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AAH2 gene is not required for dopamine-dependent neurochemical and behavioral abnormalities produced by Toxoplasma infection in mouse.

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Abstract

Infection with the protozoan parasite, *Toxoplasma gondii* (*T. gondii*), has been associated with the increased risk for several psychiatric disorders. The exact mechanisms of a hypothesized contribution of *T. gondii* infection are poorly understood. The *T. gondii* genome contains two aromatic amino acid hydroxylase genes (*AAH1* and *AAH2*) that encode proteins that can produce L-DOPA. One popular hypothesis posits that these encoded enzymes might influence dopamine (DA) production and hence DA synaptic transmission, leading to neurobehavioral abnormalities in the infected host. Prior studies have shown that deletion of these genes does not alter DA levels in the brain or exploratory activity in infected mice. However, possible effects of *AAH* gene deficiency on infection-induced brain and behavior alterations that are directly linked to DA synaptic transmission have not been evaluated. We found that chronic *T. gondii* infection of BALB/c mice leads to blunted response to amphetamine or cocaine and decreased expression of Dopamine Transporter (DAT) and Vesicular Monoamine Transporter 2 (VMAT2). Deletion of *AAH2* had no effects on these changes in infected mice. Both wild type and *aah2* strains

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A competing interests statement

The authors have no competing interests to declare

produced comparable levels of neuroinflammation. Our findings demonstrate that *AAH2* is not required for *T. gondii* infection-produced DA-dependent neurobehavioral abnormalities.

Keywords

Toxoplasma; AAH2; dopamine; amphetamine; cocaine; DAT; VMAT2

1. Background

Microbial infections may be involved in the pathogenesis of major psychiatric disorders [1]. Infection with the protozoan parasite, *Toxoplasma gondii* (*T. gondii*), has been associated with increased risk for schizophrenia, bipolar disorder, and other psychopathological conditions [2–7]. However, the exact mechanisms of how *T. gondii* infection contributes to the development of psychiatric disorders remain obscure.

There are several plausible scenarios whereby *T. gondii* infection could affect brain and behavior in the host. For example, proliferating parasites during an acute phase of infection could indirectly cause injury to brain cells because of adverse effects of the host's innate and adaptive immune response, including generation of autoantibodies against brain proteins [8]. Once a persistent infection has been established, parasitic cysts residing in the brain may also be able to affect the brain physiology [8]. Specifically, it has been proposed that brain cysts could directly affect dopamine (DA) production and DA synaptic transmission in the host's brain [9]. This hypothesis stems from the original analysis of the *T. gondii* genome that identified two genes (*AAH1* and *AAH2*) encoding for aromatic amino acid hydroxylases that convert Phenylalanine to Tyrosine and then to L-Dihydroxyphenylalanine (L-DOPA), the direct precursor of DA [10,11]. Consistent with these findings, McConkey's group reported increased levels of DA in infected brain tissue *in vivo*, and elevated DA synthesis and release by infected PC12 cells *in vitro* [12] as well as the recruitment of host DOPA decarboxylase (DDC) to the interior of cysts, which converts L-DOPA to DA [12].

Other studies report no changes in tissue levels of DA or even found reduced levels of DA and its metabolites [13]. Furthermore, the Sibley group reported that neither over-expression nor deletion of *AAH2* from parasites altered DA levels *in vitro* or *in vivo* [14]. Recently, a different group has generated a mutant strain of *T. gondii* lacking the *AAH2* gene and found no effects of mutation on the host's exploratory behaviors [15]. Collectively, these studies with *aah2* parasites have strongly suggested that *AAH2* is not required for biochemical and behavioral abnormalities observed in chronically infected mice.

