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マウスの行動変化と脳病態に関する研究）

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CONTENTS

ABBREVIATIONS

A	PC12 cells	adrenal phaeochromocytoma cells
	Arc:	activity-dependent cytoskeletal protein
C	*Cd4*:	Cd4 antigen
	Cd8:	Cd8 antigen
	CNS:	central nervous system
	cDNA:	complementary DNA
	CORT:	corticosterone
	Ct:	cycle threshold
D	Dpi:	days post infection
	DOPAC:	3,4-dihydroxyphenylacetic acid
	DA:	dopamine
E	ELISA	enzyme-linked immunosorbent assay
	EDTA:	ethylenediaminetetraacetic acid
F	*c-Fos*:	FBJ osteosarcoma oncogene
G	GABA:	gamma-aminobutanoic acid
	Glu:	glutamate
	Gapdh:	glyceraldehyde 3-phosphate dehydrogenase
	Gly:	glycine

H	Th:	helper T cell
	HPLC:	high-performance liquid chromatography
	HVA:	Homovanillic acid
	5-HIAA:	5-hydroxyindoleacetic acid
	5-HT:	5-hydroxytryptamine, serotonin
	HPA:	hypothalamic–pituitary–adrenal
I	IEG:	immediately early gene
	Cd11b:	integrin alpha M
	Ifng:	interferon-gamma
	Il:	interleukin
L	LPS	lipopolysaccharide
M	mRNA:	messenger RNA
	3-MT :	3-methoxytyramine
	Vero cells	monkey kidney adherent epithelial cells
N	NO:	nitric oxide
	NE:	norepinephrine
P	PCI:	phenol-chloroform-isoamyl alcohol
	PCR:	polymerase chain reaction
T	*TgBAG1*:	*T. gondii* Bradyzoite antigen BAG1
	TgGRA1:	*T. gondii* dense granule protein 1

TgGRA7:	*T. gondii* dense granule protein 7
TgSAG1:	*T. gondii* SAG-related sequence SRS29B
Tnfa:	tumour necrosis factor-alpha

UNIT ABBREVIATIONS

C	cm:	centimeter
D	dB:	decibel
	°C:	degree Celsius
G	g:	gravitational acceleration
H	Hz:	hertz
	h:	hour
L	lux:	lux
M	**µ**m:	micrometer
	µL:	microlitter
	µM:	micromolar
	mA	milliampere
	mg:	milligram
	min:	minute
	M:	molar
N	ng:	nano gram
P	%:	percent
S	sec:	second

GENERAL INTRODUCTION

The aim of this study is to clarify parasite-induced brain pathology in mouse models. Apicomplexan parasites, *Toxoplasma gondii* and *Neospora caninum,* can persist as their encysted stages in brain through the life of host, but the infected animals are usually asymptomatic. These parasites sometimes cause neurological disorders in the central nervous system (CNS) of the host. However, the mechanisms remain unclear on neuronal dysfunction caused by the parasite infections.

1. *Toxoplasma* and toxoplasmosis

Toxoplasma gondii, an obligate intracellular protozoan parasite, is the causative agent of toxoplasmosis. Based on the past serosurvey data, approximately one-third of the human population was infected with this parasite (85). Intermediated hosts for *T. gondii* are probably all warm-blooded animals, including many livestock animals and humans (28). Definitive hosts are members of the feline family, predominantly domestic cats (28). In the intermediate hosts, *T. gondii* undergoes two stages of asexual development. At the first stage, tachyzoites rapidly multiply by repeated endodyogeny in many different types of host cells (37). At the second stage, tachyzoites differentiate into bradyzoites, and form tissue cysts that first appear from 7 to 10 days post infection (dpi) (37). These cysts are predominantly found in the CNS and muscle tissues, where they may reside through the life of host (19). If *T. gondii* was ingested by definitive host, the bradyzoite initiate another asexual phase of proliferation in the epithelial cells of small intestine (107). Subsequently, the terminal stages of this asexual multiplication initiate a sexual phase of their life cycle. Gamogony and oocyst formation also take

place in the epithelium of small intestine (107). Unsporulated oocysts are released into the intestinal lumen and passed into the environment with the faeces. Sporogony occurs outside the host and leads to the development of infectious oocysts, which contain two sporocysts, each containing four sporozoites (28, 107).

In animal toxoplasmosis, severe clinical toxoplasmosis is generally rare, however, pig often shows clinical signs including anorexia, fever, dyspnea, and limb weakness (22). If pregnant pig get infected with *T. gondii* for the first time, *T. gondii* may transmit to the fetus and cause abortion (22). Goat also shows similar symptoms as pig (33). Additionally, ovine toxoplasmosis has been recognized as a major cause of abortion (33). *Toxoplasma gondii* also has an infectivity to cattle, but the onset is rare (33).

Infection is typically initiated by three main routes of transmission. First route is an ingestion of food contaminated with the cysts (62). Second route is a transmission from animal to human, in where people get infected by ingestion of oocysts through contact with the infected cat's feces, contact with the contaminated soil, and ingestion of contaminated water or food with the oocysts (63). Third one is a mother-to-child transmission, by which the woman who is newly infected with *T. gondii* during pregnancy can pass the infection to her unborn child (96). Generally, chronic infection is asymptomatic in immunocompetent patient (95). However, it may have a life-threating encephalitis in immunocompromised patient, and the infection in pregnant women for the first time during pregnancy may cause serious health problems when the parasite is transmitted to the fetus (74, 95, 96). While anti-toxoplasmosis drugs, such as sulfonamide and pyrimethamine, can clear the actively reproducing tachyzoites, they typically have little effect on the brain cysts, which may remain as a source of recrudescing infection, because these drugs can not across the blood-brain barrier of host (74, 106). As a result, once the parasite successes to establish their tissue cyst in

the brain, *T. gondii* can persist in the brain throughout the life of host. However, impact of the chronic infection with *T. gondii* sill remains unclear on brain function.

Recent studies have suggested that *T. gondii* infection became a risk factor for developing mental diseases, such as schizophrenia, autism, obsessive-compulsive disorder, and depression, as well as personality changes and increases of suicide rate (36, 115). Interestingly, the transmission of *T. gondii* may be facilitated by changing the host behavior. Several studies have suggested that rodents infected with *T. gondii* exhibited a decreased avoidance behavior to cat odors, indicating a manipulation of the host behavior by *T. gondii,* to facilitate the parasite's transmission and resultantly to complete their sexual replication in the definitive host (6, 23, 58, 66, 111, 114).

In addition to attenuation of odor aversion toward predator, learning and memory deficits have been demonstrated in the infected rodents with *T. gondii* (16, 46, 64, 111, 119). The effects of *T. gondii* infection on rodent behavior vary with the experimental design, including differences in rodent species, route of the infection, parasite strain, dosage and stage of the parasites, time post infection, and type of behavior test (65, 120). These differences make it difficult to clarify the characteristics of the brain pathology associated with behavioral changes following the *T. gondii* infection. Therefore, using of one behavioral paradigm and experimental design to examine both the brain histopathological and neurological changes in the infected rodents would benefit for further understanding of the mechanisms of the behavioral changes induced by *T. gondii* infection.

2. *Neospora* and neosporosis

Neospora caninum is an intracellular parasite that naturally infects dogs and cattle (27). *Neospora caninum* is genetically and morphologically related to *T. gondii*, and

was identified for the first time in 1988 (26). Past surveys indicated that a wide range of domestic and wild animals have been exposed with *N. caninum* (27). Up to now, viable parasite has been isolated from several hosts, such as cattle, sheep, water buffalo, dog, horse, and deer (29). There is no evidence of *N. caninum* infectivity in humans, but a serological evidence suggests that humans can also be exposed with *N. caninum* (110). The life cycle of the parasite consists of three infectious stages: tachyzoite, tissue cysts, and oocysts (21). Tachyzoites and tissue cysts are the stages found in the intermediate hosts (25). Tissue cysts are primarily found in the CNS, and also reported in the muscles of cattle and dogs naturally infected with *N. caninum* (90). Because domestic dog is the definitive host for *N. caninum*, viable oocysts have been isolated in the dog faces (29). Cats, mice, rats, pigs, gerbils, foxes, and monkeys may be experimental intermediate hosts (27).

Neospora caninum causes a variety of neurological symptoms, such as pelvic limb paralysis and rigid hyperextension, which are often observed in dogs (21, 29, 94). The most severe cases of neosporosis occur in young, congenitally infected pups (21). Such young dogs develop a paresis of hind limb, progressively (21, 29, 94). Neurologic signs are dependent on the parasitized sites (21). Hind limbs, forelimbs, or both may be flexed or hyperextended (3, 27). Other dysfunctions include difficulty in swallowing, paralysis of the jaw, muscle flaccidity, muscle atrophy, and even heart failure (9). Fatal neosporosis has been reported in eight to 15-year-old dogs (21). Subclinically infected bitches can transmit the parasite to their fetuses (21).

In cattle, *N. caninum* is one of the main causes of abortion, and calves vertically infected with the parasites show any neurological disorders (27). Most neosporosis-induced abortions occur at 5-6 month gestation (20). Fetuses may die in utero, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or

born clinically normal but chronically infected (20).

Clinical signs have been reported only in cattle younger than 2 month of age (20). *Neospora caninum*-infected calves may have neurologic signs, be underweight, be unable to rise, or be born without any clinical signs (20). Hind limbs or forelimbs, or the both may be flexed or hyperextended (20). *Neospora caninum* occasionally causes birth defects, including hydrocephalus and narrowing of the spinal cord (24). Abortions may be epidemic or endemic (121). In the infected areas, as many as 33% of dairy cow fetuses have been reported to abort within a few months (21).

3. Parasite location and neuroinflammation in the host brain

The location of the parasites (*T. gondii* and *N. caninum*) has been supposed to play an important role in the brain pathology of the infected host. Parasite cysts persist in many areas of the brain, and some studies have proposed specific tropisms to certain regions of mice infected with *T. gondii* and *N. caninum* (7, 50, 76, 79, 87, 111). While several studies have reported that *T. gondii* had no obvious tropisms in the brain of mouse (7, 16, 41, 104), another study found that the density of tissue cyst was twofold higher in amygdalar areas (medial and basolateral amygdala) than that in non-amygdalar areas (111). In addition, another study suggested that the presence of tissue cysts in the forebrain contributed to the attenuation of predator odor aversion and anxiety-like behavior (34). Although there are few reports about *N. caninum* location in the brain, a sporadically high parasite load were detected in the frontal lobe and periaqueductal gray of the symptomatic mice (82). Overall, these studies suggest that the parasite distribution contributes to a neurological disorder, but this still requires further investigations.

In addition to the parasite location, the characteristic feature of chronic infection is

the persistent glia activation throughout the whole CNS and the recruitment of peripheral immune cells (31, 112, 118). This prolonged neuroinflammation must directly influence the neurological disorder (88). Most of the inflammatory mediators are potentially toxic for neurons (38, 126, 127). For example, activated microglia produces pro-inflammatory cytokines, nitric oxide (NO), and excitatory amino acids (84), and thus plays a central role on the innate immune response in the CNS. These microglial responses are also strongly related to the neuronal disorders and neurodegeneration (102, 103). The neuroinflammatory activation can lead to altered levels of neurotransmitters, such as glutamate (Glu), dopamine (DA), and 5-hydroxytryptamine (5-HT, serotonin) (18).

Previous data indicated that *T. gondii* infection induced a chronic neuroinflammatory condition characterized by the predominance of helper T cell type-1 immune response, such as productions of interferon-gamma (*Ifng*), interleukin (Il) -12, *Il-1b*, Il-6, and tumour necrosis factor-alpha (*Tnfa*) (11, 57, 101). Chronic neuroinflammation following an infection with *T. gondii* can potentially elicit behavioral alterations and neurological disorders in immunocompetent hosts (88). Interestingly, however, several studies observed only limited neurodegeneration in the CNS of chronically *T. gondii*-infected mice (79, 87).

Information about pathogenicity and immune response of neosporosis has been derived mainly from the rodent models (13). Mice infected with *N. caninum* develop various nervous symptoms, such as wryneck, circular movement, and paralytic gait (69–71). Thus, mouse models seem to be suitable for analyses of brain pathology during the *N. caninum* infection. However, few studies have been done to investigate the effects of *N. caninum* infection on neuronal disorder and levels of neurotransmitters. In our previous study, gene expression profiles and histopathological changes were

demonstrated in the brain of BALB/c mice infected with *N. caninum* (82). The findings indicated that *N. caninum* infection stimulated the immune response in the brain (82). In addition, the frontal lobe and medulla oblongata were mainly affected in the symptomatic BALB/c mice infected with *N. caninum*, and some mice showed severe histopathological lesions in the cerebellum (82).

4. Dysregulation of the neurotransmission related to aversive stress in mice infected with *T. gondii*

The presence of an aversive stimulus is transmitted to the amygdala via the cortex and thalamus (72). The activated amygdala then facilitates a stimulation of the hypothalamic–pituitary–adrenal (HPA) axis (86). The HPA axis is essential for an adaptation to stressful environment (72). Activation of the HPA axis facilitates a secretion of corticosterone (CORT), which plays an important role in expressing emotional behaviors (86). The cortex, particularly the prefrontal cortex, is implicated in the stress regulation (72). Lesions in the cortex decrease or increase the CORT response to stress (55). The amygdala receives dense serotoninergic innervation from the dorsal raphe nucleus, and activation of the dorsal raphe nucleus increases both amygdala 5-HT levels and CORT secretion (54). The CORT modulates a serotonergic activity in the amygdala (100).

A previous study suggested that *T. gondii* infection caused dendritic retraction of basolateral amygdala neurons and decreased amounts of CORT, both at baseline and when induced by an aversive cat odor (78). Additionally, it has been known for decades that the norepinephrinergic system is involved in memory consolidation (49). A norepinephrinergic stimulation of the amygdala enhances the memory consolidation (35). The aversive stimuli enhances a secretion of norepinephrine (NE) from the locus

coeruleus to the cortex and amygdala, resulting in an enhanced fear memory consolidation modulated by stress hormone regulation (98). Dopaminergic pathway also plays a profound role in states of fear and anxiety, with over activation of DA transmission exacerbating the fear response and inhibiting conditioned fear (91). The cortical DA projections are particularly responsive in aversive stimulus, including a fear conditioning (91).

Research for the mechanisms underlying behavioral changes following *T. gondii* infection have focused on the effect of the infection on neuronal cell biology, including neurotransmitter synthesis, signal transduction, gene expression, and epigenetic modulation (17, 42, 99, 104, 123, 124). One study reported that dopaminergic cells were upregulated by the infection, suggesting that *T. gondii* affected the CNS to manipulate host behavior (93). In support of this finding, DA levels are higher in *T. gondii*-infected mice than those in uninfected mice (99). Furthermore, increased release of DA was observed in acutely infected mice (42), while increased levels of DA were observed in the striatum of infected mice at 6 dpi (124). Moreover, a previous study indicated that a treatment of haloperidol and valproic acid, one of the antipsychotics that are known to affect the dopaminergic system (114), to *T. gondii*-infected rats reduced their likelihood to enter the cat odour area (116), suggesting that the treatment of *T. gondii*-infected rats with antipsychotics, partially reverses the behavioral effect of *T. gondii* infection. Recently, Hari Dass and Vyas reported that *T. gondii* infection induced a hypomethylation of the arginine vasopressin promoter in the medial amygdala (17). They also showed that the decreased aversion to cat odors in the *T. gondii*-infected rat was recovered by the systemic hypermethylation (17). Despite these findings, the mechanism(s) underlying the behavioral changes induced by *T. gondii* infection remains unclear. Additionally, as compared with the results of *T. gondii*, there were few reports

about neurological dysfunction associated with *N. caninum* infection.

5. Objective

In my thesis, I focused on behavioural and brain pathological changes in mice infected with *T. gondii* and *N. caninum*.

In chapter 1, using asymptomatic C57BL/6 mice during the chronic infection with *T. gondii,* I examined the parasite distribution, histopathological lesion severity, and levels of neurotransmitters (DA, 5-HT, and NE) in brain, in order to evaluate how the latent *T. gondii* infection affects the host fear memory.

