Parasite Immunology

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Toxoplasma gondii Infection of BALB/c Mice Perturbs Host Neurochemistry

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ABSTRACT

Toxoplasma gondii infection has been associated with psychoneurological disease in humans and behavioural changes in rodents. However, the mechanisms accounting for this have not been fully described and in some cases could be argued to reflect the severe neuropathology that some mice suffer during infection. Herein we employ a multi-omics approach to extensively examine BALB/c mice that are resistant to toxoplasmic encephalitis. Using a combination of LCMS (liquid chromatography–mass spectrometry) and RNAseq we demonstrate that infection alters the neurochemistry and the transcriptome of the brains of BALB/c mice. Notable changes to tryptophan, purine, arginine, nicotinamide and carnitine metabolism were observed in infected mice and this was accompanied with changes to the levels of a number of transcripts associated with enzymes these metabolic pathways. In addition, changes were seen in transcripts of many immunologically important genes known to contribute to immunity to *T. gondii*. Changes in the levels of additional transcripts during infection have previously been associated with psychoneurological diseases. The results demonstrate that the BALB/c mouse, with its relatively mild neurological disease, is a useful model for characterising the effects of *T. gondii* infection on murine neurochemistry. The results also implicate specific biochemical pathways in mediating these changes and should inform further mechanistic studies and suggest therapeutic targets.

1 | Introduction

In the last decades a large body of evidence has emerged to highlight a relationship between a number of infections and the development of neuropsychiatric diseases [1]. A meta-analysis of studies has shown that infection by some agents, such as Human *Herpes*virus, *Borna Disease Virus*, *Chlamydophila pneumoniae* and *Toxoplasma gondii* may be associated with development of psychiatric disorders [2]. These data illustrate that the incidence of at least some infectious agents are increased in psychiatric patients compared with control populations, but do not in themselves differentiate causation from association. One disease that has received a lot of attention in recent years is toxoplasmosis and a number of individual studies have demonstrated that people with schizophrenia have an increased incidence of *T. gondii* infection compared with the general population [3–6]. Two meta-analyses support this association. First, a meta-analysis that included 23 separate reports calculated an odds ratio of 2.73 for people seropositive for *T. gondii* developing schizophrenia compared with seronegative people [7]. Second, a further meta-analysis that included 50 studies reported an odds ratio of 1.83, but reduced to 1.43 after taking into account publication bias for people seropositive for *T. gondii* having schizophrenia compared with seronegative people [8]. This association of *T. gondii* infection and schizophrenia is further strengthened by a study that demonstrated that immunological correlates of recent infection and an intense immune response was associated with the first recorded episodes of schizophrenia [7]. Furthermore, a prospective study, making use of serology at childbirth

(from 1992 to 1995) and follow up serology (2008) found that mothers developing high titres to *T. gondii* were are greater risk of schizophrenia spectrum disorders [9]. Sutterland et al. [8] also found an association between bipolar disorder (odds ratio 1.52), obsessive-compulsive disorder (odds ratio 3.4) and addiction (odds ratio 1.91). Although some studies have also reported an association of *T. gondii* with major depression this was not upheld in this meta-analysis [8]. Other studies have implicated toxoplasmosis with a spectrum of more subtle behavioural changes in humans [10–12]. However, it is difficult to demonstrate a causative relationship between any infectious agent and neuropsychiatric diseases or behavioural changes in humans and challenging to explore mechanisms.

Animal models of infection provide an obvious tool to understand and study the ability of *T. gondii* infection to affect the mammalian brain and behaviour. Indeed, studies have demonstrated that *T. gondii* infection can manipulate rodent behaviours under laboratory conditions [13–19]. One study presented evidence that *T. gondii* infection specifically ablated innate fear of cats (sensed by the smell of urine) by mice, but not other predators [20] However, a later study demonstrated that mice infected with *T. gondii* have lower anxiety levels which increases explorative behaviour [21]. Various theories have been suggested to account for these behavioural changes. Some of these potential mechanisms were articulated by Henriquez et al. [22] and include direct neuronal modulation by parasite products, parasite induced damage or death of neurones, interference with the hypothalamic–pituitary–adrenal axis or immunological induced modulation of biochemical pathways that could have a bearing on psychoneurological disease including tryptophan metabolism. In addition, and more generally, the immune processes during chronic infection may also lead to alterations in neuronal connectivity, synaptic plasticity and brain function [23, 24]. This final theory is supported by Boillat et al. [21], who found reduced anxiety in mice correlates with intensity of infection and thus inflammation. In fact, there might be validity in more than one of these theories and differences in the contribution of each might occur between the many different models used and the many different proposed effects of *T. gondii* on behaviours and development of neuropsychiatric disease.

Both host and parasite genetics are determinants of the outcome of *T. gondii* infection. Parasite determinants have been highlighted in recent years with the recognition of six clades of *T. gondii* with different disease profiles in animal models and in the clinic (reviewed in [25]). That said, the vast majority of murine studies to date have been performed with type II stains of *T. gondii* as used in this study and indeed many host determinants of infection have been well characterised using Type II strains of *T. gondii*. MHC genes largely determine the resistance of mice to encephalitis and development of large brain parasite numbers following infection with type II strains of *T. gondii*. Mice with H2-B or H2-K haplotype are susceptible and those that H2-D haplotype resistant to these aspects of disease [26]. BALB/c (H2-D) when infected with the Beverley strain of *T. gondii* undergo a short period of febrile illness from around day 8–16 which upon resolution is followed by a relatively asymptomatic period where small numbers of tissue cysts can be found in their brains [27]. Also relevant to this study, a recent study has demonstrated that BALB/c/ mice have considerably less changes to their brain transcriptome than C57BL/6 mice and that these changes are more quickly resolved [28]. We have also found that these mice despite their relatively mild disease phenotype undergo previously reported changes in behaviour in the open field test [29].

As the vast majority of humans also develop few tissue cysts and recover to carry mostly asymptomatic infections we wished to determine if this strain of mouse might provide insight into neurochemical changes during relatively mild/asymptomatic *T. gondii* infection. To achieve this, we used a combination of LCMS and RNA-Seq to detect, characterise and compare metabolites/neurochemicals and changes to transcripts in the brains of infected mice, respectively. We demonstrate that in BALB/c mice infected with *T. gondii*, a number of biochemical pathways are perturbed. These studies provide insight into the immunological and molecular processes occurring in the brains of mice with relatively mild disease and provide a basis for future studies to explore the role of each of the pathways in identifying how *T. gondii* might contribute to neuropsychiatric diseases.

2 | Materials and Methods

All animal procedures were carried out according to guidelines from The Home Office of the UK Government. All work was covered by Home Office Licence: PPL60/4568. Group sizes were determined on the basis of Roberts's laboratory previous experience and all experiments were conducted with sufficient sample sizes to give at least an 80% power to detect a difference at the significance level of 5%. In practice this entailed including additional mice in infected groups in case mice had to be euthanised due to reaching humane endpoints. All experimental groups ultimately consisted of at 4–6 mice for metabolomic studies. For RNAseq studies three control and infected mice were used. LCMS data were analysed using Ideom v19 worksheet [30], SIMCA 14 (Umetrics, Sweden) and MetaboAnalyst [31].

All mice were provided by The Biological Procedures Unit (BPU) at University of Strathclyde, which is an ethical multispecies conventional unit licensed to house animals for use in medical research. The cages were provided with bedding, water and food ad libitum. The rooms where the animals were held were on a cycle of 12 h' light and 12 h' dark.

