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Research article

# Spontaneous T. gondii neuronal encystment induces structural neuritic network impairment associated with changes of tyrosine hydroxilase expression

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ABSTRACT

Two billion people are chronically infected with *Toxoplasma gondii* worldwide with unknown consequences. Important neurological diseases have been associated to the brain infection, making essential to understand the neurophysiological changes associated with the neuronal encystment. *T. gondii* may subvert neuronal functions modifying neurotransmitter concentration in chronically infected mice but the molecular mechanisms involved are still unclear. Parasites were observed inside neuronal cells in cultures from 24 – 192 hs. The rate of infection increased with time. Neurite density decreased affecting network functionality. Neuronal survival was affected and we detected the presence of cysts inside neuronal bodies and dilated portions of neurites in association with a relative increase of TH-positive neuritic area without noticeable changes in DA immunofluorescence pattern. These results advance our knowledge of the interaction between *T. gondii* and the neuronal network of the host.

## 1. Introduction

Latent toxoplasmosis is a common chronic parasitic infection caused by *Toxoplasma gondii* with prevalence of 30–70 % among human populations worldwide [1]. This intriguing parasite is able to invade most nucleated cells but prefer to encyst in neurons.

There is recent and growing evidence of infection latency causing behavioral changes, such as schizophrenia, bipolar disorder, and other psychopathological conditions [2,3]. *T. gondii* also induced behavioral changes in rodents (increased exploratory behavior, decreased neophobia, and loss of fear of cats), which increase the risk of predation by a feline, and thus ultimately benefit the parasite [4]. The parasite crosses the biological blood brain barrier (Trojan horse mechanism) resulting in the chronic stage after the immune response establishment [5]. *T. gondii* readily infects and encysts in both astrocytes and neurons *in vitro* [6]. Differently from neurons that are not able of complete intracellular parasite clearance, accessory cells present efficient mechanisms to clear intracellular parasites [7] making it highly interesting to study the neuronal parasitism.

Infections by *Toxoplasma gondii* are widely prevalent in humans and animals in Brazil [8]. The strain #11 is an atypical strain that circulates in Brazil and is found in newborns with specific tropism to the eyes [9]. Ocular desease in children with congenital Toxoplasmosis is more frequent in Brazil than in countries where parasite has a clonal population structure. Tropism to a particular brain region remains a straightforward explanation for host behavioral manipulation in several models [10]. However, for Toxoplasmosis, there is a lack of consensus in the literature and the cyst location evidences do not support the explanation for host behavioral changes. There are several plausible scenarios whereby *T. gondii* infection could affect brain and behavior in the host. Parasites could indirectly cause acute injury to the brain cells due to adverse effects of the host's innate and adaptive immune responses, including generation of autoantibodies against brain proteins. Also, at the chronic stages parasitic cysts residing in the brain may be able to affect the central nervous system physiology affecting directly host neurotransmitters production, specially DA, and also DA synaptic

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transmission in the host [3,10]. *In vivo* measurements of neurotransmitters have primarily indicated global changes within the brain, but no studies have so far investigated cell-specific changes.

The dopaminergic system has been of major interest because dopamine is essential for locomotor activity (movement), and various forms of learning, including humor and fear [11], being also implicated in infection-induced behavioral changes [4]. McConkey's group reported increased levels of DA in infected brain tissue *in vivo*. These authors also reported increased DA synthesis and release by infected PC12 cells *in vitro* as well as the recruitment of host DOPA decarboxylase (DDC), which converts L-DOPA to DA, in the interior of non-spontaneous cysts [12]. Alterations in other markers of DA synaptic transmission, such as DA metabolites or DA receptors, in the infected host were demonstrated [13].

Our superior cervical ganglion (SCG) primary cultures resemble the natural microenvironment of the neuronal network necessary for the parasite infection and encystment. Parasite spontaneous encystment is a necessary phenomenon for the long-term maintenance of the protozoan in its host brain in order to cause clinical manifestation. There is a lack of studies on structural changes of neurite network and neurotransmitter metabolic alterations induced by the parasite spontaneous cystogenesis in neurons. Our study intends to offer an *in vitro* model for these studies, shedding light on the complex interactions between the parasite and the neuronal network.

## 2. Material and methods

Primary cultures of SCG neurons were prepared as previously described [14]. Isolated SCG cultures, in absence of antimitotic drug are constituted homogenously of neurons and glial cells in the proportion of 3:1, respectively [14]. The dissociation experiments were performed at least two times for each assay. For each experimental paradigm, at least three coverslips, in each time point were analyzed. All the experimental procedures were approved by the committee of animal experimentation (CETEA/UFMG nº 379/2012).

We used a highly virulent TgCTBr9 strain (genotype #11) isolated from a 9 months-old child presenting lethal congenital toxoplasmosis [15] and maintained in mice treated with sulfadiazine order to develop tissue cysts. Tachyzoites were collected six to eight days after intraperitoneal inoculation of 100–200 cysts per [9]. Tachyzoites were added to the SCG cultures, previously established for 24 h, at a multiplicity of infection of 10 parasites per cell during three hours. NGF was added to the cells after washing the parasites. The cultures were incubated through the experimental times of 24, 48, 72, 96 and 192 h fixed with 10 % buffered formaldehyde and used for assays, photographic documentation, staining with Gaffney's one-hour Giemsa, and immunofluorescence reactions.

