

# Toxoplasmic Infection-induced Injury in the Ileal Myenteric Plexus in Rats Depends on the Dose of *Toxoplasma gondii* Oocysts

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J Morphol Sci 2018;35:80–86.

## Abstract

**Introduction** The present study evaluated the effects of different inocula of *Toxoplasma gondii* oocysts on the ileal myenteric plexus in rats.

**Materials and Methods** Male Wistar rats, 60 days old, were distributed into four groups: control group (CG; which received saline solution) and groups that were infected with 100 (TG100), 1000 (TG1000), and 5000 (TG5000) *T. gondii* oocysts. Thirty days after infection, the rats were sacrificed, and the ileum was collected to make whole-mount preparations that were subjected to immunofluorescence staining to observe the general neuronal population (HuC/D), nitrergic neurons (nNOS), and enteric glial cells (S100). Morphometric and quantitative analyses of myenteric neurons were performed.

**Results** The infections with different *T. gondii* inocula did not cause neuronal or glial loss, but cause neuronal hypertrophy in general population and nitrergic subpopulation in infected groups. Conclusion: Changes in neuronal morphology were observed in the TG5000 group, including the presence of vacuoles, translocation of Hu protein to the nucleus, and dendritic distortions, suggesting functional alterations in these cells.

## Keywords

- ▶ dose
- ▶ oocysts
- ▶ toxoplasmosis
- ▶ enteric nervous system
- ▶ ileum

## Introduction

*Toxoplasma gondii* is an intracellular parasite that causes toxoplasmosis and is considered one of the most evolutionarily successful protozoa on Earth.<sup>1</sup> One-third of the world's population has had contact with this parasite.<sup>2</sup> In Brazil, the prevalence of anti-*T. gondii* immunoglobulin G (IgG) antibodies in the human population varies from 59% to 91% in different regions.<sup>3,4</sup> Based on SAG2 gene analysis, *T. gondii* has three main distinct clonal lineages: genotypes I, II, and III.<sup>5,6</sup> The majority of human infections is associated with type II genotype strains,<sup>5</sup> and its transmission can occur when the host ingests tissue cysts that are present in under-

cooked meat or oocysts in contaminated water and food and via the vertical transmission of tachyzoites.<sup>1,7</sup> When sporulated oocysts are ingested by any warm-blooded animal, sporozoites are released in the small intestine, and the parasite penetrates enterocytes within 30 minute.<sup>8</sup> Tachyzoites can be found in the blood 4 hour after the ingestion of oocysts, and most tachyzoites remain in the lamina propria where they multiply inside several cell types, with the exception of erythrocytes.<sup>9</sup> The presence of these parasites in the intestine can cause sloughing of the intestinal mucosa and enteritis, with edema and necrosis in the lamina propria.<sup>9</sup> Unknown is how often *T. gondii* infection causes gastrointestinal symptoms in humans.

received  
September 14, 2017  
accepted  
February 13, 2018

DOI <https://doi.org/10.1055/s-0038-1660494>.  
ISSN 2177-0298.

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## Materials and Methods

All of the procedures that involved the use of animals were approved by the Ethics Committee on the Use of Animals in Experimentation of the State University of Maringá (CEAE, Universidade Estadual de Maringá, no. 081/2012) and were in accordance with the ethical principles adopted by the Brazilian Society of Science in Laboratory Animals (SBCAL).

### Experimental Design

Twenty-five male Wistar rats (*Rattus norvegicus*), 60 days old, were randomly distributed into four groups: control group (CG;  $n = 5$ ; which received saline solution) and groups infected with 100 (TG100,  $n = 6$ ), 1000 (TG1000,  $n = 7$ ), and 5000 (TG5000,  $n = 7$ ) *Toxoplasma gondii* oocysts of the ME49 strain (genotype II). The oocysts were obtained from the Veterinary Parasitology Laboratory, Universidade Estadual de Londrina (Londrina, PR, Brazil) and previously sporulated and resuspended in 1 mL of sterile saline solution. Control rats received only the sterile saline solution. The rats were then maintained for 30 days in a animal house with controlled temperature ( $22 \pm 2^\circ\text{C}$ ) and a 12/12 hour light/dark cycle and fed standard rodent chow (Nuvilab, Quimtia SA, Colombo, PR, Brazil) and water *ad libitum*.

