Behavioral Alterations Induced By *Toxoplama Gondii* During Different Stages Of Infection In Mice

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ABSTRACT

Acute, chronic and reactivation of *Toxoplasma gondii (T. gondii)* infection is approximately found in 30-60% of population world-wide. *T. gondii* is a pathogen relevant to psychiatric disorders. We have recently found that reactivation of chronic *T. gondii* induced depressive-like behavior in mice. In the present study, we aimed to illustrated the behavioral alterations in mice during acute, chronic and after reactivation of chronic *T. gondii*. Behavioral battery included sucrose preference and forced swim, and fear conditioning tests, and measurement of locomotor activity following *T. gondii* infection in mice. First, we prepared an ethogram and confirmed that specific pathogen free-BALB/c mice exhibited sickness-like behaviors during acute infection. In addition, reduced sucrose preference and increased immobility in forced swim test (putative indicators to anhedonic and despair-like behaviors respectively) were exhibited in acute phase. While in chronic stage such symptoms were not exhibited at all. In turn, relapse of depressive- and some of sickness-like symptoms noticed after reactivation of chronic

T. gondii infection. Interestingly, increased freezing time in fear conditioning was displayed during acute and reactivated *T. gondii* but not during chronic infection. Further, despite the well-known contribution of neurotransmitters; serotonin and dopamine in major depressive disorder, low levels of these neurotransmitters were observed in the brain not only in acute but also during chronic infection. Without interpolation of these symptoms to human psychiatric disorders, collectively, our results demonstrated the crucial changes induced by acute and reactivated *T. gondii* in modifying the behavior of mice.

Key words: Depressive-and sickness-like behaviors, Mice, *Toxoplasma gondii* infection.

1. Introduction

The most successful neurotropic parasite, *Toxoplasma gondii,* chronically infects more than a third of the world's population (Montoya and Liesenfeld, 2004). And its prevalence has been linked to many psychiatric disorders including schizophrenia, bipolar mood disorder, and self –directed violence (Ling et al., 2011, Pearce et al., 2012, Pedersen et al., 2012, Zhang et al., 2012). Brain is considered an immune-privileged site for life-long bradyzoite cysts of *T. gondii*. *T. gondii* exists in two distinct forms, an immune-stimulating tachyzoite and an immune-encrypted bradyzoite. *T. gondii* can cause dysfunction of nervous system, resulting influence of host behaviour to gain the advantage of completing its own life cycle (Berdoy, 2000). Because proliferation of tachyzoites is higher than that of bardyzoites, the tachyzoites stimulate greater inflammatory responses. Therefore, the high onset of schizophrenia was reported at the acute stage of the tachyzoite infection (Carruthers and Suzuki, 2007. Major depressive disorders show a worldwide distribution pattern (Barlow and Durand. 2005). With regard to the underlying mechanisms, the tryptophan (Trp) catabolic shunt towards kynurenine (Kyn) has been proposed in depressive disorders associated with

inflammatory and comorbid conditions (Lestage et al., 2002, Wichers et al., 2005, O'connor et al. 2009a, b). Specifically, within Trp shunt to Kyn, unavailability of Trp for serotonin synthesis is due to stress-induced activation of the liver enzyme, tryptophan 2, 3-dioxygenase, and/or pro-inflammatory cytokines-induced extrahepatic enzyme, indoleamine 2, 3-dioxygenase (IDO), with both enzymes being rate limiting for Trp catabolism (Fukui et al., 1991, Lestage et al., 2002, Russo et al. 2003). In addition to the resultant low serotonergic neurotransmission, Kyn is a neuroreactive metabolite that readily crosses the blood-brain barrier (BBB), and is linked to certain neurodegenerative and depressive disorders (Fukui et al., 1991, Wichers et al., 2005, Oxenkrug, 2013). *T. gondii* infection in immunocompetent mice is nearly asymptomatic unless immunosuppression occurs, whereby *T. gondii* bradyzoites are reactivated and cause toxoplasmic encephalitis (Barlow and Durand. 2005, Dupont et al., 2012). Certain mouse strains, such as BALB/c, are considered genetically resistant to developing fatal toxoplasmic encephalitis, and instead establish latent chronic infections, as observed in humans (Brown et al. 1995, Suzuki et al., 1995, 2000). Thus, these mice may be suitable models for behavioral analyses during different stages of *T. gondii* infection (Mahmoud et al., 2016). Thus, we examined behavioral changes of BALB/c mice after *T. gondii* infection in this study. Our findings provide important information on behavioral changes, enhancement of kynurenine pathway and changes in neurotransmission during acute and reactivation stages of *T. gondii* infection.

