Supplemental material

Supplemental method

RNA extraction, reverse transcription, and real-time PCR

The levels of mRNA expression in the mouse brain were measured at 52 dpi after the brains were divided into five regions: cortex, hippocampus, amygdala, caudoputamen, and thalamus (regions related to emotional behavior and memory). The collected tissues were stored immediately at –80 °C. Total RNA was extracted from the brain samples using commercially available TRI Reagent (Sigma). The first-strand cDNA was synthesized from 0.4 μg of total RNA using the SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen, Mount Waverley, Australia). RT–PCR was performed according to the protocol supplied by the manufacturer (Applied Biosystems), and amplification was monitored with SYBR Green (Applied Biosystems). The expression of the TgSAG1, TgBAG1, and TgGRA1 mRNAs were measured to investigate the parasitic stages present in the brain (1, 2). The following primers were used: TgSAG1, 5′-TGATGCAACCGACCACAAAC-3′, 5′-CAATCGAGAAGTTCCCCGTG-3′; TgBAG1, 5′-GGGAAATGGCTGTCGCAGTA-3′ and 5′-CTTGTCCACCGGGATGTACC-3′; TgGRA1, 5′-TACAGCGAAGTCGGCAATGTT-3′ and 5′-TCGCCTTTGTTCAACGCAC-3′; IFN-γ, 5′-GAGGAACTGGCAAAAGGATG-3′ and 5′-TGAGCTCATTGAATGCTTGG-3′; CD4, 5′-GGGTTCAGGACAGCGACTTC-3′ and 5’-TTTTCTGGTCCAGGGTCACG-3’; CD8, 5′-AGGATGCTCTTGGCTCTTCC-3′ and 5′-TCACAGGCGAAGTCCAATCC-3′; and CD11b, 5′-TTCAACAAACCACAGTCCCG-3′ and 5′-TGGCTTAGATGCGATGGTGTC-3′. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was amplified

in parallel (5′-TGTGTCCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGAT-3′) and used as the internal standard. The expression level of each gene relative to that of GAPDH was calculated using the $2^{\Delta Ct}$ method, according to the manufacturer's instructions (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PC, AB Applied Biosystems). Fold expression was calculated relative to the expression levels of the corresponding gene in the hippocampi of uninfected mice.

Reference list for Supplemental materials

1. **Wu B**, **Huang B**, **Chen Y**, **Li S**, **Yan J**, **Zheng H**, **Huang S**, **Shen J**, **Lun Z-R**, **Wang Y**, **Kasper LH**, **Lu F**. 2013. Upregulated expression of Tim-3 involved in the process of toxoplasmic encephalitis in mouse model. Parasitol Res **112**:2511–2521.

2. **Mahmoud ME**, **Ihara F**, **Fereig RM**, **Nishimura M**, **Nishikawa Y**. 2016. Induction of depression-related behaviors by reactivation of chronic *Toxoplasma gondii* infection in mice. Behav Brain Res **298**:125–133.

Supplementary Table 1 Pearson's correlation coefficients for 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. S1). 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 legends

Supplementary Figure 1 Flowchart explaining the number of uninfected and infected mice, brain sampling, and methods. The figure indicates the timing of each experiment (Exp) 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 acute infection with *T. gondii*. Dpi, days post infection.

Supplementary Figure 2 Schematic diagram of the fear-conditioning test experimental design. The figure illustrates test days 1, 2, 3, and 4 (tone: 75 dB, 300 Hz; foot shock: 0.5 mA, 2 s).