### Sample Collection and Tissue Preparation

*T. gondii* infection was confirmed by the presence of anti-*T. gondii* IgG in serum using the direct agglutination method.<sup>10</sup> Thirty days after infection, the rats were deeply anesthetized with halothane vapor.<sup>11</sup> Laparotomy was performed to collect the distal ileum, which was tied at both ends and filled and distended with 4% buffered paraformaldehyde (pH 7.4) for 3 hour. The samples were opened at the mesenteric border and stored in phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.4) with 0.08% sodium azide. The whole-mount preparations were obtained by microdissection under a stereomicroscope, removing the mucosa and submucosa and keeping only the external muscle layer with the myenteric plexus.

### Immunofluorescence

The whole-mount preparations were incubated with mouse anti-HuC/D (a marker of the general population of myenteric neurons) and rabbit anti-nNOS (a marker of the nitrergic subpopulation) or rabbit anti-S100 (a marker of glial cells)

antibodies (►Table 1). Double-labeling was performed for HuC/D/nNOS and HuC/D/S100 in separate preparations. The whole-mount preparations were washed ( $2 \times 10$  minute) in PBS plus 0.5% Triton X-100 (PBS-T) and placed in a blocking solution (PBS-T, 2% bovine serum albumin [BSA] and 10% donkey serum) for 1 hour. They were then incubated for 48 hour with the primary antibodies diluted in PBS-T, 2% BSA, and 2% donkey serum at room temperature under shaking. The whole-mount preparations were then washed in PBS-T ( $3 \times 10$  minute) and incubated with secondary antibodies (►Table 1) for 2 hour at room temperature under shaking while protected from light. Lastly, they were washed in PBS-T ( $3 \times 10$  minute) and mounted on glass slides using Prolong Gold Antifade Reagent (Molecular Probes, Life Technologies, Eugene, OR, USA).

### Morphoquantitative Analysis

The neuronal density and morphometry analyses were performed on images that were captured under a fluorescence microscope with a high-resolution camera coupled to a computer. The analyses were performed using ImagePro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). The images were captured by randomly sampling across all of the whole-mount preparations, with no specific visual fields chosen, and the same field was not captured more than once. Immunoreactive neurons (HuC/D+ and nNOS+) and glial cells (S100+) were counted in 30 images per animal. The area of each image was  $\sim 0.36$  mm<sup>2</sup>, and the total quantified area was 10.82 mm<sup>2</sup> per animal. The results are expressed as the number of enteric neurons or glial cells per cm<sup>2</sup>. For morphometry, the area of 100 HuC/D+ cell bodies and 100 nNOS+ cell bodies per animal were measured, and 100 HuC/D+/nNOS- neurons per animal were also measured. Only neurons for which we could clearly delimit the cell body were chosen, and the area is expressed in  $\mu\text{m}^2$ .

### Statistical Analysis

The data were analyzed using the Statistica 7.1 and GraphPad Prism 3.1 software. The quantitative data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Morphometric data were set in delineation blocks (ANOVA) followed by Tukey's test. All of the statistical tests considered a 5% level of significance. The results are expressed as mean  $\pm$  standard error.

**Table 1** Primary and secondary antibodies used for immunofluorescence

Primary Antibodies					Secondary Antibodies			
Antibody	Host	Dilution	Company	Catalog no.	Antibody	Dilution	Company	Catalog no.
HuC/D	Mouse	1:500	Invitrogen	A21271	Anti-mouse Alexa Fluor 488	1:500	Molecular Probes, Invitrogen	A21202
nNOS	Rabbit	1:500	Zymed	61-7000	Anti-rabbit Alexa Fluor 546	1:500	Molecular Probes, Invitrogen	A11010
S100	Rabbit	1:500	Invitrogen	18-0046	Anti-rabbit Alexa Fluor 546	1:500	Molecular Probes, Invitrogen	A11010

Abbreviations: HuC/D, general neuronal population; nNOS, nitrergic subpopulation; S100, enteric glial cells.