2.1 | *Toxoplasma gondii* Infections

Toxoplasma gondii (Beverley strain) was maintained in CD2 mice by serial passage every 3–6 months. Following enumeration of tissue cysts by microscopy, homogenised brain tissue was diluted and experimental mice female BALB/c mice 20–22 g in weight, obtained from the BPU at the University of Strathclyde were assigned randomly to experimental control or infection groups. Mice were infected intraperitoneally with 10 cysts of *T. gondii* (Beverley strain) suspended in 200 μL of PBS or administered 200 μL of PBS. The Beverley strain of *T. gondii* is a type 2 stain of *T. gondii* with similar growth kinetics to the Me49

strain. Infections were confirmed by observing symptoms and weight loss in infection groups. Mice were euthanised by cervical dislocation at 4 weeks post infection.

2.2 | Sample Preparation for Metabolomics Analysis

Two independent experiments each using the brains of 4–6 infected and control mice were analysed. The right hemisphere of the brain was extracted and stored in 0.1% of formic acid. A methanol/chloroform/water two step extraction protocol was followed. Tissue samples were homogenised with a blender in 4 mL/g cold (0°C) methanol (Fisher, Optima LC/MS Grade), and 0.85 mL/g cold (0°C) water. Homogenates were transferred into chilled tubes then 4 mL/g chloroform (Fisher Chemical) and 2 mL/g water were added to the homogenate. The sample preparation was then centrifuged at 15,000 *g* for 5 min at 4°C to produce a biphasic mix. The upper polar phase was then put into HPLC vials and stored at −80°C until analysis.

2.3 | LCMS Analysis

After metabolite extraction, the samples were analysed by an LC–MS platform that consisted of an Accela 600 HPLC system in combination with an Exactive (Orbitrap) mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Two columns, with complementary abilities were used, zwitterionic ZIC-pHILIC column (150 mm × 4.6 mm; 3.5 μm, Merck, Germany) and the reverse phase ACE C18-PFP column (150 mm × 4.6 mm; 3.5 μm, Hichrom). An injection volume of 10 μL and a flow rate of 0.3 mL/min were employed. A gradient of mobile phase A, 20 mM ammonium carbonate pH 9.2, and mobile phase B, acetonitrile (I) was used to elute the ZIC-pHILIC column. The concentration of buffer A was increased from 20% to 80% over 30 min and then maintained at 92% for 5 min, before equilibrating at 20%. The mobile phases for the reverse phase column consisted of: A, 0.1% (v/v) formic acid in H_2O; B, 0.1% (v/v) formic acid in ACN. Buffer A was decreased from 95% to 10% over 30 min and maintained at 10% for 5 min.

2.4 | Data Processing

Raw data files of the metabolite standard solutions were processed using ToxID 2.1 (Thermo Fisher Scientific Inc. Hemel Hempstead, UK). After visual evaluation of extracted ion chromatograms, the retention times of the standards were used to calibrate IDEOM v19. Briefly, raw files of sample metabolites were processed and converted to mzXML open format using msConvert (ProteoWizard). Chromatograms were extracted using XCMS and stored in PeakML before aligning replicate peaks and combining them using mzMatch.R. After noise filtering and gap filling a CSV file was generated and imported into IDEOM v19 for metabolite identification based on accurate mass (±3 ppm) and retention time prediction. The lipids and peptides were excluded from the lists of putatively identified metabolites in the biological samples. The reason for that the lipid profile is subject to frequent alterations in the mouse brain and therefore it difficult to detect changes and to maintain consistency between mice groups in any experiment [32]. Peptides were removed for the same reason.

2.5 | Metabolite Identification

The metabolites were identified to metabolic standards initiative (MSI) level 1 by matching accurate mass and retention time with the mass (< 3 ppm) and retention time (±0.2 min) obtained for an authentic standard. Where no standards were available, metabolites could be identified from MS^2 spectra (fragmentation). These metabolites are referred to as 'confirmed'. Remaining metabolites were putatively identified to MSI level 2 based on calculated mass and predicted retention time. These are referred to as 'putative' metabolites.

2.6 | Metabolomics Statistics

The file produced by mzMatch were further analysed by IDEOM v19, and the student t test was performed to establish the presence of possible significant differences between the metabolites detected in different groups. *Q* values (false discovery rate [FDR] adjusted *p* values) were calculated to determine significance based on $q \leq 0.5$. *Q* values differed by several orders of magnitude and are therefore given as −log10 *q* values. A significant change has a −log10 *q* value ≥ 1.3. We chose to focus on metabolites showing relatively large differences (≥ 2-fold). These are expressed as log2 fold-change (FC) values, a 2-fold increase has a log2 FC of 1.0 and a 2 fold decrease has a log2 FC of −1.0. Further analysis was carried out using SIMCA 14 (Umetrics, Sweden) software, for processing principal components analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), which were carried out to visualise the maximal metabolic alterations between each group in all experiments. OPLS-DA ranks metabolites by Variable Importance for the Projection (VIP) scores, with a VIP value ≥ 1.0 indicating that the metabolite makes an important contribution to the difference between control and infected groups. MetaboAnalyst server and the BioCyc collection of Pathway/Genome Databases was used for further analysis.

2.7 | Isolation and Extraction of Total RNA From Mouse Brain

A sagittal slice of the left hemisphere of the brain was stored in RNA *later* at −80°C. An RNeasy purification kit (Qiagen) was used to extract total RNA from the mouse brain. RNA *later* stabiliser reagent was removed, and the mouse brain was then weighed. 15–20 mg of brain tissue was mixed with 600 μL of RLT-plus buffer containing 6 μL of 2-mercaptoethanol and homogenised. The cell lysate was transferred to a QIAshredder column (Qiagen) and centrifuged at 24,000 *g* for 2 min. The eluate was transferred to another tube and centrifuged for 3 min at 24,000 *g*. The supernatant was loaded onto to a gDNA eliminator column (Qiagen) and centrifuged for 1 min at 8000 *g* to remove genomic DNA. Total RNA was purified from the eluate using an RNAeasy column (Qiagen) according to the manufacturer's instructions. RNA was eluted in a final volume of 50 μL of RNAse free water and stored at −20°C.

2.8 | RNA-Seq

The integrity and concentration of each RNA sample was determined with a 2100 Bioanalyzer (Argilent Technologies) using the RNA 6000 Nano kit (Argilent Technologies). All samples had an RNA integrity number (RIN) ≥8.0 and an RNA concentration ≥20 ng/mL. A total of 1 μg of each RNA sample was sent to GATC biotech for RNA-Seq and transcriptomic analysis.

2.9 | Expression Analysis

The RNA-Seq analysis was carried out by GATC Biotech. Reads were aligned to the reference genome using Bowtie and potential exon-exon splice junctions were identified with TopHat. Then transcripts were identified and quantified using Cufflinks, before merging the transcript pieces to full length transcripts with Cuffmerge, The same program annotates the transcripts based on the given annotations. Finally, Cuffdiff calculated the fragments per kilobase per million mapped fragments (FPKM) for the uninfected and infected groups and then calculated the fold change, to determine which genes were differentially expressed genes (DEGs). Cuffdiff also calculated *p*-value and *q*-value (FDR adjusted *p* values) to determine if the fold change was significant. The results shown in paper are from a single experiment with three biological replicates per group (infected and uninfected). A DEG is defined as having a log2 FC ≥1 or ≤−1 and −log10 *q* ≥1.3.

