Transcriptome and Histopathological Changes in Mouse Brain Infected with *Neospora caninum*

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*Neospora caninum* is a protozoan parasite that causes neurological disorders in dogs and cattle. It can cause nonsuppurative meningoencephalitis and a variety of neuronal symptoms are observed, particularly in dogs. However, the pathogenic mechanism, including the relationship between the parasite distribution and the clinical signs, is unclear. In this study, to understand the pathogenic mechanism of neosporosis, parasite distribution and lesions were assessed in the brain of mice infected with *N. caninum* (strain Nc-1). Host gene expression was also analyzed with RNA sequencing (RNA-Seq). The histopathological lesions in the frontal lobe and the medulla oblongata were significantly more severe in symptomatic mice than in asymptomatic mice, although no association between the severity of the lesions and parasite numbers was found. In infected mice, the expression of 772 mouse brain genes was upregulated. A GOstat analysis predicted that the upregulated genes were involved in the host immune response. Genes whose expression correlated positively and negatively with parasite numbers were involved in the host immune response, and neuronal morphogenesis and lipid metabolic processes, respectively. These results suggest that changes in the gene expression profile associated with neuronal functions as well as immune responses can contribute to the pathogenesis in *N. caninum*-infected animals.

*Neospora caninum* is a protozoan parasite that causes neurological disorders in dogs and cattle. The infectivity of *N. caninum* for humans is unknown. However, serological evidence suggests that humans are exposed to *N. caninum*. Furthermore, *Neospora* DNA has been detected in experimentally infected rhesus macaques. There are three infectious stages of *N. caninum*: tachyzoites, bradyzoites in tissue cysts, and sporozoites from oocysts. Dogs probably become infected via the ingestion of tissue containing bradyzoites and cattle via the ingestion of food or drink contaminated with oocysts. Although the proliferation of tachyzoites can occur in many organs, including the heart, lung, liver, and skin, neuromuscular disorder is the clinically most important in canine neosporosis. In cattle, *N. caninum* is a major cause of abortion worldwide, and calves with congenital infections can show neurological signs. Tissue cysts are predominantly formed in the central nervous systems (CNS), and *N. caninum* is thought to show tropism for the nervous system.

*Neospora caninum* can cause fatal diseases in dogs of all ages, although most cases of clinical neosporosis are reported in puppies, which often show characteristic pelvic limb paralysis and rigid hyperextension. In contrast, a variety of neurological signs are observed in adult dogs, and neurological symptoms are thought to depend on the site that is parasitized within CNS. The intracellular multiplication of the tachyzoites and the subsequent cell rupture trigger the development of lesions in *N. caninum*-infected tissues. However, the number of parasites is not always associated with the severity of the histopathological lesions in the brain, and the mechanism of the neuronal pathogenesis of neosporosis is unclear.

*Toxoplasma gondii* is closely related to *N. caninum* and causes abortion and neuronal disorders in humans and animals. It has also been suggested that chronic infection with *T. gondii* can involve behavioral changes and psychiatric disorders in humans and animals. In a previous study, we investigated the gene expression profiles and histopathological changes in the brains of mice infected with *T. gondii*, and our findings indicated that *T. gondii* stimulated the immune response including antigen presentation and diminished signal transduction.
included small-GTPase-mediated signal transduction and vesicle formation, which both regulate neurological functions in the brain\(^1\). Therefore, changes in gene expression caused by *N. caninum* infection potentially contribute to the neuronal pathogenesis in *N. caninum*-infected animals.

Some strains of mice including BALB/c are susceptible to *N. caninum* and exhibit encephalitis caused by the parasite infection\(^2\). Therefore mice have been used widely as an infection model of neosporosis to investigate immune responses and vaccine efficacy\(^3\). In this study, to understand the mechanism of neuronal pathogenesis during subacute infection with *N. caninum*, we investigated the histopathological changes, the distribution of the parasite, and the gene expression profiles (using a whole-transcriptome shotgun sequencing approach, RNA-Seq) in the brains of mice infected with *N. caninum*.

**Results**

**Mice infected with *N. caninum* showed characteristic clinical signs and histopathological changes in the brain.** Pathological severity was evaluated in 17 mice, and 10 of the 17 mice showed clinical signs of neosporosis including febrile responses and leg paralysis 39 days after infection (See Figure 1A). Seven of 10 symptomatic mice showed neurological signs, including circling motion, head tilting, and leg paralysis. We analyzed the nine area of brain histopathologically and for the quantification of parasite load. Histopathological lesions, including perivascular cuff, mononuclear cellular meningitis, glial cell activation, and focal necrosis, were observed in the brains of all 17 mice, which are similar to the lesions found in dogs\(^4,5,14,15\). Each focal lesion was scored for severity using a scale from 1 to 4 (See Figure 2A–D). The total pathological scores for all areas in the brain are shown in Figure 2E. The total scores for the frontal lobe and medulla oblongata were significantly higher in the symptomatic mice than in the asymptomatic mice. Although there were no significant differences between the symptomatic and asymptomatic mice in the cerebellum, two symptomatic mice showed very high scores.