2. Material and methods

2.1. Mice: This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Ministry of Education,

Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 24-15, 25-59). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering. Experiments were performed using female BALB/c mice (7 weeks old) obtained from Clea Japan, Inc. (Tokyo, Japan). Animals were housed under specific pathogen-free conditions. Mice were maintained for one week in stable conditions (12-h light dark cycle; light on from 7 a.m. to 7 p.m.) with food and water *ad libitum* before starting all behavioral experiments at 09:00 a.m.

2.2. Parasite and infection: *T. gondii* (PLK strain, Type II) tachyzoites were maintained in cultured Vero (African green monkey kidney epithelial) cells and purified as previously described (Mahmoud et al., 2015). Each mouse was infected intraperitoneally with 1000 tachyzoites suspended in 0.2 mL sterile PBS. At designated time points, mice were decapitated without anesthesia, blood collected in heparinized tubes to obtain plasma, and organs harvested in liquid nitrogen and stored at −80 °C until analysis. Reactivation of *T. gondii* was performed by dexamethasone treatment. The procedure used in this study has been described to reactivate toxoplasmosis in chronically infected mice (Lyons et al., 2002, Mahmoud et al., 2016).

2.3. Behavioral Measurements:

2.3.1. Sucrose preference test: Mice were first habituated with two bottles of water for 1 week before infection followed by one bottle with 1% sucrose solution and the other with tape water. The bottle position was switched every night according to the reward test protocol (Nielsen et al., 2000, Strekalova et al., 2002). When performing sucrose

preference for experimental groups subjected to DXM treatment were also allowed to drink from two bottles within and without the drug.

2.3.2. Forced swim test (FST): FST procedures have been previously described (Kim et al., 2012, Mahmoud et al., 2016).Mice were placed individually in water, in the FST cylinder (12 cm in diameter, filled with 25 cm water depth, Coulbourn Instruments and Penlab, Harvard Apparatus, Roosvelt, Whilehall, PA, USA). Water temperature was adjusted within thermoneutral range (at 31 ± 1 °C) of rodents. The duration of immobility within a 6-min session was recorded as immobility score. The first 2 min of the test were omitted from calculations. Analysis was performed offline by an experienced observer blinded to experimental groups.

2.3.3. Despair-like behavior in fear conditioning chamber: The test is based on development of despair behavior following repeated exposure to unavoidable and unpredictable mild footshocks, and we followed standard procedures (Müller ,1997). After exposure to mild unescapable footshock (0.3 mA for 5 s), defensive freezing, defined as cessation of all movements except breathing, was assessed in animals during paired light and audible cue sessions in a commercially available fear conditioning chamber adopted for mice (Compac Act Vas, Muromachi Kikai Co., Ltd., Tokyo, Japan). A square area (17 cm^2) was provided with high unescapable walls. The paradigm consisted of two trials. Day 0 was the associative learning session, and mice were administered a series of inescapable mild footshocks (120 s accommodation, three shocks, 0.3 mA, 5 s duration, 5 s paired light and sound cues). During test trials at 10, 30, 42, and 60-days post infection (dpi), mice were exposed to day 0 conditions and assessed for despair behavior by measuring freezing during exposure (5 s) to a cued

light and tone session. Sham control mice were exposed to the fear conditioning chamber, but did not receive any footshocks or cues.