3 | Results

3.1 | *Toxoplasma gondii* Infection Induces Global Metabolomic Changes in the Brain

The pHILIC column, following manual curation and filtration successfully identified 171 metabolites with high confidence and the C18-PFP column identified 83 metabolites with high confidence. Forty-eight of these metabolites were identified using both columns adding an additional degree of confidence (this detailed in Table S1).

Data from both columns were pooled to form a single table of peak intensity values for each metabolite (Table S1). Where a metabolite was detected using both columns, the peak intensity value for the most well-defined peak was selected. Initial principal component analysis (PCA) of peak intensity values from the combined pHILIC and C18-PFP data set confirmed that infected and non-infected mouse brains formed distinct groups (Figure 1a). Principal components 1 and 2 represent 28.7% and 23.2% of the variance respectively, Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to identify metabolites associated with acquired *T. gondii* infection (Figure 1b). OPLS-DA is a supervised pattern recognition process that allows identification and ranking of metabolites that are associated with mouse infection status. This produced a list of metabolites ranked by VIP (Variable Importance for the Projection) scores, with a VIP value ≥1.0 indicating that the metabolite makes an important

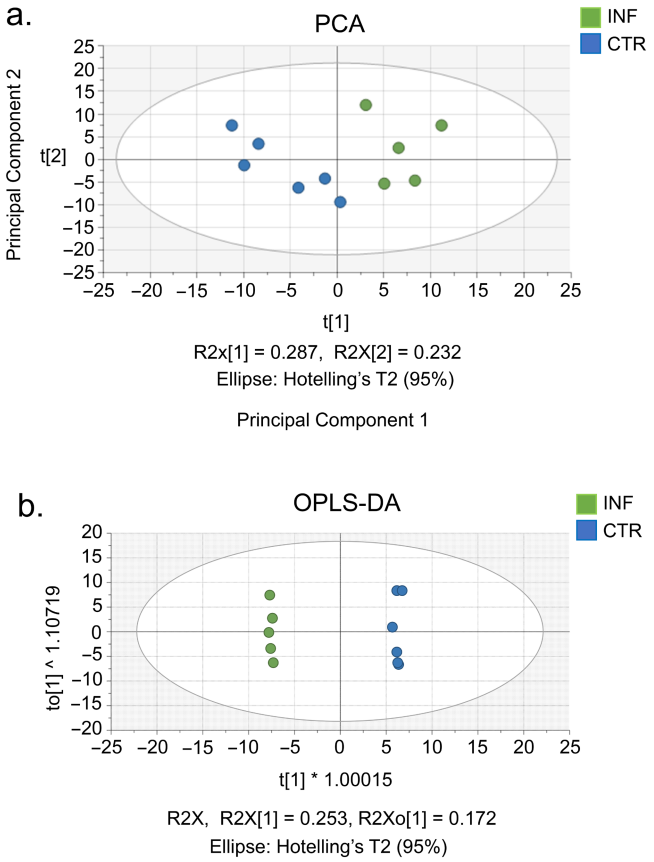

FIGURE 1 | Multivariate analysis. (a) Principal component analysis (PCA). Score plot for metabolites identified using pHILIC and C-18-PFP columns shows an excellent separation between infected (IFN) mice group and control groups (CTR), each data point represents one mouse brain sample. R2X[1] and R2X[2] indicate the proportion of the variance explained by and second principal components. Only the first and second principal components are shown. Data from experiment 1. Similar results were obtained for experiment 2. (b) Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA). Score plot for metabolites identified from pHILIC and C18-PFP columns shows an excellent separation between infected mice group and control groups, each data point represents one mouse brain sample. Data from experiment 1. Similar results were obtained for experiment 2.

contribution to the difference between the uninfected and infected groups. Fold change (infected/uninfected) and *q*-values (FDR corrected *p*-values) were also calculated for each metabolite. A volcano plot identified 27 metabolites with a significant difference in the infected animals, 22 with increased levels and 5 with decreased levels (Figure 2). All of these metabolites have a VIP value greater 1.5. A hierarchical clustering heat map for the 27 metabolites shows the variation in peak intensity between the biological replicates (Figure 3). Table S1 includes VIP, Log2 fold change (FC) and −log10 *q* values for each metabolite identified in two independent experiments. Experiment 1 included 5 infected mice and 6 uninfected control mice; experiment 2 included 4 infected and 6 control mice. The 14 metabolites showing the largest fold change are included in Table 1. Theses also had the highest VIP values and statistically significant *q* values and gave consistent results in the two experiments.

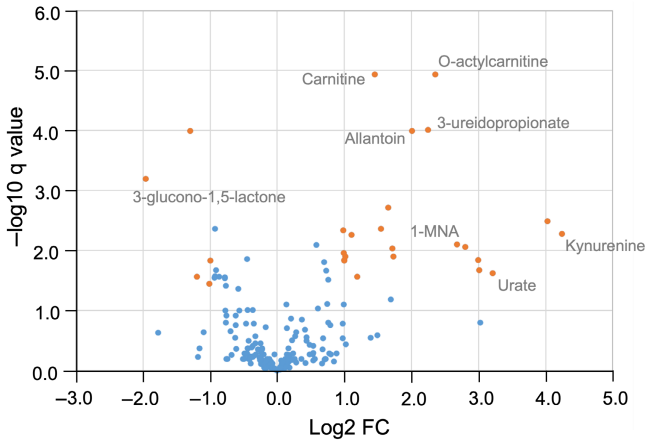

FIGURE 2 | Volcano plot of metabolomic data. Volcano plot of Log2 fold change (FC) of metabolite peak intensity (Infected animals/uninfected) against −log10 q value. Significant changes (Log2 FC ≥ 1.0 or ≤ −1.0 and −log10 q ≥ 1.3) are shown by red circles. Metabolites of interest are labelled. 1-MNA, 1-methylnicotinamide. Data from experiment 1.

3.2 | *Toxoplasma gondii* Infection Induces Transcriptomic Changes in the Brain

A volcano plot of 17,580 transcripts identified 663 DEGs between control and infected mice based on having Log2 FC ≥ 1 or ≤ −1 and −log10 q ≥ 1.3 (Figure 4). Of these, 609 genes were upregulated in infected mice relative to control mice and 54 genes were downregulated in infected mice when compared with the control group. Table S2 shows log2 fold change and −log10 q values for the DEGs and the data for all transcripts detected is in Table S3.

3.3 | Combining Transcriptomic Data and Metabolomic Data Provides Insight Into Pathways Most Perturbed by *Toxoplasma gondii* Infection

The significantly altered transcript of the DEGs were uploaded with the metabolite data to the BioCyc pathway Database Collection. This step was done to obtain comprehensive picture of the pathways affected at the transcript and metabolite level. The BioCyc software orders pathways according to pathway perturbation scores (PPS) to identify pathways that are most affected. Metabolic pathways most affected between the infected group and their controls were found to include those involved in purine metabolism, tryptophan metabolism, arginine metabolism and L-carnitine biosynthesis. Metabolites from these pathways also scored high in terms of their VIP score and were often individually statistically significant between infected and non-infected control samples. These pathways are discussed below.