One possible explanation of these contradictions is that the above studies with mutant did not employ behavioral and/or neurochemical assays that were sensitive enough to detect more subtle and specific changes in DA synaptic transmission. In order to address this possibility, we conducted a series of behavioral, pharmacological, and biochemical experiments to directly probe the status of DA synaptic transmission in mice infected with wild-type Prugniaud (PRU) strain or the *aah2* mutant in this background. We report that chronic *T. gondii* infection of BALB/c mice leads to blunted response to amphetamine or cocaine administration and decreased expression of the DA pre-synaptic markers, Dopamine

Transporter (DAT) and Vesicular Monoamine Transporter 2 (VMAT2). Deletion of *AAH2* had no effects on either behavioral or biochemical changes produced by chronic *T. gondii* infection. Notably, chronic infection with both wild-type and *aah2* parasites led to activation of microglia and astrogliosis in the brain, suggesting that neurobehavioral sequelae of infection could be explained by inflammatory processes.

2. Materials and Methods

2.1. Animals

Four-week-old male BALB/c mice, a Toxoplasma resistant strain [16], were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed five per cage with 12 h light/dark cycles with food and water *ad libitum*. Animal protocols were reviewed and approved by the Animal Care and Use Committee of Johns Hopkins University (JHU).

2.2. Toxoplasma tachyzoites purification

Prugniald (PRU) tachyzoites were maintained in human foreskin fibroblast monolayers (HFF) and purified as previously described [16]. Mice were either intraperitoneally (ip) injected with sterile Dulbecco's phosphate buffered saline (Vehicle) or received 400 tachyzoites (2 parasites/ μ L, ip) in 200 μ L vehicle at five weeks of age, typically one week after arriving at the JHU facility.

2.3. Generation of knockout *aah2* strain

Generation of transgenic PRU parasites, was accomplished after harvesting tachyzoites, as described previously [14]. They were then mixed with 5 μ g of CRISPR plasmids and 15 μ g of the appropriate homologous repair construct as plasmids linearized by restriction digest. Parasites were transfected by electroporation, and allowed to recover on HFF monolayers for 24 h. Positive selection for the (Hypoxanthine-Xanthine-Guanine PhosphoRibosyl Transferase (HXGPRT) cassette was done with 25 μ g/mL mycophenolic acid (Sigma-Aldrich, St. Louis, MO) supplemented with 50 μ g/mL xanthine (Sigma-Aldrich, St. Louis, MO) [17]. Negative selection against the HXGPRT cassette was done with 340 μ g/mL 6-Thioxanthine [18].

2.4. Behavioral testing

The behavioral tests were performed in the following order: novelty-induced activity, prepulse inhibition (PPI) of the acoustic startle response, and drug-challenge tests. The interval between different behavioral tests was 8 days.

2.4.1. Novelty-induced activity—Novelty-induced activity was assessed in the open field over a 30-min period using activity chambers with infrared beams (San Diego Instruments Inc., San Diego, CA, USA). Horizontal and vertical activities, and time spent in the center of the chamber were automatically recorded and analyzed.

2.4.2. Acoustic startle and PPI—Two identical startle chambers (San Diego Instruments Inc., San Diego, CA, USA) were used for measuring startle reactivity and plasticity. Each mouse was placed in a Plexiglas cylinder (2 cm in diameter) within each

chamber. A loudspeaker mounted 24 cm above the cylinder provided the broadband background noise and acoustic stimuli. Presentations of the acoustic stimuli were controlled by the SR-LAB software and interface system, which rectified, digitized, and recorded responses from the accelerometer. The maximum voltages within 100-ms reading windows, starting at stimulus onset, were used as the measures of startle amplitudes. Sound levels were measured inside the startle cabinets by means of a digital sound level meter (Realistic, Tandy, Fort Worth, TX, USA). The accelerometer sensitivities within each startle chamber were calibrated regularly and were found to remain constant over the test period.

