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Reassessment of the Role of Aromatic Amino Acid Hydroxylases and the Effect of Infection by Toxoplasma gondii on Host Dopamine

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Toxoplasma gondii infection has been described previously to cause infected mice to lose their fear of cat urine. This behavioral manipulation has been proposed to involve alterations of host dopamine pathways due to parasite-encoded aromatic amino acid hydroxylases. Here, we report successful knockout and complementation of the aromatic amino acid hydroxylase AAH2 gene, with no observable phenotype in parasite growth or differentiation in vitro and in vivo. Additionally, expression levels of the two aromatic amino acid hydroxylases were negligible both in tachyzoites and in bradyzoites. Finally, we were unable to confirm previously described effects of parasite infection on host dopamine either in vitro or in vivo, even when AAH2 was overexpressed using the BAG1 promoter. Together, these data indicate that AAH enzymes in the parasite do not cause global or regional alterations of dopamine in the host brain, although they may affect this pathway locally. Additionally, our findings suggest alternative roles for the AHH enzymes in T. gondii, since AAH1 is essential for growth in nondopaminergic cells.

The protozoan parasite Toxoplasma gondii is an obligate intracellular parasite that is capable of infecting most warm-blooded animals. It is a member of the phylum Apicomplexa, which also contains Plasmodium falciparum, the causative agent of malaria. Toxoplasma gondii is one of the most widely distributed parasites in the world, both in geographic location and in the diversity of its host range (1). Its only definitive hosts are members of the genus Felis. Exclusively within enterocytes of the gut, it undergoes a sexual reproductive cycle to form environmentally resistant and infectious oocysts that are shed in cat feces (2). In all other hosts, Toxoplasma gondii infection begins as a fast-growing lytic stage called the tachyzoite. Innate and adaptive immune responses restrict the growth of tachyzoites, which can respond by differentiating into bradyzoites, a semidormant stage that exists as quiescent intracellular cysts in brain and muscle tissue. This chronic infection can persist for the lifetime of the host (3). Infections spread among incidental hosts through carnivorous, vertical transmission, and ingestion of T. gondii oocysts (4).

Rodents that become infected by T. gondii exhibit an unusual behavioral response: they lose their instinctive aversion to the odor of cats and instead become mildly attracted to the scent (5–13). This behavioral manipulation appears specific to the cat (7, 8), and it has been speculated that this facilitates transmission (9). The exact mechanism of this behavioral manipulation is unknown, but parasite stimulation of host dopamine pathways in the brain has been suggested as a cause (14–16). It was observed that infection of mice by T. gondii caused a 14% increase in whole-brain dopamine levels upon establishment of chronic infection (17). Additionally, dopamine receptor antagonist drugs used for the treatment of schizophrenia block cat attraction in infected rodents (18, 19). T. gondii infection also was described to increase dopamine content and dopamine release in the dopaminergic cell line PC12 in vitro (15).

The mechanism by which infection might alter dopamine is unknown, but it has been suggested that parasite metabolism contributes to elevated dopamine levels (20). The T. gondii genome contains two genes that encode aromatic amino acid hydroxylases (AAA), which carry out the rate-limiting step of dopamine synthesis in metazoans by converting tyrosine into the dopamine precursor 3,4 dihydroxyphenylalanine (L-Dopa) (20). The two nearly identical genes AAH1 and AAH2 contain putative signal peptides targeting them for secretion, and both appear to be functional tetrahydrobiopterin-dependent aromatic amino acid hydroxylases in vitro (20). AAH1 was reported to be constitutively expressed, while the expression of AAH2 was reported to increase in the dormant bradyzoite stage (20). Because of this pattern, AAH2 was suggested to be the prime candidate effector of the parasite’s manipulation of host dopamine (20).

