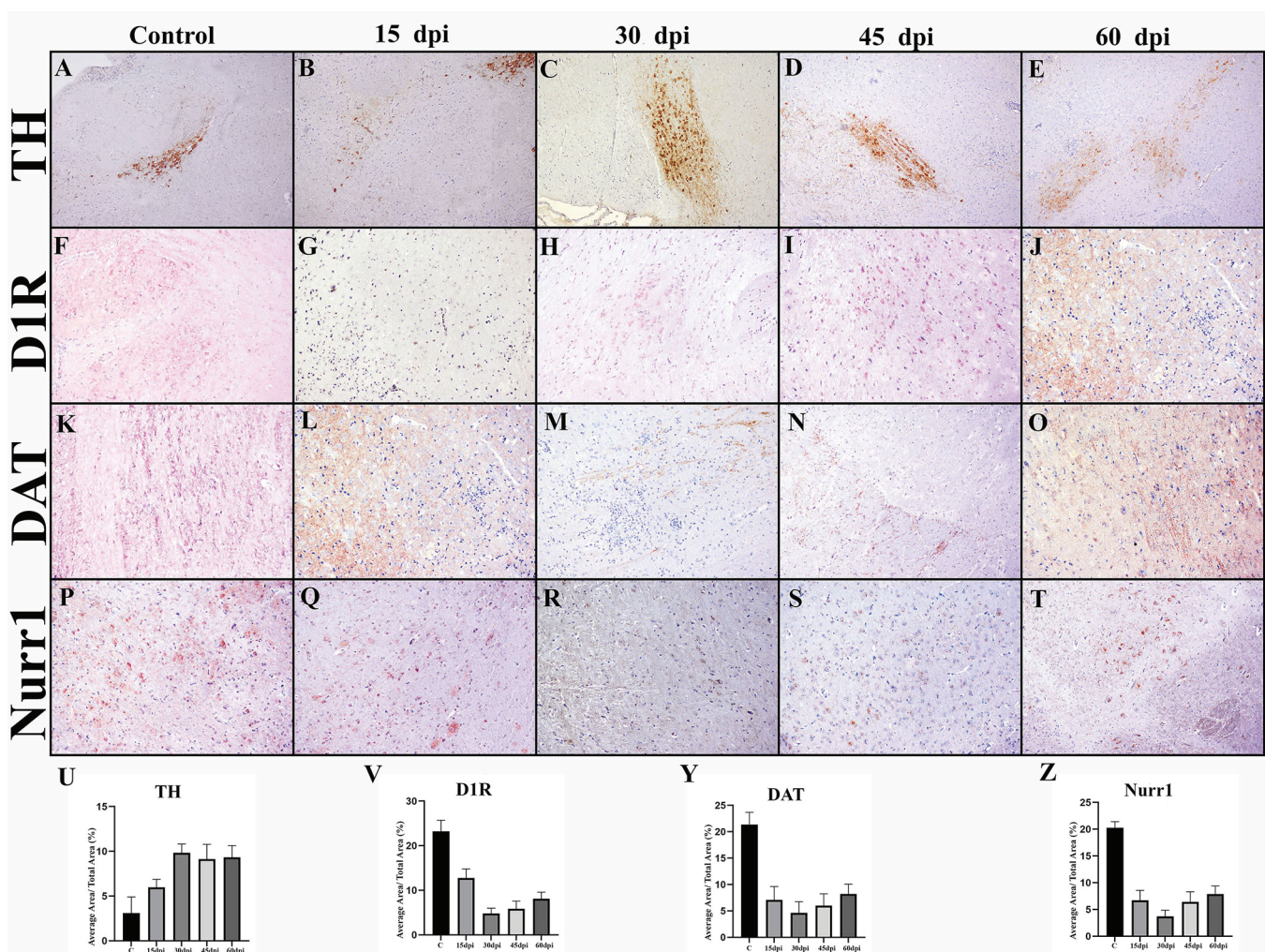


Fig. 4. Immunoperoxidase staining images and graphs of dopaminergic system markers (TH, D1R, DAT, and Nurr1) for all groups. **A-E.** Anti-TH immunoreactivities for the respective control group, 15 to 60 DPI. Mild-to-moderate TH immunopositivity was in the control group, moderate intensity was on day 15, and intense TH immunopositivity was at 30 to 60 DPI. DAB chromogen with hematoxylin counterstain. **F-J.** Anti-D1R immunoreactivities for the respective control group, 15 to 60 DPI. Intense immunopositivity was observed in the control group, with moderate intensity at 15 DPI, relatively mild at 30 and 60 DPI, and mild at 45 DPI. DAB chromogen with hematoxylin counterstain. **K-O.** Anti-DAT immunoreactivities for the respective control group, 15 to 60 DPI. Considerable intensity in the control group, mild-to-moderate intensity at 15, 45, and 60 DPI, and mild at 30 DPI. DAB chromogen with hematoxylin counterstain. **P-T.** Anti-Nurr1 immunoreactivities for the respective control groups, 15 to 60 DPI. Immunopositivity intensity in the control group was mild-to-moderate at 15, 45, and 60 DPI and mild at 30 DPI. IHC. DAB chromogen with hematoxylin counterstain. **U-Z.** Comparative immunoreactivity graphs among groups for TH, D1R, DAT, and Nurr1 markers. [X-axis: all groups, Y-axis: average immunopositive area/total area (%). A-E, x 100; F-J, P-T, x 200; K-O, x 400.

significantly lower in infected compared with healthy control groups ($p<0.0001$) (Fig. 4V-Z). In particular, the most significant differences between all groups were observed for D1R expression. A significant decrease in D1R immunopositivity was observed at 30 and 45 DPI compared with 15 DPI ($p<0.0001$), and at 60 DPI compared with 15 DPI ($p=0.0004$) (Fig. 4F-J,V). These decreases were particularly evident in the striatum. Furthermore, D1R expression showed a gradual increase after 30 DPI, and although 45 and 30 DPI showed no statistical differences, a significant increase in D1R expression was observed at 60 DPI compared with 30 DPI ($p=0.0195$) (Fig. 4F-J,V). In contrast to D1R expression, while there were differences in immunopositivity between groups, a statistically significant difference in DAT expression was only found between 30 and 60 DPI ($p=0.0371$). Compared with the healthy control group, DAT immunoreactivity, which