2.3.4. Clinical score: We customized ethograms according to the appearance of clinical symptoms during the first week of *T. gondii* infection within home and naïve cages as described previously (Mahmoud et al., 2016). Scores varied from 0 (no signs) to 10 (all signs) and included hunching, piloerection, worm-seeking behavior, ptosis, sunken eye, ataxia, reluctant movement, deficient evacuation and touch reflexes, and lying on belly.

2.3.5. Assessment of locomotor activity: It was measured in a naïve cage identical to the home cage ($16 \times 26 \times 13$ cm^3) but devoid of bedding. The number of line crossings and rearings over 12 virtual identical quadrants were counted during a period of 180 s. Test cages were kept in a testing room within the same housing room.

2.4. High-performance liquid chromatography (HPLC): Major monoamines were measured in supernatants obtained from the right halves of brains, using 5-µm octyldecyl silane columns (Eicompak SC-5ODS) and an electrochemical detector, according to the monoamine analysis application manual (Eicom, Kyoto, Japan) as described previously (Mahmoud et al., 2016). Chromatographs were quantified using PowerChrom software version 2.5 (eDAQ Pty Ltd., Densitone East, Australia).

2.5. IDO activity assay: To detect IDO activity, Kyn was measured in brain homogenate (per mg tissue) according to earlier reports (D¨aubener et al. 1994, Mahmoud et al., 2015). Equal amounts (250 µL) of homogenate and 2x IDO buffer (100 mM PBS, 40 mM ascorbate, 20 µM methylene blue and 0.8 mM L-Tryptophan, pH 6.5) were mixed, and 10 min after adding the color developing solution, Ehrlich's reagent (0.8% p-dimethylaminobenzaldehyde in acetic acid), color was detected at 490 nm.

2.6. ELISA. Plasma L-Trp was measured by fluorescence (excitation: 485 nm, emission: 665 nm) using the Bridge-It L-Trp Fluorescence Assay kit (Mediomics, LLC, St. Louis, MO, USA). Plasma L-Kyn was assayed by competitive ELISA with a mouse L-kyn monoclonal antibody (Abcam, Tokyo, Japan). ELISA readings at 490 nm were plotted against L-kyn (Sigma) standard curves.

2.7. Statistical analysis: GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) was used. Data were presented as mean ± SD. Significant differences ($P < 0.05$) were determined by Student *t*-tests or ANOVAs, with Bonferroni or Tukey's Multiple Comparison post-hoc tests used to compare means after ANOVA.

3. Results

3.1. T. gondii induced anhedonic-like behavior during acute and reactivation stages of infection. Reduced sucrose preference, putative indicative to anhedonic behavior, appeared at 7 dpi, and reaching a peak level at 10 dpi, and returning to control levels after 2 weeks (Fig. 1A). Under reactivation conditions, *T. gondii*-infected mice did not exhibit difference in sucrose consumption from the PBS-injected animals at 42 dpi (Fig. 1B). However, a highly significant reduction in sucrose consumption was observed in *T. gondii*-infected and DXM-treated mice compared with other groups at 60 dpi (Fig. 1B)

3.2.* T. gondii *increased clinical score and induced despair-like behaviors during acute and reactivation stage of the infection.

In Fig. 2 we tested the effect of *T. gondii* infection on despair-like behavior (As shown in Fig. 2A, immobile duration was increased in FST for the infected mice from day 4 to day 10 postinfection but not later. Moreover, at 42 dpi before DXM treatment, there were no significant differences between *T. gondii*-infected and PBS-injected mice in the immobile duration (Fig. 2B, upper raw). However, treatment of the infected mice

with DXM showed a significantly increased immobile duration at 60 dpi compared with the other experimental groups (Fig. 2B lower raw). This finding was confirmed by increased defensive freezing time in the fear conditioning test at 10 but not at 30 dpi (Fig. 3B). Similarly increased freezing duration was observed in *T. gondii*/DXM mice at 60 dpi, but not before DXM treatment at 43 dpi.