3.4 | *Toxoplasma gondii* Induces Purine Degradation in the Brain

Mice infected with *T. gondii* were found to have large and statistically significant increases in urate and allantoin in two independent experiments (Table 1). A smaller increase in xanthine and a decrease in adenosine were also observed, however these changes were statistically significant (− log10 q ≥ 1.3) in only one of the two experiments (Table S1). The identities of the above metabolites were confirmed using authentic standards (MSI level 1). Overall, these data indicate that *T. gondii* infection induces purine degradation in the brains of mice. Transcripts for two genes in the purine degradation pathway, adenosine deaminase (Ada) and xanthine dehydrogenase (Xdh) were found to be significantly upregulated (−log10 q = 1.90 and 2.72, respectively) in the brains of *T. gondii*-infected mice relative to non-infected control mice. These results are consistent with the depletion of adenosine and accumulation of xanthine, urate and allantoin as observed in the brains of mice infected with *T. gondii* as measured by LCMS (Figure 5).

3.5 | *Toxoplasma gondii* Induces Upregulation of the Kynurenine Pathway for Tryptophan Catabolism

Mice infected with *T. gondii* were found to have large and statistically significant increased levels of L-formylkynurenine (VIP, 1.45; log2 FC, 3.00; −log10 q 1.85) and kynurenine (VIP, 1.48; log2 FC, 4.24; −log10 q 1.85) with consistent results in an independent experiment. This data suggests that *T. gondii* infection directs tryptophan metabolism towards kynurenine rather than serotonin in the brains of mice (Figure 6). Transcripts for one gene in the tryptophan degradation pathway, arylformamidase (Afmid) were found to be significantly upregulated (log2 FC, 0.85; −log10 q, 1.53) in the brains of *T. gondii*-infected mice relative to non-infected control mouse brains. This result is consistent with the accumulation of kynurenine in the brains of mice infected with *T. gondii* as measured by LCMS.

3.6 | Levels of the Neurotransmitters Serotonin and Dopamine

Conditions of LCMS using the C18-PFP column were optimised for detection of serotonin and catecholamines including dopamine. Serotonin, produced from tryptophan (Figure 6), showed a small increase in the infected mouse brain, but this was not statistically significant (VIP, 0.99; Log2 FC, 0.68; −log10 q, 0.54). Consistent results were obtained in an independent experiment (Table S1). Dopamine was also identified but the levels detected showed no significant difference in the infected animals (VIP, 1.12; Log2 FC, 0.76; −log10 q, 0.80). This result was also confirmed in an independent experiment (Table S1).

3.7 | *Toxoplasma gondii* Induces Up-Regulation of NAD+ Consumption and Increased 1-Methylnicotinamide Synthesis

Brain extracts from *T. gondii*-infected mice showed a large increase (>4-fold) in 1-methylnicotinamide (−log10 P, 2.10; VIP, 1.4), with similar results in an independent experiment (Table 1). 1-Methylnicotinamide is produced from nicotinamide by nicotinamide N-methyltransferase (NNMT) using S-adenosylmethionine as the methyl donor. A search of the

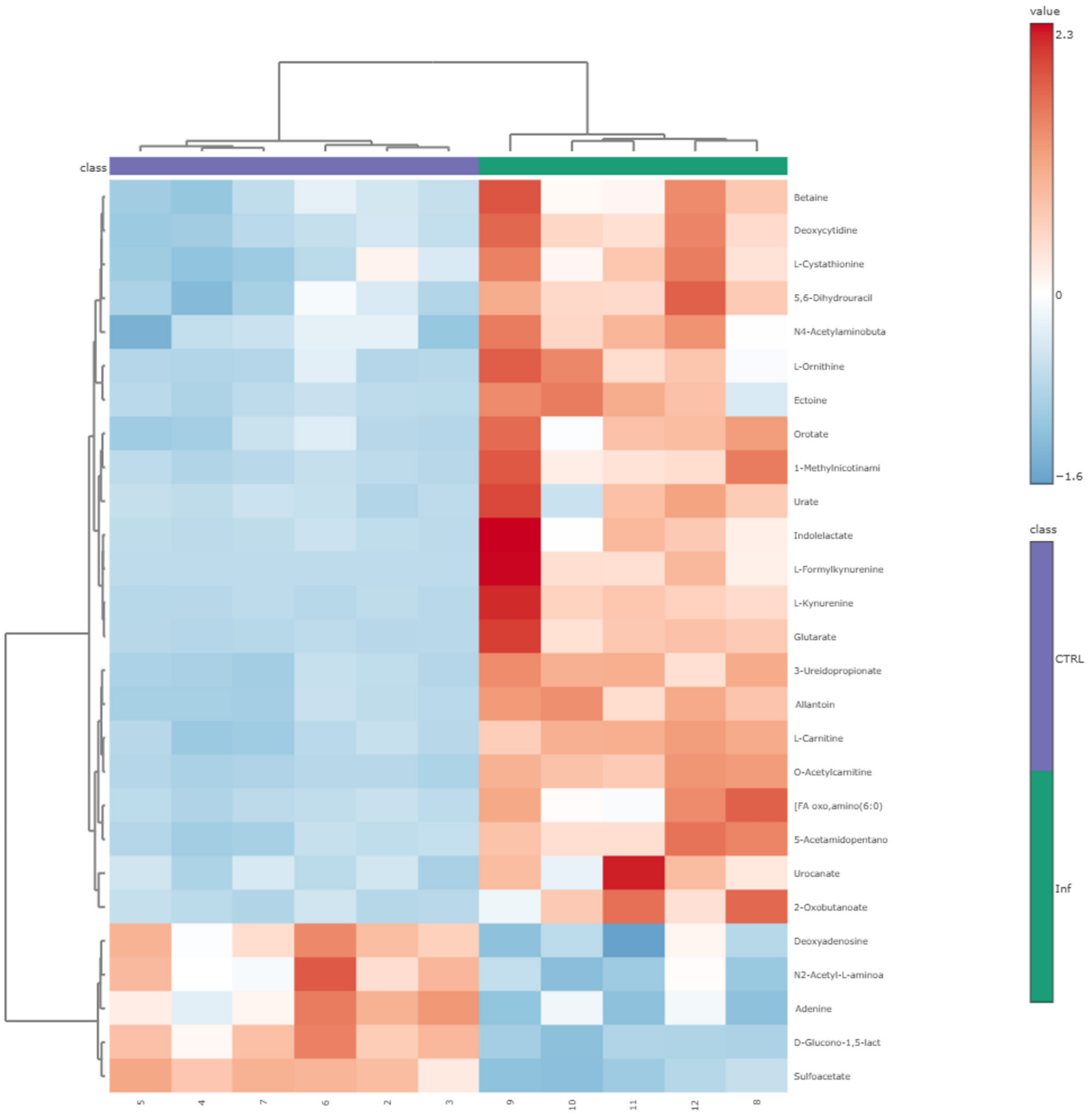

FIGURE 3 | Hierarchical clustering heatmap of metabolomic data. The 27 metabolites showed significant differences in peak intensity (Log2 FC ≥ 1.0 or ≤ −1.0 and −log10 q ≥ 1.3) in the infected mice. Data from experiment 1.

transcriptome identified genes involved in nicotinamide and NAD+ metabolism (Figure S1). NNMT was not found, however transcription of genes for enzymes that consume NAD+ and produce nicotinamide as a by-product were significantly upregulated (Figure S1), including protein mono ADP-ribosyltransferases (PARP3, PARP9, PARP11, PARP12 and PARP14) and cyclic ADP-ribose synthase (CD175). The gene encoding DTXL, an E3 ubiquitin ligase gene that forms a complex with PARP9, was also upregulated. PARP activity can have a significant effect on intracellular NAD+ availability. Analysis of the transcriptome revealed only small changes in the transcription of genes for enzymes from pathways for NAD+ biosynthesis from nicotinic acid or tryptophan and recycling from nicotinamide, but these were not statistically significant (Figure S1).