started to decrease at 15 DPI, reached its lowest level at 30 DPI (Fig. 4K-O,Y). From 30 to 60 DPI, DAT immunoreactivity remained similar and although it was still statistically significantly lower at 60 DPI compared with healthy controls, it is noteworthy that it showed a slight increase compared with other infection groups. The greatest decrease in DAT expression was observed in the striatum. Similarly, Nurr1 immunoreactivity showed a similarity between 15 and 45 DPI (Fig. 4P-T,Z). However, a statistically significant decrease was observed at 30 DPI compared with 15 and 45 DPI ($p=0.0085$ and $p=0.0200$, respectively) and 60 DPI ($p=0.0002$).

Discussion

Although infection of an immunocompetent host with *T. gondii* is usually asymptomatic, in immunocompromised patients, such as those with cancer or AIDS, encephalitis can occur with high mortality; in congenital infections, severe pathologies such as stillbirth and congenital defects can occur (Elsheikha, 2008; Pereira-Chioccia et al., 2009; Halonen and

Weiss, 2013). Previous studies have demonstrated that the neuropathology observed in TE cannot be solely attributed to tachyzoites and bradyzoites at the molecular level. It has been clearly shown that *T. gondii*-mediated elevated proinflammatory cytokines result in apoptosis, BBB damage, and neuronal necrosis/neuroparenchymal damage over time (Webster et al., 2006; Dincel and Atmaca, 2015, 2016a,b; Dincel, 2017). In this study, we demonstrated the remarkable changes in the expression of TH, D1R, DAT, and Nurr1 during the critical periods. The reduction in Nurr1 expression affected not only dopaminergic neurons in the striatum but also in the SN, similar to D1R and DAT expression. It is interesting to note that the individual TH, D1, DAT, and Nurr1 expression data of each animal also support these findings (Fig. 5). Overall, these results suggest that DAT and Nurr1 expression are affected similarly during the course of *T. gondii* infection, with a significant decrease observed at 30 DPI. The reduction in D1R, DAT, and Nurr1 expression in dopaminergic neurons in the striatum and SN may be associated with the development of dopaminergic system-related behavioral changes in TE. Further investigation is needed to determine the significance of these observations and their potential impact on the pathophysiology of TE.