**Table 2** Quantification of the general population (HuC/D +) and subpopulations (nNOS+ and nNOS-) of myenteric neurons and glial cells (S100 +) in the distal ileum in rats infected with different inocula of *T. gondii* for 30 days. The results are expressed as neurons per cm<sup>2</sup> (mean ± standard deviation)

Group	HuC/D+ General population	HuC/D + /nNOS- subpopulation	HuC/D + /nNOS+ subpopulation	S100+ Glial cells
Control	16829 ± 3966	12839 ± 3322	3988 ± 689	25752 ± 3063
TG100	15749 ± 2096	11333 ± 1981	4295 ± 742	25475 ± 2350
TG1000	19443 ± 2309	14760 ± 1533	4691 ± 790	26454 ± 2508
TG5000	17116 ± 1864	12848 ± 1251	4299 ± 685	24773 ± 1812

No significant difference was found between groups ( $p < 0.05$ ; ANOVA).

## Results

### Clinical Aspects

None of the characteristic clinical signs of toxoplasmosis were observed in the infected rats. The TG100, TG500, and TG5000 groups presented serum anti-*T. gondii* IgG 30 days after inoculation, whereas the CG remained negative.

### Neuronal and Glial Density

None of the myenteric cellular populations (neurons and glial cells) presented significant population density changes in the infected groups (TG100, TG1000, and TG5000) compared with the CG (►Table 2).

### Morphometric Analysis

The general HuC/D+ neuronal population and HuC/D + /nNOS- subpopulation presented hypertrophy in all of the infected groups compared with the CG ( $p < 0.01$ ), most prominently in the TG100 group (►Table 3). The nNOS+ subpopulation showed hypertrophy of the cell bodies in the TG100 and TG5000 groups ( $p < 0.01$ ). Some of the rats in the TG1000 group exhibited translocation of Hu protein to the nucleus, causing a reduction of cytoplasmic immunoreactivity and compromising visualization of the cell body limits. Special attention was necessary to ensure that the entire area of the neuronal body, including the nucleus and cytoplasm, was being measured.

### Qualitative Analysis

HuC/D + /nNOS+ neuronal immunoreactivity was generally weak in the infected rats (►Fig. 1). Higher HuC/D+ nuclear immunofluorescence was observed in neurons in the TG1000 group, indicating the translocation of Hu protein from the

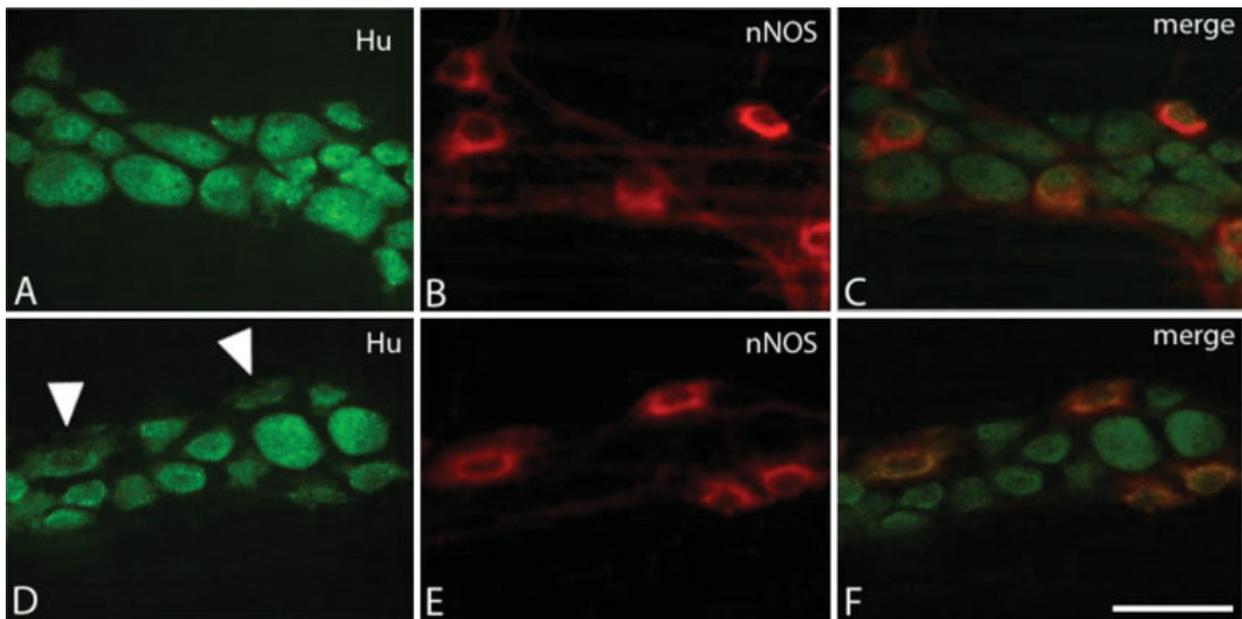
cytoplasm to the nucleus (►Fig. 2A). Some neurons in the TG5000 group presented an irregular shape and no clear definition of the cell body limits (►Fig. 2B). A substantial presence of intra-cytoplasmic vacuoles was observed in neurons in the infected groups (►Fig. 2C). Some nNOS+ neurons in the infected groups were swollen and presented distortions in the dendritic projections (►Fig. 2D). Although the HuC/D antibody that was used is considered a pan-neuronal marker, we were able to observe some nNOS+ neurons that did not show immunoreactivity to Hu protein (►Fig. 3).