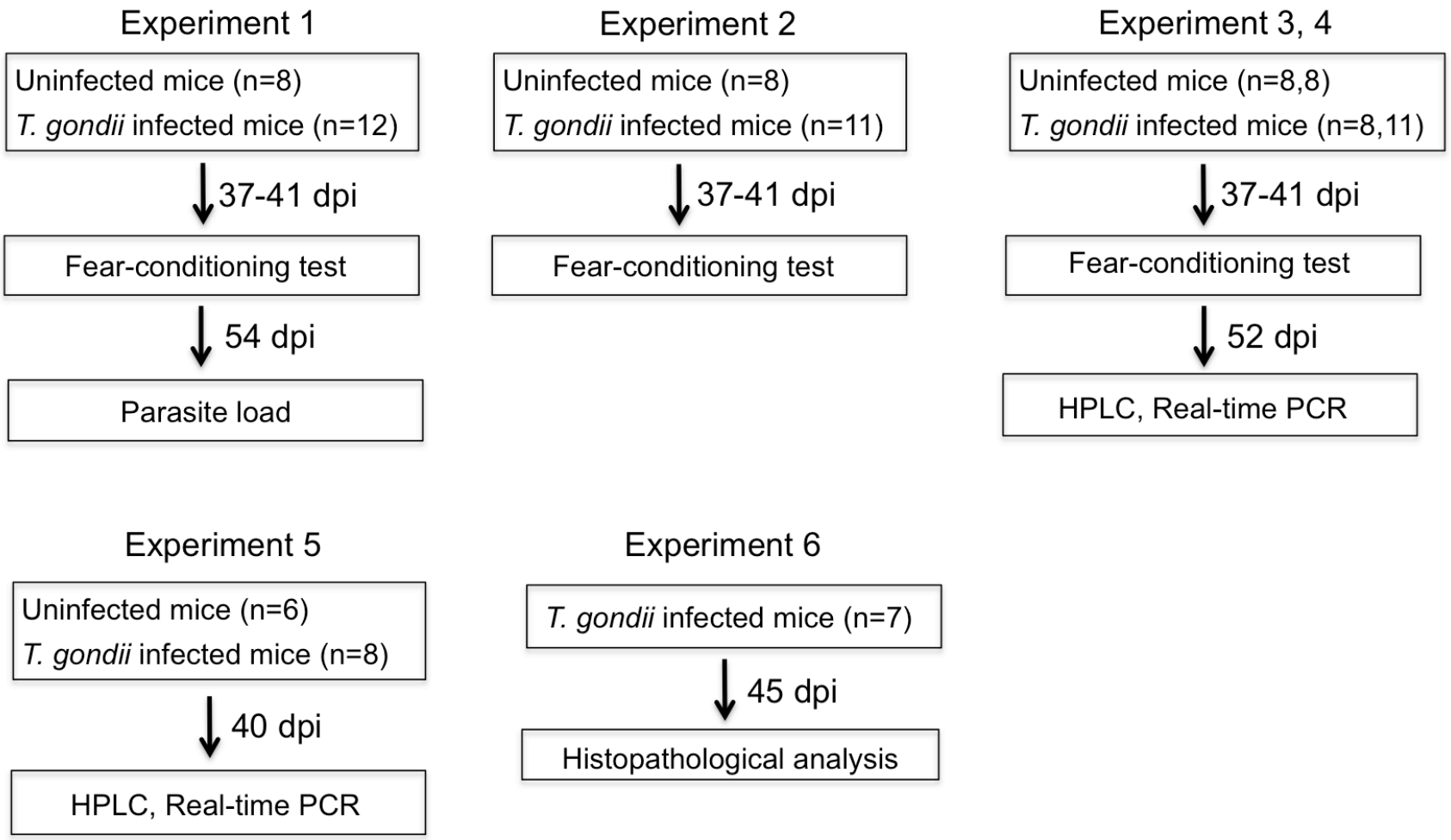
Supplementary Figure 3 Brain map of regions used in this study. (A) Sagittal section of a mouse brain. Each vertical line shows the location relative to bregma. (B) The coronal section between vertical lines 1 and 2 of panel A. The cortex and caudoputamen (CPu) were dissected from the location as shown. (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 from the pons and medulla oblongata and collected.

Supplementary Figure 4 Representative examples of histopathological lesions in brains of *T. gondii*-infected mice. The severity of the pathological lesion was 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.

