Toxoplasma gondii Infection in Mice Impairs Long-Term Fear Memory Consolidation Through Dysfunction of the Cortex and Amygdala

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Word Count for Abstract: 215

Word Count for Text: 4753

ABSTRACT

Chronic infection with *Toxoplasma gondii* becomes established in tissues of the central nervous system, where parasites may directly or indirectly modulate neuronal function. Epidemiological studies reveal that chronic infection in humans is a risk factor for developing mental diseases. However, the mechanisms underlying parasite-induced neuronal dysfunction in the brain remain unclear. Here, we examined memory associated with conditioned fear in mice and found that *T. gondii* infection impairs consolidation of conditioned fear memory. To examine brain pathology induced by *T. gondii* infection, we analyzed parasite load and histopathological changes. *T. gondii* infects all brain areas, yet the cortex exhibits more severe tissue damage than other regions. We measured neurotransmitter levels in the cortex and amygdala because these regions are involved in fear memory expression. Levels of dopamine metabolites, but not dopamine, were increased in the cortex of infected mice compared with those in uninfected mice. By contrast, serotonin levels were decreased in the amygdala and norepinephrine levels were decreased in the cortex and amygdala of infected mice. The levels of cortical dopamine metabolites were associated with the time spent freezing in the fear-conditioning test. These results suggest that *T. gondii* infection affects fear memory through dysfunction of the cortex and amygdala. Our findings provide insight into the mechanisms underlying neurological changes during *T. gondii* infection.

INTRODUCTION

Toxoplasma gondii is one of the most successful brain parasites, infecting approximately one-third of the human population (1). *T. gondii* can persist in brain and muscle throughout the host's life, and chronic infection is asymptomatic in immunocompetent humans (2). However, recent studies have suggested that *T. gondii* infection is a risk factor for developing mental diseases, such as schizophrenia and depression, as well as human behavior and personality changes and risk of suicide (3, 4). Interestingly, *T. gondii* infection increases the risk of schizophrenia roughly 2.7 times, which is higher than that for genes associated with schizophrenia (5). Several studies have also suggested that rodents infected with *T. gondii* exhibit decreased avoidance behavior to cat odors, indicating manipulation of the host's behavior by *T. gondii* to facilitate the parasite's transmission and complete sexual replication in the definitive host (6–11).

To date, research on the mechanism(s) underlying behavioral changes following *T. gondii* infection has been conducted primarily from two points of view. First, the relationship between parasite localization in the brain and behavioral changes has been investigated, with a previous study reporting that *T. gondii* has no obvious tropism in the brain (12–15). However, another study found that tissue cyst density in amygdalar areas (medial and basolateral amygdala) is twofold higher than that in non-amygdalar areas (9), whereas the presence of tissue cysts in the forebrain contributes to the attenuation of predator odor aversion and anxiety-like behavior (16). Overall, these studies suggest that *T. gondii* cyst distribution contributes to behavioral changes, but this still requires further investigation.

Second, research on the mechanisms underlying behavioral changes following *T. gondii* infection have examined the effect of the infection on neuronal

cell biology, including neurotransmitter synthesis, signal transduction, gene expression, and epigenetic modulation (14, 17–21). One study reported that dopaminergic cells are upregulated by infection, suggesting that *T. gondii* affects the central nervous system to manipulate host behavior (22). In support of this finding, dopamine (DA) levels in *T. gondii*-infected mice are higher than those in control mice (17). Furthermore, increased DA release is observed in acutely infected male mice (18), and increased DA levels are observed in the striatum of mice infected 6 days post infection (dpi) (20). Moreover, treatment of *T. gondii*-infected rats with haloperidol, an antipsychotic that is known to affect the dopaminergic system, reverses the behavioral effect of *T. gondii* infection (23). In their recent study, Hari Dass et al. indicated that *T. gondii* infection induces hypomethylation of the arginine vasopressin promoter in the medial amygdala (21). They also showed that decreased aversion to cat odors in the *T. gondii*-infected rat is recovered by systemic hypermethylation (21). Despite these findings, the mechanism(s) underlying the behavioral changes induced by *T. gondii* infection remains unclear.

The presence of an aversive stimulus is transmitted to the amygdala via the cortex and thalamus. The activated amygdala then facilitates stimulation of the hypothalamic–pituitary–adrenal (HPA) axis (24). The HPA axis is essential for adaptation to a stressful environment (25). Activation of the HPA axis facilitates secretion of corticosterone (CORT), which plays an important role in expressing emotional behavior (24). The cortex, particularly the prefrontal cortex, is implicated in stress regulation. Lesions in the cortex decrease or increase the CORT response to stress (26). The amygdala receives dense serotoninergic innervation from the dorsal raphe nucleus, and activation of the dorsal raphe nucleus increases amygdala 5-hydroxytryptamine (5-HT, serotonin) levels and CORT secretion (27). CORT

modulates serotonergic activity in the amygdala (28). A previous study suggested that *T. gondii* infection causes dendritic retraction of basolateral amygdala neurons and decreased amounts of CORT, both basal levels and those induced by aversive cat odors (29). Additionally, it has been known for decades that the noradrenergic system is involved in memory consolidation (30). Noradrenergic stimulation of the amygdala enhances memory consolidation (31). Aversive stimuli enhance secretion of norepinephrine (NE) from the locus coeruleus to the cortex and amygdala, resulting in enhanced fear memory consolidation modulated by stress hormone regulation (32).

In addition to attenuation of predator odor aversion, learning and memory deficits, as well as intact memory, have been demonstrated in rodents infected with *T. gondii* (9, 15, 33–35). The effects of *T. gondii* infection on rodent behavior vary with the experimental design, including differences in rodent species, route of infection, parasite strain, dosage and stage of parasites, time post infection, and type of behavior test (36, 37). These differences make it difficult to clarify the characteristics of the brain pathology associated with behavioral changes following *T. gondii* infection. Therefore, using one behavioral paradigm and experimental design to examine both the brain histopathological and neurological changes in infected rodents would further the understanding of the mechanisms of the behavioral changes induced by *T. gondii* infection. In this study, we investigated brain parasite distribution, histopathological lesion severity, and neurotransmitters (DA, 5-HT, and NE) to evaluate how latent *T. gondii* infection affects host fear memory.

MATERIALS AND METHODS

Ethics statement

This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine (permit number 23-64, 24-17, 25-66, 26-68). Mice were decapitated without anesthesia for brain sampling, and all efforts were made to minimize animal suffering.

Mice

Mice (C57BL/6, 8 weeks old, male) were obtained from CLEA Japan (Tokyo, Japan). Mice were housed (four to six mice/cage) under a 12-h light:dark cycle (8:00–20:00) in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All mice were treated using the guiding principles for the care and use of research animals endorsed by the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animal experiments began after 1 week of habituation.

Parasite culture

T. gondii (strain PLK; type II) parasites were passaged using monkey kidney adherent epithelial cells (Vero cells) in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA) containing 8% fetal bovine serum. Infected cells were syringe-lysed using a 27-gauge needle to release tachyzoites into RPMI-1640 medium (Sigma) and then filtered using a 5.0-μm pore size filter (Millipore, Bedford, MA, USA).

146

147 **Parasite infection and experimental groups**

148 *T. gondii* tachyzoites were intraperitoneally inoculated (1×10^3 tachyzoites) into
149 9-week-old mice. Daily body weight measurements were taken for 30 days after
150 infection. This study consisted of six experiments, and the experimental trials are
151 described in the Supplementary data (see Fig. S1). All behavioral experiments were
152 performed at 37–41 dpi, commencing at 7:00–8:30 a.m. under a light intensity of 300
153 lux. For high-performance liquid chromatography (HPLC) analysis, uninfected and
154 infected mice were sacrificed at 40 and 52 dpi. Forty and 52 dpi were selected to
155 evaluate the impact of *T. gondii* infection because these days corresponded to those
156 for the start and end of the fear-conditioning test, respectively. We examined the
157 correlation coefficients between the percentage of time spent freezing in the
158 fear-conditioning test and levels of neurotransmitters using the samples collected at
159 52 dpi. Moreover, mice were sacrificed at 45 and 54 dpi for histopathological analysis
160 and for quantitating the parasite load using quantitative PCR, respectively. Successful
161 establishment of latent infection was confirmed using an enzyme-linked
162 immunosorbent assay for detecting antibodies to the *T. gondii* dense granule protein 7
163 (TgGRA7) (38). Mice with no anti-TgGRA7 antibodies were excluded from
164 experiments.