We sought to test the hypothesis that the aromatic amino acid hydroxylases AAH1 and/or AAH2 were responsible for causing alterations in dopamine metabolism in the host. We successfully knocked out and complemented the AAH2 gene but, unexpectedly, could not observe a parasite effect on host dopamine levels either in vitro with PC12 cells or in vivo with mouse infection. Further, we observed that expression of both AAH1 and AAH2 was negligible in tachyzoites, and while they both showed increased expression in bradyzoite stages, the relative expression level still was very low. Collectively, our findings indicate that AAH enzymes in T. gondii do not cause global alterations of host dopamine; rather, they may participate in alternative pathways.
MATERIALS AND METHODS

Parasite strains. Parasites were propagated by serial passage in human foreskin fibroblast (HFF) cells grown in Dulbecco’s modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 10 mM HEPES, pH 7.4, 1 mM glutamine, 10 μg/ml gentamicin, under 5% CO₂ at 37°C (D10 medium). The PruΔku80Δhxg strain (type II) was obtained from John Boothroyd (21). The ME49 strain (type II) (ATCC 50611; American Type Culture Collection, Manassas, VA) originally was isolated from sheep diaphragm (22). The C56 strain (type III) originally was isolated from a chicken (23). PC12 cells (ATCC CRL-1271) were obtained from the ATCC and cultured in RPMI 1640 medium (ATCC) supplemented with 10% heat-inactivated horse serum (Life Technologies) and 5% FBS (PC12 medium). SH-SY5Y cells (ATCC CRL-2266), confirmed to be mycoplasma negative, also were obtained from the ATCC and cultured in a 1:1 mixture of ATCC Eagle’s minimum essential medium and F12 medium supplemented with 10% FBS (SH-SY5Y medium).

Generation of deletion plasmids. To generate a deletion plasmid targeting the AAH2 locus, regions 1.1 kb upstream and 1.8 kb downstream from the AAH2 locus were PCR amplified from ME49 genomic DNA using primers listed in Data Set S1 in the supplemental material and were cloned into pDONR-p4p1r and pDONR-p2r3 vectors, respectively (Invitrogen). Using the Gateway 3-fragment system, the upstream and downstream flanks and a central HXGPRT expression cassette cloned into pDONR-p1r2 were assembled into pDEST-R4R3 to create the plasmid pHXG::ΔaaH2.

Generation of AAH2 cleanup and complementation plasmids. To generate a plasmid to remove HXGPRT from the AAH2 locus, the 1.1 kb upstream and 1.8 kb downstream regions were PCR amplified from ME49 genomic DNA with primers that added a 20-bp overlap. The pieces then were fused by PCR and cloned into pDONR-p4p1r to create plasmid pΔaaH2. To complement AAH2, the cDNA of AAH2 was amplified from a PruΔku80Δhxg cDNA sample, and the 1.1 kb upstream region with an ~20-bp overlap into the 5’ end of the AAH2 coding sequence and the 1.8 kb downstream region with an ~20-bp overlap into the 3’ end of the AAH2 coding sequence were amplified from PruΔku80Δhxg genomic DNA (gDNA). The three pieces were fused by PCR and cloned into pDONR-p4p1r to create the plasmid p::AAH2.

Generation of AAH1/AAH2 tagging and AAH2 BAG1 overexpression plasmids. To generate plasmids to tag AAH1/AAH2, primers were designed to amplify 1.5 kb of the 3’ end of the gene with the addition of a Ty epitope (24) before the stop codon and to amplify 600 bp of the 3’ UTR of the gene with the same addition. These two pieces were fused by PCR and cloned into pDONR-p4p1r and pDONR-p2r3 vectors, respectively (Invitrogen). Using the Gateway 3-fragment system, the upstream and downstream flanks and a central HXGPRT expression cassette cloned into pDONR-p1r2 were assembled into pDEST-R4R3 to create the plasmid pBAG1::AAH2Ty.

Generation of parasite transgenic lines. To generate transgenic lines, PruΔku80Δhxg parasites were transfected with DNA PCR amplified from the critical regions of recombinant plasmids. Following the transfection of amplified DNA (5 to 15 μg), parasites were allowed to recover on HFF monolayers for 24 h before positive selection for the HXGPRT cassette with 25 μg/ml mycophenolic acid (Sigma-Aldrich) supplemented with 50 μg/ml xanthine (Sigma-Aldrich) (25) or negative selection against HXGPRT with 340 μg/ml 6-thiophone (26). Resistant parasites were cloned by limiting dilution into 96-well plates containing HFF monolayers and screened by PCR.