Images of the homogenous cell distribution and highest cell concentration areas of fixed neuronal cultures were obtained by phase contrast under 100X magnification using an Olympus IX71 microscope equipped with a Hamamatsu Orca II cooled CCD camera. Ten images were acquired for each triplicated coverslip. Neurons were distinguished from others cells by classical morphological characteristics, presenting as round cells with emission of neurites and the presence of varicosities on the neurite network [14,16]. Quantification of cell bodies was performed using ImageJ 1.48v analysis software and expressed as absolute number of neurons.

The rate of infection was evaluated using cultures fixed and stained with Giemsa solution and Periodic acid–Schiff (PAS), which stains amylopectin granules inside bradyzoits forms. In each coverslip, 300 cells were counted under immersion objective of 100X with the Olympus BX51 optical microscope. Morphological criteria were applied to discriminate non-infected or infected neurons. We validated these criteria by paired analysis of the cell markers expression in the immune-stained counterparts.

Immunofluorescence technique was performed in cultivated monolayers. Rabbit anti-NeuN (1:600, Cat: MABN91), mouse anti-tubulin β III isoform (1:1000, MAB1637), rabbit anti-tyrosine hydroxylase (1:300, Cat: MAB152, Millipore) and rabbit anti-dopamine (1:250, Cat: AB8888, Abcam) antibodies were incubated overnight at 4 °C. The secondary antibodies Alexa Fluor 546 goat anti-mouse (1:400; Cat: A11018), Alexa Fluor 594 goat anti-chicken (1:400; Cat: A11042), and Alexa Fluor 488 goat anti-rabbit (1:400, Cat: A11008) were incubated for 30 min at RT and followed by nuclear Hoechst fluorescent dye (1:200, Cat: H33342, ThermoFisher, USA).

For parasite labelling, cultures were incubated with primary rabbit anti-BAG (1:50, 7E5; kindly ceded by Dr. Rossiane C. Vommaro, UFRJ/RJ) and rabbit antiserum to *T. gondii* tachyzoites (1:1000, polyclonal; produced by R.W.A.Victor, ICB, UFMG) [17] antibodies, overnight at RT. Secondary antibody Alexa Fluor 488 was applied for 2 h at 37 °C. Technical controls were obtained by omission of the primary antibody.

In order to stain the cyst wall, we incubate the cells with *Dolichos biflorus* Agglutinin, DBA (1:5, Cat: FL103, Vector Laboratories, USA). Digital images were acquired for documentation through Image-Pro Express 4.0 (Media Cybernetics, USA). Confocal microscopy was performed using the Axio Imager Zeiss microscope and Zen software with a 40X immersion objective.

In order to access neurite network, 10 bright field images obtained from homogenous cell distribution areas of fixed neuronal cultures, under 40X magnification were used to quantify neurite density as described previously [14].

The intensity of TH expression (corrected total cell fluorescence, CTCF) in neuritic network (by excluding the cell bodies), was quantified in 10 images in triplicates of infected and control immune-stained samples under 40X magnification. The ImageJ software (NIH) was used to calculate the CTCF by applying this formula: CTCF = integrated density (indicated by the software)– (Area of selected cell x Mean fluorescence of background readings), as previously described [18]. In order to improve the accuracy of the quantification of optical density we also used the KS300 program (Zeiss, Jena, Germany) to measure digitalized TH-positive neuritic area ($mm^2$) in 10 images of each infected and control samples. The mean density of neighboring, no labeled areas in the same sections was used to normalize TH immunoreactivity. Further, we evaluated the infection-induced impact on neuritic TH expression relative to the total structural neuritic network observed by phase contrast microscopy. Ten images of superposed phase-contrast and TH-immunofluorescence fields were captures under 40X magnification and used to measure the TH-positive neuritic area ($mm^2$) and phase contrasted neuritic structure area ($mm^2$). The results were expressed as TH-positive and Total neuritic area.

In addition, we analyzed the fluorescence intensity (TH and DA) for the neuronal cell body using ImageJ software (NIH) and then measured the CTCF as previously described [18]. All analyses were performed using intra experimental controls.

The results were expressed as the mean and SD of at least two independent experiments. Two-way ANOVAs were used to compare two treatments and four time-points. When a significant *F* value was found, we performed Duncan (Neurons Infected, Fluorescence Intensity-TH and Fluorescence Intensity-DA) and Student–Newman–Keuls for the other data tests as *post hoc* analysis, according to the coefficient of variation of the variable. All tests were performed using GraphPad Prism (v 7.0) (GraphPad Software Inc., La Jolla, CA) and P values were set as $< 0.05$.