Tachyzoites were found within some lesions, but no tissue cysts were found in the present study. Therefore, the tropism of tissue cysts was not determined histopathologically. We also investigated the distribution of the parasite in the brain with real-time PCR. The numbers of parasites for each area of the brain are shown in Figure 2F. A sporadically high parasite load was detected in the frontal lobe and periaqueductal gray of the symptomatic mice. However we could not evaluate the difference of parasite number among the areas statistically because of small sample number. No association between the parasite load and the severity of the lesions was found. In an immunohistochemical analysis, the infiltration of macrophages or microglia and the production of inducible nitric oxide synthase (iNOS) were predominantly observed in the necrotic and inflammatory lesions (See Figure 3).

**Gene expression profiles are altered in their brains of mice infected with *N. caninum*.** Eight mice, consisting of four *N. caninum*-infected mice and four uninfected mice, were used in the transcriptome analysis (See Figure 1B). Two of the four *N. caninum*-infected mice showed clinical signs of neosporosis, including starry coat and hunchback, 39 days after infection. The numbers of parasites tended to be slightly higher in the symptomatic mice (See supplementary Table S1). RNA obtained from the eight mice was subjected to high-throughput sequencing on an Illumina Genome Analyzer Iix. Eight libraries were sequenced and 34–47 million raw sequence reads were obtained per sample. Of these, 63.5%–84.9% could be mapped to the mouse genome (See supplementary Table S2).

To identify the differentially expressed genes in the experimental groups, we analyzed the transcriptome data from the *N. caninum*-infected and uninfected mouse brains with DESeq. The magnitude distribution of the significantly altered genes was illustrated with an MA plot analysis (See supplementary Figure S1). We analyzed 37,306 genes including 22,661 protein-coding genes. The expression of 772 protein-coding genes (2.1% of all analyzed genes and 3.4% of protein-coding genes) in the brains of the mice infected with *N. caninum* was significantly upregulated relative to their expression in the uninfected mice. The 30 most upregulated genes after infection with *N. caninum* are listed in Table 1. These 30 genes included genes for chemokines and chemokine receptors (Cxc19, Cx18, Cx15, Cxcl10, and Cxcr6), immunoglobulins (Igk, Ig2c, and Igk), interferon (IFN)-inducible GTPase family members (Tgap2, Gbp8, and Igif1), and MHC class II antigens (Cd74, H2-Eb1, H2-Aa, and H2-Q7). The quantitative PCR analysis showed that expression of Cc18, Cx15, Cxcl10, Cxcr6, Tgap2, Gbp8, Igif1 and Saa3 were significantly upregulated in the brains of *N. caninum*-infected mice (See Figure 4). In contrast, only three genes (Fcrls, Myoc, and Gkn3) were significantly downregulated in the infected mice (See supplementary Table S3).

When the functional annotations of the genes upregulated by *N. caninum* infection were analyzed using GOSTat, gene ontology (GO) terms associated with immune system processes, immune responses, and cell activation were represented significantly more strongly in the upregulated genes compared with the reference genes (See Table 2).

**Gene expression levels correlate with the numbers of parasites in the brain.** To investigate whether the fragments/kb of transcripts/ million fragments mapped (FPKM) value correlates with the number of parasites in the infected mouse brain, the correlation coefficients were calculated. Seven hundred and twenty-two genes correlated positively with the numbers of parasites measured, whereas 320 genes showed negative correlations. There were 253 genes overlapping between genes upregulated by infection and genes correlated positively with the number of parasites (See supplementary Table S4). Ten (Cdxba, F10, F830016B08Rik, Il1rn, Lcn2, Cxcl10, Kirk1, Gimap7, Cxcr6 and Ifg1) of the 30 genes most upregulated in *N. caninum* infected mice were not overlapped with the genes correlated positively with the number of parasites. Table 3 and Table 4 show the thirty most overrepresented GO terms for the genes that correlated positively and negatively with the parasites load, respectively. The GOSTat analysis showed that the genes whose expression correlated positively with parasite numbers were associated with lysosome and ribosome in addition to immune responses. In contrast, the statistically overrepresented GO terms for the genes whose expression correlated negatively with parasite numbers involved cell and neuron projection, sterol and steroid metabolic processes, and synaptic transmission. These results indicate that *N. caninum* stimulates translational activation and intracellular digestion and reduce cell morphogenesis, including that of axons and dendrites, lipid metabolism, and neural transmission.

**Gene expression profiles of symptomatic mice differed from those of asymptomatic animals.** The severity of the clinical signs of neosporosis differed among the *N. caninum*-infected mice (See supplementary Table S1). To identify differences in the gene expression profiles associated with the development of neosporosis, the genes expressed differentially between symptomatic and asymptomatic mice were analyzed with DESeq. The expression of no gene was higher in the symptomatic mice than in the asymptomatic mice, but the expression of eight genes was lower in the symptomatic mice than in the asymptomatic mice (See Table 5). These genes encoded solute carrier family 6 (a neurotransmitter transporter, serotonin), member 4 (Slc6a4), solute carrier family 6 (a neurotransmitter transporter, glycine), member 5 (Slc6a5), tryptophan hydroxylase 2 (Tph2), and low-density lipoprotein receptor (Ldlr). Although we could not evaluate
the difference of gene expression between symptomatic and asymptomatic mice statistically because of small sample number, Slc6a4, slc6a5, Tph2 and ldlr tended to be downregulated in symptomatic mice in quantitative PCR analysis (See Figure 4).