In vitro differentiation. Parasites were differentiated using 48 h of treatment with sodium bicarbonate-free RPMI 1640 medium containing 1% FBS and 50 mM HEPES, pH 8.1, at 37°C without CO₂ (27). Parasites in PC12 cells were differentiated using 48 h of treatment with PC12 medium supplemented with 50 mM HEPES, pH 8.1. Parasites in SH-SY5Y cells were differentiated using 48 h of treatment with SH-SY5Y medium supplemented with 50 mM HEPES, pH 8.1.

Infection of mice. Parasites were harvested by syringe lysis of infected HFF cultures at 24 h postinfection using a 22-gauge needle. They then were purified by passage through a 3.0-μm polycarbonate filter (GE Water and Process Technologies, Beaumont, TX), counted by hemocytometer, and diluted in fresh D10 medium. Eight-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were injected intraperitoneally (i.p.) in a volume of 200 μl containing 10⁵, 2 × 10⁵, or 10⁶ parasites and monitored daily. One month postinfection, mice were euthanized by CO₂ asphyxiation followed by cervical dislocation serially across treatment groups to maximize consistency, and brains were removed for analysis. Animal studies were approved by the Institutional Animal Studies Committee (School of Medicine, Washington University in St. Louis).

Plaque assay. Parasites were syringe lysed from infected HFF monolayers, purified with a 3.0 μm polycarbonate filter, and counted by hemocytometer. Parasites were serially diluted, and 10⁶ parasites (100 μl) were seeded onto confluent HFF monolayers in 6-well plates and grown for 11 days at 37°C. 5% CO₂. HFFs were fixed with 70% ethanol for 10 min and then incubated with 0.5% crystal violet in distilled H₂O for 10 min, washed, and scanned with an EPSON Perfection V500 photo scanner.

Immunofluorescence microscopy. Infected monolayers cultured on glass coverslips were fixed and permeabilized with 4% formaldehyde containing 0.5% Triton X-100 for 15 min. Slips were stained with primary antibody that reacts to the Ty tag (24) and fluoroscein isothiocyanate (FITC)-conjugated Dolichos biflorus lectin (DBL) (Sigma, St. Louis, MO) diluted at 1:1,000 in phosphate-buffered saline (PBS)–5% FBS containing 5% NGS (normal goat serum; Sigma). Primary antibodies were visualized using goat anti-mouse or goat anti-rabbit IgG conjugated to Alexa-594 (Invitrogen Molecular Probes, Carlsbad, CA). Slides were analyzed with a Zeiss AxioSkop wide-field epifluorescence microscope equipped with an AxioCam charge-coupled-device (CCD) camera, and images were captured using AxioVision v3.1 (Carl Zeiss Inc., Thornwood, NY). Using Photoshop CS3 (Adobe, San Jose, CA), images were cropped, color levels were adjusted, and then images were assembled.

qPCR. Tachyzoite and bradyzoite parasites were harvested into calcium-free PBS by syringe lysis of infected HFF cultures and filtration with a 3.0 μm polycarbonate filter. RNA was harvested using a Qiagen RNeasy kit (Qiagen, Valencia, CA). cDNAs were prepared from total RNA (1.0 μg) using 50 μl oligo(dT) (20) and Superscript III reverse transcriptase (RT; Invitrogen) according to the manufacturer’s protocols. PCR primers for reference and stage-specific genes were described previously (27), and primers for AAH1 and AAH2 were designed using Primer Express software, version 1.0 (Applied Biosystems, Forster City, CA, USA). Real-time quantitative PCR (qPCR) was performed using a SmartCycler (Cepheid, Sunnyvale, CA) in a reaction volume of 25 μl containing SYBR Advantage qPCR premix (Invitrogen), 400 nM each primer, and 1 μl of cDNA. The reaction conditions were 50°C for 2 min, 95°C for 10 min, and then 45 cycles of 95°C for 15 s and 62°C for 30 s. Data analysis was conducted using SmartCycler (Cepheid) software. Relative gene expression levels were calculated as fold change using the threshold cycle (Ct) formula 2 - ΔΔCt, where ΔCt = Ct (target) – Ct (housekeeping gene) and ΔΔCt = ΔCt (tachyzoite-stage RNAs) – ΔCt (bradyzoite-stage RNAs) (28). The housekeeping gene encoding actin (ACT1) was used as a reference control.