## 3. Results

Infected cultures showed discrete morphological alteration but important loss of neurites connection in comparison with controls. At bright field microscopy, SCG control culture reveal polygonal, globoid, neuronal cells with sharp conical extensions from the end tips, with characteristic birefringence, which hardly can be focused on a single plane. The neurite network was formed from neuronal cell edges

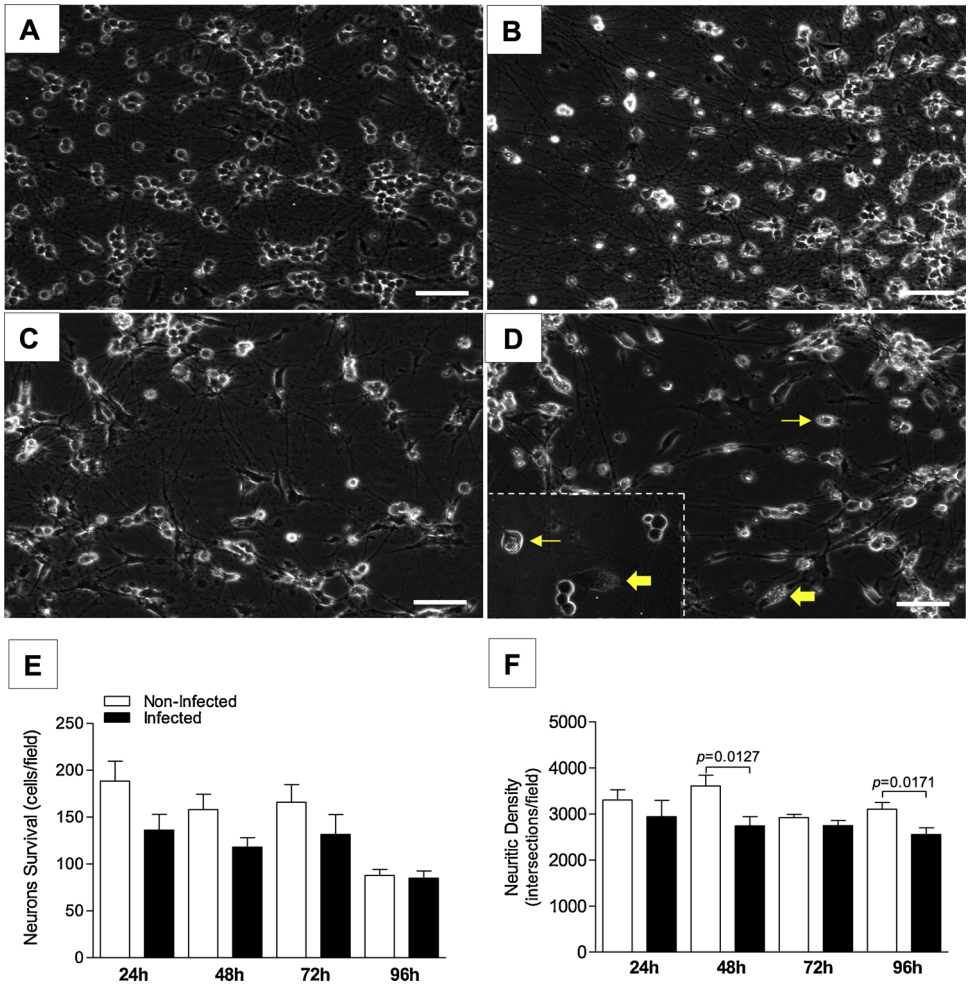


**Fig. 1.** Neuritic network functionality decreased without significant neuronal body damage in infected culture. Qualitative aspects of A 24 h and C 96 h non-infected cultures, **B** 24 h and **D** 96 h infected culture. Parasite induced enlargement of glia cells (large arrows) and neurons (thin arrows) are shown. Inset in **D** shows parasited neuron and glia. **E** Absolute number of neurons infected. **F** Neuritic density. Two-way ANOVA with post-hoc Student Newman Keuls. #Difference between 24, 48 and 72 h. n = 40 images. Bar = 20 µm.

intersected and adhered to other neurites, as well as to the surface of other neurons and accessory cells. Such extensions presented the characteristic varicosities of sympathetic neurons (rosary-bead-like formations) and were often surrounded by glial cytoplasmic processes. Glial cells were flat with large cytoplasm, reniform nucleus with evident nucleolus, adhered to the substrate, emitting radiated prolongations. Infected neuronal bodies and infected glial cells appeared enlarged (Fig. 1A–D). We also observed swelling of cell varicosities, better visualized in Fig. 4 (A–D). The parasite was present inside neuronal bodies of viable cells, and frequently inside the neuronal processes (Fig. 1B and D, Fig. 2A, PAS, 72 and 96 h). Quantitative assays confirmed decreased neuronal survival rate of infected cultures in comparison with intra experimental non-infected cultures at 24 and 48 h (Fig. 1E). SCG neuritic network stabilized and showed crescent complexity in control cultures as already described, despite of neuronal decreasing along time [14]. There were significant differences between control and infected neurite densities in the cultures of 48 h and a tendency of decrease in the cultures of 96 h (Fig. 1F).

Neurons infected with both forms of T. gondii were easily evidenced by Giemsa and the identification of bradyzoites were made by PAS stain in all the experimental times (Fig. 2). A progressive increase of the infection rate was observed overtime to both parasites forms (Fig. 2B) by PAS-labeled amylopectin granules (Fig. 2A) and confirmed by BAG (bradyzoite specific antigen) immunostaining (Fig. 2C).