The experimental session consisted of a 5 min acclimatization period to a 70-dB background noise (continuous throughout the session), followed by a pseudorandom presentation of six of each of 40-ms acoustic startle stimuli of different intensities (broadband noise of 100, 110 or 120 dB) at a 20-s inter-stimulus interval. Upon the completion of that startle-only session, each mouse was left in the enclosure for 5 min without presentations of any startle stimuli. Immediately after, the pre-pulse inhibition (PPI) session was begun. During each PPI session, a mouse was exposed to the following types of trials: pulse-alone trial (a 120 dB, 100 ms, broadband burst); the omission of stimuli (no-stimulus trial); and five pre-pulse–pulse combinations (pre-pulse–pulse trials) consisting of a 20-ms broadband burst used as a pre-pulse and presented 80 ms before the pulse using one of the five pre-pulse intensities: 74, 78, 82, 86, and 90 dB. Each session consisted of six presentations of each type of the trial presented in a pseudorandom order. PPI was assessed as the percentage scores of PPI (%PPI): $100 \times (\text{mean startle amplitude on pulse-alone trials} - \text{mean startle amplitude on prepulse–pulse trials}) / \text{mean startle amplitude on pulse-alone trials}$ for each animal separately. The percentage of PPI for each animal was used as the dependent variable in statistical analysis.

2.4.3. Stimulant-induced activity—Stimulant-induced activity in the open field was assessed using activity chambers with infrared beams (San Diego Instruments, San Diego, CA, USA). Animals were initially habituated to the activity chambers for 30 min (the habituation session). Immediately thereafter, mice received a single injection of saline and their locomotor activity was monitored for another 30 min (the saline session). Immediately after the saline session, mice were injected with D-amphetamine (3 mg/kg, ip) (Sigma-Aldrich, St. Louis, MO) and their locomotor activity was monitored for 90 min. Administration of cocaine (30 mg/kg, ip) (Sigma-Aldrich, St. Louis, MO) was done in a similar manner except for eliminating the saline session as multiple experiments with amphetamine had clearly demonstrated that saline injections *per se* did not change locomotor activity of either group of mice. Total locomotor activity was automatically recorded and analyzed.

2.4.4. Immunostaining—Upon completion of behavioral testing, mouse brains were prepared for immunostaining. Mice were anesthetized with a mixture of ketamine (100 mg/kg), xylazine (20 mg/kg), and acepromazine (10 mg/kg) and perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The harvested brain samples were postfixed at 4°C in 4% PFA, 10% sucrose, and then 30% sucrose

dissolved in PBS. Coronal brain sections were cut at 40 μ m using a sliding microtome (Microm HM 450, Thermo Scientific) and processed for immunostaining.

Sections were stained following standard procedures (3 mice per group; 3 sections per mouse). Briefly, sections were placed in blocking solution [2.5% Triton X and 5% Horse Serum in 1 $\times$ PBS] for 30 min, followed by primary antibody incubation with Rabbit anti-IBA1 (1:250, WAKO) or Chicken anti-GFAP (1:250, Abcam) overnight. Sections were incubated in secondary antibody goat α -Rb AlexaFluor568 (1:400; Life Technologies) and α -Ch AlexaFluorTM488 (1:400; Life Technologies) for 1 h and DAPI (1:3000; Invitrogen) for 10 min (Supplemental Table 1). Images were visualized using a Nikon eclipse E400 microscope and images taken with the program MetaVue version 6.2r6. Quantification of IBA1 and GFAP fluorescence staining was accomplished using ImageJ software (Build 1.51s NIH). Mean fluorescent intensity was calculated for 1800 $\times$ 2000 $\times$ 40- μ m area of the striatum. Each group consisted of three mice. For each mouse, three sections were randomly selected 80 μ m apart at Bregma levels 1.18–1.34 mm [19]. Data are presents as means $\pm$ standard deviation for each group.

2.4.5. Western blot assays—Whole striatum protein expression was measured via Western blotting following standard protocols (5–6 mice per group). Isolated striatum tissue was sonicated for 30 s in RIPA buffer (Cell Signaling Technology, 9806) containing 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF and 1 μ g/mL leupeptin. Tissue lysates were spun down at 10,000 $\times$ g for 10 min at 4 $^{\circ}$ C. The resulting supernatant was subjected to SDS/PAGE, and the separated proteins were transferred onto a nitrocellulose membrane. The membrane was washed in Tris-buffered saline solution containing 0.05% Tween 20 (TBST) and was blocked for 1 hour at RT in TBST containing 5% non-fat dry milk. The membrane was incubated overnight at 4 $^{\circ}$ C with a primary antibody. The optical density of protein bands on each digitized image was normalized to the optical density of the β -actin using the freely available ImageJ software program (version 1.49v). All primary and secondary antibodies used are listed in Supplemental Table 1.