3.8 | *Toxoplasma gondii* Induces L-Carnitine Biosynthesis

Mice infected with *T. gondii* were found to have consistently higher levels of L-carnitine in independent experiments (Table 1). There was a smaller increase in 4-trimethylammoniobutanoate, the biochemical precursor of L-carnitine, but this was statistically significant in only one of two experiments (Table S1).

TABLE 1 | Metabolites showing large and statistically significant differences in the brain extracts from infected mice.

	Experiment 1			Experiment 2		
Metabolite	**VIP**	**Log2 FC**	**−Log10 *q***	**VIP**	**Log2 FC**	**−Log10 *q***
O-Acetylcarnitine[a]	1.61	2.36	4.93	1.59	1.86	3.19
L-Carnitine[a]	1.61	1.46	4.93	1.52	1.59	2.25
Allantoin[a]	1.58	2.01	3.99	1.54	1.86	2.37
3-Ureidopropionate[a]	1.58	2.24	4.01	1.47	2.11	1.85
D-Glucono-1,5-lactone[b]	1.55	−1.96	3.20	1.38	−1.36	1.48
5-Acetamidopentanoate[b]	1.53	1.66	2.72	1.52	1.49	2.29
Glutarate[a]	1.51	4.02	2.49	1.54	3.15	2.37
Deoxycytidine[b]	1.50	1.55	2.37	1.48	1.34	1.85
5,6-Dihydrouracil[b]	1.49	1.11	2.26	1.45	1.01	1.69
L-Kynurenine[a]	1.48	4.24	2.28	1.47	4.05	1.77
L-Formylkynurenine[b]	1.45	3.00	1.85	1.54	2.66	2.06
1-Methylnicotinamide[a]	1.45	2.68	2.10	1.58	2.04	3.19
Urate[a]	1.38	3.21	1.62	1.53	3.62	2.29
Indolelactate[a]	1.37	3.01	1.68	1.44	1.38	1.64

Note: This table shows log2 fold change in metabolite peak intensities (infected/uninfected) with VIP values ≥ 1.0 and −log10 *q* values ≥ 1.3 confirming significance.
[a]Metabolites in bold were identified to MSI level 1 by accurate mass and matching retention time with an authentic standard and/or MS2 analysis (fragmentation).
[b]Metabolites were identified to MSI level 2.

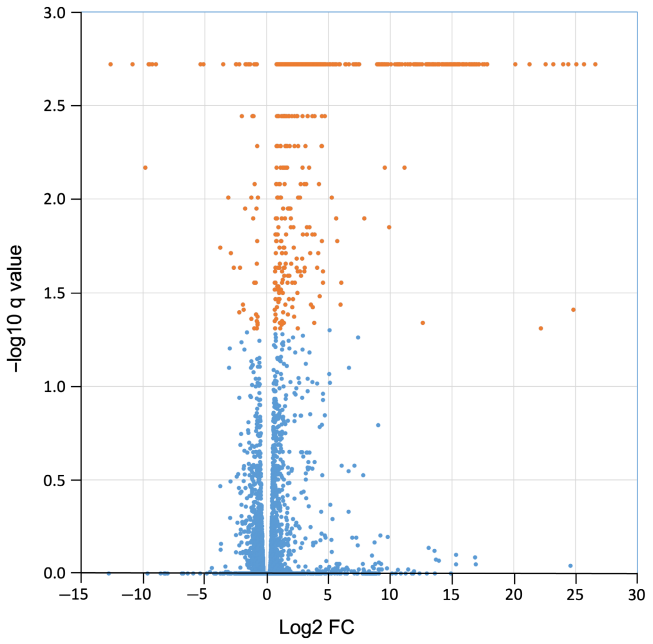

FIGURE 4 | Volcano plot of transcriptomic data. Volcano plot of Log2 fold change (FC) of FPKM values (Infected animals/uninfected) against −log10 *q* value. Significant changes (Log2 FC ≥ 1.0 or ≤ −1.0 and −log10 *q* ≥ 1.3) are shown by red circles.

Overall these results suggest the pathway for L-carnitine biosynthesis is upregulated in the *T. gondii* infected brain. The main function of carnitine is in the transfer of long chain fatty acids across the mitochondrial membrane for degradation by b oxidation. Notably, levels of O-acetylcanitine, one of the most abuntant metabolites of carnitine was significantly increased in the brains of the infected mice (Table 1).

3.9 | Upregulation of Genes Associated With *Toxoplasma gondii* Infection and Microglia Activity

Previous studies identified genes that were upregulated in *T. gondii* infection or were known to be involved in the innate immune response to the parasite [17, 33, 34]. A manual search of the RNA-Seq data revealed significant differences in expression of these genes also occurred in this study (Table 2). Transcripts of the IFN-γ inducible nitric oxide synthase 2 (NOS2) was found to be significantly upregulated in the brains on *T. gondii*-infected mice relative to non-infected control mouse brains (Table 2). Microglia play a pivotal role in the immune response to parasitic infection of the brain. Table 3 shows genes known to be involved in microglia activity that were upregulated in the *T. gondii* infected mice. These include CD36, TLR2 and TLR4.

3.10 | Transcripts Associated With Antioxidant Responses Were Altered in Infected Mice

Glutathione peroxidase 3 (Gpx3) is a glutathione dependent enzyme that catalyses the reduction of hydrogen peroxide and fatty acid peroxides. It is an extracellular enzyme found in plasma and down regulation, as seen in the *T. gondii* infected brain (Table 4) could increase oxidative stress. Glutathione-S-transferases enzymes (GSTs) conjugate reduced glutathione to target molecules and function in cellular detoxification. Glutathione S-transferase Mu1 (GSTM1) is significantly downregulated in the

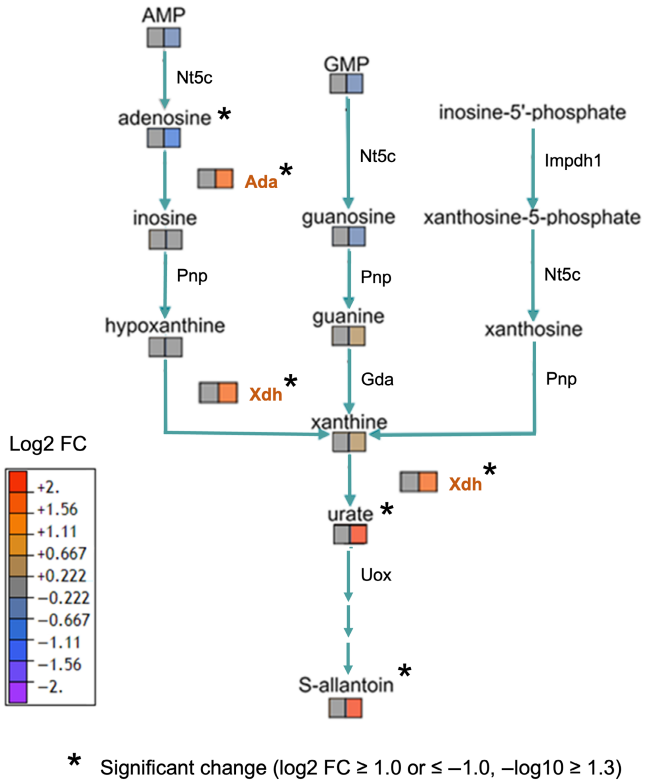

FIGURE 5 | Purine degradation pathway. Levels of metabolite (peak intensity) and transcripts (FPKM) are represented by two squares: The first is the uninfected control and the second is the infected group. The Log2 transformed data are visualised as a colour spectrum scaled from least to most abundant from −2 to 2. Purple indicates low expression, while red indicates high expression. The changes observed for adenosine, urate and allantoin were significant (Log2 FC ≥ 1.0 or ≤ −1.0 and −log10 q ≥ 1.3). Smaller changes in GMP, guanosine, guanine and xanthine were also observed but these were not significant. Metabolites shown without two squares were not detected in the metabolomic profile. The transcripts that were found to be significantly different between infected and non-infected mice are Ada (adenosine deaminase) and Xdh (xanthine dehydrogenase). Transcripts found in the data that were not significantly different are also shown. These were Nt5c (cytoplasmic nucleosidase), Pnp (purine nucleoside phosphorylase), Gda (guanine deaminase), Impdh1 (Inosine monophosphate dehydrogenase) and Uox (urate oxidase). Data from experiment 1.