## Discussion

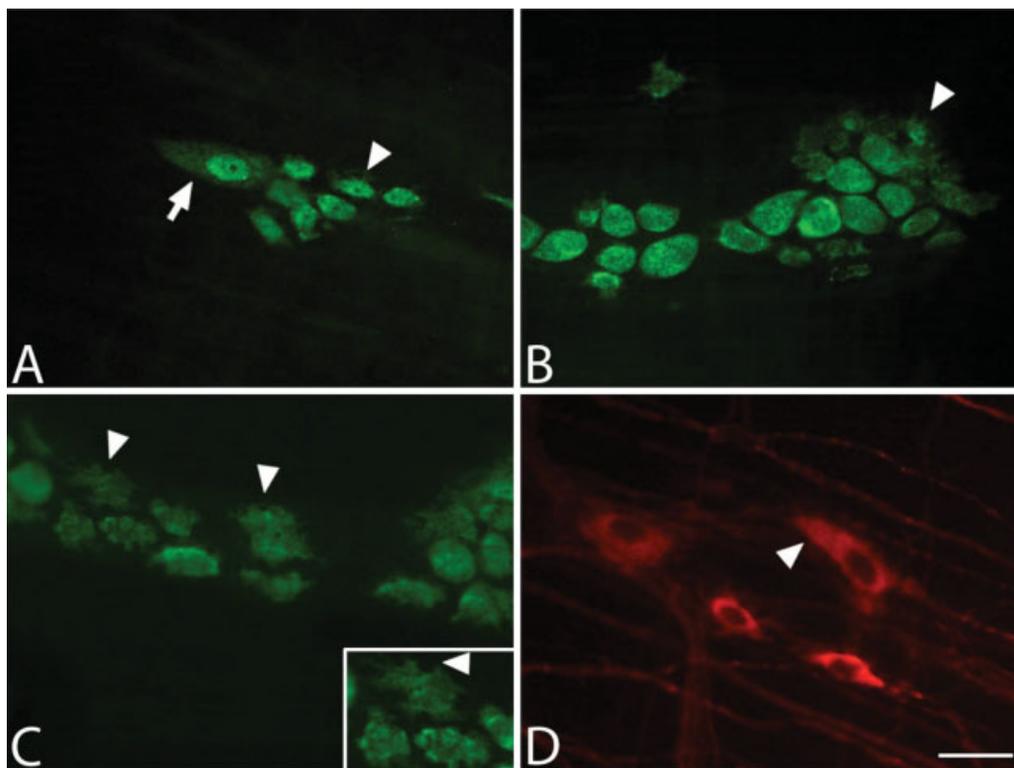
Infection with different inocula of *T. gondii* (ME49 strain, genotype II) did not cause neuronal or glial cell loss but caused morphologic changes in myenteric neurons of the ileum in rats, such as hypertrophy, the translocation and degradation of Hu protein, the presence of vacuoles, and the distortion of dendrites in nitrergic neurons. The neuronal alterations that were observed in infected animals in this experimental model were not sufficient to provoke clinical signs of toxoplasmosis, such as diarrhea. Toxoplasmosis is often a subclinical infection in both rats and humans,<sup>12</sup> which has also been observed in previous rat studies.<sup>13-16</sup> Our data showed that the infection did not cause neuronal loss in the general population (HuC/D +) or nitrergic subpopulation (nNOS +). *T. gondii* infection in rats after 30 days does not compromise the survival of myenteric neurons, regardless of the infective stage of the parasite that is used for the inoculation, which was also demonstrated in previous studies that evaluated the ileum (acute and chronic phase) and colon (chronic phase) in rats that were infected with tachyzoites.<sup>16-18</sup> The density of myenteric glial cells also did