2.4.6. Serum antibody measurements—Blood was taken by survival draw during acute infection and again during sacrifice, centrifuged at 10,000 $\times$ g for 5 min to collect serum. Absorbance values on a polyclonal anti-Toxo IgG serum ELISA kit (IBL America) were used for statistical analysis.

2.4.7. MAG1 measurement—*T. gondii* cyst burden was determined using a previously developed assay for antibodies to cyst matrix antigen 1 (MAG1) [20,21].

2.4.8. Statistical analysis—Novelty-induced or psychostimulant-induced locomotor activity was analyzed with two-way repeated measures ANOVA, with infection status and time as independent variables, and locomotor activity as a dependent variable. PPI was analyzed with two-way repeated measures ANOVA, with infection status and pre-pulse intensities as independent variables, and the percentage of PPI as a dependent variable. Other behavioral data, Western blotting data, and serology data were analyzed with one-way ANOVA or the Student two-tailed t-test. Upon determining main effects, ANOVAs were

followed by Holm-Sidak test post-hoc test. A value of $P = 0.05$ was considered significant. All data are presented as means $\pm$ Standard Error of Means (SEM).

3. Results

3.1. Behavioral testing

3.1.1. Open field test—DA synaptic transmission is critical for locomotor activity in mice [22]. We sought to evaluate the effects of chronic *T. gondii* infection on novelty-induced locomotor activity in mice in the open field test. We found that chronic infection with *T. gondii* did not significantly alter general locomotor activity in mice (SFig.1). Although infected mice exhibited moderately decreased activity in the open field test, we did not find any significant changes in the total activity, central activity or vertical (rearing) activity (SFig.1).

3.1.2. Pre-pulse inhibition of the acoustic startle—Another DA-dependent behavior is pre-pulse inhibition (PPI) of the acoustic startle [23]. Chronic toxoplasma infection significantly decreased PPI of the acoustic startle in mice (Fig.1A). Infected mice exhibited significantly decreased PPI at the pre-pulse intensities 87 and 90 dB ($P < 0.05$). Notably, impaired PPI in infected mice was unlikely related to alterations in startle responsiveness as no group-dependent differences were detected in startle response to the different magnitudes of the startle stimulus (Fig.1B).

3.1.3. Amphetamine-induced activity—One of the most robust behavior pharmacology tests for DA synaptic transmission is stimulant-evoked hyperactivity [24]. We evaluated amphetamine-induced hyperactivity in 8-month-old uninfected (control) and PRU-infected male mice, i.e., months after infection with wild-type PRU strain (Fig.2A). We found that in contrast to uninfected control mice, PRU-infected mice showed a significantly attenuated response to the stimulant.

As we observed blunted responses in 8-month-old infected mice, we could not completely rule out that aging may have confounded response to amphetamine. In order to address this question, we assessed amphetamine-induced activity in 3-month-old mice, i.e., 2 months after infection. Remarkably, blunted response to amphetamine was also observed in young adult infected mice and appeared more pronounced compared to old infected mice (Fig.2B). Thus, the blunted response to amphetamine was present regardless of how much time passed after the initial infection.

3.1.4. Cocaine-induced activity—Whether blunted response to amphetamine was specific to that stimulant or can be generalized to other stimulants remained unclear. For example, amphetamine and cocaine act through different mechanisms to enhance pre-synaptic DA release and increase locomotor activity [25]. Thus, we also assessed the effects of cocaine on locomotor activity in mice. Control and infected 3-month-old mice were given single cocaine injections (30 mg/kg ip) and locomotor activity was assessed as above except for saline injections that were not used for this experiment as our prior studies had found no group-related effects of saline injections. Similar to the amphetamine results, we observed significantly blunted response to cocaine in PRU-infected mice (Fig.3).