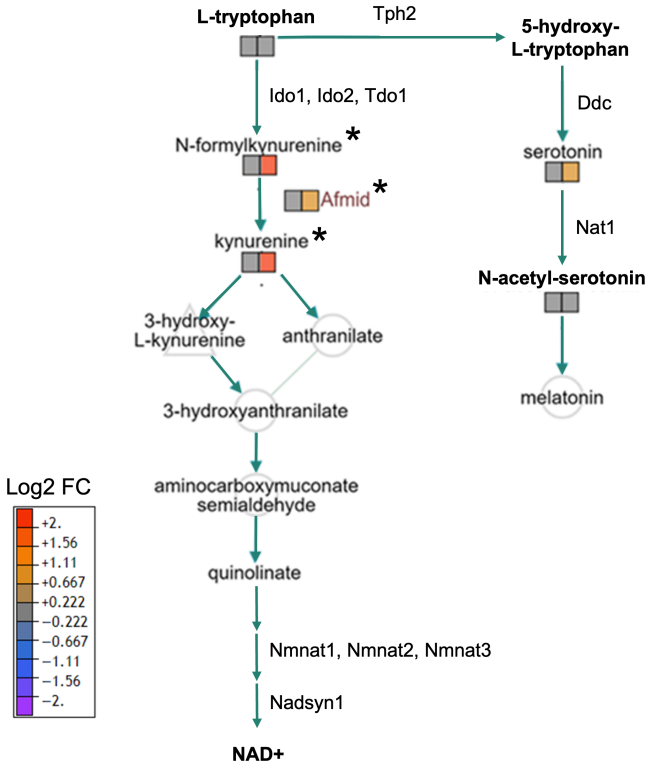

FIGURE 6 | Tryptophan degradation pathway. Levels of metabolite (peak intensity) and transcripts (FPKM) are represented by two squares: The first is the unifected control and the second is the infected group. The Log2 transformed data are visualised as a colour spectrum scaled from least to most abundant from −2 to 2. Purple indicates low expression, while red indicates high expression. Metabolites that were found to change significantly are L-formyl kynurenine and kynurenine (Log2 FC ≥1.0 or ≤−1.0 and −log10 q ≥1.3). A smaller change in serotonin was not significant. Metabolites shown without two squares were not detected in the metabolomic profile. The transcript that was found to be significantly different between infected and non-infected mice is Afmid (arylformamidase). Transcripts detected in the data that did not show a significant change are also shown. These include NMNAT (nicotinamide mononucleotide adenyltransferase), NADsyn1 (NAD+ synthetase) and Tph2 (tryptophan hydroxylase2). Data from experiment 1.

T. gondii infected mice (Table 4). GSTM1 was recently found to enhance the activation of microglia in response to TNF-α stimulation [35].

4 | Discussion

This study has integrated of transcriptomic and metabolomic data to provide a powerful investigation of the effect of *T. gondii* infection on the brains of BALB/c mice. Using these two techniques not only provides a more complete view of the effects of infection, but also provides greater confidence in results obtained. BALB/c mice were chosen for these studies as they are resistant to toxoplasmic encephalitis when infected with type-II strains of *T. gondii* as used in this study and they recover from acute infection quickly and develop relatively low cyst burdens. Despite this we have found previously that infection induces changes to their behaviour as measured in the open field test [36]. A previous study has demonstrated that *T. gondii* infected BALB/c mice have reduce neuropathological and inflammatory transcripts than infected C57BL/6 mice [28]. The results demonstrate that *T. gondii* has profound effects on a number of host metabolic pathways including purine and pyrimidine ribonucleosides and tryptophan degradation. Many of these pathways have relevance to psychoneurological and degenerative brain diseases.

4.1 | Purine Degradation Pathway

Purine metabolites are essential precursors for nucleic acid synthesis, but they also act as metabolic signals, provide

TABLE 2 | Transcripts of genes previously associated with *T. gondii* infection were significantly upregulated in infected mice.

Name	Gene ID	RefSeq ID	Log2 FC	−Log10 *q*
Nitric oxide synthase 2, inducible	Nos2	NM_010927	3.23	2.72
Complement C1Q A	C1qa	NM_007572	2.96	2.72
Complement C1Q B	C1qb	NM_009777	2.97	2.72
Complement C1Q C	C1qc	NM_007574	3.04	2.72
CD36	Cd36	NM_001159558	2.12	2.72
Guanylate binding protein 2	Gbp2	NM_010260	4.94	2.72
Guanylate binding protein 3	Gbp3	NM_001289493	4.55	2.72
Guanylate binding protein 5	Gbp5	NM_153564	4.21	2.72
Guanylate binding protein 7	Gbp7	NM_001083312	4.26	2.72
Guanylate binding protein 11	Gbp11	NM_001039647	3.77	2.72
Glial fibrillary acidic protein	Gfap	NM_010277	1.40	2.72
IFN-g inducible thiol reductase	Ifi30	NM_023065	1.59	2.72
Suppressor of cytokine signalling 1	Socs1	NM_001271603	2.64	2.72
Interferon gamma induced GTPase	Igtp	NM_018738	6.37	2.72
Interferon gamma induced GTPase 1	Iigp1/Irga6	NM_001146275	7.14	2.72

Note: Log2 FC ≥ 1.0 represents a ≥ 2-fold increase. A −log10 *q* value ≥ 1.3 is statistically significant (FDR adjusted *p*-value ≤ 0.05).

TABLE 3 | Genes known to be associated with microglia activity were significantly upregulated in infected mice.

Name	Gene ID	RefSeq ID	Log2 FC	−Log10 *q*
Allograft inflammatory factor 1	Aif1	NM_019467	2.74	2.72
CD14 antigen	Cd14	NM_009841	1.39	1.63
CD36 antigen	Cd36	NM_001159558	2.12	2.72
CD33 antigen	Cd33	NM_001111058	2.01	2.72
CD68 antigen	Cd68	NR_110993	1.30	2.72
C-X3-C motif chemokine receptor 1	Cx3cr1	NM_009987	1.29	2.44
Interleukin 1 beta	Il1b	NM-008361	3.95	2.72
Toll-like receptor 2 (TLR2)	Tlr2	NM_011905	3.07	2.72
Toll-like receptor 4 (TLR4)	Tlr4	NM_021297	1.29	2.72
Toll-like receptor 9 (TLR9)	Tlr9	NM_031178	3.52	2.72
Transmembrane protein 119	Tmem119	NM146162	1.94	2.72
Tumour necrosis factor	Tnf	NM_013693	9.11	2.72
Triggering receptor expressed on myeloid cells 2	Trem2	NM_031254	1.95	2.72

Note: Log2 FC ≥ 1.0 represents a ≥ 2-fold increase. A −log10 *q* value ≥ 1.3 is statistically significant (FDR adjusted *p*-value ≤ 0.05).