Western blotting. Western blots were done as previously described (29), with the following antibodies: rabbit aGRA2 and mouse monoclonal antibody (MAB) BB2. Primary antibodies were detected with fluorocently conjugated IRDye secondary antibodies (LI-COR, Lincoln, NE) and visualized on an Odyssey infrared imaging system (LI-COR).
ELISA. High-binding enzyme-linked immunosorbent assay (ELISA) plates (Corning, Corning, NY) seeded with parasite antigen (sonicated RH strain lysate in PBS at 10^5 parasites per well) were incubated with serum from chronically infected mice (collected 1 month postinfection) diluted 1:1,000 in PBS-Tween containing 0.1% bovine serum albumin (BSA) for 1 h at room temperature. Antibodies were detected with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson Laboratories, Sacramento, CA) for 1 h at room temperature. HRP activity was captured using BD OptEIA substrate reagent A and substrate reagent B (BD Biosciences, Franklin Lakes, NJ). Absorbance at 450 nm was read using an EL-800 universal microplate reader (Bio-Tek Instruments Inc., Winooski, VT).

**Cyst count.** Chronically infected animals were euthanized and the brains removed. Brains were homogenized and stained with DBL as previously described (27). Dilutions of stained homogenates were examined using a Zeiss wide-field epifluorescence microscope. Three separate aliquots were counted per brain sample, and total brain cyst load was determined based on the total volume of the brain homogenate (0.6 to 1.0 ml) and the average count per volume (15 to 20 μl).

**In vitro sample preparation.** Six-well plates were seeded with 300 μl of a 0.01% solution of type IV collagen (Sigma) in 0.1 M glacial acetic acid. After three 1-ml PBS washes to remove residual acetic acid, PC12 cells were seeded at 10^5 cells each into each well and allowed to adhere for 24 h. Filtered parasites were seeded onto PC12s at a multiplicity of infection (MOI) of 1 (one parasite per PC12 cell) and allowed to invade for 4 h. Cells were washed three times with PBS to remove noninvaded parasites and then returned to standard or differentiation culture conditions for 48 h. SH-SY5Y cells were seeded onto 6-well plates at 1 × 10^5 cells per well. PC12 or SH-SY5Y cells were dislodged from the plate using mechanical pressure from a pipette, counted by hemocytometer, pelleted at 5,000 RCF (relative centrifugal force), and resuspended into 500 μl ice-cold 0.1 N perchloric acid and 0.4 M sodium metabisulfite in distilled H2O (PCA buffer). Cells were homogenized on ice with a Branson sonifier cell disruptor 185 (three 5-s pulses, power of 3/10) (Danbury, CT) and centrifuged at 14,000 RCF for 15 min at 4°C. The supernatant was filtered through a 0.22-μm filter and diluted into MD-TM buffer (75 mM NaH2PO4·H2O, 1.7 mM octane sulfonic acid, 100 μM EDTA, 10% acetonitrile, pH 3.0; ESA, Bedford, MA) for analysis with high-performance liquid chromatography (HPLC).

**In vivo sample preparation.** Chronically infected mice were euthanized. The brain was removed and cut sagitally along the midline. Half of each brain was weighed, transferred to 500 μl ice-cold PCA buffer, and homogenized by a hand homogenizer, followed by sonication on ice with a Branson sonifier cell disruptor 185 (three 10-s pulses, power of 3/10). Homogenates were spun at 14,000 RCF for 15 min, and the supernatant was collected and filtered through a 0.22-μm filter. Supernatants were diluted into MD-TM buffer for HPLC analysis.

**HPLC.** HPLC analysis was performed, as described in reference 30, using a Coulson electrochemical detector (ESA, Bedford, MA) with an ESA MD 150–by-3.2-mm column. The mobile phase consisted of ESA MD-TM buffer. HPLC detection of samples was calibrated using standard samples of dopamine (DA), dopachrome (Dopac), and homovanillic acid (HVA) at 0.1, 0.5, or 1.0 ng/100 μl. The retention times of each catecholamine were determined. The total area under the HPLC trace for five replicates of each catecholamine was measured to create the reference curve for subsequent quantitative analysis of catecholamine amounts (see Fig. S1 in the supplemental material).