In chapter 2, to understand the onset mechanism of neosporosis, I examined the sickness behaviour, parasite distribution, histopathological lesions, levels of neurotransmitters (monoamines and amino acids), and expression of immediately early genes (IEGs), using asymptomatic C57BL/6 male mice during the subacute infection with *N. caninum*.

CHAPTER1

Toxoplasma gondii infection in mice impairs long-term fear memory consolidation through dysfunction of the cortex and amygdala

1. INTRODUCTION

Chronic infection of *Toxoplasma gondii* reduces an aversion of rodents to cat odors, plausibly increasing predation by its definitive host (111). Beside this, learning and memory deficits have been demonstrated in rodents infected with *T. gondii* (16, 46, 64, 111, 119). Up to now, brain pathology of the infected host, including pathological lesion, cyst location, and neuronal dysfunction, is becoming clear. However, relationships between these neurological dysfunctions and behavioral changes remain unclear. In chapter 1, I investigated the parasite distributions in brain, severities of histopathological lesions, and levels of neurotransmitters (dopamine (DA), 5-hydroxytryptamine (5-HT), and norepinephrine (NE)), in order to evaluate how the latent infection of *T. gondii* affects the host fear memory.

2. MATERIALS AND METHODS

2. 1. Ethics statement

This study was conducted 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 protocols were approved by the

Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine (permit numbers 23-64, 24-17, 25-66, and 26-68). Mice were decapitated without anesthesia for brain sampling, and all efforts were made to minimize animal suffering.

2. 2. Mice

C57BL/6 mice (8 weeks old, male) were purchased from CLEA Japan (Tokyo, Japan). Mice were housed in plastic cages (four to six mice/cage) under constant environmental conditions with 12-h light:dark cycle (8:00–20:00) in the animal facility of National Research Center for Protozoan Diseases at 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 Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animal experiments began after 1 week of habituation.

2. 3. Parasite culture

Toxoplasma gondii (strain PLK; type II) were passaged in monkey kidney adherent epithelial cells (Vero cells) in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA) containing 8% fetal bovine serum (104). Infected cells were syringe-lysed using a 27-gauge needle (Thermo, Yokohama, Japan) to release the tachyzoites into RPMI-1640 medium (Sigma), and then filtered using a 5.0-μm pore size filter (Millipore, Bedford, MA, USA).

2. 4. Parasite infection and experimental groups

The tachyzoites of *T. gondii* were intraperitoneally inoculated (1×10^3

tachyzoites/mouse) into 9-week-old mice. Body weight measurements were daily taken for 30 days after the infection. This study consisted of six experiments, and the flowcharts of experimental trials are described in Fig. 1. All behavioral experiments were performed at 37–41 days post infections (dpi), commencing at 7:00–8:30 a.m. under a light intensity of 300 lux. For a high-performance liquid chromatography (HPLC) analysis, uninfected and infected mice were sacrificed at 40 and 52 dpi. Forty and 52 dpi were selected to evaluate the impact of *T. gondii* infection, because these days corresponded to those for the start and end of fear-conditioning test, respectively. I examined the correlations between the percentages of time spent freezing in the fear-conditioning test and levels of any neurotransmitters, by using the brain samples collected at 52 dpi. Moreover, mice were sacrificed at 45 and 54 dpi for histopathological analysis and for quantitating of the parasite loads using quantitative polymerase chain reaction (PCR) analysis, respectively. Successful establishment of the latent infection was confirmed using an enzyme-linked immunosorbent assay (ELISA) for detecting the antibodies to *T. gondii* dense granule protein 7 (TgGRA7) (108). Mice with no anti-TgGRA7 antibodies were excluded from experiments.

2. 5. Fear-conditioning test

I performed contextual and cued fear-conditioning tests to evaluate learning and memory of mice. The fear-conditioning test is a behavioral experiment that assesses the ability of mice to learn the association between an environmental cue and an aversive stimulus (14). 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 the 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 the 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 an associative-type, long-term fear memory, fear-conditioning tests were performed from 37 to 41 dpi, according to methods used in earlier studies (14, 111) with some modifications as described in Fig. 2. Fear conditioning test was carried out using a fear-conditioning box MK-450 (18 cm × 17 cm × 40 cm) (Mutomachikikai, Tokyo, Japan), freezing was recorded using a video-tracking system (Comp Act VAS ver. 3.0x; Muromachikikai). The test consisted of four phases: conditioning, context, tone, and extinction, as described in Fig. 2. On test day 1, mice were placed in the box for habituation (120 sec). An auditory tone (75 dB, 300 Hz) was then presented for 28 sec, with a mild foot shock (0.5 mA) being paired with the auditory tone for 2 sec. An interval of 60 sec preceded a second identical trial. After the last foot shock presentation, mice were kept in the box for an additional 30 sec. 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 then 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 and 300 Hz for 10 sec with 50 sec interval durations). Freezing was measured during the first tone, before it was paired to a foot shock (28 sec) in the unconditioning phase, during the second tone after it was

paired to foot shock (28 sec) in the conditioning phase, during observation (300 sec) in the context test, during habituation (180 sec), and during the tone (180 sec) in the tone test. The freezing ratio (in percent) was calculated by dividing the time spent freezing by the total amount of time of each session. In the extinction test, the freezing ratio (in percent) was repeatedly calculated by diving the time spent freezing by 5 min for every 5 min of the extinction test.

2.6. DNA extraction and real-time quantitative PCR assay

To measure the parasite load in mouse brain at 54 dpi, one hemisphere from each mouse brain was separated into eight regions: cortex, hippocampus, caudoputamen, amygdala, thalamus, hypothalamus, midbrain, and cerebellum. The detail methods used for dissection of these brain regions are described in Fig. 3. The collected tissues were immediately stored at -30°C. Genomic DNAs were extracted with a standard phenol-chloroform-isoamyl alcohol (PCI) method from the brain regions, and then number of parasites in the brain was quantitatively analyzed with real-time PCR assay targeting for the B1 gene, as described previously (104). The PCR assay was performed using an ABI prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), and the amplification was monitored using the SYBR Green method (Applied Biosystems). A standard curve was constructed with tenfold serial dilutions of *T. gondii* DNA that had been extracted from 1×10^5 parasites. The curve ranged from 10,000 to 0.01 parasites (104). Parasite numbers were calculated by plotting cycle threshold (Ct) values on the standard curve (104).

2. 7. Histopathological analysis

After being fixed with 4% paraformaldehyde solution, brain samples at 45 dpi were

cut coronally, embedded in paraffin wax, sectioned at 4 μm, and then stained with hematoxylin and eosin. The severities of pathological lesions were scored using the following scheme: 0, no lesion; 1, slight lesion; 2, mild lesion; 3, moderate lesion; and 4, severe lesion (104). Representative examples of the scoring are shown in Fig. 4. 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 severities of inflammatory cells, which included glial cells, macrophages, and lymphocyte infiltration.

2. 8. RNA extraction, reverse transcription, and real-time PCR assay

The levels of messenger RNA (mRNA) expression were measured in the mouse brain at 52 dpi, after the brains were separated into five regions: cortex, hippocampus, amygdala, caudoputamen, and thalamus (regions related to emotional behavior, learning, and memory). The collected tissues were immediately stored at −80 °C. Total RNA was isolated using a TRI Reagent (Sigma). The first-strand complementary DNA (cDNA) was synthesized from 400 ng of the total RNA using the SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen, Mount Waverley, Australia). Real-time PCR assay was conducted according to manufacturer (Applied Biosystems), and amplification was monitored with a SYBR Green (Applied Biosystems). The mRNA expressions of *T. gondii* SAG-related sequence SRS29B (*TgSAG1*), *T. gondii* Bradyzoite antigen BAG1 (*TgBAG1*), and *T. gondii* dense granule protein 1 (*TgGRA1*) mRNA were measured to investigate the parasitic stages present in the brain (73, 117). The following primers were used: *TgSAG1*, 5′-TGA TGC AAC CGA CCA CAA AC-3′and 5′-CAA TCG AGA AGT TCC CCG TG-3′; *TgBAG1*, 5′-GGG AAA TGG CTG TCG CAG TA-3′ and 5′-CTT GTC CAC CGG GAT GTA CC-3′; *TgGRA1*,

5′-TAC AGC GAA GTC GGC AAT GTT-3′ and 5′-TCG CCT TTG TTC AAC GCA C-3′; *Ifng*, 5′-GAG GAA CTG GCA AAA GGA TG-3′ and 5′-TGA GCT CAT TGA ATG CTT GG-3′; Cd4 antigen (*Cd4*), 5′-GGG TTC AGG ACA GCG ACT TC-3′ and 5’-TTT TCT GGT CCA GGG TCA CG-3’; Cd8 antigen (*Cd8*), 5′-AGG ATG CTC TTG GCT CTT CC-3′ and 5′-TCA CAG GCG AAG TCC AAT CC-3′; and integrin alpha (*Cd11b*), 5′-TTC AAC AAA CCA CAG TCC CG-3′ and 5′-TGG CTT AGA TGC GAT GGT GTC-3′. A housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA, was amplified in parallel (5′-TGT GTC CGT GGA TCT GA-3′ and 5′-CCT GCT TCA CCA CCT TCT TGA T-3′), and used as the internal standard. The expression level of each gene relative to that of *Gapdh* was calculated using $2^{\Delta Ct}$ method, according to the manufacturer’s instructions (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR, AB Applied Biosystems). Fold expressions were calculated relative to the expression levels of the corresponding gene in the hippocampi of uninfected mice.

2. 9. High-performance liquid chromatography (HPLC)

Productive levels of neurotransmitters were measured in an HPLC. The brains were separated into two regions: cortex and amygdala (regions related to emotional behavior, leaning, and memory). Tissues were immediately stored at -80°C. The brain tissues were homogenized in 0.2 M perchloric acid (300 μL/10 mg tissue, containing 100 μM ethylenediaminetetraacetic acid (EDTA) -2Na). Isoproterenol HCl (Sigma) was used as a monoamine internal standard according to manufacturer (Eicom). 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 by 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 a PowerChrom software version 2.5 (eDAQ Pty Ltd., Densitone East, Australia).

2. 10. Correlation coefficient analysis

The correlation coefficients were calculated between the percentages of time spent freezing in the fear-conditioning test and the levels of cortical neurotransmitters, using the Pearson correlation coefficient (104). A previous study showed that the strength of linear association between pairs of variables could 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 (45).

2. 11. Statistical analyses

Statistical analysis was conducted with Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical differences were analyzed between two groups using two-tailed unpaired *t* tests, except for the extinction test, which were determined using repeated-measures analysis of variance (ANOVA) with Bonferroni post hoc test. Among three groups or more, statistical differences were determined using one-way ANOVA followed by Tukey's multiple comparisons test (104). For the correlation analysis, significant differences were determined using the Pearson correlation coefficient (104). The P values < 0.05 represent statistically significant differences.

3. RESULTS

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

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

3. 2. Parasite loads and pathological changes of brain regions in *T. gondii*-infected mice

Since it was thought that the distribution of *T. gondii* cyst in the brain might be an important factor affecting behavioral changes, I analyzed the expression of parasite B1 gene by real-time quantitative PCR assays in order 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. 6). In addition, to investigate the parasite stages in the brain, the mRNA expression levels of *TgSAG1* (a tachyzoite-specific gene), *TgBAG1* (a bradyzoite-specific gene), and *TgGRA1* (a nonstage-specific gene) were measured in real-time PCR assays (Fig. 7). The expressions of *TgBAG1* and *TgGRA1*

were detected in each brain region, while the expression of *TgSAG1* was very low in the infected mice. There were no significant differences in the expressions of these genes among the brain regions (Fig. 7). Histopathological analyses showed that perivascular cuffs and inflammatory cell infiltration were observed in almost all regions (Figs. 8B and C). However, the meningitis was significantly more severe in the cortex than that in other regions (Fig. 8A). The pathological scores for the perivascular cuffs in the cortex, caudoputamen, thalamus, and hypothalamus were higher than those in the midbrain (Fig. 8B), while there were no significant differences in the pathological scores for the inflammatory cell infiltration among the brain regions (Fig. 8C). In addition to the pathological analyses, the inflammatory infiltrates were assessed by real-time PCR assays for the expressions of the *Cd4*, *Cd8*, and *Cd11b* gene, which are markers of inflammatory cells (Fig. 9). The *Cd4* levels were higher in the amygdala in the infected mice than those in the other regions (Fig. 9A). The levels of *Cd8* and *Cd11b* were higher in the amygdala than those in the hippocampus (Figs. 9B and C). The level of *Ifng* was higher in the cortex than that in the hippocampus or caudoputamen (Fig. 9D).

3. 3. Neurotransmitter levels in cortex and amygdala of *T. gondii*-infected mice

I analyzed the levels of various neurotransmitters in the cortex and amygdala. Cortical DA levels were not significantly different between uninfected and *T. gondii*-infected mice at either 40 or 52 dpi (Figs. 10A and E). Amygdalar DA levels were lower in infected mice than those in uninfected animals at 40 dpi, but no difference in DA levels was detected at 52 dpi (Figs. 10A and E). I also examined the DA metabolism in these animals. Homovanillic acid (HVA) is the primary final DA metabolite produced via the intermediate products, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxytyramine (3-MT) (32). The levels of all DA metabolites

increased in the cortex of infected mice at 40 and 52 dpi, but not in the amygdala, as compared with those in uninfected animals (Figs. 10B-D, and F-H). I also determined that the levels of 5-HT in infected mice decreased in the amygdala but not the cortex at 40 and 52 dpi, as compared with uninfected mice (Figs. 11A and C). There was no difference in the level of serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cortex or amygdala following the *T. gondii* infection (Figs. 11B and D). The NE levels were decreased in both the cortex and amygdala of infected mice at 40 and 52 dpi, as compared with those in uninfected mice (Figs. 12A and B).

Next, I analyzed the correlations between the percentages of time spent freezing in the fear-conditioning test and the levels of neurotransmitters in the cortex and amygdala, by calculating Pearson correlation coefficients (Table 1, Fig. 13). I found that the level of HVA in the cortex had a moderately strong negative correlation with the percentage of time spent freezing during the context test ($r = –0.613$; Fig. 13A). 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. 13B-D). In 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; Figs. 13E and F). In addition, amygdalar 5-HT, 5-HIAA, and NE levels also showed weak-to-moderate positive correlations with freezing time during the tone test ($r = 0.371$, 0.385, and 0.388, respectively; Figs. 13G-I).

4. DISCUSSION

I showed that *T. gondii* infection in C57BL/6 male mice impaired a consolidation of fear memory consolidation, while the extinction remained intact. Vyas *et al*. observed no obvious deficits in the fear memory of *T. gondii*-infected rats in their

fear-conditioning test (111). However, Witting, P. A. showed impairment of the memory in *T. gondii*-infected mice (119). In addition, the study demonstrated that *T. gondii*-infected mice showed a higher sensitivity to learning and memory deficits than that of *T. gondii*-infected rats (119). Kannan *et al*. reported that spatial working memory was impaired in mice infected with *T. gondii* (64). In a recent study, Daniels *et al*. showed that the spatial memory recall was also impaired in rats infected with *T. gondii* (16). Thus, the effects of *T. gondii* infection on learning, memory, and emotional behavior have varied widely among the reports, because these studies used different experimental designs, which may have affected the results (65, 120). I am the first to report the impaired consolidation of fear memory in *T. gondii*-infected mice.

Consistent with previous studies in mice, *T. gondii* had no obvious preference for specific brain regions (7, 41, 104). In addition, no other study has reported a clear evidence to support the idea that the parasite localization played a critical role in the behavioral changes of mice induced by *T. gondii* infection (16, 34, 44, 111). In my study, the parasite burden was assessed by real-time PCR assay targeting for the parasite *B1* gene against the brain tissues, but cyst burden was not known. Therefore, expression levels of *TgSAG1* (a tachyzoite specific gene), *TgBAG1* (a bradyzoite specific gene), and *TgGRA1* (non-stage specific gene) were measured by other real-time PCR assays (75, 122). Although I might miss subtle cyst tropism, almost all parasites at 52 dpi were thought to be bradyzoites.