165

166 **Fear-conditioning test**

167 We performed contextual and cued fear-conditioning tests to evaluate learning and
168 memory. The fear-conditioning test is a behavioral experiment that assesses the ability
169 of mice to learn the association between an environmental cue and an aversive
170 stimulus. On the first day, the mice were placed in a conditioning chamber and given

pairings of an auditory cue and a mild foot shock. On the following 2 days, the mice were exposed to the same conditioning chamber (context test) and a differently shaped chamber, and the auditory cue was presented (tone test). Freezing behavior during the test was measured as an index of fear memory. Therefore, if a mouse normally learnt the association between the conditioned cues and the foot shocks, it spent longer in freezing than a mouse that had an incomplete memory. On the last day, the mice received 30 successive auditory cues without the foot shock (extinction test). The normal mouse spent increasingly less time in freezing during the test. However, if a mouse had a deficit in fear extinction, it showed high levels of freezing until late in the session. To measure associative-type long-term fear memory, fear-conditioning tests from 37 to 41 dpi were performed according to methods used in earlier studies (9, 39), but with some modifications (see Fig. S2). In a fear-conditioning box (18 cm × 17 cm; Muromachi, Tokyo, Japan), freezing was recorded using a video-tracking system (Comp Act VAS ver. 3.0x; Muromachi). The test consisted of four phases: conditioning, context, tone, and extinction, as described in the Supplementary data (see Fig. S2). On test day 1, mice were placed in the box for habituation (120 s). An auditory tone (75 dB, 300 Hz) was then presented for 28 s, with a mild foot shock (0.5 mA) paired with the auditory tone for 2 s. An interval of 60 s preceded a second identical trial. After the last foot shock presentation, mice were kept in the box for an additional 30 s. On test day 2, mice were placed in the same spatial and olfactory context for 5 min to measure the contextual fear-conditioned response. On test day 3, mice were placed in the box in a novel chamber and allowed to habituate for 3 min. The auditory tone was then presented for 3 min. On test day 4, to determine the extinction rate of cued fear-conditioning, mice were presented with 30 successive auditory tones (75 dB, 300 Hz, for 10 s with 50-s interval durations). Freezing was

196 measured during the first tone before it was paired to foot shock (28 s) in the
197 unconditioning phase, during the second tone after it was paired to foot shock (28 s)
198 in the conditioning phase, during observation (300 s) in the context test, during
199 habituation (180 s), and during the tone (180 s) in tone test. The freezing ratio (%)
200 was calculated by dividing the time spent freezing by the total time during each
201 session. In the extinction test, the freezing ratio (%) was repeatedly calculated by
202 dividing time spent freezing by every 5-min extinction test.
203
204 **DNA extraction and quantitative PCR**
205 To measure the *T. gondii* burden in mouse brain at 54 dpi, one hemisphere from each
206 mouse brain was divided into eight regions: cortex, hippocampus, caudoputamen,
207 amygdala, thalamus, hypothalamus, midbrain, and cerebellum (see Fig. S1). The
208 method used for dissection of these brain regions is described in detail in the
209 Supplementary data (Fig. S3). Tissue was stored immediately at −30°C. DNA was
210 isolated from the brain regions and parasite counts analyzed with real-time PCR using
211 the B1 gene, as described previously (14). PCR was performed using an ABI prism
212 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), and
213 amplification was monitored using the SYBR Green method (Applied Biosystems). A
214 standard curve was constructed with tenfold serial dilutions of *T. gondii* DNA
215 extracted from 1×10^5 parasites. The curve ranged from 10,000 to 0.01 parasites.
216 Parasite number was calculated by plotting Ct values on the standard curve.
217
218 **Histopathological analysis**
219 After being fixed with 4% paraformaldehyde solution, brain samples at 45 dpi were
220 cut coronally, embedded in paraffin wax, sectioned at 4 μm, and then stained with

hematoxylin and eosin (see Fig. S1). Pathological lesion severity was scored using the following scheme: 0, no lesion; 1, slight lesion; 2, mild lesion; 3, moderate lesion; and 4, severe lesion. Representative examples of the scoring are shown in the Supplementary data (Fig. S4). Pathological scores from 0 to 3 were determined for two types of lesions, meningitis, including ventriculitis, and perivascular cuffs. Pathological scores from 0 to 4 were also determined for inflammatory cells, which included glial cell, macrophage, and lymphocyte infiltration.

High-performance liquid chromatography

Neurotransmitter levels in the brains at 40 and 52 dpi were measured by HPLC (see Fig. S1). The brains were divided into two regions: cortex and amygdala (regions related to emotional behavior and memory) (see Fig. S3). The collected tissue was stored immediately at −80°C. Each brain sample was homogenized using a BioMasher (Funakoshi, Tokyo, Japan), and then 300 μL/10 mg tissue of 0.2 M perchloric acid (containing 100 μM EDTA-2Na) was added. Isoproterenol HCl (Sigma) was used as a monoamine internal standard. Homogenates were placed on ice for 30 min, and then centrifuged at 20,000 × g for 15 min at 0°C. Supernatants were mixed with 1 M sodium acetate to adjust the pH to 3.0 and filtered using an Ultra free MC (Millipore). The final products were injected into an HTEC-500 HPLC system (electrochemical detector; EICOM, Kyoto, Japan) equipped with an SC-5ODS column for monoamines. Chromatograms were analyzed using PowerChrom software version 2.5 (eDAQ Pty Ltd., Densitone East, Australia).

Correlation analysis

The correlation coefficients for the percentage of time spent freezing in the

fear-conditioning test and the levels of cortical neurotransmitters were calculated using the Pearson correlation coefficient. Previous studies have shown that the strength of the linear association between pairs of variables can be determined as follows using the Pearson correlation coefficient: $|r| = 0.70$, strong correlation; $0.5 < |r| < 0.7$, moderately strong correlation; and $|r| = 0.3–0.5$, weak to moderate correlation (40).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical differences between two groups were analyzed using two-tailed unpaired *t* tests, except for the extinction test, which were determined using repeated-measures analysis of variance (ANOVA) with Bonferroni as the post hoc test. With three groups or more, statistical differences were determined using one-way ANOVA followed by Tukey's multiple comparisons test. For the correlation analysis, significant differences were determined using the Pearson correlation coefficient. *P* values < 0.05 represent statistically significant differences.

RESULTS

Impaired long-term fear memory consolidation in *T. gondii*-infected mice.

We performed fear-conditioning tests to evaluate learning and memory. During the conditioning phase, there were no significant behavioral differences between infected mice and controls (Fig. 1A). However, infected mice showed significantly reduced freezing in the conditioned context (Fig. 1B) and following habituation for 3 min in a novel chamber (Fig. 1C) compared with those of control mice. The percentage of time spent in freezing behavior did not change between habituation and tone conditioning in *T. gondii*-infected mice, but freezing behavior increased with tone conditioning in uninfected mice (Fig. 1C). These results indicated that mice infected with *T. gondii* have an impaired ability to consolidate fear memory. When mice were subjected to 30 successive tones over 30 min, the percentage of time spent freezing in both control and *T. gondii*-infected mice gradually decreased (Fig. 1D). Compared with uninfected animals, *T. gondii*-infected mice exhibited significantly reduced freezing during the first 5 min. This difference might have influenced our conditioned context results.