3.1.5. Expression of synaptic markers—As we observed the blunted responses to psychostimulants that act through pre-synaptic machineries of DA vesicular transport, release and re-uptake, we sought to evaluate the effects of *T. gondii* infection on the expression of presynaptic DA markers. We found that chronic *T. gondii* infection significantly decreased expression of Dopamine Transporter (DAT) and Vesicular Monoamine Transporter 2 (VMAT2) (Fig.4). Unlike these changes, no significant alterations were found in expression of Synaptosomal-Associated Protein 25 (SNAP25), a t-SNARE protein involved in the molecular regulation of neurotransmitter release [26]; or the 2A subunit of the N-methyl-D-aspartate receptor used as a marker of glutamatergic synapse [27] (Fig.4).

3.1.6. The effects of *AAH2* deletion—Prior studies have demonstrated that deletion of the *AAH2* gene did not alter the ability of the parasite to proliferate, induce humoral immune response, alter tissue levels of DA, or alter exploratory behaviors produced by wild-type *T. gondii* infection [14,15]. Here, we evaluated the effects of the *AAH2* deletion on the behavioral and biochemical measures that are more directly associated with DA synaptic transmission. Unlike uninfected control mice, mice infected with either wild-type or *aah2* strains of the parasite exhibited comparably blunted responses to amphetamine (Fig.5). Similarly, we detected no significant alterations in expression of the DA markers in the striatum samples from mice infected with wild-type or *aah2* strains of *T. gondii* (Fig.6). The lack of any differences in the *aah2* strain on the DA-related abnormalities in infected mice were unlikely to be related to parasite strain-dependent differences in general health, weight gain, cysts formation or immune response. We found no significant differences between mice infected with wild-type or *aah2* strain in the body weight gain (SFig.2), the levels of Mag1 protein, a brain cysts marker (SFig.3) [20] or polyclonal anti-Toxo IgG response (Table 1). In addition, we observed no effects of *AAH2* deletion on *T. gondii* infection-produced neuroinflammation as assessed with IBA1⁺ or GFAP⁺ immunostaining (Fig.7).

4. Discussion

Our study demonstrates for the first time that *AAH2* gene is not required for the development and manifestation of DA-dependent behavioral and biochemical alterations produced by chronic *T. gondii* infection in mice. Instead, the present experiments suggest that neuroinflammation may be a leading cause of the pathological and behavioral abnormalities in the infected host.

The DA hypothesis posits that *T. gondii* cysts can influence the host's behavior via perturbing DA synaptic transmission and/or DA brain tissue levels [28]. Stibbs was the first one to report that, unlike acute infection, chronic *T. gondii* infection led to a modest 14% increase in DA levels when the entire mouse brain was assayed [29]. In further support of this hypothesis, Flegr and associates found that the selective DA uptake inhibitor, GBR 12909, decreased exploratory activity in infected mice and increased the same in uninfected control mice [30]. Another study demonstrated that haloperidol, a DA receptor 2 antagonist, prevented the development of parasite-associated behavioral changes [31]. A critical advancement for the DA hypothesis was provided when the McConkey lab discovered that

the *T. gondii* genome contains two genes, *AAH1* and *AAH2*. These genes encode enzymes with aromatic amino acid hydroxylase activity [11]. In follow-up studies, the group found that *T. gondii* infection of PC12 cells significantly facilitated potassium-induced release of DA and that parasitic cysts could contain DA as revealed by immunostaining with the anti-dopamine antibody [10]. These investigators also showed the presence of DOPA decarboxylase (DDC) within the parasitophorous vacuole (PV), the cytosol of infected PC12 cells and the brain tissue cysts [12]. Publications from other laboratories have also demonstrated alterations in other markers of DA synaptic transmission, e.g., DA metabolites or DA receptors, in the infected host [20,32,33].

However, all these intriguing findings provided only indirect support for the DA hypothesis. The first attempt to directly test the hypothesis was made by the Sibley lab that successfully deleted the *AAH2* gene in the parasite and found no effects of this genetic deletion on host DA *in vitro* or *in vivo*, suggesting that *AAH* enzymes may not be responsible for altered DA levels in the host brain. In fact, their studies with the *aah1* and *aah2* strains indicated that the enzymes are critically involved in the sexual cycle of the parasite in the gut of the definitive feline host where they likely play a role in oocyst wall formation [14,34]. An additional refutation of the hypothesis has recently come from Afonso et al who observed no changes in parasite-produced abnormal exploratory behaviors in the mice infected with another *aah2* strain of *T. gondii* [15]. Taken together, these studies strongly suggested that *AAH2* is not required for behavioral phenotypes in chronically infected mice.