Discussion

The BALB/c mouse is known to be susceptible to the development of encephalitis induced by N. caninum and encephalitis is observed several weeks after inoculation12,16. In a previous study of T. gondii-infected mouse brains, we showed that the frontal lobe was more affected than any other area of the brain, whereas tissue damage and parasite infection were minor in the cerebellum11. The frontal lobe and medulla oblongata were mainly affected in symptomatic mice infected with N. caninum and some mice showed severe histopathological lesions in the cerebellum. Therefore, lesion formation in the medulla oblongata and the cerebellum may be characteristic of N. caninum infection. These results suggest that damage to neuronal tissues in the frontal lobe, medulla oblongata, and cerebellum is

Figure 1 | Flow diagram illustrating the number of mice used in each analysis. (A) For the histopathological analysis, 25 mice were infected with N. caninum. Because eight of the 25 mice died before sampling or may not have been infected, the severity of the brain lesions in nine different areas (olfactory system, frontal lobe, caudate putamen, hippocampus, hypothalamus, amygdala, periaqueductal gray, medulla oblongata and cerebellum) was estimated in 17 mice (ten symptomatic and seven asymptomatic mice). (B) For the detection of N. caninum and the RNA-Seq analysis, 14 mice were used (four uninfected and 10 infected mice). Thirty-nine days after inoculation, six of the 10 infected mice showed clinical signs of neosporosis, but four animals did not. To detect N. caninum in different areas of the brain, six infected mice were selected (four symptomatic and two asymptomatic mice). The brains of these six infected mice were divided into eight different areas (olfactory system, frontal lobe, caudate putamen, hippocampus, hypothalamus, amygdala, periaqueductal gray, and cerebellum), and used for DNA extraction and quantitative PCR analysis of the parasite. Additionally, to detect N. caninum in the medulla oblongata, DNA was extracted from paraffin-embedded brain tissues containing the medulla oblongata and cerebellum (n = 17, from mice shown in Figure 1A) and subjected to quantitative PCR. For the detection of N. caninum in whole-brain samples and the RNA-Seq analysis, four uninfected and four infected (two symptomatic and two asymptomatic mice) brains were used. DNA and RNA were extracted from each brain sample and used for the quantitative PCR analysis of the parasite numbers and the RNA-Seq analysis.
associated with the pathogenesis of neosporosis. Some infectious diseases including listeriosis show brain stem tropism\(^8\). *Listeria monocytogenes*, which is able to survive and multiply in macrophages, invades the brain by centripetal migration along cranial nerve and interaction between infected myeloid cell and endothelium\(^8\). *T. gondii* and *N. caninum* are thought to be delivered to organs by infected leukocytes in the blood. Although no association between the severity of the lesions and parasite numbers was found, endothelium in brain stem may be prone to adhere to pathogen-infected leukocyte and obstruction by leukocyte adhesion may lead to the damage of parenchyma of brain. Expression of adhesion molecule in each area of brain is intriguing. In some adult cases of canine neosporosis, the cerebellum and brainstem are predominantly affected\(^8,14,15\). Those cases suggest that the cerebellum can be the most commonly affected region in adult dogs with neosporosis\(^4\). Therefore, the murine model of neosporosis could be helpful clarifying the pathology in adult dogs.

In this study, no association between the parasite load and the severity of the lesions in each area was found. In canine neosporosis,
a disassociation between parasite density and the severity of tissue damage has also been reported in adult cases. Therefore, host factors, which can cause or exacerbate tissue damage, may be involved in the pathogenesis of neosporosis. In the brains of *N. caninum* infected mice, infiltration of macrophages or microglia and the production of iNOS were observed in the lesions. Activated microglia and astrocyte produce neurotoxic factors including nitric oxide (NO). NO produced by activated microglia and astrocytes inhibits neuronal respiration and causes glutamate release from neurons, which results in neuronal death from glutamate excitotoxicity via the N-methyl d-aspartate (NMDA) receptor. Therefore, NO may be a factor that exacerbates brain tissue damage in neosporosis.

The 30 genes most strongly upregulated by *N. caninum* infection were similar to those upregulated in *T. gondii*-infected mice. IFN-inducible GTPases play an important role in the host defense against intracellular pathogens, including *T. gondii*. IIGP1 (IRGA6: immunity-related GTPase family member a6) is a member of the p47 GTPase family, and the accumulation of IIGP1 on the parasitophorous vacuole membrane of *T. gondii* resulted in the disruption of the membrane and the necrotic death of the infected cell. We showed that Iigp1 was significantly upregulated in the brain of *T. gondii* infected mice in previous study. ROP18 is a rhoptry kinase expressed highly by virulent *T. gondii* and phosphorylates and inactivates immunity-related GTPase (IRGs). However, genome sequencing and transcriptome analysis of *N. caninum* Liverpool strain revealed that ROP18 in *N. caninum* is a pseudogene and *N. caninum* is unable to prevent the host from using IRGs to disrupt the parasitophorous vacuole. This suggests that the growth of *N. caninum* could be inhibited effectively, which can lead to low parasite number in the lesions. A previous study showed that CXCL9/MIG, CXCL10/IP-10, and CCL5/RANTES are induced, dependent on IFN-γ, in the brains of BALB/c mice during chronic infection with *T. gondii*. Protective immunity against *N. caninum* involves the Th1-type immune response, including IFN-γ and interleukin 12 (IL-12) production, and CCL5 induces the migration of Th1 cells via chemokine receptors including CCR5. On the other hand, some studies have indicated that chemokines and their receptors are involved in exacerbating neuronal damage. Ischemic brain injury was exacer-
bated by a systemic increase in CCL5 during a Th1-polarized response induced by infection\textsuperscript{36} and the downregulation of CCR5 protected the brain from neuron loss, inflammation, and seizure activity\textsuperscript{37}. Therefore, the chemokines upregulated by \textit{N. caninum} infection may be involved in both protective immunity and neuronal pathogenesis. \textit{Saa3} expression was also highly upregulated in the mouse brain after \textit{N. caninum} infection. Serum amyloid A (SAA) is an acute-phase protein, and a previous study suggested that SAA3 is produced in Schwann cells and macrophages in injured peripheral nerves\textsuperscript{31}. Thus, SAA3 may act as a cytokine and be involved in the immune response and neuronal degeneration.