We detected anti-*T.gondii*-positive structures since 24 h in the infected cultures (Fig. 3B). The specificity of the anti-*T.gondii* antibody was attested by the absence of specific staining in control cultures (Fig. 3A). The anti-*T.gondii* tachyzoites (small oval structures) were inside the cells, and adhered to body and neurites, as if attached to cell membranes (Fig. 3B, 24 h–192 h – better visualized on the confocal images in the inserts). To evaluate the kinetics of spontaneous encystment and possible cell-induced functional changes associated to the chronicity of the infection, we observed DBA reactivity. DBA is a lectin that recognizes parasite cyst wall glycoprotein (CST1) (Fig. 3C). Our results demonstrated that along cystogenesis kinetic the deposition of the CST1 glycoprotein delineates the cyst wall by 48 h pi. The marker became more visible and intense by 96 and 192 h, making it possible unequivocally visualizing cystic formation at 192 h. Moreover, the presence of the partial or complete cyst seems to induce reorganization of the host cell cytoskeleton around the parasite as demonstrated by β tubulin III expression (Fig. 3C – better visualized on the confocal images in the inserts, arrow).

The infection induced structural neuritic network damage with decrease of TH immunofluorescence intensity in the preserved network (Fig. 4A–D and *J*). The enzyme expression was found decreased exclusively in the neuritic network by measuring the intensity of fluorescence (CTCF) along the experimental times from 48 h–192 h (Fig. 4J). These results were confirmed by measuring optical density (area) of neuritic network (Fig.4 E, K). In both assays, we observed impairment in the enzyme production in the network of infected cultures indicating

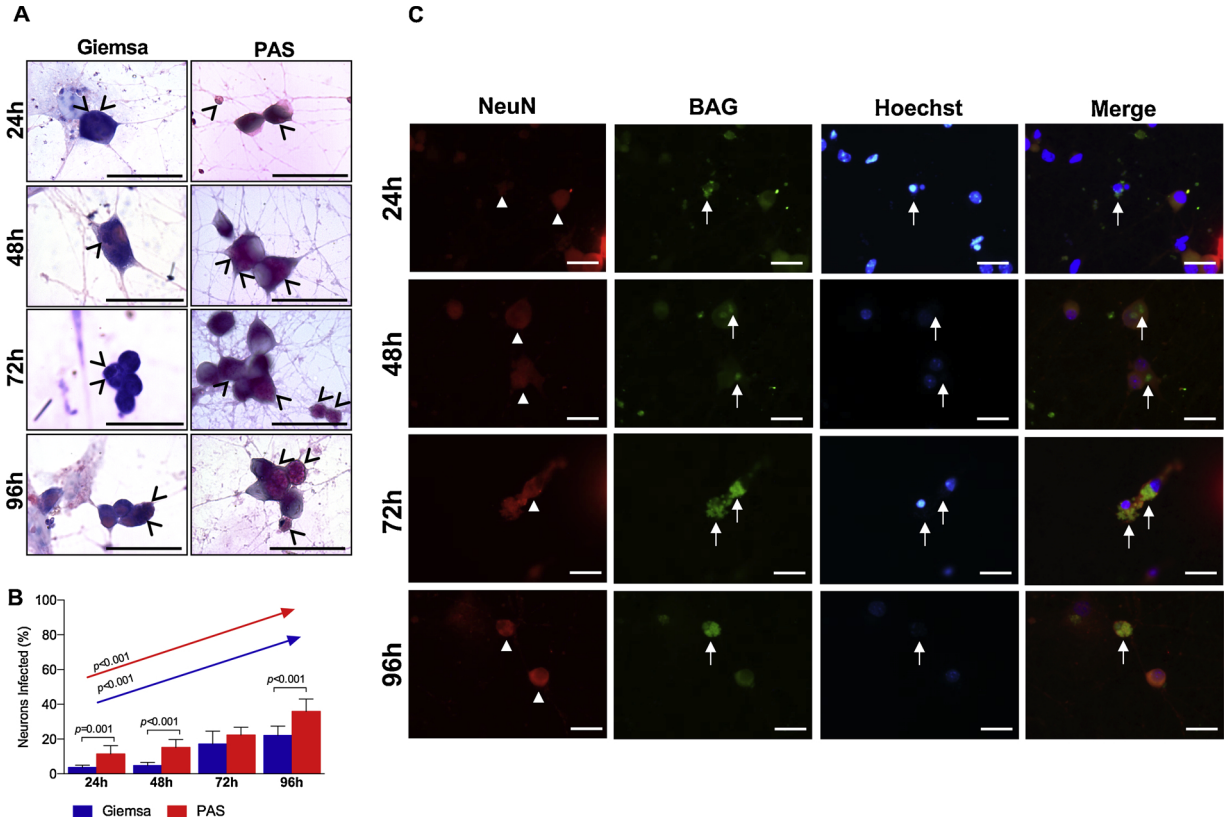


**Fig. 2. *T. gondii* infects SCG neurons.** Culture of SCG showing neuron infected with different forms of *T. gondii*. **A** Left panel indicates the cultures stained with Giemsa, which do not differentiate between parasite forms. The right panel indicates cultures stained with PAS which labels amylopectin granules of bradyzoites. **B** Percentage of infected neurons stained by Giemsa and PAS. **C** NeuN (red), BAG (bradyzoites, green) and cellular nuclei (blue). Arrowheads indicate neuronal body (red). The white arrow indicates bradyzoites marked for BAG (green) inside the cells. In the merged image the anti-BAG-positive structures are seen inside the NeuN-positive neurons as early as 24 h pi. Two-way ANOVA with post-hoc Duncan. n = 48 images. Bar = 5 μm.

a possible interference of the growing cysts with it expression and distribution.