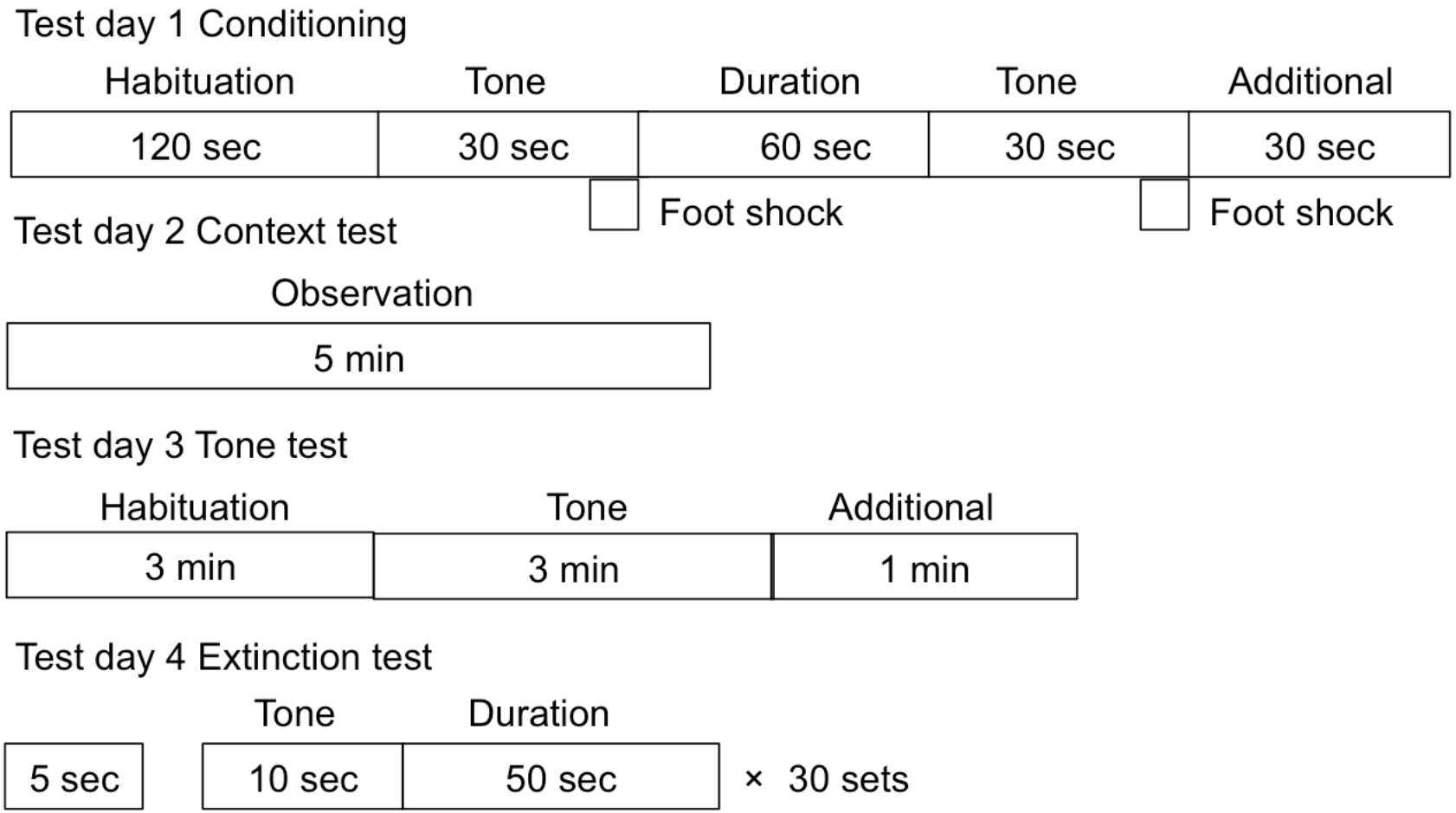
Supplemental Figure 5. *GRA1*, *BAG1*, and *SAG1* expression in the brains of *T. gondii*-infected mice. The ordinate shows the relative mean expression levels of the genes (*GRA1*, *BAG1*, and *SAG1*) in each brain region. Brain samples from uninfected and *T. gondii*-infected mice were collected at 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 ± SEM. Uninfected mice, $n = 3$; *T. gondii*-infected mice, $n = 19$. No statistically significant differences were found using one-way ANOVA with Tukey's post hoc test.