Our current study provides a new perspective for DA abnormalities in *T. gondii* infected mice. We have evaluated behavioral and biochemical changes that are directly related to DA synaptic transmission and now report that psychostimulant-induced hyperactivity can be used as a robust behavioral measure for evaluating DA synaptic transmission in *T. gondii* infected mice. Specifically, our current and prior studies demonstrate that chronic *T. gondii* infection blunts amphetamine- or cocaine-induced hyperactivity in mice irrespective of strain, sex or age of the host, time elapsed after infection or type of the parasite [20]. Psychostimulant-induced hyperactivity is a classic behavioral pharmacology test for DA-related changes [24] as both amphetamine and cocaine predominantly act through altering synaptic levels of DA, although these compounds also target other monoamine systems [35].

In order to provide a possible mechanistic explanation for the blunted response to psychostimulants in infected mice, we assessed expression of key proteins that are involved in synaptic DA transmission and are targeted by amphetamine and cocaine, i.e., Vesicular Monoamine Transporter 2 that transfers DA from cellular cytosol into pre-synaptic vesicles [36]; and Dopamine Transporter (DAT) that transfers DA from the synaptic cleft back into cytosol [37]. We found that *T. gondii* infection significantly decreased levels of VMAT2 and DAT in the striatum. We hypothesize that *T. gondii* infection decreases expression of VMAT2, leading to reduced availability of pre-synaptic DA and, as a result, decreased release of DA in response to psychostimulants. In such a case, lower levels of DAT might be a compensatory response to decreased levels of pre-synaptic DA. In addition, decreased expression of DAT will further potentiate attenuated responses to psychostimulants because of diminished availability of DAT as their primary target.

These effects appeared somewhat specific to DA, as levels of SNAP-25 or NR2A were not affected, suggesting that decreased expression of DAT and VMAT2 were unlikely associated with non-specific perturbations in synaptic transmission. The latter may have been detected had neuronal and/or synaptic loss or degeneration been present in infected mice. However, more work is clearly needed to further evaluate how *T. gondii* may affect other factors and mechanisms involved in the regulation of DA synaptic transmission. In keeping with the behavioral data, no significant effects on *T. gondii*-produced changes in expression of the DA markers were found in mice infected with the *aah2* strain. Our results convincingly demonstrate that the *AAH2* gene is not required for *T. gondii*-produced DA-dependent neurochemical and behavioral alterations. Future studies will utilize *in vivo* assays to further evaluate alterations in DA synaptic transmission in infected mice.

In contrast to the DA hypothesis, there is a growing appreciation that neuroinflammation and/or the peripheral immune response initiated by *T. gondii* infection could contribute to the various biochemical and behavioral abnormalities described in numerous studies [38]. Notably, deletion of *AAH2* did not seem to affect microglia activation or astrogliosis. Although our findings do not provide evidence for a direct, causal role of activated microglia and/or astrogliosis in DA-related alterations, it is tempting to speculate that inflammatory processes might lead to the neurobehavioral phenotypes observed in the present study. Still, future studies will directly address this possibility. In addition, since previous investigations have not found any preference by parasitic cysts for specific brain regions [39–42], it is conceivable that perturbation in other neurotransmitter systems could also result from inflammation and the immune response [8,20,43,44].

5. Conclusions

Our work demonstrates for the first time that deletion of the *AAH2* gene in *T. gondii* did not alter DA-dependent biochemical and behavioral abnormalities observed in infected mice. We found no effects of genetic ablation on infection-induced blunted responses to psychostimulants or decreased expression of DAT and VMAT2 in the striatum. This genetic manipulation had no effects on the activation of microglia or astrocytes in the brain regions containing DA synaptic terminals, suggesting a possibility that *T. gondii* produced DA-related alterations might be linked to inflammatory processes in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

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Highlights

- Chronic Toxoplasma infection blunts psychostimulant-induced hyperactivity
- Chronic Toxoplasma infection reduces levels of DAT and VMAT2 in the brain
- Deletion of parasite *AAH2* gene does not alter dopamine-dependent abnormalities
- Deletion of parasite *AAH2* gene does not affect neuroinflammation
- Parasite-produced neurobehavioral pathology may be associated with neuroinflammation

Figure 1. Chronic toxoplasma infection impairs sensorimotor gating in mice.