As the total structural neurite density was affected significantly (Fig. 1F), we also measured the TH-positive neurite area in relation to the total neurite area using superposed filters, at 48 h, as indicated in Fig. 4L. Despite the expressive effect of infection on the total neurite density, TH-positive neurites in non-infected cultures represented 25 % of the total neurite area compared to 47,5 % in the infected cultures. Infection induced a relative increase in the area of the network expressing the enzyme compared to intact cultures.

Neuronal bodies in infected cultures presented decreased TH production especially at 48 e 96 h pi (Figs. 4 A–D and 5 A and C), without changes in dopamine expression, a neurotransmitter dependent on TH (Fig. 5D).

## 4. Discussion

This study reveals important neurophysiological mechanisms in primary cultures of neurons exposed to T. gondii. Encystment is the key event implicated in the permanence of the parasite in the brain and chronic neuronal infection has been implicated in neuropsychiatric manifestation of Toxoplasmosis in humans [19]. Since other studies failed to unequivocally correlate cyst distribution in specific brain areas and alteration in neurotransmitter expression with behavioral changes [10,19,20], we intended to investigate the neuronal encystment consequences in the structural and functional network of SCG *in vitro*. Our in vitro model allowed evaluating the correlation between the neuronal encystment process and the cascade of neurotransmitter production still not described by others.

Neuronal bodies in infected cultures showed the presence of enlarged parasitized cells. The parasite was able to replicate in neurons of the SCG and presented increased rate of infection with time. The infection caused some degree of neuronal death and induced a significant neurite network disruption evidenced by a decrease in neurite density noticed at 48 and 96 h pi. β-tubulin was condensed in the cytoplasm around the parasites and the cysts without great disruption of neuronal morphology in accordance with *in vivo* studies [21].

We followed the cultures stained with PAS and anti-BAG, a small heat-shock protein that is specifically expressed in the cyst-forming bradyzoite stage of the parasite. The conversion of tachyzoites into bradyzoites and the cyst formation play a fundamental role in the maintenance of *T. gondii* in nature, especially with the transmission of the parasite through carnivorism between intermediate hosts [1]. Bradyzoites within tissue cysts contain many granules of amylopectin that stain red with PAS reagent [22], perhaps as a source of energy that is not present in tachyzoites.

Inside the neuronal processes we noticed the parasites, the growing cyst, and the concomitance of TH staining in the neurite enlarged areas. Other authors also found altered morphology, predominantly by cysts presence in neuronal processes [23]. Our results indicate progressive structural changes in the neuritic network that may account for the described functional alterations. The neuronal degenerative changes and the loss of neurite network connections induced by the presence of the various stages of the parasite could indicate a high level of parasite-host adaptation. Interestingly, dendritic spine loss and loss of dendritic function are also associated with schizophrenia and other mental health disorders [24] in an otherwise healthy patient.

Under natural conditions, *T. gondii* cysts are preferentially found in neurons and muscle cells, and this stage can persist for the whole life of the host inducing behavioral changes. Most of groups studying