Parasite load and pathological examination of brain regions in *T. gondii*-infected mice

Because the distribution of the parasite in the brain may be an important factor affecting behavioral changes, we analyzed the expression of the *B1* gene with quantitative PCR to compare the parasite counts in eight distinct brain regions: cortex, hippocampus, caudoputamen, amygdala, thalamus, hypothalamus, midbrain, and cerebellum. There were no significant differences in the parasite counts across these brain regions (Fig. 2). To investigate the parasite stages in the brain, the expression levels of *SAG1* (a tachyzoite-specific gene), *BAG1* (a bradyzoite-specific gene), and

289 *GRA1* (a nonstage-specific gene) were measured with real-time PCR (Fig. S5). The
290 expression of *BAG1* and *GRA1* was detected in each brain region, but the expression
291 of *SAG1* was very low in the infected mice. There were no significant differences in
292 the expression of these genes among the brain regions. Histopathological analysis
293 showed that perivascular cuffs and inflammatory cell infiltration were observed in
294 almost all regions. However, the meningitis in the cortex was significantly more
295 severe than that in other regions (Fig. 3A). The pathological scores for the
296 perivascular cuffs in the cortex, caudoputamen, thalamus, and hypothalamus were
297 higher than those in the midbrain (Fig. 3B). In addition to the pathological analysis,
298 the inflammatory infiltrate was assessed with real-time PCR for the expression of the
299 *CD4*, *CD8*, and *CD11b* genes, markers of inflammatory cells (Fig. S6). The CD4
300 levels were higher in the amygdala in the infected mice than in the other regions. The
301 levels of CD8 and CD11b were higher in the amygdala than in the hippocampus. The
302 level of IFN-γ was higher in the cortex than in the hippocampus or caudoputamen
303 (Fig. S6D).
304
305 **Neurotransmitter levels in cortex and amygdala of *T. gondii*-infected mice**
306 We analyzed the levels of various neurotransmitters in the cortex and amygdala.
307 Cortical DA levels were not significantly different between uninfected and *T.*
308 *gondii*-infected mice at either 40 or 52 dpi (Fig. 4A and E). Amygdalar DA levels
309 were lower in infected mice than in uninfected animals at 40 dpi, but no difference in
310 DA levels was detected at 52 dpi (Fig. 4A and E). We also examined DA metabolism
311 in these animals. Homovanillic acid (HVA) is the primary final DA metabolite
312 produced via the intermediate products 3,4-dihydroxyphenylacetic acid (DOPAC) and
313 3-methoxytyramine (3-MT) (41). The levels of all DA metabolites increased in the

cortex of the infected mice at 40 and 52 dpi, but not in the amygdala, compared with those in uninfected animals (Fig. 4B-D, and F-H). We also determined that compared with uninfected mice, the levels of 5-HT in infected mice decreased in the amygdala but not the cortex at 40 and 52 dpi, (Fig. 5A and C). There was no difference in the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) level in the cortex or amygdala following *T. gondii* infection (Fig. 5B and D). NE levels in infected mice were decreased in both the cortex and amygdala at 40 and 52 dpi compared with those in uninfected mice (Fig. 6A and B).

The correlations were examined between the percentage of time mice spent freezing in the fear-conditioning test and the levels of neurotransmitters in the cortex and amygdala by calculating Pearson correlation coefficients (Supplemental Table 1, Fig. 7). We found that the level of HVA in the cortex had a moderately strong negative correlation with percentage of time spent freezing during the context test ($r = -0.613$; Fig. 7A). Levels of DOPAC, 3-MT, and 5-HIAA in the cortex showed weak to moderate negative correlations with freezing time during the context test ($r = -0.388$, -0.378, and -0.447, respectively; Fig. 7B-D). By contrast, levels of NE displayed weak to moderate positive correlations with freezing time during the context test in both the cortex and amygdala ($r = 0.346$ and 0.414, respectively; Fig. 8E and F). In addition, amygdalar 5-HT, 5-HIAA, and NE levels were weakly to moderately positively correlated with freezing time during the tone test ($r = 0.371$, 0.385, and 0.388, respectively; Fig. 7G-I).

DISCUSSION

We showed that *T. gondii* infection in C57BL/6 male mice impaired fear memory consolidation, while extinction remained intact. Vyas et al. observed no obvious deficits in fear memory of *T. gondii*-infected rats in the fear-conditioning test (9). However, Witting showed impairment of memory in *T. gondii*-infected mice (33). In addition that study demonstrated that *T. gondii*-infected mice show higher sensitivity to learning and memory deficits than *T. gondii*-infected rats (33). Kannan et al. determined that spatial working memory is impaired in mice infected with *T. gondii* (34). In a recent study, Daniels et al. indicated that spatial memory recall is impaired in rats infected with *T. gondii* (15). Thus, the effects of *T. gondii* infection on learning, memory, and emotional behavior have varied widely among reports, although these studies used different experimental designs, which may have affected the results (36, 37). We are the first to report impaired consolidation of fear memory in *T. gondii*-infected mice.

Our results showed that *T. gondii* infection was present throughout the brain without showing marked tissue tropism. Furthermore, real-time PCR was used to determine the expression of *SAG1*, *BAG1*, and *GRA1* in the brain tissues and showed that neither the expression of the bradyzoite marker nor the low expression of *SAG1* was specific to any particular brain region, suggesting that there was no cyst tropism. Consistent with previous studies in mice, *T. gondii* had no obvious preference for specific brain regions (12–14). In addition, no other study has reported clear evidence to support the idea that parasite localization plays a critical role in the behavioral changes induced by *T. gondii* infection (9, 15, 16, 42). Here, our histopathological analysis showed that meningitis in the cortex was more severe than that in other regions. The area of meninges in cortex and cerebellum is larger than that of other

361 brain regions; however, meningitis in the cortex was more severe than that in the
362 cerebellum. Together with the results showing no marked tissue tropism for the
363 parasite, our results suggest that the immune response (indirect effects) may be more
364 brain-region specific than the parasitic cyst burden (direct effects). Similarly, our
365 previous study using BALB/c mice showed that the prefrontal cortex is more severely
366 damaged than other brain regions (14). Although the mechanism whereby the *T.*
367 *gondii*-induced pathology shows cortical specificity is unclear, these results suggested
368 that *T. gondii* caused cortical hypofunction independent of parasite distribution. In
369 addition to the pathological analysis, a real-time PCR analysis of the general makers
370 of inflammatory cells suggested that the inflammatory cell infiltration was more
371 severe in the amygdala than in the other brain regions.
372 Some drug treatments not only reduce the cyst burden but also attenuate the
373 inflammatory response in the brain. Interestingly, Bottari et al. reported that the
374 treatment of *T. gondii*-infected mice with sulfamethoxazole-trimetropim partly
375 rescued the behavioral changes associated with *T. gondii* infection, suggesting that the
376 degree of brain inflammation affects these behavioral changes (43). Therefore, to
377 investigate whether the degree of inflammation in each brain region affected the
378 behavioral changes in the infected mice, we examined the correlation between the
379 expression of IFN-γ and the percentage of time the mice spent freezing in the
380 fear-conditioning test. The level of IFN-γ was higher in the cortex than in the
381 hippocampus or caudoputamen, but there was no significant correlation between
382 IFN-γ expression and the time spent freezing in the fear-conditioning test (data not
383 shown). Furthermore, the expression levels of CD4, CD8, and CD11b in each brain
384 region did not correlate with the time spent freezing in the fear-condoning test (data
385 not shown). These results suggest that the inflammatory response was more severe in