**Statistical testing.** qPCR data were analyzed by two-way analysis of variance (ANOVA) with Tukey’s multiple-comparison test. In vitro HPLC data were analyzed by one-way ANOVA. Cyst count data and in vivo mouse dopamine data were analyzed by the Kruskal-Wallis nonparametric test and Dunn’s multiple-comparison test. Cyst count versus dopamine data were analyzed by linear regression. Statistical analysis was done in Prism 6 for Mac OSX (GraphPad Software, La Jolla, CA).

**RESULTS**

Deletion of AAH2 in the type II PruΔku80Δhxg background. Previous work has described two highly similar AAH genes in *T. gondii* (20). During the course of this work, assembly 9 of the *T. gondii* ME49 genome was released (ToxoDB.org). In this version, only one of the AAH genes is found in the chromosome (i.e., AAH2), while the other one is on an unassembled contig (i.e., AAH1 on contig TgME49_asmb1.1705). Because this differs from the genomic organization of the genes in assembly 8 (where they were found in tandem on chromosome V), we PCR mapped and sequenced the regions flanking AAH1 and AAH2 from the Pru strain. Our findings were consistent with the arrangement of the previous ME49 genome assembly 8 (see Fig. S2 in the supplemental material).

To examine the role of AAH2, a double homologous recombination strategy was employed to target the gene for deletion (Fig. 1A). We transfected knockout constructs into the background strain PruΔku80Δhxg (21) and selected for positive transfectants using HXG selection (25). The gene was successfully knocked out, creating the knockout strain PruΔku80ΔhxgΔaaah2, as shown by the absence of an expected 4.8-kb band encompassing the AAH2 gene (Fig. 1B). The knockout line then was co-transfected with the cDNA copy of the AAH2 gene driven by its endogenous 5′ and 3′ UTRs, creating the complement strain PruΔku80ΔhxgΔaaah2:AAH2. The complement was confirmed by PCR, showing the presence of a shorter 1.6-kb band consistent with the cDNA of AAH2 (Fig. 1B). Despite repeated attempts (more than 3) to delete AAH1 using a similar strategy, we were unable to obtain knockouts of this gene (data not shown), suggesting it is essential.

The parasite AAH2 is not essential for growth, differentiation, host infection, or oral transmission. To test for overall growth, we compared the ability of knockout and wild-type parasites to form plaques on HFF monolayers. A plaque assay showed no significant defect in growth rate in the knockout (Fig. 1C; also see Fig. S3 in the supplemental material). We next tested the ability of the parasite to convert from the lytic tachyzoite stage to the dormant bradyzoite stage in *in vitro* under stress induced by high pH (8.1), a well-established model for *in vitro* differentiation (31). Bradyzoite differentiation under high pH was normal, as measured by FITC-conjugated *Dolichos biflorus* lectin specific to N-acetyl galactosamine that is found in the bradyzoite cyst wall (32) (Fig. 2; also see Fig. S4 in the supplemental material).

To examine the expression of AAH1 and AAH2, we used qPCR to test for abnormalities in bradyzoite induction at the transcriptional level. We examined the change in transcription of the tachyzoite-specific genes *SAG1* and *SAG2A*, bradyzoite-specific genes *BAG1* and *LDH2* (27), as well as our genes of interest, AAH1 and AAH2. As expected, no significant differences in expression levels were seen with either the tachyzoite or the bradyzoite samples across genotypes (Fig. 3A). Of note, contrary to previous reports that AAH1 is bradyzoite specific (20), the expression of both AAH1 and AAH2 appeared to go up in bradyzoites (Fig. 3A). As expected, the transcript for AAH2 was absent from the knockout but restored in the complement (Fig. 3A). However, absolute expression of these genes remained very low; thus, relative fold change was not indicative of strong expression in the bradyzoite stage. This finding is consistent with data found in ToxoDB. Based on microarray data for the type II ME49 strain (33), the expression levels of AAH1 and AAH2 are in the 15th and

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30th percentiles among all *T. gondii* genes in tachyzoites and go up to the 35th and 40th percentiles, respectively, in bradyzoites. As a comparison, *BAG1*, a bradyzoite-specific protein that is induced in bradyzoites (34), has an expression level in the 60th percentile in tachyzoites, going up to the 100th percentile in bradyzoites.