Supplemental Figure 6. CD4, CD8, CD11b, and IFN-γ expression 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

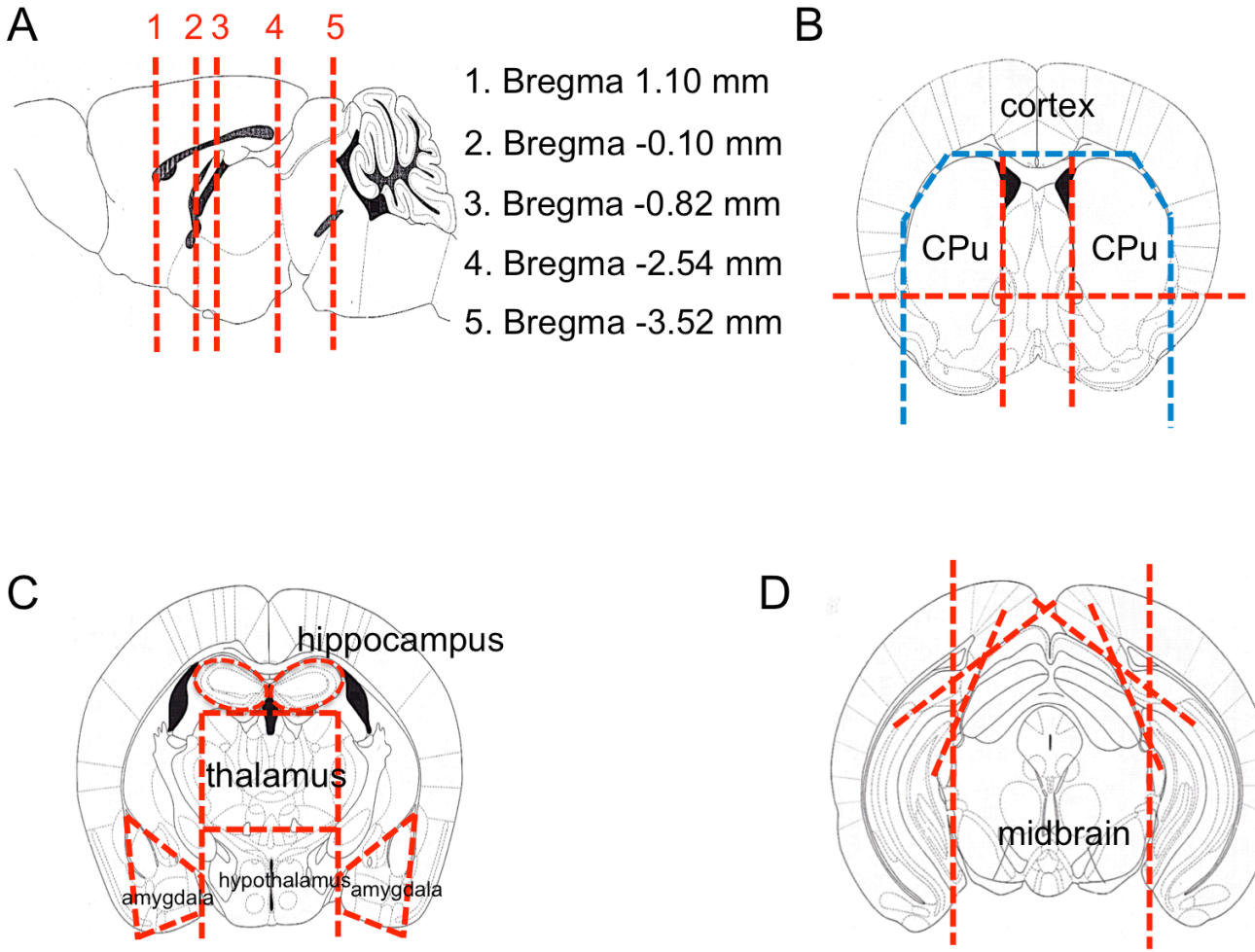
expression level of the corresponding gene in the hippocampi of uninfected mice. Data are means ± SEM. Uninfected mice, $n = 3$; *T. gondii*-infected mice, $n = 19$. Significant differences were determined using one-way ANOVA with Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).