Only three genes (\textit{Fcrs}, \textit{Myoc}, and \textit{Gkn3}) were significantly downregulated in the infected mice. \textit{Myoc} is related to myelination in the peripheral nerve\textsuperscript{38} and \textit{Fcrs} was previously known as macrophage scavenger receptor 2 (Msr2) and is upregulated by mild traumatic brain injury\textsuperscript{39}, and downregulated in brain infected \textit{T. gondii}\textsuperscript{40} and Japanese encephalitis virus\textsuperscript{41}. Although function of \textit{Fcrs} is not well known in brain, downregulation of \textit{Fcrs} is thought to be associated with host-pathogen interaction.

The GOstat analysis showed that the genes whose expression correlated negatively with parasite numbers involved cell and neuron projection, sterol and steroid metabolic processes, and synaptic transmission. Cholesterol is a component of cell membranes, and neurons and astrocytes demand high levels of cholesterol because they have the enormous membrane surfaces, encompassing their axons, dendrites, synapses, and processes\textsuperscript{42}. Cholesterol is also required for cell regeneration after CNS injury and for the formation of new synapses. Cholesterol levels can also affect the biophysical function of membranes, and changes in cholesterol levels of membrane have been shown to affect the excitability of rat hippocampal neurons\textsuperscript{43}. Therefore, changes in the expression of genes that are involved in cellular morphogenesis and lipid metabolism, including cholesterol, may be involved in the exacerbation of neuronal damage, impaired regeneration of damaged neuronal tissues, and the disruption of neurotransmission in mouse brains infected with \textit{N. caninum}.

The expression of eight genes including \textit{Hoxb5}, \textit{Slc6a5}, \textit{Tph2}, and \textit{Ldlr} was lower in the symptomatic mice than in the asymptomatic mice. \textit{Hoxb5} is a gene of gene family cording for transcription factors and \textit{Hoxb5} protein is expressed in mice lung during development\textsuperscript{44}. A previous study demonstrated that mice deficient in glycine transporter 2 encoded by \textit{Slc6a5} showed neuromotor abnormalities, such as spasticity, hind feet claspimg, and tremor\textsuperscript{45}. Tryptophan hydroxylase 2 encoded by \textit{Tph2} is the key enzyme in brain serotonin synthesis\textsuperscript{46}, and it is possible that serotonergic neurons work poorly in mice that develop neosporosis. Therefore, the reduced expression of genes for neurotransmitter transporters may contribute to the development of neosporosis by modulating serotonergic and glycnergic neurotransmission.

In this study, we demonstrated that the frontal lobe and medulla oblongata were most often affected in \textit{N. caninum}-infected mice with clinical signs and that there was no association between the parasite load and the severity of the histopathological lesions. Changes in the gene expression profile were also observed in the mouse brains after \textit{N. caninum} infection. Genes associated with the immune response,
encoding chemokines and IFN-inducible GTPase family members, were upregulated in the brains of mice infected with *N. caninum*. In contrast, the expression of genes associated with the morphogenesis of neurons, including their synapses and projections, with lipid metabolism, and with neuronal transmission correlated negatively with the number of parasites. These results suggest that neuronal tissue damage is caused by the inflammatory responses to parasite infection and can be exacerbated by the impairment of the regeneration of damaged neuronal tissue. Our results also suggest that neuronal transmission is affected by *N. caninum* infection at the level of

Figure 4 | Gene expression analysis by quantitative PCR using RNA from the brain samples used in RNA-seq analysis. (A) Expression of the genes (Saa3, Cxcl9, Cxcl10, Cxcr6, Tgtp2, Gbp8 and Ligp1) that upregulated by *N. caninum* infection. (B) Expression of the genes (Slc6a4, Slc6a5, Tph2 and Ldlr) that downregulated in symptomatic mice infected with *N. caninum*. *p < 0.05 and **p < 0.01.
gene transcription, and that neurological symptoms can be induced not only by organic damage but also by neuronal dysfunction.

**Methods**

*Ethics Statement.* This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 25–59, 25–60, 25–62). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering.

*Mice.* Mice used in this study were treated and used according to the Guiding Principles for the Care and Use of Research Animals published by the Obihiro University of Agriculture and Veterinary Medicine. Female BALB/c mice were obtained from CLEA Japan (Tokyo, Japan). The mice were housed under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan, before they were used in the experiments at 8 weeks of age.