**Table 3** Cell body area ( $\mu\text{m}^2$ ) of the HuC/D+ general population, nNOS- subpopulation, and nNOS+ subpopulation of myenteric neurons in the distal ileum in rats infected with different inocula of *T. gondii* for 30 days

Group	HuC/D+ general population	HuC/D + /nNOS- subpopulation	HuC/D + /nNOS+ subpopulation
Control	290.7 ± 117.3	289.9 ± 121.5	300.0 ± 87.82
TG100	351.1 ± 112.9a	364.6 ± 131.4a	321.1 ± 93.99a
TG1000	318.5 ± 111.0ab	333.0 ± 122.3ab	296.7 ± 87.27b
TG5000	322.9 ± 133.2ab	317.1 ± 138.4ab	325.6 ± 101.6c



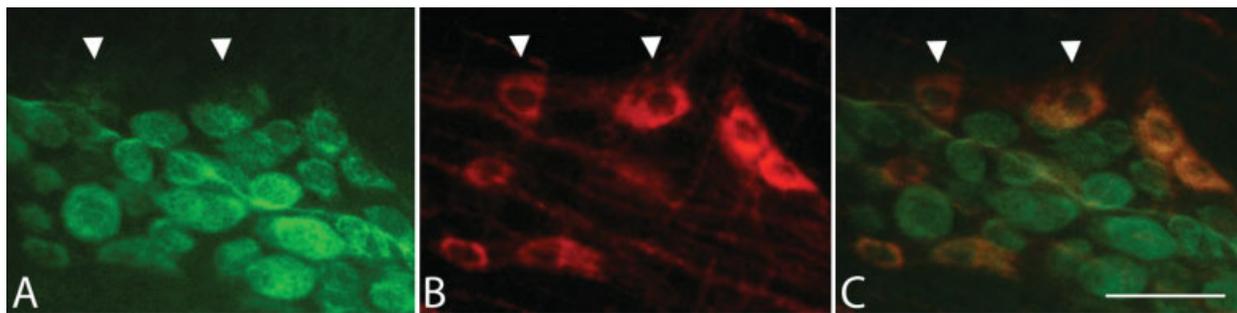
**Fig. 1** Micrographs illustrating immunoreactivity to HuC/D (green) and nNOS (red) in the myenteric plexus of the ileum in rats. (A–C) Control group; (D–F) TG5000 group. The immunofluorescence of nNOS is weaker than HuC/D protein (arrowhead). Scale bar = 50 μm.



**Fig. 2** Micrographs showing immunoreactivity to HuC/D (A–C) and nNOS (D) in the ileal myenteric plexus in infected rats. Hu protein translocated (arrow), and no labeling was observed in the cytoplasm (arrowhead in A). HuC/D+ neurons (green) in rats in the TG5000 group presented an irregular shape with no clear cellular limit (arrowhead in B) and cytoplasmic vacuoles (arrowhead in C). Nitroergic neurons (red) also showed distortions in rats in the TG5000 group (D). Scale bar = 50 μm.

not change as a result of toxoplasmic infection. The survival of myenteric glial cells may have contributed to the maintenance of the number of neurons in the general population and nitroergic subpopulation because glial cell loss usually precedes neuronal loss.<sup>19</sup>

The dose of parasites affected the myenteric neuronal population in the duodenum. Rats that were infected with *T. gondii* presented progressive, dose-dependent general neuronal loss in the duodenum in ascending order (10, 100, 500, and 5000 oocysts). The nitroergic myenteric neuronal subpopulation



**Fig. 3** Micrographs illustrating immunoreactivity to HuC/D (A) and nNOS (B) in the ileal myenteric plexus in rats in the TG100 group and a merge of both (C). The images show that not all HuC/D+ neurons were immunoreactive (arrowhead). Scale bar = 50  $\mu$ m.