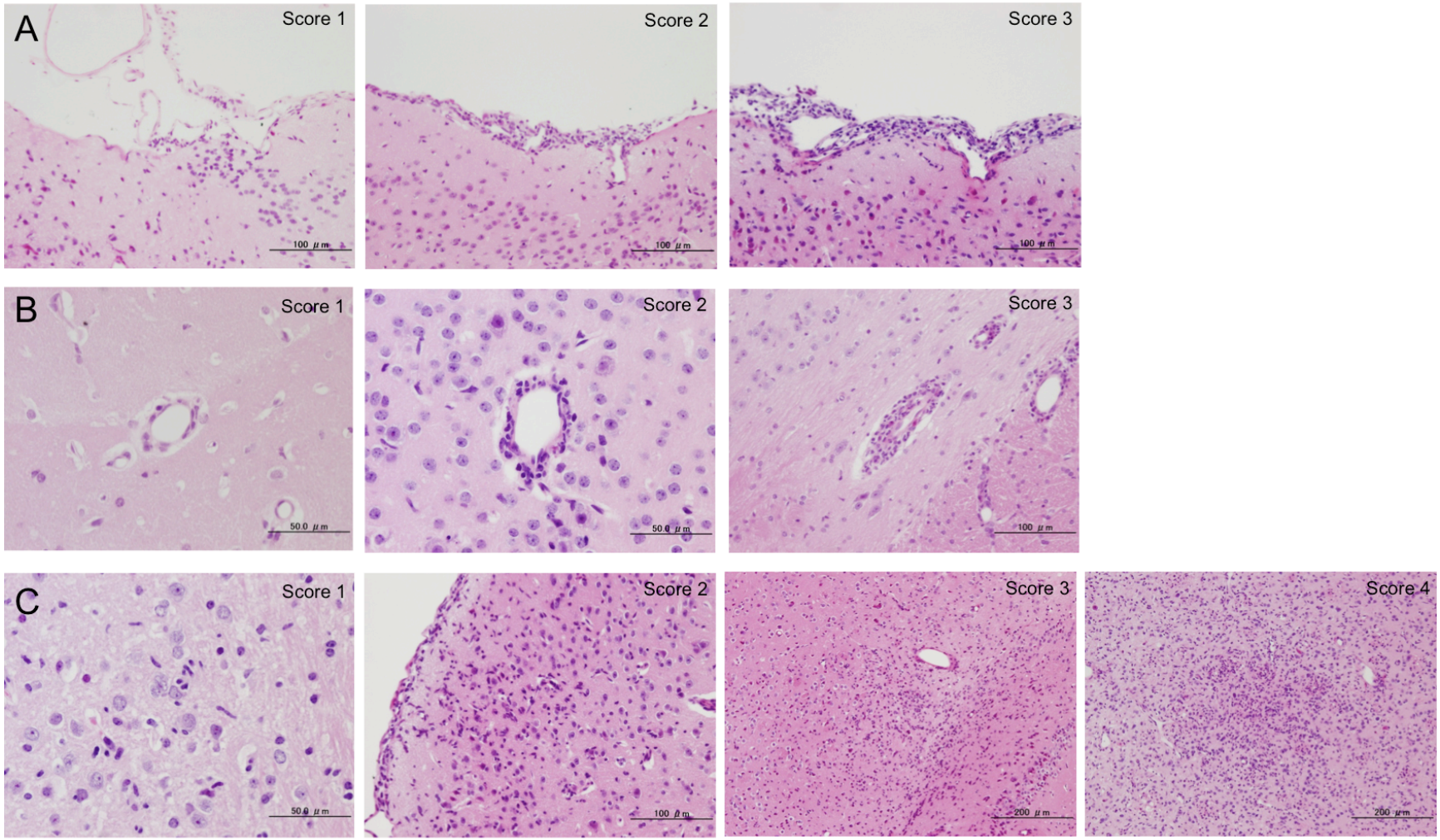
Supplementary Figure 1