386 the cortex than in the other brain regions, but that the degree of inflammation does not
387 contribute to impaired fear memory consolidation in mice infected with *T. gondii*. In
388 this study, there are no data presented directly correlating the severity of the
389 behavioral deficits with the degree of damage to the cortex and amygdala because the
390 mice used in the fear-conditioning test were different from those used for
391 histopathological analysis. Thus, more direct evidence is required before we can
392 conclude that the degree of brain inflammation affects the behavioral changes.
393 However, because the prefrontal cortex and amygdala are involved in fear memory
394 and emotional behavior (44), our results suggest that cortical and amygdalar lesions,
395 including meningitis or inflammatory infiltration, are related to the impairment of
396 neuronal function in the cortex and amygdala. We also analyzed cortical and
397 amygdalar levels of DA, 5-HT, NE, and the metabolites of DA and 5-HT, which are
398 all associated with the expression of emotional behavior, learning, and memory (45).
399 Cortical DA levels were similar in uninfected and *T. gondii*-infected mice at both 40
400 and 52 dpi. However, the levels of all DA metabolites increased at both 40 and 52 dpi.
401 Gatkowska et al. reported that dopamine turnover (HVA/DA ratio) in mice is elevated
402 in acute toxoplasmosis but not in chronic toxoplasmosis (18). By contrast, our results
403 indicated that dopamine metabolism activity was upregulated during the chronic stage
404 of *T. gondii* infection, strongly suggesting that DA metabolites were chronically
405 activated. Increased levels of DA metabolites with unaltered levels of DA itself have
406 been shown to compensate for a deficiency in available DA in the cortex (46), and
407 cortical dysfunction and dysregulation of dopamine metabolism are involved in
408 schizophrenia (47). Interestingly, *T. gondii* contains two genes encoding tyrosine
409 hydroxylase, the rate-limiting enzyme of DA biosynthesis (48). Indeed, DA levels are
410 increased in *T. gondii*-infected neurons and PC12 cells (22). These results suggest that

411 *T. gondii* may control the host's DA biosynthesis pathway.

412 In the amygdala, 5-HT levels decreased at 40 and 52 dpi. 5-HT stimulates
413 CORT secretion, and CORT modulates serotoninergic activity in the amygdala,
414 suggesting that 5-HT–CORT interactions may be involved in amygdala-dependent
415 emotional behavior (28). *T. gondii* infection reduces CORT levels (29), suggesting
416 HPA axis dysfunction mediated through the amygdala in mice infected with *T. gondii*.
417 We found that NE levels in the infected mice were decreased at both 40 and 52 dpi in
418 the cortex and amygdala. Aversive stimuli enhance secretion of NE from the locus
419 coeruleus in the cortex and amygdala, resulting in enhanced fear memory
420 consolidation modulated by stress hormone regulation (32). Thus, our results
421 suggested that decreased NE levels in the infected mice also contributed to the
422 dysfunction of the cortex and amygdala. Moreover, imbalance in the amygdala
423 serotonergic system has been linked to anxiety and depression (49). Therefore, our
424 results suggest that *T. gondii* infection causes highly characteristic brain pathology in
425 these neurological diseases and that lower levels of 5-HT and NE in the cortex and
426 amygdala following *T. gondii* infection may be associated with neurological
427 dysfunction.

428 Lastly, we found a negative correlation between the levels of all DA
429 metabolites in the cortex and freezing behavior during the context test, meaning that
430 the higher the cortical DA metabolites, the less time the animal spent freezing. In
431 other words, fear memory consolidation was impaired in mice showing high levels of
432 DA metabolites. By contrast, levels of 5-HT and 5-HIAA in the amygdala and NE in
433 the cortex and amygdala were positively correlated with freezing behavior, indicating
434 that the lower the 5-HT, 5-HIAA, and NE levels, the less time the mouse spent
435 freezing. Auditory stimulus information in the amygdala is regulated by

436 neurotransmitters, including DA, 5-HT, and NE (45). A fear-conditioned tone
437 increases levels of these neurotransmitters and influences excitatory and inhibitory
438 neuron interactions. Thus, loss of serotonergic and adrenergic neurons impairs
439 acquisition of conditioned fear (45), suggesting that the lower levels of brain DA,
440 5-HT, and NE we detected in *T. gondii*-infected mice were associated with diminished
441 fear memory. This is the first report to demonstrate a connection between altered
442 neurotransmitter levels and behavioral changes following *T. gondii* infection.

443 Here, we used one behavioral paradigm and experimental model to examine
444 the connection between *T. gondii*-induced inflammatory and neuronal damage to
445 specific brain regions and subsequent behavioral change. Even though our model
446 using C57BL/6 male mice is commonly used in this field, further investigation will be
447 needed to determine whether these findings remain consistent across several rodent
448 models.

449 In conclusion, *T. gondii* infection in mice impaired long-term fear memory
450 consolidation through dysfunction of the cortex and amygdala. In the infected mice,
451 the cortex was more severely damaged than other brain regions, with dysfunction
452 likely occurring in the brain. Dopamine metabolism was increased to compensate for
453 a deficiency in available cortical DA in the infected mice due to hypofunction of the
454 cortex. In addition, we showed imbalances in neurotransmitters associated with
455 modulating the stress response (5-HT and NE) in the amygdala. These data support
456 the hypothesis that the modification of responsiveness to stress mediated via the
457 limbic–hypothalamic–pituitary–adrenal axis causes behavioral changes following *T.*
458 *gondii* infection. Thus, our findings not only provide insight into the mechanisms
459 underlying central nervous system changes during *T. gondii* infection, but also
460 elucidate the underlying mechanism of the relationship between *T. gondii* infection

and onset of mental disease.

FUNDING INFORMATION

This research was supported by the Japan Society for the Promotion of Science through the Funding Program for Next Generation World-Leading Researchers (NEXT Program), initiated by the Council for Science and Technology Policy (2011/LS003) (Y. N.). This work was also supported by JSPS KAKENHI Grant Numbers 15K15118 (Y. N.), 23117007 (K. N.). F. Ihara is a research fellow of Japan Society for the Promotion of Science (15J03171). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

ACKNOWLEDGMENTS

We thank Dr. Toshiaki Ishii for advice on behavioral science. We also thank Youko Matsushita, Megumi Noda, Hikaru Takenaka, and Yoshie Imura for their technical assistance.

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AUTHORS CONTRIBUTIONS

F.I., M.N., and Y.N. conducted the experiments. F.I., M.E.M., N.Y., K.N., and Y.N. designed the experiments. F.I. and Y.N. performed the data analysis. F.I., M.E.M., and Y.N. wrote the manuscript. All authors revised the manuscript and approved the final version.

631 **FIGURE LEGENDS**

632 **FIG 1** Impaired long-term fear memory consolidation in uninfected and *T.*
633 *gondii*-infected mice. The ordinate shows the percentage of time spent freezing. (A)
634 Unconditioned trial shows freezing during the first tone before pairing to foot shock,
635 and conditioned trial shows freezing during the second tone after pairing to foot shock
636 on test day 1. (B) Contextual conditioned freezing time. (C) Tone conditioned
637 freezing time. (D) Extinction of tone conditioned freezing time. (A-C) Significant
638 differences were determined by unpaired *t* tests (****$p < 0.0001$). (D) Significant
639 differences were determined using repeated measures ANOVA with the post hoc
640 Bonferroni test. Significant main effects were shown for *T. gondii* infection ($F_{(1, 70)}$ =
641 75.90, $p < 0.0001$) and time ($F_{(5, 350)} = 117.5$, $p < 0.0001$), and their interaction was
642 also significant ($F_{(5, 350)} = 4.410$, $p < 0.001$). Freezing (%) was calculated by dividing
643 freezing time into the times for observation (300 s) in the context test, habituation
644 (180 s), tone (180 s) during the tone test, and every 5 min in the extinction test. Data
645 represent mean ± SEM. Uninfected mice, $n = 32$; *T. gondii*-infected mice, $n = 42$.
646 Data were summarized from four independent experiments.
647
648 **FIG 2** Parasite load in the brain of *T. gondii*-infected mice. The ordinate shows
649 parasite number per 50 ng of tissue DNA. Brain samples were collected at 54 days
650 post infection. Each circle represents data for one mouse, and bars represent the
651 average value of all data points (*T. gondii*-infected mice, $n = 10$). No statistically
652 significant differences were found using one-way ANOVA with Tukey's post hoc test.
653
654 **FIG 3** Histopathological changes in the brains of *T. gondii*-infected mice. The
655 ordinate shows the pathological score for each brain region. Brain samples were

collected at 45 days post infection. Histopathological lesions were scored as follows: 0, no lesion; 1, slight lesion; 2, mild lesion; 3, moderate lesion; and 4, severe lesion. Each circle represents the data for one mouse, and bars represent the average value for all the data points (*T. gondii*-infected mice, $n = 7$). Significant differences were determined using one-way ANOVA with Tukey's post hoc test. Different letters (a, b) indicate statistically significant differences among groups ($*p < 0.05$). (A) The hippocampus, caudoputamen, thalamus, and midbrain were excluded because they lack meninges.