was shown to increase at doses of 100, 500, and 5000 *T. gondii* oocysts.<sup>20</sup> Although infection with different doses of *T. gondii* oocysts in mice is more harmful to the ileum than the duodenum,<sup>21</sup> we did not know whether this holds true for rats. Studies by our group suggest that duodenal myenteric neurons in rats are more sensitive than ileal myenteric neurons to toxoplasmic infection.<sup>20</sup> Within the general neuronal population (HuC/D +), we found that ~25% of the neurons were nitrergic (nNOS +) in all of the groups. The different doses of *T. gondii* oocysts did not cause any changes in the proportion. Although HuC/D is considered a pan-neuronal marker, we observed the absence of Hu protein immunoreactivity in some neurons that were immunoreactive to nNOS protein (**→ Fig. 3**). Similar results have also been reported in humans.<sup>22</sup> Although we did not observe myenteric neuronal loss, toxoplasmic infection caused hypertrophy in HuC/D +, nNOS +, and HuC/D + /nNOS- neurons. This hypertrophy might have occurred in response to the inflammation that was caused by *T. gondii* in the intestine, a remarkable feature of this parasitic infection.<sup>21</sup> In fact, the cell body area increased in all of the groups but in descending order of the dose of the parasites. This indicates that higher parasite doses might impair neuronal plasticity. The mechanisms that are involved in these effects should be explored in further studies. Other studies by our group found morphometric alterations in myenteric neurons during toxoplasmic infection. For example, we observed hypertrophy in myenteric neurons in the distal ileum in rats that were infected with tissue cysts of the ME-49 strain of *T. gondii* for 24 hour.<sup>18</sup> We also observed hypertrophy in nitrergic subpopulation and total population of myenteric neurons of proximal jejunum in rats that were infected for 36 days with tissue cysts of the ME-49 strain of *T. gondii*.<sup>23</sup>

Despite the fewer morphometric alterations in myenteric neurons in rats in the TG1000 and TG5000 groups, we suggest that toxoplasmic infection compromises neuronal plasticity in the ileum when it is exposed to higher doses of the parasites. Considering that neuronal plasticity is important for the survival and physiologic balance of neurons,<sup>24</sup> myenteric neurons in rats that received higher doses of the parasites may be more impaired because of the infection. The qualitative analysis appears to support this hypothesis.

We observed the nuclear translocation of Hu protein especially in the TG1000 group. This could indicate an

imbalance in the trafficking of molecules between the cytoplasm and nucleus, thus compromising cellular metabolism. Hu protein is an RNA-binding protein that is present in both the cytoplasm and nucleus, with many functions in neuronal biology, such as cellular development and plastic adaptations.<sup>25</sup> Changes in the localization of Hu protein may occur in response to stimuli that change the mRNA regulation by Hu<sup>26</sup> or modify or selectively degrade Hu protein, thus modifying its immunoreactivity.<sup>25</sup>

The consequences of translocation of this protein are not yet completely understood. This event may be indicative of neuronal injury (Rivera et al., 2011),<sup>27</sup> and Hu protein loss in the cytoplasm may indicate changes in general protein synthesis. Some HuC/D+ neurons presented a reduction of cytoplasmic immunoreactivity, including almost no labeling in some cases in the TG1000 group. Some HuC/D +, nNOS-, and nNOS+ neurons in the infected groups also showed the presence of vacuoles in their cytoplasm. Although cytoplasmic vacuolation occurs in neurons that are exposed to injury, the presence of these vacuoles is not necessarily related to neuronal death.<sup>28</sup> Additionally, HuC/D +, nNOS-, and nNOS+ neurons with an abnormal aspect were frequently observed in the TG5000 group. These neurons presented distortion and no distinct demarcation of the cytoplasm.

## Conclusion

The different doses of *T. gondii* oocysts did not cause glial or neuronal death in the ileal myenteric plexus in rats, but they provoked hypertrophy of their cell bodies. The higher doses (1000 and 5000 oocysts) caused less hypertrophy. The appearance of cellular injury signals, such as cytoplasmic vacuoles, Hu nuclear translocation, and dendritic distortion, may indicate less neuronal plasticity that is caused by this infection.

## Acknowledgments

This work was supported by the governmental agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Programa de Apoio à Pós-graduação (PROAP). The authors thank Professor João Luis Garcia (Universidade Estadual de Londrina – Londrina, PR, Brazil) for donating the oocysts used in this study.

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