The areas of meninges in the cortex and cerebellum are larger than those of other brain regions; however, meningitis in the cortex was more severe than that in the cerebellum. Together with the results showing no marked tissue tropism for the parasite, my results suggested that the immune response (indirect effects) might be more brain-region specific than the parasitic cyst burden (direct effects). Similarly, our

previous study using BALB/c mice showed that the prefrontal cortex was more severely damaged than other brain regions (104). Although the mechanism whereby the *T. gondii*-induced pathology shows a cortical specificity is still unclear, these results suggested that *T. gondii* might cause a cortical hypofunction independent on the parasite distribution.

In addition to the pathological analysis, my real-time PCR assays for the general makers of inflammatory cells suggested that the inflammatory cell infiltration was more severe in the amygdala than those in the other brain regions. The level of *Ifng* was higher in the cortex than that in the hippocampus or caudoputamen. In my study, however, there are no data directly correlating the severity of the behavioral deficits with the degree of damage to the cortex and amygdala. Therefore, more direct evidence is required before I can conclude that the degree of brain inflammation affects the behavioral changes. However, because the prefrontal cortex and amygdala are involved in the fear memory and emotional behavior (97), our results suggest that cortical and amygdalar lesions, including meningitis or inflammatory infiltration, are related to the impairment of neuronal function in the cortex and amygdala.

I also analyzed cortical and amygdalar levels of DA, 5-HT, NE, and the metabolites of DA and 5-HT, which are all associated with the expressions of emotional behavior, learning, and memory (68). Gatkowska *et al*. reported that DA turnover (HVA/DA ratio) in mice was elevated in acute toxoplasmosis, but not in chronic one (42). In contrast, my results indicated that DA metabolism activity was up-regulated during the chronic stage of *T. gondii* infection, strongly suggesting that DA metabolites were chronically activated. Increased levels of DA metabolites with unaltered levels of DA itself have been shown to compensate for a deficiency in available DA in the cortex (80), and cortical dysfunction and dysregulation of DA metabolism are involved in

schizophrenia (67). Interestingly, *T. gondii* contains two genes encoding tyrosine hydroxylase, which is the rate-limiting enzyme of DA biosynthesis (40). Indeed, DA levels are increased in *T. gondii*-infected neurons and the adrenal phaeochromocytoma cells (PC12 cells) (93). These results suggest that *T. gondii* may control the host's DA biosynthesis pathway.

The amygdala is a key integrative site regulating anxiety and fear (92). An imbalance of the amygdala serotonergic system links to anxiety and depression (5). In the amygdala, 5-HT stimulates CORT secretion, and the CORT modulates serotoninergic activity in the amygdala, suggesting that 5-HT–CORT interactions may be involved in the amygdala-dependent emotional behavior (100). The *T. gondii* infection reduces the CORT levels (78), suggesting a dysfunction of HPA axis mediated through the amygdala in mice infected with *T. gondii*. Therefore, I considered that dysfunction of amygdalar serotonergic system was involved in the impaired fear memory consolidation in the infected mice with *T. gondii*. Aversive stimuli enhances a secretion of NE from the locus coeruleus in the cortex and a1mygdala, resulting in an enhanced fear memory consolidation modulated by stress hormone regulation (98). Thus, I considered that decreased levels of NE also contributed to the dysfunction of cortex and amygdala in the infected mice.

Lastly, I found a negative correlation between the levels of all DA metabolites in the cortex and freezing behavior during the context test. In other words, fear memory consolidation was impaired in mice showing high levels of DA metabolites. In contrast, levels of 5-HT and 5-HIAA in the amygdala and NE in the cortex and amygdala were positively correlated with the freezing behavior. Auditory stimulus information in the amygdala is regulated by neurotransmitters, including DA, 5-HT, and NE (68). A fear-conditioned tone increases levels of these neurotransmitters and influences

excitatory and inhibitory neuron interactions (92). Thus, loss of serotonergic and adrenergic neurons impairs an acquisition of conditioned fear (68), suggesting that the lower levels of brain DA, 5-HT, and NE detected in *T. gondii*-infected mice might be associated with the diminished fear memory. This is the first report to demonstrate a connection between the altered neurotransmitter levels and behavioral changes following the *T. gondii* infection.

In this study, I used one behavioral paradigm and experimental model to examine the connection between *T. gondii*-induced inflammatory and neuronal damage to specific brain regions and the subsequent behavioral change. Even though our model using C57BL/6 male mice has commonly been used in this field, further investigation will be needed to determine whether these findings remain consistent across several rodent models.

In conclusion, *T. gondii* infection impaired the long-term fear memory consolidation through the dysfunctions of cortex and amygdala in mice. In the infected mice, the cortex was more severely damaged than other brain regions, with dysfunctions likely occurring in the brain. The DA metabolism was increased to compensate for a deficiency in available cortical DA in the infected mice due to the hypofunction of the cortex. In addition, I showed imbalances in neurotransmitters modulating the stress response (5-HT and NE) in the amygdala. These data support a hypothesis that the modification of responsiveness to stress mediated via the limbic–hypothalamic–pituitary–adrenal axis might cause the behavioral changes following *T. gondii* infection. Thus, my findings not only provided an insight into the mechanisms underlying central nervous system (CNS) changes during *T. gondii* infection, but also elucidated the underlying mechanism of the relationship between the *T. gondii* infection and onset of mental disease.

Table 1 Pearson's correlation coefficients between the percentage of time spent freezing in the fear-conditioning test and levels of neurotransmitters.

	Cortex		Amygdala	
	Context test (r)	Tone test (r)	Context test (r)	Tone test (r)
DA	-0.212	-0.121	0.065	0.201
DOPAC	-0.388 *	-0.112	0.132	0.299
3-MT	-0.378 *	-0.073	-0.183	0.032
HVA	-0.613 *	-0.277	0.147	0.285
5-HT	-0.267	-0.222	0.231	0.371 *
5-HIAA	-0.447 *	-0.117	0.017	0.385 *
NE	0.346 *	0.153	0.414 *	0.388 *

After the fear-conditioning test, some mice were used for the correlation analysis (Experiments 3 and 4, see Fig. 1). Each value represents the correlation coefficient (Pearson's r): $|r| = 0.70$, 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. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 3-MT, 3-methoxytyramine; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; NE, norepinephrine. Significant differences were determined using two-tailed t tests ($*p < 0.05$).

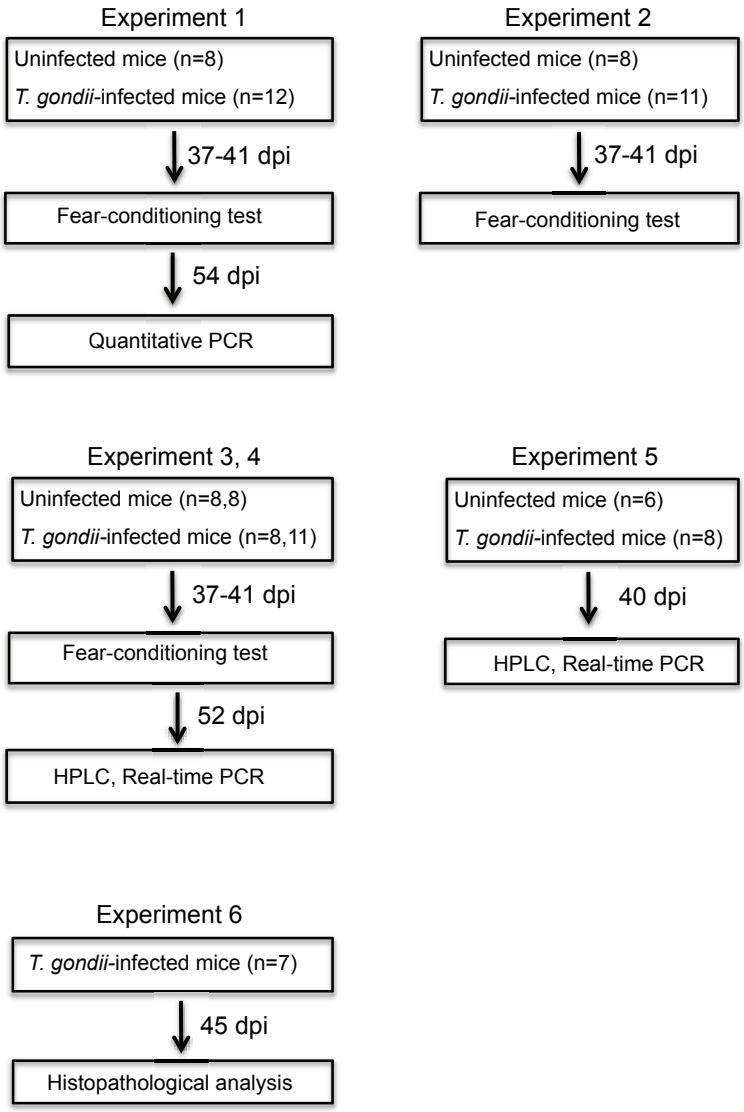

Figure 1 Flowcharts explaining the number of uninfected and infected mice, and methods.

The figure indicates the timing of each experiment and experimental group. For the behavioral study, a total of 32 uninfected and 42 *T. gondii*-infected mice were used, because several mice died of the acute infection with *T. gondii*. Dpi, days post infection.

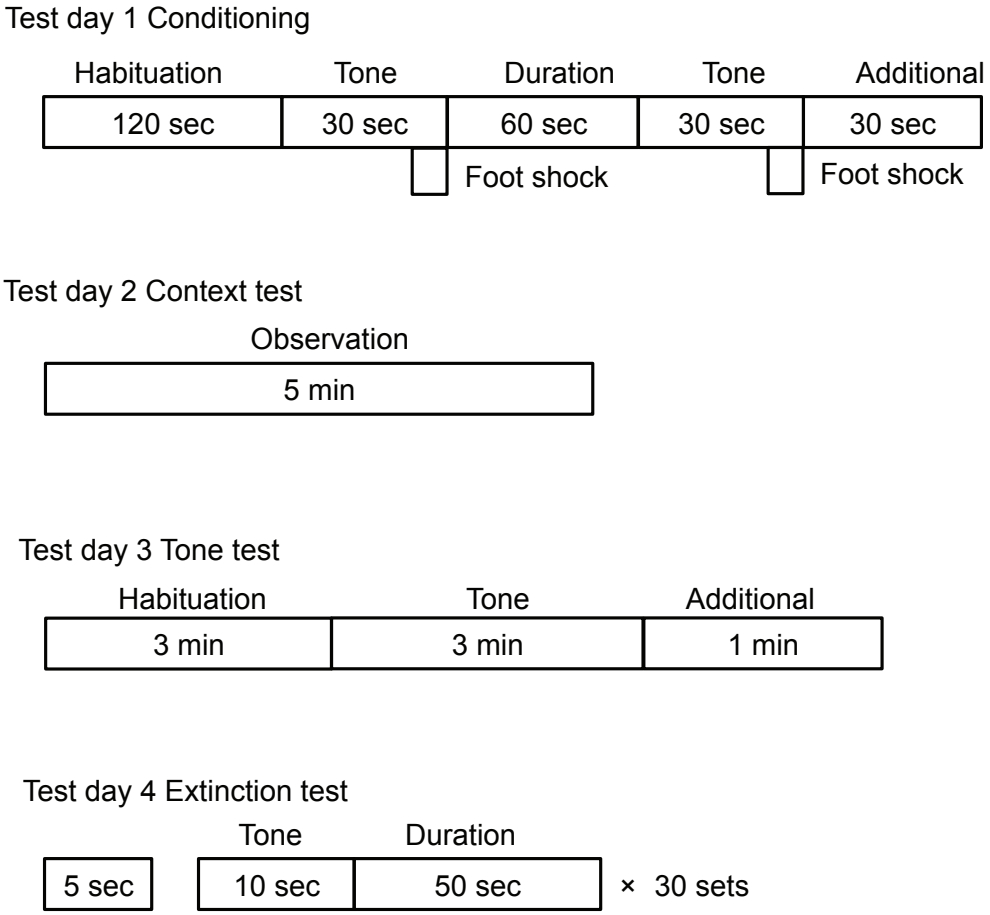

Figure 2 Schematic diagrams of the experimental designs for fear-conditioning tests.
The figure illustrates test days 1, 2, 3, and 4 (tone: 75 dB, 300 Hz; foot shock: 0.5 mA, 2 sec).

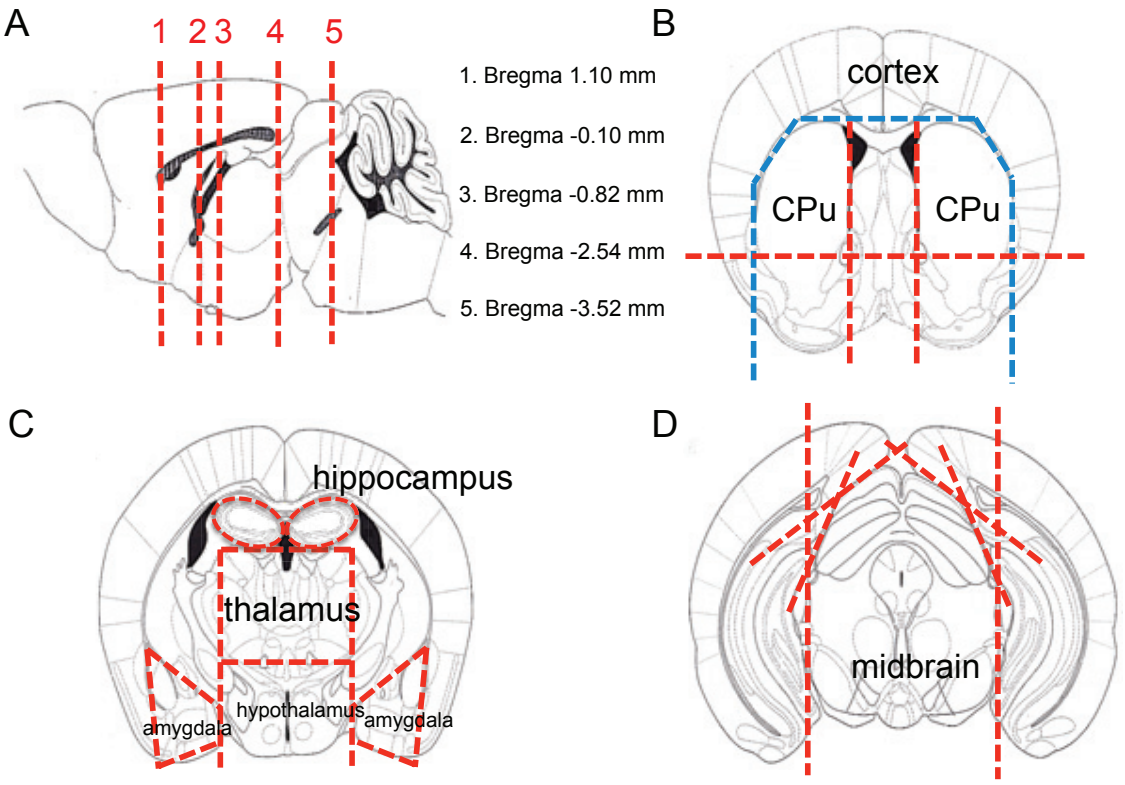

Figure 3 Brain map of regions used in this study.
(**A**) Sagittal section of a mouse brain. Each vertical line shows the location relative to bregma. (**B**) The coronal section between vertical lines 1 and 2 of panel **A**. The cortex and caudoputamen (CPu) were dissected from the location as shown. (**C**) The coronal section between vertical lines 3 and 4 of panel **A**. The thalamus, amygdala, and hypothalamus were collected as shown. The hippocampus was collected from the remaining slice after removing the cortex etc. (**D**) The coronal section between vertical lines 4 and 5 of panel **A**. The midbrain was collected as shown. The cerebellum was separated and collected from the pons and medulla oblongata.