The infected mice showed some clinical symptoms after *T. gondii* infection starting from the day 3 to day 18 postinfection (Fig. 4A) and infected mice after DXM treatment such as piloerection and warmth-seeking behavior (Fig 4C). Consequently, reduced locomotor activity was dispaled by infected mice from day 4 to day day 23 postinfection (Fig. 4B). However, non-different locomotor activity was found among all groups at 60 dpi (Fig. 4D). The results obtained showing no difference in locomotor activity among groups, coupled with minimal clinical signs observed in ethogram analysis, suggest that enhanced immobility and freezing in the FST and fear test, respectively, is unlikely due to motor defects. Therefore, the resulting depressive-like behavior is not likely to be associated with generic symptoms of sickness.

3.3.* T. gondii *reduced levels of serotonin and dopamine (DA) in brain: To determine whether the observed behavioral changes were associated with the levels of serotonin and DA, we examined the production of these neurotransmitters in the brain collected at the end of behavioral experiments. Serotonin and DA levels were significantly reduced at 10 and 30 dpi compared with PBS-injected group (Fig. 5A). Although lower levels of these neurotransmitters were observed in mice at 60 dpi, treatment of the infected mice with DXM did not change the levels of these neurotransmitters from those of the control (Fig. 5B). Hence, low levels of 5-HT and DA were also observed in chronic stage, these

results indicated that the low levels of serotonin and DA did not participated in the exhibited behavioral changes at least during chronic stage of the infection.

3.4.* T. gondii *enhanced tryptophan turnover to kynurenine in brain during acute and reactivation stage of the infection: Next, we examined Trp catabolites, monoamines and their metabolites. In *T. gondii*-infected mice, we found that the calculated Kyn/Trp ratio in brain at 10 dpi was approximately 3.7-fold that at 30 dpi, whereas in plasma it was nearly 1.9-fold (Fig. 5A). Similarly, Kyn/Trp ratio in the brain of the infected and DXM-treated mice was significantly higher than that of the other groups (Fig. 5B). However, plasma Kyn/Trp ratio in the infected and DXM-treated group was not different from the infected and untreated group while it was still significantly higher than that of uninfected group (Fig. 5B).

Altogether, enhancement of IDO activity in brain indicated by the elevated Kyn/Trp ratio was commonly observed at the acute and reactivation stage of the infection.

Discussion

Despaired mice do not display escape tendencies in the FST and fear conditioning test (Borsini et al., 1986). We found in parallel with the observed anhedonic-like behavior in the tachyzoite/reactivated tachyzoite stages, despair-like behavior is also displayed. Nevertheless, specificity of the behavioral effect has not yet been investigated in either tachyzoite stages (both acute and reactivation stage), because mice at acute stages show comparable despair in the FST and fear conditioning test together with high clinical score and reduced locomotor activity. However during the chronic stage, mice do not display such behavior in the FST or fear conditioning test (Fig. 2 and 3). We found that in parallel with the reduced sucrose consumption, increased

immobility duration in FST and freezing time in fear conditioning test were also observed in the infected mice at acute and reactivation stages of the *T. gondii*. However, mice did not exhibit such despair-like behavior in the FST and fear conditioning test during the chronic stage. Altogether, it is feasible that anhedonic- and despair like behaviors induced by *T. gondii* is specific to acute and reactivation stages of the infection.

Decreased levels of Trp and increased levels of Kyn were observed in the serum, lung and brain from mice infected with T. gondii, furthermore maximum induction of IDO expression and activity were observed during acute infection (Fujigaki et al., 2002). Our findings support a role of Trp turnover to Kyn in depression pathophysiology induced by *T. gondii* infection. Low levels of brain serotonin induced by *T. gondii* infection at the chronic stage were neither stage specific nor associated with depressive-like symptoms. Anhedonic and despair-like behaviors were present when low serotonin level was associated with a high Kyn/Trp ratio in the plasma and the brain, and with or without clinical score. In addition, anhedonic and despair-like behaviors at the reactivation stage were also associated with an enhanced Kyn/Trp ratio in the brain, but not in the plasma Kyn/Trp ratio. These results suggest induction of anhedonic and depressive-like behaviors via enhanced brain IDO activity as indicated by an elevated Trp/Kyn ratio in the plasma and brain during acute stage and in the brain only at the reactivation stage. Hence, anhedonic and depressive-like behaviors are associated with an elevated Kyn/Trp ratio as in reported other studies (Lestage et al., 2002, Wicher et al., 2005, O Conor et al., 2009 a, b, Mahmoud et al., 2016).