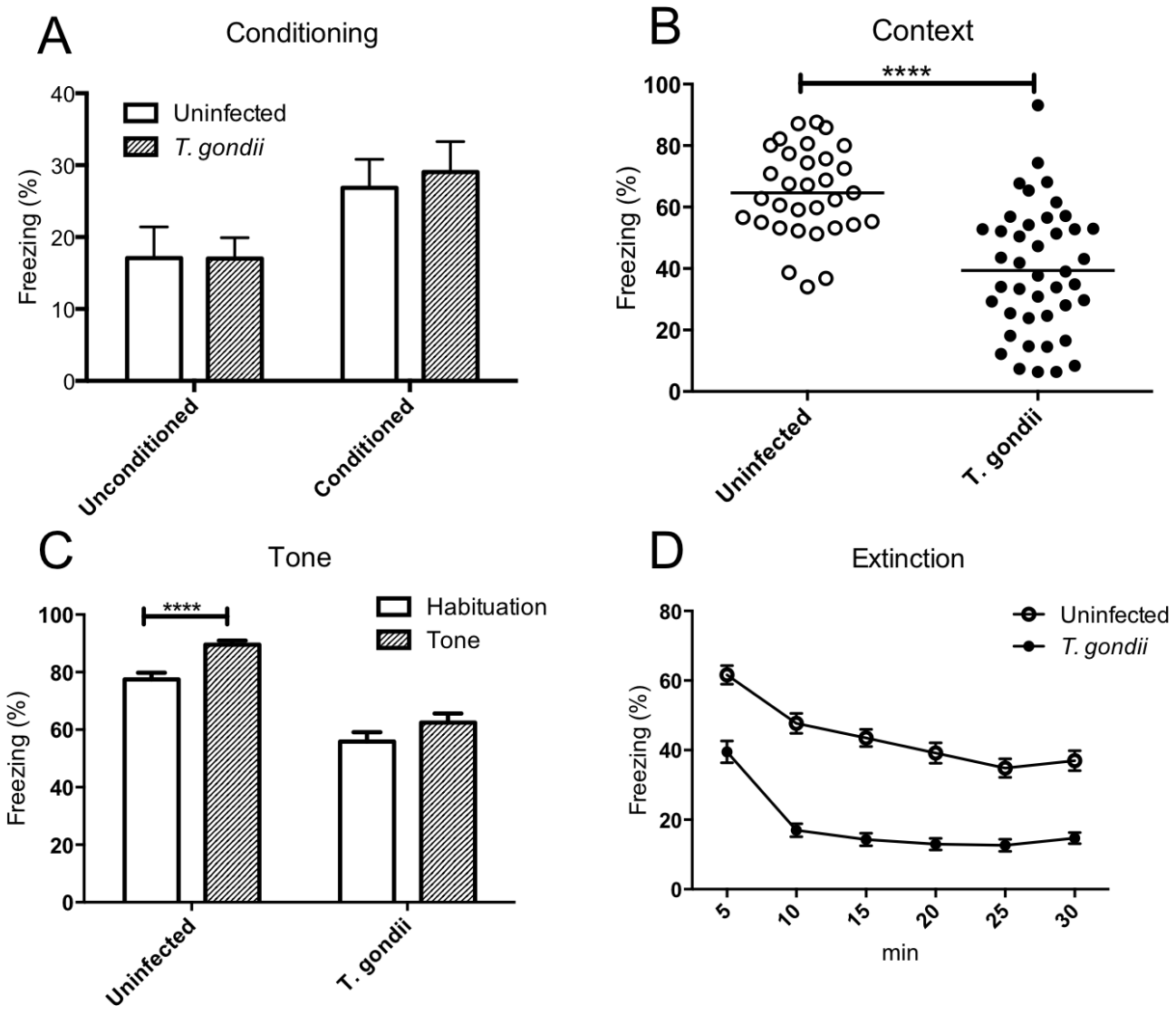
FIG 4 Levels of dopamine and its metabolites in the cortex and amygdala of uninfected and *T. gondii*-infected mice. The ordinate shows levels of the neurotransmitter dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), and homovanillic acid (HVA) in the cortex at 40 (A-D) and 52 (E-H) days post infection. Data represent mean ± SEM. (A-D) Uninfected, $n = 6$; *T. gondii*-infected mice, $n = 8$. (E-H) Uninfected, $n = 16$; *T. gondii*-infected mice, $n = 19$. Significant differences were determined by unpaired *t* tests ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

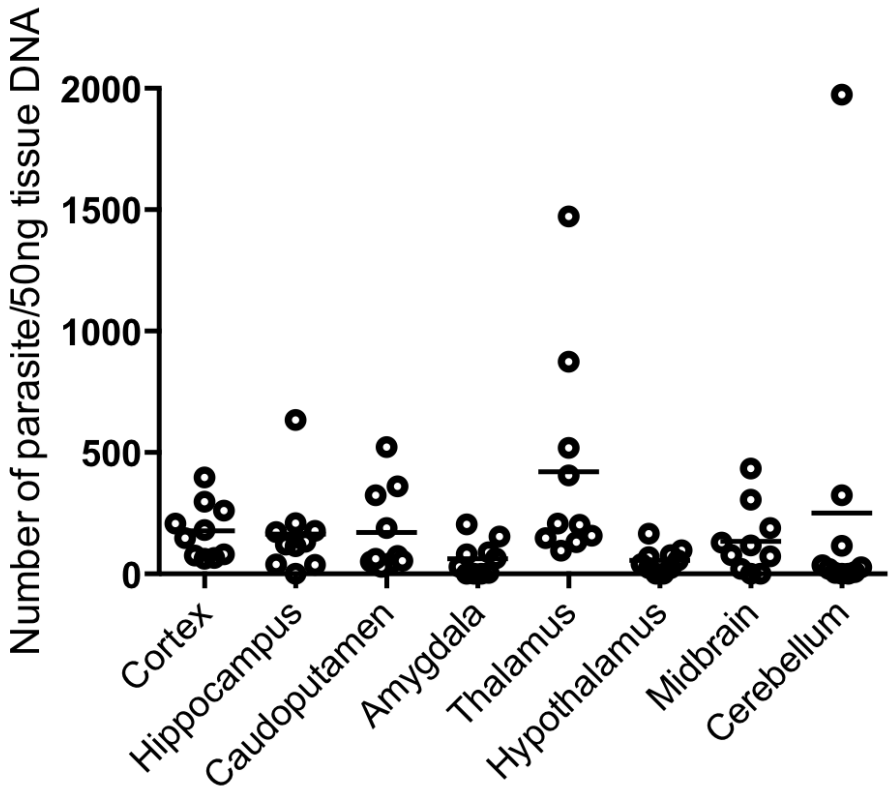
FIG 5 Levels of serotonin and its metabolite in the cortex and amygdala of uninfected and *T. gondii*-infected mice. The ordinate shows levels of the neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cortex and amygdala at 40 (A and B) and 52 (C and D) days post infection. Data represent mean ± SEM. (A and B) Uninfected, $n = 6$; *T. gondii*-infected mice, $n = 8$. (C and D) Uninfected, $n = 16$; *T. gondii*-infected mice, $n = 19$. Significant differences were determined by unpaired *t* tests ($*p < 0.05$, $**p <$