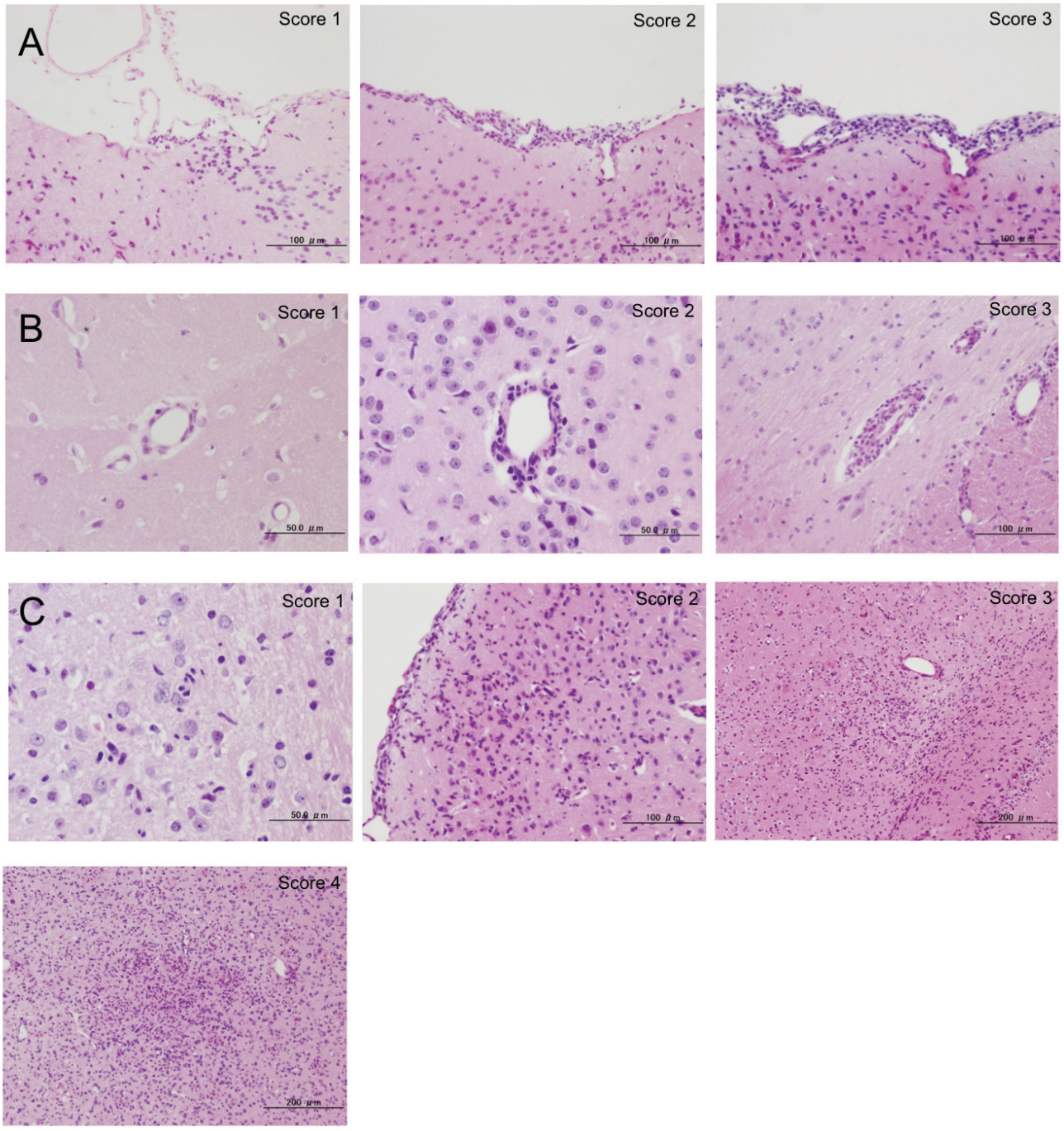

Figure 4 Representative examples of histopathological lesions in the brains of *T. gondii*-infected mice.
The severities of the pathological lesions were scored using the following scheme: 0, no lesion; 1, slight lesion; 2, mild lesion; 3, moderate lesion; and 4, severe lesion. (**A, B**) Because no mouse showed severe lesions (Score 4) for meningitis or perivascular cuffs, the pathological scores were determined from 0 to 3. (**A**) Meningitis, (**B**) Perivascular cuffs, and (**C**) Inflammatory cell infiltration were shown.

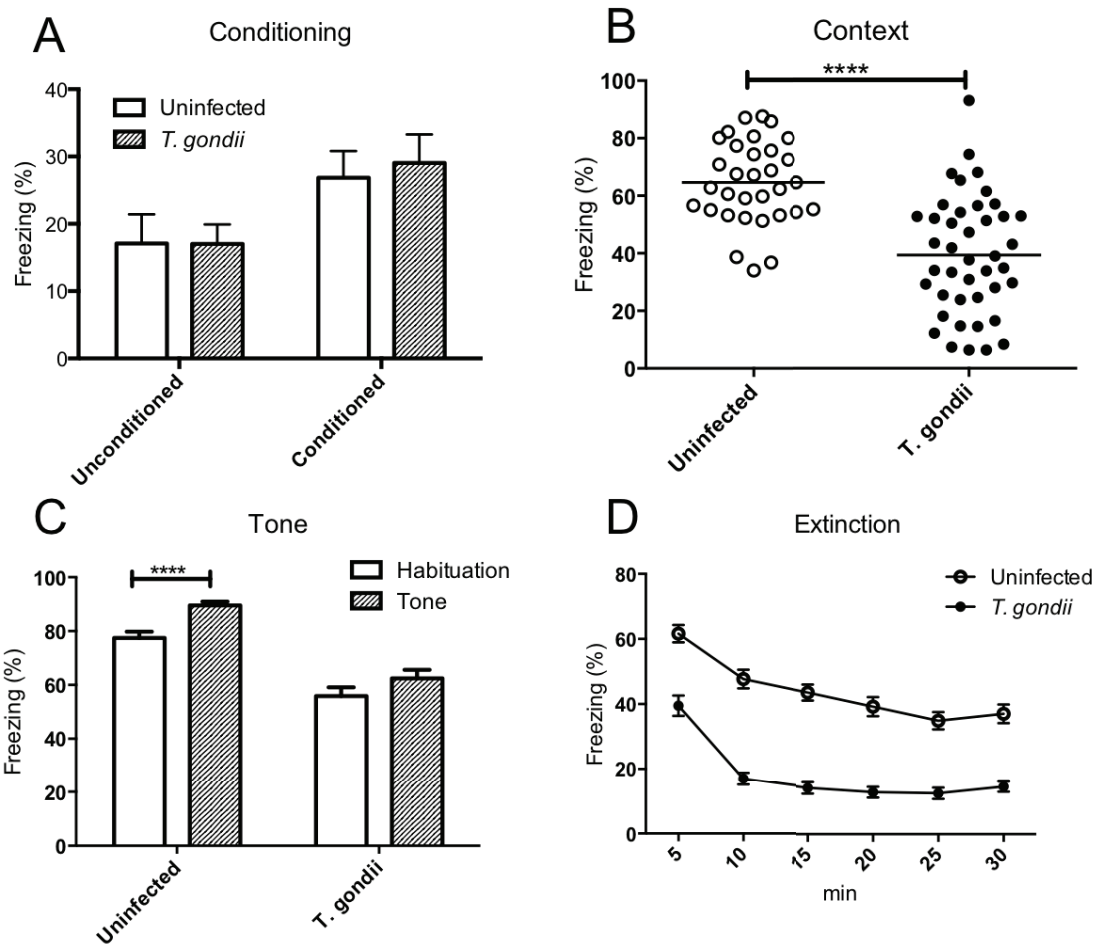

Figure 5 Impaired consolidation of long-term fear memory in *T. gondii*-infected mice.

The ordinate shows the percentage of time spent freezing. (**A**) Unconditioned trial shows a freezing during the first tone before pairing to foot shock, while conditioned trial shows the freezing during the second tone after pairing to foot shock on test day 1. (**B**) Contextual conditioned freezing time. (**C**) Tone conditioned freezing time. (**D**) Extinction of tone conditioned freezing time. (**A-C**) Significant differences were determined by unpaired *t* tests (****$p < 0.0001$). (**D**) Significant differences were determined using repeated measures of ANOVA with the post hoc Bonferroni test. Significant main effects were shown for *T. gondii* infection ($F_{(1, 70)} = 75.90$, $p < 0.0001$) and time ($F_{(5, 350)} = 117.5$, $p < 0.0001$), and their interaction was also significant ($F_{(5, 350)} = 4.410$, $p < 0.001$). Freezing (%) was calculated by dividing freezing time into the times for observation (300 s) in the context test, habituation (180 s), tone (180 s) during the tone test, and every 5 min in the extinction test. Data represent mean ± standard error of the mean. Uninfected mice, $n = 32$; *T. gondii*-infected mice, $n = 42$. Data were summarized from four independent experiments.

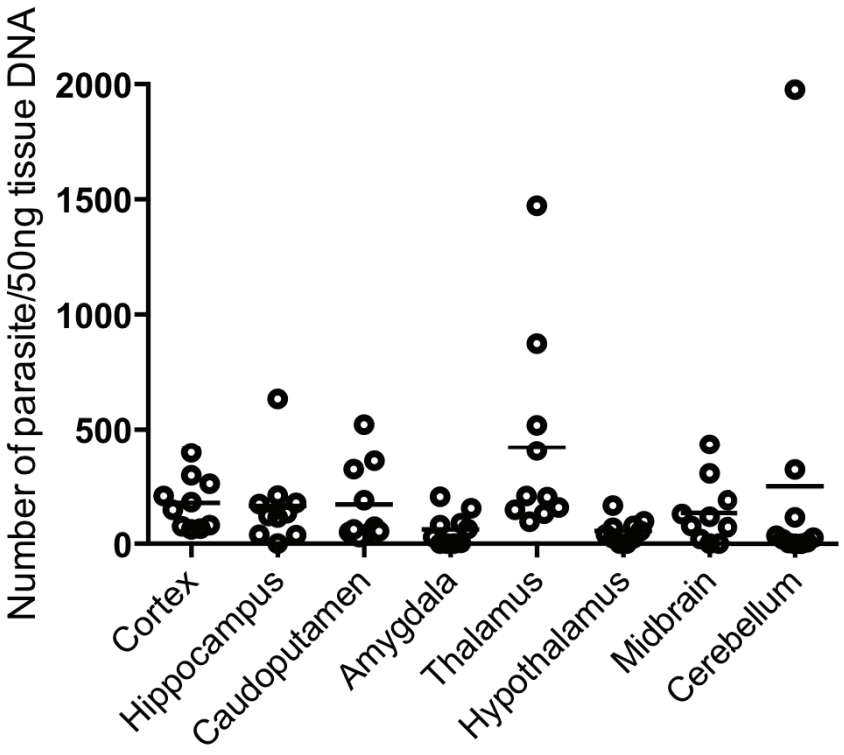

Figure 6 Parasite loads in the brain regions of *T. gondii*-infected mice.
The ordinate shows parasite number per 50 ng of tissue DNA. Brain samples were collected at 54 days post infection. Each circle represents data for one mouse, and bars represent the average value of all data points (*T. gondii*-infected mice, n = 10). No statistically significant differences were found in one-way ANOVA with Tukey's post hoc test.

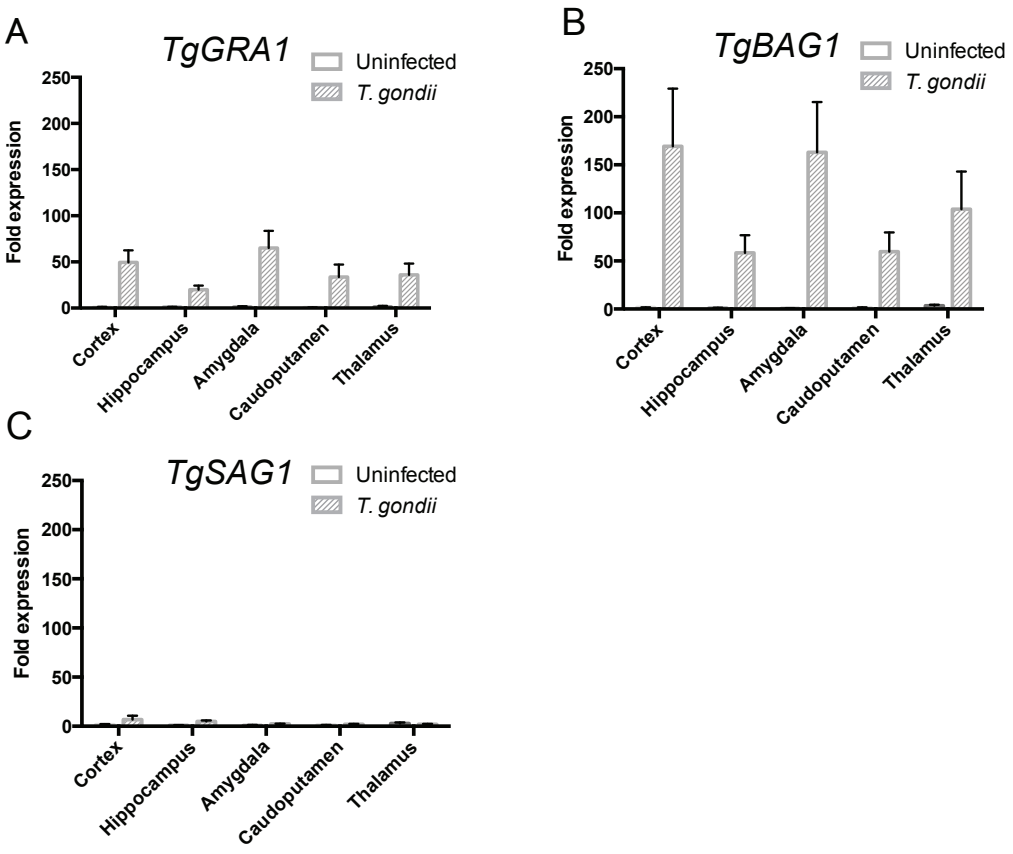

Figure 7 *TgGRA1*, Tg*BAG1*, and Tg*SAG1* expressions in the brains of *T. gondii*-infected mice.

The ordinate shows the relative mean expression levels of the genes (*TgGRA1*, *TgBAG1*, and *TgSAG1*) in each brain region. Brain samples from uninfected and *T. gondii*-infected mice were collected at 52 days post infection. The expression level of each gene relative to *Gapdh* was calculated with the $2^{\Delta Ct}$ method. Fold expression was calculated relative to the expression level of the corresponding gene in the hippocampi of uninfected mice. Data are means ± standard error of the mean. Uninfected mice, n = 3; *T. gondii*-infected mice, n = 19. No statistically significant differences were found in one-way ANOVA with Tukey's post hoc test.