The induction of depression symptoms may be explained by IDO activity enhanced by proinflammatory cytokines, thereby increasing Trp catabolism to Kyn within the

brain and causing reduced bioavailability of Trp and thus low serotonin level. The levels of serotonin and DA after *T. gondii* infection were neither specific to acute stage nor despair-and anhedonic-like symptoms, at least under our conditions. Thus, there may be a need to extensively examine the lowered serotonergic and dopaminergic neurotransmission in distinct brain regions following *T. gondii* infection. Our study shows that infection with and reactivation of *T. gondii* tachyzoites induces alterations in behavior of mice, possibly via enhancement of Trp catabolism to Kyn by IDO activity within the brain.

Conclusion: Altered behaviors induced by *T. gondii* infection in terms of anhedonic- and despair-like behaviors were exhibited at the acute and reactivated stages plus elevated clinical score during acute stage. On other hand, no such behavioral alterations were displayed during chronic infection.

Conflict of interest: All authors disclose that there is no any actual or potential conflict of interests.

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Figure Captions

Fig. 1: Reduced sucrose preference following *T. gondii* infection. (**A**) Reduced sucrose preference, a hall mark of anhedonic behavior, was calculated as the percentage of total fluid intake over 24 hours for 1 month after *T. gondii* infection or PBS injection (n = 8). (**B**) In *T. gondii*-infected or PBS-injected mice, dexamethasone (DXM) was continuously administered in drinking water from 43 to 58 dpi. Sucrose preference was measured in 2 groups before DXM treatment at 42 dpi (n = 12) and in 4 groups after DXM treatment at 60 dpi (n = 6). * indicate significant differences between PBS-injected and *T. gondii*-infected groups and data represent mean ± SD.

Fig. 2: Examination of sedentary time in the forced swim test (FST). (**A**) In *T. gondii*-infected or PBS-injected mice, sedentary time (immobility) was examined for 14 dpi. (n = 10). (**B**) Immobility in FST was also examined before DXM treatment in *T. gondii*-infected and PBS-injected groups at 42 dpi (n = 24, left) and after DXM treatment in 4 groups at 60 dpi (n = 12, right). Data represent mean ± SD.

Fig. 3: Examination of freezing duration in fear conditioning test (**A**) In *T. gondii*-infected or PBS-injected mice, freezing duration was examined at 10 and 30 dpi. (**B**) Freezing duration was also examined before DXM treatment in *T. gondii*-infected and PBS-injected groups at 42 dpi (n = 24) and after DXM treatment in 4 groups at 60 dpi (n = 12). Data represent mean ± SD.

Fig. 4: Clinical scores and locomotor activity in *T. gondii*-infected mice during acute chronic and reactivation stages. Clinical score (**A**) and locomotor activity (**B**) were measured once a day for 30 days. (**C**) In addition, clinical score was also measured for 3 days before and for 14 days during treatment with DXM. (**D**) Similarly locomotor activity was measured at 60 dpi (n =12). Data represent mean ± SD.

Fig. 5: Reduction of serotonin and dopamine levels, and enhanced tryptophan catabolism following *T. gondii* infection. (**A**) In *T. gondii*-infected or PBS-injected mice, brain serotonin, dopamine (DA) and Trp turnover to L-kynurenine (Kyn) /Trp ratio, and plasma Kyn/Trp ratio were determined at 10 and 30 days post-infection (dpi) (n = 10). (**B**) Similarly these variables were measured before and after DXM treatment (n = 10).