**Preparation of *N. caninum* tachyzoites and parasite infection of mice.** Neospora caninum tachyzoites of strain Nc-1TM were propagated in monkey kidney adherent fibroblasts (Vero cells) cultured in Eagle’s minimum essential medium (Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum. For the purification of the tachyzoites, the parasites and host-cell debris were washed in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and passed through a 27-gauge needle and a 5.0-µm pore filter (Millipore, Bedford, MA, USA). Eight-week-old mice were inoculated intraperitoneally with *N. caninum* parasites purified from an in vitro culture. All the mice were checked regularly for clinical signs of *N. caninum* infection, such as febrile responses (e.g., a starry stiff coat), hunched back, and limb paralysis.

**Brain sampling.** For the histopathological analysis, 25 mice were infected with 1 × 106 *N. caninum*. Because six mice died or were euthanized before the scheduled date as a result of emaciation, 19 mice were sampled. Thirty-nine days after inoculation, at which time histopathological changes have occurred, the experimental mice were killed and their brains rapidly removed (See Figure 1). The brains were fixed with 4% paraformaldehyde for 6 days. We used these brains in the histopathological and immunohistochemical analyses. Two of 19 mice showed no clinical symptoms and no pathological changes. Because it was possible that the parasite had not infected these two mice, the distribution and severity of the brain lesions were estimated in the remaining 17 of 19 mice. Of the 17 mice, 10 mice showed clinical signs of neosporosis 39 days after infection (See Figure 1).

Fourteen mice (four uninfected mice and 10 infected mice) were used for the detection of *N. caninum* and the RNA-Seq analysis (See Figure 1). Thirty-nine days after infection, six infected mice showed the clinical signs of neosporosis and four animals did not. To detect *N. caninum* in different areas of the brain, six infected mice were selected (four symptomatic and two asymptomatic mice). The brains of these six infected mice were divided into eight different areas, the olfactory system, frontal lobe, caudate putamen, hippocampus, hypothalamus, amygdala, periaqueductal gray, and cerebellum. The samples were used for DNA extraction and quantitative PCR of the parasites were stored at −20°C until analysis.

We used the other eight mice (four uninfected and four infected mice) to detect *N. caninum* in whole brain samples and in the DNA-Seq analysis. Of the four infected mice, two were symptomatic and two were asymptomatic. The brains of four infected mice and four uninfected mice were individually homogenized in 1 ml of TRI Reagent (Sigma-Aldrich, Tokyo, Japan). Each brain sample was then divided for DNA extraction (for quantitative PCR of the parasite numbers) or RNA extraction (for the RNA-Seq analysis).

**Pathological analysis.** After fixation, the brain samples were cut coronally, embedded in paraffin wax, sectioned to 4 µm, and then stained with hematoxylin and eosin (HE). To estimate the severity of the histopathological lesions, they were scored with the following scheme: 0, no lesion; 1, minimal lesions limited to localized perivascular cuffs; 2, minimal lesions with occasional lymphohistiocytic infiltrates; 3, moderate lesions, including perivascular cuffs and infiltrates; 4, severe lesions, including perivascular cuffs and infiltrates; 5, marked lesions, including edema, necrosis, or inflammation; 6, extensive lesions, including edema, necrosis, or inflammation.

**Table 2 | GO terms for the most significantly upregulated genes in mouse brains after infection with *N. caninum***

| Accession | GO term                  | # Genes | %   | # Genes | %   | FDR    |
|-----------|--------------------------|---------|-----|---------|-----|--------|
| GO:0002376 | immune system process   | 566     | 3.8 | 184     | 28.4| 0      |
| GO:0006952 | defense response         | 353     | 2.4 | 104     | 16.0| 0      |
| GO:0006955 | immune response          | 346     | 2.3 | 138     | 21.3| 0      |
| GO:0006954 | inflammatory response    | 178     | 1.2 | 64      | 9.9 | 5.92E-64|
| GO:0009897 | external side of plasma membrane | 121   | 0.8 | 51      | 7.9 | 6.28E-59|
| GO:0009986 | cell surface             | 168     | 1.1 | 58      | 8.9 | 2.06E-55|
| GO:0045321 | leukocyte activation     | 189     | 1.3 | 60      | 9.2 | 1.41E-52|
| GO:0001775 | cell migration           | 182     | 1.2 | 60      | 9.2 | 1.41E-52|
| GO:0009961 | response to wounding     | 252     | 1.7 | 69      | 10.6| 1.10E-51|
| GO:0002682 | regulation of immune system process | 92   | 0.6 | 41      | 6.3 | 1.50E-49|
| GO:005776  | regulation of immune response | 90     | 0.6 | 40      | 6.2 | 3.46E-48|
| GO:0051239 | regulation of multicellular organismal process | 250 | 1.7 | 65      | 10.0| 1.01E-45|
| GO:009605  | response to external stimulus | 393 | 2.6 | 83      | 12.8| 1.57E-45|
| GO:0046649 | lymphocyte activation    | 173     | 1.2 | 53      | 8.2 | 1.36E-44|
| GO:0042110 | T cell activation        | 112     | 0.7 | 39      | 6.0 | 5.58E-37|
| GO:0022352 | immune effector process  | 113     | 0.8 | 38      | 5.9 | 1.10E-34|
| GO:0051240 | positive regulation of multicellular organismal process | 126 | 0.8 | 38      | 5.9 | 1.10E-34|
| GO:001816  | cytokine production      | 95      | 0.6 | 33      | 5.1 | 6.63E-31|
| GO:0002520 | immune system development | 228   | 1.5 | 49      | 7.6 | 1.39E-26|
| GO:0006935 | chemotaxis               | 92      | 0.6 | 30      | 4.6 | 4.22E-26|
| GO:0042330 | taxis                    | 92      | 0.6 | 30      | 4.6 | 4.22E-26|
| GO:0048534 | hemopoietic or lymphoid organ development | 212 | 1.4 | 45      | 6.9 | 6.89E-24|
| GO:0019882 | antigen processing and presentation | 43 | 0.3 | 33      | 5.1 | 2.91E-33|
| GO:0006950 | response to stress       | 666     | 4.5 | 87      | 13.4| 2.07E-22|
| GO:0003907 | hemopoiesis              | 190     | 1.3 | 40      | 6.2 | 8.01E-21|
| GO:0005883 | plasma membrane          | 147     | 0.9 | 143     | 21.8| 1.76E-20|
| GO:0051249 | regulation of lymphocyte activation | 76  | 0.5 | 36      | 5.5 | 3.31E-20|
| GO:0050865 | regulation of cell activation | 79  | 0.5 | 36      | 5.5 | 8.86E-20|
| GO:0048002 | antigen processing and presentation of peptide antigen | 28  | 0.2 | 26      | 4.0 | 1.33E-19|
| GO:0002521 | leukocyte differentiation | 118     | 0.8 | 30      | 4.6 | 1.33E-19|
Immunohistochemical analysis of macrophages/microglia and iNOS.