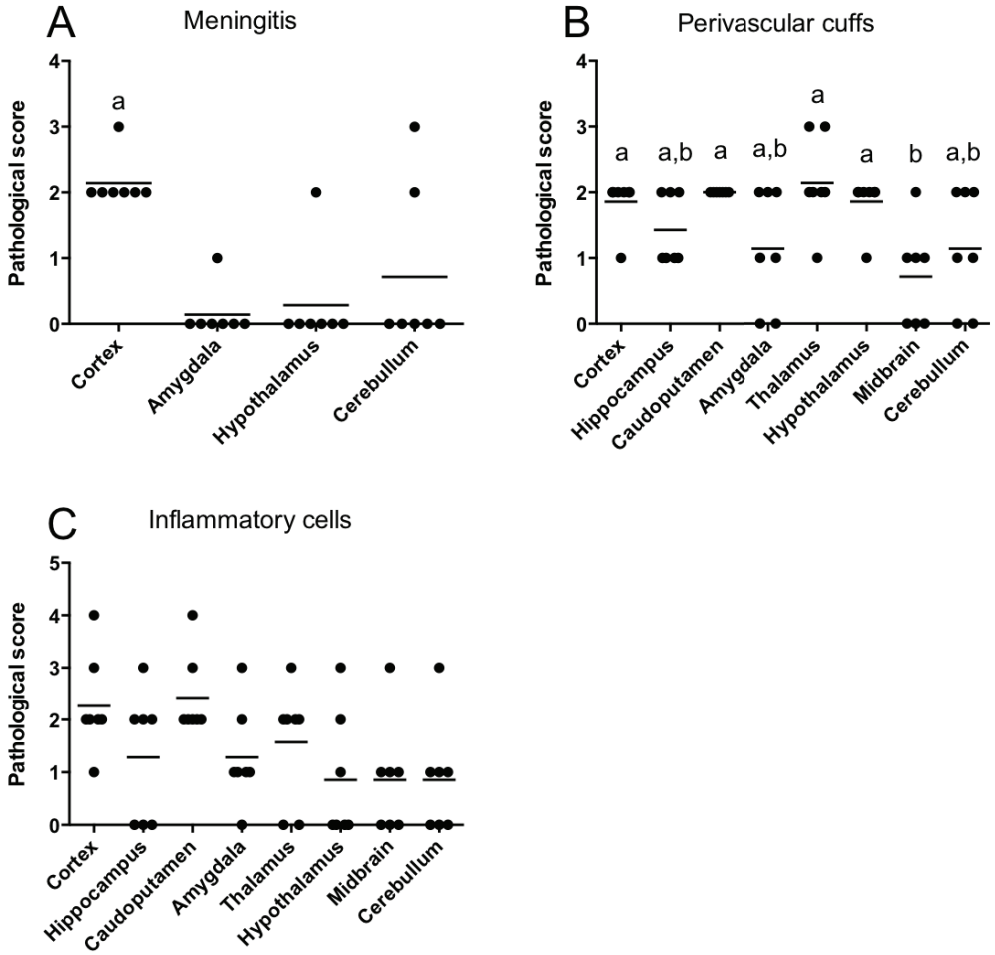

Figure 8 Histopathological changes in the brains of *T. gondii*-infected mice.
The ordinate shows the pathological score for each brain region. Brain samples were collected at 45 days postinfection. Histopathological lesions were scored as follows: 0, no lesion; 1, slight lesion; 2, mild lesion; 3, moderate 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 .

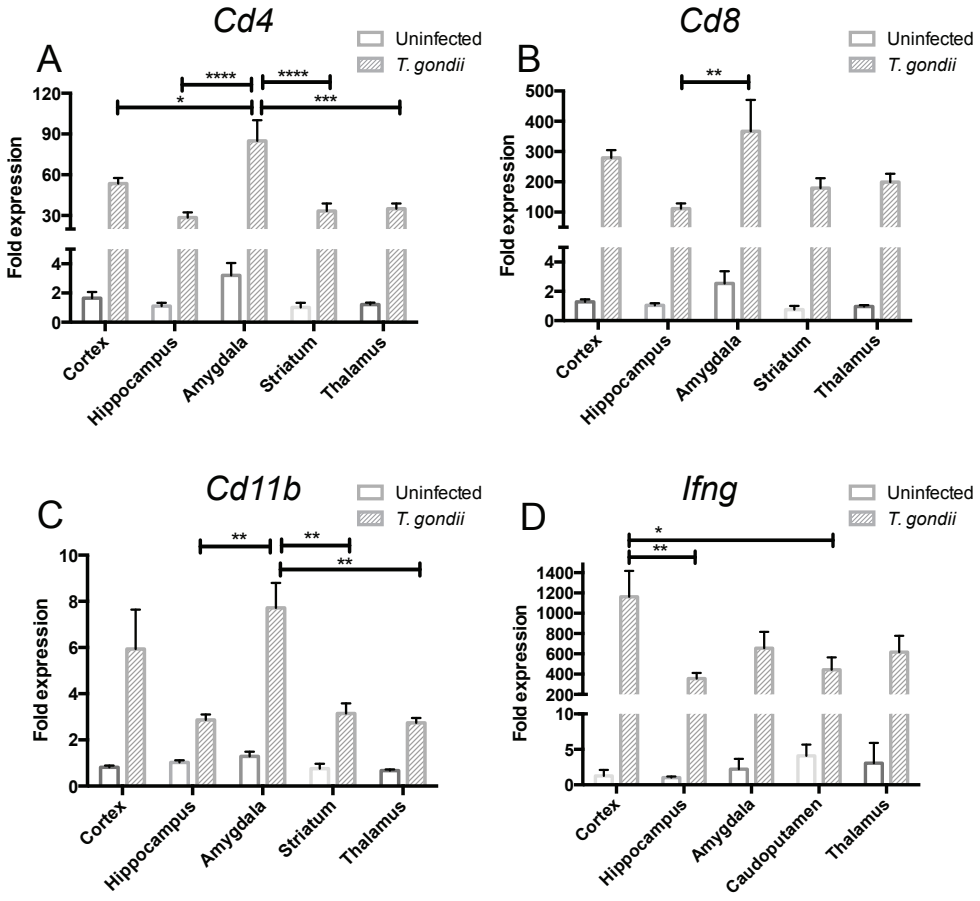

Figure 9 *Cd4*, *Cd8*, *Cd11b*, and *Ifng* expressions in the brains of *T. gondii*-infected mice.

The ordinate shows the relative mean expression levels of the genes in each brain region. Brain samples of uninfected and *T. gondii*-infected mice were collected at 52 days post infection. The expression level of each gene relative to *Gapdh* was calculated using the $2^{\Delta Ct}$ method. Fold expression was calculated relative to expression level of the corresponding gene in the hippocampi of uninfected mice. Data are means ± standard error of the mean. Uninfected mice, $n = 3$; *T. gondii*-infected mice, $n = 19$. Significant differences were determined using one-way ANOVA with Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

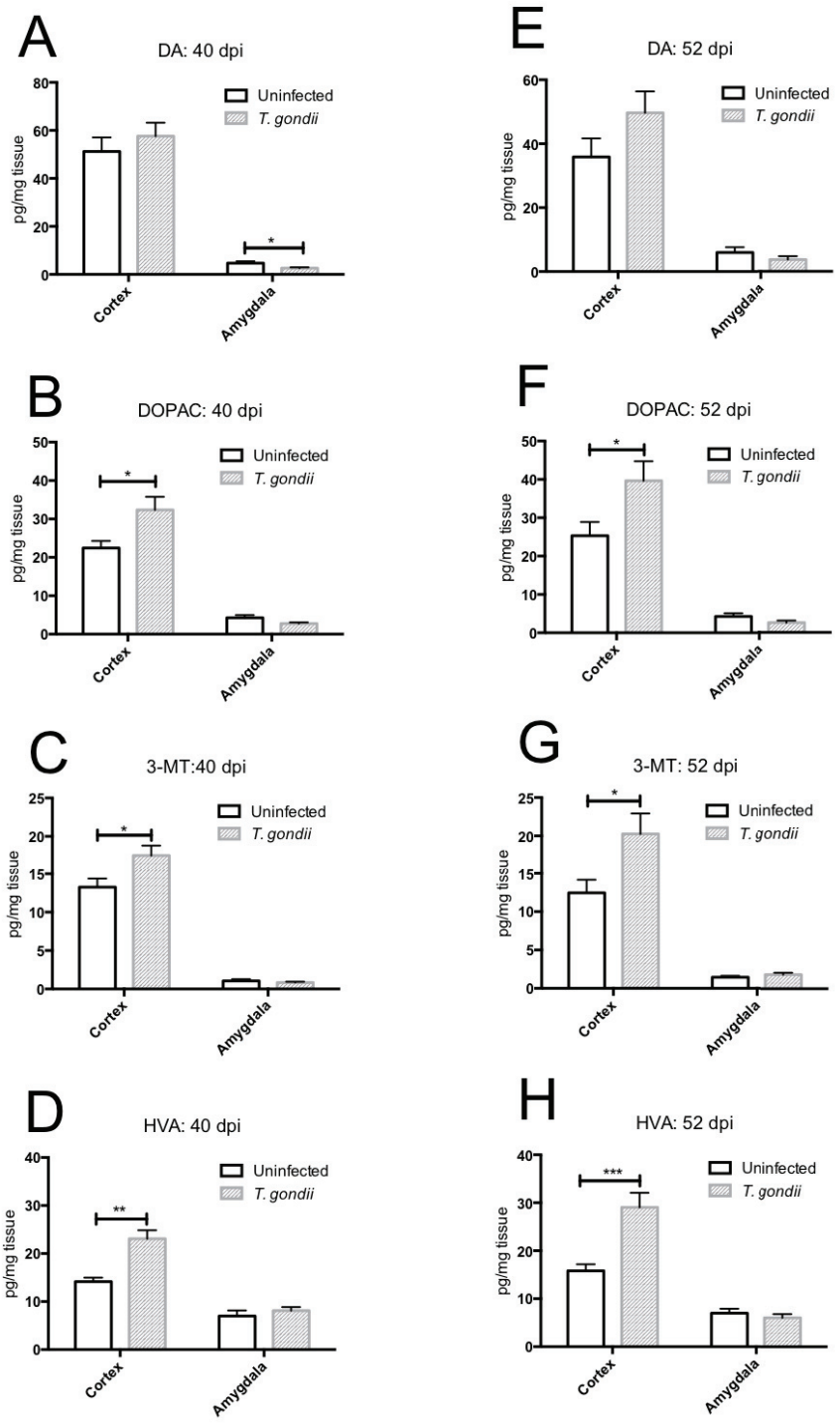

Figure 10 Levels of dopamine and its metabolites in the cortex and amygdala of uninfected and *T. gondii*-infected mice.

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 (Dpi). Data represent mean ± standard error of the mean. (**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 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

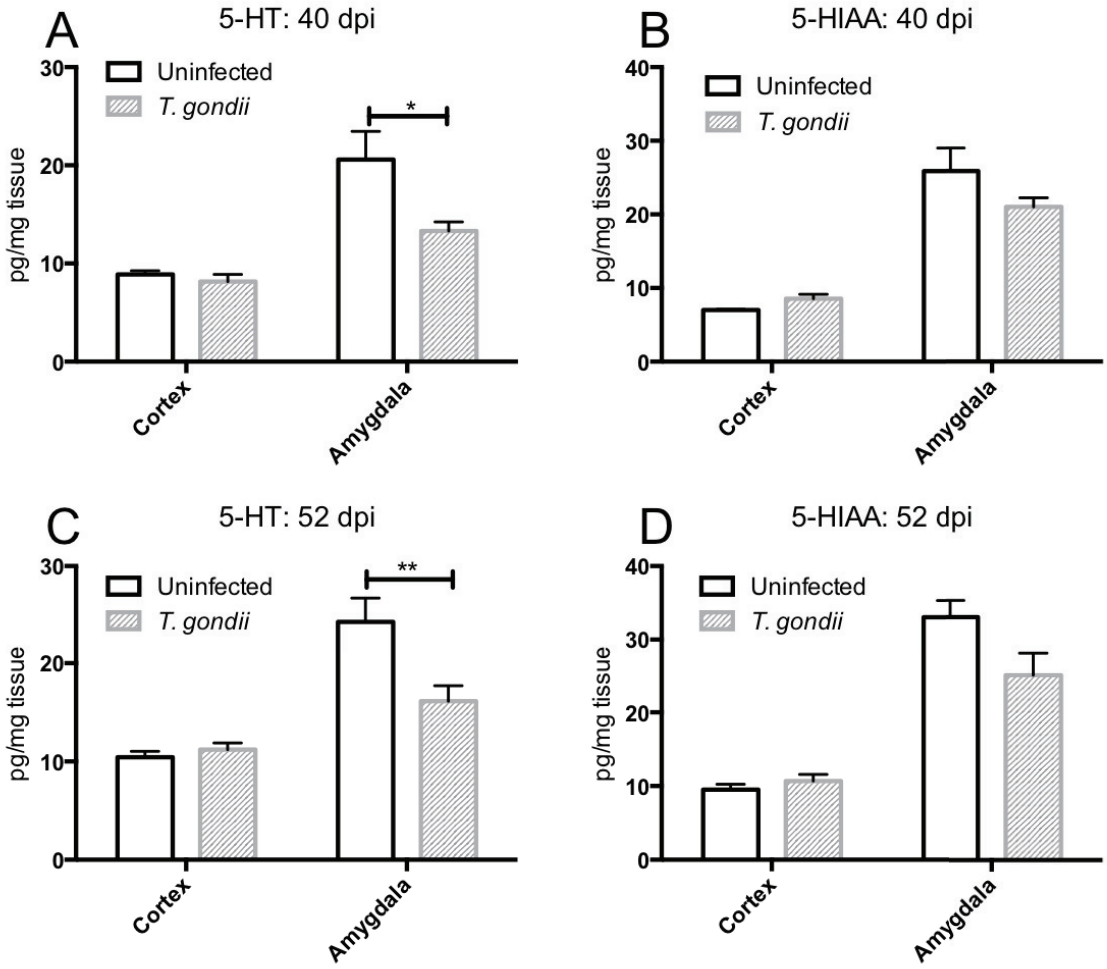

Figure 11 Levels of serotonin and its metabolite in the cortex and amygdala of uninfected and *T. gondii*-infected mice.

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 (dpi). Data represent mean ± standard error of the mean. (**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 ($^{*}p < 0.05$, $^{**}p < 0.01$).

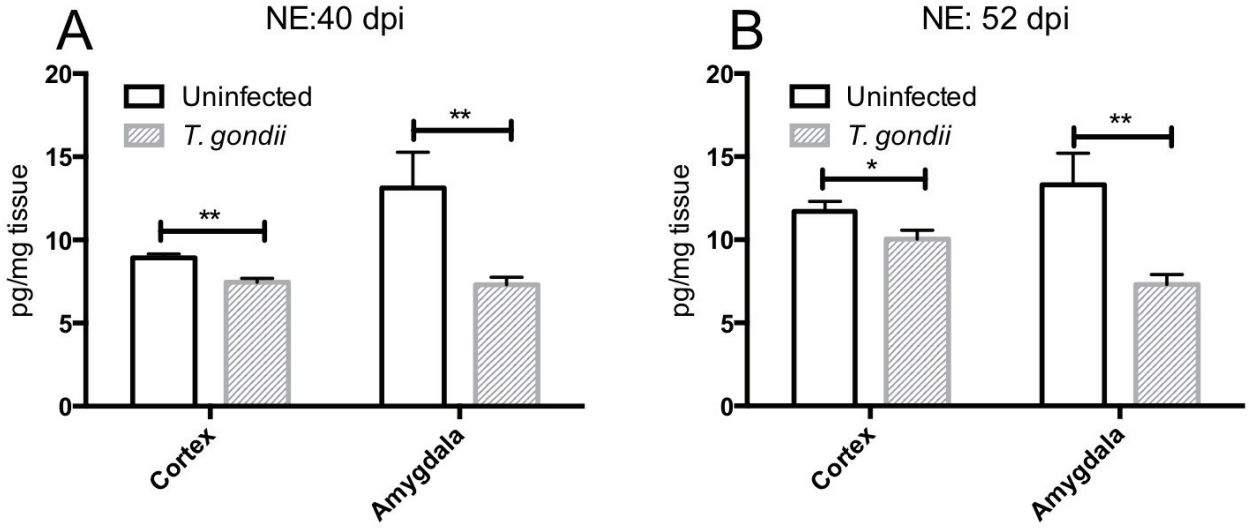

Figure 12 Norepinephrine levels in the cortex of uninfected and *T. gondii*-infected mice.

The ordinate shows levels of the neurotransmitter norepinephrine (NE) in the cortex and amygdala at 40 (**A**) and 52 (**B**) days post infection (dpi). Data represent mean ± standard error of the mean. (**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 ($*p < 0.05$, $**p < 0.01$).