TABLE 4 | Transcripts associated with antioxidant responses were decreased in infected mice.

Name	Gene ID	RefSeq ID	Log2 FC	−Log10 *q*
Glutathione S-transferase mu1	Gstm1	NM_010358	−0.78	2.28
Glutathione peroxidase 3	Gpx3	NM_008161	−1.34	2.72

Note: Log2 FC ≥ 1.0 represents a ≥ 2-fold increase. A −log10 *q* value ≥ 1.3 is statistically significant (FDR adjusted *p*-value ≤ 0.05).

energy, control cell growth, form part of essential coenzymes, contribute to sugar transport and donate phosphate groups in phosphorylation reactions [37]. Purine metabolites such as (Adenosine triphosphate) ATP and adenosine have been demonstrated as significant neuronal co-transmitters with GABA, acetylcholine, glutamate, noradrenaline and dopamine [38]. A previous study showed that hypoxanthine levels were upregulated in the brains of toxoplasma infected mice [39]. Herein, the results demonstrate that *T. gondii* infection increases purine catabolism, leading to a reduction in adenosine and accumulation of uric acid and allantoin in the brains of infected mice. In humans where the urate oxidase gene Uox is a pseudogene the end product of purine catabolism is uric acid rather than allontoin [40]. Humans do not synthesise allantoin and uric acid is the end product of the purine degradation pathway. As a compensatory adaptation to reduce the risk of uric acid over production, xanthine dehydrogenase expression in humans is strongly repressed and the purine salvage pathway from hypoxanthine is upregulated [40, 41]. However, IFN-γ is known to be produced during a *T. gondii* infection and has been shown to increase uric acid production in human hepatocytes by upregulating xanthine dehydrogenase activity via the JAK/STAT1 pathway [42]. Thus, despite the significant differences in purine catabolism described above, *T. gondii* infection in humans could also result in upregulation of xanthine dehydrogenase and favour uric acid production.

Consistent with these observations the transcriptomic data found transcripts for adenosine deaminase (Ada) and xanthine dehydrogenase to be raised in the brains of infected mice. Changes to purine levels could have neurological consequences as adenosine is known to have neuromodulatory effects throughout the brain, affecting fundamental processes such as normal neuronal signalling, learning and memory, astrocytic function, anxiety and stress response [43]. Along with these normal physiological processes, adenosine and its receptors are also involved in neuropathologies such as Parkinson's disease [44] and schizophrenia [45]. Therefore, any alteration resulting in changes to adenosine levels could have profound effects. In addition to alteration to purine metabolism, transcripts for the purinergic receptors adenosine A3 receptor (Adora3); purinergic receptor P2X, ligand-gated ion channel, 7 (P2rx7); and pyrimidinergic receptor P2Y, G-protein coupled, 6 (p2ry6) were increased (Table S2).

Previous studies assessed the purine levels and adenosine deaminase (Ada) activity in the brain of mice (BALB/c) experimentally infected with *T. gondii*. The results of chronic infection were similar to our results, since purine metabolites were catabolised and uric acid accumulated and increased. Ada activity also was increased in the chronic stage of infection [46]. Ada has been demonstrated to be involved in the development and maintenance of the immune system in mammals including humans and associated with differentiation of epithelial and monocytes [47]. A study has implicated abnormalities of immune functions including T cell activation in people with Parkinson's Disease with high serum Ada activity [48]. The ability of *T. gondii* infection to alter Ada levels in humans and any potential consequence for neurodegenerative diseases remains to be determined.

Alteration of purine levels could also have direct effects on *T. gondii* as it is a purine auxotroph and therefore relies on scavenging host cell purines. Although it has the capability to use host adenine, hypoxanthine, xanthine, guanine, guanosine and inosine, adenosine is thought to be the most important salvaged purine [49–51]. Thus, degradation of purine might deprive *T. gondii* of this essential resource. Importantly studies in different organs of mice demonstrate that IFN-γ is capable of inducing xanthine oxidase (XO) which catalyses the oxidation of xanthine to uric acid [52]. Consequently, purine degradation might represent a host evolved mechanism to limit the growth of pathogens such as *T. gondii*, but with potential neurological consequences.

4.2 | Tryptophan Degradation Pathway

Tryptophan metabolism is important for production of the neurotransmitters, serotonin and tryptamine and the neurotransmitter-like substance, melatonin. Tryptophan can also be degraded through the kynurenine metabolism in the brain to give rise to a number of products, some of which are known to be neuro-active or even neurotoxic. In a number of studies, depletion of tryptophan has been demonstrated to result in reduction of brain serotonin levels and depression [53]. In the current study serotonin levels were not found to be altered which is consistent with a previous study of *T. gondii*-infected [54]. However, levels of kynurenine were found to be significantly increased in the brains of infected mice compared with control mice indicating degradation of tryptophan via the kynurenine pathway. Consistent with this, transcripts for arylformamidase (Afmid) were raised in the brains of infected mice compared with brains from control non-infected mice. In the literature, there is a growing body of evidence that the kynurenine metabolite can contribute to the pathophysiology of several diseases such as acquired immunodeficiency syndrome (AIDS)-related dementia, Alzheimer's disease and schizophrenia [55–60]. These studies demonstrated that overproduction of kynurenic acid (KYNA), another kynurenine derived metabolite, is associated with cognitive impairment in brain diseases. The kynurenine pathway is initiated by the oxidative ring opening of tryptophan by either indoleamine 2, 3-dioxygenase (IDO) or tryptophan 2, 3 dioxygenase (TDO). Importantly, in the context of *T. gondii* infection, IDO is known to be induced by IFN-γ and the process of tryptophan degradation has been implicated in host defence as *T. gondii* is a tryptophan auxotroph [61, 62]. In the brain, the L-kynurenine is enzymatically converted in microglial cells and astrocytes. In schizophrenia, there is a persistent reduction of microglial kynurenine 3-monooxygenase activity, along with increased L-kynurenine influx from the circulation. This results in increased kynurenic acid formation in astrocytes which leads to inhibition of α-7 nicotinic (α7nAch) and the N-methyl-D-aspartate (NMDA) receptors which leads to cognitive impairments [63].

Induction of the kynurenine pathway during *T. gondii* infection is a likely result of the immune response and IFN-γ production. The resultant tryptophan degradation is likely to be host protective through depriving the parasite of tryptophan, but the accumulation potentially neurotoxic metabolites could be responsible for adverse effects and trigger brain diseases and dysfunction.

4.3 | NAD+ Consumption and PARP Activity

In cells that express the kynurenine pathway, tryptophan degradation can lead to NAD+ synthesis. In this study, transcripts for enzymes that convert quinolinate to NAD+ were identified, but expression did not change in the *T. gondii* infected mice. However, a group of enzymes that consume NAD+ were significantly upregulated. Poly ADP ribose polymerases (PARPs) are regulatory proteins that transfer one or more ADP ribose residues from NAD+ to a target nucleotide or protein generating nicotinamide (NAM) as a by-product. In this study PARP3, PARP4, PARP9, PARP11, PARP12 and PARP14 were upregulated in the brains of the *T. gondii* infected mice. PARP9 and PARP14 are interferon sensitive genes that have opposing effects on IFN-γ induced macrophage activation. PARP14 is anti-inflammatory, it suppresses IFN-γ induced responses by catalysing ADP-ribosylation of STAT1, inhibiting its activation by phosphorylation [64]. PARP9 is catalytically inactive but forms a complex with PARP14 inhibiting its activity. The upregulation of PARP9 and PARP14 observed in this study is consistent with an inflammatory response in the *T. gondii* infected brains.