Further transmission of *T. gondii* and re-regulation of behavioral changes in the intermediate host may allow the parasite to complete its life cycle. One study has shown that anti-dopaminergic drugs effectively prevent behavioral and cognitive changes associated with *T. gondii* (Webster et al., 2006). Another study has demonstrated that *T. gondii* directly alters dopamine signaling to influence behavioral changes in the host. This suggests that manipulating the dopaminergic system is an important mechanism for *T. gondii*-induced changes in host behavior (Prandovszky et al., 2011). A recent study has demonstrated that *T. gondii* directly modifies dopamine signaling to induce behavioral changes in the host. This implies that manipulating the dopaminergic system is a crucial mechanism for *T. gondii*-induced alterations in host behavior

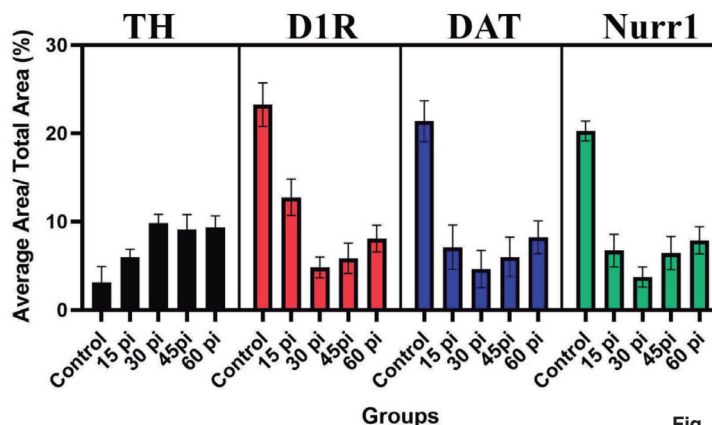


Fig. 5. Individual TH, D1, DAT, and Nurr1 expression data for each animal.

(Prandovszky et al., 2011). In the present study, TH expression was found to increase in correlation with the increase in cyst number and size at 30 and 45 DPI. These findings suggest that TH expression and tissue cyst formation may be correlated with the progression of TE. Further investigation is needed to determine the significance of these observations and their potential impact on the development of severe neuropsychiatric outcomes. The immune system's response to *T. gondii* infection is believed to play a crucial role in host neurochemistry and neuropathogenesis by altering neurotransmitter levels. This alteration may impact behavior through increased dopamine. Pathologically elevated levels of TH expression have been shown to play a critical role in TE neuroimmunopathogenesis.

It has been described in detail that chronic *T. gondii* infection causes behavioral changes, such as increased physical activity, learning and memory disorders, increased anxiety, and decreased Bobcat urinary escape in mice with tissue cysts in their brains (Abo-Al-Ela, 2019; Wana et al., 2023). Although it is widely accepted that the change in such behavior is simply a behavioral modulation of the parasite to ensure its own life cycle, the fact that this behavioral modulation is not limited to rodents and that there are differences according to host gender makes it difficult to explain in a simple way. The mammalian TH gene located in the *T. gondii* genome is active in bradyzoites at the cystogenic stage, which is the resting phase of the parasite, and high TH activity is observed in the infected and immediate environment (Prandovszky et al., 2011). Since TH is the enzyme that synthesizes dopamine, very high levels of DOPA expression are also formed in the bradyzoites that form the tissue cyst. Synthesized DOPA is rapidly transported to the axons by intracellular vesicles and consumed. In this study, it is a well-known fact that TH levels were significantly higher in the brains of mice with chronic TE than in uninfected mice. However, a situation that deserves particular attention is that the localization of high TH activity in the brains of mice with toxoplasmosis parallels the presence of a large proportion of infected neurons and neuroglia containing tissue cysts. More interestingly, TH immunopositivity was frequently found in bradyzoites within tissue cysts. In cell culture studies, the simultaneous increase in the number of bradyzoites in the cell cytoplasm and the increase in the amount of DOPA in the supernatant supports this observation (Prandovszky et al., 2011). According to the results of this study, the expression of Nurr1, a transcription factor that plays important roles in dopaminergic neuron development, differentiation, and protection, decreased in infected mouse brains. In Parkinson's disease, a decrease in Nurr1 expression occurs in midbrain dopaminergic neurons, leading to striatal dopamine activity and motor behavior disorders. Interestingly, in mice with experimental Nurr1 ablation, a decrease in DAT expression is also observed in dopaminergic neurons (Kadkhodaei et al., 2013). In light of these findings, the decreases in Nurr1 and DAT