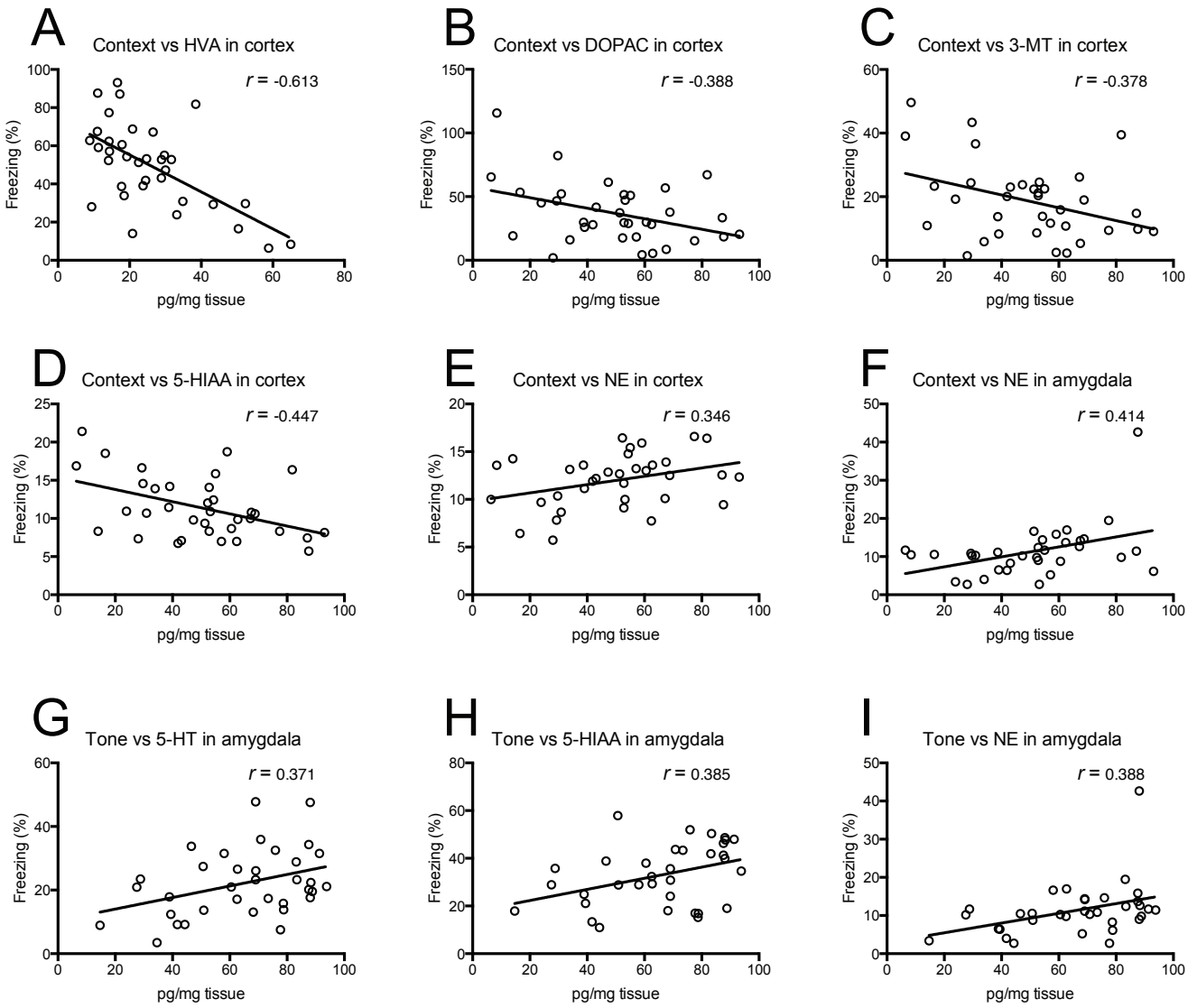

Figure 13 Correlation coefficients between neurotransmitter levels and the percentage of time spent freezing during the context test and tone test.
After the fear-conditioning test, some mice were used for the correlation analyses (Experiments 3 and 4, see Fig. 1). The ordinate shows the percentage of 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.

CHAPTER 2

Changes in neurotransmitter levels and expression of immediate early genes in brain of mice infected with *Neospora caninum*

1. INTRODUCTION

Neospora caninum causes neurological disorders, including wryneck and hind limb paralysis, in the infected animals (21, 29, 94), but the onset mechanism of the neurological symptoms of neosporosis is almost unknown. To understand the neurological disorders of neosporosis, examining of the brain pathology in the asymptomatic stages would be benefit.

In this study, I examined sickness behaviour, parasite distribution, histopathological lesions, levels of neurotransmitters (monoamines and amino acids), and mRNA expressions of immediately early genes (IEGs), using asymptomatic C57BL/6 mice during the subacute infection with *N. caninum*. Our findings would provide an insight into neosporosis associated with the brain dysfunction via inflammation, dysregulation of neurotransmitters, and downregulation of IEGs.

2. MATERIALS AND METHODS

2. 1. Ethics Statement

This study was performed in a strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of Ministry of Educations, Culture Sports,

Science and Technology, Japan. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit numbers 25–59, 25–60, and 25–62). Mice were decapitated without anesthesia for brain sampling, and all efforts were made to minimize animal suffering.

2. 2. Mice

Male C57BL/6 mice (8 weeks old) were purchased from CLEA Japan. Mice were housed (four to six mice/cage) in 12-h light (8:00-20:00) in the animal faculty of National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All mice were cared in accordance with the guidelines approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animal experiments began after 1 week of habituation.

2. 3. Parasite culture

Neospora caninum (strain Nc-1) were maintained in Vero cells with Eagle's minimum essential medium (Sigma) containing 8% fetal bovine serum. Infected cells were syringe-lysed using a 27-gauge needle (Thermo) to release the tachyzoites into RPMI-1640 medium (Sigma), and then filtered using a 5.0-μm pore size filter (Millipore).

2. 4. Parasite infection of mice

As same as described in chapter 1, *N. caninum* were intraperitoneally inoculated (1×10^5 tachyzoites) into mice (9 weeks old). To check clinical condition, body weight measurements were daily taken for 30 days after the infection.

2. 5. Open field test

To investigate a locomotor-activity behavior of *N. caninum*-infected mice, mice were tested only one time. Exploration in open field, which is a circular area with a diameter of 50 cm (Muromachikikai), was recorded for 5 min, using a video tracking system (Comp Act VAS ver. 3.0x, Muromachikikai). Total travelled distance, average speed, and rearing counts were measured. Behavioural experiments were performed at 30 days post infection (dpi), and commenced at 7:00 am, under 300 lux light intensity.

2. 6. Brain sampling

For histopathological analyses, five infected mice were sacrificed at 45 dpi, and then their brains were rapidly removed. The brains were perfused with 4% paraformaldehyde solution (Wako, Osaka, Japan). For quantitative polymerase chain reaction (PCR) assays of the parasite, two uninfected mice and 12 infected mice were sacrificed at 54 dpi. One hemisphere from each mouse brain was separated into eight different areas: cortex, hippocampus, caudoputamen, amygdala, thalamus, hypothalamus, midbrain, and cerebellum. The samples were stored at −20°C to use for DNA extraction and the subsequently quantitative PCR assays. For quantitative real-time PCR and high-performance liquid chromatography (HPLC) analyses, six uninfected mice and six infected mice were sacrificed at 40 dpi. The half brains were divided into five regions: cortex, hippocampus, caudoputamen, amygdala, and thalamus, and then frozen at −80°C until use. The samples from the right and left brains were used for the real-time PCR and HPLC analyses, respectively. To measure glycine (Gly) levels in the hindbrain regions, eight uninfected mice and 12 infected mice were sacrificed at 54 dpi. The brain samples were divided into three hindbrain regions, cerebellum, midbrain, and medulla

oblongata plus pons, and then frozen at −80°C until HPLC analysis.

2. 7. Histopathological analyses

After fixation with 4% paraformaldehyde solution, brain samples were coronally cut, embedded in paraffin wax, sectioned at 4 μm, and then stained with hematoxylin and eosin (82). The severities of pathological lesions were scored by the following scheme: 0, no lesion; 1, slight lesion; 2, mild lesion; 3, moderate lesion; and 4, severe lesion. An example of the scoring is shown in Fig. 15A. Total scores (from 0 to 4) for four types of lesions (meningitis, perivascular cuffs, inflammatory cells including glial cells, macrophage, and lymphocyte infiltration, and necrosis) were determined in each region by the severe degree. Pathological scores were calculated by adding each score for different lesions, with total scores ranging from 0 to 16 (82).

2. 8. Real-time quantitative PCR assay for the detection of *N. caninum* DNA

DNA was extracted with a standard standard phenol-chloroform-isoamyl alcohol (PCI) method from each brain region, and the parasite counts were analysed by a real-time PCR assay targeting the parasite *Nc5* gene, as described previously (1). The primers are complementary to the *Nc5* gene of *N. caninum*: the forward primer spanning nucleotides 248–257 (5'-ACT GGA GGC ACG CTG AAC AC-3') and the reverse primer spanning nucleotides 303–323 (5'-AAC AAT GCT TCG CAA GAG GAA-3'). The PCR was performed using an ABI prism 7900HT sequence detection system (Applied Biosystems), and the amplification was monitored using the SYBR Green method (Applied Biosystems). A standard curve was constructed with tenfold serial dilutions of *N. caninum* DNA that had been extracted from 1×10^5 parasites. The curve ranged from 10,000 to 0.01 parasites. Parasite number was calculated by plotting cycle

threshold (Ct) values on the standard curve.

2.9. RNA extraction, reverse transcription, and real-time PCR assays

Total RNA was extracted from mouse brain using TRI reagent (Sigma). First-strand cDNA was synthesised from 0.4 μg of the total RNA by a SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was then performed, according to Applied Biosystems (Applied Biosystems), and the amplification was monitored using the SYBR Green (Applied Biosystems). The IEG primers have been described previously (89): Activity-dependent cytoskeletal protein (*Arc*), 5′-GGA GGG AGG TCT ACC GTC-3′ and 5′-CCC CCA CAC CTA CAG AGA CA-3′; FBJ osteosarcoma oncogene (*c-fos*), 5′- CGA AGG GAA CGG AAT AAG-3′ and 5′-CTC TGG GAA GCC AAG GTC-3′; interferon-gamma (*Ifng)*, 5′-GAG GAA CTG GCA AAA GGA TG-3′ and 5′-TGA GCT CAT TGA ATG CTT GG-3′; tumour necrosis factor alpha (*Tnfa)*, 5′-GGC AGG TCT ACT TTG GAG TCA TTG C -3′ and 5′-ACA TTC GAG GCT CCA GTG AA -3′. A housekeeping gene, *Gapdh* mRNA, was also amplified in parallel (5′-TGT GTC CGT GGA TCT GA-3′ and 5′-CCT GCT TCA CCA CCT TCT TGA T-3′) and used as an internal standard. The optimal reference gene was selected, based on the Cotton EST database (http://www.leonxie.com). Expression levels of each gene relative to the *Gapdh* were calculated using $2^{\Delta Ct}$ method (User Bulletin no. 2; Perkin-Elmer). Fold expression levels among brain regions were calculated relative to expression levels of the corresponding genes in the hippocampus of uninfected mice.

2. 10. High-performance liquid chromatography (HPLC)

Each brain sample was homogenised using a bio-masher (Funakoshi), and then 300 μL/10 mg tissue of 0.2 M perchloric acid (containing 100 μM EDTA-2Na) was added. Both isoproterenol HCL (monoamine internal standard) and homoserine (amino acid internal standard) (both Sigma) were added in homogenates according to manufacturer (Eicom). The 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 then filtered by Ultra free MC (Millipore). Final products were injected into the HPLC system HTEC-500 (electrochemical detector; EICOM), equipped with a SC-5ODS column for monoamines or a SA-5ODS column for amino acids. Chromatographs were analysed using a PowerChrom software version 2.5 (eDAQ Pty Ltd.).

2. 11 Statistical analyses

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software). Statistical differences between two groups were analysed using two-tailed unpaired *t* tests (104). With three groups or more, statistical differences were determined using one-way ANOVA followed by Tukey's multiple comparison test (104). The *P* values < 0.05 represent statistical differences.

3. RESULTS

3. 1. Effect of *N. caninum* infection on behaviors of mice

In a experimental model using C57BL/6 male mice for *N. caninum* infection, any typical acute stage symptoms were not observed in the infected animals, during the

experimental period. However, the infected mice showed differences in the locomotor activities on an open field test (Fig. 14). Total distance travelled, average speed, and rearing counts were significantly decreased in the infected mice, as compared with those of infected mice. These results suggest that *N. caninum* infection may affect the brain function of asymptomatic mice.

3. 2. Histopathological lesions and parasite distributions in distinct brain regions

I analysed eight regions of the collected brains, histopathologically (Fig. 15). Histopathological lesions, including perivascular cuff, mononuclear cellular meningitis, glial cell activation, and focal necrosis, were observed in the brains of infected mice. Although there were no significant differences among the brain regions, the pathological score tended to be lower in the cerebellum, as compared with those of other regions (Fig. 15B). Next, parasite loads in distinct brain regions were examined by a quantitative PCR assay (Fig. 15C). The *N. caninum* was observed in all of the brain regions examined, but there were no significant differences among the brain regions. These results suggest that *N. caninum* infection may induce wide-ranging histopathological lesions in the brains of asymptomatic mice.

3. 3. Expression levels of *Ifng* and *Tnfa* in brains of *N. caninum*-infected mice

I examined the expression levels of *Ifng* and *Tnfa* in five brain regions, which are involved in neuroinflammation and central nervous system (CNS) disorders mediated via activated microglia (102, 103). Expressions of *Ifng* were significantly upregulated in the cortex, caudoputamen, hippocampus, and thalamus of *N. caninum*-infected mice, as compared with those of uninfected mice (Fig. 16A). Additionally, the expression levels of *Tnfa* were significantly upregulated in the cortex, caudoputamen, hippocampus,

thalamus, and amygdala of the infected mice, as compared with those of uninfected mice (Fig. 16B). However, there were no significant differences in the levels of *Ifng* and *Tnfa* expressions amongst brain regions. Although the expression level of *Tnfa* in hippocampus was down as compared with those of the other regions, the mean *Tnfa* expression level in the hippocampus of infected mice was over 30 fold as compared with that of uninfected animals. Increased expressions of *Ifng* and *Tnfa* might be associated with the histopathological lesions observed in the brains of infected mice.

3. 4. Neurotransmitter levels in distinct brain regions of *N. caninum*-infected mice

Next, I measured neurotransmitters in the brains. Levels of several neurotransmitters were altered in distinct brain regions of infected mice, as compared with those of uninfected mice (Fig. 17). Glutamate (Glu) levels were significantly higher in the cortex, caudoputamen, and hippocampus of *N. caninum*-infected mice than those of uninfected animals (Fig. 17A). Glycine (Gly) levels were also higher in the cortex, hippocampus, thalamus, and amygdala of infected mice than those of uninfected animals (Fig. 17B). Since Gly is known to be an inhibitory neurotransmitter in the hindbrain (8), the levels of Gly were also analysed in the pons and oblongata, midbrain, and cerebellum (Fig. 17C). As the results, Gly levels of the infected mice were increased in these regions, as compared with those of uninfected animals. These findings suggested that excess Gly neurotransmission occurred in the brain due to the *N. caninum* infection. In comparison to uninfected mice, higher levels of gamma-aminobutyric acid (GABA) in the amygdala and the lower levels in the cortex were observed in the infected mice (Fig. 17D). In the case of the monoamine neurotransmitter dopamine (DA), the levels were higher in the caudoputamen and amygdala than those of uninfected animals. However, lower levels of DA were seen in the hippocampus and thalamus of infected mice with *N. caninum*

(Fig. 17E). In addition, higher 5-hydroxytryptamine (5-HT) levels were observed in the cortex, caudoputamen, amygdala, and thalamus of infected mice (Fig. 17F). Together, *N. caninum* infection may trigger a difference in the neurotransmitter levels in several brain regions of mice.

3. 5. Expression levels of *c-Fos* and *Arc* in distinct brain regions of *N. caninum*-infected mice

Finally, I examined expression levels of *c-Fos* and *Arc,* because they have been used as biological markers for neuronal activations (47, 53, 84, 109). Expression of *c-Fos* decreased in all regions of *N. caninum*-infected mice (Fig. 18A), which *Arc* expression also decreased in the cortex, caudoputamen, hippocampus, and amygdala, but not in the thalamus of *N. caninum*-infected mice (Fig. 18B). These data indicated that *N. caninum* infection reduced expressions of IEGs in the brain.