Expression of AAH1 and AAH2 is very low in tachyzoites or bradyzoites. We sought to identify the localization of the parasite hydroxylases using both immunofluorescence and Western blot analysis. Using a double homologous recombination strategy with HXG selection as previously described (15), we generated parasite lines in which the *AAH2* genomic locus was used to knock out the *AAH2* gene in the PruΔku08Δhxg background by double homologous crossover. MPA, mycophenolic acid; Xa, xanthine, which was used for selection. (Center) The deletion strain (*Δaah2::HXG*) was transfected with cleanup construct to remove the HXGPRT drug marker. (Right) The deletion strain (*Δaah2::HXG*) was complemented by replacing HXGPRT with a cDNA copy of AAH2. (B) Diagnostic PCR analysis confirmed the presence of bradyzoite vacuoles within PC12 cells (see Fig. S7 in the supplemental material). Expected product sizes were the following: AAH1, 4.820 kb; AAH2, 4.820 kb; AAH2 cDNA, 1.698 kb. (C) Plaque assay measuring in vitro growth of strains on HFF monolayers stained with crystal violet.

**Infection with *T. gondii* does not affect global or regional host dopamine levels in vivo.** We initially tested for changes in the ability to form cysts in host brains in vivo. One thousand tachyzoites of each strain were injected i.p. into 6- to 8-week-old CD1 female mice, and brains were assayed 1 month postinfection for cyst formation. Both mutant and complement strains showed normal parasite cyst burden relative to that of wild-type parasites (Fig. 5A). Additionally, there was no difference in average cyst size between wild-type and *Δaah2* knockout parasites (see Fig. S8 in the supplemental material). We also tested the ability of these cysts to survive digestion to induce oral transmission of infection. Five cysts were fed by oral gavage to naive 6- to 8-week-old CD1 mice, and serum was collected 1 month postinfection to assay infectivity by ELISA. All strains caused seroconversion in mice upon...
Infection by *Toxoplasma gondii* Does Not Alter Dopamine

**FIG 2** Differentiation into bradyzoites *in vitro*. (A) Formation of cysts by wild-type, Δaah2, and Δaah2::AAH2 parasites in tachyzoite and bradyzoite conditions *in vitro*. Partial and fully formed cysts were enumerated based on staining with DBL. There was no significant difference in cyst formation in bradyzoite-induced parasites (P = 1.00) and no significant difference in cyst formation (P = 0.66) or partial cyst formation (P = 0.88) in tachyzoite conditions (both determined by one-way ANOVA; n = 3 experiments). (B) Representative pictures of intact, partial, and absent cyst formation in parasite vacuoles. Blue, 4’,6-diamidino-2-phenylindole (DAPI); red, GRA7; green, DBL. Scale bar, 10 μm.

We observed no significant difference in whole-brain dopamine levels between mice infected with wild-type, Δaah2, or Δaah2::AAH2 parasites compared to PBS controls (Fig. 5B). However, because the overall cyst burden was relatively low, −100 cysts per brain, we repeated whole-brain dopamine assays with the more cyst-competent ME49 strain. At 1 month postinfection, cyst burden in ME49-infected mice was significantly higher (Fig. 5A), but brain dopamine levels were 20% lower than those of uninfected mice (Fig. 5B). This likely was due to persistent acute infection, as concurrent illness also was observed. Consequently, infection with ME49 parasites was repeated at a lower dose (200 tachyzoites), and brain dopamine was assayed 2 months postinfection, after acute illness was no longer visible. Despite slightly increased cyst burdens relative to those of Pru infection, brain dopamine levels still were not significantly different from those for uninfected mice (Fig. 5B). Overall there was no correlation relationship between cyst density and whole-brain dopamine for ME49-infected mice (Fig. 5C). We further investigated the possibility of changes in dopamine at the regional level, examining the striatum of infected and uninfected mice, and again we found no difference in dopamine levels between control and infected mice (see Fig. S10 in the supplemental material).

Finally, because all of the previous experiments were done with type II strains, we repeated the experiment using the type III C56 strain, reported previously (17). Five mice were infected i.p. with our standard dose (10^3 tachyzoites) and five mice with the dose used by Stibbs (10^4 tachyzoites) (17). Using either method, the brain cyst density was significantly lower at 1 month postinfection than with the type II Pru or ME49 strains (e.g., 2/5 mice infected with 10^3 parasites and 3/5 mice infected with 10^4 parasites showed brain cyst densities below the detection limit of 20 cysts per brain [Fig. 5A]). Brain dopamine levels were not significantly different from those of uninfected mice at either dose (10^3 tachyzoites, P = 0.13; 10^4 tachyzoites, P = 0.20; Mann-Whitney test) (Fig. 5B).