The effects of chronic *T. gondii* infection on the acoustic startle and sensorimotor gating were evaluated.

A - PPI was measured in control (vehicle) and infected (PRU) mice at different pre-pulse intensities (74–90 dB); the background noise was 70 dB, and the startle stimulus was 120 dB. The Y-axis depicts the percentage of PPI; the X-axis shows different intensities of pre-pulses.

Two-way repeated measures ANOVA shows a significant effect of pre-pulse intensity, $F(4,84)=25.8$, $p<0.001$ and the group by pre-pulse intensity interaction, $F(4,84)=3.34$, $p=0.016$. Post-hoc Bonferroni t-test shows significant group-related differences for 87 dB and 91 dB pre-pulses, $P<0.05$; * - denotes $P<0.05$ vs. PRU mice; $n=10$ mice/group.

B - Startle responses were measured in control (vehicle) and infected (PRU) mice at three different intensities of the startle stimulus (100, 110 and 120 dB); the background noise was 70 dB. The Y-axis depicts the startle magnitudes (artificial units); the X-axis shows different intensities of the startle stimulus. No group differences were found in responses to the increasing intensities of the startle stimulus, $P>0.05$; $n=10$ mice/group.

Figure 2. Chronic toxoplasma infection blunts response to amphetamine

Locomotor activity was evaluated in control (vehicle) or infected (PRU) mice during the habituation session (intervals 1–6), the saline session (intervals 7–12) and the amphetamine session (intervals 13–30). The Y-axis depicts locomotor activity as assessed by counting beams breaks; the X-axis shows 5-min intervals for each session. Thin arrow points to the time of saline injection, thick arrow points to the time of amphetamine injection (5mg/kg, ip).

A – The effects of *T. gondii* on amphetamine-induced activity in 8-month-old mice. Two-way repeated measures ANOVA shows a significant effect of time, $F(29,599)=14.84$, $P<0.001$ and the group by time interaction, $F(29,599)=3.06$, $P=0.016$. Post-hoc Holm-Sidak test showed significant group-related differences at intervals 13 and 14, $P<0.05$; * - denotes $P<0.05$ vs. PRU mice; n=10 mice/group;

B - The effects of *T. gondii* on amphetamine-induced activity in 3-month-old mice. Two-way repeated measures ANOVA shows a significant effect of time, $F(29,599)=16.6$, $P<0.001$ and the group by time interaction, $F(29,599)=2.03$, $P=0.001$. Post-hoc Holm-Sidak test showed significant group-related differences at intervals 15–19, $P<0.05$; * - denotes $P<0.05$ vs. PRU mice; n=10 mice/group - check this

Figure 3. Chronic toxoplasma infection blunts response to cocaine.

Locomotor activity was evaluated in control (vehicle) or infected (PRU) mice during the habituation session (intervals 1–6) and the cocaine session (intervals 7–24). The Y-axis depicts locomotor activity as assessed by counting beams breaks; the X-axis shows 5-min intervals for each session. Thick arrow points to the time of cocaine injection (30 mg/kg dose, ip). Two-way repeated measures ANOVA shows a significant effect of time, $F(23,215)=2.35$, $P=0.001$ and the group by time interaction, $F(23,215)=2.23$, $P=0.002$. Post-hoc Holm-Sidak test showed significant group-related differences at intervals 7–8 and 10–13, $P<0.05$; * - denotes $P<0.05$ vs. PRU mice; $n=10$ mice/group.

Figure 4. Chronic toxoplasma infection alters expression of DA synaptic markers.