4. DISCUSSION

Generally, *N. caninum* infection causes encephalomyelitis, polyradiculoneuritis, pelvic limb paralysis, rigid hyperextension, and muscle atrophy in dogs (21, 29, 94). *Neospora caninum* infection not only causes similar neurological signs in cattle, but is also associated with abortions in cows (21). Moreover, *N. caninum*-infected rodents develop various kinds of neurological signs, such as wryneck, circular movement, and paralytic gait (69–71). However, the mechanism of onset of neosporosis is largely unknown. Because neurological signs appear asymptomatic and gradually become symptomatic (21, 29, 94), it is important to study the brains of *N. caninum*-infected, asymptomatic animals.

In this study, *N. caninum*-infected mice showed a low locomotors activity behavior in a novel environment at 30 dpi. To our knowledge, our study is the first report for the behavioral change of mice infected with *N. caninum*. The behavioral change of rodents infected with *T. gondii* has been well studied in the past decade (120). The effects of *T. gondii* infection on rodent behavior vary with experimental designs. Time post infection may also affect the activity level of mice (120). For example, decreased locomotor activity of infected animals was found within 2 to 3 months post infection (119). In contrast, increased locomotor activity of infected animals was observed at a later clinical stage (3–7 months post infection) (51, 52, 64, 113). In addition, another study did not find any significant differences in the activities at 3 months post infection (56, 111). Consequently, hypoactivities of mice infected with *T. gondii* were observed from the acute to sub-acute stage. Hyperactivity of infected mice observed in my study was thought to be a result of histopathological damage associated with an immune response against *N. caninum* infection in the brains (56). In this study, I have carried out the open field tests at 4 weeks post infection, and showed a mild decrease of locomotor activity in the mice infected with *N. caninum*. Although *N. caninum*-infected mice showed no typical clinical symptoms or loss of body weight, they expressed high levels of inflammatory cytokines and histopathological damages in the brains. Thus, our results suggested that at 4 weeks post infection, *N. caninum*-infected mice had less activity levels due to the sustained inflammatory responses and histological damages of brains.

Several studies have indicated that the brain stem and cerebellum show a high sensitivity to *N. caninum* infection (2, 10, 39, 73). However, there was no association between the parasite density and the pathological lesion severity in those regions (82). In this study, both of the parasite loads and tissue damages did not show any tropisms. These results suggested that tissue damages associated with *N. caninum* infection had

no regional specificity in the brain during the chronically infections stage. Previously, I found that the frontal lobe and medulla oblongata were mainly affected in the symptomatic BALB/c mice infected with *N. caninum*, and some mice showed severe histopathological lesions in the cerebellum (82). Therefore, lesion formation in the medulla oblongata and the cerebellum might be characteristic of the neosporosis in mice. In this study, the lesions in the cerebellum were less, as compared with those in other regions, indicating the importance of histopathological lesions in the cerebellum for onset of neosporosis.

Our previous study showed that activation of microglia and increased expression level of inducible nitric oxide (NO) synthase were observed in the brains of mice infected with *N. caninum* (82). In this study, expressions of *Ifng* and *Tnfa* were highly upregulated in the brains of *N. caninum*-infected mice, suggesting a CNS disorder via inflammation by immune cells. Generally, *Ifng* controls *N. caninum* proliferation in the macrophage cultures (4, 81, 105). Treatment of microglia and astrocytes with *Ifng* and *Tnfa* showed an inhibition of the parasite growth (60). Moreover, the *Ifng* and *Tnfa* inhibited the *N. caninum* growth in a bovine cerebellar cell culture (125). These responses induce neuronal disorder and neuronal cell death, although they can eliminate the parasites in tissues (4, 60, 61, 125). The *Ifng* and *Tnfa* produced by immune cells disrupt mitochondrial adenosine triphosphate production and cause a Glu excitotoxicity (102, 103). Excess release of Glu induces a neuronal cell death via N-methyl d-aspartate receptor (102, 103). Together, my results demonstrated a chronic inflammation in the brain of asymptomatic C57BL/6 mice infected with *N. caninum*.

Pathogenic infection into the brain can alter the neurotransmission, and cause a dysfunction of this organ (30). Some studies have suggested that *T. gondii* can alter DA metabolism and host behavior (40, 93). Stibbs, H. H. showed that DA levels were 14 %

higher in mice chronically infected with *T. gondii* than controls (99). Gatokowska *et al*. suggested that increases levels of 5-HT and DA might be responsible for the low locomotor-activity behaviour of mice (42). These results have indicated differences in several neurotransmitter levels between uninfected and infected mice with *T. gondii*. In this study, I examined the changes in neurotransmitter levels in brain tissues following the *N. caninum* infection for the first time. My results showed that *N. caninum* infection induced marked changes of excitatory and inhibitory neurotransmitter levels in wide brain areas, suggesting that these responses might induce sickness behavior in the infected mice. The amount of Glu was increased in the cortex, caudoputamen, and hippocampus of mice infected with *N. caninum*. Level of GABA, a major inhibitory neurotransmitter, was decreased in the cortex of infected mice, suggesting that elevated levels of Glu might be due to a lack of GABA effect. Our previous study has showed an activation of microglia in the brain of mice infected with *N. caninum* (82). The activated microglia induces the excess Glu, and causes an excitotoxicity to neuronal cells via the N-Methyl-D-Aspartate receptor (102, 103). Because GABA levels showed no difference in the caudoputamen and hippocampus between the infected and uninfected mice, the elevated Glu might be a result of an immune reaction mediated via the activated microglia.

A release of *Ifng* leads to a tryptophan degradation via indoleamine-2,3-dioxygenase and elevated 5-HT (12). In contrast, our data showed an increased level of 5-HT in almost all regions examined, while the expression level of *Ifng* was highly up-regulated in those areas, suggesting a possibility that the tryptophan levels might also be increased in the brains of mice infected with *N. caninum*. It has been suggested that the increased levels of tryptophan were associated with the infection and accompanied by 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of 5-HT (30). In fact, *T.*

gondii-infected male mice showed an increased serotonergic activity at 3 weeks post infection (42). Interestingly, interleukin (*Il*) *-1* and lipopolysaccharide (LPS) administration also increase the concentrations of tryptophan and 5-HT throughout the brain (30). In addition, LPS administration increased levels of DA and 5-HT (117). Therefore, *N. caninum* infection was considered to induce a marked change of monoamine levels in wide brain areas via the long-lasting immune reactions.

Since noradrenergic and serotonergic systems function to inhibit active behavior (30), elevated brain serotonergic neurons by *N. caninum* infection may contribute to the behavioral changes. Furthermore, DA levels in the caudoputamen were increased in the mice infected with *N. caninum*, while that in the thalamus was decreased. Both the caudoputamen and thalamus are part of the basal ganglia circuit that control voluntary motor movements (77). Thus, dysregulation of dopaminergic neurons in these regions may contribute to the behavioral changes associated with *N. caninum* infection.

Although some differences were observed in changes of neurotransmitter levels among brain regions, further researches are required to clarify the relationships among the neurotransmitter level, parasite load, and severity of brain lesions. In this study, there were no data concerning the correlation between severity behavioral deficits and the amount of neurotransmitter change, because mice tested in the open field were different from mice prepared for HPLC and qPCR analyses. However, I analyzed the correlation between the behavioral changes and parasite load (Table 2). I found that the number of parasites in the thalamus had a negative correlation with the total distance travelled and average speed in the open field test. In addition, the number of parasites in the caudoputamen showed a negative correlation with average speed in the open field test. The thalamus and caudoputamen have a connection to the motor cortex (77). Thus, the parasites in these regions might contribute to the behavioral changes in *N.*

caninum-infected mice. Furthermore, the number of parasites in the cerebellum had a negative correlation with rearing counts in the open field test. Normal cerebellum processing is necessary for motor control (77). Therefore, our results suggested that the parasite load in the cerebellum might affect the total number of rearing counts.

Hind limb paralysis and rigid hyperextension are characteristic symptoms of *N. caninum* infection in dogs (21, 29, 94). The *N. caninum*-infected mice also show a similar type of neurological symptom (69–71). However, the onset mechanism of neurological symptom was unknown. In this study, Gly levels were altered by *N. caninum* infection in several brain regions. In addition, levels of Gly were increased in the hindbrain, including pons and oblongata, midbrain, and cerebellum of the infected mice. Gly is a major inhibitory neurotransmitter in the hindbrain and the spinal cord (8). Increased level of Gly in cerebrospinal fluid caused severe hyperkinesia in humans (59). Thus, these results implicate that increased level of Gly in the progressive stage of neosporosis might cause hind limb paralysis.

The expression of IEGs is induced by neuronal activity (53, 83, 109). The *c-Fos* is a regulatory transcription factor and known as a marker of neuronal activity. *Arc* is an effector protein, a key protein implicated in synaptic plasticity and synapse strength and hence in learning and memory (48). In this study, expression levels of *c-Fos* and *Arc* were decreased in *N. caninum*-infected mice, indicating that *N. caninum* infection down-regulated the gene expression associated with neuronal cell function. Thus, *N. caninum* infection may impair the learning and memory capacity of the host. Because I confirmed the up-regulation of *Ifng* and *Tnfa* expressions and alteration of neurotransmitter levels in the brains of *N. caninum*-infected mice, down-regulation of *c-Fos* and *Arc* expressions in the brain may be triggered by neurological pathology following the *N. caninum* infection.

In conclusion, *N. caninum* infection affected the brain function of asymptomatic mice. In the infected mice, histopathological lesions were observed everywhere, except for the cerebellum, while previous studies showed that hindbrain areas, including the cerebellum, were preferably damaged in the symptomatic animals (2, 10, 39, 73, 82). These data supported an idea that lesion formation in hindbrain area was associated with neosporosis (82). This is the first report to alter neurotransmitter levels in *N. caninum*-infected animals. However, the relationships between altered neurotransmitter levels and neurological disorder remain unclear. Overall, my results suggested that chronic neuroinflammation following *N. caninum* infection might cause neurological dysfunction and neuronal cell death, resulting in decrease of locomotor activity. Further investigation is needed to understand the critical neuronal dysfunction related to onset of neurological symptoms.

Table 2 Pearson's correlation coefficients between the behavior in the open field test and parasite load in different brain regions.

	Total distace travelled (r)	Average speed (r)	Rearing (r)
Cortex	-0.299	-0.303	-0.337
Caudoputamen	-0.283	-0.604 *	-0.518
Hippocampus	-0.229	-0.384	-0.001
Thalamus	-0.579 *	-0.658 *	-0.360
Amygdala	0.008	-0.293	-0.491
Hypothalamus	-0.167	-0.432	-0.277
Midbrain	-0.129	-0.424	-0.565
Cerebellum	-0.092	-0.381	-0.629 *

The correlation coefficients between the behavior in the open field test and the parasite load in different brain regions were calculated using the Pearson correlation coefficient, two-tailed t tests (*$p < 0.05$). After the open field test, some mice were used for the correlation analyses. The strength of the linear association between the 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. *N. caninum*-infected mice, n = 12.

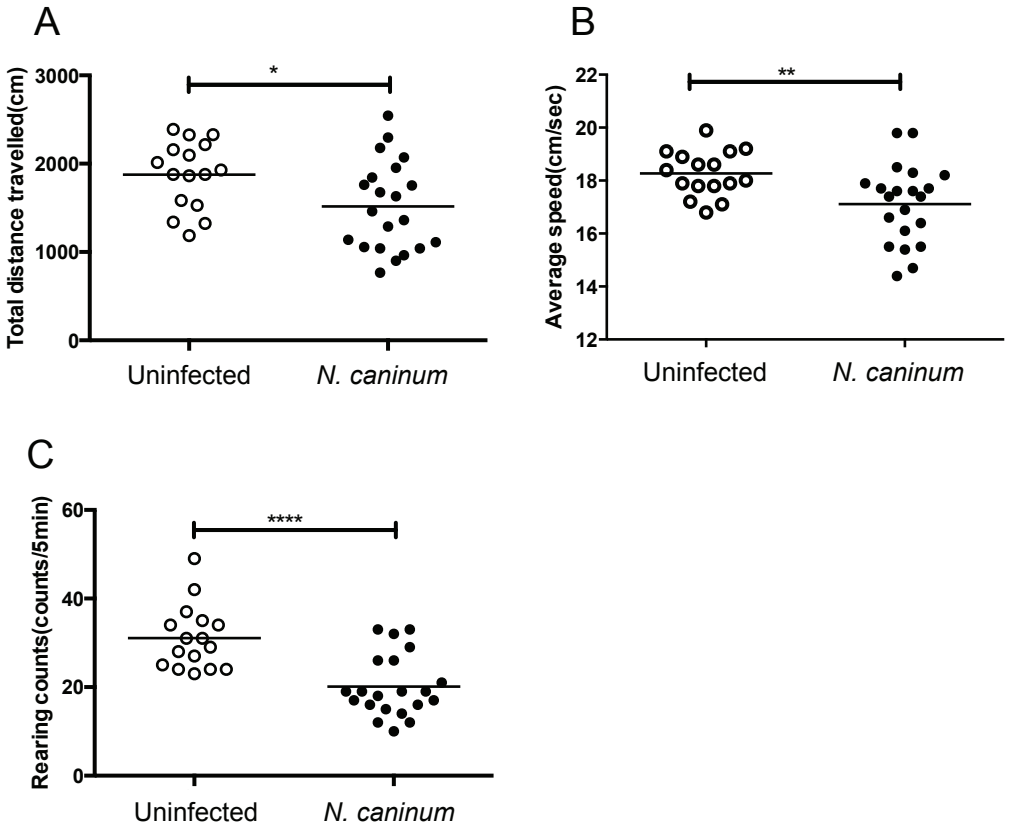

Figure 14 Effects of *N. caninum* infection on behaviors of mice.
Changes in the locomotor activities and exploration parameters at 30 days post infection: (**A**) total distance travelled (cm), (**B**) average speed (cm/sec), and (**C**) rearing counts (counts/5min). Data were summarised from two independent experiments. To reserve the number of mice required in *N. caninum*-infected group, each experiment used uninfected, n = 8; *N. caninum*-infected mice, n = 12. Because one mouse died due to rare severe neurological injury at 28 days post infection, the total numbers of uninfected mice were n = 16; *N. caninum*-infected mice, n = 23. Significant differences were determined by unpaired t tests (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

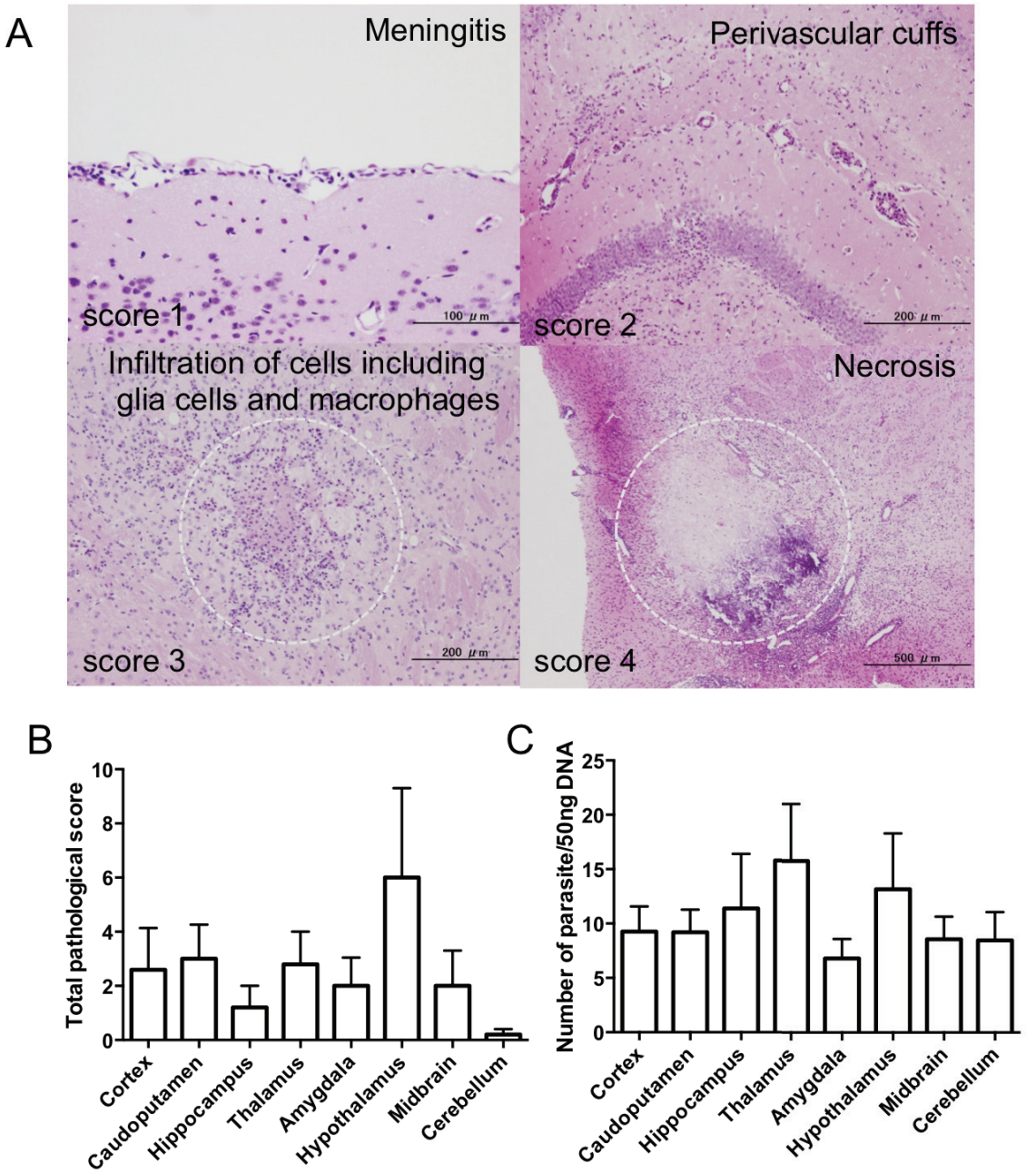

Figure 15 Histopathological lesions and parasite loads in different brain regions of *N. caninum*-infected mice.