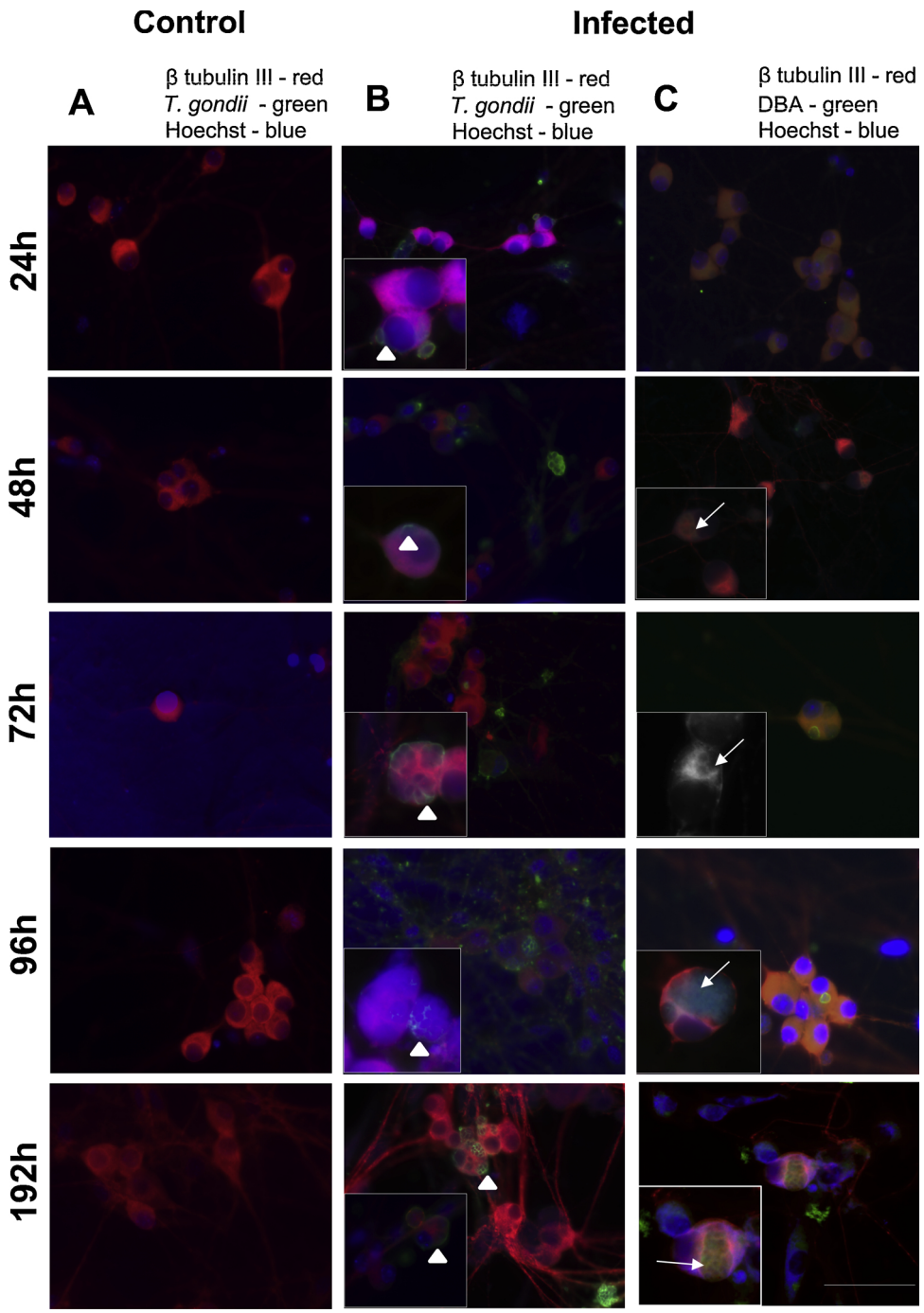


**Fig. 3. *T. gondii* spontaneous encystment kinetic. A** Control culture showing merged images for β tubulin III (red) and Hoechst (blue). **B** Neurons (red) Infected were stained with anti-*T. gondii* (green). Inserts indicate confocal images and the arrowheads points to parasites inside the cells**. C** DBA (green) indicates parasite cyst wall glycoprotein deposition with a progressive intensification of DBA staining from 48 h. Inserts indicate confocal images and the arrows points to cysts inside the cells. n = 40 images. Bar = 5 μm.

encystation use some type of stimuli, pH and temperature alteration or addition of exogenous IFN-γ [25].

Our results indicated the spontaneous conversion of infective forms to the chronic encysted forms 24 h pi for bradyzoites and 192 h pi for the complete cyst detection. This is the first study of the process of spontaneous *in vitro* encystation of the virulent, human, atypical TgCTBr9 strain Genotype #11 of *T. gondii* [20] followed for 192 h. This atypical strain was isolated in a very vulnerable population that usually develops retinochoroiditis, indicating the relevance of this study model. For the first time this process was followed in a primary neuronal culture, the cell type that is the natural host cell for this complex parasite.

Only a few studies evaluated *in vitro* spontaneous encystment using diverse immortalized cell types [26] and neurotransmitters changes during the *in vitro* infection by the *T. gondii* parasite [12,27]. Paredes-Santos et al., studied the infection of fibroblast by EGS strain (virulent strain) and found cyst formation by 96 h [26], while we found it as early as 72 h. Halonen et al., studied the cystogenic strain ME49 in human neurons and astrocytes and found induced cystogenesis with 48 h pi [28]. These differences might be due to parasite genetic background and or, host cell environment, implicating in encystation process and overall behavior of the strain.