**DISCUSSION**

Although *T. gondii*’s ability to manipulate rodent behavioral responses to the cat is well documented (5–13) and several studies suggest that the parasite manipulates dopamine signaling in the host brain (15, 17, 19, 20, 35), no parasite effectors have been found for these host alterations. We sought to test the hypothesis that the parasite genes AAH1 and/or AAH2, which encode the catecholamine biosynthetic enzyme tyrosine hydroxylase, were responsible for the alterations in host dopamine levels observed *in vitro* (15) and *in vivo* (17). However, we were unable to demonstrate *T. gondii* infection having any effect on dopamine levels in the catecholaminergic cell line PC12 or in infected mouse brains. Furthermore, contrary to previous reports that described tyrosine hydroxylase within parasite bradyzoite cysts (15), we observed that levels of both AAH1 and AAH2 were extremely low and undetectable at native expression levels in both tachyzoites and bradyzoites, consistent with low expression levels observed in microarray data (previously reported in ToxoDB). Collectively, these studies indicate that AAH genes do not lead to global changes in dopamine production in the host, although they may contribute to local differences. Additionally, our findings suggest that AAH genes are involved in a different function, since AAH1 is essential for growth in nondopaminergic cells.

We did not observe changes in global brain dopamine levels with either the Pru strain or the C56 strain, unlike what was previously reported (17). However, closer examination of this original study indicates that the significant difference reported can be attributed to very low variance in the sample rather than dramatic differences in the average values (17). Brain dopamine values in our sample values approximate those reported previously yet show higher variance, consistent with the expectation for animal studies. Our results are consistent with other reports that investigated dopamine and neurotransmitter levels in infected rodents. For example, Goodwin et al. (36) described minimal changes observed in dopamine, norepinephrine, and serotonin concentrations in the frontal cortex and striatum of chronically infected
mice. Similarly, Gatkowska et al. (37) noted profound changes in
the ratios of dopamine, serotonin, and norepinephrine to their
metabolites during acute infection, but which returned to baseline
upon the establishment of chronic infection. Additionally, we did
not observe differences in dopamine or related metabolite levels in
the striatum, a region of the brain rich in dopaminergic terminals.
It was recently suggested that expression of AAH enzymes in bra-
dyzoites (detected with a commercial anti-tyrosine hydroxylase
[TH] antibody) leads to elevated dopamine surrounding tissue
cysts in vivo (15). We were unable to replicate these findings here,
as the commercial antibody to TH did not react to T. gondii in our
hands, even in the strain that overexpressed AAH2. Additionally,
atin experiments attempts to localize dopamine in PC12 cells were not successful,
presumably because this small metabolite rapidly diffuses in fixed
cells and tissues. Despite not observing global or regional changes,
our experiments cannot rule out an effect of AAH enzymes on
localized or transient dopamine levels in the host, which could in
turn affect behavior. Further experiments using microdialysis
monitoring may reveal highly localized changes to dopamine in
the CNS and may be useful in studying the role of parasite infec-
tion and AAH enzymes on host signaling pathways.

Previous studies have indicated that infection with T. gondii

FIG 3 Differentiation and expression levels in tachyzoites and bradyzoites. (A) Quantitative real-time PCR comparing gene expression of bradyzoites relative to
that of tachyzoites of wild-type, knockout (Δaah2), and complemented (Δaah2::AAH2) parasites. Stage-specific markers SAG1, BAG1, SAG2A, and LDH2, along
with AAH1 and AAH2, were monitored with gene-specific primers (see Table S1 in the supplemental material). Results are means ± standard deviations (SD)
(n = 3 experiments). Excluding the expected differences in AAH2 expression, expression differences between the genes probed were not significant (P = 0.19 by
two-way ANOVA). (B) Western blot of bradyzoites expressing a Ty epitope-tagged AAH1 or AAH2 or a tagged copy of AAH2 driven by the BAG1 promoter. Red,
GRA2; green, Ty. Expected protein sizes: AAH1 and AAH2, 55 kDa; GRA2 (dense granule protein 2), 28 kDa. (C) Immunofluorescent assay of AAH1-Ty-
and AAH2-Ty- parasites differentiated into bradyzoites. Blue, DAPI; green, DBL; red, Ty. Scale bar, 10 μm.