(A) Representative examples of histopathological lesions from brains of *N. caninum*-infected mice. score 1: slight meningitis, score 2: mild perivascular cuffs, score 3: moderate inflammatory cell infiltration, score 4: extensive necrosis with rarefaction. **(B)** Total pathological score for each brain region. Brain samples were collected at 45 days post infection. Histopathological lesions were scored as above. Minimum value = 0 and maximum value = 16. *N. caninum*-infected mice, $n = 5$ from one experiment. (**C**) Parasite number per 50 ng tissue DNA. Brain samples were collected at 54 days post infection. *N. caninum*-infected mice, $n = 12$ from one experiment. Significant differences were analysed by one-way ANOVA with Tukey's post hoc test, but there were no significant differences.

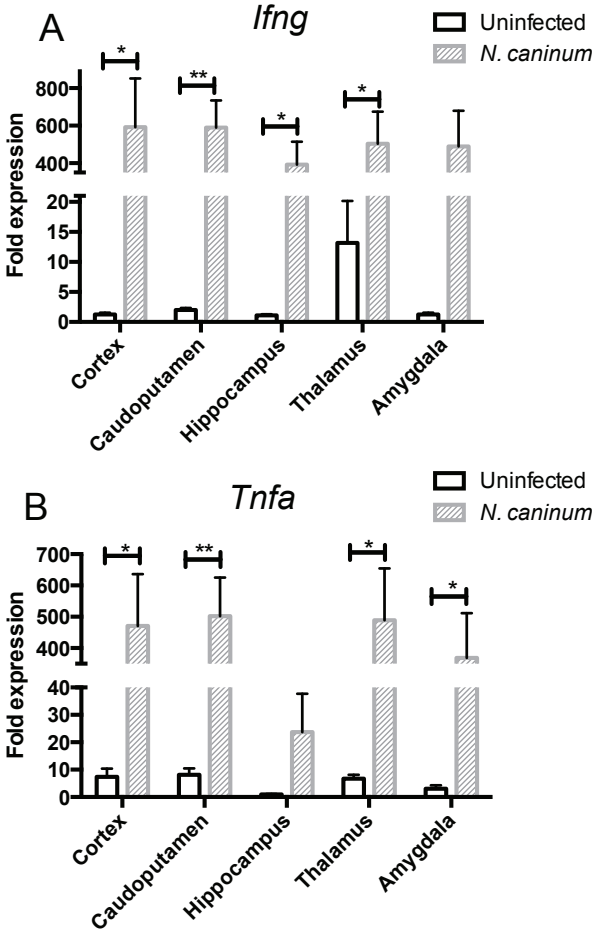

Figure 16 Expression levels of *Ifng* and *Tnfa* in the brain of *N. caninum*-infected mice.

Brain samples of uninfected and *N. caninum*-infected mice were collected at 40 days post infection. Fold expression was determined by real-time PCR assays, and calculated as the relative to expression levels of the *Ifng* and *Tnfa* in the hippocampus of uninfected mice. Significant differences in each brain region group were determined between uninfected and *N. caninum*-infected by unpaired t tests (*$p < 0.05$, **$p < 0.01$). Significant differences among brain regions were analysed by one-way ANOVA with Tukey's post hoc test, but there were no significant differences. Data represent mean ± standard error of the mean. Control, $n = 6$; *N. caninum*-infected mice, $n = 6$. Data are representative of two independent experiments.

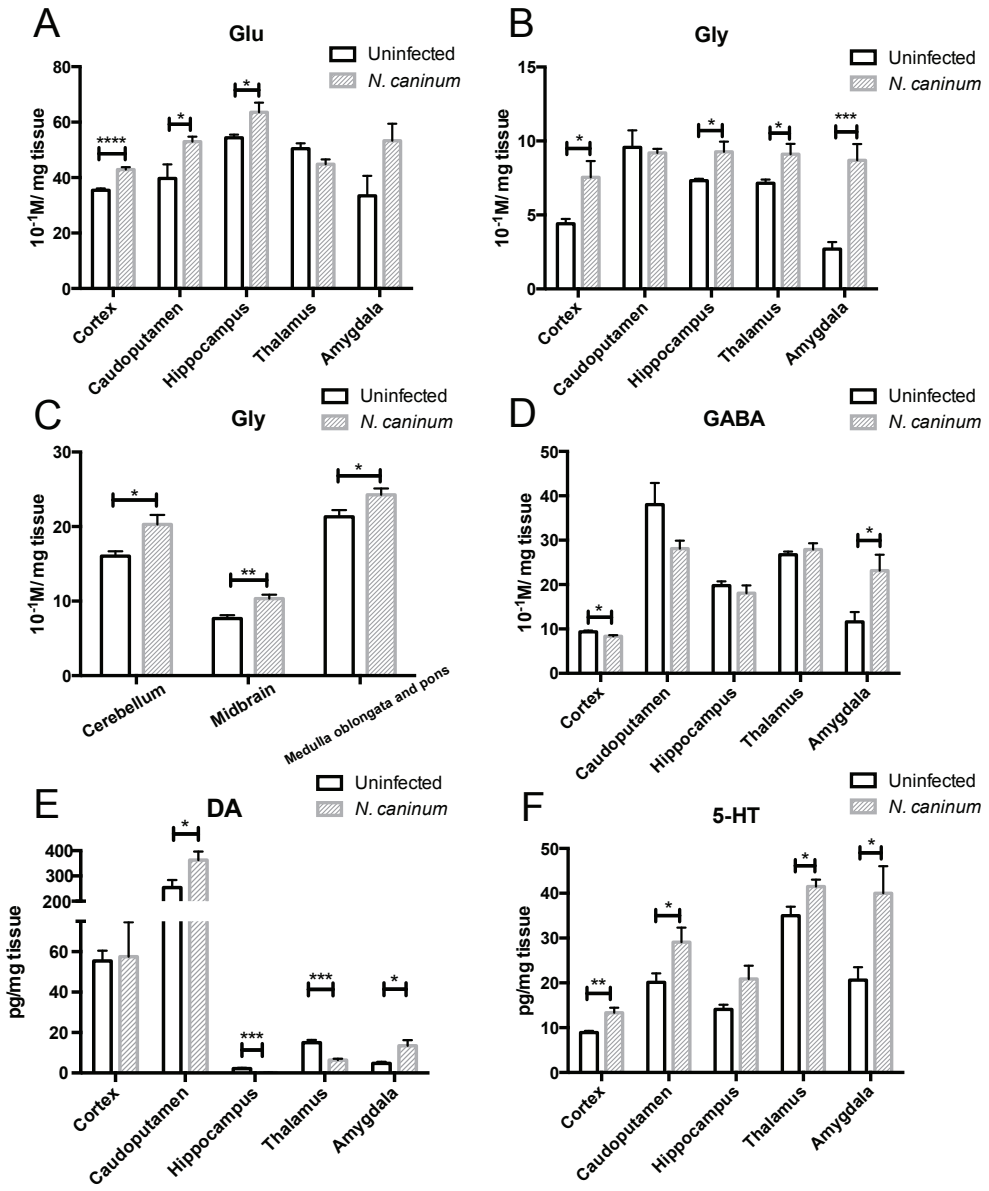

Figure 17 HPLC analyses of amino acids and monoamines.
(**A**, **B**, **D**, **E,** and **F**) Brain samples were collected at 40 days post infection and analysed by HPLC for glutamate (Glu), glycine (Gly), gamma-aminobutyric acid (GABA), dopamine (DA), 5-hydroxytryptamine (5-HT). Data represent mean ± standard error of the mean. Control, $n = 6$; *N. caninum*-infected mice, $n = 6$. Data are representative of two independent experiments. (**C**) Brain samples were collected at 52 days post infection and analysed by HPLC for Gly in hindbrain areas. Data represent mean ± standard error of the mean. Control, $n = 8$; *N. caninum*-infected mice, $n = 12$. Significant differences in each brain region were determined between uninfected and *N. caninum*-infected groups, using unpaired *t* tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

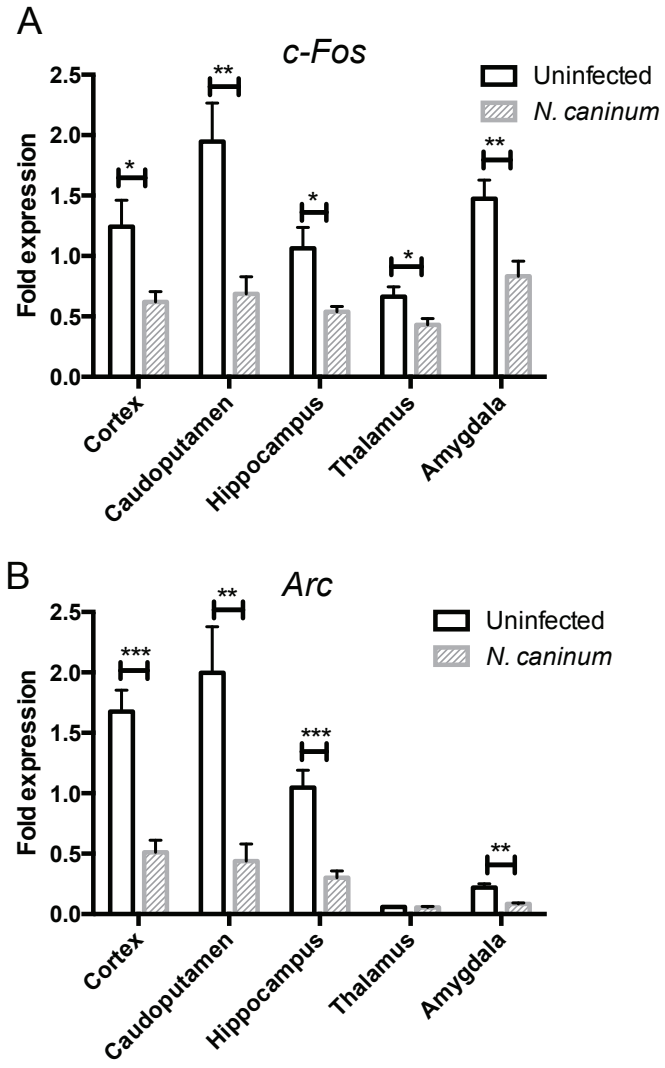

Figure 18 Expression levels of *c-Fos* and *Arc* in the brains of *N. caninum*-infected mice.

Brain samples of uninfected and *N. caninum*-infected mice were collected at 40 days post infection. Fold expression was determined by real-time PCR analyses, and calculated as the relative to expression levels of *c-Fos* and *Arc* in the hippocampus of uninfected mice. Significant differences were determined in each brain region between uninfected and *N. caninum*-infected groups by unpaired t tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Data represent mean ± standard error of the mean. Control, $n = 6$; *N. caninum*-infected mice, $n = 6$. Data are representative of two independent experiments.

GENERAL CONCLUSIONS

Two protozoan parasites, *T. gondii* and *N. caninum,* show a high similarity in their morphological characterization and genetic background. Furthermore, both the parasites form tissue cysts in the central nervous system (CNS) of hosts. However, infections with these parasites cause different neurological disorders with different neurological symptoms in the hosts. In my thesis, I focused on the behavioral and brain pathological changes in the mice infected with *T. gondii* and *N. caninum*. I summarized the main findings of this study.

In chapter 1, *T. gondii* infection impaired consolidation of fear memory in mice. While *T. gondii* did not show obvious tropism in the brain, the cortex was preferably damaged by the *T. gondii* infection. In addition, the neurotransmission related to fear response was impaired in the cortex and amygdala of mice infected with *T. gondii*. Overall, these results indicated that impaired fear memory consolidation in the infected mice with *T. gondii* was mediated by the neuronal dysfunction of the cortex and amygdala. This study revealed a novel neuronal dysfunction associated with the behavioral change of host. Interestingly, the neuronal dysfunctions were similar to the brain pathology of the human mental diseases, such as schizophrenia and the depression. Thus, this study provided insights not only into the mechanism of behavioral manipulation of host by the parasite infection, but also into the relationship between the *T. gondii* infection and mental diseases of human.

In chapter 2, I demonstrated that *N. caninum*-infected mice showed a mild decrease of locomotor activity. The *N. caninum* did not show obvious tropism in the brain. However, it expanded all over the brain. Histopathological lesions were observed everywhere, except for the cerebellum of mice infected with *N. caninum*.

Neuroinflammation following the *N. caninum* infection impaired the balance of neurotransmitters, and decreased a neuronal activity in the brain. These results suggested that *N. caninum* infection caused a neuronal dysfunction with neuronal cell death via chronic neuroinflammation. In my knowledge, this is the first report for neuronal dysfunction following asymptomatic *N. caninum* infection. Thus, this study provided the fundamental observations for evaluating the onset mechanism of neurological disorders associated with neosporosis.

Finally, I considered about specificities of behavioral changes of mice infected with *T. gondii* and *N. caninum*. I suggested that a decrease of locomotor activity was caused by non-region specific tissue damage and immune response in mice infected with *N. caninum*. In addition, I demonstrated that *T. gondii*-infected mice also showed a decrease of locomotor activity (data not shown). Thus, behavioral changes, observed in *N. caninum*-infected mice, seemed to be non-specific changes. As far as I know, there were no reports that demonstrated impaired consolidation of fear memory following infection, while my data displayed that *N. caninum* infection did not affect fear memory (data not shown). This suggested that impaired fear memory consolidation in mice infected with *T. gondii* was a specific behavioral change. Moreover, these results indicated that behavioral changes following *T. gondii* infection were not caused (at least not directly) by non-specific neuroinflammation.

Several other pathogens have been reported that these pathogens were also related to behavioral changes of host, while the mechanisms remain unclear. Herpes viruses, such as cytomegalovirus and herpes simplex virus, prefer CNS of host and establish latent infection (43). If the host immune system was compromised, then the virus could reactivate and cause severe encephalitis (43). Encephalitis caused by reactivated virus sometimes impaired memory, and affected personality (15). The lesions in limbic

system might be related to these symptoms (15). Thus, there is a possibility that other pathogen infections, which form lesions in similar location in *T. gondii* infection, can cause similar behavioral changes observed in *T. gondii* infection.

In conclusion, I elucidated that the chronic infections with *T. gondii* and *N. caninum* caused neuronal dysfunctions related to behavioral changes. My studies provided fundamental insights regarding the parasite-induced brain pathology and neurological disorders.

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