Immunohistochemistry was performed with antibodies directed against ionized calcium-binding adaptor molecule 1 (Iba1; Wako, Tokyo, Japan; diluted 1: 200) or inducible nitric oxide synthase (iNOS; Abcam, Cambridge, MA, USA; diluted 1: 200) as the primary antibody and a secondary antibody conjugated with horseradish-peroxidase-labeled polymer (EnVision ×1 kit, DAKO, Copenhagen, Denmark). To immunostain for Iba1 and iNOS, deparaffinized sections were subjected to heat exposure to each primary antibody at 4 °C for 30 min at room temperature to prevent nonspecific reactions. The sections were subjected to 5 µM EDTA with 100 µg/mL proteinase K at 55 °C for 30 min at room temperature to prevent nonspecific reactions. The sections were incubated in goat serum for 30 min at room temperature. The tissue sections were then incubated in goat serum for 30 min at room temperature. The sections were then exposed to each primary antibody at 4 °C overnight and then incubated with the secondary antibody for 40 min at 37 °C. The signals were visualized using diaminobenzidine (ImmPACT DAB®, Vector Laboratories Inc., Burlingame, CA, USA), followed by counterstaining with Mayer’s hematoxylin.

DNA extraction and quantitative PCR for the detection of N. caninum DNA. To prepare DNA, the brain of each N. caninum-infected mouse was thawed in 10 times its volume of DNA extraction buffer (0.1 M Tris–HCl [pH 9.0], 1% SDS, 0.1 M NaCl, 1 mM EDTA) with 100 µg/mL protease K at 55 °C. DNA was extracted with a commercial kit (DNeasy Blood & Tissue Kit; Qiagen, Hilden, Germany) with a standard protocol recommended by the manufacturer. In addition to eight areas of brain (olfactory system, frontal lobe, caudate putamen, hippocampus, hypothalamus, amygdala, periaqueductal gray, and cerebellum), we estimate the parasite load in the medulla oblongata because relatively severe histopathological changes were observed in the area. To evaluate the parasite load in the medulla oblongata, we used the DNA extracted from paraffin-embedded tissues sections from the 17 mice used for the pathological analysis. The sections contained the medulla oblongata and cerebellum because we could not remove the cerebellum from the samples. DNA was extracted from the paraffin-embedded tissues with a commercial kit (QiAamp® DNA FFPE Tissue Kit; Qiagen). The parasite DNA was amplified as described previously.

Table 3 | Thirty most overrepresented GO terms for the genes that correlated positively with the parasites load