<table>
<thead>
<tr>
<th>Stage and strain</th>
<th>Dopac a</th>
<th>Dopamine</th>
<th>HVA b</th>
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<tbody>
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<td>Tachyzoite</td>
<td></td>
<td></td>
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<td>PruΔku80Δhxg</td>
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<td>2.28 ± 0.86</td>
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<tr>
<td>Uninfected</td>
<td>0.25 ± 0.37</td>
<td>17.48 ± 5.68</td>
<td>1.99 ± 0.56</td>
</tr>
<tr>
<td>Bradyzoite</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PruΔku80Δhxg</td>
<td>0.02 ± 0.03</td>
<td>0.96 ± 0.21</td>
<td>1.38 ± 0.10</td>
</tr>
<tr>
<td>Δaah2</td>
<td>0.07 ± 0.01</td>
<td>1.37 ± 0.78</td>
<td>2.55 ± 0.31</td>
</tr>
<tr>
<td>Δaah2::AAH2</td>
<td>0.25 ± 0.19</td>
<td>1.54 ± 0.12</td>
<td>1.88 ± 0.17</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.01 ± 0.01</td>
<td>0.71 ± 0.00</td>
<td>1.09 ± 0.82</td>
</tr>
</tbody>
</table>

a 3,4-Dihydroxyphenylacetic acid.
b Homovanillic acid.
c Results are reported as ng catecholamine/10^5 cells. Means ± standard deviations are
shown from 3 technical replicates each (tachyzoites) and 2 each (bradyzoites) for one
representative experiment.
Infection by Toxoplasma gondii Does Not Alter Dopamine

FIG 5 Dopamine content in the brain of control and infected mice. (A) Parasite cyst burden in whole brains of CD1 mice examined at 1 (1 mo) or 2 months (2 mo) postinfection. Infection with ME49 at 1 mo showed significantly higher cyst burden (\(P = 0.0003\) by Kruskal-Wallis test with Dunn’s multiple comparisons for Pru strains and ME49). Infection with the type III C56 strain showed significantly lower cyst burden (\(P = 0.0003\) by Kruskal-Wallis test with Dunn’s multiple comparisons for Pru strains and C56), with 5 of 10 mice showing cyst burdens below the detectable limit of 20 cysts/brain (not plotted). (B) Dopamine levels in total brain homogenates in uninfected and infected mice at 1 (1 mo) or 2 (2 mo) months postinfection. Dopamine levels were not significantly different between infected or uninfected animals or between infection strains (\(P = 0.075\) by Kruskal-Wallis test with Dunn’s multiple comparisons test). (C) Linear regression analysis between cyst density and brain dopamine concentration in mice infected with ME49 for 1 month (1mo) or 2 months (2mo). \(R^2 = 0.1333\) (red). If the highest point in the linear regression was removed, \(R^2 = 0.1175\) (blue).

Infection with the type III parasite cysts showed significantly higher cyst burden (\(P = 0.0003\) by Kruskal-Wallis test with Dunn’s multiple comparisons for Pru strains and ME49). Infection with the type III C56 strain showed significantly lower cyst burden (\(P = 0.0003\) by Kruskal-Wallis test with Dunn’s multiple comparisons for Pru strains and C56), with 5 of 10 mice showing cyst burdens below the detectable limit of 20 cysts/brain (not plotted). Dopamine levels in total brain homogenates in uninfected and infected mice at 1 (1 mo) or 2 (2 mo) months postinfection. Dopamine levels were not significantly different between infected or uninfected animals or between infection strains (\(P = 0.075\) by Kruskal-Wallis test with Dunn’s multiple comparisons test). Linear regression analysis between cyst density and brain dopamine concentration in mice infected with ME49 for 1 month (1mo) or 2 months (2mo). \(R^2 = 0.1333\) (red). If the highest point in the linear regression was removed, \(R^2 = 0.1175\) (blue).
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REFERENCES