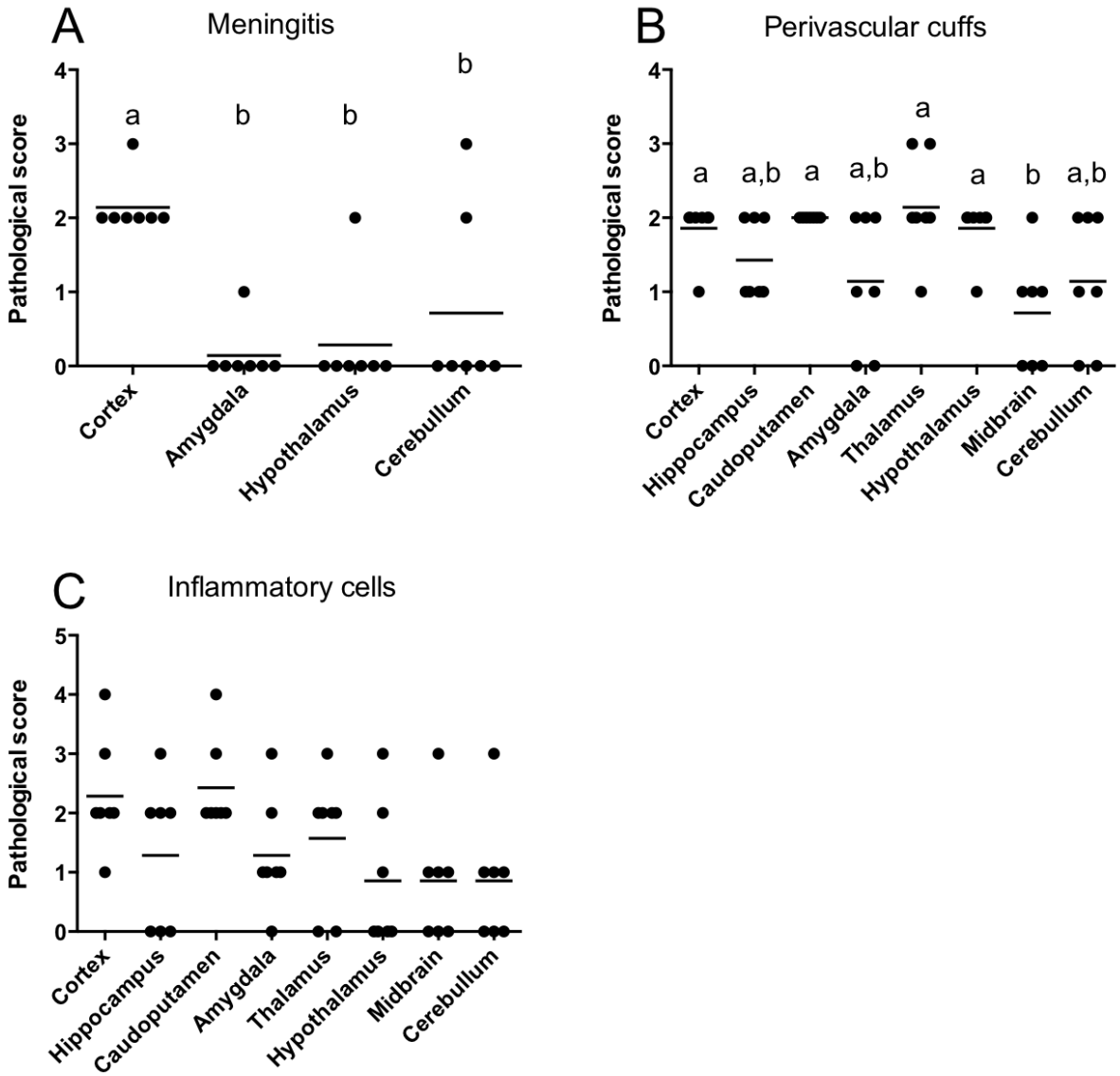
0.01).

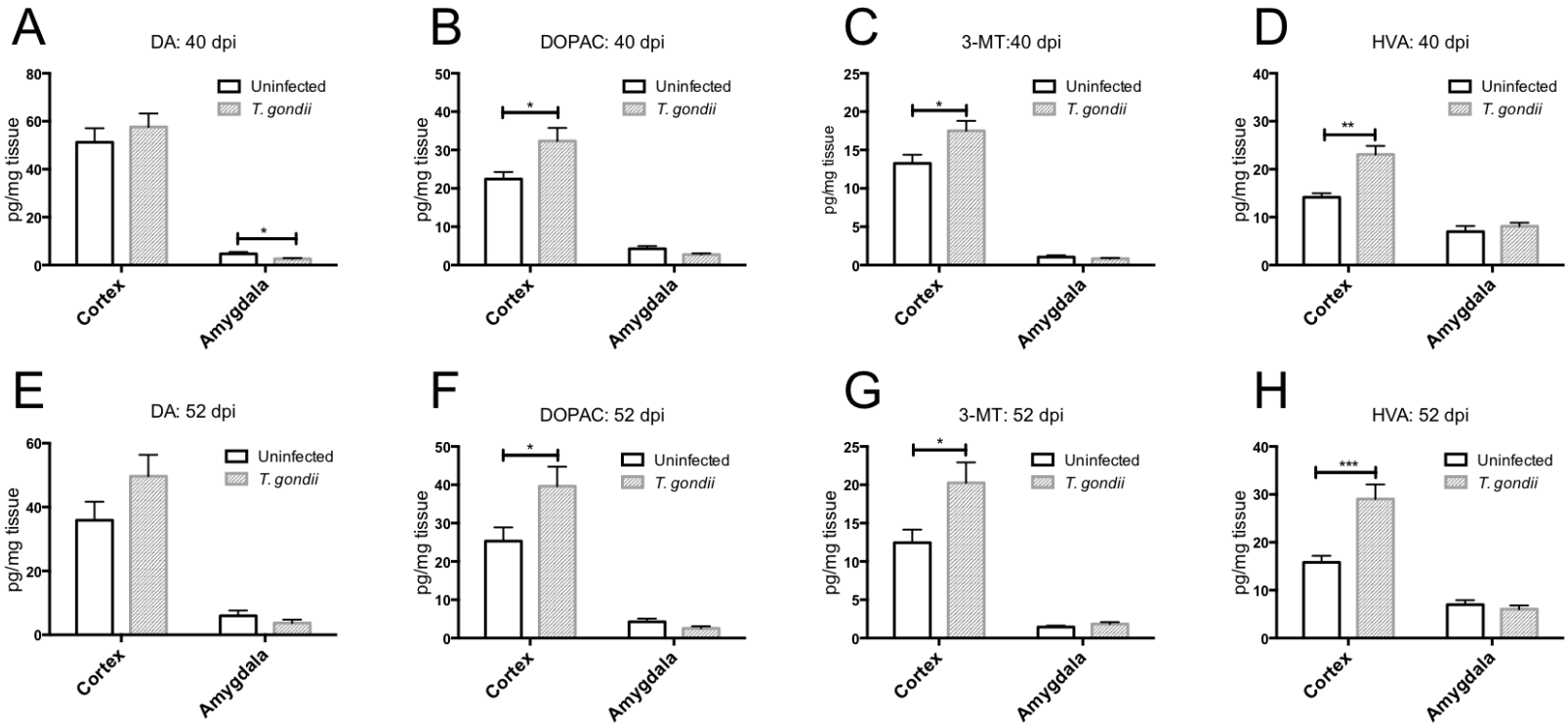
FIG 6 Norepinephrine levels in the cortex of uninfected and *T. gondii*-infected mice. The ordinate shows levels of the neurotransmitter norepinephrine (NE) in the cortex and amygdala at 40 (A) and 52 (B) days post infection. Data represent mean ± SEM. (A) Uninfected, $n = 6$; *T. gondii*-infected mice, $n = 8$. (B) Uninfected, $n = 16$; *T. gondii*-infected mice, $n = 19$. Significant differences were determined by unpaired *t* tests (*$p < 0.05$, **$p < 0.01$).