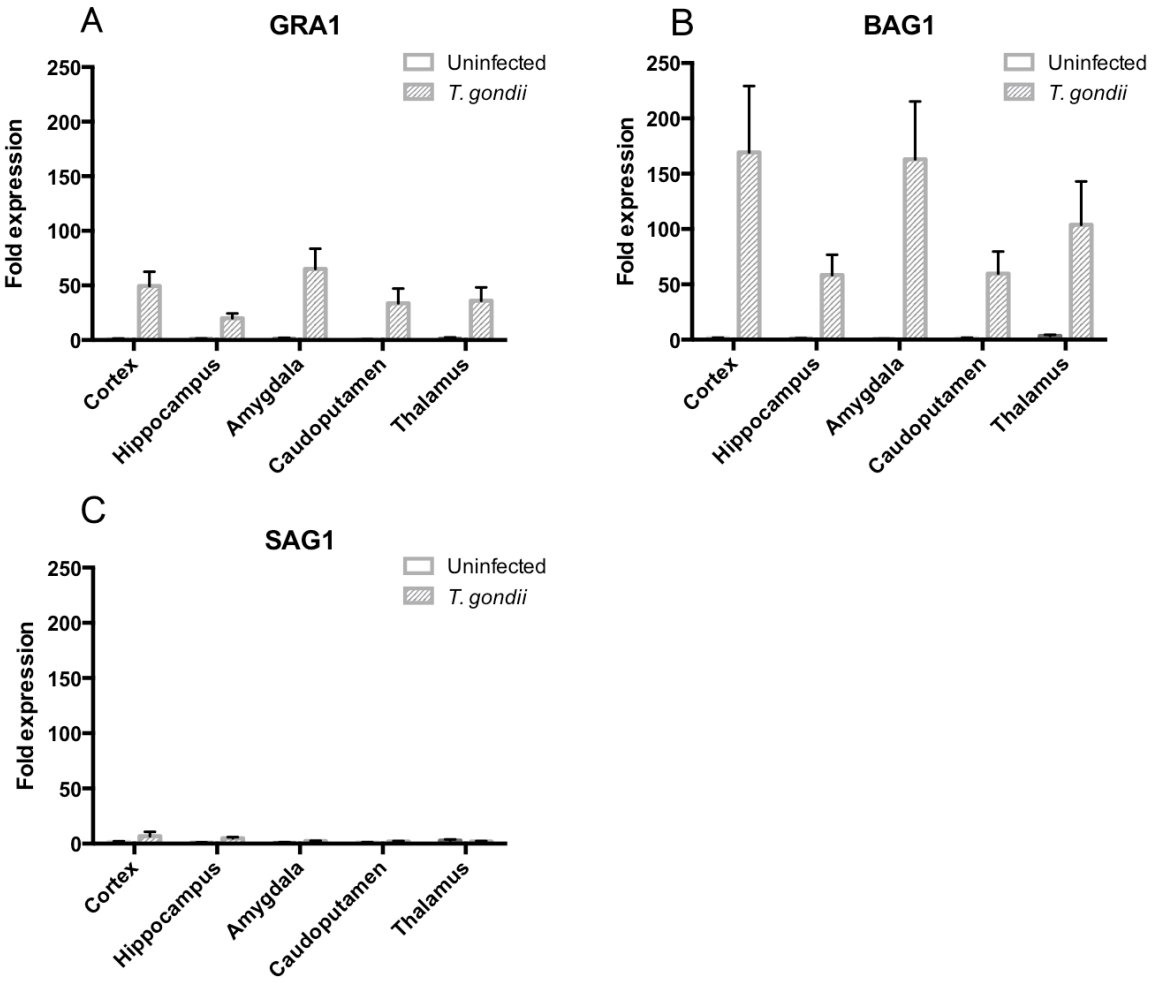
Supplementary Figure 2