Expression of the DA and synaptic markers were assessed in striatal samples of control (vehicle) or infected (PRU) mice. Chronic *T. gondii* infection significantly decreased levels of Dopamine Transporter (DAT) and Vesicular Monoamine Transporter 2 (VMAT) but had no significant effects on expression of Synaptosomal-Associated Protein 25 (SNAP25) or the NMDA receptor subunit 2A (NR2A); * - indicates for DAT $P=0.015$, $t=3.082$, two-tailed Student test; for VMAT2 $P=0.012$, $t=3.258$, two-tailed Student test; $n=5$ per samples.

Figure 5. Deletion of *AAH2* does not alter blunted response to amphetamine

Locomotor activity was evaluated in control (vehicle) mice, mice infected with wild-type laboratory strain (PRU), or mice infected with the KO strain (PRU-KO) during the habituation session (intervals 1–6), the saline session (intervals 7–12) and the amphetamine session (intervals 13–30). The Y-axis depicts locomotor activity as assessed by counting beams breaks; the X-axis shows 5-min intervals for each session. Thin arrow points to the time of saline injection, thick arrow points to the time of amphetamine injection. Two-way repeated measures ANOVA shows a significant effect of time, $F(29,899)=4.13$, $P<0.001$ and the group by time interaction, $F(29,899)=1.69$, $P<0.001$. Post-hoc Holm-Sidak test showed significant difference between control mice and PRU or PRU-KO mice at intervals 16–18, and control and PRU mice at intervals 15–19; $P<0.05$; * - denotes $P<0.05$ vs. PRU and PRU-KO mice; ** - denotes $P<0.05$ vs. PRU only; $n=10$ mice/group

Figure 6. Deletion of *Aah2* does not change expression of synaptic DA markers.

Expression of the DA and synaptic markers were assessed in striatal samples of control (vehicle) mice, mice infected with wild-type laboratory strain (PRU), or mice infected with the KO strain (PRU-KO). Chronic *T. gondii* infection with PRU or PRU-KO strains significantly decreased levels of Dopamine Transporter (DAT) and Vesicular Monoamine Transporter 2 (VMAT) but had no significant effects on levels of Synaptosomal-associated protein 25 (SNAP25) or the NMDA receptor subunit 2A (NR2A); one-way ANOVA for DAT - $F(2,7)=6.39$, $P<0.05$; and for VMAT2 - $F(2,7)=19.64$, $P<0.01$; Holm-Sidak Post Hoc test showed significant difference between controls and PRU or PRU-KO; * - denotes $P<0.05$ vs. PRU or PRU-KO mice.

Figure 7. Deletion of *Aah2* does not affect activation of microglia or astrocytes

Expression of the microglia marker, IBA1, and the astrocyte marker, GFAP, was assessed in the striatum of control (Vehicle) mice, mice infected with wild-type laboratory strain (PRU), or mice infected with the KO strain (PRU-KO). Brain sections were immunostained with anti-IBA1 (red); anti-GFAP (green) or DAPI (blue, the nuclear marker). 3 equally distanced sections were used for each mouse in each group (n=3 mice/group).

Panel A shows representative images with the outlined areas depicted at a higher magnification on Panel B; scale bars - 1mm (A) and 50 μ m (B). Compared to control mice, chronic *T. gondii* infection with PRU or PRU-KO strains produced a significant activation of microglia and astrogliosis in the striatum (C); one-way ANOVA for IBA1 - $F(2,7)=6.39$, $P<0.05$; and for GFAP - $F(2,7)=19.64$, $P<0.01$; Holm-Sidak Post Hoc test showed significant difference between controls and PRU or PRU-KO; * - denotes $P<0.05$ vs. PRU or PRU-KO mice.

Table 1.

Polyclonal Anti-Toxo IgG Response

	0 wpi	2 wpi	3 wpi
Vehicle	0.0277± 0.004	0.0270±0.004	0.0247±0.003
PRU	0.0177± 0.002	0.3183±0.014	0.514±0.067
Tg-THAAH2	0.0134± 0.001	0.3982±0.022	0.5646±0.054

Data represent absorbance values ± SEM, n=4 in each group, wpi – weeks post infection.