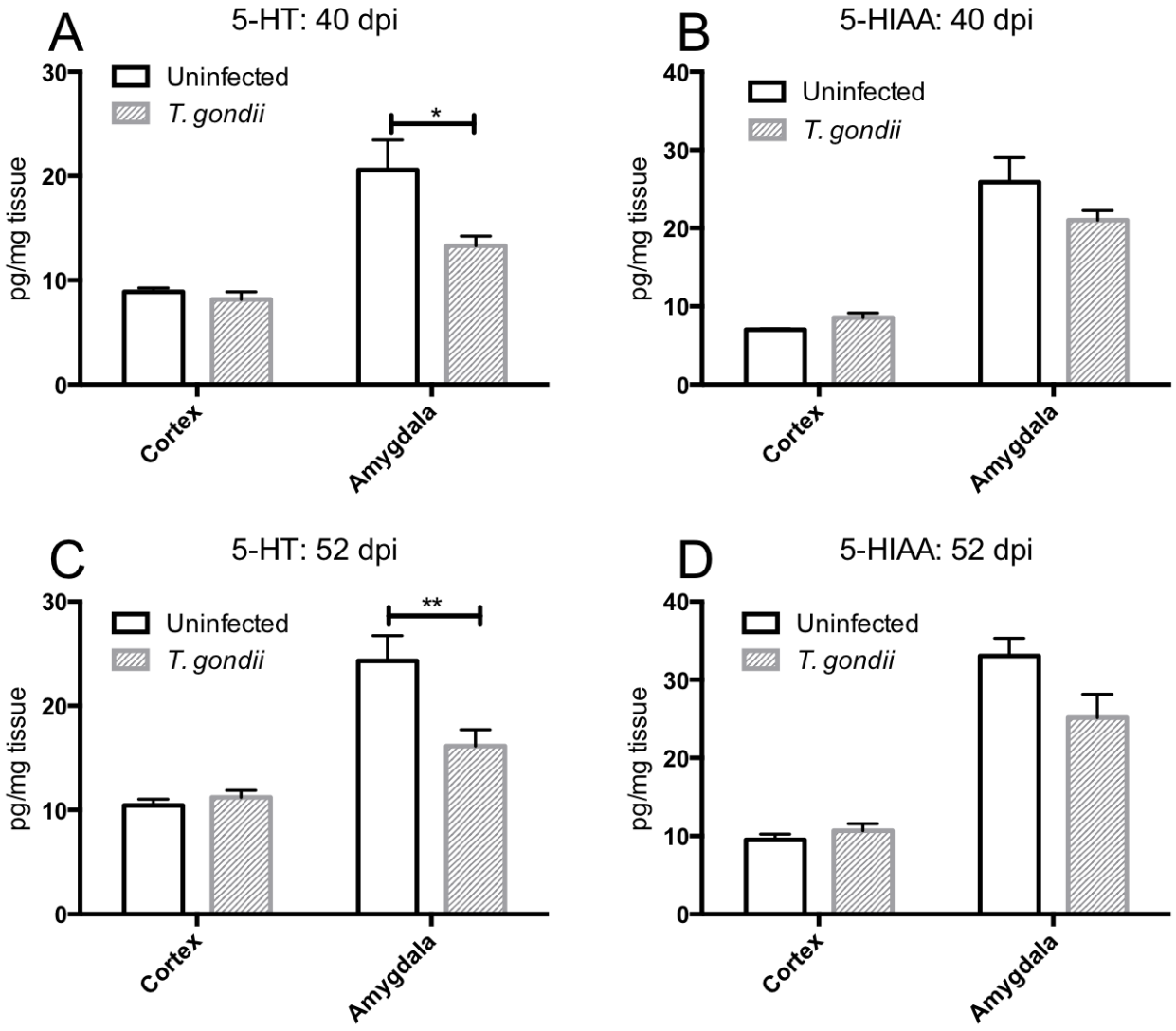
FIG 7 Correlation coefficients between neurotransmitter levels and the percentage of time spent freezing during the context test and tone test. After the fear-conditioning test, some mice were used for the correlation analysis (Experiments 3 and 4, see Fig. S1). The ordinate shows the percentage of time spent freezing during the context test. The abscissa shows the level of each neurotransmitter in the cortex or amygdala at 52 days post infection. Solid line represents the calculated line of best fit. Correlation coefficients were calculated using Pearson's correlation coefficient: $|r| = 0.70$, strong correlation; $0.5 < |r| < 0.7$, moderately strong correlation; and $|r| = 0.3$–0.5, weak to moderate correlation. Uninfected, $n = 16$; *T. gondii*-infected mice, $n = 19$. HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; 3-MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindoleacetic acid; NE, norepinephrine; 5-HT, 5-hydroxytryptamine.

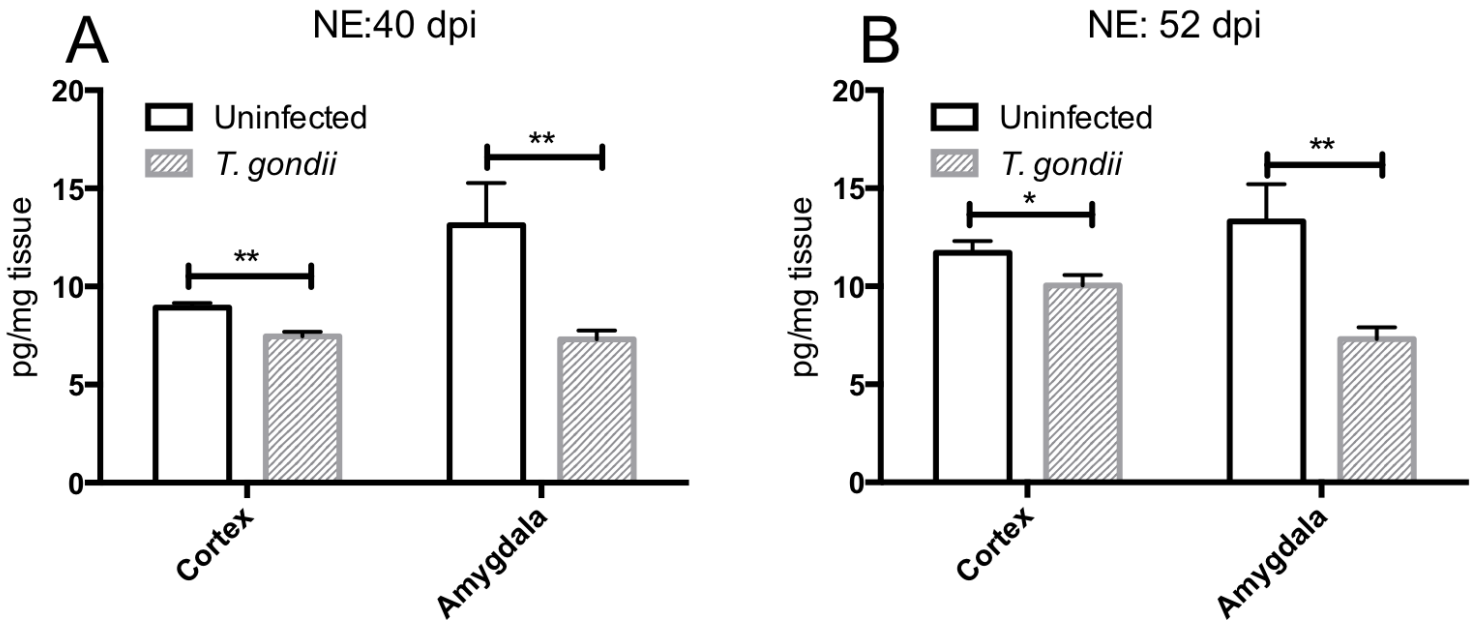

A
Conditioning
Uninfected
T. gondii
Freezing (%)
40
30
20
10
0
Unconditioned
Conditioned
B
Context
****
100
80
60
40
20
0
Freezing (%)
Uninfected
T. gondii
C
Tone
****
Habituation
Tone
100
80
60
40
20
0
Freezing (%)
Uninfected
T. gondii
D
Extinction
Uninfected
T. gondii
80
60
40
20
0
Freezing (%)
5
10
15
20
25
30
min