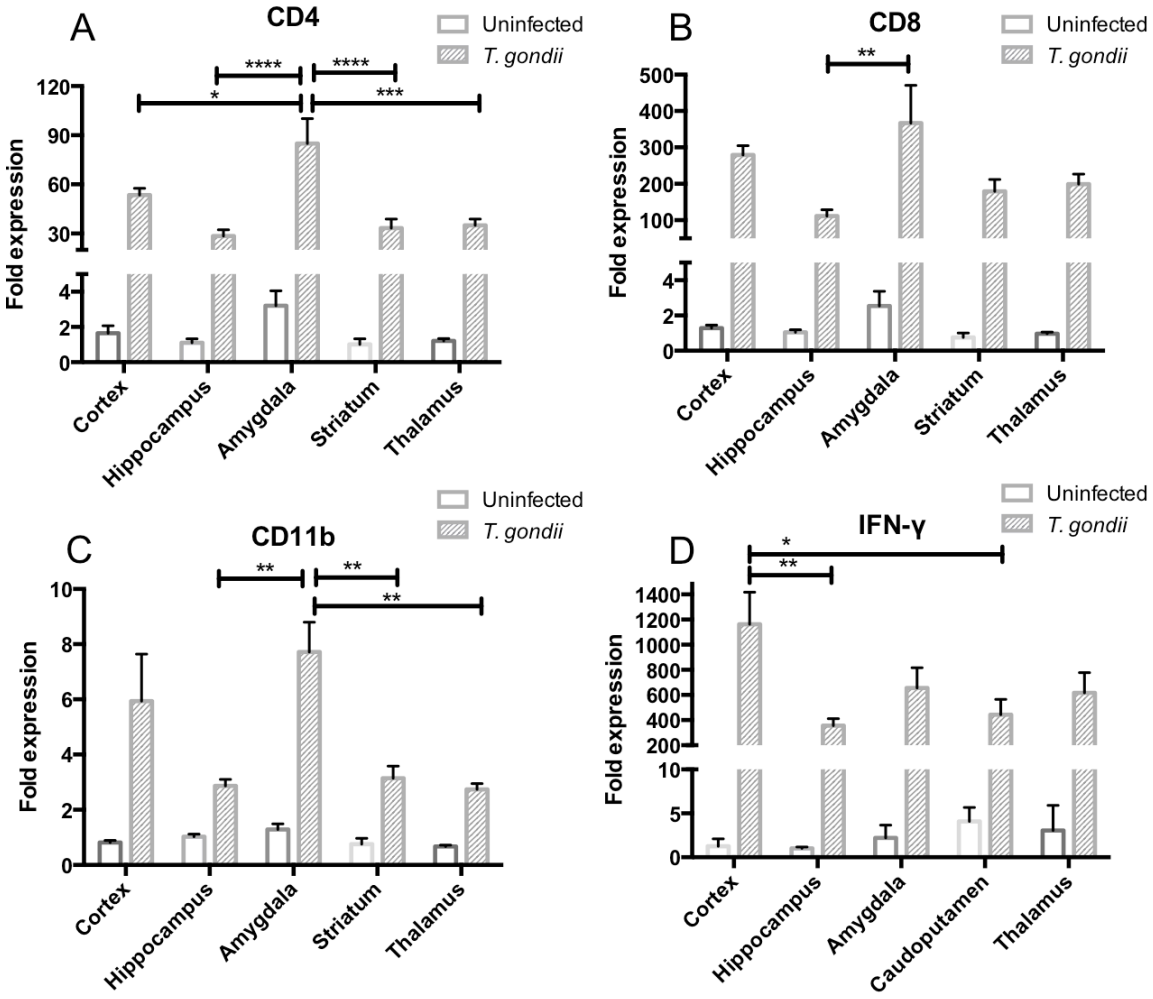
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6