| Accession | GO term                      | # Genes | %   | # Genes | %   | FDR  |
|-----------|------------------------------|---------|-----|---------|-----|------|
| GO:0002376 | immune system process        | 566     | 3.8 | 93      | 15.0| 1.29E-37|
| GO:0006955 | immune response              | 346     | 2.3 | 65      | 10.5| 3.06E-31|
| GO:0000323 | lytic vacuole                | 153     | 1.0 | 40      | 6.5 | 8.65E-29|
| GO:0005764 | lysozyme                     | 153     | 1.0 | 40      | 6.5 | 8.65E-29|
| GO:0005773 | vacuole                      | 173     | 1.2 | 41      | 6.6 | 3.18E-26|
| GO:0019882 | antigen processing and presentation | 43 | 0.3 | 28      | 4.5 | 4.23E-18|
| GO:0048002 | antigen processing and presentation of peptide antigen | 211 | 1.7 | 33      | 5.21E-16|
| GO:0069522 | defense response             | 353     | 2.4 | 51      | 8.2 | 6.81E-16|
| GO:0044444 | cytoplasmic part             | 2791    | 18.7| 196     | 31.7| 1.28E-12|
| GO:0057377 | cytoplasm                    | 4861    | 32.5| 295     | 47.7| 5.58E-12|
| GO:0019884 | antigen processing and presentation of exogenous antigen | 22 | 0.1 | 16      | 2.6 | 2.34E-10|
| GO:0024788 | antigen processing and presentation of exogenous peptide antigen | 17 | 0.1 | 14      | 2.3 | 1.89E-09|
| GO:0042611 | MHC protein complex          | 19      | 0.1 | 14      | 2.3 | 4.69E-09|
| GO:0066412 | translation                  | 385     | 2.6 | 44      | 7.1 | 1.60E-08|
| GO:0024747 | antigen processing and presentation of peptide antigen via MHC class I | 14   | 0.1 | 12      | 1.9 | 3.47E-08|
| GO:0019886 | antigen processing and presentation of exogenous peptide antigen via MHC class II | 15   | 0.1 | 12      | 1.9 | 4.97E-08|
| GO:0024959 | antigen processing and presentation of peptide antigen via MHC class II | 15   | 0.1 | 12      | 1.9 | 4.97E-08|
| GO:0037375 | structural constituent of ribosome | 143   | 1.0 | 23      | 3.7 | 7.41E-08|
| GO:0025054 | antigen processing and presentation of peptide or polysaccharide antigen via MHC class II | 16   | 0.1 | 12      | 1.9 | 7.57E-08|
| GO:0002252 | immune effector process      | 113     | 0.8 | 20      | 3.2 | 7.57E-08|
| GO:0069554 | inflammatory response        | 178     | 1.2 | 26      | 4.2 | 1.05E-07|
| GO:0058400 | ribosome                     | 148     | 1.0 | 23      | 3.7 | 1.83E-07|
| GO:0035029 | ribonucleoprotein complex    | 350     | 2.3 | 39      | 6.3 | 3.78E-07|
| GO:0098979 | external side of plasma membrane | 121 | 0.8 | 20      | 3.2 | 4.47E-07|
| GO:0016064 | immunoglobulin mediated immune response | 55 | 0.4 | 17      | 2.7 | 5.25E-07|
| GO:0019724 | B cell mediated immunity     | 56      | 0.4 | 17      | 2.7 | 6.41E-07|
| GO:0005829 | cytosol                      | 316     | 2.1 | 36      | 5.8 | 6.81E-07|
| GO:0009611 | response to wounding         | 252     | 1.7 | 31      | 5.0 | 7.65E-07|
| GO:0024499 | lymphocyte mediated immunity | 75      | 0.5 | 19      | 3.1 | 8.81E-07|
| GO:0024443 | leukocyte mediated immunity  | 81      | 0.5 | 19      | 3.1 | 2.52E-06|

(FDR < 0.05)

Briefly, we used the SYBR Green PCR Core Kit (PE Applied Biosystems, Foster City, CA, USA) and primers 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 AAG GAG GAA-3′). Amplification, data acquisition, and data analysis were carried out in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), and the calculated cycle threshold values (Ct) were exported to Microsoft Excel for analysis as described previously. The limit of detection was 0.1 parasites in 50 ng of tissue DNA.

Transcriptome sequencing. Transcriptome sequencing was conducted as described previously. Briefly, samples obtained from eight mice underwent poly-A selection with 1 µg of total RNA. Sequencing libraries were constructed with the RNA Sample Prep Kit (Illumina, San Diego, CA, USA); 36-bp single-end sequencing was performed using the Illumina Genome Analyzer Ix (Illumina) with the TruSeq SBS Kit v5-GA (36 cycle) (Illumina), according to the manufacturer’s instructions. All treatment and following analyses associated with transcriptome sequencing were done individually.

Aligning sequence tags to the mouse genome and RefSeq. Aligning sequence tags was conducted as described previously. Briefly, the raw sequence reads were mapped to the mouse genome (mm10) using TopHat (ver. 1.3.3) using the calculated cycle threshold values (Ct) were exported to Microsoft Excel for analysis as described previously. The MGI ID and gene ontology (GO) were used to classify the estimated expression profiles, together with the gene biotypes extracted from the gtf data.
Identification of differentially expressed genes. Aligning sequence tags was conducted as described previously11. Briefly, differentially expressed genes were identified with the DESeq package in the R software43, using a two-fold change (log2 fold-change 1 or 2) and a 5% false discovery rate (FDR) cut-off for the thresholds. The expression intensity values were analyzed with an MA plot-based method after data normalization and the calculation of FDR.

Gene ontology (GO) analysis. The function of individual genes can be analyzed with GOstat (http://gostat.wehi.edu.au/), as described previously11. Using GOstat, we identified GO terms statistically overrepresented in the selected genes compared with the reference genes (all genes; 14938 genes). The statistical analysis was performed with a Benjamini correction, which controls FDR44. FDRs < 0.05 were considered statistically significant.

Correlation between gene expression levels and parasite numbers. The correlation coefficients for the FPKM values and the numbers of parasites in the mouse brain were calculated using Pearson’s correlation coefficient, as previously described11. The range of correlation coefficients for positive correlations was 0.7 to 1.0, and the no-correlation range was 0.7 to 0.7, whereas the range for negative correlation was 0.7 to 1.0. We analyzed genes that correlated positively or negatively with the number of parasites in the mouse brain using GOstat.