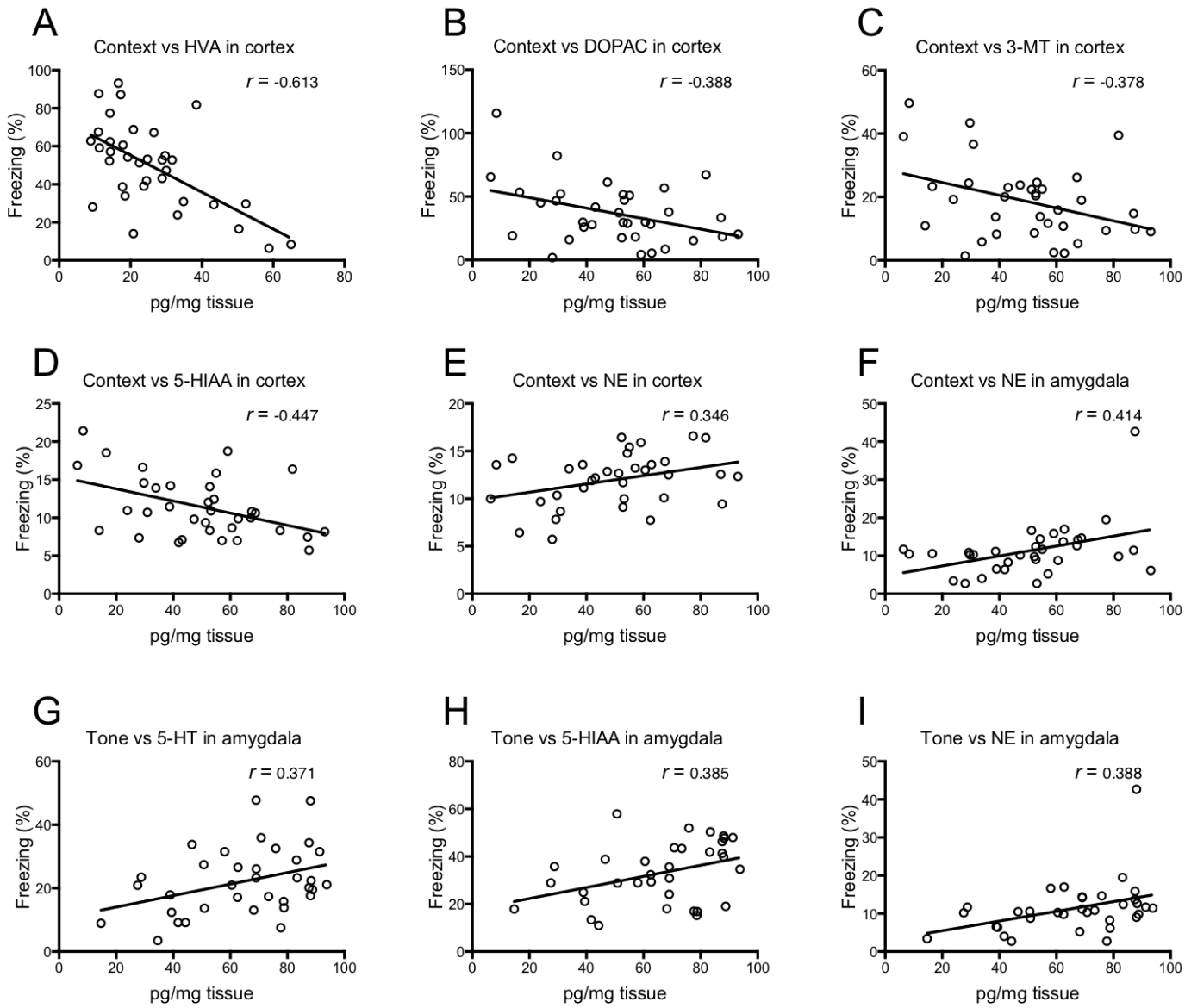

Number of parasite/50ng tissue DNA
2000
1500
1000
500
0
Cortex
Hippocampus
Caudoputamen
Amygdala
Thalamus
Hypothalamus
Midbrain
Cerebellum


A
Meningitis
Pathological score
0
1
2
3
4
a
b
b
b
Cortex
Amygdala
Hypothalamus
Cerebullum
B
Perivascular cuffs
Pathological score
0
1
2
3
4
a
a,b
a
a,b
a
a
b
a,b
Cortex
Hippocampus
Caudoputamen
Amygdala
Thalamus
Hypothalamus
Midbrain
Cerebullum
C
Inflammatory cells
Pathological score
0
1
2
3
4
5
Cortex
Hippocampus
Caudoputamen
Amygdala
Thalamus
Hypothalamus
Midbrain
Cerebullum


A
DA: 40 dpi
B
DOPAC: 40 dpi
C
3-MT:40 dpi
D
HVA: 40 dpi
E
DA: 52 dpi
F
DOPAC: 52 dpi
G
3-MT: 52 dpi
H
HVA: 52 dpi
Uninfected
T. gondii
pg/mg tissue
Cortex
Amygdala
*
**
***


A
5-HT: 40 dpi
Uninfected
T. gondii
pg/mg tissue
30
20
10
0
*
Cortex
Amygdala
B
5-HIAA: 40 dpi
Uninfected
T. gondii
pg/mg tissue
40
30
20
10
0
Cortex
Amygdala
C
5-HT: 52 dpi
Uninfected
T. gondii
pg/mg tissue
30
20
10
0
**
Cortex
Amygdala
D
5-HIAA: 52 dpi
Uninfected
T. gondii
pg/mg tissue
40
30
20
10
0
Cortex
Amygdala


A
NE:40 dpi
Uninfected
T. gondii
pg/mg tissue
20
15
10
5
0
**
**
Cortex
Amygdala
B
NE: 52 dpi
Uninfected
T. gondii
pg/mg tissue
20
15
10
5
0
*
**
Cortex
Amygdala


A
Context vs HVA in cortex
r = -0.613
Freezing (%)
pg/mg tissue
B
Context vs DOPAC in cortex
r = -0.388
Freezing (%)
pg/mg tissue
C
Context vs 3-MT in cortex
r = -0.378
Freezing (%)
pg/mg tissue
D
Context vs 5-HIAA in cortex
r = -0.447
Freezing (%)
pg/mg tissue
E
Context vs NE in cortex
r = 0.346
Freezing (%)
pg/mg tissue
F
Context vs NE in amygdala
r = 0.414
Freezing (%)
pg/mg tissue
G
Tone vs 5-HT in amygdala
r = 0.371
Freezing (%)
pg/mg tissue
H
Tone vs 5-HIAA in amygdala
r = 0.385
Freezing (%)
pg/mg tissue
I
Tone vs NE in amygdala
r = 0.388
Freezing (%)
pg/mg tissue