4.4 | Methylnicotinamide Synthesis

PARP activity could reduce local NAD+ levels and increase NAM levels. In this study there was a significant increase in methylnicotinamide (MNAM) which is produced from NAM and S-adenosyl methionine in a reaction catalysed by nicotinamide N-methyltransferase (NNMT). MNAM has been shown to act as an inhibitor of NLRP3 inflammasome activation in LPS and treated macrophages [65]. NMAN has also been shown to have a neuroprotective effect on LPS induced mice, attenuating LPS-induced cognitive impairment and inflammation [66]. Thus the observed increased levels of MNAM seen in *T. gondii* infected animals could be host protective.

4.5 | Carnitine Metabolism

It is acknowledged that the glucose is the primary energy source for the brain. However, under some conditions such as fasting, fatty acids work as an alternative energy metabolism for the brain. Significant alterations of carnitines and phospholipids indicate variation of fatty acids oxidations metabolism. In this experiment, L-carnitine and its derivative, Acetyl carnitine were found to be increased in the brains of infected mice. Up regulation of Acetyl-carnitine is consistent with previous findings [39]. Acetyl-carnitine regulates the activity of many mitochondrial enzymes which are involved in the citric acid cycle, the gluconeogenesis, the urea cycle and the fatty acids oxidation, as its acetyl groups are incorporated into brain lipid metabolism [67, 68]. In addition, acetyl carnitine plays a significant role in neuroprotection and has beneficial effects in major depressive disorders and Alzheimer's disease [69].

4.6 | Dopamine

The dopamine synthesis pathway involves several enzymes and cofactors, any one of which could be manipulated to yield alteration in dopamine levels. A previous study in the literature demonstrated that *T. gondii* has the ability to directly up regulate the dopamine levels of infected neuronal cells [70]. However, in the current study, no significant differences in dopamine levels were detected in the brains of infected mice. Differences between these studies might reflect the timing of sampling.

4.7 | Immune Response to *Toxoplasma gondii* and Microglial Activation

In a previous study, gene expression analysis of female Swiss Webster mice brain infected with *T. gondii* using full genome microarrays demonstrated that expression of genes such as those encoding suppression of cytokine signalling 1 (SOCS1), CD36, complement component 1 (C1q), and glial fibrillary acidic protein (GFAP) were highly expressed [17]. Another analysis of gene expression of female BALB/c mice brain infected with *T. gondii* using RNA-Seq showed that a similar set of genes Socs1, Cd36, C1q and Gfap were increased [33]. Consistent with these findings, our results demonstrated that particular genes were upregulated (Socs1, Cd33, Cd36, C1qa, C1qb, C1qc, and Gfap). GFAP is a transmembrane receptor mainly expressed by microglial cells in the brain that regulates innate immune responses are upregulated in the brains of mice with *T. gondii* infection. Upregulation of this receptor has been correlated with high risk of AD. In mouse models of AD an increase in CD33 levels has been shown to slow microglial phagocytosis by increasing plaque deposition in the brain [71, 72]. A meta-analysis in human studies has demonstrated that different variants in CD33 were associated with AD [73].

Microglial phenotype regulation is mainly dependent on their interaction with molecules released by surrounding cells or associated with pathogens, through membrane bound pattern recognition receptors (PRRs) [74]. DEGs identified in this study include components of TLR meditated signalling pathways (CD14, CD36, TLR2, TLR4 and TLR9), all significantly upregulated in the brains of the *T. gondii* infected mice. TLRs are a major family of PRRs, and many of them are expressed by microglia. TLR-mediated signalling has been suggested to be both beneficial and detrimental depending on the receptor involved in the response [75]. For instance, TLR9 stimulation has a positive role to reduce neurodegenerative pathology in mice [76]. However, chronic microglia activation through ligation of TLR4 expressed on microglia, increases neuroinflammation and pathology [77]. In contrast, TLR4 may play a protective role in AD and enhance cognitive function [78]. These conflicting results regarding TLR4 could be explained by understanding other parameters in its function and whether it is in complex with TLR2 or co-receptors CD14 and CD36 and thus how it initiates an intracellular signal cascade, leading to the expression of the pro-inflammatory molecules. Of note, CD36, TLR2 and 4 are all upregulated in the brains of *T. gondii*-infected mice.

Collectively, the data obtained demonstrates that microglial activation is a feature of *T. gondii* infection. Besides the genes mentioned above, other genes that have been reported to be involved in microglial activation such as Aif-1 (allograft inflammatory

factor 1), CD68, and Tmem119 (transmembrane protein 119) [79–81] were also shown to be significantly upregulated in this study. Importantly, some of these gene products are known to be important in control of *T. gondii* infection. However, there is increasing evidence that many are also associated with neuropathology and neurological diseases and dysfunction.

4.8 | Oxidative Stress

Another interesting observation was the down regulation of glutathione S-transferase, mu1 (GSTM1) and glutathione peroxidase because both enzymes function in defence against oxidative stress. Oxidative stress has been suggested to contribute to the pathology in schizophrenia, AD, PD, stroke and epilepsy [82, 83].

4.9 | Limitations of This Study

Careful optimisation of metabolite extraction and LCMS analysis was carried out before commencing this study. To our knowledge this is the most complete description of changes in the levels of soluble metabolites that occur in the adult mouse brain following infection with *T. gondii*. Putative functions of metabolites showing the largest changes were predicted by referring to previous studies but these need to be verified by further experimentation. This study was carried out using whole brain extracts and the regions of the brain where metabolic and transcription changes occur have not been identified. Significant changes in a minor sub-population of cells might not be detected by comparing whole brain extracts, particularly when the metabolite or transcript is present at high levels throughout the brain or where the metabolite or transcript are associated with infiltrating immune cells. This study focuses on a single time-point (4 weeks following infection) and therefore the temporal dynamics of transcripts and metabolites are not explored. This study aimed to characterise host metabolites rather than parasite metabolites. However, as it used infected tissue, parasite metabolites although logically present in small amounts, could still in theory contribute to analytes detected. A recent study has demonstrated that parasite metabolites vary according to the cells they infect [84].

5 | Conclusion

Overall, the studies described herein demonstrate that *T. gondii* infection alters a number of host processes in the brains of BALB/c mice even after acute disease has resolved. Notably, BALB/c mice are not susceptible to Toxoplasmic encephalitis when infected with the strain of *T. gondii* used in this study and have relatively few brain cysts present at this time-point. While many of the changes are in keeping with host defence such as microglial activation, tryptophan degradation and potentially purine degradation, their effects are still evident after active overt disease. Some of these effects and other changes identified through use of the Mouse Genome Database Informatics (MGI) [85] are known to be involved in human neurological diseases. Clearly, further studies are required to determine if these changes are immune driven or directly influenced by parasite products. It will be challenging to determine if similar changes occur in humans infected with *T. gondii*. Techniques including Magnetic resonance spectroscopy at present offer only limited potential for non-invasive in vivo analysis of human brain metabolites [86].

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.