expression observed in *T. gondii*-infected mice in our study could be interpreted as intracellular infection in dopaminergic neurons first causing a decrease in Nurr1 transcription and subsequently affecting DAT activity. Decreased Nurr1 expression does not have a direct connection with TH activity in neurons, but causes long-term neuron mitochondrial dysfunction and deterioration in axonal microstructure and fiber integrity. In short, decreasing Nurr1 levels is a negative factor for dopaminergic neuron longevity, and the shortening of the lifespan in *T. gondii*-infected cells can be considered as a situation against the parasite. On the other hand, behavioral experiments conducted on Nurr1 +/- heterozygous transgenic female mice showed that *T. gondii*-infected male mice were not afraid of cat urine and remained longer in the open arm of the elevated plus maze due to decreased Nurr1 transcription and DOPA neurotransmission disruptions (Eells et al., 2015). In our study, which has findings supporting that decreased Nurr1 and DAT activity and disruptions in dopaminergic neurotransmission can be induced by *T. gondii*, although behavioral tests were not performed, significantly decreased Nurr1 and DAT expression were associated with similarly decreased dopaminergic neuron health and disruptions in axon-dendrite fiber structure and anxiety. An interpretation could be that this may be associated with depression and even schizophrenia.

However, a very interesting finding in the present study is that D1R and DAT expression increased in a correlated manner on days 30, 45, and 60, and showed a second peak on day 60 after day 15. Studies have explained that D1R activation increases dopamine signaling and this effect is terminated by DAT (Chen et al., 2006; Cartier et al., 2010; Beaulieu and Gainetdinov, 2011). This may explain why dopamine activity may be lower on 30 and 45 DPI compared with day 60, and even why dopamine does not remain at very high levels throughout life in *T. gondii*-positive individuals.

A transgenic mouse study demonstrated that directly increasing TH activity induces oxidative stress (OS) in the striatum. This finding is remarkable because directly increasing TH activity alone can cause OS *in vivo*, generate autotoxic dopamine metabolites, and increase toxicity (Ogawa et al., 2005; Vecchio et al., 2021). The precursor of dopamine, L-DOPA, is associated with increased dopamine levels in rodents with *T. gondii*-infected brains (Stibbs, 1985). L-DOPA has been implicated in causing oxidative damage in cells by inducing OS (Pattison et al., 2002). Our previous research has shown that oxidative/nitrosative stress plays a significant role in the pathogenesis of the neuropathology described in TE (Dincel and Atmaca, 2015, 2016a). Additionally, *T. gondii* exacerbates oxidative damage by causing a significant decrease in Cu/Zn superoxide dismutase expression (Dincel and Atmaca, 2016a). This study explains that increased TH expression in all infected groups increases dopamine levels. The observed OS can also be caused by *T. gondii*-mediated manipulation of L-DOPA. Therefore, both *T.*

gondii and the dopaminergic system it manipulates contribute to neuropathogenesis and impact behavioral changes. This study demonstrates that *T. gondii*-produced L-DOPA contributes to initiating OS by increasing reactive oxygen species in host cells.

Gaskell et al. (2009) and Prandovszky et al. (2011) have shown that tissue cysts express TH and stimulate dopamine release in chronic phase TE induced in infected mice. However, in this study, strong TH expression was not observed in tissue cysts at 15 and 60 DPI. Nevertheless, significantly increased TH expression was observed in the walls of tissue cysts at 30 and 45 DPI. Furthermore, we discovered that TH expression was consistently increased in all infected groups. After tissue cysts caused degeneration and necrosis in midbrain neurons, TH expression continued to grow despite a decrease in the number of neurons. The fact that tissue cysts showed TH expression, particularly at 30 and 45 DPI, suggests that TH expression is involved in other mechanisms that have not yet been identified, independent of dopaminergic system receptor and Nurr1 expression. Understanding the distribution and size of tissue cysts in various brain regions during *T. gondii* infection can provide valuable insights into the pathophysiology of TE. The presence of *T. gondii* immunopositivity in neurons and tissue cysts suggests an active infection, while the appearance of bradyzoites may indicate a dormant parasite stage. Further investigation is needed to determine the significance of these findings in the context of TE.

This study presents information on the impact of *T. gondii* on the dopaminergic system and the reasons for behavioral changes in the mouse brain. The investigation of behavioral and neuropsychiatric changes in chronic TE is ongoing but still needs to be completed. The study examines changes in TH, D1R, DAT, and Nurr1 expression at critical periods following infection. These markers are shown to play significant roles in TE neuroimmunopathogenesis. Additionally, TH expression and the resulting dopamine may be linked to apoptosis and OS, which explains the significance of these markers in the development of the neuropsychiatric disorders caused by *T. gondii*.

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numbered 28141 and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123).

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Dopaminergic system in toxoplasmic encephalitis neuroimmunopathogenes

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