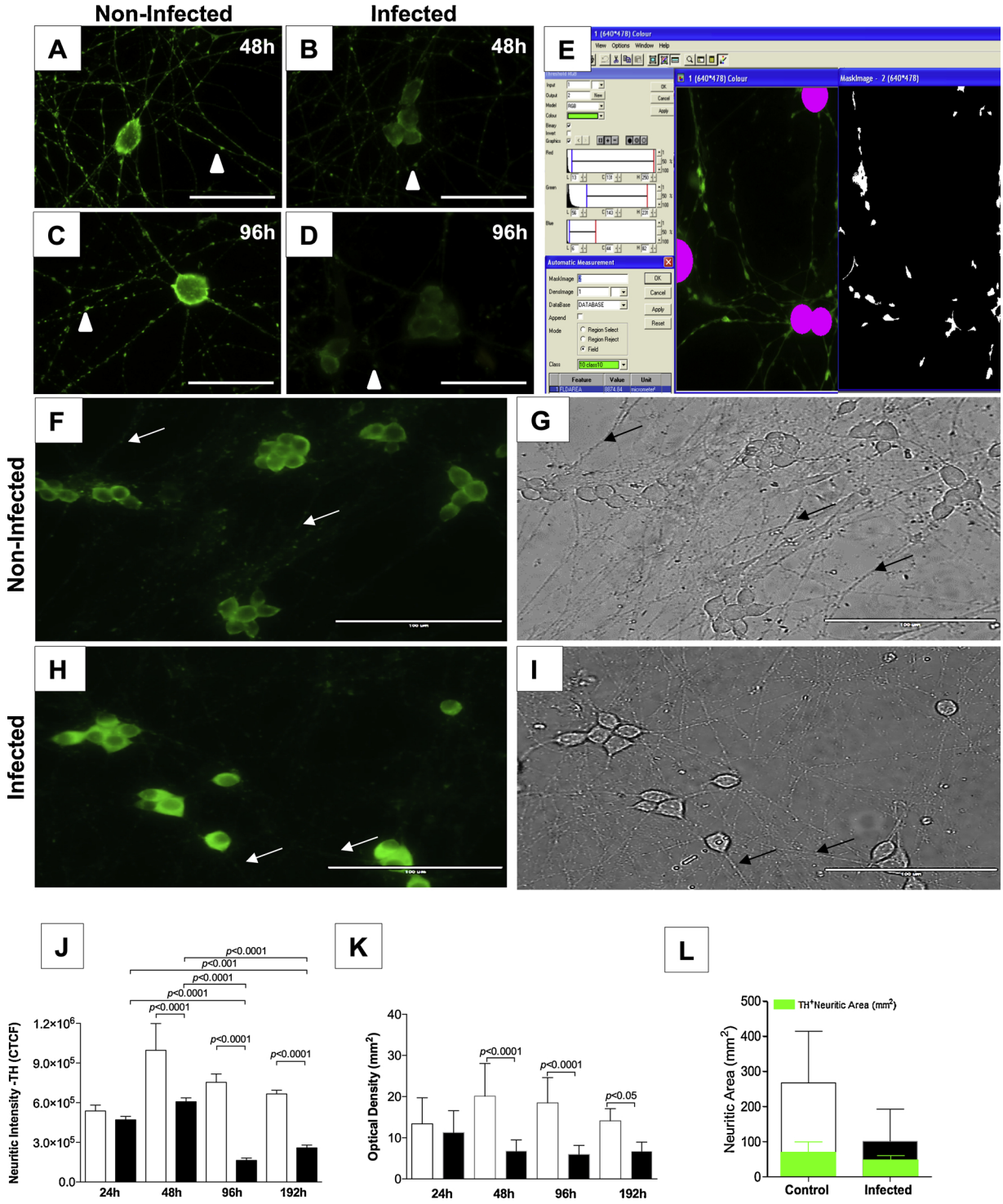


**Fig. 4. Neuritic density overlapped with TH immunostaining.** Non infected cultures **A** and **C,** and infected cultures **B** and **D**, Representative photomicrographs (40X) of the TH staining by immunofluorescence technique of the measurements represented in **J-L.** Arrow-heads indicate varicosities on the neuritic network. **E** An example of the software image process is provided. Immunoreactivity was evaluated in terms of optical density, excluded the neuron bodies. **F** Control and **H** Infected cultures showing TH staining by immunofluorescence. **G** Control and **I** Infected cultures showing total neuritic network by phase contrast microscopy. **J** quantification of TH neuritic intensity fluorescence. **K** Optical density in neurite network. **L** Morphometric quantification of TH-positive area relative to total neuritic network area measured by KS300. Two-way ANOVA with post-hoc Student Newman Keuls. n = 40 images. Bar = 20 μm.

The functional status of the model was evaluated by the correlation between infection and the expression of the TH enzyme. Our results indicate that the optical density (area) and the intensity of TH-immunofluorescence decreased in the neuritic network of infected cultures. Both measurements indicated interference of infection and encystation of the parasite in the TH production in the neuronal bodies and neuritic network. For the neurite network, we detected decreased TH expression from 48 – 192 hs, indicating a long-term disruption of TH production and probably reflecting the significant damage and reduction in the structure of this network. TH-positive area in non-infected cultures (basal conditions) represented 25 % of total neurite area compared to 47 % in the infected cultures. The kinetics of TH