Quantitative reverse-transcription-PCR (qRT-PCR). Steady state mRNA expressions of the genes that upregulated by N. caninum infection and downregulated in symptomatic mice infected with the parasites were measured by qRT-PCR. Total RNA was extracted using TRI Reagent (Sigma, St Louis, MO, USA), from the left halves of brains. Reverse transcription was performed using Superscript IITM Reverse

| Accession | GO term                      | Reference gene | Genes negatively correlated with parasite number |
|-----------|------------------------------|----------------|-----------------------------------------------|
| GO:0042995 | cell projection              | 333 2.2         | 23 8.9 6.75E-08                               |
| GO:0043005 | neuron projection            | 108 0.7         | 16 6.2 1.27E-06                               |
| GO:0005886 | plasma membrane              | 1476 9.9        | 56 21.7 2.07E-06                              |
| GO:0011179 | localization                 | 2430 16.3       | 78 30.2 7.01E-06                              |
| GO:0016192 | vesicle-mediated transport   | 375 2.5         | 21 8.1 9.05E-05                               |
| GO:0006810 | transport                    | 2099 14.1       | 65 25.2 6.52E-04                              |
| GO:0011625 | steroid metabolic process    | 67 0.4          | 10 3.9 6.52E-04                               |
| GO:0051234 | establishment of localization| 2152 14.4       | 66 25.6 6.52E-04                              |
| GO:0008202 | steroid metabolic process    | 130 0.9         | 13 5.0 6.52E-04                               |
| GO:0048667 | neuron morphogenesis         | 153 1.0         | 14 5.4 6.52E-04                               |
| GO:0048812 | neurite morphogenesis        | 153 1.0         | 14 5.4 6.52E-04                               |
| GO:0016126 | sterol biosynthetic process  | 26 0.2          | 7 2.7 6.52E-04                                |
| GO:0050808 | synapse organization and biogenesis | 39 0.3       | 8 3.1 6.52E-04                               |
| GO:0007268 | synaptic transmission        | 179 1.2         | 15 5.8 6.52E-04                               |
| GO:0000267 | cell fraction                | 422 2.8         | 21 8.1 6.80E-04                               |
| GO:0019226 | transmission of nerve impulse| 211 1.4         | 16 6.2 8.53E-04                               |
| GO:0030424 | axon                        | 58 0.4          | 9 3.5 8.72E-04                                |
| GO:0016020 | membrane                    | 6123 41.0       | 144 55.8 9.91E-04                             |
| GO:0008324 | cation transmembrane transporter activity | 435 2.9       | 21 8.1 1.08E-03                              |
| GO:0008203 | cholesterol metabolic process| 61 0.4         | 9 3.5 1.08E-03                               |
| GO:0007409 | axonogenesis                 | 146 1.0         | 13 5.0 1.10E-03                               |
| GO:0006836 | neurotransmitter transport   | 46 0.3          | 8 3.1 1.10E-03                                |
| GO:0000904 | cellular morphogenesis       | 172 1.2         | 14 5.4 1.10E-03                               |
| GO:0030030 | cell projection organization and biogenesis | 252 1.7       | 17 6.6 1.10E-03                              |
| GO:0048858 | cell projection morphogenesis | 252 1.7        | 17 6.6 1.10E-03                              |
| GO:0032990 | cell part morphogenesis      | 252 1.7         | 17 6.6 1.10E-03                               |
| GO:0006695 | cholesterol biosynthetic process | 20 0.1         | 6 2.3 1.16E-03                               |
| GO:0031175 | neurite development          | 183 1.2         | 14 5.4 1.99E-03                               |
| GO:0048699 | generation of neuron         | 299 2.0         | 16 6.2 2.12E-03                               |
| GO:0015075 | ion transmembrane transporter activity | 595 4.0       | 25 9.7 2.46E-03                              |

Table 5 | Genes significantly altered in brain samples from mice exhibiting clinical signs of neosporosis

| Gene symbol | Description                             | Average of FPKM |
|-------------|-----------------------------------------|-----------------|
| Hoxb5       | homeobox B5                             |                 |
| Slc6a4      | solute carrier family 6 (neurotransmitter transporter, serotonergic) |                 |
| BC037034    | cDNA sequence BC037034                 |                 |
| Tph2        | tryptophan hydroxylase 2               |                 |
| Slc6a5      | solute carrier family 6 (neurotransmitter transporter, glycine) |                 |
| Mab21I2     | mab-21-like 2 (C. elegans)             |                 |
| Fos         | FBJ osteosarcoma oncogene              |                 |
| Ldrl        | low density lipoprotein receptor        |                 |

| | FPKM Fold change | FDR |
|------------------|-----------------|-----|
| No signs (n = 2) | Clinical signs (n = 2) | 0.08 | 7.33E-04 |
| Hoxb5            | 1.09            | 0.09 | 0.08 | 7.33E-04 |
| Slc6a4           | 2.94            | 0.38 | 0.13 | 3.04E-09 |
| BC037034         | 10.04           | 1.44 | 0.14 | 9.05E-03 |
| Tph2             | 4.03            | 0.88 | 0.22 | 6.85E-06 |
| Slc6a5           | 4.15            | 0.95 | 0.23 | 3.84E-06 |
| Mab21I2          | 3.26            | 1.08 | 0.33 | 2.41E-02 |
| Fos              | 33.97           | 12.06| 0.36 | 5.30E-03 |
| Ldrl             | 7.42            | 2.89 | 0.39 | 9.05E-03 |

(=two-fold change and FDR < 0.05)
Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instruction. Real-time PCR was performed using an Applied Biosystems Prism 7700 Sequence Detection System with SYBR Green master mix (AB Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The fold change Ct method was used, where Ct is the threshold concentration (User Bulletin no. 2; Perkin-Elmer, Boston, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control. Specific primer sequences were shown in Table S5.

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