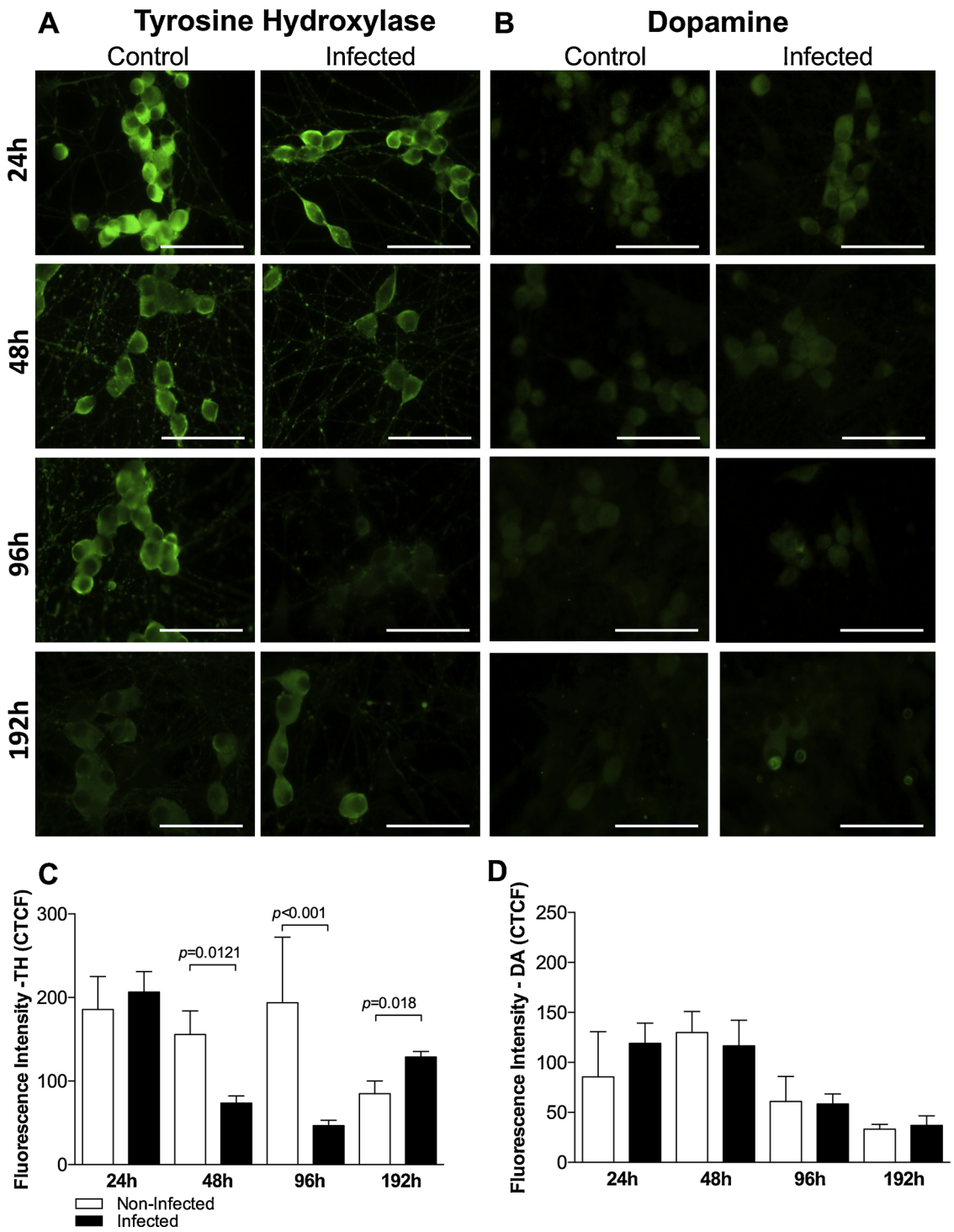


**Fig. 5. TH fluorescence intensity but not dopamine fluorescence intensity is compromised in infected cultures**. **A** and **C** show respectively the qualitative and quantitative TH kinetic expression in infected and non-infected neuron bodies. **B** and **D** show the qualitative and quantitative expression of DA kinetic in infected and non-infected neuron bodies. Two-way ANOVA with post-hoc Duncan. n = 40 images. Bar = 10 μm.

expression are compartmentalized, indicating cell body reestablishment of the enzyme expression in the later time point (192 h pi) while the expression in the cellular prolongations is still significantly reduced possibly implicating the neurite localization of the cysts. These changes could be evidence for damage, remodeling and compensation by the host feedback mechanism, and ultimately responsible for the initiation of behavioral-associated neurotransmitter production that deserves further elucidation.

Interestingly the dopamine, a product of the catecholamine cascade and a neurotransmitter associated with neuro-psychiatry alterations in chronically infected patients, did not present neuronal body alteration in any of the analyzed time points. Several studies that directly measured dopamine levels or dopamine metabolites in brain tissues suggest increase of CNS dopamine levels [12,29] while others were unable to confirm these changes [30] in behavioral changes. One could hypothesize that the proven production of dopamine by the parasite [29] in the chronic form of the infection plus the neuronal production of the dopamine itself could be inducing negative feedback, shutting down the

enzyme temporarily and locally, as an adaptation mechanism. By the other side, L-tyrosine is the substrate of TH for Dopamine production, and knowing that the parasite does not produce L-tyrosine directly [27], we could suggest that L-tyrosine from the host cell is used in order to make its own parasitic TH.

One explanation for these contradictory findings is that each group used different mouse strains, which can affect the immune response to *T. gondii* [31]. Indeed, there are growing evidences that non-neuronal cells can be responsible by the dopamine increase detected post infection. Our findings indicate that *in vitro* infection of neurons in the absence of immune cells did not induce dopamine changes despite of the TH decrease in some moments post-infection.

In addition, the parasite tyrosine hydroxylase has a high degree of homology (53 % identity) with mammalian tyrosine hydroxilases. Further on, *Toxoplasma* tachyzoites and bradyzoites were shown to be auxotrophic to L-tyrosine, L-arginin and L-tryptophan, and require exogenous tyrosine for cyst formation *in vitro*, despite keeping the ability to invade [27].

Changes in the neurotransmitter output (and neurite network integrity and function along the parasite infection, and its cystogenesis have never been discussed in any cell culture system and urge to be better elucidated in the context of the behavioral problems attributed to the host-parasite interaction in infected patients. Given the diverse range of disorders associated with infection, it is clear that the neurophysiological changes induced are exceptionally complex. Unravelling the mechanisms involved in the host-parasite interaction will be essential to further understanding the neurobiological aspects of this disease

## Author statement

Each author contributed individually and significantly to the development of this study and approved the final version submitted for publication.

**JLB:** Methodology Development, conducting a research and investigation process, performing the experiments, data collection, analyze or synthesize study data, edited and approved the manuscript; **SRB:** Conceptualization, performed experiments, analyze data, approved the manuscript; **MFR:** Methodology Development, validation, performing the experiments, synthesize study data, application of statistical to analyze study data, edited and approved the manuscript; **MLMN:** Performed experiments, analyzed data, approved the manuscript; **CTC:** Performed experiments, analyzed data, approved the manuscript; **BVP**: Performed experiments, analyzed data, approved the manuscript; **RWAV:** Provided laboratory support, isolated and provided parasites, edited and approved the manuscript; **RMEA:** Provided laboratory support, conceptualized the work, analyzed data, synthesize study data, wrote, edited and approved the manuscript, supervision, project administration, funding acquisition.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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